Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

Conducting and Characterizing Femto Flow Electrospray Ionization

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Experimental:

Reagents and materials

Acetylcholine, amitriptyline hydrochloride, cytochrome c (C2506), MRFA (Met-Arg-Phe-Ala acetate salt), melezitose, maltoheptaose, methanol (HPLC grade) and water (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO). All analytes were prepared in the aqueous solution unless mentioned otherwise. The blank solvent was a mixture of methanol and water in 1:1 (v:v).

SEM

The spray emitters were imaged by scanning electron microscope (Lyra 3 GMU FIB, Tescan, Pleasanton, CA) with sputter coating of Au-Pd in 20 nm thickness. The inner diameters of submicron emitter tips were ranging from 30–160 nm. Except for SEM analysis, emitter tips without metal coating were used in this work.

Microscope

Photos and videos were taken by the microscope (Olympus IX73, Palatine, IL) for the measurement, such as emitter tip size and sample volume.

Mass spectrometry

An LTQ-XL linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA) was used in this work. The mass spectrometer was operated with the following parameters: Intel temperature 125℃, inlet capillary voltage 46 V, tube lens voltage 110V. The maximum injection time typically used in this work varied from 100 ms to 8 s. In this work, the automatic gain control (AGC) was on and set to be 3E4. In all experiments, the sample containing emitter tips were placed 5–10 mm away from the instrument inlet.

Confirmed by correspondences with manufacturer and reported in previous works,[1,2] the "intensity" reported by the Thermo Xcalibur software for the LTQ-XL instrument is a "current" intensity value as the detected ion counts divided by the injection time (ion counts/IT). The labeled NL (normalized level) is the current intensity for the highest datapoint in a spectrum. For the LTQ-XL, the data density is ~10 data points per Thomson (Dalton/e). One could estimate the number of analyte ions using above information.[1]

Ion source and emitters

Two types of ESI emitters were used in this work, one with micrometer emitter tip and another with submicron emitter tip. Borosilicate glass capillaries (B150-86-10, Sutter Instrument Co., Novato, CA) were pulled by a micropipette puller (Model P-1000, Sutter Instrument Co., Novato, CA) with a trough filament. The puller parameters are listed in **Table. S2**. It typically takes 15 min to pull 15 capillaries into 30 submicron spray emitters.

Wire-in electrospray: A gold wire (0.1 mm diameter, 99.998%, Premion) was used as the electrode inserted in the spray emitter. The high voltage power supply (Stanford Research Systems, Inc. Model

PS350) was connected to this electrode via a copper clip. A 100 MOhms resistor was used in the wire-in ESI experiments to prevent the occurrence of air breakdown.

Relay electrospray: A wire-in ESI was the primary emitter and was used as the primary ion source. Blank solvent (MeOH, H_2O, 1:1) was loaded in the primary emitter. A submicron emitter tip containing the sample and hexane (if applicable) was placed in front of the primary emitter with its backend at 5–8 mm from the tip of the primary emitter.

Ionization Current Measurement

The current measurements were performed inside a homebuilt Faraday cage, which was made from finely woven metal mesh, to reduce the electromagnetic radiation interference. A high voltage power supply was connected to the wire-in electrode, and a conductive thumbtack was used as the ground electrode. The electrometer (Keithley Instruments 6514 System, Cleveland, OH) was connected to the ground electrode to collect data, the output of which was recorded by LabVIEW program (National Instruments) with a sampling rate at 100 Hz. A more detailed description could be found in our previous work.[1]

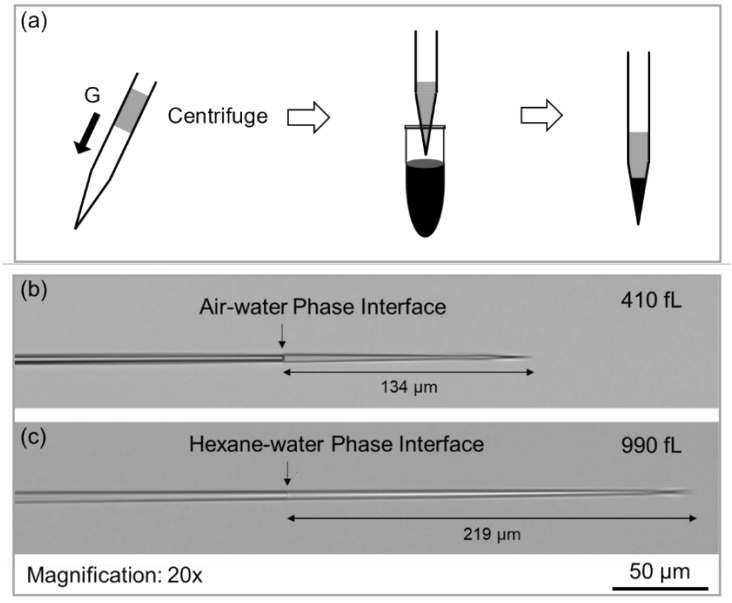

Figure S1. (a) Scheme showing loading aqueous sample into an emitter tip pre-filled with hexane. **(b)** Microscope photo of aqueous sample in submicron emitter tip without hexane. **(c)** Microscope photo of aqueous sample in submicron emitter tip with hexane. Hexane has less contrast than air but is still easily distinguishable.

As shown in **Fig. S1a**, backfill 10 μL hexane at the backend opening by micropipette and centrifuge the capillary for 1 min using a homemade holder (Centrifuge 5415C, Eppendorf, Germany). The hexane was centrifuged to fill the emitter tip, which was checked using a microscope to confirm that no air bubbles were present. Then the front of emitter tip was dipped into aqueous sample for 3–5 min and approximately 0.1–10 pL aqueous solution was loaded at emitter tip, forming a visible phase interface between water and hexane, **Fig. S1c**.

Hexane effectively reduced the evaporation of aqueous sample by reducing the interfacial area between the sample and air. In addition to the sealing by hexane, other methods have been tried to minimize the evaporation. For example, evaporation was expected to be reduced by controlling the vapor pressure inside the capillary, which was achieved by adding a drop of water at the back end of the glass emitter. It was assumed that this water droplet would evaporate and saturate the air inside the capillary, which would prevent the evaporation of aqueous sample loaded at the front tip. In the experiment, however, it was found that this would lead to condensation of the water in the taper (typically around 30 μm in diameter).

Table S1. Evaporation rates for varied droplet sizes, obtained from literature.[2]

Shape	R (μm)	Volume (pL)	Interface Area (cm^2)	Evaporation Rate (pL/min)	Evaporation Rate (#/s·cm^2)
Droplet	7.5	1.77	7.07E-06	2164	7.59E+19
	9.8	3.94	1.21E-05	3639	7.47E+19
	15	14.14	2.83E-05	6896	6.05E+19

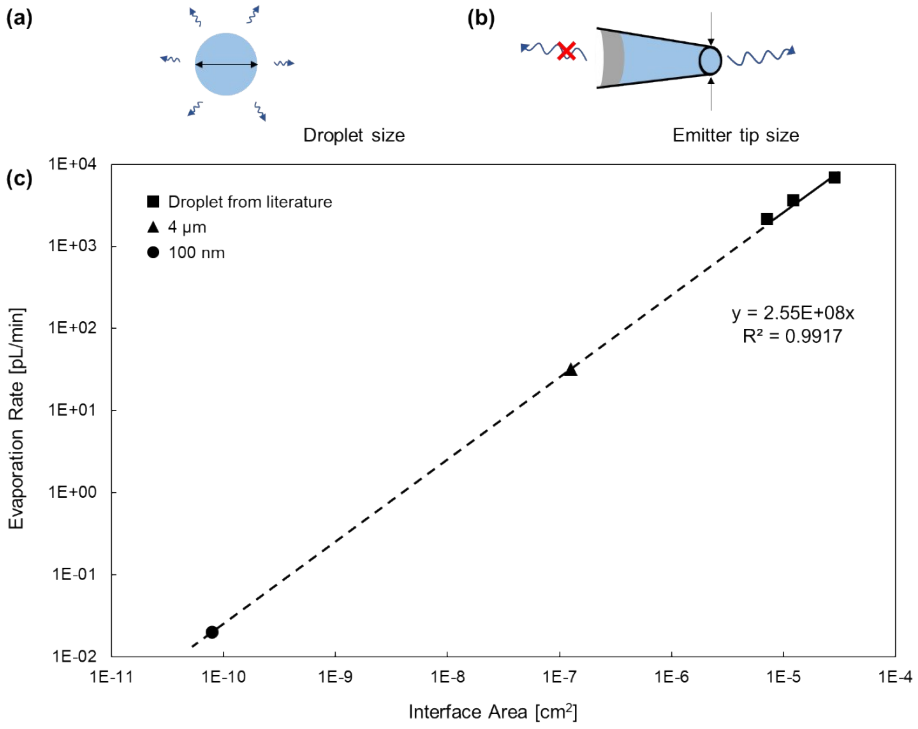

Figure S2. Estimating evaporation rates of sample solution in emitter tips. **(a)** Evaporation scheme of a droplet. Evaporation data was taken from literature[2] to establish a per surface area rate. **(b)** Aqueous sample (**blue**) under hexane (**grey**) in emitter tip. **(c)** Correlation between evaporation rate (pL/min) and interface area (cm^2) for the reported droplet evaporation rates. Both *x* and *y* axis are on the logarithmic scale. The slope of the linear regression is 2.55E8 pL/(min·cm^2). For a 100 nm, 4 µm emitter tip, the tip opening interface area are 7.85E-11 cm^2, 1.26E-7 cm^2, respectively. Calculated evaporation rates are 32 pL/min and 20 fL/min using slope value 2.55E8 pL/(min·cm^2).

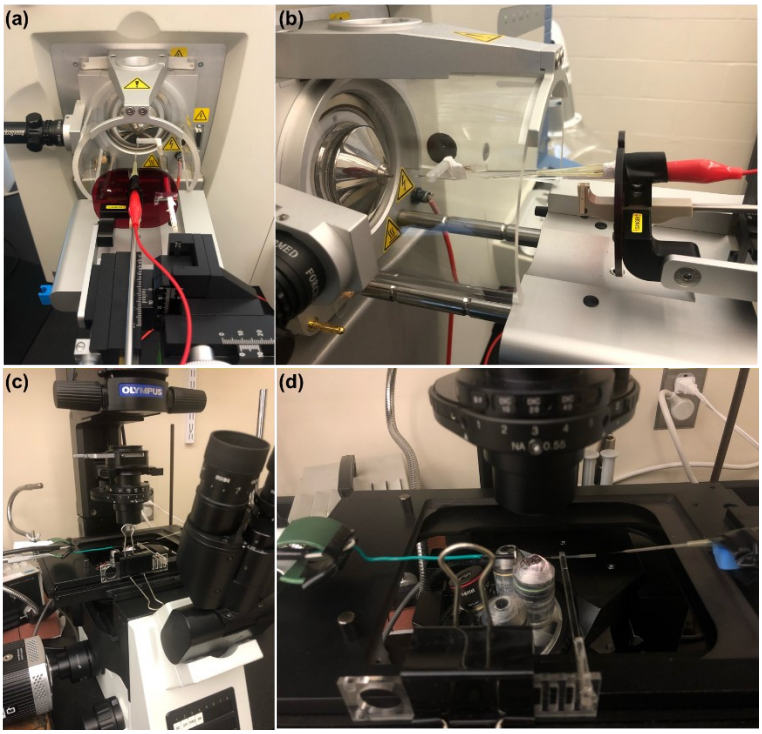

Figure S3. Photos of the relay ESI setup mounted in front of mass spectrometer **(a) (b)**, and on top of an optical microscope **(c) (d)**.

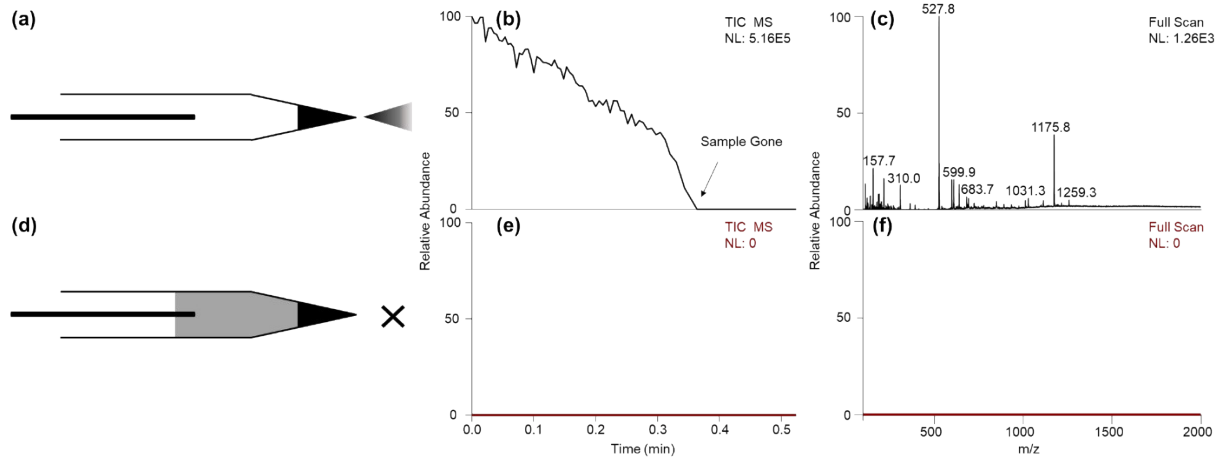

Figure S4. Scheme **(a)** air gap between electrode wire and sample solution (**black color**), **(b)** electrode wire in hexane layer (**grey color**) behind sample**.** Around 0.3 nL aqueous sample solution was loaded in each micrometer emitter tip. **(b, c)** and **(e, f)** are TIC and full scan mass spectra of 100 µM melezitose and maltoheptaose mixture using setup **(a)** under 2 kV and setup **(b)** under 4 kV, respectively. No ion signal was observed using set up **(d)**, suggesting the charge transport is prevented by hexane.

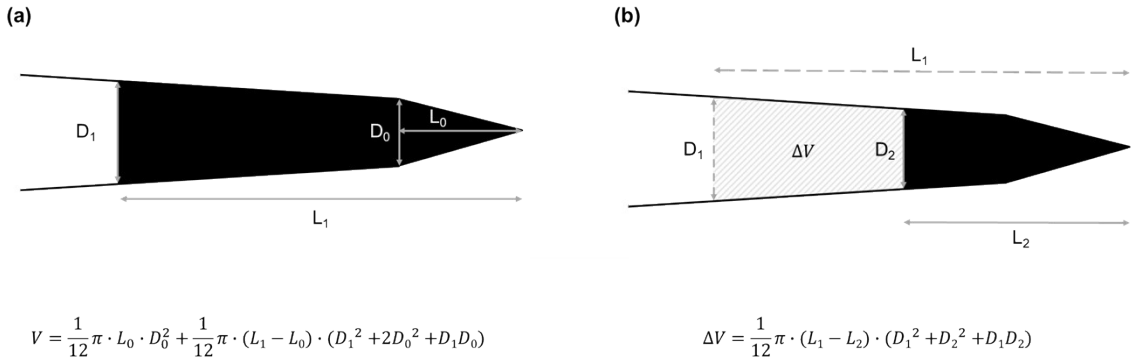

Figure S5. (a) Sample volume calculation, and **(b)** calculation for volume consumption.

The total volume of sample in the submicron emitter tip was measured using the equation in **Figure S5a**. The slope of the taper shank was not perfectly constant. Therefore, the total volume was approximated as consisting of a truncated cone and a circular cone. The narrow taper of the submicron emitter tip enables facile tracking the consumption of sample. Due to the relatively high error percent caused by the thickness of the wall inside the tip (shown in SEM images), and by the potential miscounting pixels when dealing with such a sharp tip area, the flow rate was calculated based on the consumption volume of sample (i.e., a truncated cone) **(Figure S5b)**.

Take one experiment data as an example, the L_1, L_2, D_1, D_2 were 726, 691, 20, 19 pixels. Assuming each measurement miscounts by 1 pixel, the error in L_1, L_2, D_1, D_2 measurement would be 1/726, 1/691, 1/20, 1/19, which are 0.14%, 0.14%, 5%, 5%, respectively. The propagated error in the consumption volume would be 7.2%, according to equation $\Delta V = \frac{1}{12}\pi \cdot (L_1 - L_2) \cdot ({D_1}^2 + {D_2}^2 + D_1 D_2)$.

Both phase boundaries between air and water (**Figure S1b**) and between hexane and water (**Figure S1c**) are obvious to distinguish due to the difference in refraction indexes.

Several methods of tracking volume changes have been tried. To better visualize the interfacial motion, iodine was added to hexane to increase the contrast between hexane phase and aqueous phase. To our surprise, the addition of saturated iodine in hexane (0.03016 g added into 200 μL and not dissolved completely) did not improve the contrast. Another method was to use fluorescein solution and fluorescent mode microscopy. Due to lower light intensity, fluorescent microscopy was slower and was not as ideal for motion tracking. In conclusion, bright-field microscopy and the addition of pure hexane were found to be the most effective way for tracking interface among other approaches.

Under the microscope, one evaporation rate was observed to be 29 fL/min and 30 fL/min when the illumination light was continuously ON or mostly OFF, respectively. In comparison, the same emitter tip

had an evaporation rate of 150 fL/min when mounted in front of the mass spectrometer, suggesting the increased evaporation is likely due to the air flow and elevated temperature (~3.0 °C) in front of the mass spectrometer inlet. Nevertheless, all these evaporation rates are much smaller than the ESI flow rates measured in this work.

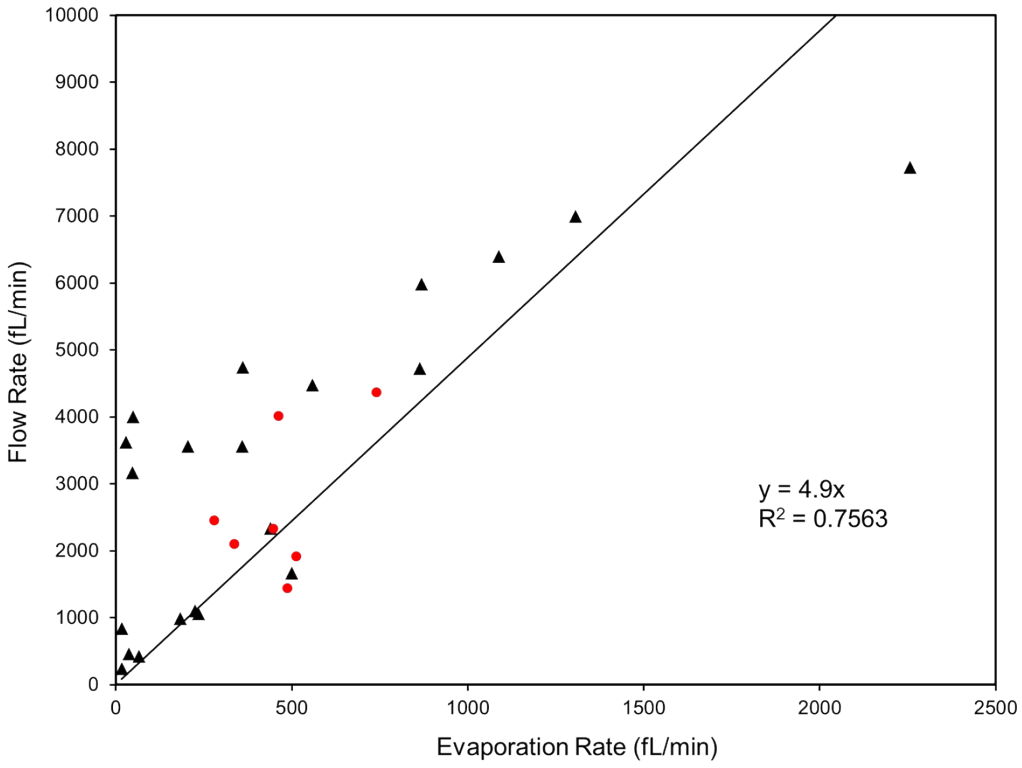

Figure S6. Measured ESI flow rates plotted against the corresponding evaporation rates in the multiple experiments each using a new emitter tip. The black triangles represent the flow rates of secondary emitter when using a submicron electrospray emitter tip as the charge supply. The red dots represent the flow rates of secondary emitter when using a plasma ion source[3] as the charge supply. Generally, higher ESI Flow rates were observed with increasing evaporation rates. One data point (1306, 6994) was observed without hexane sealing.

Table S2. Puller programs for borosilicate glass emitters pulled in this work.

Type	Tip size (O.D.)	Taper length (mm)	Heat	Pull	Vel.	Delay/Time	Pressure	Ramp
Micrometer	4 µm	3.6	296	6	18	50 (delay)	590	286
Submicron	~100 nm	14	286	70	70	250 (time)	500	286

To evaluate the stability of the puller and the reproducibility of pulling programs. The outer diameters of the taper at 150 µm from the tip were recorded for the emitter tips. For the micrometer emitter tip pulling program, an average diameter of 36.7 µm was obtained with a standard deviation of 1.2 µm (5 repeats), i.e., 3.1% RSD. For the submicron emitter tip pulling program, measuring the outer diameter of the taper at 500 µm returned an average diameter of 10.3 µm with a standard deviation of 2.6 µm (5 repeats), i.e., 25.6% RSD. There is a higher variation when pulling submicron emitter tips, despite the good stability indicated by the constant Ramp value and micrometer

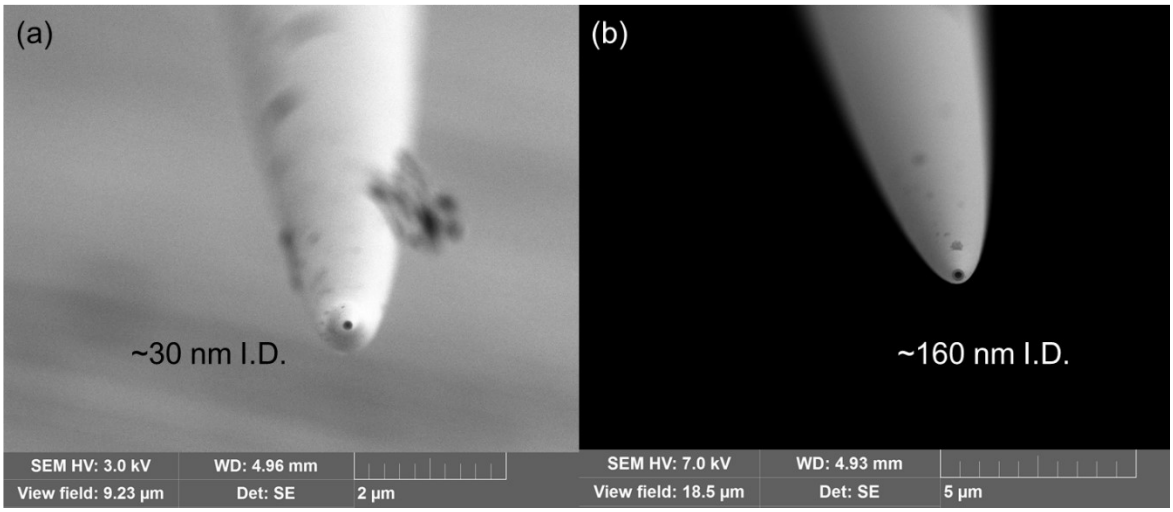

Figure S7. Scanning electron microscope (SEM) images of the submicron emitter tip showing one with relatively smaller internal diameter around 30 nm **(a)** and one with relatively larger internal diameter around 160 nm **(b)**.

Table S3. Summary of observed ESI flow rates and evaporation rates

Method	Sample	Flow Rate (fL/min)	Evaporation Rate (fL/min)	Notes
Multiple time points	1 mM Acetylcholine	4741	360	
	10 μM neurotensin and maltoheptaose in 50 mM ammounium acetate	6994	1306	Without adding hexane at end
	100 μM Amtriptyline	235	18	
	50 μM MRFA and Cyt c	7729	2257	
	100 μM MRFA	6399	1089	
		3562	359	
		2331	439	
		841	17	
		1057	236	
		425	65	
		2103	335	Plasma ion source as charge supply
		2339	447	
		4369	739	
	100 μM melezitose and maltoheptaose in water	4323	N/A*	*Three trials were using the same emitter. No evaporation rate measurement as the sample solution was depleted by ESI. Used ~500 fL/min as evaporation rate.
		2099		
		1665		
		4475	558	Same emitter as above three trials, another loading was made in this round
		3164	48	
		3620	150	Evaporation in front of MS
			29	Evaporation under microscope with light OFF
			30	Evaporation under microscope with light ON
		3164	48	
		3558	205	
Continuous Monitoring	water	4722	863	
		4001	55	
		450	37	
	100 μM melezitose and maltoheptaose in water	5982	868	
		1097	225	
		980	183	
		1924	511	Plasma ion source as charge supply
		4015	461	
		1447	487	
		2458	279	

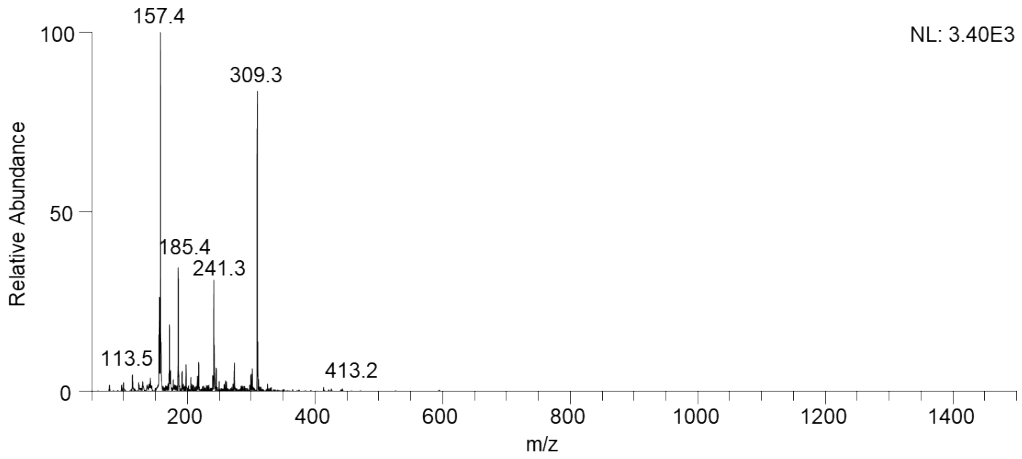

Figure S8. Full scan mass spectrum of relay ESI when the secondary emitter was empty, and the primary emitter was loaded with blank solvent.

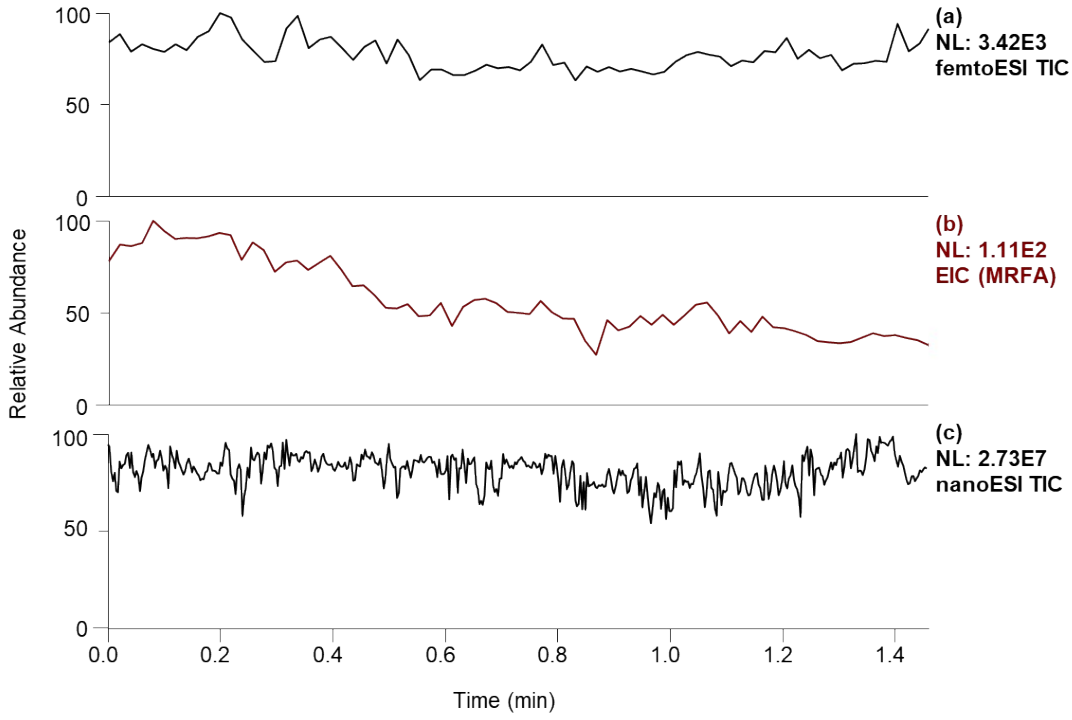

Figure S9. When analyzing a 100 μM MRFA aqueous solution using relay ESI of submicron emitter tip, the TIC **(a)** and EIC of protonated MRFA **(b)** had continuous signals. **(c)** A typical nanoESI TIC for the same solution was obtained using micrometer emitter tip.

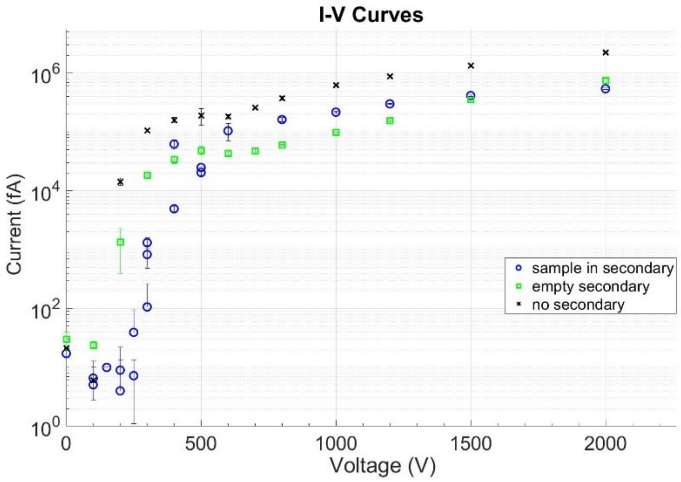

Figure S10. I-V curves for relay ESI under different conditions, (**blue**) 100 µM MRFA aqueous solution loaded in the secondary emitter, (**green**) an empty secondary emitter and (**black**) with no secondary emitter. The primary emitter was loaded with blank solution and was placed in same position in these three cases, which is ~ 6 cm away from the ground, 5–8 mm away from the backend of the secondary emitter (if present).

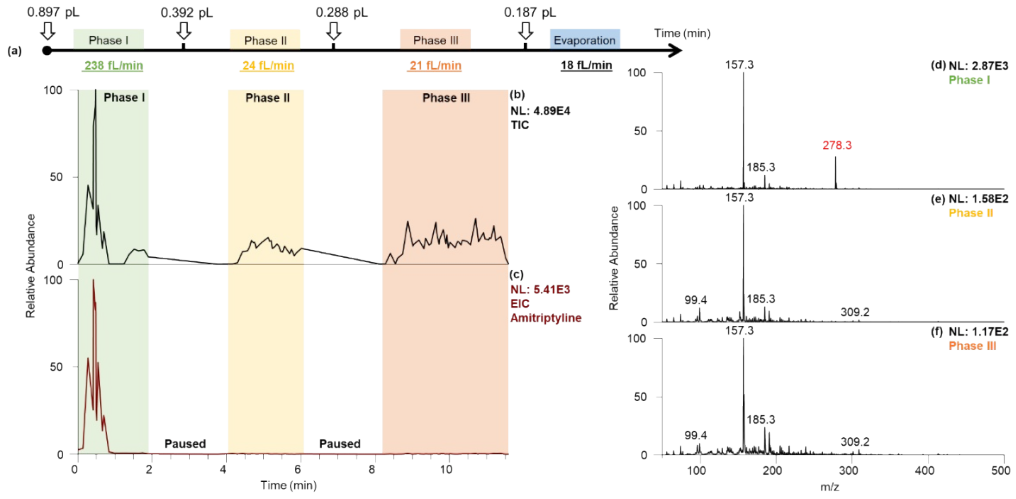

Figure S11. (a) Timeline of one experiment with volume measurement and calculated flow rates. TIC **(b)** and EIC of pronated amitriptyline **(c)** of 100 µM amitriptyline aqueous sample. **(d), (e), (f)** were full scan mass spectra of three trails using the same emitter and the same sample. In phases II and III, there was no signal of amitriptyline. However, according to the microscope photos, the sample was still consumed at approximately the rate of evaporation (18 fL/min).

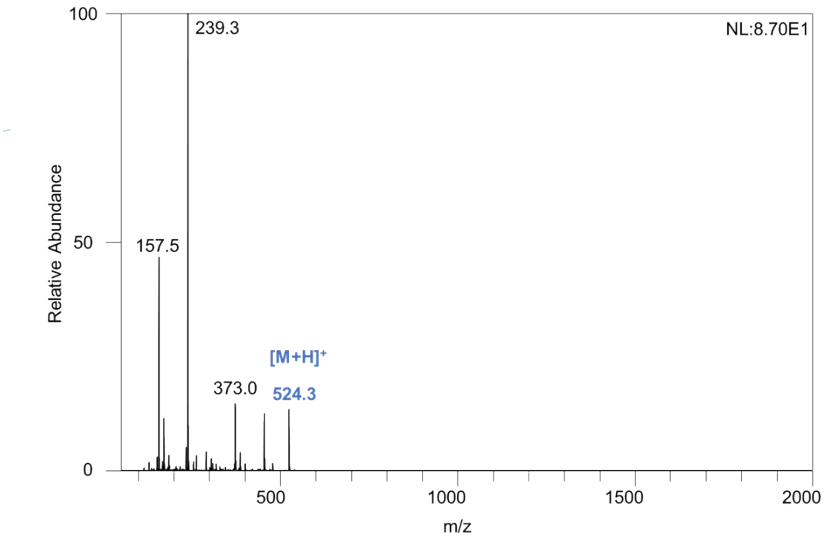

Figure S12. Full scan mass spectrum of 50 μM cyt c and 50 μM MRFA aqueous mixture sample using relay ESI and a submicron emitter tip. Only protonated MRFA ion was observed, suggesting the cyt c was likely filtered out by the partially clogged emitter tip. This is not likely due to the migration effect along the tip[4] since the sample solution was loaded from tip front opening with only ~ 100s μm in length. More likely, the tip opening somehow filtered out the cyt c protein, either during sample loading or electrospray analysis. This suggests that the nanopipettes could be controlled to perform size-selective sampling in future studies.

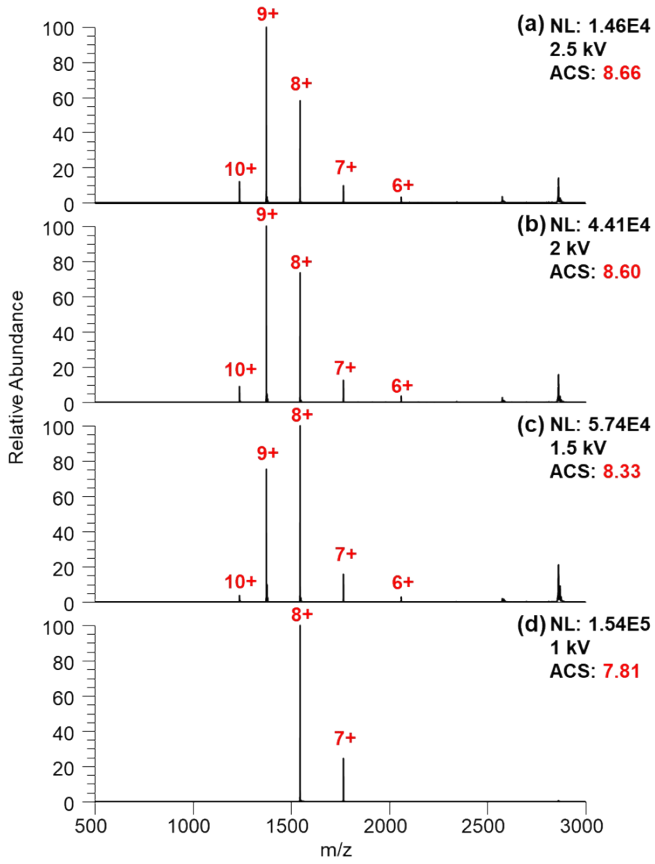

Figure S13. Full scan mass spectra of 10 µM cyt c aqueous sample using the same micrometer emitter tip showing varied charge state distribution under voltages **(a)** 2.5 kV, (**b)** 2 kV, **(c)** 1.5 kV, **(d)** 1 kV. The average charge state decreased as the applied voltage decreased.

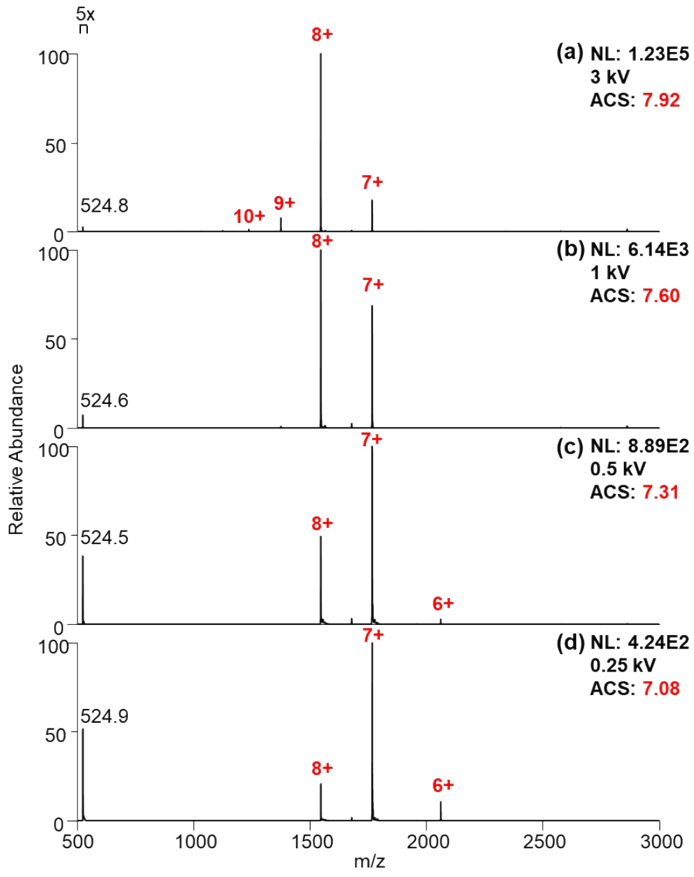

Figure S14. Full scan mass spectra of 50 μM cyt c and 50 μM MRFA aqueous mixture sample using the same submicron emitter tip showing varied charge state distribution under voltages **(a)** 3 kV, **(b)** 1 kV, **(c)** 0.5 kV, **(d)** 0.25 kV. The average charge state (ACS) decreased as the applied voltage decreased. The protonated MRFA peaks in **(a)–(d)** were amplified by a factor of 5.

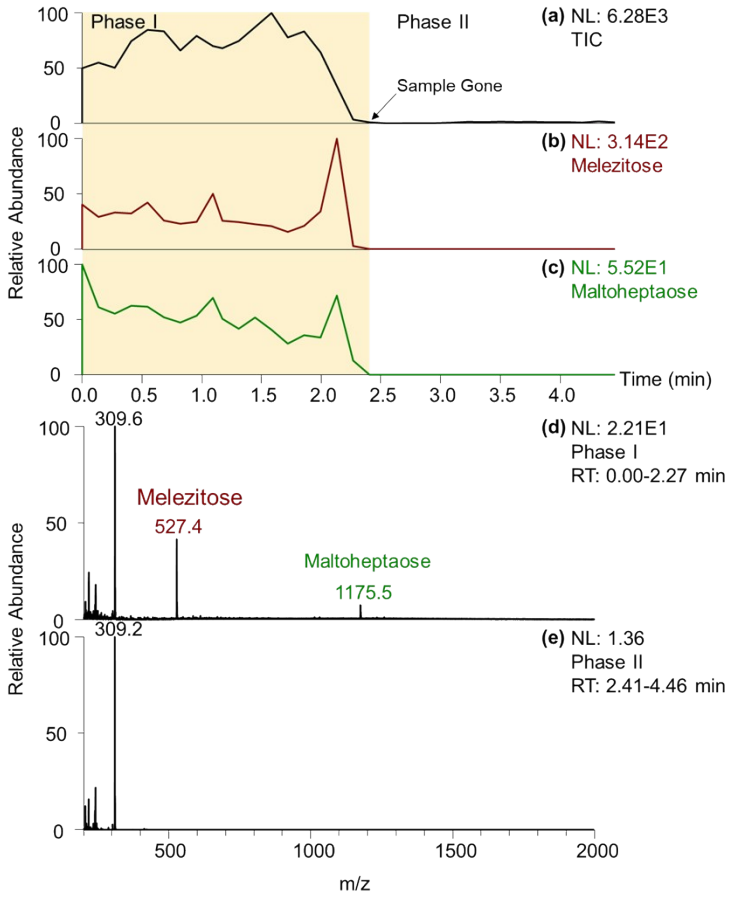

Figure S15. TIC **(a)** & EIC of sodiated melezitose **(b)** and sodiated maltoheptaose **(c)** of a 100 μM melezitose and 100 μM maltoheptaose aqueous mixture sample solution (6.35 pL) using relay ESI. Full scan mass spectra of phase I **(d)** and phase II **(e)** showed the presence and absence of analyte peaks, respectively.

As can be seen above, after 2.27 min, the TIC diminished and the EIC of both glycans dropped to zero. In the mass spectra, by comparing the mass spectrum for the initial 2.27 min (d) and the subsequent times (e), the disappearance of the analytes signal could be due to the depletion of the sample. The microscopic photo taken also confirmed that there was no sample solution at the emitter tip. With an initial sample volume of 6.35 pL, the estimated flow rate for the first 2.27 min was 2.8 pL/min without considering evaporation in the process; with evaporation (~ 500 fL/min, obtained by loading the same emitter again) considered, the estimated flow rate for the first 2.27 min was 1670 fL/min. The consumed sample amount was 0.635 femtomole (6.35 pL, 100 μM) and there were 19 scans in the first 2.27 min. Therefore, the average consumed sample amount rate was 33.4 attomole per scan.

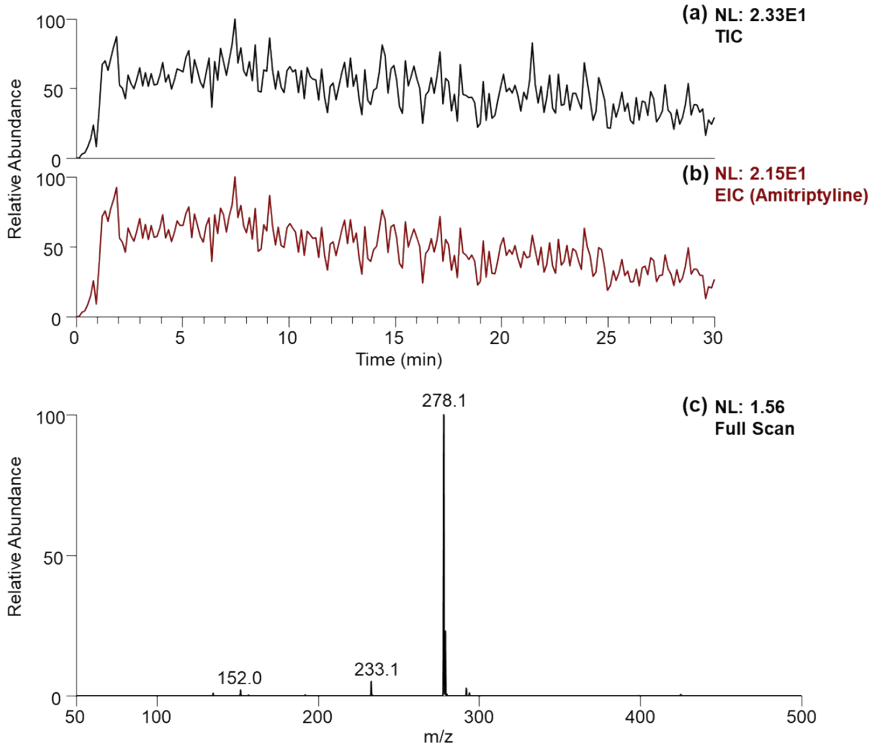

Figure S16. TIC **(a)** and EIC of protonated amitriptyline **(b)** and full scan mass spectrum **(c)** of 100 µM Amitriptyline aqueous sample when using submicron relay ESI. After there was no observable (under the microscope) sample solution inside the emitter tip, this "empty" emitter still produced the amitriptyline signal for up to 30 min.

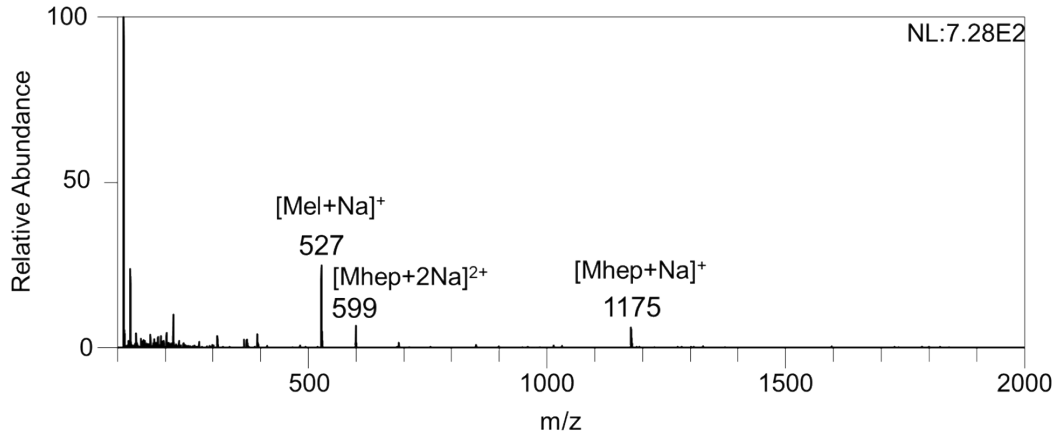

Figure S17. Full scan mass spectrum of the emitter tip after front-loading 8.4 pL of aqueous sample mixture solution of 50 µM melezitose, 50 µM maltoheptaose, 50 µM ubiquitin and 50 µM cytochrome c. The simultaneous absence (intensity <1% NL) of ubiquitin and cyt c signal, and presence of glycan signal

suggest that the protein molecules are more prone to filtration effect during the loading and electrospray through the submicron emitter tip. Note the PI of ubiquitin and cyt c are 6.8 and 9.6, respectively.

(a)

Total Ion Current:	12815.10
Scan Low Mass:	100.00
Scan High Mass:	4000.00
Scan Start Time (min):	1.36
Scan Number:	71
Base Peak Intensity:	2772.45
Base Peak Mass:	1546.55
Scan Mode:	ITMS + p NSI Full ms [100.00-4000.00]
LTQ XL Data:	
==========	
AGC:	On

(b)

Total Ion Current:	107.79
Scan Low Mass:	100.00
Scan High Mass:	4000.00
Scan Start Time (min):	0.00
Scan Number:	1
Base Peak Intensity:	5.09
Base Peak Mass:	524.64
Scan Mode:	ITMS + p NSI Full ms [100.00-4000.00]
LTQ XL Data:	
==========	
AGC:	On

Figure S18. Screenshots of the scan header data from **(a)** nm-sized relay in **Figure 3a** and **(b)** μm-sized fA mode in **Figure 3d**. The total ion current (TIC) was 12815 and 108 ion counts per second, respectively.

Video S1. Continuous monitoring a lower evaporation and ESI flow rate. (Video S1.1 Evaporation, Video S1.2 Flow rate)

Video S1.1 Sample evaporation with hexane sealing at the backend. The video was taken by microscope under 40 x magnification at 10 frames per second. The length and width of the field of view are 312 μm and 174.2 μm, respectively. At the beginning, 0.9 pL of aqueous sample solution was present in the emitter tip, which occupied a length of 163 μm to the right of the liquid-liquid interface (3.25 μm in diameter). The voltage was OFF in this video. the consumption rate (the movement of phase boundary) of sample solution represents evaporation. 0.09 pL of solution was consumed in 2 min 25 s, corresponding to an evaporation rate of 37 fL/min.

Video S1.2 Continuous monitoring sample ESI flow by microscope. The video was taken by microscope under 40 x magnification at 10 frames per second. The length and width of the field of view are 312 μm and 174.2 μm, respectively. At the beginning, 0.8 pL of aqueous sample solution was present in the emitter tip, which occupied a length of 126 μm. This video was taken after the evaporation (**Video**

S1.1) and the primary ESI source was turned ON in this video. 0.8 pL of solution was consumed in 1.8 min, corresponding to an average consumption rate of 450 fL/min, which represents the ESI flow rate.

Video S2. Continuous monitoring a higher evaporation and ESI flow rate

Online measurement of the evaporation and electrospray flow rate for a 100 μM MRFA aqueous solution loaded in a nanopipette. The video was taken by microscope under 20 x magnification at 10 frames per second. The length and width of the field of view are 624 μm and 348.4 μm, respectively. At the beginning, 3.4 pL of aqueous sample solution was present in the emitter tip, which occupied a length of 236 μm. To the left of the aqueous sample was hexane. The liquid-liquid interface (6.5 μm in diameter) bends because of different surface tensions. The primary ESI source was ON from 24.8 s to 40.8 s. When the voltage was OFF at the other time, the movement of phase boundary represents the evaporation of water through the cross-section of tip. By calculating the volume change over time, the average evaporation rate during 0–24.8 s was 870 fL/min. The solution consumption rate of 5980 fL/min during ON time was believed to represent the ESI flow rate.

Video S3. Continuous monitoring evaporation and ESI flow rate using plasma ion source as the charge supply

Plasma ion source[3] was utilized as the charge supply to trigger the electrospray of secondary emitter in this video. The video was taken by microscope under 20 x magnification at 10 frames per second. The video is played at 3x speed. The length and width of the field of view are 624 μm and 348.4 μm, respectively. At the beginning, 6.4 pL of aqueous sample solution 100 μM melezitose and maltoheptaose was present in the emitter tip, which occupied a length of 502.2 μm. To the left of the aqueous sample was hexane. The liquid-liquid interface (6.2 μm in diameter) bends because of different surface tensions. The primary ESI source was ON from 59 s to 94 s. When the voltage was OFF at the other time, the movement of phase boundary represents the evaporation of water through the emitter tip opening. By calculating the volume change over time, the average evaporation rate during 0–59 s and 94–146 s were 487 fL/min and 481 fL/min, respectively. The solution consumption rate of 1447 fL/min during ON time was believed to represent the ESI flow rate.

In the experiments, dust outside the emitter tip had no observable effect on the ESI. As the interface migrates, necessary focus adjustment caused brightness changes in the videos.

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