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Electrowetting-on-dielectric actuation of droplets with capillary electrophoretic zones for off-line mass spectrometric analysis

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ABSTRACT

Present article describes a novel technique based on digital microfluidics that allows collecting fractions of interest after electrophoretic separation and detection for further ESI-MS investigation. In this technique, a mixture is injected into a capillary electrophoresis (CE) apparatus, and microliter droplets are generated at the CE outlet at a frequency high enough to fraction each compound into several droplets, compartmentalizing the CE zones into a sequence of droplets. The droplets are transported from the CE outlet to a storage tube inlet using electrowetting-on-dielectric (EWOD) for droplet actuation. By applying a vacuum at the other end of the storage tube, the droplets form a sequence of plugs separated by air gaps. The plugs stored in the tubing are later analyzed using a standalone spectrometric device. Off-line electrospray ionization mass spectrometry (ESI-MS) was used to characterize the corresponding vitamin and was performed by pumping the segmented plugs directly into a spray emitter tip. The technique could be of interest to laboratories without access to well-equipped facilities (e.g. clean-rooms or lab robots).

1. Introduction

Capillary electrophoresis (CE) is an alternative technique to liquid chromatography for performing separations in proteomic [1], metabolomic [2] and pharmaceutical [3] research. The ability to reliably interface CE separations with mass spectral (MS) analysis has therefore become increasingly important [4]. However, the nature of CE gives rise to some particular challenges with regard to on-line MS detection. The principal method of on-line CE-MS interfacing is electrospray ionization (ESI). Both the CE and ESI processes require stable electrical contact of the solution with an electrode at the capillary outlet without interrupting the electro-osmotic flow from the CE separation. In addition, the low volumetric flow rates used in CE impose restrictions on the geometry of the tip, if a stable electrospray is to be maintained. The compatibility of the background electrolyte with the electrospray process and on the resulting mass spectra must also be considered.

Off-line analysis of CE fractions is somewhat simpler. Although this is not a new approach, the problem is still relevant and novel solutions are actively being sought. The use of capillary electrophoresis to collect fractions was first demonstrated by Hjerten and Zhu in 1985 on nucleosides, pH markers and IEF ampholytes [5]. Therefore, off-line coupling systems, which allowed automated protein spotting, were introduced [6]. Zamfir et al. suggested the use of off-line high-performance capillary electrophoresis in combination with nanospray ESI Q-TOF [7,8]. In the first microchip presented by Effenhauser et al., the separation channel of the CE fractionator had two exits: one was used for collection, while the other served as a waste channel. By alternating the applied potentials at predetermined times and closing an electric circuit, the fractions could be drawn through either channel [9]. A microfabricated device capable of selecting and collecting multiple components from a mixture separated by CE was recently described by Zalewski et al. [10]. In the work by Barbula et al., effluent from capillary columns is deposited on a rotating Teflon disk that is covered with paper. As the surface rotates, the temporal separation of the eluting analytes (i.e., the electropherogram) is spatially encoded on the surface [11]. Desorption electrospray ionization is then used for ionization and MS analysis of the deposited sample.

However, off-line analysis and characterization of samples separated by CE has been problematic even with conventional approaches to fraction collection [12–17]. Droplet-based microfluidics could provide a novel means for using MS to analyze the separated compounds. Droplets or plugs within multiphase microfluidic systems are rapidly attracting interest as a way to manipulate samples and chemical reactions on the scale of femtoliters to microliters. Developments in droplet manipulation such as passive and active fusion, splitting and sorting have enabled more complicated and integrated experiments [18,19]. There has been substantial recent interest in integrating CE and LC separations

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with droplet-based microfluidics. Edgar was the first to identify a concept based on the use of droplets to compartmentalize the separated CE bands [20]. An approach by Niu et al. efficiently integrates two separation dimensions: the first dimension – capillary HPLC separation – generates, segments and recombines droplets in a continuous flow, followed by the second dimension – CE separation [21]. Doshi et al. described the formation of heterogeneous libraries of droplets using fractionation of a sample into droplets by means of a C18 cartridge [22,23]. By interfacing nanoflow ultra-performance liquid chromatography with droplet-based microfluidics, Theberge et al. developed a method to produce libraries of picoliter droplets of chromatographic zones [24].

Challenges remain with chemical analysis of droplets in general and MS analysis in particular. Helmja et al. demonstrated droplet-based fraction collection in capillary electrophoresis for various stand-alone mass spectrometers [25]. Kennedy's group used oil segmented flow fraction collection from capillary LC and off-line ESI-MS [26,27]. Collections of nanoliter fractions are stored in tubing as plugs that can later be used to manipulate the samples. Off-line ESI-MS was used to characterize samples by pumping the segmented plugs directly into a nanospray emitter tip.

In most of these systems, droplets form spontaneously when a laminar stream of aqueous effluent is injected into an immiscible carrier fluid, either at a T-junction or in a flow-focusing geometry [28]. Oil must be depleted prior to MS analysis, and this requires a special step. Kennedy et al. [26] siphoned the oil away from the ESI tip. Electrowetting on dielectric (EWOD) can be used to actuate droplets instead of using a carrier liquid. According to [29,30] electrowetting was firstly discovered by French physicist Gabriel Lippman in 1875. Further, this technique has been developed to electrowetting on dielectric [31–35] and found many applications in digital microfluidics, where discrete droplets of conductive aqueous solutions could be manipulated by electrostatic forces on an array of electrodes coated with an insulating dielectric. Recent developments in this field [36,37], include proteomics analyses [38–40], immunoassays [41–44], and microfluidic platforms capable of all the steps required for mammalian cell culture: seeding, growing, detaching, and re-seeding the cells on a fresh surface [45].

However, despite much enthusiasm for this approach, access to digital microfluidics devices continues to be a barrier for researchers without access to well-equipped facilities (mainly, clean-rooms). This has spurred research into methods of fabrication that are robust and easy to implement [46], including prototyping on copper laminates using commercial printers [47,48]. Here, we report an interface for off-line CE-MS coupling which is based on a robust EWOD platform. It is used to compartmentalize CE zones into droplets, which are stored as plugs in a Teflon tube for ESI-MS analysis for further MS analysis. In our system, CE zones that emerge at the capillary outlet are dissolved in microlitre droplets of pure (MS compatible) solvent generated by an external droplet generator and transported away from the outlet of the separation capillary by EWOD. This is a novel approach in the field of fraction collection. As above, droplets are transported to a storage tube for ESI-MS analysis. The concept has been proven by an ESI-MS analysis of vitamins in a standard solution as well as in commercial formulations.

2. Materials and methods

2.1. Chemicals

Thiamin hydrochloride (vitamin B1), thiamin monophosphate (phosphorylated vitamin B1), and pyridoxine (vitamin B6) and the reagents (acetic acid, methanol, acetonitrile, and sodium hydroxide) were purchased from Sigma–Aldrich (Germany). The purity of the chemicals was 98% or higher. Deionized water (pH 7.0) purified with a MilliQ system (Millipore Corporation, Bedford, USA) was used for the preparation of the standard solutions.

Three commercial vitamin solutions purchased from a local pharmacy were tested. The first sample was a Milgamma® N injection solution (a medication prescribed for diabetics) with concentrations of B1 (148 mmol/L), B6 (243 mmol/L) and lesser amounts of interfering compounds such as lidocaine (43 mmol/L), cyanocobalamin, and benzyl alcohol. The second sample was Apovit "Lion Boy" (a potable liquid food supplement manufactured by Nycomed SEFA AS Estonia) that contains B1 (300 μmol/L), B6 (710 μmol/L), as well as various other components. The third sample was "Doppelherz" (a liquid food supplement comprised of an enriched mixture of herbal extracts, from Queisser Pharma) containing B1 (189 μmol/L), B6 (296 μmol/L).

Epoxy single-sided copper clad laminate (copper layer 17.5 μm; Elfa Elektroonika AS) was used as the substrate for fabricating the digital microfluidics actuator (DMFA). Other fabrication materials included silicone oil (PRF silicon oil, Taerosol Oy, Finland), food wrap with an estimated thickness of 10 μm (Lindner Haushaltsprodukte GmbH, D-51149, Köln), poly[4,5-difluoro-2,2-bis(trifluoromethyl)-1,3-dioxole-co-tetrafluoroethylene] dioxole 87 mol% (Teflon AF, DuPont), and Fluorinert FC-75. Gold paint (Glanzgold NF 12% solution of gold, Schjerning, Denmark) and silver paint (Silver Conductive Paint, Electrolube, England) were used to treat the outlet of the separation capillary as well as the grounded electrodes.

2.2. Instrumentation

In present study the authors suggest a new application of the previously described in [53] CE-DMF plate instrumentation, where DMF platform has been combined with CE instrumentation for repeated computer controlled sampling from a droplet (using EWOD for droplet manipulation on the surface of the DMF platform) and electrophoretic analysis with C^4D. On contrary to earlier research here we use DMF platform not as a sampler but as a collector and transporter of CE fractions either into the storage tube (if they contain analyte) or to waste (if empty). Selection was made according to the C^4D detector signal. Air plugs instead of a carrier liquid were used for encapsulation of the selected fractions. Air segmentation solves several problems encountered in on-line connection of storage tube with ESI-MS. First, there is no risk for the MS source to be contaminated since the zones are separated only by air. Second, no complicated instrumentation modifications are needed for purification of fractions from the carrier liquid.

2.3. Capillary electrophoresis

The CE instrument with a capacitively coupled contactless conductivity detector (C^4D) and the DMFA for the droplets were purpose-built instruments. A schematic diagram of the instrument is provided in Fig. 1A. The CE system requires power supply (Spellmann, Hauppauge, NY) capable of delivering voltages up to +25 kV. The C^4D was constructed according to the ideas outlined by da Silva et al. [49] and Zemann et al. [50] and has already been used for various applications [51,52]. It operates at 60 V peak-to-peak sine wave oscillating in a frequency 200 kHz. The instrument is controlled and the signal obtained via a USB connection with software developed in-house. The separation was effected in a 70 cm (effective length 35 cm) fused-silica capillary (Polymicro Technologies, Phoenix, AZ), with an outer diameter of 150 μm and an inner diameter of 75 μm. The surface of outlet (ground) end of the separation capillary was covered first by gold paint and heated at 600 °C for 2 min. After cooling the outlet end of capillary was painted by silver paint, leaving the end of the capillary covered only with gold to enable

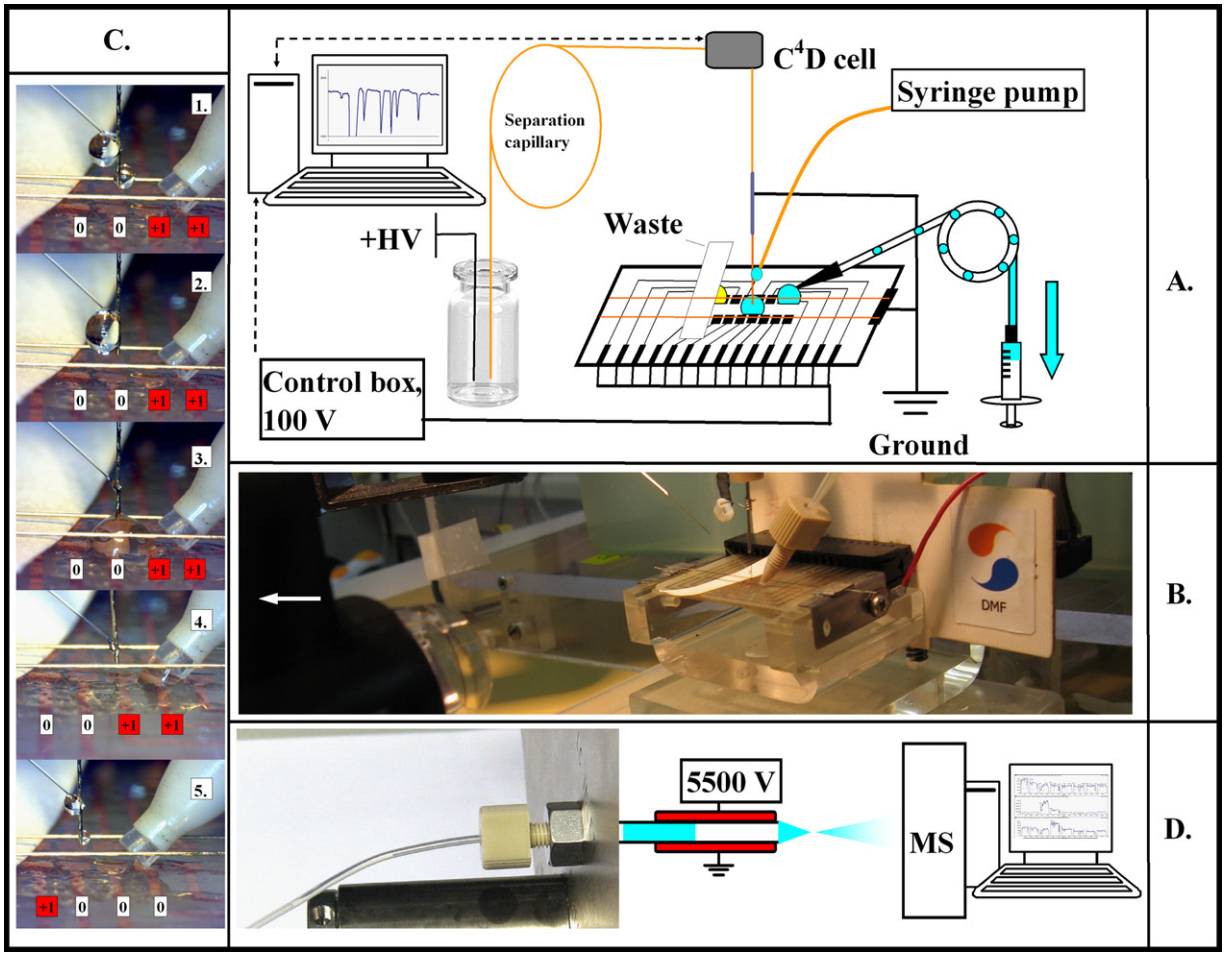

Fig. 1. Schematic diagram of coupling CZE–DMFA with a storage tube and ESI-MS. (A) Instrumentation scheme of on-line coupling of CZE–DMFA with a storage tube, (B) photo of the outlet end of the CE analyzer, the DMFA platform and storage tube, (C1), (C2) droplet generation, (C3) droplet detachment, (C4) droplet transport to the storage tube, (C5) discarding unused droplets. The numbers indicate the potential of the control electrodes (0 = "off"; +1 = "on"). Two horizontal leading electrodes are visible in the photographs beside the vertical separation capillary and the inclined droplet generator capillary. (D) Storage tube on-line connection to ESI-MS inlet.

electrical contact with the ground. The separation voltage was +18 kV. The outlet end of the separation capillary was directed through a grounded piece of vertical syringe needle located to the center of the DMFA electrodes array at a height of 2 mm above the platform (Fig. 1B).

Prior to the first use, the capillary was rinsed with 1 mol/L NaOH for 10 min, 0.1 mol/L NaOH for 20 min, MilliQ water for 30 min, and finally with a background electrolyte (BGE) 0.05 mol/L acetic acid solution for 10 min. Before each analysis sequence, the capillary was manually washed with approximately 100 column volumes of 0.1 mol/L NaOH, 150 column volumes of MilliQ water and 150 column volumes of the BGE, using a syringe. The rinsing step was followed by high-voltage conditioning of the capillary for 2 min. The standards and samples were injected into the capillary gravitationally from 25 cm at a fixed time of 15 s.

2.4. Digital microfluidic actuator (DMFA)

The fabrication of the "one-plate" DMFA used for transporting droplets from the CE capillary outlet to the inlet of the storage tube has been fully described by Gorbatsova et al. [53]. Briefly, it consists of a printed circuit board with an H-shaped set of 14 electrodes designed for various applications (only four electrodes were activated for this study). The electrodes measure 1 mm × 1 mm and the gaps between electrodes are approximately 200 μm. In this study, the food wrap was spin-coated with 0.1% Teflon AF solution in Fluorinert FC-75 at 500 rpm for 20 s and 2000 rpm for 20 s, and then annealed on a hot plate (100 °C, 30 min). Two grounded wire electrodes are situated above the DMFA at a height of 2 mm. The DMFA electrodes were powered by 100 V AC using a custom made control box and software [53].

The electrodes of the DMFA platform were activated during fraction collection, and when the droplet made contact with the DMFA platform, it was immediately transported away from the separation capillary to the proximity of the storage tube (if the fraction contained analytes) or to waste (if the fraction did not contain analytes). The selection was made according to the C^4D detector signal. The droplets were manually aspirated by means of a 500 μL Hamilton syringe into the storage tube (a fluorinated ethylene propylene tube with dimensions of 0.5 mm I.D. × 1.6 mm O.D. × 304.8 cm). Approximately 15 plugs of fractions were created in this experiment. Using air instead of oil to encapsulate the plugs prevented the risk of MS source contamination. CE experiment time varied from 10 to 20 min depending on the sample. The droplet actuation was monitored and recorded by a digital microscope (Digital Microscope, Dino-Lite, Anmo Electronics Corporation, Taiwan).

2.5. Droplet generation, actuation and collection into a storage tube

A sheath liquid composed of 0.1% v/v acetic acid in a 35% v/v methanol/water solution was delivered to the proximity of the CE

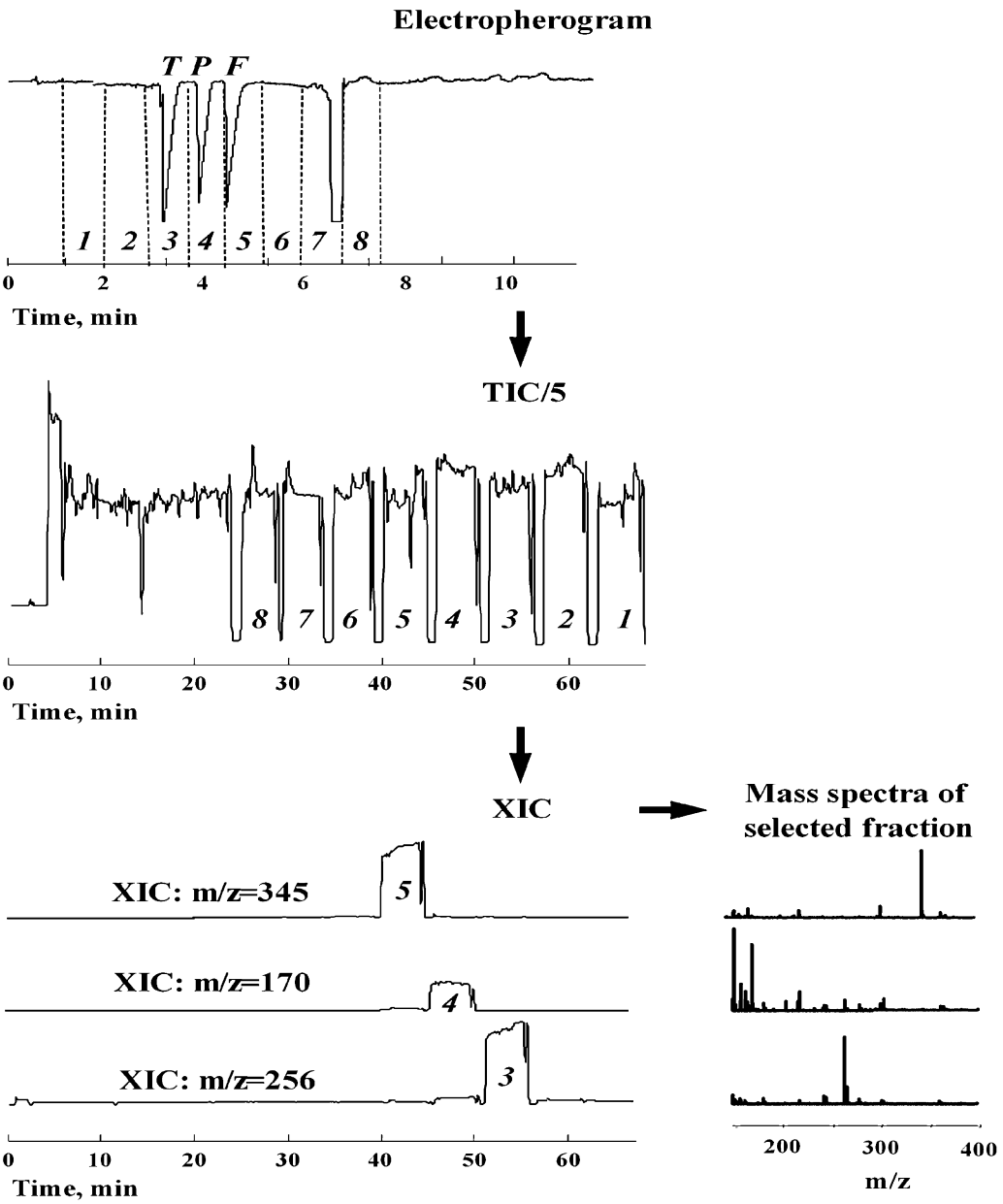

Fig. 2. Analysis of the vitamin standard solution; T – thiamin, P – pyridoxine, F – thiamin phosphate. CZE: BGE 0.05 mol/L acetic acid, separation voltage +18 kV, injection gravitationally 25 cm 15 s. Sheath liquid: 0.1% v/v acetic acid in a 35% v/v methanol/water, flow rate of 4 μL/min. MS: syringe pump speed 2–4 μL/min, positive ion mode, needle voltage, 5.5 kV; auxiliary gas (N2), 172.37 kPa; and curtain gas (N2), 137.9 kPa. TIC – total ion current chromatogram, XIC – extracted ion current chromatogram.

capillary outlet using a syringe pump (KD Scientific, USA) at a flow rate of 4 μL/min through a 75 μm I.D. capillary, thus enabling the CE effluent to be dissolved into the droplet before it detached onto the DMFA platform as illustrated in Fig. 1A–C. The resolved analytes in the CE capillary were sequentially fractionated into droplets that were transported to the storage tube by means of a DMFA. The composition of the sheath liquid was selected to match both the ionic strength of the separation buffer and MS. Two capillaries, 100 μm (I.D.) × 350 μm (O.D.), and 75 μm (I.D.) × 150 μm (O.D.) were tested in order to find the most suitable conditions for fractionation. It was found that the former capillary created 8 μL volume droplets and the latter produced 3 μL droplets for delivery to the DMF platform. The latter capillary was used throughout the experiments. The fraction volume was kept constant by varying the syringe pump flow rates, which influence the fractionation rate of the separation. A flow rate of 4 μL/min created a fractionation rate of 1.33 droplets/min. By calculating average EOF speed in Figs. 2 and 3 as 350/5.6 = 62.5 mm/min and taking into account the 75 μm I.D. of the separation capillary, it can be seen that the eluted fraction from the capillary during 0.6 min has an approximate volume of 0.3 μL. This indicates that our fractionation process results in a CE zone dilution of approximately tenfold.

2.6. MS analysis of fractions

After the separation was completed, the storage tube was disconnected from the separation equipment and connected to the sample input of the mass-spectrometer (the QStar Elite ESI-Q-TOF-MS from Applied Biosystems) (Fig. 1D). The contents of the storage tube were pumped directly to the ESI emitter tip by a syringe pump at a speed 2–4 μL/min. The mass spectrometer was set to acquire data in the positive ion mode, with a selected mass range of 150–900 *m/z*. The following conditions apply to operation in positive ion mode: needle voltage, 5.5 kV; auxiliary gas (N2),

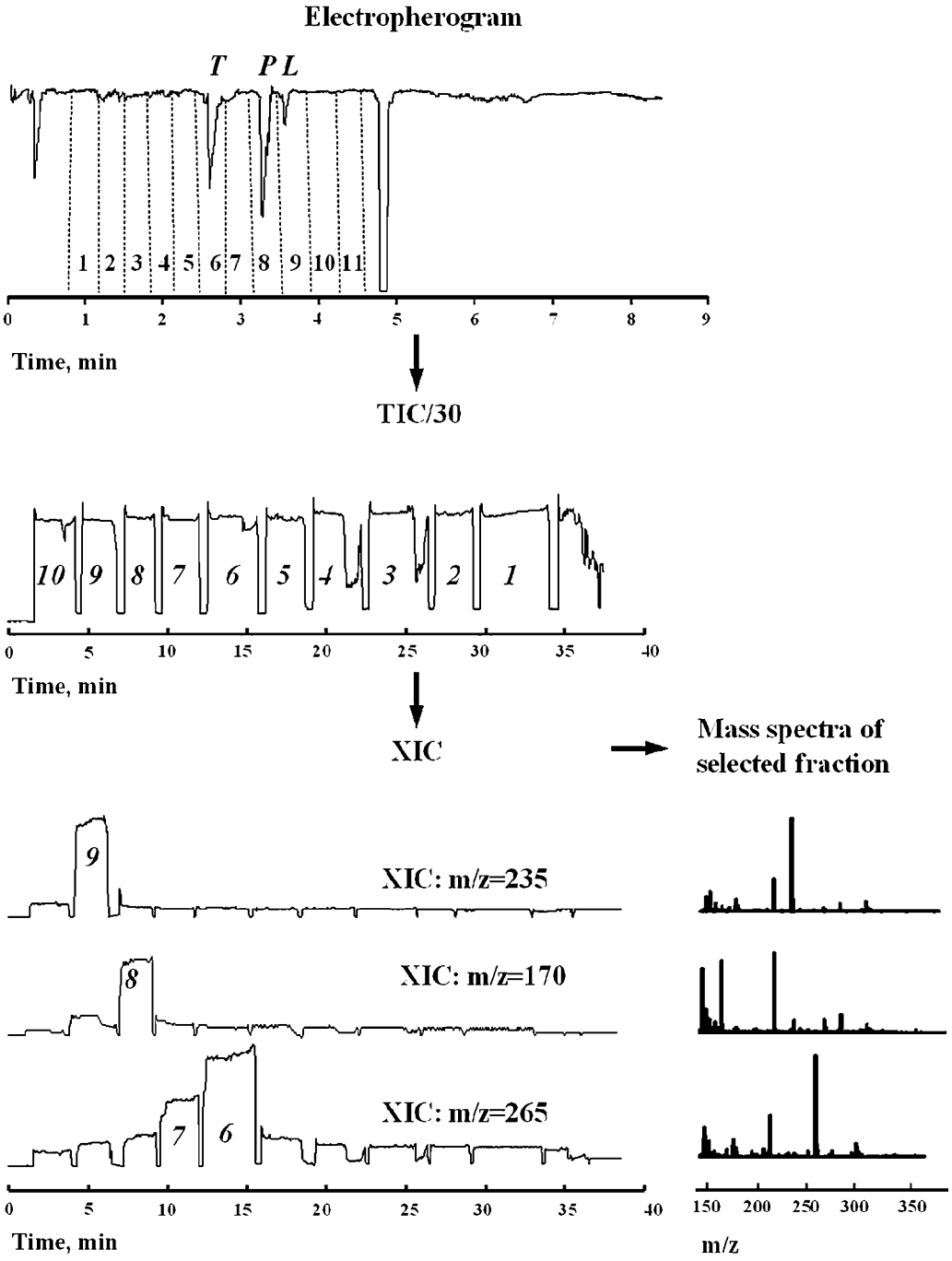

Fig. 3. Analysis of the Milgamma injection solution; T – thiamin, P – pyridoxine, L – lydocaine. CZE: BGE 0.05 mol/L acetic acid, separation voltage +18 kV, injection gravitationally 25 cm 15 s. Sheath liquid: 0.1% v/v acetic acid in a 35% v/v methanol/water, flow rate of 4 μL/min. MS: syringe pump speed 2–4 μL/min, positive ion mode, needle voltage, 5.5 kV; auxiliary gas (N2), 172.37 kPa; and curtain gas (N2), 137.9 kPa. TIC – total ion current chromatogram. XIC – extracted ion current chromatogram.

172.37 kPa; and curtain gas (N2), 137.9 kPa. Extracted ion-current strategies were used for detecting the vitamins in CE fractions.

The experimental procedure was as follows:

1. Preconditioning the separation capillary.
2. Activating the droplet-generating syringe pump at a speed of 4 μL/min.
3. Injecting the sample hydrodynamically (raising the capillary inlet to 25 cm for 15 s).
4. Applying high voltage to perform CE analysis.
5. Transporting the droplets (with dissolved CE fraction) to the storage tube or to waste (filter paper is used to absorb the droplet) after they detach from the capillary outlet.
6. Aspirating the droplet into the storage tube by pulling the plunger of a syringe connected to the other end of the tube.
7. Repeating actions 5 and 6 for each droplet.
8. Disconnecting the storage tube from the equipment after the CE run was completed and transporting it to the QStar Elite MS.

3. Results and discussion

For this research, we used 3 μL droplets, which generated approximately 10–25 fractions per electropherogram, depending

on the separation. Only 10–15 fractions consisting of separated compounds were presumably collected in the storage tube. Sample plugs of a consistent 9.23 mm length (RSD of 6.1% for 10 plugs, observed by microscope) were obtained for all fractions collected under the specified CE separation conditions.

3.1. CE-ESI-MS analysis of collected fractions

A mixture of three low molecular mass vitamins – thiamin, pyridoxine and thiamin phosphate – was analyzed at concentrations of 50 μmol/L to 1 mmol/L of injected sample. A typical electropherogram of the vitamins is shown in Fig. 2 (the C^4D detector signal is for 500 μmol/L). The fractions were collected as segmented plugs for off-line analysis and later infused through the ESI emitter tip to a QStar Elite MS operating in full scan mode. The individual plug trace signal can hardly be identified in the total ion current (TIC) (Fig. 2) because of chemical noise dominating the TIC. However, in the extracted ion currents (XIC), trace signals of the plugs that contained the corresponding analyte are clearly visible. There is excellent correspondence between the fraction numbers outlined in the electropherogram and the plug numbers indicated by the XIC signals. Their identity is further confirmed by the mass spectra of the corresponding fractions. Detection of the same vitamins in the commercial products is more challenging. Fig. 3 shows the electropherogram, TIC, XIC and mass spectra for Milgamma. The results for the two other commercial products ("Lion Boy" and "Doppelherz") were quite similar to that of Milgamma and are not shown here. Although those samples are more complex, the target analytes can be clearly identified by the corresponding XIC signals. Here as well, chemical noise contributes significantly to the TIC signal, making that of the target analytes difficult to see.

Several characteristics of the ion current traces can be observed from Figs. 2 and 3. The order of plugs detected by MS is the reverse of that of the fraction numbers in the electropherogram, which is due to the "first in, last out" principle of filling the storage tube. Analytes can sometimes be identified in two or more adjacent fractions. The ESI signal of a plug is nonstationary (e.g., features spikes), which is not surprising, since ESI is a rather unstable phenomenon. Nevertheless, identification of the analytes in the sample is unanimous. The dispersion of plug widths and distances can be attributed to the manual aspiration of the droplets.

4. Discussion

It is clear that there are sample possibilities to fractionate CE output into a storage tube. The use of robotic manipulators or programmable *x*, *y*, *z*-stages are usually involved. EWOD-based actuators provide an attractive alternative, because they possess no mechanically moving parts. Dilution of the CE zone in the droplet increases the typical μM detection limit of the CE-ESI-MS method by approximately one order of magnitude, but this is counterbalanced by the opportunity for MS analysis. The advantage of present instrumentation is fraction size 3 μL in comparison with 12 or 18.5 μL demonstrated earlier in [25]. A green analytical chemistry [54] aspect of the approach should be emphasized as well: the results from a simple instrument (e.g., a portable CE device) can be improved significantly by taking advantage of core lab facilities (e.g., MS), thereby obviating the need to acquire new instruments. The fractionation rate could obviously be increased, if desired, and many fractions produced per CE zone (an approach that has been taken in several previous studies), but this is obviously not necessary. If the identification of the peak is the goal, the whole zone can be compartmentalized into the droplet to increase the sensitivity of detection.

The lowest sample concentration introduced into CE with detectable MS signal was 50 μM constituting the LOD of the present approach.

5. Conclusion

Capillary electrophoretic separations can be fractionated into robust droplet-collecting storage tubes. The high detection limit is the obvious disadvantage of the proposed method, but we have attempted to demonstrate that this can be tolerated if it simplifies the equipment. The proposed design of the experiment is simple and cheap enough to be set up in virtually every CE laboratory that has access to core laboratory mass-spectrometry which is not online with separation instruments.

Acknowledgments

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