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Achieving Stable ESI-MS Detection from Microfluidic Chips

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Abstract

The past two decades have witnessed remarkable advances in the development of microfluidic devices as bioanalytical platforms for the analysis of biological molecules. The implementation of mass spectrometry (MS) detection systems on these devices has become inevitable, and various chip-MS ionization interfaces have been developed. As electrospray ionization (ESI) is particularly relevant for the analysis of large biological molecules such as proteins or peptides, efforts have focused on advancing interfaces that meet the demands of nano-separation techniques that are typically used prior to MS detection. Achieving stable ESI conditions that enable sensitive MS detection is, however, not trivial, especially when the spray is generated from a microfabricated platform. This chapter is aimed at providing a step-by-step protocol for producing stable and efficient electrospray sample ionization from microfluidic chips that are used for capillary electrophoresis (CE) separations.

Keywords

microfluidics; mass spectrometry; electrospray ionization; signal stability

1. Introduction

To date, a variety of microchip-ESI/MS interfaces [1–19], relying mostly on nano-ESI microfabricated nebulizers, liquid sheath or liquid junction approaches, and recently on surface acoustic wave nebulizers and electrostatic-spray ionization [20–22], have been developed. In the simplest form, electrospray can be generated directly from the chip edge [1–3], from inserted capillaries [4–9], or from chip-integrated microfabricated emitters [10–12, 15–19]. Interfacing CE separations to mass spectrometry detection is particularly challenging due to the fact that optimal CE separation conditions, that involve high concentrations of non-volatile buffer systems or additives (100–200 mM), are not tolerated by the electrospray ionization process. Approaches relying on the use of a liquid sheath or liquid junction, that dilute the CE separation buffer prior to ESI, have proven to be successful. Nevertheless, the inability to use non-volatile buffer systems, potentially the best for a given separation, continues to be a challenge. Channel coatings that bleed and generate intense background ions add to the list of concerns. Moreover, additional challenges relate to the chip and MS interface design and operation, i.e.,: (a) the chip must produce sufficient EOF to sustain a stable spray, therefore, separations must be conducted in high pH buffers;

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(b) organic solvent modifiers (CH_3OH, CH_3CN) must be added to the CE buffer to enable a stable and efficient ESI process, but this can alter separation efficiency; (c) the electrical field strengths on the chip must be set properly to maintain balanced electroosmotic flows (EOFs) throughout all channels, to not alter the flat EOF profiles and separation efficiency [23, 24]; and (d) the chip-MS interface contribution to peak broadening must be minimal, therefore the dead volumes associated with the interface should be very small, at the pL level.

It is beyond the purpose of this work to present in detail the various ESI-MS interfaces that have been developed for CE separations on a chip. Therefore, for the protocol that is described in this chapter, we will consider the case of a simple cross-channel glass chip configuration that has been equipped with a fused silica capillary emitter for the generation of an electrospray, and that has the CE terminus/ESI voltage applied through a porous glass junction [9–25] – a design that interferes minimally with the CE separation performance (see Fig. 1). Glass chips are preferred for such applications due to the native ability to generate EOF (300–400 nL/min) at pH~7–8, a flow that is an ideal match for nano-ESI sources that operate at 50–300 nL/min for achieving sensitive, amol-fmol detection. For microfluidic chips that comprise various other implementations of an ESI-MS interface, the contribution of the interface to peak broadening, spray stability and sensitivity should be explored by experiments that are specific to that particular interface. The principles that are described in here, however, are applicable for chip preparation and testing of the ESI signal quality.

2. Materials

Use only high purity reagents and HPLC grade organic solvents and water.

2.1 Microfluidic chip preparation for analysis

1. Microfluidic chips prepared from glass.
2. Stand for mounting the microfluidic chips.
3. Binocular benchtop microscope.
4. Microanalytical balance (e.g., Mettler Toledo).
5. Ultrasonic bath (e.g., Branson ½ gallon).
6. Syringe infusion pump (e.g., Harvard Pump 11 Elite).
7. Syringe: 250 μL (e.g., Hamilton 1725 RN).
8. Vacuum system.
9. Disposable hypodermic syringe: 1 mL, gauge 26, ½ inch needle.
10. Conical polypropylene tubes: 15 mL.
11. Fused silica capillary tubing: 20 μm i.d. × 90 μm o.d. (Polymicro Technologies).
12. Fused silica capillary tubing: (50–100) μm i.d. 360 μm o.d. (Polymicro Technologies).

13. Sodium hydroxide solution: 0.1 M NaOH. Weigh 40 mg NaOH with the analytical microbalance and dissolve in 10 mL water in a 15 mL conical tube. Disperse by sonication and filter the solution, if necessary, by using a 0.2 μm filter. Store the stock solution at room temperature.
14. Epoxy glue (e.g., Epo-Tek, two component glue, Epoxy Technology).
15. E6000 glue (Eclectic Products).
16. Methanol.

2.2 Fluidic flow testing and optimization

1. Nikon Eclipse TE-2000U inverted epi-fluorescence microscope with 4X, 10X or 20X objectives and appropriate fluorescence filter cubes. Other fluorescence microscope systems of similar performance can be used. CCD camera and image processing software are optional.
2. LabSmith microfluidics high voltage power supply. Other microfluidics power supplies of similar performance can be used.
3. Picoammeter (e.g., Keithley 6487).
4. Digital multimeter (e.g. Fluke 179).
5. High voltage probe (e.g., Fluke 80K-40).
6. Tabletop microcentrifuge (e.g., Eppendorf 5424).
7. Amber glass sample vials: 4 mL.
8. Polypropylene vials: 1.5–2 mL.
9. Fused silica capillary tubing: 200 μm i.d. × 360 μm o.d. (Polymicro Technologies).
10. CE buffer solution: 10 mM NH_4HCO_3. Weigh 8 mg NH_4HCO_3 with the analytical microbalance and dissolve in 10 mL aqueous/organic solvent mixture (e.g., H_2O/CH_3OH 70:30 v/v), in a 15 mL conical tube. Disperse by sonication and filter the solution, if necessary, by using a 0.2 μm filter. Store the stock solution at room temperature (*see* Notes 1 and 2).
11. Rhodamine 610 stock solution: 2.6 mM. Weigh 5 mg Rhodamine 610 with the analytical microbalance and dissolve in 4 mL water, in an amber glass vial. Disperse by sonication and filter, if necessary, by using a 0.2 μm filter.
12. Acetic acid, glacial.

2.3 ESI-MS from the chip

1. Mass spectrometer equipped with nano-ESI source, e.g., LTQ-XL (Thermofisher Scientific). Other MS systems of similar performance can be used.
2. XYZ stage.
3. Thermoshaker (e.g., Eppendorf ThermoMixer® C).

4. Ammonium bicarbonate solution: 50 mM NH_4HCO_3, pH~7.8. Weigh 40 mg NH_4HCO_3 with the analytical microbalance and dissolve in 10 mL water in a 15 mL conical tube. Disperse by sonication and filter, if necessary, by using a 0.2 μm filter (*see* Note 2).

5. Bovine serum albumin (BSA) stock solution: 1.25 mg/mL BSA. Weigh 5 mg BSA with the analytical microbalance and dissolve in 4 mL ammonium bicarbonate solution (50 mM) in an amber glass vial. Disperse by sonication and filter, if necessary, by using a 0.2 μm filter (*see* Note 3).

6. Peptide standard stock solutions: 1 mg/mL. Weigh 4 mg of one or more standard peptides (e.g., angiotensin II, bradikynin, leucine encephalin or gramicidin S) with an analytical microbalance and dissolve in 4 mL water in an amber glass vial. Disperse by sonication and filter, if necessary, by using a 0.2 μm filter (*see* Note 3).

7. Trypsin, mass spectrometry grade: 0.1 μg/uL. Prepare the trypsin solution by dissolving 20 μg sequencing-grade trypsin in 200 μL ammonium bicarbonate solution (50 mM). Disperse briefly by sonication (*see* Note 4).

2.4 Data analysis

1. Computer with operating system compatible with the MS data processing software.

2. Software package for analyzing MS raw files: Proteome Discoverer (Thermofisher Scientific). Other software packages that handle the raw data files generated by the mass spectrometer can be used.

3. Statistical analysis software. Any software package that handles basic statistical data analysis can be used.

3. Methods

3.1 Microfluidic chip preparation for analysis

1. Prepare microfluidic chips in-house [26, 27] or purchase from various manufacturers, e.g., Micronit, Micralyne, Agilent, etc. We note that specialized designs, with electrospray ionization capability, must be fabricated. It is beyond the purpose of this chapter to detail the fabrication of microfluidic devices, however, for describing the procedure, we will consider the use of: (a) glass chips that enable either pressure or electrically driven fluid flows (see Fig.1), (b) capillary emitters inserted in a microfluidic channel for generating an electrospray (see Fig. 2), and (c) a nanoporous glass junction for applying the ESI high voltage (see Fig.1).

2. Attach reservoirs to the microfluidic chips. The reservoirs can be prepared from glass tubing (e.g., 2–5 mm i.d. × 4–7 mm o.d.), cut in 7–8 mm length, and attached with a two-component epoxy glue. Cure at 90–110 °C for 1 h.

Alternatively, commercial polymeric unions can be used that enable the connection of a syringe pump to the chip (*see* Note 5).

3. Insert a piece of a fused silica capillary tubing (20 μm i.d. × 90 μm o.d. × 1 cm long) in the end of the microfluidic channel that will deliver the fluid to the ESI-MS interface, seal with E6000 glue, and cure at room temperature overnight (*see* Note 6). This will be the ESI emitter.

4. Secure the chip in a stand. For in-house made chips, the stands are also in-house prepared. Such stands can be fabricated from PEEK sheets and braces that help fasten the chips. The stand can also accommodate fittings for fluid delivery [28].

5. Clean/re-hydrate the microfluidic channels with NaOH solution (0.1 M). Dispense the solution with the aid of a hypodermic syringe in one reservoir, and allow the channels to fill by capillary action. Fill the chip from only one reservoir to avoid trapping gas bubbles in the microchannels. Apply a slight vacuum from the other reservoirs to help the NaOH solution flow through the channels. Continue the process for 30 min (*see* Note 7). Alternatively, if unions are bonded to the chip, the channels can be flushed by using a 250 μL syringe connected to the chip via a (50–100) μm i.d. × 360 μm o.d. fused silica capillary. The syringe can be actuated manually or by a syringe pump.

6. Remove the solution of NaOH from all reservoirs and channels through vacuum suction, and rinse the chip with copious amounts of water. Next, fill the channels with water, as described above, and rinse sufficiently to completely remove the NaOH solution. Check with pH paper the pH in the chip reservoirs.

7. Remove the water from all reservoirs and fill the reservoirs and channels with methanol. Remove the methanol from all microfluidic channels through vacuum suction and allow the chip to dry.

3.2 Fluidic flow testing and optimization

1. Fill the chip with CE buffer solution, as described in section 3.1, step 5.

2. Visually check the chip under a benchtop microscope to ensure that air bubbles are not trapped in the microfluidic channels or reservoirs.

3. Secure the chip in a stand and introduce Pt electrodes in all reservoirs.

4. Connect the high voltage power supply and test for electrical continuity by applying a low electric field strength, e.g., 100–250 V/cm, between various chips reservoirs. Measure the electrical current through the microfluidic channels. This can be accomplished by applying the voltage to one reservoir and grounding the opposite reservoir through the picoammeter. For channel sizes of ~(10–50) μm depth × (50–100) μm wide, the electrical currents should be ~10–100 μA (*see* Note 8).

5. If the current measurements are satisfactory and reproducible, increase progressively the field strength to the maximum value that will be used during

analysis (e.g., 400–500 V/cm), repeat the above current measurements, and check reproducibility (*see* Note 9).

6. Measure the fluid flow (EOF) that is delivered through the ESI emitter. This can be accomplished with a simple experimental setup that involves sliding a 200 μm i.d. capillary (~10 cm long) over the 90 μm o.d. ESI emitter and monitoring the displacement of the liquid meniscus vs. time. For stable ESI generation from 20 μm i.d. × 90 μm o.d capillary emitters, the flow rate should be at least ~100 nL/min. If the chip is equipped with a fine silicon spraying nozzle, e.g., ~(5–10) μm i.d × 20 μm o.d., lower flow rates (~50 nL/min) will be tolerated (*see* Note 10).

7. Prepare a Rhodamine 610 solution of 10–20 μM by diluting an appropriate volume of the stock solution with CE buffer solution (*see* Note 11).

8. Place the chip on the fluorescence microscope and fill the sample reservoir with Rhodamine 610 solution (20 μM). Perform either a gated or pinched CE injection [29] of Rhodamine 610 by applying the appropriate high voltages to the corresponding reservoirs (see Fig. 3) (*see* Note 12 and 13).

9. Optimize the sample injection/analysis voltages and check again the electrical currents between different reservoirs. Monitor the exit EOF through the ESI emitter, as described above, and adjust all voltages proportionally to ensure the presence of sufficient EOF for stable ESI-MS operation (*see* Note 14).

3.3 ESI-MS from the chip

1. Prepare a BSA digest solution by mixing 800 μL of the BSA stock solution (1.25 mg/mL) with the 200 μL trypsin solution in a 1.5 mL Eppendorf polypropylene vial. Mix by vortexing or brief sonication, and place the vial in the thermomixer for overnight digestion, at 37 °C. Quench the next day with glacial acetic acid, 10 μL/mL digest solution (*see* Note 15).

2. Prepare dilute solutions of standard peptides or BSA digest (1–10 μM) by diluting the stock peptide or BSA digest solutions with appropriate volumes of CE buffer (NH_4HCO_3 10 mM). Mix by vortexing.

3. Create an MS and a tandem MS data acquisition method. The MS method will be used for optimizing the ESI baseline signal from the chip, and the tandem MS method for collecting collision induced dissociation (CID)-MS data from sample injections and separations on the chip.

4. Burn off with a lighter the polyimide coating from the chip ESI emitter for a length of 1–2 mm (*see* Note 16).

5. Fill the microfluidic device with CE buffer, place the device in the stand, and secure the stand on an XYZ stage positioned in front of the mass spectrometer. Adjust the position the chip such that the tip of the ESI emitter is ~2 mm away from the mass spectrometer inlet capillary (*see* Note 17).

6. Apply the optimized voltages (see section 3.2, step 9) to the chip reservoirs and double-check the presence of flow at the tip of the ESI emitter.

7. Next, raise all voltages, proportionally, until the level of the ESI voltage is sufficient for generating a stable Taylor cone and ESI signal from the emitter. For the emitter used in this study (20 μm i.d. × 90 μm o.d.), the optimal ESI voltage is ~1800–2000 V. If the ESI source is equipped with a CCD camera, the onset of the spray can be monitored visually.

8. Allow for the spray to stabilize for 4–5 min, and assess thereafter the stability of the signal by acquiring data with the MS acquisition method. Process the results and display the total ion chromatogram (TIC). For a stable ion trace, the RSD is within +/− 10 % of the signal average. If signal spikes are present and frequent, the flow rate, the position of the ESI emitter in the source, or the ESI voltage should be adjusted (see Fig. 4A). Averaging 3–10 mass spectra improves the stability of the signal (*see* Notes 18–20).

9. Perform an injection, initially of a single peptide or a mixture of 3–5 standard peptides, then of a more complex sample such as the BSA digest. Use the tandem MS method for data acquisition.

10. Process the raw MS files with the software package at hand. Identify the peptide sequences and assess separation efficiency.

11. Perform multiple injections and evaluate sensitivity, sample migration reproducibility and chip reusability. Low fmol range detection of peptides from simple mixtures should be routine. If the results are satisfactory, the overall microfluidic method can be used for further separations.

4. Notes

1. The CE buffer solutions must be optimized for a given separation. Nevertheless, when CE separations are interfaced to ESI-MS detection, only volatile buffer systems can be used for separation, such as NH_4HCO_3, CH_3COOH or HCOOH. Non-volatile salts, such as phosphate buffers, deposit on the MS inlet ion optics elements, deteriorate the electrical fields, and very rapidly suppress the ESI signal. Such salts can also contribute to irreversible corroding the MS ion optics components. In addition, the volatile buffers must be dissolved in an aqueous/organic solvent mixture that has at least 20 % organic solvent (e.g., CH_3OH), preferably more (30–50 %), to ensure stable ESI operation. In case that the chip has a design that enables the delivery of an organic solution that is mixed with the CE buffer just prior to electrospraying the buffer, then the CE buffer solution can be prepared with water alone. The pH of the CE buffer can be adjusted to a range of 6–8, however, lower pH values should be avoided as the EOF will be too small to sustain stable ESI.

2. All solutions that are electrosprayed from the chip and that contain volatile components, must be prepared fresh, daily, prior to analysis.

3. The solubility of protein and peptide stock solutions in water (or other solution of choice) should be checked prior to preparation. The stock solutions can be

preserved in a solid-frozen state at −20 °C. In case that the solutions contains organic solvents, freeze at −80 °C.

4. The trypsin solution should be prepared fresh, right before performing the digestion of the protein, to avoid losing its enzymatic activity.

5. Any epoxy glue that is used in the fabrication of microfluidic chips should be evaluated for possible bleeding of components that generate background noise in the ESI mass spectra of the aqueous/organic eluents that are used during analysis.

6. The E6000 glue continues to be soft after curing. This enables the removal of the glue and the ESI emitter, in case of damage, and replacement. If this procedure is attempted, the glue should be peeled off carefully such that its removal extracts the emitter.

7. Do not allow the chip to dry out while filled with NaOH solution, as this may result in channel clogging. Replenish the reservoirs continuously with solution while the chip is treated with NaOH, and/or preserve the chip in a water-saturated chamber.

8. The presence of organic solvents reduces the magnitude of the electrical current.

9. Avoid the use of high electric field strengths that produce high current, Joule heating, bubble formation and possible catastrophic failure of the experiment.

10. Very fine ESI emitters, of a few micrometer i.d., are capable of pulling their own flow in the presence of a strong electrical field. No pumping or EOF is necessary. Clogging of such fine emitters is, however, frequent.

11. The concentration of the Rhodamine 610 solution that will be injected in the chip should be sufficient to enable visualization with the available fluorescence microscope.

12. Rhodamine 610 is a fluorescent dye that at neutral and high pH values has a zwitterionic structure. It is neutral, and can be used as a tracer dye to monitor the direction of the fluidic flows. At low pH, Rhodamine 610 is (+) charged, therefore, in addition to its electroosmotic mobility it will also display electrophoretic mobility.

13. When the chip is interfaced to an MS detector and flow is necessary for the generation of an electrospray, gated injections are preferred, as such injections do not interrupt the flow towards the ESI emitter and do not destabilize the spray. Pinched injections can be used if can be performed fast. Small perturbations during injection, due to voltage switching, are expectable, however, in either case.

14. If the experimental setup in the lab includes a laser point detection system from the chip, the movement of a fluorescent plug of Rhodamine 610 through the microfluidic separation channel can be observed and used for the calculation of

the flow rate. Rhodamine 610 can be used for this purpose only at neutral or basic pH, when it does not possess electrophoretic mobility.

15. Typically, to improve the enzymatic digestion process, the proteins are first denatured in a urea solution (6–8 μM), the disulfide bonds are reduced in the presence of dithiothreitol (5 mM), and the free cysteine thiol groups are alkylated with iodoacetamide to block the random regeneration of disulfide linkages. In such cases, the protein digest solutions should be first cleaned with C18 desalting cartridges before attempting injection on the chip. For the purpose of this demonstration, reduction and alkylation were ignored, as these steps did not affect the outcome of the optimization process.

16. The polyimide coating should be removed from the ESI emitter, as during operation it can peel off and destabilize the electrospray ionization process.

17. In most cases the ESI-MS interface of the mass spectrometer must be modified to accommodate the microfluidic chip. An XYZ stage represents a simple solution. The chip and the stand should be enclosed in a transparent plastic chamber to prevent accidental exposure to high voltages.

18. Averaging mass spectra is useful for improving signal stability, however, the number of mass spectra that can be averaged is limited by the data acquisition speed of the particular MS platform that is used in the experiment. The time needed to generate the averaged mass spectrum should be kept short, e.g., <1 s, to avoid contributions to peak broadening and loss of separation resolution.

19. It is useful to have “practice” chips that encompass only a sample infusion channel that can be connected through a polymeric union to a syringe pump. With such a setup, a known, stable flow rate can be generated, and the sample solution can be continuously infused for optimizing the ESI signal and the MS data acquisition parameters.

20. Whether using a regular CE or an infusion chip, EOF or syringe pumping for fluid delivery, the performance of the chip ESI-MS platform can be also evaluated with the data dependent acquisition method, while infusing a peptide mixture (see Fig. 4B). A stable signal will result in equally spaced “signal spikes” of approximately the same height. The signal spikes in Fig. 4B represent occurrences of full mass spectra when a TIC is recorded, while the valleys represent the period of time spent on acquiring tandem mass spectra when a TIC is not recorded.

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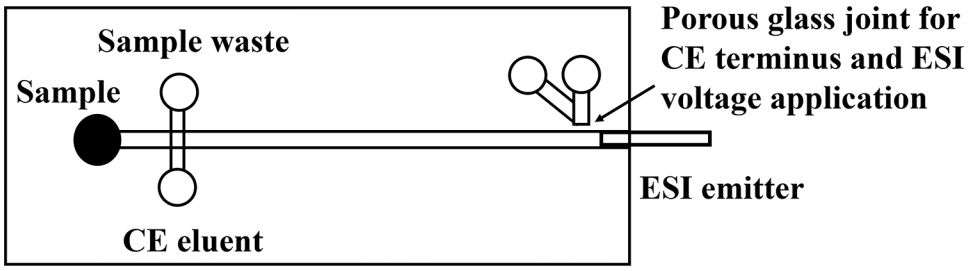

Figure 1.

Schematic diagram of a CE microfluidic chip with an ESI emitter inserted in the CE separation channel. The ESI voltage is applied through a porous glass junction placed at the end terminus of the CE separation channel.

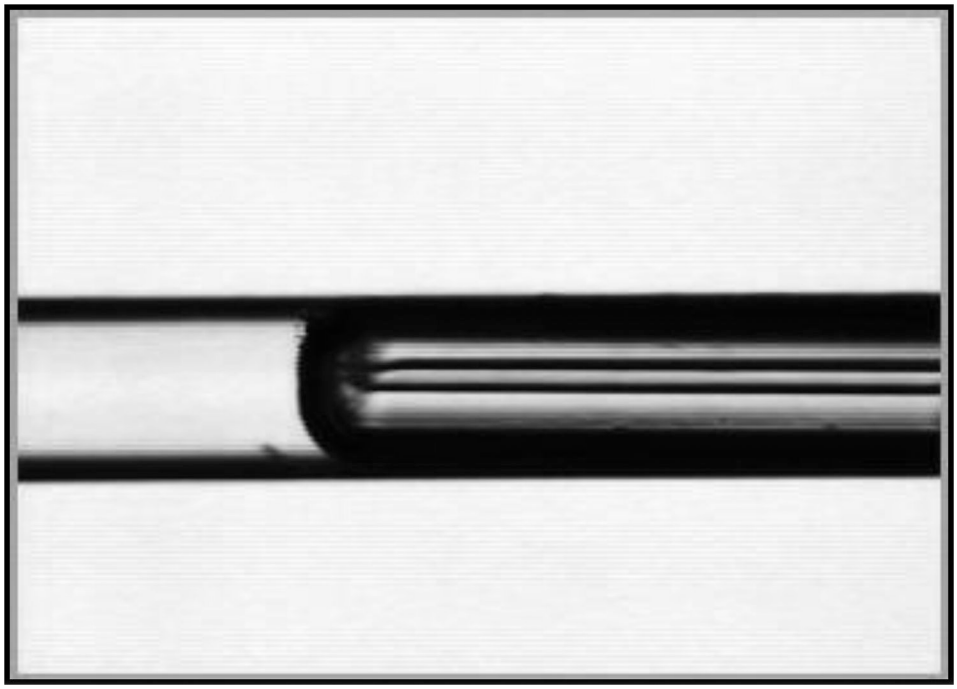

Figure 2.
Picture of a fused silica capillary (20 μm i.d. × 90 μm o.d.) inserted in the CE separation channel.

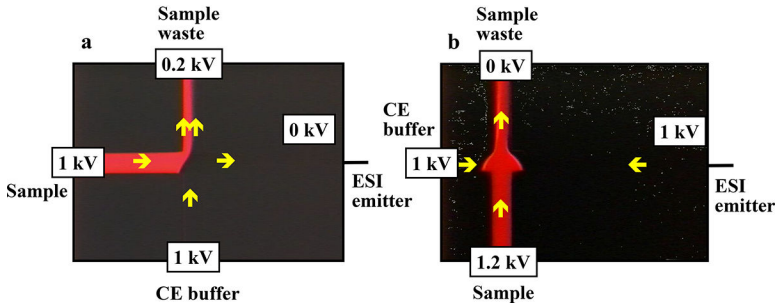

Figure 3.

Pictures of Rhodamine 610 injection in a microfluidic CE channel. The shown voltages enable either a (a) gated injection or (b) pinched injection.

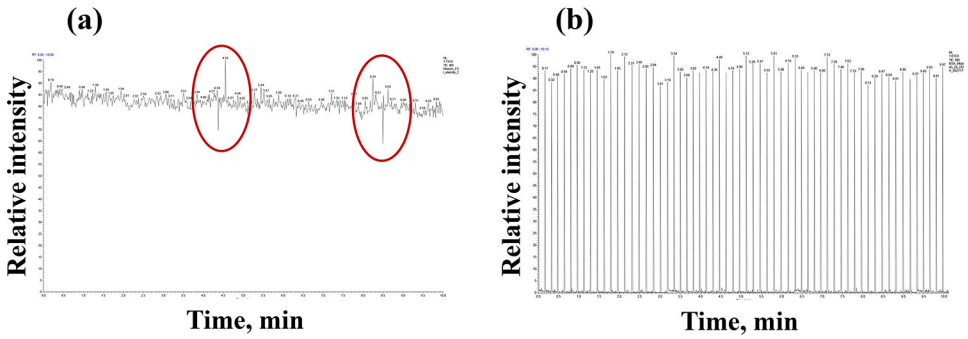

Figure 4.

Chromatograms displaying the stability of the ESI signal during an infusion experiment. **(a)** TIC generated with an MS acquisition method: the spikes represent instances of spray instability; **(b)** TIC generated with a data dependent acquisition method: the peaks represent instances of full mass spectra, while the valleys represent instances of tandem mass spectra.