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Research Article

A robust sheath-flow CE-MS interface for hyphenation with Orbitrap MS

The hyphenation of capillary electrophoresis with high-resolution mass spectrometry, such as Orbitrap MS, is of broad interest for the unambiguous and exceptionally sensitive identification of compounds. However, the coupling of these techniques requires a robust ionization interface that does not influence the stability of the separation voltage while coping with oxidation of the emitter tip at large ionization voltages. Herein, we present the design of a sheath-flow CE-ESI-MS interface which combines a robust and easy to operate set-up with high-resolution Orbitrap MS detection. The sheath liquid interface is equipped with a gold coated electrospray emitter which increases the stability and overall lifetime of the system. For the characterization of the interface, the spray stability and durability were investigated in dependence of the sheath-flow rate, electrospray voltage, and additional gold coating. The optimized conditions were applied to a separation of angiotensin II and neurotensin resulting in LODs of 2.4 and 3.5 ng/mL.

Keywords:
CE-MS / Electrospray ionization / Peptide separation
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1 Introduction

Since its development in the late 1980s [1], the hyphenation of high-resolution separations by CE with highly sensitive mass detection has proven to be a powerful tool in a broad range of research [2–6]. CE-ESI-MS is the most frequently used interface for online coupling, however, the interface set-up must overcome some challenging experimental restrictions. To start, the capillary outlet also has to serve as the electrospray emitter and it has been challenging to provide a stable electrical contact without using complicated assemblies or provoking side reactions at the capillary tip. Furthermore, the low flow of CE separations often hampers the successful establishment of a steady electrospray. Finally, limitations in the buffer selection have to be considered in order to ensure mass compatibility [7].

Several interface set-ups have been introduced and they can be categorized by the presence or absence of a flow assisting sheath liquid [8–10]. With the sheathless design, the CE effluent is usually sprayed directly from the capillary outlet into the mass spectrometer. This way sample dilution and dead volume can be reduced to a minimum to achieve very high sensitivities. Generating the electrical contact involves sophisticated fabrication at the capillary end, which is achieved by coating the outer tip of the capillary with conductive materials [11, 12], including an electrode inside the capillary outlet [13, 14] or using porous etched capillary walls [15]. However, sheathless interfaces often experience spray instabilities due to the low flow rates of CE. The separation buffer directly influences the spray quality which can result in a limited choice of BGE. For the very sensitive set-up and the special fabrication, adept operators are required limiting a broader application in CE-MS.

The second approach improves the spray performance by adding a flow enhancing sheath liquid to the BGE as soon as it exits the separation capillary. The sheath liquid needs to be mechanically or electrokinetically pumped and provides the electrical contact between electrode and CE effluent. Analyte ionization and mass compatibility of the separation buffer benefit by this approach. Such sheath-flow interfaces tend to be more physically robust and usually have simple set-ups. Two different constructional designs are possible for merging the sheath liquid and separation mixture. With liquid junction interfaces merging takes place in a small gap between capillary outlet and emitter, decoupling the CE separation and electrospray process [16]. Therefore, analyte dilution can be reduced and the efficient mixing results in a stable electrospray [17]. However, the junction also causes dead volume effects which can result in peak broadening and a loss of separation efficiency [18]. Moreover, low reproducibility of the gap adjustment poses a serious problem in the routine laboratory practice. A much wider range of applications can be found in the coaxial sheath-flow interface which combines a robust set-up and reproducible results. Here, the terminal separation capillary is surrounded by a tube that delivers the sheath liquid and usually acts as the

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Abbreviations: **EIE**, extracted ion electropherogram

Color online: See article online to view Figs. 1–3 in color.

electrospray emitter as well [19]. In this way, dead volume is reduced. However, high sheath-flow rates lead to analyte dilution resulting in a reduced sensitivity [20]. Several interfaces have been developed for minimizing the amount of sheath liquid required including electrokinetically driven sheath liquid [21], a flow-through microvial strategy [22], and a pressurized liquid junction [23]. Other developments include a nanoflow sheath liquid interface with a blunt metal needle for sheath liquid delivery and a protruding separation capillary [24]. Fang et al. developed a similar interface with a tapered sheath-flow capillary made of glass and an extendable separation capillary which can serve as the emitter [25].

Because of the highly efficient separations with sharp analyte peaks and small sample amounts common for CE analyses, mass spectrometers coupled to CE must provide fast scan rates and high sensitivities. Although an extensive variety of different mass spectrometers has been used so far, TOF-MS is the most prevalent [26, 27]. High sampling rates combined with adequate sensitivity, great resolution, and mass accuracy make it appealing for the application in CE-MS. Furthermore, hybrid mass spectrometers like QTOF enable the integration of MS/MS experiments for targeted and nontargeted approaches. Even higher mass accuracies and resolution can be achieved by Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) [28, 29]. Unfortunately, FT-ICR achieves only slow scan speeds and is expensive in purchase and maintenance compared to other instruments. Orbitrap mass analyzers provide an attractive compromise between the accurate FT-ICR and fast TOF-MS. Their major advantages are their high resolution even at lower molecular weight compounds and their superior and reproducible mass accuracy along the whole mass range. They are extremely sensitive and the scanning rates are fast enough for analyzing even high-speed separations [30, 31]. Although CE-Orbitrap MS is a highly promising and powerful method, so far there are only very few sheath-flow interfaces which implement this hyphenation. Amenson-Lamar et al. recently demonstrated the outstanding sensitivity of the Orbitrap mass analyzer with their electrokinetically driven sheath-flow interface (commercialized by CMP Scientific) by detecting 1 zmol of angiotensin II in a BSA background [32]. The interface uses an etched separation capillary which is positioned in a tapered glass emitter. A joint orifice of emitter and capillary is not possible, however, the narrow emitter orifice of not more than 35 μm enables very low sheath-flow rates of a few nanoliters per minute. Wang et al. used their homebuilt flow-through microvial interface coupled to an Orbitrap Fusion Lumos mass spectrometer for high-resolution cIEF-MS analysis of protein pI markers and infliximab [33]. New approaches for CE-Orbitrap MS include a cost-efficient sheath-flow interface with a blunt-tip fused silica emitter [34] and a tapered-tip CE-nanoESI interface by Nemes' group [35].

To date, the developed interfaces require a high level of expertise, and commercial interfaces lack the sensitivity and robustness to exploit the full potential of CE-MS. Herein, we report a very basic, reliable, but at the same time highly sensitive sheath-flow CE-ESI-MS interface. The presented system is characterized by its straightforward and robust design which still provides extremely accurate analyses and the integration of MS/MS experiments in combination with the Q Exactive Orbitrap mass spectrometer. In this system, high voltage is directly applied to a tapered stainless steel emitter which can also be subjected to a gold coating to increase lifetime and resistance against oxidation. The sheath liquid closes the electrical circuit and provides a stable electrospray. No nebulizing gas is applied, to avoid loss of efficiency.

2 Materials and methods

2.1 Materials and reagents

All reagents were purchased from the following commercial sources and were used without further purification. Angiotensin II, neurotensin, formic acid, and sodium hydroxide were obtained from Sigma-Aldrich (Steinheim, Germany), acetic acid from VWR (Radnor, USA). Isopropanol (HPLC grade) was obtained from Thermo Fisher Scientific (Waltham, USA). Ultrapure water was provided by a Puranity PU purification system from VWR. The peptide stock solutions were prepared in ultrapure water and were stored at −20°C. PEEK tee, ferrules and tubing were purchased from Sigma-Aldrich and Fisher Scientific (Schwerte, Germany). Bare fused silica capillaries were obtained from MicroQuartz (Munich, Germany). The isocratic pump and splitter assembly were purchased from Agilent (Waldbronn, Germany). The linear translation stage was obtained from Thorlabs GmbH (Dachau, Germany).

2.2 Instrumentation

All separations were performed using an Agilent 7100 CE system. ChemStation software (version C.01.07) was utilized for data acquisition and instrument control. All analyses were conducted at 25°C in positive polarity mode (anode at the capillary inlet) with an aqueous acetic acid solution (2 M) as BGE. For the experiments, bare fused silica capillaries (od 0.36 mm, id 0.05 mm) with a total length of 80 cm were used. The polyimide coating was removed (2 mm) at the MS end of the capillary. Prior to the first use capillaries were conditioned with water (2 min), followed by 0.1 M NaOH (5 min), water (2 min), and separation buffer (2 min), all injected under 1 bar pressure. Between runs the capillaries were flushed (1 bar pressure) with 0.1 M NaOH (30 s) followed by water (1 min) and separation buffer (2 min). In all cases, samples were injected hydrodynamically by applying a pressure of 30 mbar for 10 s. The level of the CE system was adjusted in such a way that separation capillary entry and ion transfer capillary inlet were at the same horizontal level to avoid suction effects.

Mass spectrometric analysis was performed with a Q Exactive Plus mass spectrometer by Thermo Fisher Scientific where the standard ESI source was replaced by the handmade CE-ESI-MS interface described in section 2.3. The mass

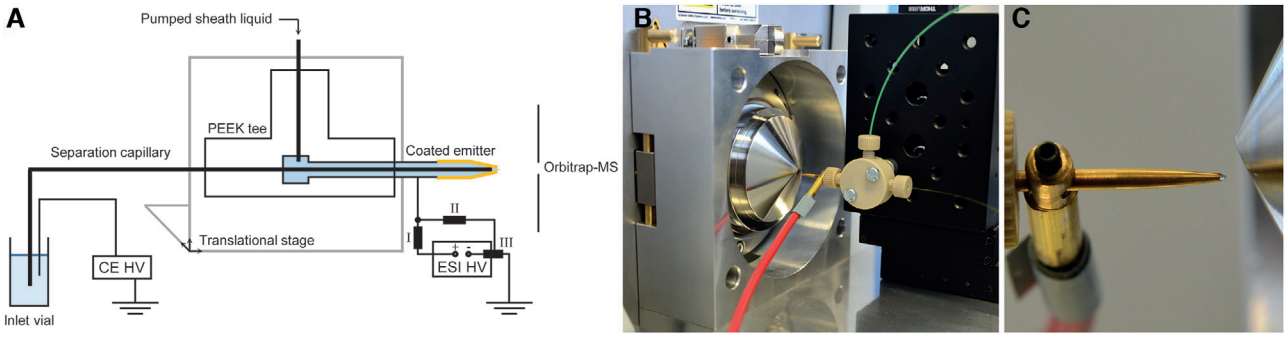

Figure 1. Schematic diagram (A) and images of the coaxial sheath-flow interface (B) including a close-up of the gold-coated emitter with Taylor cone (C). I, II, and III: 10, 50, and 0 MΩ resistor.

spectrometer inlet was set to ground potential and only a minimal flow of sweep gas was selected. The temperature of the ion transfer capillary was adjusted to 140°C and S-lens RF level was set to 50. For optimal spraying conditions, the distance between emitter tip and heated capillary inlet was fine-tuned prior to each run and was approximately 2 mm. The sheath liquid was a mixture of ultrapure water and isopropanol (50:50) with 0.05% formic acid.

2.3 Interface

The design of the interface is shown in Fig. 1. The separation capillary is threaded through a PEEK tee using a PEEK fitting for 1/16" od tubing and a graphite ferrule (id 0.4 mm). The capillary passes through the stainless steel electrospray emitter which is also attached to the tee by a PEEK fitting and silicon ferrule (id 1/16"). The tubing of an Agilent splitter assembly (G1607-60000) is attached to the orthogonal port of the tee and is connected to an Agilent 1260 Infinity II Isocratic Pump (G7110B) which is providing the sheath liquid. High voltage is applied to the injection end of the capillary (CE HV) and directly to the emitter (ESI HV). Separation was achieved by the difference in these potentials.

Emitters were made from a stainless steel tubing (od 1/16", id 0.5 mm) obtained from Altmann Analytik KG (Munich, Germany). The stainless steel tubing was cut into smaller pieces whose tip was tapered using a Dremel 8100 (Racine, USA). Evaluation of tip size, diameter, and quality were conducted using a Kruess MSZ5600 microscope (Hamburg, Germany). The gold coating procedure was performed as described elsewhere [36]. Briefly, the tapered emitter tip was dipped into an aqueous solution of gold(III)-chloride, potassium cyanide, sodium hydrogenphosphate, and sodium sulfite. A voltage of 1.5 to 2.2 V was applied to the emitter for 5 min. The resulting gold layer was rinsed with water to remove traces of cyanide.

The ESI HV was established with a high voltage power supply (13673-93) by Phywe Systeme KG (Goettingen, Germany). The PEEK tee was attached to a linear (*x*-*y*-*z*) translation stage to enable fine-tuning of the position and distance between emitter tip and heated capillary inlet. The translation stage was fixed on a custom-made stainless steel framework which in turn was joined to the mass spectrometer using the existing system and replacing the standard Ion Max Source.

3 Results and discussion

3.1 Design of the CE-ESI-MS interface

Here, we present the development of a sheath-flow CE-ESI-MS interface which combines a robust and easy to operate set-up with highly sensitive analyses. The basic and straightforward design is assembled from inexpensive, readily accessible parts that were adapted to our needs and can easily be renewed or replaced. The interface replaces the standard ESI source of the mass spectrometer and is attached through a custom-made stainless steel framework. A linear (*x*-*y*-*z*) translation stage allows for the fine-tuning of the position of the emitter tip and, thus, enables the adjustment of the optimal spraying conditions prior to each run. The sheath liquid was added to the system in a coaxial manner to enable post-separation reactions and modifications of the BGE for optimal electrospray conditions. Furthermore, coaxial sheath-flow interfaces tend to provide more stable and reproducible analyses compared to sheathless and liquid junction interfaces. There are no restrictions regarding the solvents that can be employed as sheath liquid in the introduced interface. High voltage was directly applied to a tapered stainless steel emitter connected to a resistor network to stabilize simultaneously the separation and spray voltage (cf. Fig. 1). To emulate the presence of an ESI source in the Thermo Orbitrap MS software, a 15 pin male adapter was modified with a resistor of 2.15 kΩ between pins 1 and 7, and the safety interlock by connecting pins 10 and 11. The quality of the emitter tip poses an important aspect for successfully establishing a stable electrospray. A symmetrical, hydrophobic, and tapered emitter is a prerequisite for a sharp Taylor cone which is mandatory for satisfactory sensitivities and resolution [37, 38]. In the presented system stainless steel emitters were used, since they are more stable than those made from glass. In case of wear, they can be newly tapered by hand and reused. A common reason for a limited lifetime are electrochemical reactions on

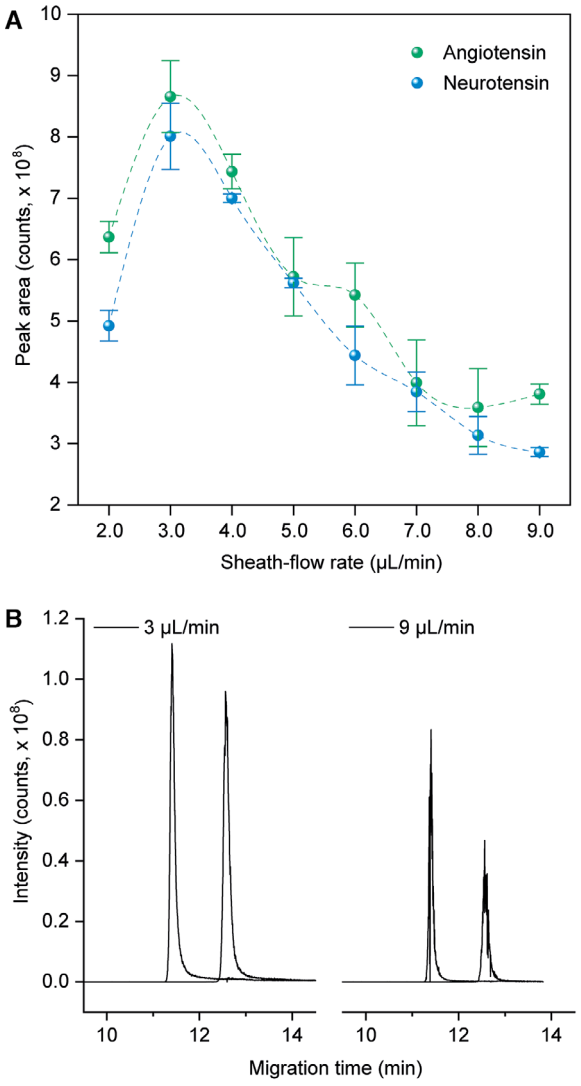

Figure 2. Peak areas of doubly charged angiotensin II (*m/z* 523.7745) and triply charged neurotensin (*m/z* 558.3105) as a function of the applied sheath-flow rate (A) and representative extracted ion electropherograms (EIEs) of 3 and 9 µL/min (B). All of the experiments were performed three times. The error bars display the standard deviation of the experiments. The dotted line is for illustration purposes only. BGE, acetic acid (2 M); sheath liquid, 50% v/v isopropanol aqueous solution with 0.05% formic acid; sample (angiotensin II and neurotensin each 10 µM in BGE) injected at 30 mbar for 10 s; CE inlet, 30 kV; emitter, 3.2 kV.

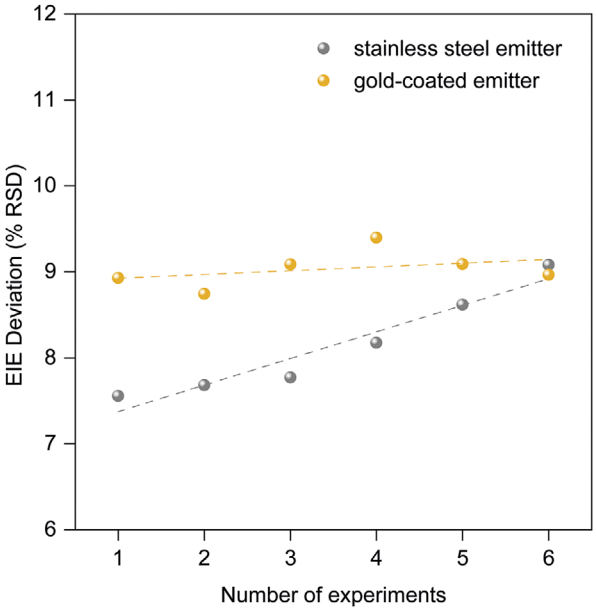

Figure 3. Deviation of the extracted ion electropherogram (EIE) as a function of the number of experiments executed with either the stainless steel emitter or the gold-coated emitter. Each experiment was a continuous infusion of 1 µM solution in BGE of angiotensin II for 50 min. BGE, acetic acid (2 M); sheath liquid, 50% v/v isopropanol aqueous solution with 0.05% formic acid; constant assisting pressure of 80 mbar; emitter, 3.2 kV.

the metal surface. Gas formation caused by solvent oxidation, corona discharge, and corrosion of the metal surface leads to mechanical and oxidative stress on the emitter [39]. Inert noble metals have a higher resistance against oxidative stress and therefore an additional gold coating [36] was applied to the emitter tip to increase stability and lifetime in our system. A possible decrease of the interface performance caused by sample adsorption on the gold layer was not investigated. The use of a nontransparent metal emitter makes repeatable and precise adjustments of the distance between capillary exit and emitter tip difficult. Since it has been shown that cavities between capillary and emitter tip are often the reason for band broadening and loss of both sensitivity and separation efficiency [21], a joint orifice of emitter and capillary was manually adjusted. For this purpose, emitters whose inner diameters were larger than the outer diameter of the separation capillary were used. This also significantly improved the ease of use, since a risk of emitter clogging was practically nonexistent. While in principle the enduring and stable interface design can be hyphenated to any mass analyzer, it was hyphenated to a Q Exactive Plus Orbitrap mass spectrometer for highly accurate analyses.

3.2 Characterization of the CE-ESI-MS interface

3.2.1 Sheath-flow rate and electrospray voltage

In the presented interface, high voltage is directly applied to the emitter and can be adjusted to an arbitrary value between 0 and 10 kV. In a first set of experiments, the influence of the ESI voltage on the separation of angiotensin II and neurotensin and their corresponding MS signals was examined. High voltage was varied between 2 and 4 kV and the resulting signal intensities, peak areas, and *S/N* ratios were compared. While low values provided only poor spray stability, high values exposed the emitter to potential oxidation. Apart from that, no significant influence could be observed and in further experiments the ESI voltage was adjusted to a value around 3 kV which supplied the most stable conditions.

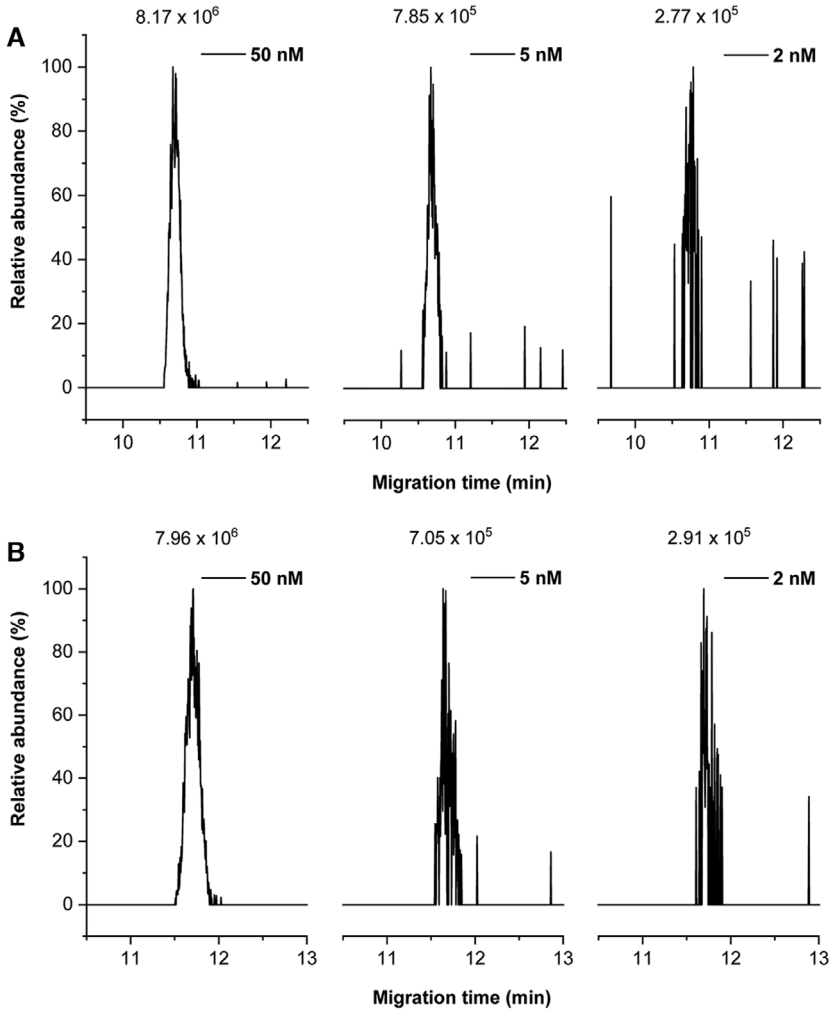

Figure 4. Extracted ion electropherograms (EIEs) of doubly charged angiotensin II (*m/z* 523.7745, A) and neurotensin (*m/z* 558.3105, B) at 50, 5, and 2 nM sample concentrations. The value above the respective MS signals represents the absolute intensity counts. The respective LOD was calculated from the separation of the 2 nM samples. For separation conditions, see Table 1.

The sheath liquid enhances the stability of the electrical contact at the ESI emitter tip. Furthermore, merging of the BGE with sheath liquid usually results in improved mass compatibility and ionization efficiency. However, dilution effects can also reduce the detection sensitivity of analyses. Thus, the objective is minimizing the sheath-flow rate while maintaining a stable electrospray. To evaluate dilution effects in our system, we applied different sheath-flow rates on a separation of a solution of angiotensin II and neurotensin (each 10 μM). The rate was varied between 2.0 and 9.0 μL/min and the corresponding peak areas were compared (Fig. 2A). With decreasing sample dilution, peak areas were gradually increasing until they reached their maximum value at 3.0 μL/min. Even lower sheath-flow rates produced only minor peak areas due to deficient spraying conditions. Furthermore, signal intensities varied together with the peak areas throughout the experiments depending on the sheath-flow rate. In contrast to extremely high or low values, the flow rate of 3.0 μL/min gave sharp, much more stable signals with significantly higher *S/N* (Fig. 2B). For further experiments, sheath-flow rates between 3 and 4 μL/min were usually adopted.

3.2.2 Effect of the gold-coated emitter on the long-term spray stability

Metal emitters provide a physically robust basis for analyses. They can be sharpened manually with little effort and give the opportunity for structural adjustments and reutilization. To increase the resistance against oxidative stress and lifetime, an additional gold coating was applied to the stainless steel emitters. The effect on the spray stability was evaluated by continuous injection of a 1 μM solution of angiotensin II while using either an uncoated or coated emitter. In both cases, a stable electrospray was produced throughout the whole run time of 50 min. The experiment was repeated six times so that the spray stability could be observed over a total time of 300 min. The RSD of the intensity in the extracted ion electropherograms (EIEs) of both emitters was examined (Fig. 3). Throughout the total number of analyses, the gold-coated emitter produced a slightly higher average value of 9% compared to that of the uncoated emitter (8%). However, a slight increasing trend of the RSD could be observed in the measurements of the uncoated emitter. Especially after experiment 3 (corresponding to 150 min of analysis time),

Table 1. Peak areas, signal intensities, and LODs of test proteins obtained with sheath-flow CE-ESI-MS interface under optimized conditions[a]

Protein	Peak area (x 10^5)[b]	Intensity (x 10^4)[b]	LOD (nM)[c]	LOD (ng/mL)[c]
Angiotensin	1.7	2.8	2.3	2.4
Neurotensin	1.3	2.9	2.1	3.5

a) CE capillary, 50 μm id, 80 cm; BGE, acetic acid (2 M); sheath liquid, 50% v/v isopropanol aqueous solution with 0.05% formic acid; sheath-flow rate 3.0 μL/min; sample injected at 100 mbar for 10 s; constant assisting pressure of 10 mbar; CE inlet, 30 kV; emitter, 3.2 kV; MS inlet capillary, 140°C; scan range: *m/z* 450–1300.
b) Protein concentration, 2 nM.
c) Calculated from the separation of the 2 nM solution.

the stability of the electrospray decreased relative to the previous experiments. In contrast, a comparably stable electrospray could be maintained with the gold-coated emitter over the entire period. The RSD values show no increasing trend remaining at the same level of 9%. Therefore, the coating reduced side reactions on the metal surface and lead to an enhanced long-term spray stability.

3.2.3 Limit of detection

The optimized conditions were applied to the separation of angiotensin II and neurotensin and the LOD of both compounds was determined. Usually the LOD is estimated by the $S/N = 3$. However, the EIEs of the Q Exactive Plus show no classic continuous background signal and instead, scarce single noise peaks occur independent of time (Fig. 4). The same problem was observed by Amenson-Lamar et al. who coupled a homebuilt CE-ESI-MS interface to a Q Exactive HF mass spectrometer to determine the LOD of angiotensin [32]. They used an alternative method described by Knoll [40] which estimates the LOD from the intensity and full width at half maximum of the sample peak, the duration of a blank segment, and the peak intensity of the largest noise fluctuation therein. As described by Amenson-Lamar et al. [32], we applied the same formula to the separation of angiotensin II and neurotensin (sample concentration in both cases 2 nM). Therefore, the estimated LODs of angiotensin II and neurotensin was 2.4 and 3.5 ng/mL (Table 1). Thus, the LODs obtained with this basic interface set-up are in the typical range of small peptides as they are also obtained by sheathless and nanoflow interfaces with lower analyte dilution [22, 41, 42].

4 Concluding remarks

We presented the design of a robust and stable coaxial sheath-flow CE-ESI-MS interface for highly sensitive analyses. The straightforward design is set-up from low cost components that can be easily modified for special experiments. The interface enables the utilization of various types of capillaries. Although any kind of coating or interior surface modification can be employed, in this study only uncoated capillaries were used. The gold coating enhanced the overall lifetime of the stainless steel emitters. The sheath liquid supplied further stabilization of the spraying conditions and increased ionization efficiency and mass compatibility. The interface was coupled to a high-performance and high-resolution Q Exactive Plus Orbitrap mass analyzer. The performance of the presented CE-ESI-MS system was demonstrated in a CE separation of angiotensin II and neurotensin. Although sheath-flow rates of a few microliters per minute were employed, increased ionization efficiency outweighed dilution effects resulting in remarkable detection sensitivity. The obtained LODs of 2.4 and 3.5 ng/mL are typical for small peptides even for interfaces with lower analyte dilution [22, 41, 42]. Due to its simple construction, convenient operation and reproducibly accurate analyses, we envision a great potential of the developed interface in a wide scope of application.

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The authors have declared no conflict of interest.

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