

Hyphenation of Production-Scale Free-Flow Electrophoresis to Electrospray Ionization Mass Spectrometry Using a Highly Conductive Background Electrolyte

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Supporting Information

ABSTRACT: In this technical note, we demonstrate the hyphenation of production-scale free-flow electrophoresis (FFE) and sheathless electrospray ionization mass spectrometry (ESI-MS). In contrast to previous hyphenation approaches, we used a highly conductive background electrolyte (BGE) required for production-scale FFE. We found that this kind of BGE as well as a production-scale setup leads to significant electric interference between FFE and MS. This interference prevents steady-state FFE operation. We examine this interference in detail and discuss possible solutions to this issue. We demonstrate that the straightforward grounding of the transfer line removes the influence of ESI-MS on FFE, but



creates a current leak from the ESI interface, which adversely affects the ESI spray. Furthermore, we show that only the electrical disconnection of the ESI probe from the FFE-MS transfer line suppresses this undesirable current. In order to facilitate the electrical disconnection we used a low conductivity, silica-based ESI probe with withdrawn inner capillary. This approach allowed the interference-free hyphenation of production-scale FFE (using a highly conductive BGE) with ESI-MS.

ree-flow electrophoresis (FFE) allows the separation of multiple analytes in a continuous flow.¹ It is an attractive technique for downstream purification of products from continuous flow synthesis (CFS).² In order to build a continuous production system, FFE-CFS should be complemented by continuous monitoring for system control and quality assurance. An analytical method used for this monitoring process should ideally be universal, fast, sensitive, specific, and easy-to-implement. Optical techniques such as spectrophotometry, fluorometry, refractometry, and Raman spectroscopy fulfill most of these criteria and, thus, were implemented by a number of groups.³⁻¹¹ However, the universality of these methods is limited since they require analytes with respective optical properties or require chemical derivatization with chromophores or fluorophores. The specificity of optical techniques is also restricted since they provide only very limited information about the structure of analytes. Structural information, however, is crucial for identification, differentiation, and proper quality assurance in CFS-FFE, since educts and products naturally share a similar structure and are comprised of similar functional groups.

Mass spectrometry (MS) provides the universality and specificity that optical methods are lacking. MS is also fast and sensitive.¹² However, the combination of FFE with MS is not as straightforward as with optical techniques. FFE and MS will interfere electrically with each other. This issue has been

solved well for a related hyphenated technique: capillary electrophoresis-MS (CE-MS). $^{13-16}$ For FFE-MS, however, researchers break new ground. There are only three research groups that have developed and reported strategies for the online coupling of FFE to MS so far. All groups worked with microscale FFE (μ FFE). Chartogne et al. built a μ FFE chip for interfacing capillary isoelectric focusing with MS to characterize protein mixtures.¹⁷ Benz et al. coupled their μ FFE chip to MS for monitoring a multicomponent [3 + 2]-cycloannulation.¹⁸ Park et al. demonstrated the online coupling of free-flow isotachophoresis with mass spectrometry.¹⁹ These groups did not report any electrical interference between their chips and MS. We attribute the absence of interference to the microscale setup and low conductive electrolyte (low μ S cm⁻¹) used in these works (see Results and Discussion for details). In general, the electrolyte conductivity should be high enough not to be affected by the analyte conductivity. In the mentioned works, the analyte concentrations (μ M range) and, thus, their conductivities were very low; this justified the use of low conductive electrolytes.

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Outputs of production-scale CFS (up to mmol min⁻¹) are achieved by using high concentrations (mM range) and large flow rates (mL min⁻¹).²⁰⁻²² The downstream FFE must be stable at high concentrations of a variety of species present in the CFS effluent. The stability requires that the background electrolyte (BGE) be highly conductive. If MS follows FFE, the BGE should be compatible with MS. Buffer compounds such as acetates, formates, ammonia, HEPES, and MES are MS-compatible.²³⁻²⁵ From these, HEPES and MES can be used to create highly conductive BGEs.²⁶

In this work, we report on the first approach (to our best knowledge) to connecting highly conductive BGE-FFE with MS. We found significant electric interference between FFE and MS, which disturbs the operation of FFE. This phenomenon was studied in detail, and a simple-to-implement solution to suppress the interference was found.

EXPERIMENTAL SECTION

Reagents. The details are listed in the Supporting Information.

FFE-MS Setup. A schematic of our FFE-MS setup is depicted in Figure S1. The setup consists of an FFE-device described elsewhere,^{27–29} in which the sample outlet 2 is directed toward the electrospray ionization (ESI) interface of the MS instrument by a transfer line (polyethylene tubing; inner diameter of 1/16''; 38 cm long). The effluent of outlets 1 and 3 is collected. Transfer line 2 and collection lines 1 and 3 (same material, diameter, and length as transfer line) are grounded by small metallic connectors (1 cm long) between the two pieces of tubing. The metallic connectors were connected to the case of the MS instrument, which is grounded. The lengths from the FFE outlets to the metal connector of the transfer line to the MS was 14 cm.

Experimental Parameters. We used HEPES (10 mM, pH 7.5) as BGE in FFE and a mixture of rhodamine and fluorescein (100 μ M each) as a well-studied model pair of analytes.²⁷ The details are listed in the Supporting Information.

Mass Spectrometry. An AB Sciex API 5000 equipped with Turbo V source (Vaughan, ON, Canada) was used for the experiments. For the initial experiments, we used a standard stainless steel probe/electrode configuration. For later experiments (see Results and Discussion) we used a fused silica probe (wide bore, 0.5 mm inner diameter) plumbed from the FFE coupling line all the way to the end of the probe. The MS instrument was calibrated before the first experiments and optimized for detection of the two analytes: fluorescein and rhodamine. Detailed experimental conditions for MS measurements can be found in the Supporting Information.

RESULTS AND DISCUSSION

Note. In the following P_x (roman P) refers to the node/ point in the electric circuit, while P_x (italic P) refers to the corresponding potential.

Interference. In our initial FFE-MS setup, we connected the middle outlet of FFE to the ESI interface of the MS instrument by an ungrounded transfer line. Also, the collection lines were not grounded. We found that the ESI-MS instrument creates a significant electrical field in the FFE device. This was manifested by the shift of an analyte stream without any separation voltage (V_{FFE}) applied and by arcing when mounting

the clamps to connect the FFE high voltage source. Grounding the transfer line removes this interference.

Grounding, however, is not a sufficient solution. We found, without any separation voltage ($V_{\rm FFE}$) applied, that grounding adversely affects the MS signals. This was indicated by the dependence of the analyte signals on the presence of grounding (Figure S2). On the one hand, both analyte signals could be observed without grounding. On the other hand, grounding suppressed the fluorescein signal almost completely while immensely increasing the rhodamine signal. In further experiments with applied separation voltage in FFE, we did not find any evidence that grounding does affect FFE separation nor that the applied separation voltage has any effect on the analyte signals.

Source of Interference. We examined the electric circuits of our setups to find the source of the interference described above. The diagrams are depicted in Figure 1A (no grounding)



Figure 1. Electric circuit diagrams of FFE-ESI-MS depicting the cases without (A) and with (B) grounding of the transfer line.

and B (grounding applied). In general, FFE can electrically be described as a set of two resistors (R_1 and R_2), which are linked to each other (P_2) and connected $(P_1 \text{ and } P_3)$ to a high voltage source (V_{FFE}) . The connection point P₂ is the physical connection point (outlet 2) for the transfer line to the ESI probe: it is located in the middle of the separation chamber. Thus, R_1 and R_2 are equal. The ESI interface can be depicted as a high voltage source $(V_{\rm ESI})$ connected to a high-impedance resistor (R_{ESI}). One connection point (P_6) is grounded, while the other one (P_5) corresponds to the electrical contact at the ESI probe. The transfer line between the FFE chip and the ESI interface is either a single resistor $(R_3, without grounding,$ Figure 1A) or two resistors (R_4 and R_5 , with grounding, Figure 1B, $R_3 = R_4 + R_5$ linked by a grounding point (P₄). Electrical connectivity in all cases is maintained by the highly conductive BGE. Currents and potentials can be calculated by applying Kirchhoff's circuit laws (see Supporting Information for more details).

In the setup without grounding of the transfer line (Figure 1A), point P_5 bears a high potential ($P_5 = V_{ESU}$ since P_6 is grounded). Since P_2 and P_5 are connected, there is also a



Figure 2. Schematics of the standard steel probe (A) and fused silica probe (B) configurations. The standard steel probe possesses a metallic, inner capillary (1). Thus, the liquid inside this capillary is connected directly with the ESI potential through a metal contact (2). Furthermore, the capillary is contacted at the tip of the probe by the effluent itself (3). The fused silica probe (4) is nonconductive, that is, the liquid is no longer connected directly via the metal contact (5). Furthermore, the capillary was withdrawn by about 1 mm. This creates a microspray (6) just before the actual electrospray. This microspray restricts the flow of electrical charges to the direction of the flow, that is, no current can leak from the tip against the flow anymore.

potential at P₂ (39 V) and current flow (about 2.4 mA for I_{R1} and I_{R2}) in the chip even if no separation voltage (V_{FFE}) has been applied yet. The potential difference between P2 and the FFE electrodes (P_1 and P_3) creates an electric field (7.6 V cm⁻¹) in the chip. With an applied FFE separation voltage, this potential adds to the FFE-created potential resulting in a potential of -334 V instead of -375 V (sole chip). Although this change seems minuscule, it causes an asymmetry in both potential distribution and current flow in the chip. Furthermore, there is a potential difference of 5.8 kV between P_2 and P_5 , which creates a large electric field (150 V cm⁻¹) in the tubing between FFE and MS. The magnitude of the potential created by the ESI power supply in the transfer line and FFE device unambiguously suggests that the ESI potential is responsible for the aforementioned effects of stream shifting and arcing.

In order to suppress these effects, grounding is introduced at P_4 (Figure 1B). Effectively, grounding divides the circuit into two subcircuits, namely one for the FFE device and one for the ESI interface, which can be examined independently. If there is no FFE separation voltage applied ($V_{FFE} = 0$), all potentials in the FFE subcircuit become zero and no current is flowing, regardless of the applied ESI potential. With FFE separation voltage applied ($V_{FFE} = 750$ V), the potential at P_2 is -371 V, which resembles the potential in the sole chip (-375 V) and preserves an almost symmetrical potential distribution and current flow. The current flow between P_2 and P_4 is relatively small (approximately 500 μ A compared to 23 mA in the chip). Conclusively, the grounding removed every perceptible, negative effect on FFE separation as desired and expected.

In the ESI subcircuit, the potential applied to the ESI spray between P_5 and P_6 is exactly the same as between P_5 and P_4 . However, the resistance of R_5 (416.1 k Ω) is 2 orders of magnitude smaller than that of $R_{\rm ESI}$ (110 M Ω). This leads to a high current (13.2 mA) flowing through the transfer line away from the ESI probe. The high voltage source of an ESI interface is usually loaded with a high-impedance resistor (several M Ω) and supports currents in the nA or low μ A scales. Therefore, it most likely cannot stably maintain the potential at P_5 , which is one of the main factors contributing to the stability and the performance of the ESI spray itself. The higher the load, indicated by the current in the transfer line, attached to the ESI interface, the less stable the potential at P_5 is.

Conclusively, while grounding improves the situation for FFE by symmetrizing the potential distribution, it impairs the situation for the ESI spray. This means that grounding is not a sufficient solution. **Dependencies of Interference Effects.** In order to find dependencies of and solutions to eliminate these interferences, we examine and describe them mathematically. For this, we define two descriptors, H_{asym} and H_{leak} , as measures for the asymmetric potential distribution and the current leak at the ESI probe, respectively (see Supporting Information for details).

We found that the main contributor to H_{asym} is the ratio of geometries (length over cross-section) of FFE and the tubing of the transfer line. However, the sensitivity of H_{asym} to this contribution changes with applying grounding. $\dot{H}_{\rm asym}$ is a steeply growing function when no grounding is applied; small changes in the geometry ratio can lead to very large changes in symmetry. In contrast, H_{asym} is a linear function when grounding is applied; it is less sensitive to changes in the geometry ratio. Due to the change in sensitivity the overall value of H_{asym} also changes. In our specific case, it decreases from 0.25 to 0.023 when grounding is applied, suggesting that the potential distribution in the chip is almost perfectly symmetrical after grounding, and further modifications to the setup are not necessary. Therefore, grounding is the most straightforward and most practical way of ensuring a symmetrical potential distribution in FFE.

Furthermore, we found that H_{leak} depends on both the conductivity of the BGE as well as the geometry of the transfer line. This means that the highly conductive BGE and the large dimensions of the transfer line, which are both required for a production-scale setup, cause the current leak. We assume that for $H_{\text{leak}} \leq 0.1$, that is, the current leaking through the transfer line is at a maximum only a tenth of the ESI current, the influence of the leak on the potential of the ESI spray is insignificant. In our setups, H_{leak} is about 100 for the nongrounded circuit and about 270 for the grounded circuit. The large values indicate that the current leak significantly influences the electrical potential on the ESI probe. Any changes that minimize H_{leak} lead to minimizing the current leak. This can be done by either adjusting the geometries of the transfer line or the conductivity κ of the BGE. Changing the geometries (here: increasing the length or decreasing the crosssection area of the transfer line) is not feasible. In our case, to reach a H_{leak} value of 0.1, the transfer line should be either 1000 times longer (i.e., about 380 m) or 1000 times narrower (i.e., about 1.6 μ m inner diameter) for the setup without grounding in the transfer line. The factor is even larger, namely, 2700, for the setup with grounding. In principle, the current leak can be suppressed by drastically decreasing the conductivity κ . The later could be done by reducing the concentration of BGE in the transfer line by dialysis or dilution. However, this solution is

Analytical Chemistry

elaborate and also undesirably reduces the concentration of analytes. For certain combinations of analytes and BGEs, chemical or electrolytical suppressors used in ion chromatography might be an option to remove the electrolyte without affecting the analytes; ¹² although it is also not a straightforward procedure. To conclude, it appears that adjusting the geometries of the transfer line or the conductivity κ of the BGE are not feasible strategies. Rather, another method is required, which can hopefully prevent current leakage at the ESI completely.

Preventing Current Leakage at ESI Interface. Preventing current leakage is not straightforward, though. The resistivity of the transfer line has to be drastically decreased while maintaining the flow of material from the transfer line to ESI. We achieved this by two modifications to the ESI source. First, we replaced the standard steel probe with a low conductive, fused silica probe. Second, we withdrew the inner capillary of the ESI probe by about 1 mm. These modifications electrically disconnected the liquid effluent of FFE from the ESI probe (Figure 2). Such an uncommon configuration caused the mixing of effluent and nebulizer gas before the tip of the probe, which created a microspray before the actual electrospray. A spray can be thought of as a conglomeration of droplets. Such droplets, which are surrounded by inert gas, possess a limited ability to exchange charges. Of course, they still carry charges. However, these charges can only move in the direction of the physical flow of the droplets, that is, only to the tip of the ESI probe. Thus, there is no current of charges coming from the probe tip toward the FFE (or the ground at P_4) anymore. This is indicated by the independence of the dye signals on the state of grounding (Figure S5). Also, alternating the separation voltage during experiments did not influence the ESI-spray (see Figure S6 and text in the Supporting Information for details). It should be noted that this configuration provides nonoptimal conditions for the electrospray. However, in our model case, the quality of the spray only decreased marginally resulting in slightly higher noise (compare Figure S5 to Figure S2).

Superficially, the modified probe configuration renders the grounding of the transfer lines redundant. However, there is still a current flowing from FFE (P_2) through the tubing (to P_4). With the grounding removed, this current might reach and influence the microspray and, in turn, the electrospray. Due to this and electrical safety reasons, the transfer line(s) should stay grounded.

Interpretation of Results of Previous μ FFE-MS Works. As mentioned earlier in the paper, Chartogne et al., Benz et al., and Park et al., who previously reported on μ FFE-MS, did not report any interferences between FFE and MS.^{17–19} As a result, they did not need to use any kind of preventive measures for their interfacing FFE-MS. In order to compare our nongrounded, unmodified system to theirs, we calculated the currents and potentials in their systems using the electric circuit diagram from Figure 1A. The calculated values for the conductivity of the BGEs and the H_{assym} and H_{leak} descriptors can be found in Table 1 (see Supporting Information for details).

Although the separation zone of our FFE chip is about 100–1000 times larger in volume, our geometry (ratio of length $L_{\rm FFE}$ over cross-section $A_{\rm FFE}$) is comparable to those of the other works. The only exception is the chip of Park et al., which has a very small cross-section. In contrast, the cross-section of our transfer line is about 150–250 times larger, resulting in a likewise smaller geometry (ratio of length $L_{\rm trans}$ over cross-

Table 1. Calculations for Different FFE-MS Approaches (Using the Circuit Scheme of Figure 1A)

background electrolyte ^a	FFE geometry ^b	transfer line geometry c	ĸ	$H_{ m asym}$	$H_{ m leak}$	ref
	$L_{ m FFE}/A_{ m FFE}$	$L_{ m trans}/A_{ m trans}$	${ m mS}~{ m cm}^{-1}$			
1% acetic acid, pH 2.7	$10 \text{ mm}/2.5 \text{ mm}^2$	$20 \text{ cm}/0.0044 \text{ mm}^2$	3.6×10^{-2}	4.8×10^{-3}	8.8×10^{-3}	17
2 mM ammonium acetate, pH 4 in 70% methanol	$11.7 \text{ mm}/1.0 \text{ mm}^2$	$3 \mathrm{cm}/0.0010 \mathrm{mm}^2$	6.4×10^{-3}	9.7×10^{-4}	2.9×10^{-3}	18
10 mM formic acid, 7 mM propionic acid, pH $3.4-4.3~(\mathrm{NH_4^+})$	$23 \text{ mm}/0.075 \text{ mm}^{2d}$	$30 \text{ cm}/0.0079 \text{ mm}^2$	2.6×10^{-1}	3.1×10^{-2}	8.5×10^{-2}	19
10 mM HEPES, pH 7.5 (NaOH)	$100 \text{ mm}/18 \text{ mm}^2$	$38 \text{ cm}/2.0 \text{ mm}^2$	1.7	2.5×10^{-1}	1.0×10^{2}	this work ^f
10 mM HEPES, pH 7.5 (NaOH)	$100 \text{ mm}/18 \text{ mm}^2$	FFE: $24 \text{ cm}/2.0 \text{ mm}^2$	1.7	2.3×10^{-2}	2.7×10^{2}	this work ^g
		ESI: 14 cm/2.0 mm ²				
10 mM HEPES, pH 7.5 (NaOH)	$100 \text{ mm}/18 \text{ mm}^2$	FFE: $24 \text{ cm}/2.0 \text{ mm}^2$	1.7	2.3×10^{-2}	0.0	this work ^h
		ESI: $14 \text{ cm}/2.0 \text{ mm}^2$				
sground electrolyte composition including pH and titrator (in ¹	orackets if known). ^b Geome	try of the chip: $L_{ m FFE}$ is the dist	ance between the e	lectrodes and A _{FFE} is	s the cross-section	of the separation

subcircuit, respectively. ^dThis value results from the narrow geometry (5 μ m etching height of the chip). ^eConductivity of the background electrolyte. ^fValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figure 1. ^hValues calculated using the circuit scheme of Figur chamber. c Geometry of the transfer line: $L_{
m trans}$ is the length and $A_{
m FFE}$ is the cross-section of the transfer line. For the last two rows, two values for length are given, which correspond to the FFE and the ESI ^aBack

8418

section A_{trans}). In turn, H_{asym} becomes much larger in our case, leading to an asymmetrical potential distribution in FFE ($H_{\text{asym}} = 0.25$). In contrast, the other groups have almost perfect symmetric potential distribution ($H_{\text{asym}} \leq 0.031$) in their μ FFE chips. Furthermore, our BGE is up to 280 times more conductive, which results, together with the large geometry of the transfer line, in a likewise larger H_{leak} compared to the other works. This causes a large current flow from the ESI tip toward the FFE chip in our setup ($H_{\text{leak}} = 100$), while for the other groups, a current flow is barely perceptible ($H_{\text{leak}} \leq 0.085$). Conclusively, all previous works did not perceive nor examined any of the interference reported in this article because the effects are negligible at their conditions (geometry and BGE conductivity).

Furthermore, we can track and compare the changes of the descriptors when applying grounding only and when applying grounding and additionally using the modified ESI probe configuration (last two rows in Table 1). It is obvious that with grounding our $H_{\rm asym}$ value (0.023) is comparable to the ones of the other works ($H_{\rm asym} \leq 0.031$). However, $H_{\rm leak}$ almost tripled ($H_{\rm leak} = 270$) because the current leaking from the ESI probe through the transfer line almost tripled as described before. The modified ESI probe configuration suppresses this current leak completely and, subsequently, sets $H_{\rm leak}$ to near zero, which is again comparable to the other works ($H_{\rm leak} \leq 0.085$). Conclusively, by applying grounding and using the modified ESI probe configuration we approached the low level of interference characteristic of μ FFE–MS in a production-scale FFE setup.

CONCLUSIONS

In summary, we described an approach to connect highly conductive BGE-FFE with MS. We found a significant electric interference between FFE and MS, which has two substantial effects. First, it causes an asymmetric current and electric potential distribution in FFE. Second, it creates a current leak at the ESI probe, which negatively influences the spray. We examined this interference and its effects in detail and found that the asymmetric current distribution only depends on the geometry, while the electric current at the ESI interface depends on the geometry as well as the conductivity of the BGE. For the asymmetric current distribution, we have shown that not only grounding the transfer line is the most straightforward, but it is also the only practical solution. However, there is no feasible and straightforward method to suppress the electric current leakage at the ESI interface. Therefore, we used an uncommon ESI probe configuration to prevent charges flowing from the ESI probe tip toward FFE. We achieved this by using a low-conductivity, fused silica probe with a slightly withdrawn inner capillary. While this uncommon probe configuration might be not the ultimate solution, it allows the interference-free hyphenation of highly conductive, production-scale FFE to ESI-MS.

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.anal-chem.6b02235.

Experimental details (PDF).

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Notes

The authors declare no competing financial interest.

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The hyphenation of production-scale free-flow electrophoresis to electrospray ionization mass spectrometry using a highly conductive background electrolyte

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Supporting information

TABLE OF CONTENTS

- Reagents, FFE-MS setup, experimental parameters, and experimental conditions for mass spectrometry (S2)
- Calculating the conductivity of the electrolyte (S1)
- Calculating the potentials and currents of the electric circuits (S3)
- Calculating of the descriptors H_{asym} and H_{leak} (S7)
- Effect of grounding on analyte signals with new ESI-probe configuration (S10)
- Examining the influence of FFE on ESI-spray with new ESI-probe configuration (S10)
- Previous µFFE-MS works (S11)

Note: In the following text, P_x (roman P) refers to the node/point in the electric circuit, while P_x (italic P) refers to the electrical potential in the corresponding point. The values of potentials are with respect to ground.

Safety considerations. The performed experiments involve high voltage sources, which interfere. This can lead to unpredictable effects and should, therefore, be performed with caution (danger of electrical shock). Therefore, appropriate precautions (e.g. protective gear, presence of an assistant) should be taken.

REAGENTS

All solutions were prepared using analytical grade reagents. 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES), rhodamine 6G hydrochloride (rhodamine), fluorescein sodium salt (fluorescein), and sodium hydroxide were purchased from Sigma Aldrich (Oakville, ON, Canada). Deionized water (18.2 M Ω cm⁻¹) was used for preparation of all solutions.

FFE-MS SETUP

A schematic of our FFE-MS setup is depicted in **Figure S1**. The setup consists of an FFE-device, in which the sample outlet 2 is directed towards the electrospray ionization (ESI) interface of the MS instrument by a transfer line (polyethylene tubing; inner diameter of 1/16"; 38 cm long). The effluent of outlets 1 and 3 are collected. Transfer line 2 and collection lines 1 and 3 (same material, diameter, and length as transfer line) are grounded by small metallic connectors (1 cm long) between the two pieces of tubing. The metallic connectors were connected to the case of the MS instrument, which is grounded. The lengths from the FFE outlets to the metal connector of the transfer line to the MS was 14 cm.

EXPERIMENTAL PARAMETERS

We used HEPES (10 mM, pH 7.5) as BGE and a mixture of rhodamine and fluorescein (100 μ M each) as a well-studied model pair of analytes. Additionally, the individual dyes are visible to the naked eye, which allows their easy observation in FFE. Flow rates of the BGE and the sample solution were 5 mL min⁻¹ and 5 μ L min⁻¹, respectively. Liquid level of the chimneys was controlled by adjusting the flow rates at the electrolyte outlets by means of clamps (photo in **Figure S1**). The flush outlet at the far end of the chip was used only for purging the chip but was closed for the experiments. For purging, a 10% ethanol solution was passed through the device to wet the entire surface prior to the introduction of BGE. The FFE chip was cooled by a precooled metal block (-20°C, $10 \times 8 \times 4$ cm), which was periodically exchanged (every 20–30 min).

EXPERIMENTAL CONDITIONS FOR MS

An AB Sciex API 5000 equipped with Turbo VTM source (Vaughan, ON, Canada) was used for the experiments. For the initial experiments, we used a standard stainless steel probe/electrode configuration. For later experiments (see *Results and Discussion* section in the main text) we used a fused silica probe (wide bore, 0.5 mm inner diameter) plumbed from the FFE coupling line all the way to the end of the probe. The MS instrument was calibrated before the first experiments and optimized for detection of the two analytes: fluorescein and rhodamine. Experimental conditions for MS measurements are listed in **Table S1**.

Parameter	Value(s)					
Vacuum Gauge	3.3×10^{-5} Torr (4 mPa)					
Polarity	Positive					
Collision gas	6 psi (413 hPa)					
Curtain Gas [™] supply	10 psi (689 hPa)					
Nebulizer gas (GS1)	55 psi (3792 hPa)					
Heater gas (GS2)	15 psi (1034 hPa)					
Ionspray voltage	5500 V					
Source temperature	100 °C					
Collision cell exit potential	15 V					
Declustering potential	50 V					
Mass ranges	Q1 (m/z), Q3 (m/z), Dwell (ms)					
Fluorescein	333.1, 287.0, 25.0					
	333.1, 271.0, 25.0					
	333.1, 231.1, 25.0					
Rhodamine	444.2, 416.0, 25.0					
	444.2, 387.0, 25.0					
	444.2, 327.0, 25.0					

Table S1. Parameters of MS measurements.



Figure S1. Photo and schematic of the FFE-MS setup. For clarity, only sample outlets 1 to 3 are depicted.



Figure S2. Influence of grounding state on fluorescein and rhodamine signals. The dashed lines represent the corresponding zero lines (baselines). For this experiment, no separation voltage was applied ($V_{FFE} = 0$).

CALCULATING THE CONDUCTIVITY OF THE ELECTROLYTE

The electrical conductivity κ of the background electrolyte (BGE) can be calculated as:

$$\kappa = \frac{1}{\rho} = \frac{1}{R} \frac{L}{A} \quad (S1)$$

where ρ is the electrical resistivity, *R* is the electrical resistance between the two electrodes of the free flow electrophoresis (FFE) chip. The separation zone of the chip is defined by the cross-section area *A* and the distance *L* between the two electrodes. In our case, the cross-section area was $A = 9 \text{ cm} \times 0.2 \text{ cm} = 1.8 \text{ cm}^2$ and the distance between the electrodes was L = 10 cm. For our measurements, we found that for an applied voltage of 750 V, the average current I_{avg} was 23.0 mA resulting in a resistance $R = 33 \text{ k}\Omega$. The electrical resistivity and conductivity were $\rho = 5.9 \Omega \text{m}$ and $\kappa = 1.7 \text{ mS cm}^{-1}$, respectively.

CALCULATING THE POTENTIALS AND CURRENTS OF THE ELECTRIC CIRCUITS

Resistors. P_2 can refer to the physical point of any outlet. Thus, the resistances R_1 and R_2 depend on the location of P_2 . Using Equation (S1), it is possible to express the ratio of the resistances R_1 and R_2 as a simple ratio r_L of lengths since the conductivity κ and the cross-section area A is the same for both resistors:

$$\frac{R_1}{R_2} = \frac{L_1}{L_2} = r_L$$
 (S2)

where L_1 is the distance between P_1 and P_2 and L_2 is the distances between P_2 and P_3 . Obviously, if P_2 is exactly in the



Figure S2. Electric circuit diagrams of FFE-ESI-MS depicting the situation without (A) and with (B) grounding of the transfer line.

middle between the two electrodes (*i.e.* $L_1 = L_2$), r_L becomes equal to unity and R_1 and R_2 become equal to each other. For outlet 1 and 3 in our setup, r_L is 0.46 ($L_1 = 3.15$ cm, $L_2 = 6.85$ cm) and 2.17 ($L_1 = 6.85$ cm, $L_2 = 3.15$ cm), respectively. For all following equations, we assume that P_2 is always located between P_1 and P_3 (*i.e.* $P_1 \neq P_2 \neq P_3$), so that $R_1 > 0$ and $R_2 > 0$. Using r_L , R_1 can be expressed by a fraction of the total resistance R in the FFE chip:

$$R_{\rm I} = \frac{r_{\rm L}}{1+r_{\rm I}}R\tag{S3}$$

 R_2 is simply $R-R_1$. If P_2 is exactly in the middle between the two electrodes, the resistances R_1 and R_2 are exactly half of the total resistance R (32.6 k Ω), namely $R_1 = R_2 = 16.3$ k Ω . The remaining resistances R_3 , R_4 and R_5 can be calculated using Equation (S1) whilst taking into account the tubing dimensions: diameter of 1/16" = 1.5875 mm (resulting in a cross-section area of 1.979 mm²) and lengths of 38, 24, and 14 cm for R_3 , R_4 , and R_5 , respectively.

Circuit of a sole FFE chip. No matter where P_2 is located, the currents can be calculated by using Kirchhoff's circuit laws as:

$$I_{R_1} = I_{R_2} = \frac{U_{21}}{R_1} = \frac{U_{32}}{R_2} = \frac{U_{31}}{R_1 + R_2} = \frac{U_{31}}{R} = I_{avg}$$
(S4)

where I_{Rx} and R_x are the current through and the resistance of the corresponding resistor. U_{xy} is the potential difference between points P_x and P_y :

$$U_{\rm xy} = P_{\rm x} - P_{\rm y} \qquad (S5)$$

This, of course, should result in a current of 23.0 mA since this is just the back-calculation of our measurement, which was used to determine the conductivity of the electrolyte. From Equation (S4) we can find an expression for P_2 :

$$\frac{U_{21}}{R_1} = \frac{U_{32}}{R_2}$$
$$U_{21}R_2 = U_{32}R_1$$
$$R_2P_2 - R_2P_1 = R_1P_3 - R_1P_2$$
$$P_2(R_2 + R_1) = R_1P_3 + R_2P_1$$

This leads to the following general expression for P_2 in a sole chip:

$$P_2 = \frac{R_2 P_1 + R_1 P_3}{R_1 + R_2}$$
(S6)

We can express R_1 by r_L and R_2 using Equation (S2):

$$P_2 = \frac{R_2 P_1 + r_L R_2 P_3}{r_L R_2 + R_2} = \frac{P_1 + r_L P_3}{r_L + 1}$$
(S7)

In our case P_3 is grounded and its potential P_3 is zero. Therefore, P_2 becomes a simple fraction of P_1 :

$$P_2^{\text{sole}} = \frac{1}{r_{\text{L}} + 1} P_1$$
 (S8)

The resulting equation is very simple and easy to interpret. For example, if P₂ is located in the middle between the two electrodes (*i.e.* $r_{\rm L} = 1$), its potential P_2 becomes exactly half of the potential at P_1 .

FFE-MS circuit without grounding (Figure S3A). All resistances and all potentials except for potential P_2 are known. There is a large, positive potential at P_5 , a zero potential at P_6 , another zero potential at P_3 , and a zero or negative potential at P_1 . Therefore, the current flows from P_5 towards P_1 (or P_6) and from P_3 towards P_1 . This results in the following relationship of the currents:

$$I_{R_1} = I_{R_2} + I_{R_3}$$
 (S9)

The currents I_{R_1} , I_{R_2} , and I_{R_3} can be expressed *via* the potential differences and resistances:

$$\frac{U_{21}}{R_1} = \frac{U_{32}}{R_2} + \frac{U_{52}}{R_3}$$
(S10)

or potential values using Equation (S5):

$$\frac{P_2 - P_1}{R_1} = \frac{P_3 - P_2}{R_2} + \frac{P_5 - P_2}{R_3}$$
(S11)

We can solve Equation (S11) for P_2 :

$$\frac{R_2 R_3 (P_2 - P_1) - R_1 R_3 (P_3 - P_2) - R_1 R_2 (P_5 - P_2)}{R_1 R_2 R_3} = 0$$

$$R_2 R_3 (P_2 - P_1) - R_1 R_3 (P_3 - P_2) - R_1 R_2 (P_5 - P_2) = 0$$

$$R_2R_3P_2 + R_1R_3P_2 + R_1R_2P_2 = R_1R_3P_3 + R_1R_2P_5 + R_2R_3P_1$$

$$P_2(R_2R_3 + R_1R_3 + R_1R_2) = R_1R_3P_3 + R_1R_2P_5 + R_2R_3P_1$$

Finally, we get a general expression for P_2 :

$$P_2 = \frac{R_2 R_3 P_1 + R_1 R_3 P_3 + R_1 R_2 P_5}{R_2 R_3 + R_1 R_3 + R_1 R_2}$$
(S12)

We can express R_1 via r_L and R_2 using Equation (S2):

$$P_{2} = \frac{R_{2}R_{3}P_{1} + r_{L}R_{2}R_{3}P_{3} + r_{L}R_{2}^{2}P_{5}}{R_{2}R_{3} + r_{L}R_{2}R_{3} + r_{L}R_{2}R_{3} + r_{L}R_{2}} = \frac{R_{3}P_{1} + r_{L}R_{3}P_{3} + r_{L}R_{2}P_{5}}{R_{3} + r_{L}R_{3} + r_{L}R_{2}} = \frac{R_{3}\frac{P_{1} + r_{L}P_{3}}{R_{3} + r_{L}R_{3} + r_{L}R_{2}}}{R_{3} + r_{L}R_{2}}$$

The first term in the numerator resembles the term of P_2 for the sole chip in Equation (S7). Therefore, we factorize the whole fraction as follows:

$$P_{2} = \frac{P_{1} + r_{L}P_{3}}{r_{L} + 1} \frac{R_{3} + \frac{r_{L} + 1}{P_{1} + r_{2}P_{3}} \frac{r_{L}}{r_{L} + 1} R_{2}P_{5}}{R_{3} + \frac{r_{L}}{r_{L} + 1}R_{2}} =$$

$$= \frac{P_{1} + r_{L}P_{3}}{r_{L} + 1} \frac{1 + \frac{P_{5}}{P_{1} + r_{L}P_{3}} \frac{r_{L}R_{2}}{R_{3}}}{1 + \frac{1}{r_{L} + 1} \frac{r_{L}R_{2}}{R_{3}}}$$

From this equation it is easy to see that the very first factor is indeed the expression for P_2 of the sole chip in Equation S7. Again, in our case P_3 is grounded and its potential P_3 is zero. Furthermore, $r_L R_2$ can be replaced by R_1 giving the following expression for P_2 in the **n**on-grounded system:

$$P_{2}^{\rm ng} = P_{2}^{\rm sole} \frac{1 + \frac{P_{5}}{P_{1}} \frac{R_{1}}{R_{3}}}{1 + \frac{1}{r_{\rm L}} + 1} \frac{R_{1}}{R_{3}}} = P_{2}^{\rm sole} T^{\rm ng}$$
(S13)

The currents can then be calculated using the corresponding potential differences and resistances.

Difference in P_2 **between setup without grounding and sole chip.** Furthermore, Equation (S13) reveals the difference between the sole chip and the non-grounded, hyphenated setup. The difference (influence) is expressed by the modifierterm T^{ng} multiplied to P_2^{sole} . Because of this term, the potential P_2^{ng} depends on the ESI potential P_5 and the resistance R_3 of the transfer line. If we look closely at both the numerator and denominator in Equation (S13), it becomes clear that they only differ in the factor in front of the ratio of resistances. Given that the ESI potential (here: 5.5 kV) is practically always a lot larger than the FFE potential (here: -750 V) and r_L is a positive, real number, we get the following expressions for both ratios:

$$\frac{P_5}{P_1} < -1 \qquad \qquad 0 < \frac{1}{r_L + 1} < 1$$

Furthermore, the resistances R_1 and R_3 are always positive, real numbers. R_3 can be considered much larger than R_1 given that the resistance of the transfer line is larger than of the FFE chip.

$$0 \le \frac{R_1}{R_3} \ll 1$$

This assumption is reasonable since the cross-section of the tubing is a lot less than the cross-section of the FFE chip. Due to all this, T^{ng} gives a value between zero and one, *i.e.* the connected ESI source reduces the absolute value of P_2 compared to the sole chip. In the ideal case, it would be approaching unity meaning that the ESI source has no influence on FFE. For this, the denominator and the numerator have to be equal:

$$1 + \frac{P_5 R_1}{P_1 R_3} = 1 + \frac{1}{r_L + 1} \frac{R_1}{R_3}$$

$$\frac{P_5}{P_1} = \frac{1}{r_L + 1}$$

$$P_5(r_1 + 1) = P_1$$
(S14)

Again, $r_{\rm L}$ is a positive, real number, i.e. the expression $r_{\rm L}+1$ is always greater or equal to 1. This means that i) P_1 and P_5 have to have the same sign, ii) P_1 (FFE potential) has actually to be a multiple of P_5 (ESI potential) for $T^{\rm ng}$ to approach unity. Furthermore, the ratio of both potentials depends on the outlet connected to the ESI interface. Thus, it is practically impossible to fulfill the criteria given in Equation (S14).

FFE-MS circuit with grounding (Figure S3B). Introduced grounding at P_4 divides the circuit into two subcircuits, which can be calculated independently. Again, all resistances and all potentials except for potential P_2 are known. The ESI subcircuit is very easy to calculate. The current of I_{R_5} can be easily expressed by the following relation. Since P_4 is grounded its potential P_4 is zero:

$$I_{\rm R_5} = \frac{U_{54}}{R_5} = \frac{P_5 - P_4}{R_5} = \frac{P_5}{R_5}$$
(S15)

The currents in the FFE subcircuit are as follows:

$$I_{R_1} = I_{R_2} + I_{R_4}$$
 (S16)

The currents I_{R_1} , I_{R_2} , and I_{R_4} can be expressed by the potential differences and resistances

$$\frac{U_{21}}{R_1} = \frac{U_{32}}{R_2} + \frac{U_{42}}{R_4}$$
(S17)

and the potential differences can be expressed by the actual potentials using Equation (S5):

$$\frac{P_2 - P_1}{R_1} = \frac{P_3 - P_2}{R_2} + \frac{P_4 - P_2}{R_4}$$
(S18)

We can solve Equation (S18) for P_2 :

$$\frac{R_2 R_4 (P_2 - P_1) - R_1 R_4 (P_3 - P_2) - R_1 R_2 (P_4 - P_2)}{R_1 R_2 R_4} = 0$$

 $\begin{aligned} R_2 R_4 (P_2 - P_1) - R_1 R_4 (P_3 - P_2) - R_1 R_2 (P_4 - P_2) &= 0\\ R_2 R_4 P_2 + R_1 R_4 P_2 + R_1 R_2 P_2 &= R_1 R_4 P_3 + R_1 R_2 P_4 + R_2 R_4 P_1\\ P_2 (R_2 R_4 + R_1 R_4 + R_1 R_2) &= R_1 R_4 P_3 + R_1 R_2 P_4 + R_2 R_4 P_1\\ \end{aligned}$ Finally, we get a general expression for P_2 :

$$P_2 = \frac{R_2 R_4 P_1 + R_1 R_4 P_3 + R_1 R_2 P_4}{R_2 R_4 + R_1 R_4 + R_1 R_2}$$
(S19)

We can express R_1 by r_L and R_2 using Equation (S2):

$$P_{2} = \frac{R_{2}R_{4}P_{1} + r_{L}R_{2}R_{4}P_{3} + r_{L}R_{2}^{2}P_{4}}{R_{2}R_{4} + r_{L}R_{2}R_{4} + r_{L}R_{2}^{2}} =$$

$$= \frac{R_{4}P_{1} + r_{L}R_{4}P_{3} + r_{L}R_{2}P_{4}}{R_{4} + r_{L}R_{4} + r_{L}R_{2}} =$$

$$= \frac{R_{4}\frac{P_{1} + r_{L}P_{3}}{r_{L} + 1} + \frac{r_{L}}{r_{L} + 1}R_{4}P_{4}}{R_{4} + \frac{r_{L}}{r_{L} + 1}R_{2}}$$

The first term in the numerator resembles the term of P_2 for the sole chip in Equation (S7). Therefore, we factorize the whole fraction as follows:

$$P_{2} = \frac{P_{1} + r_{L}P_{3}}{r_{L} + 1} \frac{R_{4} + \frac{r_{L} + 1}{P_{1} + r_{2}P_{3}} \frac{r_{L}}{r_{L} + 1} R_{2}P_{4}}{R_{4} + \frac{r_{L}}{r_{L} + 1}R_{2}} = \frac{P_{1} + r_{L}P_{3}}{r_{L} + 1} \frac{1 + \frac{P_{4}}{P_{1} + r_{L}P_{3}} \frac{r_{L}R_{2}}{R_{4}}}{1 + \frac{1}{r_{L} + 1} \frac{r_{L}R_{2}}{R_{4}}}$$

From this equation it is easy to see that the very first factor is indeed the expression for P_2 of the sole chip in Equation S7. Furthermore, this equation and its derivation equal the ones for the non-grounded system (see last subsection). As before, P_3 is grounded and its potential P_3 is zero. Furthermore, $r_L R_2$ can be replaced by R_1 . The key difference is that P_4 is grounded, *i.e.* its potential is zero. This leads to the following general expression for P_2 in the **a**pplied-grounded system:

$$P_2^{\rm ag} = P_2^{\rm sole} \frac{1}{1 + \frac{1}{r_L + 1} \frac{R_1}{R_4}} = P_2^{\rm sole} T^{\rm ag}$$
(S20)

The currents can then be calculated using the corresponding potential differences and resistances.

Difference in P_2 **between all three setups.** Similar to the nongrounded case, Equation (S20) reveals the difference between the sole chip and the applied-grounded, hyphenated setup. The difference is expressed by the modifier-term T^{ag} multiplied to P_2^{sole} . However, in contrast to T^{ng} , which is expressed in Equation (S13), T^{ag} has 'lost' its numerator and, thus, does not depend on the ESI potential anymore. Still, there is a dependency on the resistance R_4 of the transfer line. However, we can make the assumption that the resistance of the transfer line is much larger than of the FFE chip. Therefore, the denominator of T^{ag} and, in turn, T^{ag} itself approaches unity:

Conclusively, applied grounding renders the influence of the hyphenation on the potential P_2 negligible:

$$P_2^{\rm ag} \approx P_{2,\rm approx}^{\rm ag} = P_2^{\rm sole} T_{\rm approx}^{\rm ag} = P_2^{\rm sole}$$
(S22)

Calculated values of circuits. All values calculated with the above formulas above are listed in **Table S2**. For discussion, see main text.

Table S1. Calculated values of electrical potentials and currents for electric circuits in Figure S3 with P_2 being the sample outlet 2. The potentials at P_3 , P_6 , and P_4 (when present) are always 0 V and, therefore, not listed. ^aCalculated values for an FFE chip without any additional connections or hyphenations for comparison. ^bFor clarity, the absolute values of the currents are given only.

	Configuration	Voltages and potentials, V					Current ^b , mA				
		$V_{\rm FFE}$	$V_{\rm ESI}$	P_1	P_2	P_5	$I_{\mathrm{R}_{1}=16.3\mathrm{k}\Omega}$	$I_{\text{R}_2 = 16.3 \text{ k}\Omega}$	$I_{\mathrm{R}_3=1.13\mathrm{M}\Omega}$	$I_{\rm R_4 = 713 \ k\Omega}$	$I_{\mathrm{R}_{5}=416\mathrm{k}\Omega}$
1	sole FFE chip ^a	750	-	-750	-375	-	23.0	23.0	-	-	-
2	Fig. 3A (no ground)	-	5500	0	39	5500	2.4	2.4	4.7	-	-
3	Fig. 3A (no ground)	750	5500	-750	-334	5500	25.5	20.4	5.0	-	-
4	Fig. 3B (grounding)	-	5500	0	0	5500	0	0	-	0	13.2
5	Fig. 3B (grounding)	750	5500	-750	-371	5500	23.2	22.7	-	0.5	13.2

 $T^{\mathrm{ag}} \approx T^{\mathrm{ag}}_{\mathrm{approx}} = 1$ (S21)

CALCULATING DESCRIPTORS HASYM AND HLEAK

In order to find dependencies of and solutions to eliminate the described interferences (see main text), we examine them mathematically. For this, we introduce two descriptors.

Descriptor of potential asymmetry. First, we define H_{asym} as a measure of the asymmetry of potential distribution in the FFE chip as

$$H_{\rm asym} = \frac{U_{21}}{U_{32}} - r_L \tag{S23}$$

The ratio of potential differences can be expressed by the potentials themselves using Equation (S5):

$$\frac{U_{21}}{U_{32}} = \frac{P_2 - P_1}{P_3 - P_2} \quad (S24)$$

The potential at P_3 is zero since it is grounded. Substituting this expression into Equation (S23) gives:

$$H_{\text{asym}} = \frac{P_2 - P_1}{-P_2} - r_L = \frac{P_1}{P_2} - (r_L + 1)$$
(S25)

 H_{asym} is zero in a non-disturbed FFE-system (*i.e.* no asymmetry), which indicates the absence of any shifting effects as it is the case of the sole FFE chip (see below). For disturbed systems, the asymmetry factor deviates from zero and becomes a measure for the disturbance of the system.

Potential asymmetry in the sole chip. For the sole chip we can replace the second term of Equation (S25) by using Equation (S8) giving

$$H_{\text{asym}}^{\text{sole}} = \frac{P_1}{P_2^{\text{sole}}} - \frac{P_1}{P_2^{\text{sole}}} = 0$$

Obviously, there is no hyphenation-induced potential asymmetry in the sole chip because there is no hyphenation, *i.e.* H_{asym}^{sole} is always zero.

Potential asymmetry in non-grounded setup. The potential P_2 in Equation (S25) can be expressed by P_2^{ng} from Equation (S13):

$$H_{\text{asym}}^{\text{ng}} = \frac{P_1}{P_2^{\text{ng}}} - (r_L + 1) = \frac{P_1}{P_2^{\text{sole}}} \frac{1}{T^{\text{ng}}} - (r_L + 1)$$

Plugging in the expression for P_1 over P_2^{sole} from Equation (S8) gives

$$H_{\text{asym}}^{\text{ng}} = (r_L + 1) \frac{1}{T^{\text{ng}}} - (r_L + 1)$$

Plugging in the expression for T^{ng} from Equation (S13) gives after some rearranging a general expression for the asymmetric descriptor in the non-grounded setup:

$$\begin{split} H_{\text{asym}}^{\text{ng}} &= \frac{(r_L + 1) \left(1 + \frac{1}{r_L + 1} \frac{R_1}{R_3} \right)}{1 + \frac{P_5}{P_1} \frac{R_1}{R_3}} - (r_L + 1) &= \\ &= \frac{r_L + 1 + \frac{R_1}{R_3}}{1 + \frac{P_5}{P_1} \frac{R_1}{R_3}} - (r_L + 1) &= \\ &= \frac{r_L + 1 + \frac{R_1}{R_3} - (r_L + 1) - (r_L + 1) \frac{P_5}{P_1} \frac{R_1}{R_3}}{1 + \frac{P_5}{P_1} \frac{R_1}{R_3}} &= \\ &= \frac{\frac{R_1}{R_3} \left(1 - (r_L + 1) \frac{P_5}{P_1} \right)}{1 + \frac{P_5}{P_1} \frac{R_1}{R_3}} &= \\ &= \frac{1 - (r_L + 1) \frac{P_5}{P_1}}{\frac{R_3}{R_1} + \frac{P_5}{P_1}} \end{split}$$

 H_{asym}^{ng} now depends solely on the ratio of R_3 over R_1 . r_L is a parameter describing the position of P_2 . In theory, H_{asym}^{ng} depends also on the ratio of the two potentials at P_1 and P_5 . However, influencing the descriptor by adjusting the potentials is very limited since the potentials have to be in certain ranges for the FFE-MS tandem to be useful. Thus, this ratio is considered to be a constant. Further, the resistances can be expressed by

$$R_{\rm n} = \frac{1}{\kappa} \frac{L_{\rm n}}{A_{\rm n}} \equiv \frac{G_{\rm n}}{\kappa}$$
(S26)

where A_n and L_n are the area of the cross-section and the length of the conductor, respectively, κ is the conductivity of the BGE, and G_n is the geometry factor (the ratio of L_n over A_n). Conductivity κ is the same for both resistors since it is the same BGE flowing through the FFE as well as the tubing. Thus, it cancels itself out when substituting the resistances by Equation (S26). This means that H_{asym}^{ng} only depends on the ratio of the geometry factors G_1 and G_3 .

$$H_{\text{asym}}^{\text{ng}} = \frac{1 - (r_L + 1)\frac{P_5}{P_1}}{\frac{G_3}{G_1} + \frac{P_5}{P_1}}$$

Since G_1 describes the geometry of the FFE chip and G_3 the geometry of the transfer line (from P₂ to P₅), we replace them

by the more descriptive symbols of G_{FFE} and G_{trans} , respective-ly:

$$H_{\text{asym}}^{\text{ng}} = \frac{1 - (r_L + 1)\frac{P_5}{P_1}}{\frac{G_{\text{trans}}}{G_{\text{FFE}}} + \frac{P_5}{P_1}}$$
(S27)

It is important to point out that due to this, asymmetric potential distribution would also happen when a low conductive electrolyte, such as acetic acid, is used for a production-scale setup. H_{asym}^{ng} can be seen as a function of the ratio of geometry factors G_{FFE} and G_{trans} :

$$H_{\text{asym}}^{\text{ng}}(x) = \frac{1 - (r_L + 1)\frac{P_5}{P_1}}{\frac{1}{x} + \frac{P_5}{P_1}}$$
(S28)

where x is:

$$\frac{G_{\rm FFE}}{G_{\rm trans}} \equiv x$$

The shape of the function depends on the position of the outlet, which is described by the parameter $r_{\rm L}$ (Figure S4).

Potential asymmetry in grounded setup. The potential P_2 in Equation (S25) can be expressed by P_2^{ag} from Equation (S20):

$$H_{\text{asym}}^{\text{ag}} = \frac{P_1}{P_2} - (r_L + 1) = \frac{P_1}{P_2^{\text{sole}}T^{\text{ag}}} - (r_L + 1)$$

Plugging in the expression for P_1 over P_2^{sole} from Equation (S8) gives

$$H_{\text{asym}}^{\text{ag}} = (r_L + 1) \frac{1}{T^{\text{ag}}} - (r_L + 1)$$

Plugging in the expression for T^{ag} from Equation (S20) gives after some rearranging a general expression for the asymmetric descriptor in the applied-grounded setup:

$$H_{\text{asym}}^{\text{ag}} = (r_L + 1) \left(1 + \frac{1}{r_L + 1} \frac{R_1}{R_4} \right) - (r_L + 1) =$$
$$= r_L + 1 + \frac{R_1}{R_4} - (r_L + 1) = \frac{R_1}{R_4}$$

Again, if we substitute the resistances R_1 and R_4 by Equation (S26) we see that the descriptor only depends on the ratio of the geometry factors:

$$H_{\rm asym}^{\rm ag} = \frac{G_1}{G_4}$$

Since G_1 describes the geometry of the FFE chip and G_4 the geometry of the transfer line (from P₂ to P₄), we replace them – analogously to H_{asym}^{ng} – by the more descriptive symbols of G_{FFE} and G_{trans} , respectively:

$$H_{\rm asym}^{\rm ag} = \frac{G_{\rm FFE}}{G_{\rm trans}} \quad (S29)$$

Similar to H_{asym}^{ng} , the conductivity of the BGE plays no role for the asymmetric potential distribution. In contrast, however, H_{asym}^{ag} has no – even theoretical – dependency on the potentials and the dependency on the ratio of geometry factors is very simple – linear proportional. Again, H_{asym}^{ag} can be seen as function of the ratio of geometry factors G_{FFE} and G_{trans} :

$$H_{\rm asym}^{\rm ag}(x) \equiv x$$
 (S30)

where x is the same as for H_{asym}^{ng} :

$$\frac{G_{\rm FFE}}{G_{\rm trans}} \equiv x$$

 H_{asym}^{ag} has no parameter and, therefore, its shape does not depend on the position of the outlet.

Comparing H_{asym} for non-grounded and grounded setup. The descriptor for the asymmetrical potential distribution is quite different for both setups. However, in both cases the main contributor is the ratio of geometry factors of the FFE chip and the transfer line $(G_{FFE}/G_{trans} = x)$. Therefore, we describe both descriptors as functions of x in order to compare and examine the influence of grounding. Applying grounding to the setup means to exchange H_{asym}^{ne} by H_{asym}^{ag} , *i.e.* the type of function changes dramatically with the state of grounding. It is a steeply growing function H_{asym}^{ng} when no grounding is applied (see Equation (S28) and **Figure S4**); small changes in x can lead to very large changes in symmetry. In contrast to H_{asym}^{ng} , H_{asym}^{ag} is a linear function (see Equation (S30)); it is less sensitive to changes in x. Both functions are plotted in **Figure S4**.

It is clear that the primary strategy to minimize H_{asym} is to increase the geometry factor of the transfer line (G_{trans}) as much as possible relative to the geometry factor of FFE (G_{FFE}). When grounding is applied, G_{trans} decreases and G_{FFE} does not change; however, H_{asym} still decreases significantly (from 0.25 to 0.023 in our example, see inset of **Figure S4**) due to the change of function $H_{asym}(x)$. In our specific case, $H_{asym}^{ag} = 0.023$ suggests that the potential distribution in the chip is almost perfectly symmetrical, and further modifications to the setup are not necessary. Therefore, grounding is the most straightforward and most practical way of ensuring a symmetrical potential distribution in FFE.

Descriptor for current leak at the ESI interface. We define a second descriptor, H_{leak} , as a measure of current leak from the ESI probe (P₅) into the transfer line:

$$H_{\text{leak}} = \frac{I_{\text{R}_{\text{trans}}}}{I_{\text{R}_{\text{ESI}}}} = \frac{R_{\text{ESI}}U_{\text{trans}}}{R_{\text{trans}}U_{\text{ESI}}} (S31)$$

 H_{leak} is zero if there is no current leak and non-zero otherwise.

Current leak in non-grounded setup. For this setup, H_{leak} corresponds to the geometry factor G_3 as well as the P_2^{ng} , P_5 , and P_6 :

$$H_{\text{leak}}^{\text{ng}} = \frac{I_{\text{R}_3}^{\text{ng}}}{I_{\text{R}_{\text{ESI}}}} = \frac{R_{\text{ESI}}U_{52}}{R_3U_{56}} = R_{\text{ESI}}\frac{\kappa}{G_3}\frac{P_5 - P_2^{ng}}{P_5 - P_6} \quad (S32)$$

Since P₆ is grounded its potential is zero.

Current leak in the applied-grounded setup. Here, H_{leak} corresponds to the geometry factor of G_5 :

$$H_{\text{leak}}^{\text{ag}} = \frac{I_{\text{R}_{5}}^{\text{ag}}}{I_{\text{R}_{\text{ESI}}}} = \frac{R_{\text{ESI}}U_{54}}{R_{5}U_{56}} = R_{\text{ESI}}\frac{\kappa}{G_{5}}\frac{P_{5} - P_{4}}{P_{5} - P_{6}}$$
(S33)

Since P₄ and P₆ are grounded their potentials are zero.

Comparing H_{leak} **for non-grounded and grounded setup.** In order to compare H_{leak}^{ng} and H_{leak}^{ag} we define a ratio r_{f} as

$$r_{f} = \frac{H_{\text{leak}}^{\text{ng}}}{H_{\text{leak}}^{\text{ag}}} = \frac{G_{5}}{G_{3}} \frac{P_{5} - P_{2}^{ng}}{P_{5}} = \frac{A_{5}L_{3}}{A_{3}L_{5}} \frac{P_{5} - P_{2}^{ng}}{P_{5}}$$
(S34)

Grounding the transfer line does not change the type of tubing used for the transfer line. Hence, the cross-section is the same for both, *i.e.* $A_3 = A_5$:

$$r_{f} = \frac{L_{3}}{L_{5}} \frac{P_{5} - P_{2}^{ng}}{P_{5}} = \frac{I_{R_{3}}^{ng}}{I_{R_{5}}^{ag}}$$
(S35)

However, its effective length is reduced greatly (by a factor of 2.7, from 38 to 14 cm, in our case) while the potential difference changes to a much smaller extent (5834 to 5500 V in our case). Thus, it is clear why the current leak increases upon applying grounding by the same factor as the decrease of the transfer line length (a factor of 2.6, from 5.0 to 13.2 mA, in our case).

Similar to H_{asym} , H_{leak} depends in both cases on the corresponding geometry factor (G_3 or G_5) of the transfer line. In contrast to H_{asym} , however, H_{leak} depends further on κ .

Significance of H_{leak} . The high voltage source of an ESI interface is usually loaded with a high-impedance resistor (several M Ω) and supports currents in the nanoscale or low microscale.



Figure S4. The two H_{asym} functions, H_{asym}^{ng} (shown for the corresponding $r_{\rm L}$ for outlet 1–3 of our setup) and H_{asym}^{ag} , for the non-grounded and grounded setups, respectively. H_{asym}^{ag} is much flatter and insensitive to changes in the geometry factors than H_{asym}^{ng} . The inset is an enlargement of the area marked by the dashed line. The arrow in the inset shows the change in our setup when grounding is applied for the middle outlet 2. The shift on the x-axis reflects the shortening of the transfer line from 38 to 24 cm (to the ground).

Therefore, it most likely cannot stably maintain the potential at P_5 , which is one of the main factors contributing to the stability and the performance of the ESI spray itself. The higher the load – indicated by the current in the transfer line – attached to the ESI interface, the less stable is the potential at P_5 .

Conclusively, while grounding improves the situation for FFE by symmetrizing the potential distribution, it impairs the situation for the ESI spray. This means that grounding is not a sufficient solution. Both descriptors, H_{asym} and H_{leak} , have to be minimized in order to have a functional production-scale FFE-MS setup.

EFFECT OF GROUNDING ON ANALYTE SIGNALS WITH NEW ESI-PROBE CONFIGURATION

The new configuration caused the mixing of effluent and nebulizer gas before the tip of the probe. This created a microspray before the actual electrospray. A spray can be thought of as a conglomeration of droplets. Such droplets, which are surrounded by inert gas, possess a limited ability to exchange charges. Of course, they still carry charges. However, these charges can only move into direction of the physical flow of the droplets, i.e. only to the tip of the ESI probe. Thus, there is no current coming from the probe tip towards the FFE (or the ground at P_4) anymore. This is indicated by the independence of the dye signals on the state of grounding (**Figure S5**).



Figure S5. With this new probe configuration, the grounding state no longer influences the analyte signals; no separation voltage was applied for this experiment ($V_{\rm FFE} = 0$). The dashed lines represent the corresponding zero lines (baselines).

EXAMINING THE INFLUENCE OF FFE ON ESI-SPRAY WITH NEW ESI-PROBE CONFIGURATION

On our final setup (with the low conductive probe and withdrawn inner capillary, see main text), we conducted experiments in which we turned on ($V_{FFE} = 750$ V) and off



Figure S6. Influence of the separation voltage (V_{FFE}) on the ESI spray. The dashed lines show the corresponding average signal at equilibrium before applying any voltage.

 $(V_{\text{FEE}} = 0 \text{ V})$ the separation voltage in order to see if FFE has any influence on the ESI spray. An exemplary experiment is depicted in Figure S6. The experiment starts without any separation voltage, *i.e.* both analytes flow into outlet 2, which is connected to the ESI interface. The signals were allowed to reach steady state (10-15 min) before turning on the separation voltage. It takes about 3-4 min for the signal to start decreasing. After about 5-7 min the signals for both analytes drop to their minima. At this point the two analytes are fully separated on the FFE chip and flow to outlets 1 and 3, respectively. After turning off the separation voltage, the analytes will again head to outlet 2 increasing the signal of both dyes again. After 13-15 min the signal is usually fully recovered. Furthermore, the signal to noise ratio compared to its original state is exactly the same. This indicates that neither the separation voltage itself nor turning it on or off affects the ESI spray in a quantifiable way.

PREVIOUS µFFE-MS WORKS

For all studies mentioned in the main text²⁻⁴, we used the same electrical circuit depicted in **Figure S3A** (with P₂ as the middle outlet, i.e. $r_L = 1.00$) to calculate the currents, potentials, and descriptors. It should be noted that, while this model circuit suits our setup very well, it might not be the optimal model for the other systems. The design of the ESI sources may differ between different MS models; in particular, the ESI potential or the ground might be applied at the sprayer. Here, we calculate a worst-case scenario in which the potential is fully applied at the sprayer (capillary), i.e. it can fully 'reach' for the µFFE. This model appears to be the most suitable for comparing the previous studies on µFFE-MS with our study.

All used parameters extracted from the publications as well as our own (for comparison) are listed in **Table S3**. The geometry factors for FFE were calculated as

$$G_{\rm FFE} = \frac{L_{\rm FFE}/2}{A_{\rm FFE}} = \frac{L_{\rm FFE}}{2W_{\rm FFE}h_{\rm FFE}}$$
(S36)

where $L_{\text{FFE}}/2$ is half the distance between the electrodes (since we calculate for P₂ in the middle), W_{FFE} is the length of the electrodes, and h_{FFE} the height of the separation chamber. By using the reported voltages U_{FFE} applied and currents I_{FFE} observed, the conductivity of the background electrolyte (BGE) can be calculated by the following formula (see also Equation (S1)). Since the geometry factor only reflects half of the chip (see above) we have to introduce a factor of 2 here again.

$$\kappa_{\rm BGE} = \frac{2G_{\rm FFE}}{R_{\rm FFE}} = \frac{2G_{\rm FFE}I_{\rm FFE}}{U_{\rm FFE}} \tag{S37}$$

The geometry factor for the transfer lines were calculated using

$$G_{\text{trans}} = \frac{L_{\text{trans}}}{A_{\text{trans}}} = \frac{L_{\text{trans}}}{\pi (d_{\text{trans}} / 2)^2}$$
(S38)

where L_{trans} and d_{trans} are the length and the inner diameter of the transfer line, respectively. *Benz et al.* used a self-built onchip-nanosprayer. The on-chip transfer line between the separation zone and the sprayer had a width of 50 µm and height of 20 µm, so that in this case the cross-section A_{trans} is just the product of these two.

With these geometry factors and the conductivities the descriptors were calculated by using Equation S27 and S32. 110 M Ω was used for R_{ESI} to calculate H_{leak} .

For calculating the last two rows of **Table 1** in the main tex of the manuscript, *i.e.* applying grounding and the modifying the ESI probe, we used Equation S29 and Equation S33 with the geometry factors for the corresponding part of the transfer line (0.12 for the FFE subcircuit and 0.071 for the ESI subcircuit). These factors were calculated using Equation S38 and the corresponding length of the transfer line from the FFE outlet or the ESI probe to P_4 , respectively (24 cm for the FFE subcircuit and 14 cm for the ESI subcircuit).

Note: All cited studies did not *report* any interference between μ FFE and MS. This does *not* mean that there were not any, which the respective authors had to overcome first to attain a working setup with the geometry and design as reported.

Table S3. Parameters used for calculations. ^a*Benz et al.* used a self-built on-chip-nanosprayer. The on-chip transfer line between the separation zone and the sprayer had a width of 50 μ m and height of 20 μ m. The cross-section A_{trans} is the product of these. ^bThis value was taken from Reference 1 not from the μ FFE-MS paper directly.

		Ref. 3	Ref. 2	Ref. 4
	This work	Chartogne et al.	Benz et al.	Park et al.
Voltage $U_{\rm FFE}$	-750 V	-56 V	-602 V	-1200 V
Current I _{FFE}	-23 mA	-50 μΑ	-33 μΑ	-100 μΑ
Resistance <i>R</i> _{FFE}	33 kΩ	1.1 MΩ	18 MΩ	12 MΩ
FFE Dimensions	$90 \text{ mm} \times 100 \text{ mm}$	$25 \text{ mm} \times 10 \text{ mm}$	20 mm × 11.7 mm	$15 \text{ mm} \times 23 \text{ mm}$
$(W_{\rm FFE} \times L_{\rm FFE} \times h_{\rm FFE})$	\times 200 μ m	\times 100 μ m	\times 50 μ m	\times 5.0 μ m
FFE profile A _{FFE}	$1.8 \times 10^{-5} \text{ m}^2$	$2.5 \times 10^{-6} \text{ m}^2$	$1.0 \times 10^{-6} \text{ m}^2$	$7.5 \times 10^{-8} \text{ m}^2$
Geometry factor $G_{\rm FFE}$	2.8 mm^{-1}	2.0 mm^{-1}	5.9 mm^{-1}	$1.5 \times 10^2 \text{ mm}^{-1}$
Conductivity $\kappa_{_{\rm BGE}}$	1.7 mS cm^{-1}	$3.6 \times 10^{-2} \text{ mS cm}^{-1}$	$6.4 \times 10^{-3} \text{ mS cm}^{-1}$	$2.6 \times 10^{-1} \text{ mS cm}^{-1}$
Length L_{trans} of transfer line	38 cm	20 cm	3 cm	30 cm
Diameter d_{trans} of transfer line	1/16" = 0.0625" = 1.59 mm	75 µm	$50 \ \mu m \times 20 \mu m^a$	100 µm
Cross-section A_{trans} of transfer line	$2.0\times 10^{-6}~m^{\text{2}}$	$4.4\times10^{-9}~m^{2}$	$1.0\times 10^{-9}~m^{\rm 2}$	$7.9\times10^{-9}~m^2$
Geometry factor G_{trans}	$1.9 \times 10^5 \text{ m}^{-1}$	$4.5\times10^7~\text{m}^{-1}$	$3.0 \times 10^7 \text{ m}^{-1}$	$3.8 imes 10^7 \ { m m}^{-1}$
ESI voltage $V_{\rm ESI}$	5500 V	3000 V	1200 V ^b	4000 V
Descriptor H _{asym}	2.5×10 ⁻¹	4.8×10 ⁻³	9.7×10 ⁻⁴	3.1×10 ⁻²
Descriptor H _{leak}	1.0×10 ²	8.8×10 ⁻³	2.9×10 ⁻³	8.5×10 ⁻²

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