**Evaluation of on-line solid-phase extraction capillary electrophoresis–mass spectrometry with a nanoliter valve for the analysis of peptide biomarkers**

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**ABSTRACT**

On-line solid-phase extraction capillary electrophoresis-mass spectrometry (SPE-CE-MS) is a powerful technique for high throughput sample clean-up and analyte preconcentration, separation, detection, and characterization. The most typical design due to its simplicity and low cost is unidirectional SPE-CE-MS. However, in this configuration, the sample volumes introduced by pressure depend on the dimensions of the separation capillary and some matrix components could be irreversibly adsorbed in its inner walls. Furthermore, in many cases, the requirements of on-line preconcentration are incompatible with the background electrolyte necessary for an efficient separation and sensitive MS detection. Here, we present SPE-CE-MS with a nanoliter valve (nvSPE-CE-MS) to overcome these drawbacks while keeping the design simple. The nvSPE-CE-MS system is operated with a single CE instrument and two capillaries for independent and orthogonal SPE preconcentration and CE separation, which are interfaced through an external and electrically isolated valve with a 20 nL sample loop. The instrumental setup is proved for the analysis of opioid and amyloid beta peptide biomarkers in standards and plasma samples. NvSPE-CE-MS allowed decreasing the limits of detection (LODs) 200 times with regard to CE-MS. Compared to unidirectional SPE-CE-MS, peak efficiencies were better and repeatabilities similar, but total analysis times longer and LODs for standards slightly higher due to the heart-cut operation and the limited volume of the valve loop. This small difference on the LODs for standards was compensated for plasma samples by the improved tolerance of nvSPE-CE-MS to complex sample matrices. In view of these results, the presented setup can be regarded as a promising versatile alternative to avoid complicated matrix samples entering the separation capillary in SPE-CE-MS.

## 1. Introduction

The analysis of biomarkers of different diseases or physiological processes in biological samples is difficult, in general, due to their low concentration and the complexity of the sample matrix [1–3]. Capillary electrophoresis-mass spectrometry (CE-MS) is a proficient technique for the highly efficient separation and characterization of polar and charged biomarkers, including peptides and proteins [4–6]. However, as in many other microscale separation techniques, the limited concentration sensitivity of CE for most analytes, due to the reduced sample injection volumes needed for an appropriate separation (typically 1-2% of the capillary volume), very often hinders a more widespread application [7–9]. To lower the limit of detection (LOD), on-line solid-phase extraction capillary electrophoresis (SPE-CE) is recognized as an excellent alternative [8–10]. Unidirectional SPE-CE is the most typical design due to its simplicity and low cost [8,9]. In this configuration, an extraction microcartridge is inserted near the inlet of the separation capillary and contains an appropriate sorbent to retain the analytes from a large volume of sample (~50-100 μL). After sample loading, the capillary is rinsed to eliminate non-retained molecules and filled with background electrolyte (BGE). Then, the analytes are eluted in a small volume of an appropriate solution (~25-50 nL), resulting in sample clean-up and concentration enhancement before the electrophoretic separation and detection [11]. As can be noted from this method, the introduction of the sample and the washing of the microcartridge before the elution of the retained compounds are conducted in the same direction as the subsequent separation. This could be detrimental when loading complex samples, because some of the matrix components could be irreversibly adsorbed in the inner wall of the separation capillary, or when the requirements of on-line preconcentration are incompatible with the BGE necessary for an efficient separation and sensitive MS detection. In addition, the sample volumes introduced by pressure depend on the small internal diameter (id) and the total length ($L_T$) of the separation capillary. In order to overcome these drawbacks, other designs have been proposed, in which the sample is introduced in an orthogonal direction to the separation [3,8–10]. These orthogonal designs with microfluidic devices or capillaries require the use of valves and are more difficult to construct and operate than the typical unidirectional SPE-CE microcartridges.

In 1987, Tsuda et al. [12] devised a rotary-type valve suitable for sample injection in CE. This concept was considered by Debets et al. [13] to develop an on-line SPE-CE system that needed a liquid chromatography (LC) pump to load the sample in a SPE column contained in a rotary-type valve. After switching the valve position, the analytes were eluted and separated in the CE capillary. Later, Tempels et al. [14] demonstrated on-line SPE-CE-MS via a valve interface. However, the system implied certain complexity because a LC pump and three valves were required. In the following years, the contributions about SPE-CE using capillaries and valves were scarce, due to the complexity of the instrumental setups and poor performance of

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the valves [8,15,16]. Recently, Kohl et al. [17] introduced a novel 4-port valve with an internal nanoliter loop for the direct coupling of two capillaries. This setup has shown high versatility for two-dimensional separation with on-line MS detection, having been successfully applied for two-dimensional capillary zone electrophoresis [18], capillary isoelectric focusing[19], imaged capillary isoelectric focusing [20], and capillary sieving electrophoresis with sodium dodecyl sulphate [21]. In these applications, the analytes are separated with high efficiency in the first dimension, using in most of the cases a BGE containing non-compatible components with on-line MS detection. Then, the peaks of interest are heart-cut, transferred, separated from the co-transferred MS interfering compounds and detected in the second dimension. Taking into account that capillaries used in both dimensions are independent, this setup for bidimensional CE separations could also be suitable for developing SPE-CE-MS with a nanoliter valve (nvSPE-CE-MS) methods for purification and preconcentration of biomarkers. The clean-up and preconcentration step will take place in the first-dimension capillary (loading capillary) and the separation and MS detection in the second-dimension (separation capillary).

In this study, we investigate for the first time the potential of nvSPE-CE-MS in comparison with unidirectional SPE-CE-MS for the analysis of peptide biomarkers in standards and plasma samples. Specifically, opioid peptides and amyloid beta (Aβ) peptide fragments were analyzed because of their importance in different neurological disorders, including chronic pain and Alzheimer's disease, respectively [22,23].

## 2. Materials and methods

### 2.1. Chemicals and reagents

Ultrapure water (18 MΩ*cm at 25 ℃) from an SG UltraClear UV system (Siemens Water Technologies, Günzburg, Germany) was used to prepare all background electrolytes (BGEs), samples, and solutions. Acetic acid (HAc; ROTIPURAN, 100%, p.a.), acetonitrile (ACN; MS grade), ammonium hydroxide solution (ROTIPURAN, 30%, p.a.), formic acid (HFor; ROTIPURAN, ≥98%, p.a., ACS), methanol (MeOH; MS grade), propan-2-ol (MS grade), sodium formate (≥99% ACS), and sodium hydroxide (≥98%) were supplied by Carl Roth (Karlsruhe, Germany). Dynorphin A (1–7) (Dyn A), endomorphin 1 (End 1), methionine enkephalin (Met), and human plasma (P9523) were provided by Sigma-Aldrich (St. Louis, MO, USA). Aβ peptide fragments 1-15, 10-20, 20-29, and 25-35 were provided by Bachem (Bubendorf, Switzerland). Sep-Pak C18 cartridges (55–105 µm particle diameter, 125 Å pore size) were supplied by Waters (Milford, MA, USA). PEG (average $M_r$=8,000) was purchased from Fluka (Buchs, Switzerland).

## 2.2. Electrolyte solutions, sheath liquid, and standards

The method for the analysis of opioid peptides by unidirectional SPE-CE-MS was adapted from a previous work [11,24,25]. The separation BGE contained 50 mM HAc:50 mM HFor, pH 3.0 adjusted with ammonium hydroxide solution. The sheath liquid solution consisted of a hydroorganic mixture of 60:40 (v/v) propan-2-ol:water with 0.05% (v/v) of HFor and was delivered at a flow rate of 4.0 µL·min$^{-1}$ by a syringe pump (Cole-Parmer, Vernon Hills, IL, USA). The eluent consisted of 60:40 (v/v) MeOH:water with 50 mM HAc:50 mM HFor. Elution conditions were reoptimized for nvSPE-CE-MS, and the optimized eluent consisted of 80:20 (v/v) ACN:water with 50 mM HAc:50 mM HFor.

An aqueous standard solution of each peptide at 2,500 mg·L$^{-1}$ was prepared and stored in a freezer at -20ºC when not in use. Working standard solutions containing the three opioid peptides or the four Aβ peptide fragments were obtained by mixing and diluting with water. These mixtures were used to spike human plasma samples in order to obtain the fortified samples. Diluted solutions were discarded at the end of the day.

## 2.3. Apparatus, instrumentation and procedures

pH measurements were made with a pH-meter 691 (Metrohm, Herisau, Switzerland). Agitation was performed with a vortex mixer 7-2020 (NeoLab, Heidelberg, Germany). Centrifugations were performed in a Minispin plus (Eppendorf, Hamburg, Germany).

CE-MS experiments were performed on a PA 800 Plus pharmaceutical analysis system (Beckman Coulter, Brea, CA, USA) coupled to a micrOTOF-Q (Bruker Daltonics, Bremen, Germany) through a G1607A orthogonal sheath flow electrospray interface (Agilent Technologies, Waldbronn, Germany). CE control and data analysis were carried out using 32 Karat version 10.1 software (Beckman Coulter). MS control and data analysis were carried out using micrOTOF control version 2.3 software (Bruker Daltonics). The mass spectrometer was operated in ESI+ mode. MS parameters were optimized by infusion of the analytes at 0.7 psi through the capillary to the MS: end plate -500 V, capillary voltage 4500 V, nebulizer gas 0.5 bar, gas temperature 170 ºC, dry gas flow rate 2 L·min$^{-1}$, transfer time 40 µs, prepuls storage 10 µs, scan range 100-1250 m/z.

Calibration was performed every day by infusing a 5 mM sodium formate solution in sheath liquid.

### 2.3.1. CE-MS

Separations were performed at 25°C in a 72 cm $L_T$ × 50 µm id × 365 µm od bare fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA). All capillary rinses were performed at 14 psi. For new capillaries or between workdays, the capillaries were activated flushing off-line to avoid the unnecessary contamination of the MS system, with 1 M NaOH (15

or 5 min, respectively), water (15 or 10 min), and BGE (30 or 15 min). Samples were hydrodynamically injected at 0.7 psi for 10 s (12 nL, estimated using the Hagen–Poiseuille equation [26]) and a separation voltage of +15 kV (normal polarity, cathode in the outlet) was applied. Between runs, the capillary was conditioned flushing with water (2 min) and BGE (2 min). The capillary was stored overnight filled with water.

### 2.3.2. CE-MS with a nanoliter valve (nvCE-MS)

The C4N-4354-.02D microinjector four-port valve, including rotors (internal sample loop of 20 nL) and stators both made from plastic material (PAEK and Valcon E, respectively), was obtained from VICI AG International (Schenkon, Switzerland). A detailed description of the general valve design can be found elsewhere [17]. Prior to assembling the valve, all channels and surfaces were carefully cleaned to avoid contamination and particles that may cause obstructions. The valve could be switched between two positions, loading position (Figure 1-A, with no SPE microcartridge installed for nvCE-MS) and separation position (Figure 1-B), using an actuator controlled via two buttons.

Capillaries were connected to the valve by 1/32″ fingertight fittings in combination with appropriate 1/32″ sleeves for the use of 365 µm od capillaries. The loading capillary (65 cm $L_T$ × 75 µm id × 365 µm od) was cut into two fragments 1a and 1b ($L_{cap1a}$ 50 cm and $L_{cap1b}$ 15 cm) (Figure 1). Capillary 1a inlet was placed in the outlet of the CE instrument to apply pressure for washing, injecting and mobilizing the sample, while the outlet was connected to the valve port S. Capillary 1b was connected from port W to a waste vial. The separation capillary (100 cm $L_T$ × 50 µm id × 365 µm od) was cut into two fragments 2a and 2b ($L_{cap2a}$ 55 cm and $L_{cap2b}$ 45 cm) (Figure 1). Capillary 2a inlet was placed in the inlet of the CE instrument, to apply voltage for the electrophoretic separation, and the outlet was connected to the valve port P. Capillary 2b was connected from port C to the MS interface.

Capillary 1a possessed a window at 8 cm before the valve ($L_{eff}$ 42 cm). An external UV detector ECD2000 (ECOM spol, Chrastany u Prahy, Czech Republic) was used to monitor UV signal at 200 nm wavelength. Control and data processing were carried out by ECOMAC v0.281 software (ECOM spol).

The transfer of the sample from the loading to the separation capillary was made switching the valve when the sample reached the center of the valve sample loop (switching time, $t_2$). This time was calculated before each analysis taking into account the mobilization velocity ($L_{eff}$ / $t_1$, where $t_1$ is the time measured when the sample plug was detected by UV) and the total distance covered by the sample plug up to the loop center (i.e., $L_{cap1A}$ + 0.25 cm, if the 20 nL valve loop was assumed to be equivalent to a 0.5 cm $L_T$ x 75 µm id capillary).

While in loading position, the nebulizer gas and the ESI capillary voltage were switched off to avoid bubble formation in the separation capillary during the sample transfer. First, the

separation capillary was equilibrated flushing with BGE at 14 psi for 2 min. After that, the loading capillary was flushed with water at 14 psi for 2 min. Then, the sample was hydrodynamically injected in the loading capillary at 0.7 psi for 30 s (190 nL [26]) and pushed at 1 psi with water. The mobilization was stopped at $t_2$ and the valve was switched to the separation position to transfer 20 nL of sample to the separation capillary. Separation was conducted applying a voltage of +15 kV (normal polarity). After the first 0.2 min of the separation, the nebulizer gas and the ESI capillary voltage were switched on. The capillaries were stored overnight filled with water.

It is worth highlighting that the setup for nvCE-MS using a PA 800 Plus pharmaceutical analysis system was straightforward because this CE instrument allows applying pressure up to 70 psi in both the inlet (separation capillary) and the outlet (loading capillary). Other CE instruments may require some modifications to work with a single instrument. For example, in a classical Agilent HP 3DCE instrument (Agilent Technologies) the low pressure (i.e., ≤50 mbar) used for sample injection and mobilization could be only applied in the inlet, hence the inlet and outlet tubings coming from the CE pump must be exchanged, and also the power and data cables of both lifts, to adapt the instrument for nvCE-MS.

### 2.3.3. Unidirectional SPE-CE-MS

The construction of the double-frit particle packed SPE microcartridge was monitored under a stereomicroscope and was carried out as described elsewhere [11,24,25] with some modifications due to the configuration of the cartridge cassette of the PA 800 Plus instrument. A capillary (72 cm $L_T$ × 75 µm id × 365 µm od) was activated and, then, cut into two pieces of 30 and 42 cm to insert in between the microcartridge (0.7 cm $L_T$ x 250 µm id x 365 µm od). Before, a polyethylene frit obtained from a small fragment of the original filters found in the C18 Sep-Pak cartridges was introduced in one of the ends of the microcartridge. This microcartridge end was coupled to the 30 cm capillary (inlet) using a 0.5 cm Tygon® tube of 250 µm id. Then, the microcartridge was vacuum-filled with C18 sorbent from the Sep-Pak cartridge until it was completely full. Finally, a second polyethylene frit was inserted in the end side and the microcartridge was connected to the 42 cm capillary (outlet) using another Tygon® tube.

The method for unidirectional SPE-CE-MS was adapted from our previous works [11,24,25]. SPE-CE-MS column was conditioned flushing at 14 psi consecutively with water (1 min), MeOH (1 min), water (1 min), and BGE (3 min). The sample was loaded by flushing at 14 psi for 10 min (approximately 60 µL). The non-retained molecules were eliminated and the capillary was equilibrated by rinsing with BGE for 2 min. Retained peptides were desorbed by injection of the eluent solution at 0.7 psi for 10 s (60 nL). Separation was conducted applying a voltage of +15 kV (normal polarity). Between runs, the capillary was rinsed with water (2 min)

and ACN (2 min) to avoid carry-over between consecutive analyses. The SPE-CE-MS capillary was stored overnight filled with ACN.

### 2.3.4. SPE-CE-MS with a nanoliter valve (nvSPE-CE-MS)

The microcartridge was prepared as described for unidirectional SPE-CE-MS (section 2.3.3) and inserted in the loading capillary 1a at 30 cm from the inlet (Figure 1).

While in loading position, as in nvCE-MS, the nebulizer gas and the ESI capillary voltage were switched off. First, the separation capillary was equilibrated flushing with BGE at 14 psi for 2 min. After that, the loading capillary was conditioned as in unidirectional SPE-CE-MS, flushing at 14 psi consecutively with water (1 min), MeOH (1 min), water (1 min), and BGE (3 min). The sample was loaded by flushing at 14 psi for 10 min (approximately 60 µL). After rinsing with water for 2 min, retained peptides were desorbed injecting the eluent solution at 1 psi for 30 s (280 nL). The eluent plug was pushed at 1 psi with water until stopping the mobilization at $t_2$. Then, the valve was switched to the separation position to transfer 20 nL of the eluent plug to the separation capillary. Three additional 20 nL heat-cuts were consecutively transferred repeating thrice the following two steps: first, the valve was switched back to the loading position (Figure 1-A) and another fraction of the elution plug from the loading capillary was pushed with water to the sample loop at 0.5 psi for 5 s (23 nL). Second, the valve was switched to the separation position (Figure 1-A) and the elution plug inside the sample loop was pushed to the separation capillary at 1 psi for 20 s (24 nL). Once the transfer of four heart-cuts was completed, separation was conducted applying a voltage of +15 kV (normal polarity). The nebulizer gas and the ESI capillary voltage were switched on after the first 0.2 min of separation. Between runs, the loading capillary was rinsed with water (2 min) and ACN (2 min), to avoid carry-over between consecutive analyses. The SPE-CE loading capillary was stored overnight filled with ACN. The separation capillary was stored overnight filled with water.

### 2.3.5. Plasma samples pretreatment

Lyophilized plasma was dissolved in 5 mL of water, aliquoted and frozen at -20 ℃. Aliquots of the frozen plasma were thawed at room temperature when required. In order to prepare spiked samples, an appropriate amount of the standard peptide mixture was added to each plasma aliquot at a ratio 99:1 (v/v) plasma:standard solution and was vortexed thoroughly. Precipitation with ACN was done as previously described [27] starting with 200 µL of spiked plasma. The pooled supernatant was evaporated using a Savant DNA 120 SpeedVac (Thermo Fisher Scientific, Massachusetts, USA) and was reconstituted with 100 µL of water. The solution was subjected to ultrafiltration using Amicon Ultra-0.5 10,000 $M_r$ cut-off cellulose acetate filters (Millipore, Bedford, MA, USA) passivated with 5% (v/v) of PEG in water [27].

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## 2.3.6. Quality parameters

All quality parameters with MS detection were calculated from data obtained by measuring peak area and migration time ($t_m$) from the extracted ion electropherogram (EIE) of each peptide. The m/z values of the molecular ions considered for each peptide are shown in Table 1. Repeatability was evaluated as percentage of the relative standard deviation (%RSD) of peak areas and $t_m$ for triplicate analysis (n=3). The LOD (S/N=3) for each peptide was obtained analyzing low-concentration samples.

## 3. Results and discussion

## 3.1. CE-MS

In previous works, the acid-base properties of opioid peptides [28] and Aβ peptide fragments [29] were characterized by CE-UV to establish electrophoretic migration models and systematically optimize their separation. Accurate $pK_a$ values were determined, which can be used to easily obtain charge-to-mass ratios ($q/M_r$) of the peptides and to calculate the multicriterium optimization function T' for a simple, experiment-free and reliable selection of optimized separation conditions [30]. Figure 2A shows the plot of $T'(q/M_r^{1/2})$ vs pH for the mixture of the three opioid peptides (Dyn A, End 1, and Met) where a maximum indicates that the best separation will be obtained at pH values ranging from 3.0 to 3.5. Figure 2B-i shows the EIEs for the analysis of standards at 10 mg·L$^{-1}$ by CE-MS using 50 mM HAc:50 mM HFor, pH 3.0, as BGE demonstrating that the three opioid peptides were separated. At this concentration, %RSD values ranged from 8.7 to 10% for peak areas and 1.5 to 2.7% for $t_m$ (Table S-1). The LOD for the analysis of the opioid peptides by CE-MS was 100 µg·L$^{-1}$ (Table 1), similar to the value obtained in previous studies [24,31].

The same strategy was followed to select pH 3.0 for the separation of a mixture of the four Aβ peptide fragments (Aβ 1-15, 10-20, 20-29, and 25-35) [29]. Figure S-1A shows the EIEs for the analysis of standards at 10 mg·L$^{-1}$ by CE-MS using 50 mM HAc:50 mM HFor, pH 3.0, as BGE and the four Aβ peptide fragments were separated. At this concentration, %RSD values ranged from 7.4 to 11% for peak areas and 0.92 to 1.3% for $t_m$ (Table S-1). The LOD was slightly higher than for the opioid peptides, probably due to the larger $M_r$ of the Aβ peptide fragments, and values ranged between 500 and 1,500 µg·L$^{-1}$ (Table 1).

## 3.2. nvCE-MS

In order to evaluate the instrumental setup, nvCE-MS was tested, using the same separation conditions as in CE-MS. Sample was hydrodynamically injected in the loading capillary and transferred to the separation capillary through the valve. This transfer step is a crucial part of the nvCE-MS methodology because precise positioning of the sample in the loop is mandatory. A simple approach is to estimate the time required to mobilize the sample until it

reaches the center of the valve sample loop ($t_2$), as described in section 2.3.2. Mobilization of the sample through the loading capillary pushing with BGE was first tested, but UV detection of the sample plug was difficult due to the intense UV absorption of the HFor and HAc in the BGE. Therefore, it was decided to mobilize the sample by pushing with water. A pressure of 1 psi was selected because it resulted in a $t_2$ of around 7 min, which was a good compromise between the flow rate necessary for a precise peak cutting and total analysis time. Pressure was stopped at $t_2$ and the valve was switched from the loading to the separation position (Figure 1). Then, the analytes were electrophoretically separated, detected and characterized by CE-MS. Separation voltage was limited to +15 kV to prevent any current leakage due to the material properties of the valve and the close distance of the rotor channels [18]. Figure 2B-ii shows the analysis of the opioid peptides at 10 mg·L$^{-1}$ by nvCE-MS. Compared to the analysis by CE-MS (Figure 2B-i), the total analysis time was shorter mainly due to the shorter separation distance (45 cm capillary 2b in nvCE-MS vs 72 cm capillary in CE-MS). The peak areas and repeatability were similar to those in CE-MS, %RSD values ranged from 5.9 to 8.3% for peak areas and 0.74 to 1.8% for $t_m$ (Table S-1).

### 3.3. Unidirectional SPE-CE-MS

In order to lower the LODs of CE-MS, on-line preconcentration was used. First, the unidirectional SPE-CE-MS method developed in previous works for the analysis of opioid peptides [11,24,25] was adapted to the available CE-MS-instrumentation for a meaningful comparison with subsequent nvSPE-CE-MS. Figure 3A-i shows the analysis of the opioid peptides at 1 µg·L$^{-1}$ by unidirectional SPE-CE-MS. Compared to CE-MS (Figure 2B-i), the peaks were slightly wider mainly due to the use of a wider capillary (75 vs 50 µm id), but the three peaks were still baseline separated. Repeatabilities were similar, with %RSD values from 6.9 to 12% for peak areas and 1.2 to 1.6% for $t_m$ (Table S-1). The LOD was around 0.1 µg·L$^{-1}$ (Table 1), an improvement of 1000 times compared to CE-MS.

For the analysis of plasma samples by unidirectional SPE-CE-MS, a double-step sample clean-up pretreatment consisting of precipitation with ACN followed by ultrafiltration through 10,000 $M_r$ cut-off filters was necessary to prevent the microcartridge blockage due to saturation of the limited amount of C18 sorbent contained inside [27]. Figure 3B-i shows the analysis of a pretreated plasma sample spiked at 10 µg·L$^{-1}$ by unidirectional SPE-CE-MS. Compared to the analysis of standards (Figure 3A-i), the separation resolution and repeatabilities for peak areas were similar, with %RSD values from 11 and 13% (Table S-1). %RSD values for $t_m$ were slightly increased, ranging from 3.7 to 4.6%. The LODs were 1 µg·L$^{-1}$ for End 1 and Met (Table 1), which supposed 10 times increase compared to standards due to the complexity of the sample matrix. Furthermore, Dyn A presented the highest LOD (10 µg·L$^{-1}$) because its $M_r$ was larger (Table 1) and recovery after the sample pretreatment was lower [27].

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Unidirectional SPE-CE-MS is simple, low cost and valve-free operated and has been widely and successfully demonstrated [8,9]. However, it has several drawbacks due to performing both SPE preconcentration and electrophoretic separation in the same capillary. Consequently, capillary dimensions (i.e., $L_T$ and id) must be selected as a compromise between sample loadability at a certain pressure and efficient electrophoretic separation. In addition, loaded sample matrix components could irreversible contaminate the separation capillary or the MS, if the ionization voltage or the nebulizer gas values are not set to zero. Finally, for an appropriate separation it is required to fill the loading capillary with an MS compatible BGE, which could promote undesired elution of the analytes in some applications. In order to overcome these disadvantages, we investigated nvSPE-CE-MS.

### 3.4. nvSPE-CE-MS

The starting point for the optimization of the nvSPE-CE-MS methodology was the optimized conditions for unidirectional SPE-CE-MS. The instrumental setup of nvSPE-CE-MS was the same as for nvCE-MS with the addition of a microcartridge in the loading capillary. The methodology was as follows: in loading position (Figure 1-A), the sample was loaded hydrodynamically in the loading capillary and the analytes were retained in the microcartridge. As loading and separation capillaries were independent, there was no contamination of the separation capillary or the MS due to the loaded sample matrix. Then, the loading capillary was washed to eliminate the non-retained compounds. The eluent was injected, mobilized by pressure pushing with water, and transferred to the separation capillary by switching the valve. In separation position (Figure 1-B), the voltage was applied and the eluted analytes were separated, detected, and characterized by CE-MS.

In nvSPE-CE-MS, the loading and separation capillaries were independent, hence their dimensions could be different. A loading capillary with wide id (i.e., 75 µm id) was selected for an adequate loadability (i.e., load a larger volume of sample for a given time or decrease the loading time for a certain sample volume) whereas a separation capillary with narrow id (i.e., 50 µm id) was used to obtain better peak efficiency. As the microcartridge was not inserted in the separation capillary, potential issues derived from current breakage due to backpressure were also avoided. In addition, the separation capillary could be always kept full with BGE while all the SPE pressure-based steps were done in the loading capillary.

Preliminary results by nvSPE-CE-MS showed that, of the three opioid peptides, only Dyn A was detected when the elution conditions of unidirectional SPE-CE-MS were applied (injection of 60:40 (v/v) MeOH:water with 50 mM HAc:50 mM HFor at 0.7 psi for 10 s). This poor elution was probably because the volume of eluent injected was lower than expected, due to the increased backpressure caused by the presence of the valve. Quantitative desorption of the analytes was achieved when the eluent was injected at 1 psi for 30 s. The eluent plug reached

the center of the valve after about 8 min of pushing with water at 1 psi, a slight increase compared to nvCE-MS (i.e., 7 min) due to the presence of the microcartridge.

The elution profile of the three opioid peptides using different hydroorganic eluents was investigated and characterized by the analysis of several consecutive heart-cuts of the elution plug, because of the limited volume of the valve sample loop (20 nL). Figure 4-A shows the elution profile using 60:40 (v/v) MeOH:water with 50 mM HAc:50 mM HFor as eluent. In the first two heart-cuts, mainly Dyn A was detected, indicating that Dyn A elution was faster than for End 1 and Met, coherently with the previous preliminary results. End 1 and Met were mainly detected in the third and fourth heart-cuts. Therefore, using an eluent of 60:40 (v/v) MeOH:water with 50 mM HAc:50 mM HFor at least four or more heart-cuts would be needed for a quantitative transfer of the eluted opioid peptides. To elute the analytes in a narrower zone, the eluotropic strength of the eluent was increased. Figure 4-B shows the elution profile using an eluent of 80:20 (v/v) ACN:water with 50 mM HAc:50 mM HFor. In this case, although Dyn A still eluted slightly faster, the elution profile of all the opioid peptides was coincident and more than 85% of the total sum of peak areas of the three opioid peptides was detected considering the second and third heart-cuts. However, it was decided to apply four consecutive heart-cuts (4x20 nL=80 nL) in order to ensure a complete and repeatable transfer of the eluted peptides. Figure 3A-ii shows the analysis of the three opioid peptides at 1 $\mu g \cdot L^{-1}$ by nvSPE-CE-MS in the optimized conditions. Compared to the analysis by unidirectional SPE-CE-MS (Figure 3A-i), the total analysis time was shorter, mainly due again to the shorter separation distance (45 cm capillary 2b in nvSPE-CE-MS vs 72 cm capillary in unidirectional SPE-CE-MS). In addition, the peak efficiency was better because a narrower separation capillary was used for the separation (50 vs 75 µm id in nvSPE-CE-MS and unidirectional SPE-CE-MS, respectively). The differences in the peak intensities may be due to the lower volume of sample loaded because of the extra backpressure promoted by the valve or because of partial loss of the elution plug in the multiple heart-cut transfer. For 1 $\mu g \cdot L^{-1}$, repeatability was similar compared to CE-MS and unidirectional SPE-CE-MS, with %RSD values from 9.0 to 11% for peak areas and 1.4 to 2.4% for $t_m$ (Table S-1). The LOD for the analysis of the opioid peptides by nvSPE-CE-MS was 0.5 $\mu g \cdot L^{-1}$ (Table 1), 200 times lower than by CE-MS and 5 times higher than by unidirectional SPE-CE-MS.

The analysis of pretreated plasma samples was also evaluated by nvSPE-CE-MS. Figure 3B-ii shows the analysis of a pretreated plasma sample spiked at 10 $\mu g \cdot L^{-1}$. Similarly to the analysis of standards, the total analysis time was shorter and the peak intensities were lower in nvSPE-CE-MS than in unidirectional SPE-CE-MS (Figure 3B-i), and %RSD values were similar, ranging from 5.3 to 13% for peak areas and 4.2 to 5.9% for $t_m$ (Table S-1). This similarity in the %RSD of peak areas and $t_m$ suggested that the separation capillary was not apparently contaminated during the sample loading in unidirectional SPE-CE-MS. Otherwise

the higher %RSD values compared to standards would be related in both cases to the impurities from the complex sample matrix eluted with the peptides, which did not affect separation resolution. The LODs for the opioid peptides in plasma samples ranged from 1 to 10 $\mu g \cdot L^{-1}$ (Table 1), hence 2 to 20 times higher compared to standards. This increase of the LODs could be explained by the complex sample matrix and the recoveries of the sample pretreatment, as also indicated before for unidirectional SPE-CE-MS. Nevertheless, as the LODs of both methodologies for plasma samples were similar, this would indicate a lower sample matrix effect in nvSPE-CE-MS.

To further validate the nvSPE-CE-MS approach with a different set of peptide biomarkers, a mixture of A$\beta$ peptides at 10 $\mu g \cdot L^{-1}$ was analyzed (Figure S-1B). Compared to CE-MS at 10 $mg \cdot L^{-1}$ (Figure S-1A), the relative peak area of A$\beta$ 10-20 decreased probably due to the lower retention of this peptide in the C18 sorbent. Repeatability was similar to the obtained for the analysis of opioid peptides, with %RSD values from 5.3 to 12% for peak areas and 1.8 to 2.7% for $t_m$ (Table S-1). The LOD for the analysis of A$\beta$ peptides by nvSPE-CE-MS was 5 $\mu g \cdot L^{-1}$ (Table 1), around 200 times lower than by CE-MS.

Figure S-1C shows the analysis of a pretreated plasma sample spiked at 50 $\mu g \cdot L^{-1}$ by nvSPE-CE-MS. Compared to the analysis of standards (Figure S-1B), the separation resolution and the repeatability for peak areas were similar, with %RSD values from 5.1 to 13%, and the %RSD for $t_m$ was again slightly higher, ranging from 2.7 to 4.0% (Table S-1). The LODs were 10 to 50 $\mu g \cdot L^{-1}$ (Table 1), 2 to 10 times higher than for standards.

In the current nvSPE-CE-MS setup, the small valve loop volume (20 nL) limited the volume transferred in each heart-cut. The slightly lower sensitivity enhancement compared to unidirectional SPE-CE-MS suggests that the transfer of the eluent plug in nvSPE-CE-MS should be further improved. In the future, a valve with a larger internal loop volume or a valve for the direct connection of the loading and separation capillaries is desirable to ensure a more efficient transfer of an appropriate eluent plug to the separation capillary. If this issue is addressed, the segregation of SPE preconcentration from CE separation by using a nanovalve shows potential for improving SPE-CE-MS applications with immunoaffinity [32–34], immobilized metal affinity [35,36], ion exchange [37] or hydrophilic interaction liquid chromatography sorbents. NvSPE-CE-MS will enable the use of the most appropriate loading conditions for optimum analyte retention and sorbent stability (e.g. salty and neutral pH conditions for immunoaffinity or immobilized metal affinity sorbent), in combination with the typical formic or acetic acid separation BGEs necessary to maximize ionization efficiency and sensitivity in positive ESI-MS mode. Moreover, nvSPE-CE-MS will enable a wider use of high hydroorganic content or non-aqueous BGEs, which is currently very limited, especially with the typical reversed-phase chromatographic sorbents (e.g. C18).

## 4. Concluding remarks

We have developed a nvSPE-CE-MS setup with a single CE instrument and demonstrated the applicability for purification, preconcentration, separation, detection, and characterization of opioid peptides and Aβ peptide fragments using a C18 sorbent. An improvement of the LODs of 200 times was obtained for standards compared to CE-MS. In the analysis of pretreated plasma samples, the LODs were slightly higher than for the standards. In comparison with unidirectional SPE-CE-MS, the LODs for standards were five times higher. However, they were similar for plasma samples, indicating an improved tolerance of nvSPE-CE-MS to complex sample matrices. The results with unidirectional SPE-CE-MS were very similar, but LODs were five times lower. Further optimization of parameters such as sorbent particle size, microcartridge dimensions or valve design can further improve the nvSPE-CE-MS method. In nvSPE-CE-MS, the loading and the separation capillaries, and, hence, SPE preconcentration and clean-up and CE separation, are independent. Therefore, it is possible to use a loading capillary with a wide id to maximize loadability of the sample and a separation capillary with a narrow id to obtain adequate peak efficiencies. There is no possibility of contamination of the separation capillary when loading a complex biological sample or during the washing step. In addition, the separation capillary can be filled with the optimized BGE for the separation and the detection without potential elution of the retained peptides, enabling additional modes of SPE-CE-MS. These advantages compared to unidirectional SPE-CE-MS have the cost of a more complex setup and methodology. The mobilization step transferring the eluted plug from the loading to the separation capillary adds to the total analysis time. In addition, an UV detector is required for method development. Furthermore, improved transfer volumes of the valve are desired in order to gain flexibility and ease-of-use of the nvSPE-CE-MS-setup. In summary, nvSPE-CE-MS is a promising technique for the analysis of low-concentration analytes in complex samples such as biological fluids that should be further investigated to exploit its full potential.

The authors have declared no conflict of interest.

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**Table 1.** Relative monoisotopic molecular mass ($M_r$) and mass-to-charge (m/z) ratios of the molecular ions for the opioid peptides and amyloid beta (Aβ) peptide fragments. Limit of detection (LOD) for the analysis of the peptides by CE-MS, unidirectional SPE-CE-MS, and nvSPE-CE-MS.

| Peptide | $M_r$ | $[M + n*H]^{n+}$ | | LOD of standards (µg·L$^{-1}$) | | | LOD in spiked plasma (µg·L$^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| | | m/z | n | CE | SPE-CE [a] | nvSPE-CE | SPE-CE [a] | nvSPE-CE |
| Dyn A | 867.4715 | 434.7430 | 2 | 100 | 0.1 | 0.5 | 10 | 10 |
| End 1 | 610.2904 | 611.2976 | 1 | 100 | 0.05 | 0.5 | 1 | 5 |
| Met | 573.2257 | 574.2330 | 1 | 100 | 0.1 | 0.5 | 1 | 1 |
| | | | | | | | | |
| Aβ 1-15 | 1825.7768 | 457.4515 | 4 | 1500 | - | 5 | - | 10 |
| | | 609.5996 | 3 | | | | | |
| Aβ 10-20 | 1445.7456 | 482.9225 | 3 | 500 | - | 5 | - | 50 |
| | | 723.8801 | 2 | | | | | |
| Aβ 20-29 | 1022.4669 | 512.2407 | 2 | 1000 | - | 5 | - | 10 |
| Aβ 25-35 | 1059.5747 | 530.7946 | 2 | 500 | - | 5 | - | 10 |

[a] The analysis of Aβ peptide fragments by unidirectional SPE-CE-MS was not performed (-).

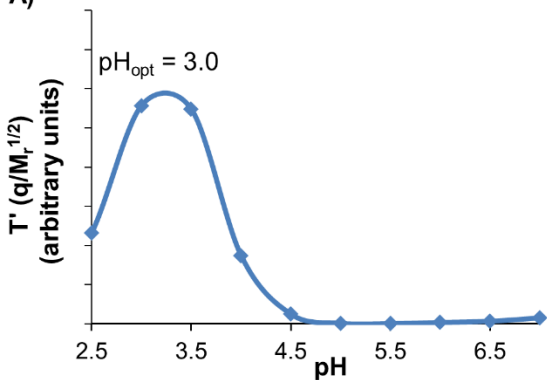**nvCE-MS or nvSPE-CE-MS**

**A) Loading position**

**B) Separation position**
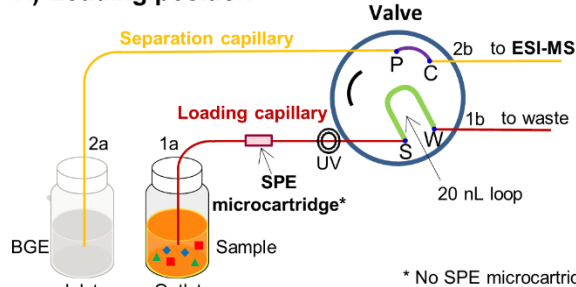
* No SPE microcartridge installed for nvCE-MS
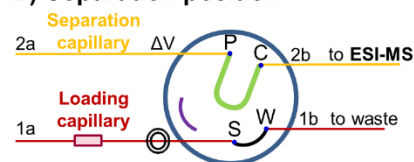
**Figure 1**. Setups for nvCE-MS or nvSPE-CE-MS. (A) Loading (no SPE microcartridge installed for nvCE-MS) and (B) separation positions.
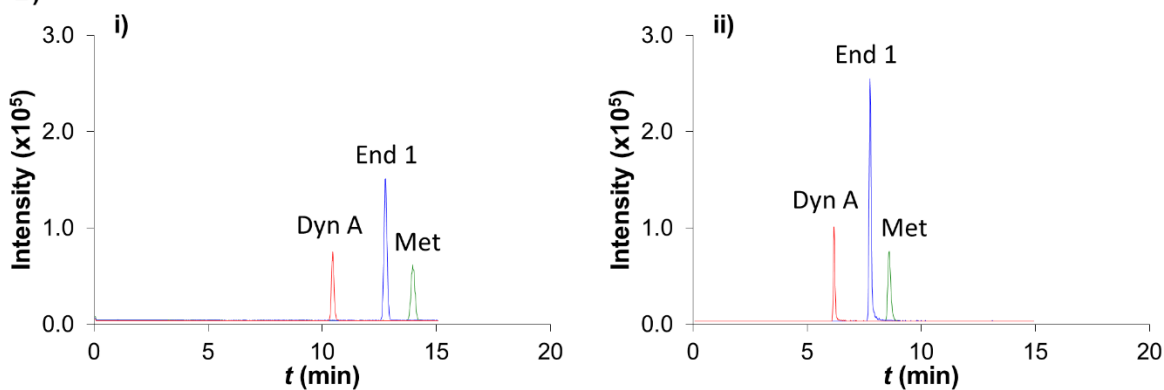


**Figure 2**. Separation of the opioid peptides. A) Plot of T' vs pH of the BGE (a BGE of 50 mM HAc:50 mM HFor, pH=3.0 was finally selected for the analyses). B) Extracted ion electropherograms in the optimized conditions for the analysis of standards at 10 mg·L$^{-1}$ by (i) CE-MS, and (ii) nvCE-MS.
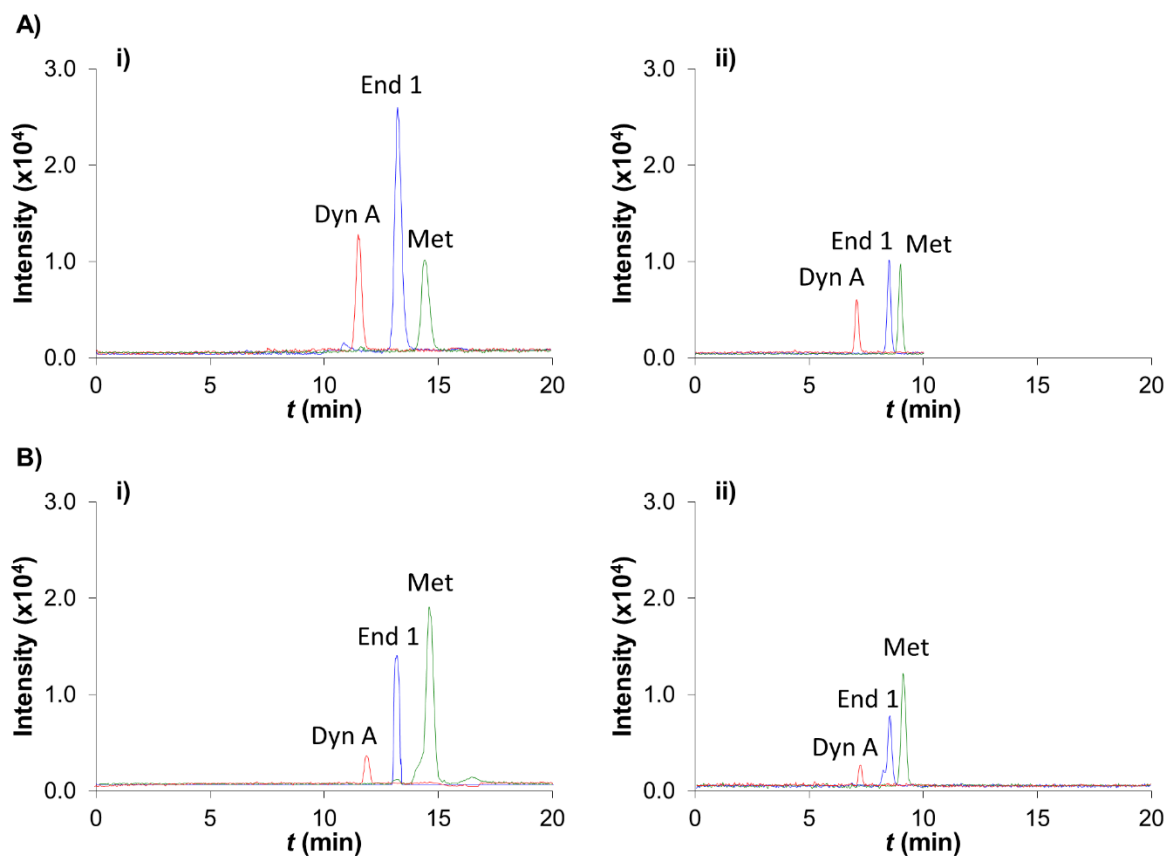
**Figure 3**. Extracted ion electropherograms of the opioid peptides in the optimized conditions for the analysis of A) a standard mixture at 1 µg·L$^{-1}$ and B) a plasma sample spiked at 10 µg·L$^{-1}$ by (i) unidirectional SPE-CE-MS, and (ii) nvSPE-CE-MS.
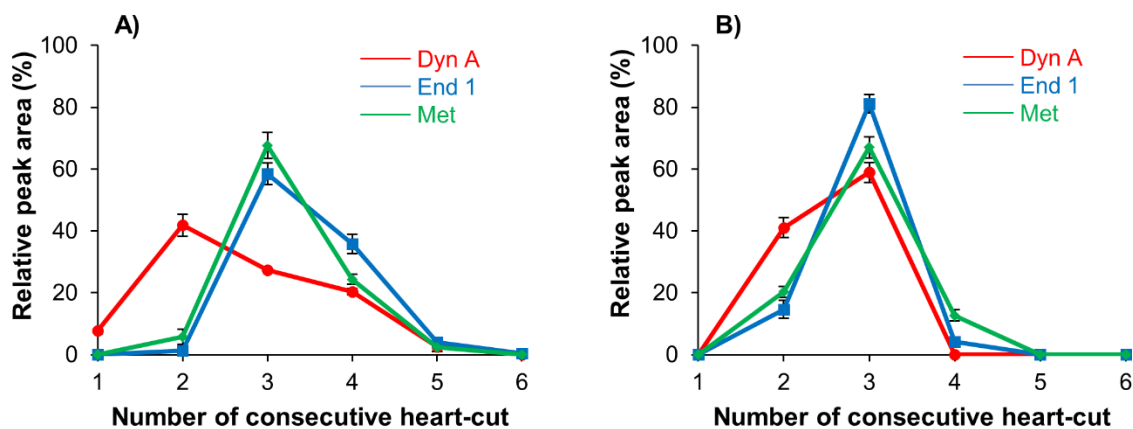


**Figure 4**. Characterization of the opioid peptide elution profile by analyzing several consecutive heart-cuts in nvSPE-CE-MS using different eluents containing 50 mM HAc:50 mM HFor and (A) 60% (v/v) MeOH and (B) 80% (v/v) ACN. Percent relative peak area was calculated normalizing to the sum of the peak areas of all the analyses for the peptide under consideration.

**Table S-1.** Repeatability (n=3) for the analysis of the opioid peptides and amyloid beta (Aβ) peptide fragments by CE-MS, nvCE-MS, unidirectional SPE-CE-MS, and nvSPE-CE-MS.

| | %RSD (n=3) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Peak area | | | | | | Migration time | | | | | |
| | CE (standards) [a] | nvCE (standards) [a] | SPE-CE (standards) [b] | SPE-CE (plasma) [c] | nvSPE-CE (standards) [b] | nvSPE-CE (plasma) [c] | CE (standards) [a] | nvCE (standards) [a] | SPE-CE (standards) [b] | SPE-CE (plasma) [c] | nvSPE-CE (standards) [b] | nvSPE-CE (plasma) [c] |
| **Opioid peptide** | | | | | | | | | | | | |
| Dyn A | 8.7 | 8.0 | 11 | 11 | 11 | 13 | 2.7 | 1.8 | 1.2 | 4.0 | 1.4 | 5.9 |
| End 1 | 10 | 5.9 | 6.9 | 13 | 11 | 5.3 | 1.7 | 0.74 | 1.6 | 3.7 | 2.4 | 4.2 |
| Met | 10 | 8.3 | 12 | 11 | 9.0 | 5.6 | 1.5 | 1.3 | 1.4 | 4.6 | 2.4 | 4.7 |
| **Aβ peptide fragment** | | | | | | | | | | | | |
| Aβ 1-15 | 11 | - | - | - | 12 | 5.1 | 1.0 | - | - | - | 1.8 | 2.7 |
| Aβ 10-20 | 10 | - | - | - | 8.8 | 13 | 1.1 | - | - | - | 2.7 | 2.9 |
| Aβ 20-29 | 7.4 | - | - | - | 5.3 | 8.3 | 0.92 | - | - | - | 2.4 | 4.0 |
| Aβ 25-35 | 8.0 | - | - | - | 9.0 | 8.7 | 1.3 | - | - | - | 2.4 | 3.3 |

Analysis at

[a] 10 mg·L$^{-1}$

[b] 1 µg·L$^{-1}$ for opioid peptides and 10 µg·L$^{-1}$ for Aβ peptide fragments

[c] 10 µg·L$^{-1}$ for opioid peptides and 50 µg·L$^{-1}$ for Aβ peptide fragments

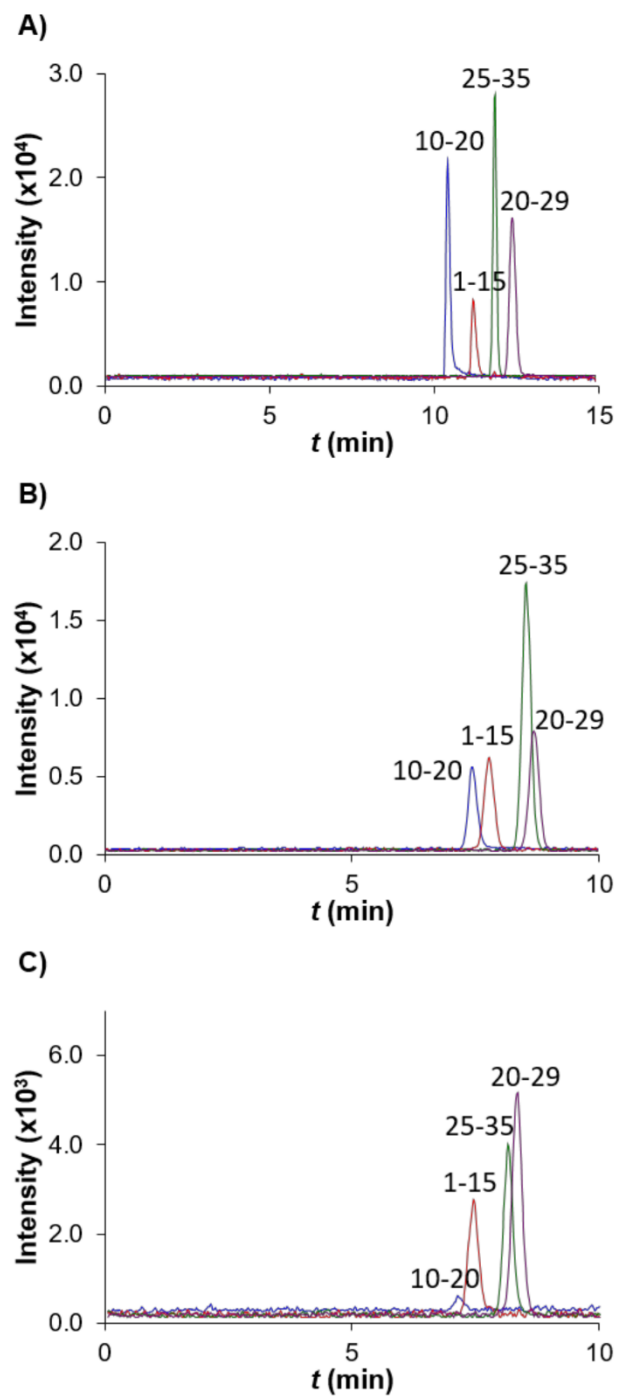**Figure S-1.** Extracted ion electropherograms of the Aβ peptide fragments in the optimized conditions. A) A standard mixture at 10 mg·L$^{-1}$ by CE-MS. B) A standard mixture at 10 μg·L$^{-1}$ and C) a plasma sample spiked at 50 μg·L$^{-1}$ by nvSPE-CE-MS.