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On-line solid-phase extraction to enhance sensitivity in peptide biomarker analysis by microseparation techniques coupled to mass spectrometry: Capillary liquid chromatography versus capillary electrophoresis

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ABSTRACT

In this study, on-line solid-phase extraction capillary liquid chromatography-mass spectrometry (SPE-CapLC-MS) and on-line solid-phase extraction capillary electrophoresis-mass spectrometry (SPE-CE-MS) were compared for the analysis of the opioid peptide biomarkers dynorphin A (1–7) (DynA), endomorphin 1 (End 1), and methionine-enkephalin (Met). First, a capillary liquid chromatography-mass spectrometry (CapLC-MS) method was established, which allowed limits of detection (LODs) of 0.5 μg/mL for Dyn A and Met, and 0.1 μg/mL for End 1. Then, a column switching setup operated by a 2-position/6-port micro-valve with a C_{18} enrichment column was assembled for SPE-CapLC-MS. Under optimized conditions, the LODs for the three peptides were lowered up to 1000-fold compared to CapLC-MS, until detecting 0.5 ng/mL concentrations. Repeatability (<0.2 % and <11 % RSD for retention times and peak areas, respectively), linearity (0.5–100 ng/mL), and durability (20 runs) of the enrichment column were appropriate, and the method was applied to analyze human plasma samples. Finally, the established SPE-CapLC-MS method was compared with a valve-free C_{18}-SPE-CE-MS method previously described by our group for the analysis of these opioid peptides, using the same mass spectrometer. Both methods presented an evident difference regarding the need of a valve for the operation and allowed high preconcentration factors and quite similar LODs (until 0.5 and 0.1 ng/mL by SPE-CaLC-MS and SPE-CE-MS, respectively). Some other distinctions related to the instrumental set-up, procedure and method performance were also disclosed and discussed in detail.

1. Introduction

Liquid chromatography (LC) has become the most commonly applied analytical technique for the highly efficient separation, identification and quantification of a broad range of compounds, including peptides in complex biological samples, such as biological fluids, tissues, and protein digests for bottom-up proteomics [1–7]. In these bioanalytical applications, sample availability is typically limited, and the different types of small-scale LC coupled on-line with mass spectrometry detection (i.e. micro, capillary and nanoLC-MS) are essential for microseparation [8–10]. The downsizing of the column diameter allows injecting smaller sample volumes, as well as reducing the mobile phase

Abbreviations: ACN, Acetonitrile; C_{18}, Octadecylsilane-bonded silica; C_8, Octyl silane-bonded silica; CapLC-MS, Capillary liquid chromatography-mass spectrometry; Dyn A, Dynorphin A (1–7); EIC, Extracted ion chromatogram; EIE, Extracted ion electropherogram; End 1, Endomorphin 1; ESI-MS, Electrospray ionization mass spectrometry; FA, Formic acid; HAc, Acetic acid; ID, Internal diameter; IPA, Isopropyl alcohol; LC, Liquid chromatography; LC-MS, Liquid chromatography-mass spectrometry; LOD, Limit of detection; L_T, Total length; M_r, Relative molecular mass; *m/z*, Mass-to-charge ratio; Met, Methionine-enkephalin; MS, Mass spectrometry; nLC-MS, Nano-liquid chromatography-mass spectrometry; OD, Outer diameter; PVDF, Polyvinylidene difluoride; PEG, Polyethylene glycol; %RSD, Percent relative standard deviation; S/N, Signal-to-noise ratio; SPE, Solid-phase extraction; SPE-CapLC, On-line solid-phase extraction capillary liquid chromatography; SPE-CapLC-MS, On-line solid-phase extraction capillary liquid chromatography-mass spectrometry; SPE-CE, On-line solid-phase extraction capillary electrophoresis; SPE-CE-MS, On-line solid-phase extraction capillary electrophoresis-mass spectrometry; SPME, Solid-phase microextraction; TFA, Trifluoroacetic acid.

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flow rates while increasing sensitivity due to the improved ionization efficiency. Thereby, the different small-scale LC types are typically categorized on the basis of the low-flow rate ranges required for the operation [8–10]. Among them, capillary LC-MS (CapLC-MS, typical flow rates = 1–10 μL/min [10]) represents the best compromise between sample volume requirement and sensitivity, providing good separation efficiency, short analysis time, instrumental simplicity, full automation, as well as greater throughput and robustness than nanoLC-MS (nLC-MS, typical flow rates = 100–500 nL/min [10]), which is recognized as the most sensitive small-scale LC [3–6,10]. However, in any case, the reduced injection volumes result in an important limitation in terms of limit of detection (LOD) (in concentration units). To overcome this issue, solid-phase extraction (SPE), which includes solid-phase microextraction (SPME), can be used for analyte purification and preconcentration before CapLC-MS analyses [3,9,11–15]. In on-line solid-phase extraction capillary liquid chromatography-mass spectrometry (SPE-CapLC-MS), the sample pretreatment step is performed on-line before the separation and detection, allowing minimum sample handling, avoiding sample loss or contamination, preventing analyte degradation, and increasing analytical throughput [3,9,11–15]. SPE-CapLC-MS system requires an additional column and pump for the on-line extraction and a microvalve for the column-switching process between sample loading and elution, separation, and detection. In addition, there are different commercial pre-columns, guard columns or cartridges that can be used as enrichment columns. First, a large volume of sample is loaded into the enrichment column to retain the analyte(s) of interest, while weakly retained compounds (e.g. potential interferences) are washed to waste. Second, the microvalve is set to the elution position and the mobile phase pass through the enrichment column to release the retained analyte molecules into the analytical column for separation and detection [3,9,11–15].

Capillary electrophoresis-mass spectrometry (CE-MS) is also an excellent alternative for the highly efficient separation and characterization of peptides at small-scale [16–20]. The electroseparation mechanism in capillary zone electrophoresis (CZE) is complementary to the hydrophobicity-based mechanism in reversed-phase LC, which are the typical modes applied in peptide analysis by CE-MS and LC-MS, respectively [21–23]. CE-MS, which uses 50 and 75 μm internal diameter (ID) capillaries, is inherently a nanoflow technique [21,24,25]. However, the total flow rates with triple-tube coaxial sheathflow CE-MS (hereinafter CE-MS), which is currently the most popular, robust, and versatile CE-MS setup, are similar to those in CapLC-MS [24,25]. As in CapLC-MS, the LODs in CE-MS are compromised by the limited loading capacity [26]. In addition, the typical injected sample volumes in CE-MS are ten times lower than in CapLC-MS columns due to the narrower capillary diameters. On-line solid-phase extraction capillary electrophoresis-mass spectrometry (SPE-CE-MS) has demonstrated its potential to obtain high preconcentration factors, good reproducibility and broad applicability, including in peptide analysis [26–32]. In the most simple and popular SPE-CE configuration, a microcartridge, filled with an appropriate sorbent to retain the compound(s) of interest, is integrated in-line near the inlet of the separation capillary, and no valves are necessary for the operation [26]. First, the target analytes are retained in the microcartridge while loading a large volume of sample. After washing to remove non-retained molecules, they are eluted in a smaller volume of an appropriate solution, resulting in sample clean-up and concentration enhancement before electrophoretic separation and detection. A limitation of SPE-CE is that microcartridges are not commercially available, hence they must be fabricated in house. However, a wide variety of microcartridge designs have been described and construction is relatively simple and low-cost in most cases [26].

Indeed, SPE-CapLC-MS and SPE-CE-MS have been broadly applied in peptide analysis, however systematic and direct comparisons, using the same analytes and mass spectrometers are very scarce [33], promoting misconceptions. M. Pelzing et al [33] showed that nLC-MS was only about five times more sensitive than SPE-CE-MS for the analysis of bovine serum albumin (BSA) tryptic digests, despite the difference was less pronounced than expected. In contrast, SPE-CE-MS allowed better peptide recoveries and faster analyses. This study aims at continue filling this gap by comparing SPE-CapLC-MS and SPE-CE-MS for the analysis of opioid peptide biomarkers, which are a group of neuropeptides related to analgesia, pain modulation, neurological disorders, and cancer [7,34]. First, CapLC-MS and SPE-CapLC-MS were developed and validated for the analysis of dynorphin A (1–7) (DynA), endomorphin 1 (End 1), and methionine-enkephalin (Met) in standard solutions and human plasma samples. Then, the opioid peptides were analyzed by SPE-CE-MS with the same mass spectrometer, applying a method previously described in our group [27,28]. Similarities and differences between the instrumental set-ups and procedures were disclosed, as well as those related to method performance. The presented direct and fair comparison may help to an appropriate, wise, and consciously technique selection in peptide analysis and other fields.

2. Materials and methods

2.1. Reagents

All reagents were analytical grade or better. Sodium hydroxide (99.0 %, pellets), formic acid (FA, 98–100 %), trifluoroacetic acid (TFA, ≥ 99.0 %), glacial acetic acid (HAc, 100 %) and ammonium hydroxide (25 % (v/v)) were purchased from Merck (Darmstadt, Germany). Acetonitrile (ACN, ≥ 99.9 %), isopropyl alcohol (IPA, ≥99.9 %), methanol (MeOH, ≥99.9 %) and water were all LC-MS grade (Honeywell, Seelze, Germany). Dynorphin A (1–7) (Dyn A) was provided by Bachem (Bubendorff, Switzerland). Endomorphin 1 (End 1), methionine-enkephalin (Met), and polyethylene glycol (PEG) with an average molecular mass (M_r) of 8000 (PEG8000, 40 % (m/m)) were supplied by Sigma (St. Louis, MO, USA).

2.2. Apparatus and procedures

CapLC-MS experiments were performed in a 1200 series capillary liquid chromatograph coupled to a LC/MSD ion trap SL mass spectrometer with an orthogonal G1385-44300 interface (Agilent Technologies, Waldbronn, Germany). The SPE-CapLC-MS system was composed of a 1260 Infinity conventional liquid chromatograph (Agilent technologies) for sample loading (pump A in Fig. 1) and the same CapLC-MS system. The CapLC chromatograph was used to deliver the mobile phase for the elution, separation and detection (pump B in Fig. 1). Separation and enrichment were carried out in a Zorbax 300 SB-C_8 column (3.5 μm particle diameter, 300 Å pore diameter, 150 mm × 0.3 mm total length (L_T) × ID, Agilent Technologies) and a Zorbax 300 SB-C_{18} column (5 μm, 300 Å, 5 mm × 0.3 mm, Agilent Technologies), respectively.

CE-MS and SPE-CE-MS experiments were performed as described in our previous studies [27,28], in a HP^{3D} CE system coupled to the same mass spectrometer with an orthogonal G1603A sheathflow interface (Agilent Technologies). The sheath liquid was delivered at a flow rate of 3.3 μL/min by a K_D Scientific 100 series infusion pump (Holliston, MA, USA).

The ion trap mass spectrometer was operated in positive mode using the following parameters, which were optimized in our previous study by CE-MS and SPE-CE-MS [27,28]: capillary voltage, 4000 V; capillary exit voltage, 150 V; skimmer voltage, 50 V; octopole voltage,15.41 and 3.34 V; octopole radiofrequency, 267.21 Vpp; voltage lenses, −11.32 and −100 V; and trap drive 70.41 (arbitrary units). Due to the slight differences on the total flow rates between the optimized electrophoretic and chromatographic approaches (~3.3 vs 8 μL/min, respectively), the nebulizer gas (N_2) pressure, drying gas (N_2) flow rate and drying temperatures were slightly different: 7 vs 10 psi, 2 vs 4 L/min, and 300 vs 325 °C, respectively. Full scan mass spectra were acquired in the *m/z* range from 100 to 1,250 *m/z*, averaging every-seven spectra (speed = 5500 *m/z*/s). ChemStation A.10.02 software (Agilent Technologies) was

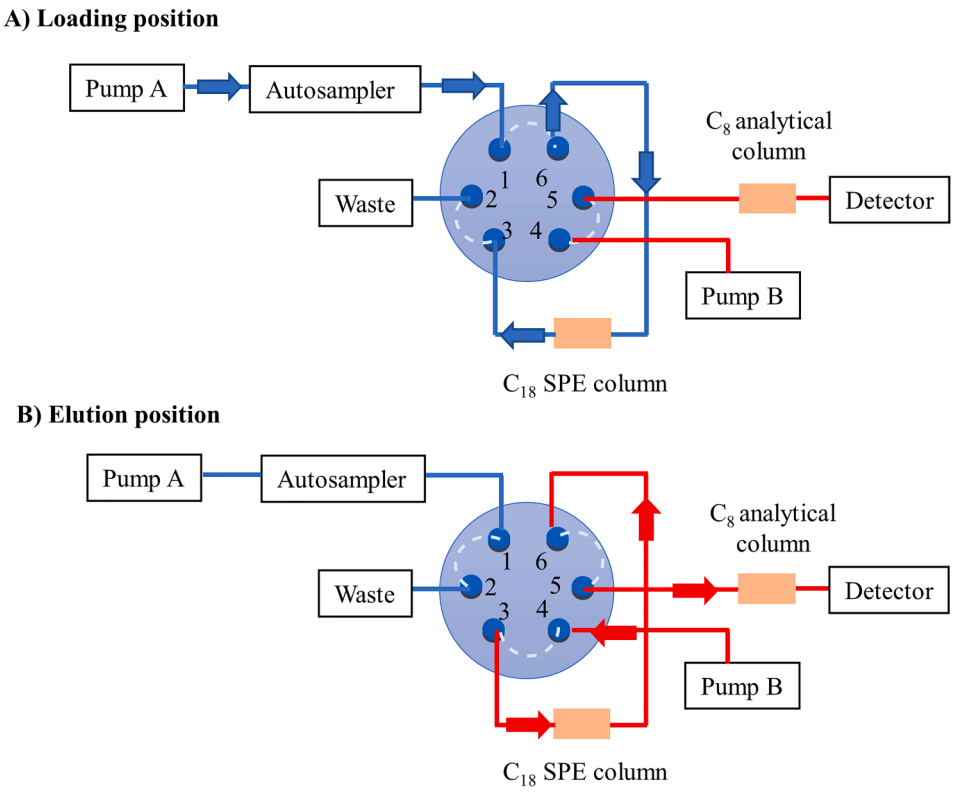

Fig. 1. SPE-CapLC-MS system with a 2-position/6-port microvalve, a conventional pump (pump A), a capillary pump (pump B), and the analytical and enrichment columns in (A) loading and (B) elution positions.

used for the CapLC pump and CE instrument control and separation data acquisition, which was run in combination with LC/MSD trap 3.2 software (Agilent Technologies) for control, data acquisition, and data analysis of the mass spectrometer.

A SPE vacuum manifold and a drying adapter of 12 slots designed for the vacuum distributor Visiprep (Supelco, Bellefonte, PA, USA) were used for sample drying. Centrifugation was performed in a cooled Rotanta 460 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). pH was measured with a MicropH 2002 potentiometer and a 52–03 combined pH electrode (Crison instruments, Barcelona, Spain).

2.3. Samples

Aqueous individual stock solutions of each peptide (Dyn A, End 1 and Met) at 2500 μg/mL were prepared and stored in the freezer at −20 °C until its use. A standard mixture solution of the three peptides at 100 μg/mL was prepared using individual stock solutions and stored in the freezer at −20 °C until its use. Diluted standard mixture solutions were freshly prepared from the 100 μg/mL standard mixture before the analyses. Samples were diluted in water and water with 0.1 % (v/v) FA, for electrophoretic and chromatographic analyses, respectively. Before chromatographic analysis, standard mixture solutions were filtered using 0.22 μm polyvinylidene difluoride (PVDF) centrifugal filters (Ultrafree®-MC, Millipore) at 12,000 g for 4 min at 25 °C.

Venous blood from a healthy volunteer was collected in standard 4 mL clinical vials (BD Vacutainer, 7.5 mg K_2EDTA, Becton and Dickinson, Madrid, Spain). Plasma was separated from the blood cells by centrifugation at 8000 g for 5 min at 4 °C and stored at −20 °C until its use. Plasma samples were processed as we described in previous studies to remove salts and high molecular mass proteins [27,28,30]. The off-line double-step pretreatment of plasma samples consisted of protein precipitation with cold ACN (plasma/ACN, 500:3000 μL) followed by centrifugal filtration with 10,000 M_r cutoff cellulose acetate filters (Amicon® Ultra-0.5, Millipore, Bedford, MA, USA). Centrifugal filters were passivated before the first use with 5 % (v/v) of PEG in water [30]. After filtration, the filtrate volume was adjusted to 200 μL (SPE-CapLC-MS) or 500 μL (SPE-CE-MS) with water. Only before SPE-CapLC-MS samples were further purified using 2 mg Oasis HLB μElution plates (Waters, Milford, MA, USA) [35]. Briefly, the sorbent was first conditioned with 200 μL of ACN followed by 200 μL of water with 0.1 % (v/v) TFA. Two hundred μL of sample was then loaded into the cartridges and washed with 800 μL of 0.1 % (v/v) TFA solution followed by 200 μL of water. The peptides were finally eluted using 100 μL of a 70 % (v/v) ACN solution. The solvent was evaporated to dryness and the obtained residue was reconstituted with 200 μL of water with 0.1 % (v/v) FA. Spiked plasma samples were prepared at 5 ng/mL by adding a mixture of the three peptides before making up the final volume.

2.4. CapLC-MS and SPE-CapLC-MS

CapLC-MS experiments were performed at room temperature with gradient elution at a flow rate of 8 μL/min and injecting 0.15 μL of sample. Eluting solvents were (A) water and (B) ACN (both with 0.1 % (v/v) FA). Solvents were degassed by sonication for 10 min before use. The optimized gradient of solvent B was as follows: from 20 % to 50 % (v/v) (0–5 min, linear), 50 % (v/v) (5–10 min), from 50 % to 20 % (v/v) (10–15 min, linear) and 20 % (v/v) (15–35 min).

SPE-CapLC-MS experiments were performed at room temperature. A 2-position/6-port microvalve (1200 Infinity 2/6 Microswivalve, G1162A, Agilent Technologies) was used for sample loading and column switching. A schematic drawing of the complete system is presented in Fig. 1. The microvalve was set to loading position (position 1, Fig. 1A) and 100 μL of sample (full-loop) was loaded at a flow rate of 150 μL/min (pump A). When necessary, the loading process was repeated twice to load 200 μL of sample. Loading was extended for 2.5 min to ensure washing of interferences, then the microvalve was switched to the elution position (position 2, Fig. 1B) and the retained analytes were eluted into the analytical column. Note that elution was performed in

the back-flush mode, pumping the mobile phase from the outlet to the inlet of the enrichment column at a flow rate of 8 μL/min (pump B). Optimized gradients for pump A and pump B are presented in Table 1. After 20 min of separation, the microvalve was switched again to the loading position to re-equilibrate the enrichment column at 150 μL/min (pump A) with loading solvent (100 % (v/v) water with 0.1 % (v/v) FA). Meanwhile, the analytical column was also re-equilibrated to the initial conditions (100 % (v/v) water with 0.1 % (v/v) FA) using pump B.

All quality parameters were calculated from data obtained by measuring the peak area and retention time from the extracted ion chromatograms (EICs) of the three opioid peptides (considering the *m/z* of the most abundant molecular ions, i.e. 434.7 (+2) Dyn A, 611.3 (+1) End 1 and 574.2 (+1) Met)). Intra-day repeatability studies (n = 8) were performed with a standard mixture solution of the three peptides at a concentration of 10 μg/mL and 10 ng/mL for CapLC-MS and SPE-CapLC-MS, respectively. Repeatability was calculated as percent relative standard deviation (%RSD) of peak areas and retention times. The linearity range and LODs (signal-to-noise ratio (S/N) >3) were investigated by analyzing standard mixture solutions of peptides at concentrations ranging from 0.1 to 50 μg/mL for CapLC-MS and from 0.1 to 100 ng/mL for SPE-CapLC-MS.

2.5. CE-MS and SPE-CE-MS

All fused silica capillaries were supplied by Polymicro Technologies (Phoenix, AZ, USA). CE-MS and SPE-CE-MS experiments were performed as described in our previous study using 72 cm L_T × 75 μm ID × 360 μm outer diameter (OD) fused silica capillaries. In SPE-CE-MS, a double-frit particle packed fused silica microcartrige (7 mm L_T × 250 μm ID × 360 μm OD, 0.1 cm frits), filled with the sorbent found in C_{18} Sep-Pak cartridges (Waters, 55–105 μm particle diameter and 125 Å pore size), was inserted at 7.5 cm from the inlet of the separation capillary [27]. Samples were loaded during 10 min at 930 mbar (65 μL as estimated with the Hagen Poiseuille equation [36], which is a volume 6500 times larger than the volume injected by CE-MS [27], i.e. 10 nL = 3 s at 33.5 mbar [36]). The eluent was injected during 10 s at 50 mbar (60 nL [36]). The quality parameters obtained for the opioid peptides in our previous study [27] were experimentally confirmed again to ensure an accurate and fair comparison between SPE-CapLC-MS and SPE-CE-MS.

3. Results and discussion

3.1. Analysis of opioid peptides by CapLC-MS and SPE-CapLC-MS

The development of a sensitive SPE-CapLC-MS method is not straightforward, and a CapLC-MS method was firstly established to obtain appropriate separation efficiency, separation resolution, total analysis time, LODs, and repeatabilities of peak areas and retention times.

Table 1
Optimized conditions for SPE-CapLC-MS.

Time [a] (min)	Gradient A (pump A)			Gradient B (pump B)[c]	
	Flow rate A[b] (μL/min)	H_2O[c] (%v/v)	ACN (%v/v)	H_2O[c] (%v/v)	ACN[c] (%v/v)
0	150	100	0	100	0
2.5	8	100	0	100	0
5	8	100	0	100	0
10	–	–	–	50	50
15	–	–	–	50	50
19	8	100	0	–	–
19.5	150	100	0	–	–
25	150	0	100	–	–
30	150	100	0	90	10
60	150	100	0	100	0

a) Under optimized conditions the switching valve times were 2.5 and 20 min.
b) The flow rate of pump A was 150 μL/min and was reduced to 8 μL/min during most of the time the valve was in the elution position to avoid excessive waste. The flow rate of pump B was always 8 μL/min.
c) This mobile phase contained 0.1% (v/v) FA.

3.1.1. CapLC-MS

The chromatographic separation was optimized with a mobile phase flow rate of 8 μL/min in order to maintain a reasonable total analysis time without exceeding the maximum pressure recommended by the manufacturer in the chromatographic system (i.e. 400 bar). Isocratic elution experiments employing different ACN compositions (20, 25, 30 and 35 % (v/v)) were firstly tested, and acceptable results were obtained by using 25 % (v/v) of ACN (data not shown). Then, gradient elution assays were conducted to improve peptide separation while decreasing total analysis time. The best results were achieved by using the gradient elution program of 35 min described in the experimental section. In this gradient, the last 20 min were used for re-equilibration of the system to the starting ACN composition (20 % (v/v) ACN). Gradient programs with shorter re-equilibration times (e.g. 10 and 15 min) were assessed to further reduce the total analysis time, but poor separations and repeatabilities were obtained.

It is worth mentioning that as in CapLC-MS the flow rate values were slightly higher than in CE-MS (8 vs ~3.3 $\mu L \cdot min^{-1}$), the ESI-MS interface parameters related to the nebulizer and drying gases (i.e. nebulizer gas pressure, drying gas temperature and flow rate) needed a little adjustment compared to our previous work by CE-MS [27]. Low nebulizer gas pressures (7 psi) allowed detecting Met and End 1, although with poor repeatabilities, while an excessive increase in the nebulizer gas pressure (10 and 15 psi) and temperature (350 °C) hindered Met and Dyn A detection. The three studied opioid peptides were only detected by using 10 or 15 psi as nebulizer gas pressure and 325 °C, while maintaining the drying gas at 4 L/h. As better peptide intensities were achieved with a nebulizer gas pressure of 10 psi, these conditions were selected for further experiments.

Fig. 2 presents an overlay of the extracted ion chromatograms (EICs) and mass spectra of the three opioid peptides (Dyn A, Met, and End 1) for the analysis of a 10 μg/mL peptide mixture under the optimized conditions. Separation was completed in less than 7 min (Fig. 2A) and single protonated molecular ions were mainly detected for End 1 and Met, whereas double protonated molecular ions were found for Dyn A (Fig. 2B). Although some peak tailing was observed, good separation resolution between peptides was achieved. The method was validated in terms of intra-day repeatability, linearity and LODs. Table 2A summarizes %RSD values for repeatability of peak areas and migration times. The %RSD values ranged from 0.2 to 1.2 % for retention times and from 2.7 to 5.8 % for peak areas. Linearity was evaluated in the concentration range between 0.1 and 50 μg/mL. The method was linear between 5 and 50 μg/mL for Dyn A and Met and 1–50 μg/mL for End 1 ($r^2 > 0.99$). Dyn A and Met presented LOD values of 0.5 μg/mL, while End 1 could be detected until 0.1 μg/mL.

3.1.2. SPE-CapLC-MS

Once the CapLC-MS method was established, SPE-CapLC-MS was investigated with the aim of lowering the LODs while maintaining the separation resolution. The SPE-CapLC-MS set-up (Fig. 1) was operated by a 2-position/6-port microvalve. It included a conventional pump (pump A) to load in the enrichment column a large sample volume (100 μL at full-loop) and wash potential interferences at high flow rate (150 μL/min), as well as a capillary pump (pump B) to elute the preconcentrated peptides into the analytical column at the flow rate established for CapLC-MS (8 μL/min).

Initial SPE-CapLC-MS experiments were performed loading 100 μL of peptide mixture prepared in water with 0.1 % (v/v) FA, using water as mobile phase, and a valve switching time from loading to elution positions of 5 min (see Table 1 for final optimized conditions). The EICs of the three opioid peptides under these conditions for a 10 ng/mL peptide mixture are shown in Fig. 3C. The three peptides could be detected, but

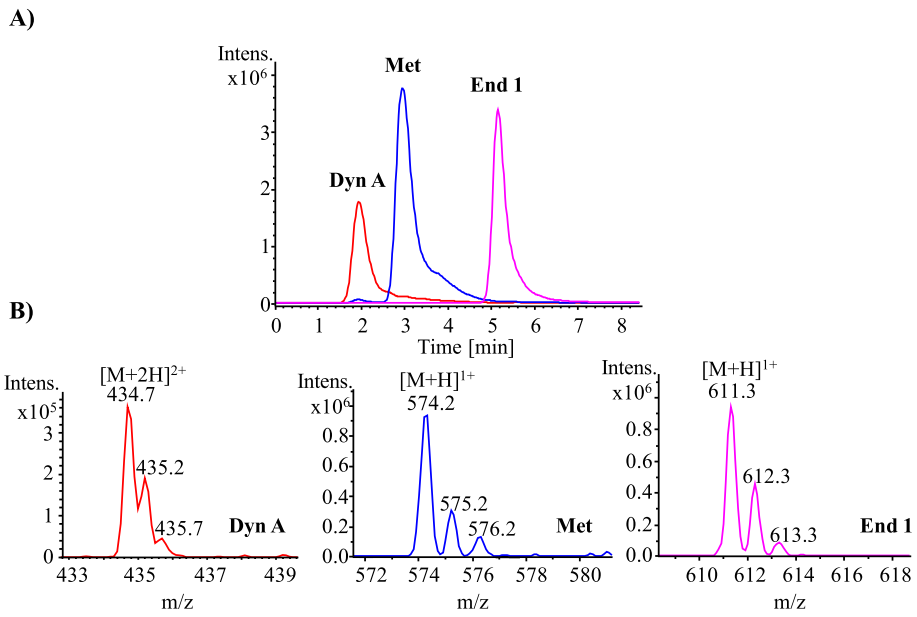

Fig. 2. A) Extracted ion chromatograms (EICs) (434.7, 574.2 and 611.3 *m/z*) and B) mass spectra by CapLC-MS for the analysis of a 10 μg/mL standard peptide mixture.

Table 2
Intra-day repeatability, linearity and LOD values for the analysis of the opioid peptides by A) CapLC-MS, B) SPE-CapLC-MS, and C) SPE-CE-MS.

Peptide	Repeatability[a] (n = 8), %RSD		Linearity		LOD
	Time	Peak Area	Regression line ($R^2 > 0.990$)	Concentration range	Experimental
A) CapLC-MS				(μg/mL)	(μg/mL)
Dyn A	0.7	2.7	A = 146845356C + 1400083	5–50	0.5
Met	1.2	5.8	A = 9362234C + 3923536	1–50	0.5
End 1	0.2	3.8	A = 5210114.20C − 1299057	1–50	0.1
B) SPE-CapLC-MS				(ng/mL)	(ng/mL)
Dyn A	0.1	11	A = 2999304C − 21009163	0.5–100	0.5
Met	0.1	0.6	A = 11448728C + 21866285	0.5–100	0.5
End 1	0.2	2.4	A = 9943474C − 14359317	0.5–100	0.5
C) SPE-CE-MS				(ng/mL)	(ng/mL)
Dyn A	1.9	10	A = 4629499282C − 7076	0.1–100	0.1
Met	2.3	12	A = 772834076C + 3803118	1–100	1.0
End 1	2.2	11	A = 3455054518C + 43697	0.1–100	0.1

a) 25 μg/mL (CapLC-MS) or 10 ng/mL (SPE-CapLC-MS and SPE-CE-MS) peptide mixtures were analyzed on the same day.

the relative area of the peak corresponding to Dyn A was lower than before by CapLC-MS (Fig. 2A). In order to increase Dyn A recovery, the loaded sample volume was increased from 100 μL to 200 μL and 300 μL, by consecutively performing two and three full-loop injections, respectively. Fig. 3 shows the EICs of the three opioid peptides after loading

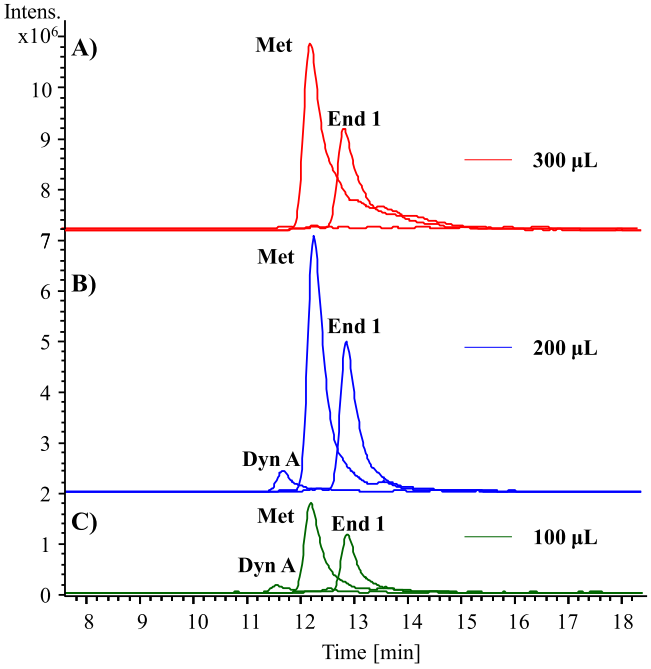

Fig. 3. Extracted ion chromatograms (EICs) (434.7, 574.2 and 611.3 *m/z*) by SPE-CapLC-MS for the analysis of a 10 ng/mL standard peptide mixture loading different sample volumes: A) 300 μL (3 consecutive full-loops), B) 200 μL (2 consecutive full-loops), and C) 100 μL (1 full-loop). Sample loading time was 5 min.

different sample volumes.

As can be seen, with 200 μL there was a significant increase in peak area for all peptides compared to 100 μL. In contrast, with 300 μL, peak areas were lower than with 200 μL, suggesting that the breakthrough volume of the enrichment column was exceeded [27,28]. Therefore, a sample volume of 200 μL was selected. Regarding sample loading time, a slight increase in the three peptide peak areas was observed when valve switching time was decreased from 5 to 2.5 min (Fig. S1). This suggested that longer loading times promoted peptide washing out. Consequently,

a sample loading time of 2.5 min was selected for the rest of experiments. Fig. 4A shows the EICs of the three peptides for the loading of 200 µL of a 10 ng/mL peptide mixture during 2.5 min (see Table 1 for final optimized conditions). As can be observed, separation was completed in less than 14 min and all three opioid peptides eluted at longer retention times compared to CapLC-MS analyses (Fig. 2A). Regarding peak tailing and separation resolution, both were very similar compared to CapLC-MS. Fig. S2A shows, as an example, the mass spectra obtained for the time region corresponding to elution of End 1 and Met under the optimized conditions. In order to obtain these clean mass spectra, samples were filtered before loading with PVDF membrane filters. Fig. S2B shows the mass spectra when typical nylon membrane filters were used instead. As can be observed, two other high abundant molecular ions were detected with 453.4 and 679.5 *m/z* values. These interfering signals were related to cyclic oligomers from the nylon membrane filters used for mobile phases filtration, as reported by other authors in MS analyses [37]. In our case, theses impurities were only observed by SPE-CapLC-MS as they were also preconcentrated with the target peptides and eluted at similar retention times. Using PVDF membrane filters, these impurities were no longer detected (Fig. S2A).

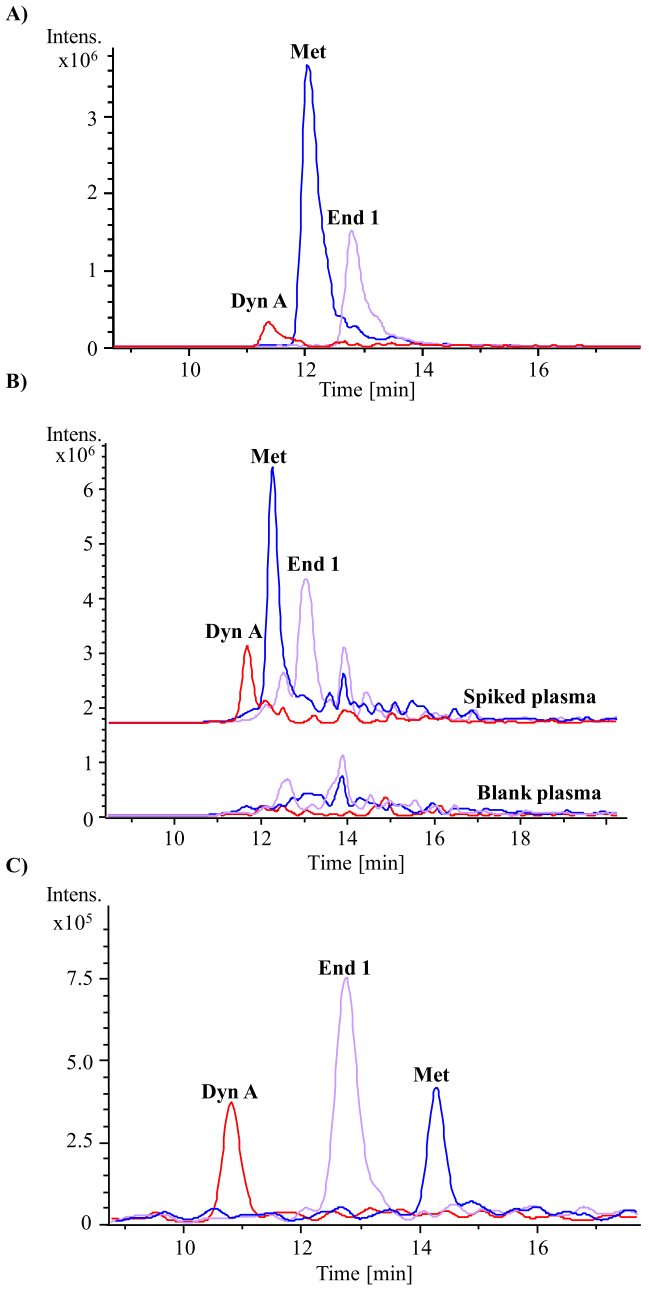

Fig. 4. Extracted ion chromatograms (EICs) or electropherograms (EIEs) (434.7, 574.2 and 611.3 *m/z*) for the analysis of A) a 10 ng/mL standard peptide mixture by SPE-CapLC-MS, B) a blank plasma sample and a plasma sample spiked at 5 ng/mL with peptides by CapLC-MS and C) a 10 ng/mL standard peptide mixture by SPE-CE-MS. In SPE-CapLC-MS 200 µL were loaded and loading time was 2.5 min.

The method was validated in terms of intra-day repeatability, linearity and LODs. Table 2B summarizes %RSD values for repeatability of peak areas and retention times. The %RSD values ranged from 0.1 to 0.2 % for retention times and from 0.6 to 11 % for peak areas. The slightly higher %RSD values in peak areas compared to CapLC-MS (Table 2A) were expected due to the complexity of the instrumental set-up. Linearity ranges were slightly broader than in CapLC-MS, from 0.5 to 100 ng/mL for the three studied opioid peptides. The obtained LODs were 1000 times lower for Dyn A and Met, and 200 times lower for End 1 than by CapLC-MS. The enrichment columns could be used for 20 runs without deterioration in the extraction or system pressure performance.

The developed SPE-CapLC-MS method was applied to the analysis of opioid peptides in plasma samples. Preliminary experiments were performed processing the samples with an off-line double-step pretreatment, based on protein precipitation with ACN followed by centrifugal filtration with 10,000 M_r cutoff filters, developed in our previous study for SPE-CE-MS [27,28,30]. However, the enrichment column was blocked after a couple of analyses. In order to increase the durability of the enrichment column to at least 10 injections with plasma samples, we further purified the obtained filtrates by SPE with HLB µElution plates [35]. Fig. 4B shows the EICs of the three opioid peptides for the analysis of a blank plasma sample and a plasma fortified with 5 ng/mL of Dyn A, Met and End 1. As can be observed, the opioid peptides could be successfully detected in a plasma sample spiked at a concentration of 5 ng/mL, with similar retention times and separation resolutions than in the analysis of the standard mixture (Fig. 4A). These results demonstrate the potential of SPE-CapLC-MS for enhancing CapLC-MS sensitivity for peptide analysis also in complex biological samples.

3.2. *SPE-CapLC-MS versus SPE-CE-MS*

Results by SPE-CapLC-MS were compared with those previously obtained by SPE-CE-MS [27]. The quality parameters for the opioid peptides of our previous study [27] were experimentally confirmed again to ensure an accurate and fair comparison. Table 2B and C show repeatability, linearity and LODs by both methods.

In terms of LODs, the values by SPE-CapLC-MS were in the same order of magnitude to those obtained by SPE-CE-MS, but five times higher in the case of Dyn A and End 1 and two times lower in the case of Met. One would expect a much better performance of SPE-CapLC-MS, as the enrichment column and microcartidge had similar dimensions with both techniques (5 mm L_T × 300 µm ID vs 7 mm L_T × 250 µm ID for SPE-CapLC-MS and SPE-CE-MS, respectively) but, in case of SPE-CapLC-MS, the C_{18} sorbent had smaller particle size (5 µm vs 55–105 µm) and the loaded sample volume was larger (200 µL vs 65 µL). The highly efficient preconcentration in SPE-CE-MS may be related to the extremely large volume loaded compared to CE-MS and the elution in a nanoliter volume (eluent volume ~60 nL), even though the elution in SPE-CapLC-MS was performed in the back-flush mode to maximize recoveries. Regarding repeatability, the %RSD values of times and peak areas by SPE-CapLC-MS were in general significantly lower than by SPE-CE-MS, excepting for DynA, which was detected as a smaller peak than Met and End 1, hence it was more difficult to integrate. It is well known the better repeatability of retention time in LC than migration time in CE, something that, if necessary, can be approached using electrophoretic mobility or relative migration time as an alternative to raw migration time for identification purposes. The better repeatability of peak areas in SPE-CapLC-MS may be related to the better repeatability of the full-loop

loading and the elution operated by the CapLC pump. In terms of linearity, concentration ranges were similar in both cases, but slightly wider for Dyn A and End 1 by SPE-CE-MS and for Met by SPE-CapLC-MS. In Fig. 4A and C can be observed the typical chromatograms and electropherograms obtained at 10 ng/mL by SPE-CapLC-MS and SPE-CE-MS under optimized conditions. As can be seen, total separation times were similar in both cases, but separation resolution was better by SPE-CE-MS. The improved resolution could be attributed to the greater peak efficiencies and better peak shapes by SPE-CE-MS. In addition, variation in the retention/migration order of the studied peptides was observed. Indeed, it is widely accepted that the separation mechanism based on differential analyte charge-to-hydrodynamic radius ratios of CZE is orthogonal to the hydrophobicity-based mechanism of reversed phase LC, hence providing complementary selectivities [21–23]. Regarding durability of the enrichment columns and microcartidges, with standards was similar (20 analyses), but in SPE-CapLC-MS an extra purification step was needed with plasma samples to perform a similar number of analyses than in SPE-CE-MS (10 analyses).

Beyond the specific performance of both methods, one of the main advantages of SPE-CE-MS is the simplicity of the instrumental set-up and easiness for the operation, as no valves are required to automate the procedure. In addition, the total analysis time, including the equilibration, loading, elution, separation, detection and reequilibration steps, is much shorter than by SPE-CapLC-MS (15 min vs 30 min). Attention may also be drawn to the reduced solvent consumption in SPE-CE-MS, as well as the lower cost of the involved instrumentation, separation capillaries and microcartridges, which can be easy homemade prepared when needing replacement. Despite, the non-commercial availability of the microcartridges may be regarded by many as a limitation. Other potential advantage of SPE-CE is that, if necessary, it can be combined with on-line electrophoretic preconcentration techniques such as transient isotachophoresis [29] or with nanoflow sheathless CE-MS [31] to further improve sensitivity. The many positive arguments make SPE-CE-MS very recommendable. Unfortunately, nowadays SPE-CE-MS has less potential to be widely applied than SPE-CapLC-MS, since CE-MS is less popular than LC-MS. This could be attributed to the common erroneous belief that CE operation is more complex, especially to the fact that the CE-MS coupling is less reproducible and robust than the LC-MS coupling. This may be definitely true for those users who do not receive an appropriate expert training.

4. Conclusions

In this study, an SPE-CapLC-MS method was developed for the analysis of low molecular mass opioid peptide biomarkers (Dyn A, End 1, and Met). First, a CapLC-MS method was established for the analysis of the studied peptides in standard solutions, as a starting point for the optimization and validation of an SPE-CapLC-MS method with improved sensitivity. SPE-CapLC-MS allowed an enhancement in sensitivity up to 1000-times in comparison to CapLC-MS, leading to LOD values of 0.5 ng/mL for the studied peptides. The applicability of the SPE-CapLC-MS method was also demonstrated for the analysis of plasma samples. Results obtained by SPE-CapLC-MS were compared with those obtained by SPE-CE-MS. Based on the comparison of method performances, instrumental set-ups and procedures, SPE-CE-MS presented many advantages compared to SPE-CapLC-MS for the sensitive detection of peptide biomarkers in biological fluids. In addition, SPE-CE-MS combined with on-line electrophoretic preconcentration or nanoflow sheathless CE-MS have potential to further sensitivity improvements beyond current SPE-nLC-MS, which is widely thought to be the most sensitive analytical approach for peptide analysis, especially in bottom-up proteomics. This conclusion may also be extended to other analytes and application fields, and may help to renew the interest and expand the applicability of SPE-CE-MS over SPE-LC-MS approaches.

CRediT authorship contribution statement

Oumaima El Ouahabi: Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing. **Montserrat Mancera-Arteu:** Supervision, Investigation, Writing – original draft, Writing – review & editing. **Laura Pont:** Methodology, Validation, Writing – review & editing. **Estela Giménez:** Investigation, Writing – review & editing. **Victoria Sanz-Nebot:** Investigation, Writing – review & editing, Funding acquisition. **Fernando Benavente:** Conceptualization, Supervision, Investigation, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on reasonable request

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