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Automated sequential injection-capillary electrophoresis for dried blood spot analysis: A proof-of-concept study

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Keywords

Automation; Dried blood spots; Sequential injection analysis; Capillary electrophoresis; Capacitively coupled contactless conductivity detection; Clinical analysis

ABSTRACT: A hyphenated analytical platform that enables fully automated analyses of dried blood spots (DBSs) is proposed by the at-line coupling of sequential injection (SI) to capillary electrophoresis (CE). The SI system, exploited herein for the first time for unattended DBS handling, serves as the 'front end' mesofluidic platform for facilitating exhaustive elution of the entire DBS by flow programming. The DBS eluates are thus free from hematocrit and non-homogeneity biases. The SI pump transfers the resulting DBS eluates into CE sample vials through an internal port of the CE instrument and homogenizes the eluates, whereupon the eluted blood compounds are automatically injected, separated, and quantified by the CE instrument. The SI and CE are commercially available off-the-shelf instruments, and are interconnected through standard nuts, ferrules, and tubing without additional instrumental adjustments. They are controlled by dedicated software and are synchronized for a fully autonomous operation. The direct determination of endogenous (potassium and sodium) and exogenous (lithium as a model drug) inorganic cations in DBS samples has been used for the proof-of-concept demonstration. The hyphenated SI-CE platform provides excellent precision of the analytical method with RSD values of peak areas below 1.5% and 3.5% for intra-day and inter-day analyses, respectively, of the endogenous concentrations of the two inorganic cations. For the determination of lithium, calibration is linear in a typical clinical range of the drug (R^2 better than 0.9993 for 2 – 20 mg/L), RSD values of peak areas are below 4.5% (in the entire calibration range), limit of detection (0.4 mg/L) and limit of quantification (1.3 mg/L) are well below the drug's minimum therapeutic concentration (4 mg/L), and total analysis time is shorter than 5 min. The SI-CE platform reflects the actual trends in the automation of analytical methods, offers rapid and highly flexible DBS elution/analysis processes, and might thus provide a general solution to modern clinical analysis as it can be applied to a broad range of analytes and dried biological materials.

INTRODUCTION

Micro-sampling of dried blood spots (DBSs) has been suggested as a viable alternative to venous blood collection and has been accepted for specific clinical assays.[1] Standard DBS sampling involves the collection of a microliter volume of capillary blood onto a paper-based sampling card from a finger or a heel prick. The collected blood is then dried up in ambient air for several hours to form the DBS.[2] Since the collected biological material is dry, DBSs are considered non-biohazardous, can be transported by mail, and can be stored at very simple and inexpensive conditions. Analytes in DBSs exhibit better stability in comparison to wet blood samples because enzymes and other reactive compounds are deactivated during the drying process.[2] Moreover, the collection of capillary blood is more acceptable for most clinical subjects (specifically for individuals with severe anemia, infants, and children), and DBS sampling might thus open new horizons in clinical analysis[3-4] and personalized healthcare.[5]

Besides the formerly-evidenced advantages, collection and analysis of DBS face some challenges, too. DBSs are typically pretreated by multiple-step processes, which are tedious, time-consuming, and costly. They include sub-punching of a small part of the DBS, which is then eluted by vigorous shaking, and the resulting eluate is extracted, centrifuged, evaporated, and reconstituted with a solvent compatible with the subsequent analytical technique.[2] In addition, DBS analyses are associated with sensitivity issues due to the minute initial blood volumes and with compromised reliability of quantitative data due to the hematocrit effects and non-homogenous analyte distribution.[6-7] Recently, novel concepts based upon blood volume-related corrections,[8] volumetric absorptive microsampling (VAMS),[9] and end-to-end capillaries[10-11] were proposed to avoid the detrimental effects of DBS collection on quantitative DBS analyses and to simplify DBS collection. Even though these alternatives have improved and simplified DBS collection, the subsequent DBS treatment has remained the major challenge in contemporary DBS analysis because it is performed manually in most assays.[2]

To avoid the manual DBS treatment, semi(automated) robotic stations for the hyphenation of the DBS to liquid chromatography (HPLC)[2, 12] and for the direct injection from DBS into mass spectrometry (MS)[2, 13] have been presented. However, the automation of the DBS processing/analysis is not mature yet and there is still a quest of easier, cheaper, and fully unmanned systems. The major deficiencies of the current (semi)automated systems identified so far are: (i) the transfer of the DBS cards to the robotic systems by laboratory staff, (ii) the elution of a sub-section of the original DBS, (iii) the low elution efficiency, (iv) the coelution of matrix components into the separation/detection system, (v) the high complexity and rigidity of the processes, and (vi) the high cost of the robotic analytical systems.[2] As a consequence of (i), manual handling of biological material is necessary, of (ii), quantitative analyses are hematocrit-dependent, of (ii and iii), costly analytical systems with high sensitivity are employed, of (iv), separation/spectral interferences and ion suppression are encountered, and of (v and vi), two stand-alone instruments (for the DBS elution and the eluate analysis) are required, which make the system highly complex and not affordable for most laboratories.

Flow injection (FI) and related mesofluidic systems were originally conceived to simplify the analytical workflows and to lower the burden of routine laboratories while outperforming robotic stations in terms of affordability and versatility.[14-15] Flow-through approaches are based on monitoring reactions under non-steady-state conditions for high-throughput assays, yet assuring repeatable timing of events with minimal operator intervention.[16] The second generation of FI, so-called sequential injection (SI), capitalizes on programmable flow under user-friendly software control, i.e., a single system can be programmed for a plethora of unit operations and reaction schemes without the need for system reconfiguration, by exploiting a bidirectional pump and a multi-position selection valve.[17] As a result, SI-based fluidic systems are regarded as the most appropriate vehicles for automation of sample preparation, including liquid-phase (micro)extraction, sorptive (micro)extraction, and leaching procedures.[18-19]

Indeed, the handling and leaching/extraction of solid and dried samples (usually foodstuff or environmental matrices) is greatly simplified and accelerated by resorting to FI/SI approaches[20-21] that are readily tailor-made to the user's demands. Yet, to our best knowledge, the exploitation of FI/SI as a front end to modern analytical instrumentation for autonomous processing of DBSs has not been reported to date.

Capillary electrophoresis (CE) offers a cheap, simple, and highly efficient instrumental configuration, which is perfectly suited to simplify sample processing and analyze minute volumes of biological samples.[22-24] Besides, commercial CE systems are equipped with an internal port, which can straightforwardly connect the CE autosampler with an external liquid handling device.[25] Thus, samples processed with, e.g., an SI system, can be directly transferred into sample vials in the CE autosampler for at-line CE analyses. The reagents/sample volumes handled by SI and those typically used in CE are perfectly compatible and this is one of the key aspects for the ease of coupling of these two techniques. Another pivotal issue of such coupling is the use of CE as the analytical end for (i) rapid separations with high separation efficiencies, (ii) high tolerance to common interferences encountered in biological samples, and (iii) potential on-capillary concentration.[26-28] CE has been recently also shown suitable for an all-in-one concept enabling processing and analyses of DBSs using a single off-the-shelf instrument.[11] Despite this achievement, it is still rather difficult to perform fully automated, flexible, and comprehensive DBS pretreatment by a single CE instrument. We believe that the high flexibility of the DBS treatment can be obtained by the direct coupling of an autonomous liquid handling device, such as SI, to CE. The first SI-CE couplings were reported at the turn of the millennium.[29-30] Nevertheless, the SI systems were merely employed for liquid sample delivery to the separation capillary end for split-mode injections. It should be also noted that the hyphenation has been realized preferably with lab-made CE instruments, has mostly been applied to "clean" samples and has not been used for handling solid/dry samples.[31-32] In fact,

coupling of flow-through DBS processing by mesofluidic platforms to CE analysis has not been described as of yet.

In order to resort to the favorable synergetic aspects of SI and CE and to complement/broaden the portfolio of automated DBS analytical set-ups, the actual contribution presents the proof-of-concept of a novel, fully automated SI-CE platform for DBS analysis. To this end, we aim at the autonomous elution of the entire DBS (thus free from hematocrit and non-homogeneity effects) with an SI system that will be at-line coupled to the internal autosampler of a CE instrument. Investigation of critical parameters for flow-through DBS elution will be investigated in details. Performance characteristics of the automated SI-CE system for DBS analysis will be compared with the standard DBS elution methodology by the determination of endogenous and exogenous ionic species in DBSs at physiologically relevant concentrations.

EXPERIMENTAL SECTION

Reagents, Standard Solutions, and DBS Samples. Details on reagents and standard solutions are described in *Supporting Information*. DBS samples were formed by spotting 10 μL of capillary blood from a finger prick onto Whatman™ 903 Protein Saver sampling card (GE Healthcare Ltd, Cardiff, UK) and by drying the spots at laboratory temperature for 3 h. The DBS samples were analyzed the day after collection. Written informed consent was signed by all donors of the DBS samples. Other details on DBS sampling can be found in an earlier contribution.[8]

Capillary Electrophoresis Apparatus. CE analyses were performed with a 7100 CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with an Admet capacitively coupled contactless conductivity detector (C^4D) (Admet, Prague, Czech Republic). Other details can be found in *Supporting Information*.

Sequential Injection System. The components of the SI system are schematically illustrated in the right panel of **Figure 1**. The mesofluidic MicroSIA system was purchased from FIALab Instruments Inc. (Seattle, WA, USA) and employed a low-pressure metal-free 8-position selection valve and a 30 mm-stroke bi-directional syringe pump. A 3-way head valve allowed the connection of the syringe pump to the carrier solution (deionized (DI) water), air, and the flow pathway via a 65 cm-long holding coil (HC1, 1.0 mm i.d./1.6 mm o.d. perfluoroalkoxy (PFA) tubing, Vici-Jour, Schenkon, Switzerland). A 500 µL-borosilicate glass syringe (XC/XP with PTFE plunger tip seal, Tecan Systems, Inc., San Jose, CA, USA, P/N 20725590) was used for automatic liquid handling. The peripheral ports #2 and #3 of the selection valve served for the autonomous aspiration of air and elution solution through the communication channel into HC1, and port #1 was used for liquid disposal to waste. Tubing connected to ports #1, #2, and

#3 were 10 cm-long segments of PFA tubing (1.0 mm i.d/1.6 mm o.d., Vici-Jour). Unattended control of all flow system units (syringe pump, head valve, selection valve) was accomplished via USB using the open-source software CocoSoft (version 5.15) written in Python programming language.[33] An initial SI system flushing sequence was carried out at the beginning of each working day and is presented in **Table S1** in *Supporting Information*.

DBS Elution Device. The DBS elution device (disassembled and assembled) is depicted in **Figure 2** and is based on a commercial dialysis unit (Harvard Apparatus, Holliston, MA, USA, P/N 74-0400). The connection between the MicroSIA and the DBS elution device was accomplished through a 10 cm-long holding coil (HC2, 0.5 mm i.d/1.6 mm o.d. PFA tubing, Vici-Jour) connected to port #4 of the selection valve. A leak-free connection at the DBS elution device inlet was achieved by pushing the end of the HC2 into a 1 cm-long segment of silicone tubing (1 mm i.d./3 mm o.d., Gumex, Strážnice, Czech Republic). A DBS disc (11 mm) was placed into the DBS holder together with a silicone O-ring (8 mm i.d./11 mm o.d., 1 mm thick, Zlíntech, Zlín, Czech Republic) and the two parts of the device (holder + nut) were screwed together. The connection between the outlet of the device and the CE replenishment needle assembly was realized by a 15 cm-long holding coil (HC3, 0.5 mm i.d./1.6 mm o.d. PFA tubing, Vici-Jour), which acted as the transfer line from SI to CE. A leak-free connection at the DBS elution device outlet was achieved by pushing the end of the HC3 into a 1 cm-long segment of silicone tubing (1 mm i.d./3 mm o.d., Gumex).

Off-line DBS Elution Procedures. ***SI Elution***. Preliminary tests involving off-line SI elution of DBS samples were performed according to the following procedure. (i) The entire DBS was punched from the sampling card using an 11 mm cork-borer. (ii) The resulting disc with the DBS and a silicone O-ring were placed into the DBS holder and the holder and the nut were

screwed together. (iii) The DBS was eluted with DI water using the MicroSIA and the resulting eluate was collected into a 250 μL plastic vial. The eluate was homogenized by agitation at 1000 rpm for 60 s and 50 μL was transferred to a CE micro-vial (Agilent Technologies, P/N 9301-0978) for injection.

DBS Agitation. Details on the standard protocol for DBS elution by agitation can be found in *Supporting Information*.

At-line SI-CE Coupling. Schema and components of the platform for the autonomous SI-CE analyses of DBSs are graphically presented in **Figure 1**. The DBS elution device was assembled identically to the procedure reported in section *DBS Elution Device*. The operation of the SI system was identical to that described in section *Off-line DBS Elution Procedures. SI Elution* with the exception that the DBS eluate was autonomously transferred to an empty glass snap-cap vial in the replenishment lift of the CE instrument through the transfer line (HC3). The eluate was then autonomously homogenized by a stream of air delivered by the MicroSIA pump directly to the CE vial and the replenishment lift moved the vial to the autosampler carousel for subsequent CE injection/analysis. The connection between the SI and the CE system is graphically presented in **Figure S1** in *Supporting Information*. The original tubing connecting the CE replenishment needle with the CE replenishment system was disconnected from port A of the replenishment needle assembly. Subsequently, the outlet of HC3 was screwed into port A. Connection to port B (liquid level sensor) of the replenishment needle assembly was not modified.

RESULTS AND DISCUSSION

Manual DBS Elution. Manual DBS elution[8] was used as a reference procedure for the comparison with the newly developed DBS elution procedure using the SI system and is detailed in **Figure S2** and the corresponding text in *Supporting Information*. Maximum DBS elution efficiency was achieved in 15 min and was used for all manual DBS elutions. Interestingly, the calculated elution efficiency showed an unexpected positive bias for Na^+ eluted from DBSs with maximum efficiencies ≥ 100%. This discrepancy has been investigated in detail in *SI Elution of Inorganic Cations from DBS* and has been identified as a cross-contamination resulting from the DBS sampling material.

Configuration of the Flow-Through SI system. The settings and connections of the SI system and the related flow pathways were comprehensively examined for reliable flow-through elution of DBS. This included investigation of the connections between the head valve and the selection valve of the SI system, and between the selection valve of the SI system and the DBS elution device. Moreover, the DBS disc size and the internal chamber layout of the DBS elution device were explored as well as the parameters of the outlet tubing from the DBS elution device, i.e. the transfer line to CE. The selected dimensions and lengths were 1.0 mm i.d. and 65 cm for the tubing interconnecting the head valve and the selection valve of the SI system (HC1), 0.5 mm i.d. and 10 cm for the tubing interconnecting the selection valve and the DBS elution device (HC2), and 0.5 mm i.d. and 15 cm for the outlet tubing from the DBS elution device (HC3, transfer line). The dimensions/lengths were chosen to ensure sufficient volume of HC1 (~ 510 μL) for a full-stroke operation of the 500 μL syringe pump, and the minimum feasible volume of HC2 and HC3 for the transfer of the eluent to and the eluate from the DBS elution device, respectively. More details can be found in *Supporting Information*.

The DBS holder used in this work has an internal chamber that accommodates DBS discs with a diameter up to 11.6 mm. The 10 μL volume of capillary blood forms a DBS with a 6-7 mm diameter. A DBS disc size of 11 mm that ensures a whole DBS punch and a constant position of the disc inside the holder was, therefore, selected. Initial experiments with the holder, the DBS disc, and the nut screwed together revealed (leak-free) elution of the central part of the DBS only (see **Figure S3** in *Supporting Information*). This was caused by the fact that the eluent stream did not efficiently wet and elute the peripheral parts of the DBS disc covered by the nut. As we have aimed at the DBS elution free from hematocrit/non-homogeneity effects, the layout of the DBS holder was slightly modified to achieve exhaustive DBS elution. A 1 mm thick silicone O-ring (8 mm i.d./11 mm o.d.) was placed onto the DBS disc before the DBS elution device was screwed together. This formed an internal cavity above the DBS (with a constant volume and a diameter larger than the DBS), which ensured the intimate contact of the eluent with the DBS card and thus fostered the elution of the entire DBS.

SI Hydrodynamic Characteristics for Off-line DBS Elution. The hydrodynamic parameters of the SI setup for the DBS elution were initially examined with an eluate volume of 250 μL. For the DBSs formed by spotting 10 μL of capillary blood, the dilution factor was 25 and was selected based on our previous experience; the 25-diluted DBS eluates ensured repeatable and interference-free CE analyses of target analytes with minimum capillary maintenance.[8] The ruggedness of the SI system for DBS processing under flow-through conditions was examined using different configurations and flow rates, and the resulting performance is summarized in **Table 1**. First, the DBS elution device was used without the DBS disc and 250 μL of DI water was flushed through the SI pathway followed by 250 μL of air. A flow rate of 300 μL/min was selected as a suitable liquid transfer speed that offers a reasonably short elution time but a sufficiently long contact time based on the results presented in *SI elution of inorganic cations*

from DBS. The transferred liquid was collected at the outlet of HC3, weighted, and the liquid volume was calculated according to equation (1) in *Supporting Information*. Second, a DBS disc with no capillary blood was placed in the DBS elution device, the SI flushed 250 μL of DI water and 250 μL of air consecutively through the device at 300 μL/min, and the transferred liquid was collected and its volume calculated as previously. Finally, a DBS disc with 10 μL of blood was placed in the DBS elution device, consecutively flushed with 250 μL of DI water and 250 μL of air, and the transferred liquid was collected and its volume calculated as previously. DBSs were eluted at five different flow rates ranging from 150 to 2000 μL/min (see **Table 1**). For a comparison, DBSs were also eluted by the standard protocol according to *Manual DBS elution* (see above), the eluate was recovered by pipetting out all free liquid from the vial and its volume was calculated as previously.

The results demonstrate an accurate liquid transfer by SI (246.8 μL) through the empty DBS holder with excellent repeatability (0.3% RSD). The slightly lower absolute volume can be ascribed to the precision of glass syringe manufacturing, which is usually around 1%. The eluate volumes collected after elution of blank and blood-spotted DBS discs were 20.8 and 24.0 μL less, thus indicating some adsorption of the eluent solution by the cellulose-based DBS sampling material. Similarly, DBSs eluted at different flow rates indicate similar adsorption of the eluent (20.2 – 22.6 μL) by the DBS discs regardless of the eluent flow rate. The repeatability of the elution process was slightly worsened whenever DBS discs were processed and this might be attributed to the manual sub-punching of the discs and the slight differences in homogeneity of the sampling material. Nevertheless, RSD values were in all instances better than 1.9%, thereby again demonstrating excellent repeatability of the SI-driven DBS elution process. In addition, the volume collected after the DBS elution with the SI system is in all instances higher and more repeatable than that after the manual DBS elution. These results also suggested that

the SI-based automated elution is more amenable to DBS processing with lower elution volumes for improved sensitivity (see **Figure 4** later in the manuscript).

SI Elution of Inorganic Cations from DBS. To further evaluate the efficiency of the SI system for flow-through DBS elution at different flow rates, the collected eluates were analyzed by CE-C^4D for the quantitative determination of endogenous inorganic cations. Capillary blood contains ~ 100 mM concentrations of K^+ and Na^+ and 2 – 3 orders of magnitude lower concentrations of Ca^{2+}, Mg^{2+}, and NH_4^+.[8] The two major cations were considered in our experiments and their concentrations in DBS eluates after SI treatment were compared with their concentrations in DBS eluates prepared according to *Manual DBS elution* reported earlier. The elution efficiency values achieved at 150, 300, 600, 1200, and 2000 μL/min flow rates are depicted in **Figure 3** along with the duration of the total elution procedure. The increase of the flow rate from 150 to 2000 μL/min decreased the elution time by a factor of 5.6. The total elution procedure at 2000 μL/min took 40 s, which is 22.5-fold faster than the manual DBS elution. On the other hand, elution efficiency at 2000 μL/min was slightly compromised (~ 80%) because the contact time of the elution solution with the DBS had been reduced to 7.5 s only. The incomplete DBS elution was clearly observed by visual inspection of the DBS discs and is demonstrated in **Figure S4** in *Supporting Information*. Elution efficiencies were rather consistent (94 – 98%, RSD ≤ 3.1%) for 150 – 600 μL/min flow rates, and 600 μL/min was selected for subsequent experiments (with 250 μL elution volume) due to the faster elution procedure (merely 75 s).

The elution volume determines the actual blood dilution factor of the final DBS eluate. In fact, various dilution factors might be required based on the analyte's blood concentration and the complexity of the resulting eluate. The flexibility of the SI system for the DBS elution was demonstrated by the autonomous handling of various elution volumes (75 – 250 μL), which

resulted in blood dilution factors within the range of 7.5 – 25. The elution flow rate was here decreased down to 300 μL/min to ensure a reasonable contact time with the DBS for the smallest eluent volumes and was later further investigated in a separate procedure. The results in **Figure 4** demonstrate a linear increase of collected eluate volumes in the 75 – 250 μL range. The collected eluate volumes were lower by approximately 25 μL in comparison to the original elution volumes, and the volume reduction was consistent with the volumes adsorbed by the DBS sampling discs reported in **Table 1**. Concentrations of the inorganic cations in the DBS eluates increased for reduced elution volumes and peak areas for K^+ and Na^+ were 2.9 and 2.8-fold higher for 75 vs. 250 μL eluate volumes, respectively. These values were slightly lower than the theoretically calculated increase (3.33-fold) and were caused by the non-exhaustive elution of DBS compounds at the herein selected SI conditions (elution volume and flow rate). The repeatability of the DBS elution protocol for the 75 μL elution volume (RSD 10.1 – 11.6%) worsened considerably compared to 250 μL (RSD 1.3 – 1.4%) and was also ascribed to the incomplete elution of the DBS compounds at the selected SI conditions. The SI system was capable of handling even lower volumes of the DBS elution solution and the minimum volume was approx. 35 μL due to the liquid absorption by the DBS disc (approx. 25 μL). Nevertheless, application of such low volumes resulted in an even more compromised elution repeatability, minute volumes and increased matrix complexity of collected eluates, excessive saponification during at-line eluate homogenization, and required additional adjustments of the SI-CE set-up. A comprehensive investigation of all these aspects was beyond the scope of the actual proof-of-concept study and DBS elution with minute eluate volumes will be elaborated in detail in a subsequent study.

The effect of the SI operational conditions on the DBS elution was further examined for just a 10-fold dilution factor using an elution volume of 100 μL and elution flow rates of 150 – 1200 μL/min. The dilution factor can be automatically adjusted by programming adequate elution

volume in the SI script. The resulting elution times and elution efficiency values for K^+ and Na^+ are depicted in **Figure 5**. Higher flow rates demonstrate faster elution procedures (0.8 min for 1200 μL/min vs. 1.4 min for 150 μL/min), however, at the expense of reduced elution efficiency and repeatability. The elution efficiency values dropped down to 80% (RSD ~ 4.1%) and 60% (RSD ~ 8.1%) for 600 μL/min and 1200 μL/min, respectively. Elution of DBSs at low dilution factors is therefore recommended at low flow rates ($\leq$ 300 μL/min) so as to enable nearly exhaustive elution of K^+ and Na^+ from the DBS (93 – 95%) with excellent repeatability (RSD $\leq$ 2.4%) and elution times $\leq$ 85 s.

An interesting artifact was observed in the CE-C^4D electropherograms resulting from the two elution procedures (manual vs. automated DBS elution). Analytical signals for the low abundant inorganic cations (NH_4^+ and Ca^{2+}) were considerably higher for the eluates prepared by the manual DBS elution. To prove our hypothesis that their increased concentrations are caused by the DBS sampling material and/or by the DBS processing procedure, five different samples were prepared and analyzed. CE-C^4D electropherograms of the five samples are depicted in **Figure S5** in *Supporting Information*. In brief, a standard blood sample was prepared by diluting 10 μL of liquid capillary blood with 240 μL of DI water. One blank eluate was prepared by the automated and another one by the manual elution of blood-free sampling discs. One DBS eluate was prepared by the automated and another one by the manual elution of sampling discs with 10 μL DBSs. In comparison to the standard blood sample, increased peak areas were observed for NH_4^+, Ca^{2+}, and Na^+ in the manually prepared DBS eluate. Manual elution of a blood-free DBS disc revealed a considerable release of NH_4^+, Ca^{2+}, and Na^+ into the eluate, which rationalized the observed increase of their CE peak areas in the DBS eluate. Under the DBS elution conditions employed, the peak areas increased by 107%, 1%, 226%, and 11% for NH_4^+, K^+, Ca^{2+}, and Na^+, respectively, and had a significant effect on the quantitative DBS analysis. On the other hand, the automated SI system resulted in a considerably milder elution

process. In comparison to the manually eluted blood-free DBS disc, the released amounts of NH_4^+, K^+, Ca^{2+}, and Na^+ were 4, 3, 8, and 4-fold lower, respectively. Leaching of the DBS sampling material components into the eluates has not been reported earlier and might not be critical for clinical analyses of drugs and other exogenous compounds because they will very likely not be present in sampling materials. However, it can be detrimental for inorganic analysis as many inorganic ions are present at trace concentrations in blood and their determination can be impaired by the DBS material leaching. DBS elution by SI might thus be advantageous due to the milder and easily controllable elution process. Furthermore, sampling on alternative materials (such as VAMS[9] or soluble foams[34]), which might be characterized by reduced leaching of intrinsic inorganic cations, could be beneficial.

DBS Elution Device Orientation. Three different orientations of the DBS holder were tested and the corresponding results are depicted in **Figure S6** and **Table S3** in *Supporting Information*.

At-line SI-CE Coupling for a Fully Autonomous DBS Analysis. After the initial examination of the SI characteristics for the off-line DBS elution, the SI system and the DBS elution device were coupled to the inlet port of the replenishment needle assembly (port A, see *Experimental Section*, **Figure 1**, and **Figure S1**) of the commercial CE. The coupling required only standard nuts, ferrules, and tubing. No adjustment other than unscrewing the original tubing from port A of the replenishment needle assembly was required. Length (15 cm) and i.d. (0.5 mm) of the transfer line were carefully selected to ensure minimum dead volume and backpressure.

Both autonomous units (the SI and the CE instrument) were controlled by a single personal computer, which enabled a full synchronization of SI elution and CE analysis steps. Facile adjustments of the operational parameters of the SI system during method developments were

performed by CocoSoft freeware. Examples of selected scripts for the initial SI cleaning and the SI-controlled DBS elution are shown in *Supporting Information*. Full control of the CE instrument was achieved by ChemStation software, which enabled autonomous manipulation of CE vials within the autosampler carousel and the replenishment lift. The entire analytical process ran fully unattended and the description of all program steps and details of the SI-CE synchronization are shown in **Table S4** and **Table S5** in *Supporting Information*. To examine the suitability of the at-line SI-CE coupling for the automated flow-through DBS elution, 5 unique DBSs were eluted with 250 μL of DI water at 600 μL/min. The eluates transferred to CE sample vials were weighted and recalculated to volume. The average collected eluate volume was 222.4 μL (2.7% RSD) and was consistent with the volume (and repeatability) of the off-line DBS eluate collection.

The major advantage of the proposed at-line coupling is the synchronization of the overall DBS analysis steps. DBS elution, eluate transfer to the sample vial, and eluate homogenization (see next section) were carried out by the SI system and were controlled by CocoSoft. Simultaneously with the DBS elution, ChemStation performed preconditioning of the separation capillary (flushing with NaOH and BGE solutions) for the CE analysis. Once the eluate was ready for analysis and the CE capillary preconditioned, the sample vial was moved to the CE carousel for injection and the quantitative analysis was immediately initiated. A considerable reduction of analysis time was thus achieved because the DBS elution/homogenization and capillary preconditioning were performed simultaneously. Duration of all respective steps of a typical SI-CE procedure is specified in **Table S4** (75 s for DBS elution/homogenization, 120 s for capillary preconditioning, 120 s for CE analysis) and the total DBS analysis time was 280 s per sample (**Table S5** in *Supporting Information*). A 20 s flush with 100 mM NaOH was sufficient for the removal of blood matrix components from the capillary inner walls (e.g., proteins, after the previous DBS analysis) and ensured excellent

repeatability of migration times of K^+ and Na^+ at their endogenous concentrations. RSD values for intra-day (5 DBSs in one day, $n = 5$) and inter-day (5 DBSs in one month, $n = 5$) measurements were ≤ 0.3% and 1.1%, respectively.

Autonomous Homogenization of the Collected DBS Eluate. The DBS eluate collected in a CE sample vial after the SI elution is rather non-homogenous. This is caused by the gradual dissolution of the dried blood during the DBS elution procedure that generates a saturated and diluted DBS eluate at the beginning and at the end of the procedure, respectively (see **Figure S7** in *Supporting Information*). Quantitative analyses might thus be significantly biased if CE injections are performed from the non-homogenous eluates. The at-line SI-CE coupling offers a flexible tool for an attractive, quick, and efficient eluate homogenization by SI pumping of a given volume of air at a given flow rate through the entire SI-CE system until the CE replenishment needle. The two parameters were investigated in separate procedures detailed in **Table 2** and in *Supporting Information*. Experimental results revealed that the eluate was well homogenized, as compared to vortex mixing, by flushing 250 μL of air at 600 μL/min after the eluate. Quantitative determination of K^+ and Na^+ at their endogenous concentrations in five distinct DBS eluates demonstrated intra-day repeatability and inter-day reproducibility of peak areas better than 1.5% and 3.5%, respectively.

A comprehensive description of the SI-CE platform operation and operator's steps during the autonomous DBS elution/analysis are reported in *Supporting Information.*

Model Clinical Application. The proposed at-line SI-CE coupling for fully autonomous DBS analyses has been further evaluated by the determination of lithium as a clinically relevant analyte. Lithium is determined in human blood as a drug for the treatment of bipolar disorders. Lithium therapeutic concentrations are in the 4 – 8 mg/L range and the borderline between the

maximum therapeutic concentration, toxicity (8 – 13 mg/L), and poisoning (16 mg/L) is relatively narrow.[35] DBSs for the determination of lithium were prepared by spotting and drying out 10 μL of drug-free capillary blood and the same capillary blood spiked with 2, 5, 10, and 20 mg/L of lithium. The SI elution was performed with 250 and 100 μL of DI water and the resulting eluates were at-line transferred to CE for the autonomous homogenization, injection, and analysis. The results are summarized in **Table S6** in *Supporting Information* and demonstrate excellent repeatability (RSD less than 4.5%) and linearity (coefficients of determination better than 0.9993) of the analytical technique. Further improvement of the quantitative parameters might be achieved by the application of an internal standard. The limits of detection and quantification (LOD and LOQ, defined as 3S/N and 10S/N, respectively) were 1.0 and 3.3 mg/L for the 250 μL and 0.4 and 1.3 mg/L for the 100 μL elution volume, respectively. These results imply 2.5-fold better LOD/LOQ for the latter elution conditions, which are consistent with the reduced dilution factor. Sufficient sensitivity for the SI-CE-C^4D determination of lithium in clinical samples was observed for both elution volumes. Zoomed sections of the electropherograms for the five DBSs eluted with 100 μL of DI water are depicted in **Figure 6** and a full-scale electropherogram demonstrating the separation efficiency, baseline stability and matrix-related peaks is depicted in **Figure S8** in *Supporting Information*.

CONCLUSIONS

A novel hyphenated analytical platform capable of autonomous DBS analyses is herein presented. An SI system is used as the 'front end' manifold for handling minute volumes of solutions and facilitating the fully unattended DBS elution from a customized DBS elution device. The flow manifold is furnished with a multi-position selection valve and bidirectional syringe pump for flexible SI-based manipulations of elution solutions. Their volumes and flow rates might be investigated at will by flow programming to ensure the elution of the entire DBS

in the shortest possible time. The outflow of the DBS elution device is connected to an internal port of a CE instrument for the at-line SI-CE coupling. This coupling enables the automated transfer of the resulting DBS eluate to a sample vial in the CE autosampler by the SI pump, followed by the autonomous injection, separation, and quantification of the eluted blood components by the CE system. The SI and CE are commercially available off-the-shelf instruments, and are interconnected through standard nuts, ferrules, and tubing. The only adjustment to the original instruments is the disconnection of the internal tubing from the CE replenishment assembly device and its replacement with the outflow from the DBS elution device. The instruments are controlled by dedicated software and are synchronized for a fully unattended operation. Moreover, the SI-CE coupling offers reliable liquid handling, rapid analysis, and sufficient sensitivity for the determination of endogenous and exogenous DBS compounds. The proposed proof-of-concept study reflects the actual trends in automation of analytical techniques and provides a general solution to modern clinical analysis as it can be applied to a broad range of analytes and dried biological materials. Moreover, sensitivity and selectivity of this concept might be further enhanced by the at-line coupling of SI to CE with ESI-MS detection because it has been proven recently that interferences from blood matrix were not observed and DBS eluates were fully compatible with CE-ESI-MS in isotachophoretic mode.[36]

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The authors declare no conflict of interest.

SUPPORTING INFORMATION

(i) Experimental Section details, (ii) DBS elution by agitation, (iii) SI-CE connection, (iv) off-line DBS elution by SI, (v) electropherograms for cross-contamination/interference study, (vi) CocoSoft scripts, (vii) orientation of the DBS elution device, (viii) homogenization of DBS eluates, (ix) SI-CE synchronization flow-charts, (x) SI-CE operation.

REFERENCES

1. Guthrie, R.; Susi, A., A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics* **1963,** *32* (3), 338-343.

2. Li, W.; Lee, M. M., *Dried Blood Spots. Applications and Techniques.* Wiley: East Hanover, NJ, USA, 2014; p 363.

3. Milosheska, D.; Grabnar, I.; Vovk, T., Dried blood spots for monitoring and individualization of antiepileptic drug treatment. *Eur. J. Pharm. Sci.* **2015,** *75*, 25-39.

4. Wilhelm, A. J.; den Burger, J. C. G.; Swart, E. L., Therapeutic Drug Monitoring by Dried Blood Spot: Progress to Date and Future Directions. *Clin. Pharmacokinet.* **2014,** *53* (11), 961-973.

5. Tanna, S.; Lawson, G., Dried blood spot analysis to assess medication adherence and to inform personalization of treatment. *Bioanalysis* **2014,** *6* (21), 2825-2838.

6. de Vries, R.; Barfield, M.; van de Merbel, N.; Schmid, B.; Siethoff, C.; Ortiz, J.; Verheij, E.; van Baar, B.; Cobb, Z.; White, S.; Timmerman, P., The effect of hematocrit on bioanalysis of DBS: results from the EBF DBS-microsampling consortium. *Bioanalysis* **2013,** *5* (17), 2147-2160.

7. De Kesel, P. M.; Sadones, N.; Capiau, S.; Lambert, W. E.; Stove, C. P., Hemato-critical issues in quantitative analysis of dried blood spots: challenges and solutions. *Bioanalysis* **2013,** *5* (16), 2023-2041.

8. Dvořák, M.; Ryšavá, L.; Kubáň, P., Capillary Electrophoresis with Capacitively Coupled Contactless Conductivity Detection for Quantitative Analysis of Dried Blood Spots with Unknown Blood Volume. *Anal. Chem.* **2020,** *92* (1), 1557-1564.

9. Protti, M.; Mandrioli, R.; Mercolini, L., (T)utorial: Volumetric absorptive microsampling (VAMS). *Anal. Chim. Acta* **2019,** *1046*, 32-47.

10. Neto, R.; Gooley, A.; Breadmore, M. C.; Hilder, E. F.; Lapierre, F., Precise, accurate and user-independent blood collection system for dried blood spot sample preparation. *Anal. Bioanal. Chem.* **2018,** *410* (14), 3315-3323.

11. Ryšavá, L.; Dvořák, M.; Kubáň, P., Dried Blood Spot Self-Sampling with Automated Capillary Electrophoresis Processing for Clinical Analysis. *Angew. Chem. Int. Ed.* **2021,** *60* (11), 6068-6075.

12. Luginbühl, M.; Gaugler, S., The application of fully automated dried blood spot analysis for liquid chromatography-tandem mass spectrometry using the CAMAG DBS-MS 500 autosampler. *Clin. Biochem.* **2020,** *82*, 33-39.

13. Wagner, M.; Tonoli, D.; Varesio, E.; Hopfgartner, G., The use of mass spectrometry to analyze dried blood spots. *Mass Spectrom. Rev.* **2016,** *35* (3), 361-438.

14. Kolev, S.; McKelvie, I. D., *Advances in Flow Injection Analysis and Related Techniques*. Elsevier: Amsterdam, 2008; Vol. 54, p 783.

15. Ruzicka, J., Flow Injection Analysis-Tutorial & News on Flow Based micro Analytical Techniques, https://www.flowinjectiontutorial.com/. **2019**.

16. Zagatto, E. A. G.; Rocha, F. R. P., The multiple facets of flow analysis. A tutorial. *Anal. Chim. Acta* **2020,** *1093*, 75-85.

17. Lenehan, C. E., Flow Injection Analysis/Sequential Injection Analysis. In *Encyclopedia of Analytical Science (Third Edition)*, Worsfold, P.; Poole, C.; Townshend, A.; Miró, M., Eds. Elsevier: Amsterdam, 2019; pp 227-232.

18. Horstkotte, B.; Miró, M.; Solich, P., Where are modern flow techniques heading to? *Anal. Bioanal. Chem.* **2018,** *410* (25), 6361-6370.

19. Hansen, E. H.; Miró, M., On-line sample processing methods in flow analysis. In *Advances in Flow Methods of Analysis*, Trojanowicz, M., Ed. Wiley-VCH: Weinheim, Germany, 2008; pp 291-320.

20. Frenzel, W.; Miró, M., Flow Analysis/Flow Injection Analysis: Environmental and Agricultural Applications. In *Encyclopedia of Analytical Science (Third Edition)*, Worsfold, P.; Poole, C.; Townshend, A.; Miró, M., Eds. Elsevier: Amsterdam, 2019; pp 164-194.

21. Rosende, M.; Miró, M., Recent trends in automatic dynamic leaching tests for assessing bioaccessible forms of trace elements in solid substrates. *TrAC - Trends Anal. Chem.* **2013,** *45*, 67-78.

22. Almeda, S.; Arce, L.; Valcarcel, M., The More and Less Common Approaches to Enhancing Sensitivity in Capillary Electrophoresis. *Curr. Anal. Chem.* **2010,** *6* (2), 126-143.

23. Xie, H. Y.; He, Y. Z., Green analytical methodologies combining liquid-phase microextraction with capillary electrophoresis. *TrAC-Trends Anal. Chem.* **2010,** *29* (7), 629-635.

24. Kubáň, P.; Boček, P., Direct coupling of supported liquid membranes to capillary electrophoresis for analysis of complex samples: A tutorial. *Anal. Chim. Acta* **2013,** *787*, 10-23.

25. Santos, B.; Simonet, B. M.; Ríos, A.; Valcárcel, M., Direct automatic determination of biogenic amines in wine by flow injection-capillary electrophoresis-mass spectrometry. *Electrophoresis* **2004,** *25* (20), 3427-3433.

26. Kubáň, P.; Dvořák, M.; Kubáň, P., Capillary electrophoresis of small ions and molecules in less conventional human body fluid samples: A review. *Anal. Chim. Acta* **2019,** *1075*, 1-26.

27. Breadmore, M. C.; Grochocki, W.; Kalsoom, U.; Alves, M. N.; Phung, S. C.; Rokh, M. T.; Cabot, J. M.; Ghiasvand, A.; Li, F.; Shallan, A. I.; Keyon, A. S. A.; Alhusban, A. A.; See, H. H.; Wuethrich, A.; Dawod, M.; Quirino, J. P., Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2016-2018). *Electrophoresis* **2019,** *40* (1), 17-39.

28. Šlampová, A.; Malá, Z.; Gebauer, P., Recent progress of sample stacking in capillary electrophoresis (2016-2018). *Electrophoresis* **2019,** *40* (1), 40-54.

29. Fang, Q.; Wang, F. R.; Wang, S. L.; Liu, S. S.; Xu, S. K.; Fang, Z. L., Sequential injection sample introduction microfluidic-chip based capillary electrophoresis system. *Anal. Chim. Acta* **1999,** *390* (1-3), 27-37.

30. Wu, C. H.; Scampavia, L.; Ruzicka, J., Microsequential injection: anion separations using 'Lab-on-Valve' coupled with capillary electrophoresis. *Analyst* **2002,** *127* (7), 898-905.

31. Kubáň, P.; Karlberg, B., Flow/sequential injection sample treatment coupled to capillary electrophoresis. A review. *Anal. Chim. Acta* **2009,** *648* (2), 129-145.

32. Clavijo, S.; Avivar, J.; Suarez, R.; Cerda, V., Analytical strategies for coupling separation and flow-injection techniques. *TrAC - Trends Anal. Chem.* **2015,** *67*, 26-33.

33. Cocovi-Solberg, D. J.; Miró, M., CocoSoft: educational software for automation in the analytical chemistry laboratory. *Anal. Bioanal. Chem.* **2015,** *407*, 6227-6233.

34. Ask, K. S.; Pedersen-Bjergaard, S.; Gjelstad, A., Dried Blood Spots on Carboxymethyl Cellulose Sheets: Rapid Sample Preparation Based on Dissolution and Precipitation. *Chromatographia* **2016,** *79* (7-8), 509-514.

35. Schulz, M.; Schmoldt, A.; Andresen-Streichert, H.; Iwersen-Bergmann, S., Revisited: Therapeutic and toxic blood concentrations of more than 1100 drugs and other xenobiotics. *Crit. Care* **2020,** *24* (1), 195.

36. Malá, Z.; Gebauer, P., Capillary isotachophoresis with electrospray-ionization mass-spectrometric detection: Cationic electrolyte systems in the medium-alkaline range for selective analysis of medium strong bases. *J. Chromatogr. A* **2020,** *1618*, 460907.

Figure Captions

Figure 1. Schematic illustration of the at-line SI-CE coupling for the automated DBS elution and analysis. HC1 – holding coil 1, HC2 - holding coil 2, HC3 – holding coil 3.

Figure 2. Sketch of the components of the DBS elution device (disassembled and assembled).

Figure 3. The effect of the flow rate on the DBS elution efficiency and the total elution time. DBS parameters and SI conditions as for **Table 1**; CE conditions, see *Experimental Section* in *Supporting Information*, $n = 5$.

Figure 4. The effect of the elution volume on the peak areas of inorganic cations eluted from DBSs and the collected eluate volume. SI conditions: elution flow rate, 300 μL/min; elution solution, DI water; DBS parameters and CE conditions as for **Figure 3**, $n = 5$.

Figure 5. The effect of the flow rate on the DBS elution efficiency and the total elution time. DBS parameters and CE conditions as for **Figure 4**, SI conditions: elution solution, 100 μL of DI water, $n = 5$.

Figure 6. Autonomous SI-CE-C^4D determination of lithium in DBS samples. DBS parameters and CE conditions as for **Figure 4**. SI conditions: elution flow rate, 300 μL/min; elution solution, 100 μL of DI water; spiked lithium concentrations: a – 0 mg/L, b – 2 mg/L, c – 5 mg/L, d – 10 mg/L, e – 20 mg/L.

Table 1. Hydrodynamic parameters of the SI system for the DBS elution as compared with the standard agitation procedure. DBS parameters: blood volume, 10 μL; disc size, 11 mm. SI conditions: elution solution, 250 μL of DI water; for full SI program, see **Table S2** in *Supporting Information*; *n = 5*.

Eluent flow rate (μL/min)	300[a]	300[b]	150[c]	300[c]	600[c]	1200[c]	2000[c]	n.a.[e]
Total elution time (s)	125	125	225	125	75	50	40	900[e]
Contact time[d] (s)	n.a.	50	100	50	25	12.5	7.5	900[e]
Collected eluate volume (μL)	246.8	222.8	226.6	226.0	226.2	225.6	224.2	204.2[e]
RSD (%)	0.3	1.1	1.8	1.2	1.9	1.4	1.6	3.7[e]

[a] – SI elution with no DBS disc
[b] – SI elution of a blank DBS disc with no capillary blood
[c] – SI elution of a DBS disc with 10 μL of capillary blood
[d] – Contact time of eluent with the DBS
[e] – DBS agitation at 1000 rpm for 15 min
n.a. – not applicable

Table 2. SI-driven homogenization of the collected DBS eluates. DBS parameters and CE conditions as for **Figure 3**, SI conditions: elution flow rate, 600 μL/min; elution solution, 250 μL of DI water; *n = 3*.

Elution volume (μL)	Eluent flow rate (μL/min)	Air volume (μL)	Air flow rate (μL/min)	Duration[a] (s)	Average difference[b] K^+ (%)	Average difference[b] Na^+ (%)
250	600	250	150	150	0.1	0.4
250	600	250	300	100	1.0	2.1
250	600	250	600	75	1.6	1.9
250	600	250	900	67	3.0	3.7
250	600	100	600	55	12.6	10.5
250	600	0[c]	0[c]	40[c]	25.2	25.7

[a] – Duration includes total time for SI elution of DBS and homogenization of the eluate.
[b] – Difference (%) denotes the difference of analyte's peak area in an eluate, which was homogenized by air only, vs. analyte's peak area in the same eluate, which was first homogenized by air and subsequently agitated at 1000 rpm for 3 min.
[c] – Eluate was injected immediately after transfer to the CE vial with no homogenization by air.

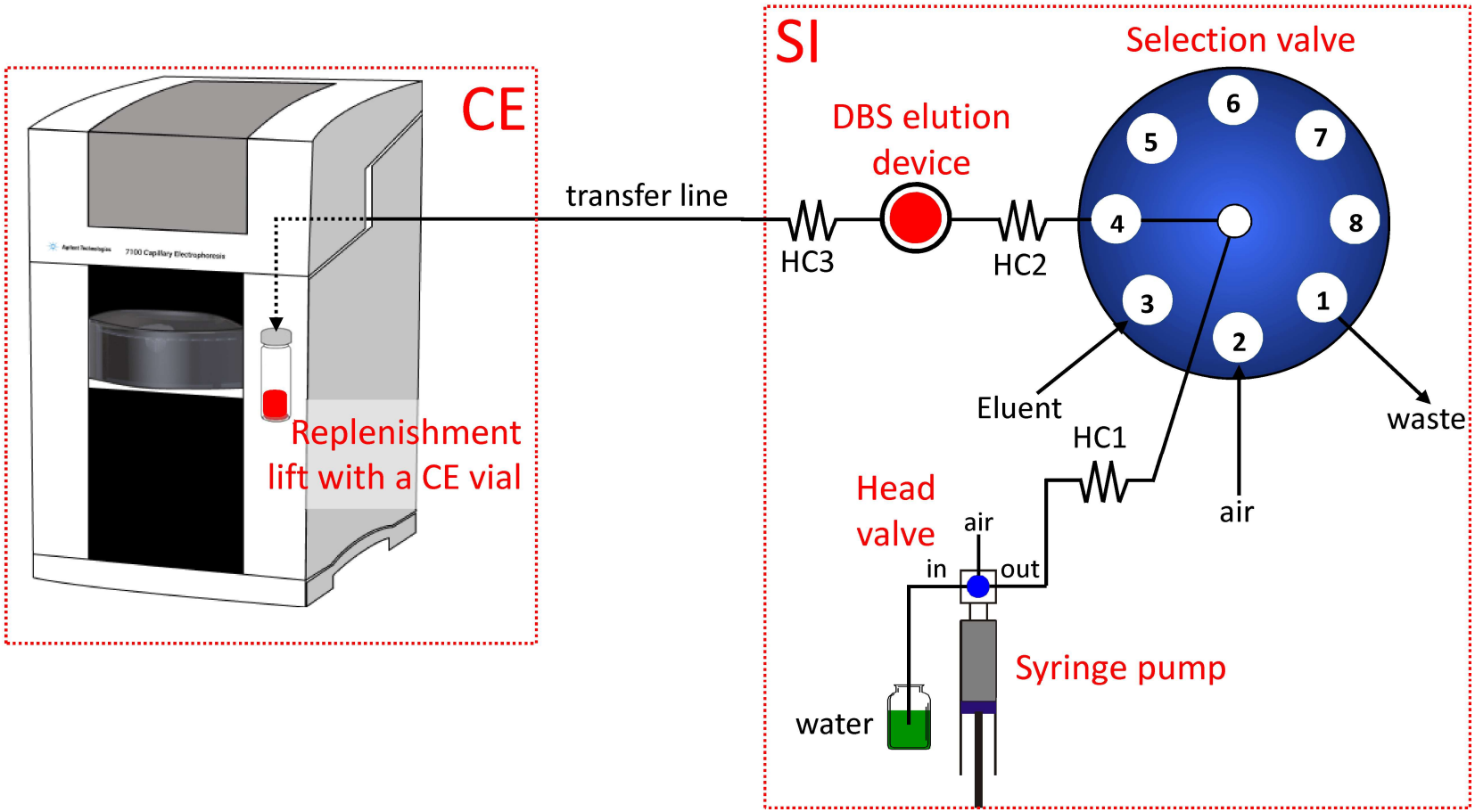

CE
Replenishment
lift with a CE vial
transfer line
SI
Selection valve
DBS elution
device
HC3
HC2
6
5
7
4
8
3
1
2
Eluent
waste
HC1
air
Head
valve
air
in
out
Syringe pump
water

Figure 1

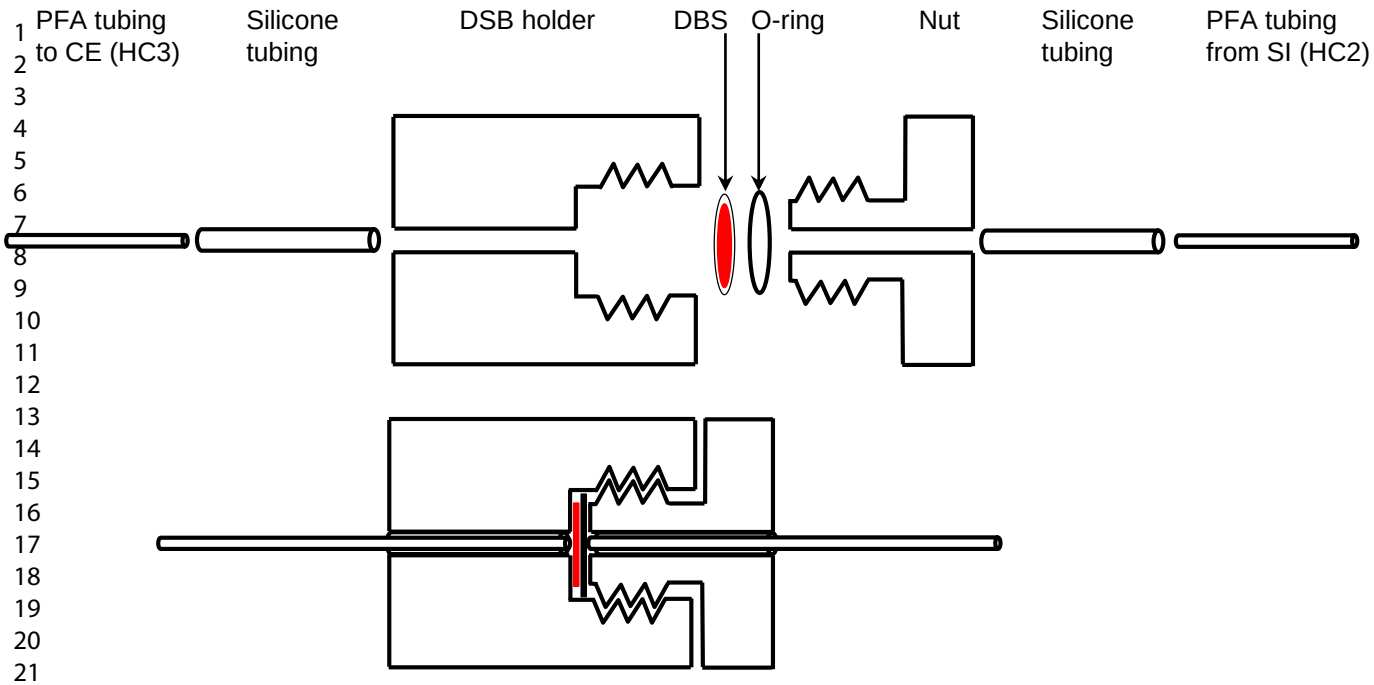

PFA tubing to CE (HC3)
Silicone tubing
DSB holder
DBS
O-ring
Nut
Silicone tubing
PFA tubing from SI (HC2)

Figure 2

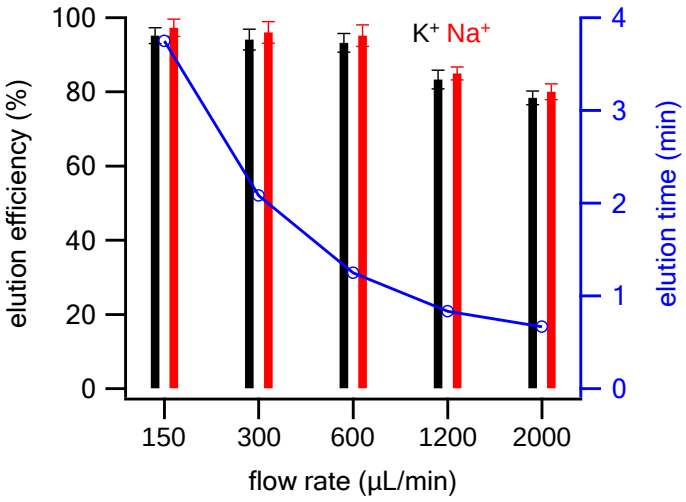

100
80
60
40
20
0
elution efficiency (%)
K+ Na+
4
3
2
1
0
elution time (min)
150
300
600
1200
2000
flow rate (µL/min)

Figure 3

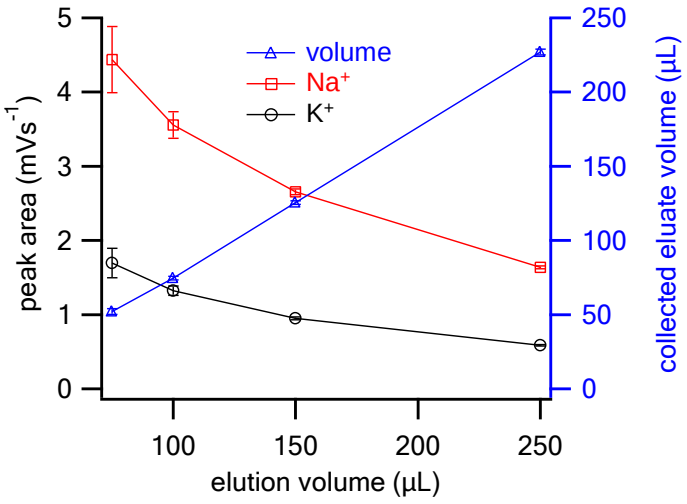

volume
Na+
K+
peak area (mVs-1)
collected eluate volume (µL)
elution volume (µL)
0
1
2
3
4
5
0
50
100
150
200
250
100
150
200
250

Figure 4

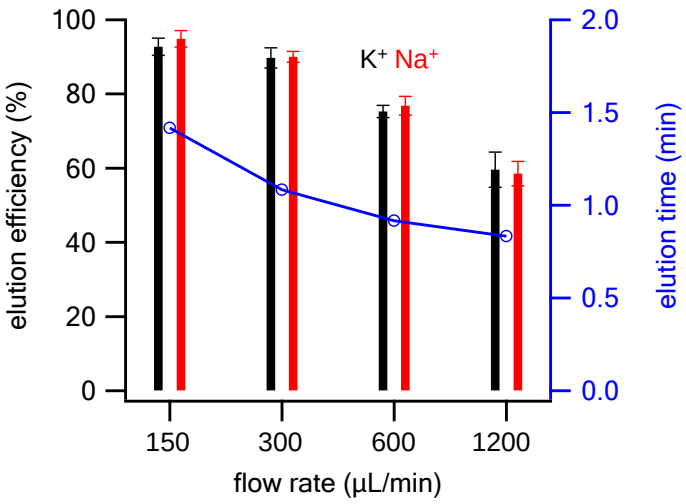

100
80
60
40
20
0
elution efficiency (%)
K+ Na+
2.0
1.5
1.0
0.5
0.0
elution time (min)
150
300
600
1200
flow rate (µL/min)

Figure 5

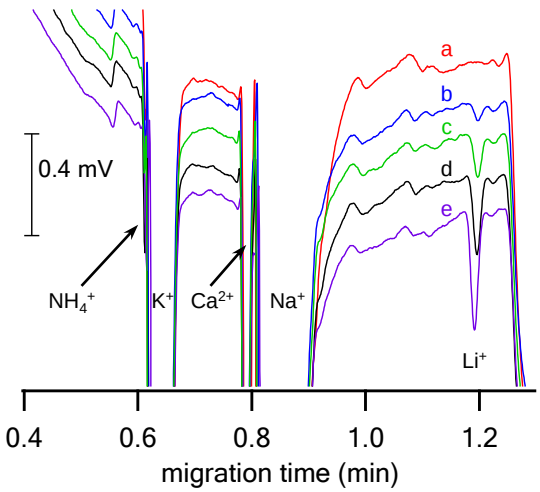
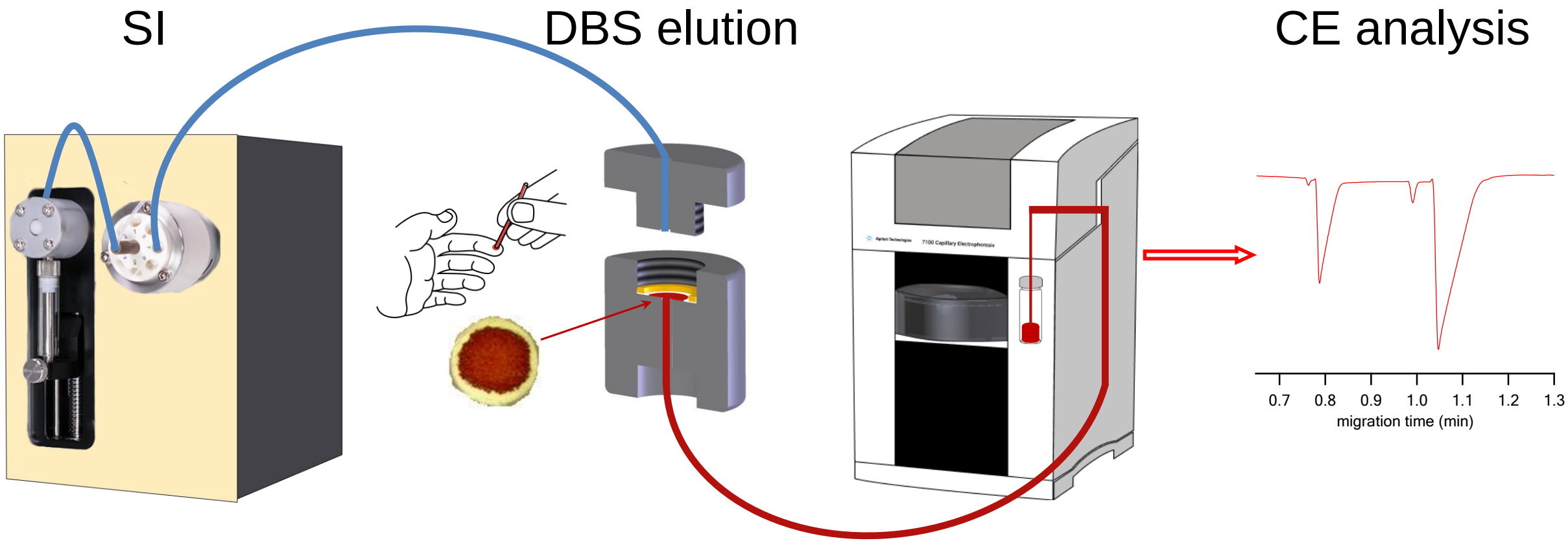


Figure 6


SI
DBS elution
CE analysis
0.7
0.8
0.9
1.0
1.1
1.2
1.3
migration time (min)