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On-line aptamer affinity solid-phase extraction capillary electrophoresis-mass spectrometry for the analysis of protein biomarkers in biological fluids and food: A tutorial

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ABSTRACT

The analysis by capillary electrophoresis (CE) of low abundant proteins in complex samples, such as biological fluids and food, is especially challenging, due to the poor concentration sensitivity of microscale separation techniques and the sample matrix complexity. In order to overcome these major drawbacks, microextraction sample preparation techniques based on on-line solid-phase extraction capillary electrophoresis (SPE-CE) are regarded as an excellent alternative for sample matrix clean-up and analyte preconcentration with minimum sample handling. In this study, we present, as a tutorial, a valve-free on-line aptamer affinity solid-phase extraction capillary electrophoresis-mass spectrometry (AA-SPE-CE-MS) method for purification, preconcentration, separation, detection, and characterization of intact protein biomarkers in biological fluids and food using as cases of study α-synuclein (α-syn), concanavalin A (Con A), and β-lactoglobulin (β-LG), which are related to Parkinson's disease and food allergy, respectively. This tutorial is not limited to the description of the analytical method, but it also provides ready-to-use preparation procedures for sorbent and microextraction devices, and introduces strategies to overcome undesired effects, allowing a straightforward implementation and optimum performance of AA-SPE-CE-MS, as a platform to develop further applications.

1. Introduction

Capillary electrophoresis (CE) is a very suitable technique for the efficient separation of ionizable biomolecules such as peptides and proteins [1–7]. However, the reduced sample volume typically injected in CE (1-2% of the total capillary volume) compromises the concentration sensitivity, as in other microscale separation techniques [8–13]. To solve this issue, many strategies based on using selective and sensitive detectors (e.g., fluorescence and mass spectrometry (MS) detectors), as well as different electrophoretic and microextraction techniques for on-line preconcentration [8–15] have been proposed. Among these microextraction techniques, on-line solid-phase extraction capillary electrophoresis (SPE-CE) has been broadly recognized as a powerful tool to enhance sample loadability of CE, while cleaning-up and preconcentrating the target peptides and proteins to achieve until 10,000 times lower limits of detection (LODs) [9,16–27]. In the most simple and convenient valve-free SPE-CE configuration [9], a microcartridge integrated in-line near the inlet of the separation capillary contains a suitable sorbent to selectively retain the target analytes. After loading a large volume of sample (~50–100 μL), the capillary is rinsed to remove non-retained molecules and filled with the separation background electrolyte (BGE). Then, the retained 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.

Traditionally, due to their versatility and commercial availability, the most widely used sorbents in SPE-CE have been typical chromatographic sorbents, such as C8, C18, HLB, or ion exchange [9]. However, they offer limited selectivity, which makes the analysis of proteins in complex samples, such as biological fluids and food, a challenging task. To overcome this major drawback, sorbents with improved selectivity have been proposed, such as those based on immobilized metal affinity chromatography (IMAC) [23], lectins [28], antibodies [20–22], and aptamers [24–27,29–31]. In the last years, aptamer-based sorbents have been demonstrated to be an excellent alternative for the analysis of small molecules and proteins by SPE-CE with fluorescence and MS detection (AA-SPE-CE-LIF [29] and AA-SPE-CE-MS [24–27,30,31], respectively). Aptamers are generally single-stranded RNA or DNA oligonucleotides with less than 100 nucleotides, capable of selectively recognizing tar-

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get molecules with high affinity [32,33]. They are typically selected through a long and expensive iterative procedure called SELEX (systematic evolution of ligands by exponential enrichment), which uses large combinatorial libraries of oligonucleotides [32,33]. However, once their sequence has been established, they can be chemically synthesized at a low price in an automated, rapid, and reproducible way, avoiding the inconveniences that exist, for instance, in antibody production, such as the need for animals or cells, and variations between batches [34]. Other interesting advantages of aptamers include robustness, thermal stability, tolerance to wide ranges of pH and salt concentrations. Furthermore, their terminal residues can be easily derivatized to reproducibly prepare highly efficient extraction sorbents [24–27,31].

In this tutorial, we provide a detailed procedure for the preparation of aptamer affinity sorbents by covalent immobilization of aptamers onto the surface of commercially available magnetic beads (MBs). We also include all the necessary relevant information for the successful construction of fritless microcartridges, the optimization of the most critical steps to develop an AA-SPE-CE-MS method, as well as a troubleshooting guide for non-expert users. We selected as illustrative examples the analysis in blood of α-synuclein (α-syn), which is a protein biomarker of Parkinson's disease, and the analysis in food of concanavalin A (Con A) and β-lactoglobulin (β-LG), which are allergenic proteins. We intend to present a general guide for the straightforward implementation and optimum performance of AA-SPE-CE-MS, as a platform to expand its applicability beyond of protein biomarkers, biological fluids, food, and MS detection.

2. Materials and procedures

2.1. Reagents and solutions

All solutions are prepared using LC-MS grade water and analytical grade reagents or better. Except for the sheath liquid, they are filtered through 0.22 μm nylon filters before AA-SPE-CE-MS analysis. The BGE, the sheath liquid, and the eluent are also degassed in an ultrasonic bath for 10 min. All solutions are stored at 4 ºC and allowed standing at room temperature before use.

Protein stock solutions (1 $mg{\cdot}mL^{-1}$) are prepared in water or phosphate buffered saline (PBS), aliquoted, and stored in a freezer at -20 ˚C. If necessary, low molecular mass (M_r) excipients are removed from the stock solution by passing through low-protein binding centrifugal filters of appropriate M_r cut-off, depending on the M_r of the target protein (a detailed protocol using Amicon® Ultra-0.5 centrifugal filters from Millipore (Bedford, MA, USA) can be found in [35]). Aliquots of the stock solution are thawed before use, and working standard solutions are freshly prepared by diluting with proper volumes of water or PBS.

The aptamer sequence against the target protein can be selected after a SELEX procedure [32,33] or it can be found in the literature to be later synthesized in automated platforms by manufacturers of custom oligonucleotides (e.g., Integrated DNA Technologies, Coralville, IA, USA [24–27,31]). Aptamers are recommended to be ordered purified by LC and modified with a C6 spacer arm terminated by a 5′-amino group. This modification allows the covalent immobilization of the aptamer with an adequate orientation and minimal steric hindrance onto an appropriately activated solid support. Aptamer solutions are prepared in PBS at a concentration of 100 µM, aliquoted, and stored in a freezer at -20 ˚C.

2.2. Preparation of the AA sorbent

The AA sorbent is prepared as described in our previous studies [25–27,31] using commercially available activated MBs (MAGicBeads ACT magnetic beads (MBs) of 45–165 μm diameter were provided by MAGic BioProcessing (Uppsala, Sweden)). Note that these MBs and the provider company underwent different name changes during the last years. These MBs are functionalized for the covalent immobilization of molecules with primary amino groups (e.g., the amino-modified aptamer). The preparation of the AA sorbent is very simple and easily reproducible thanks to the use of a magnet during all the washing steps to recover completely the MBs:

- Pipette 200 μL of MBs into a clean vial. Vortex and remove the supernatant after magnetic separation with a neodymium cube magnet (e.g., 12 mm, N48) to sediment the particles (20 μL of sedimented MBs). Wash the MBs (three times) with 200 μL of PBS containing 0.1% (v/v) Tween 20 (PBS-T) and resuspend in 200 μL of PBS-T.
- Add 10 μL of the activation buffer provided with the MBs kit to the MBs suspension. Moderately shake the mixture for 15 min at room temperature using, for example, a TS-100 thermoshaker (Biosan, Riga, Latvian Republic). Remove the supernatant by magnetic separation, wash the MBs (three times) with 200 μL of PBS-T, and resuspend in 150 μL of PBS-T.
- Add 50 μL of the amino-modified aptamer solution (100 µM) and moderately shake the mixture for 40 min at room temperature. Remove the supernatant by magnetic separation, wash the AA-MBs (three times) with 200 μL of PBS, and remove the supernatant by magnetic separation.
- Add 200 μL of blocking buffer (5% bovine serum albumin (BSA) in PBS-T) and moderately shake for 2 h at 37 ºC. Remove the supernatant by magnetic separation, wash the AA-MBs (three times) with 200 μL of PBS, and resuspend in 200 μL of PBS for short-term use. For long storage periods, it is recommended to store the AA-MBs in PBS with 20% (v/v) ethanol at 4 ˚C to prevent microbial action.
- Do not allow to dry the activated MBs or the AA-MBs. This preparation procedure results in enough AA-MBs to prepare more than 50 AA microcartridges. Anyway, if the procedure is systematically followed the reproducibility of the preparation is excellent.

2.3. Construction of the AA microcartridge

The construction of fritless particle-packed AA microcartridges is carried out as described in previous studies [9,24,26,27,31], taking advantage of the larger size of the sorbent particles compared to the inner diameter (id) of the separation capillary. Fritless microcartridges avoid the potential issues with frits, such as complex preparation or handling, increased flow restriction, counter-pressures, and bubble formation. The capillaries can be purchased, for example, from Polymicro Technologies (Phoenix, AZ, USA).

Before constructing the AA microcartridge, it is recommended to prepare:

- Separation capillary: Cut a 72 cm total length (L_T) x 75 μm id x 360 μm outer diameter (od) capillary. Activate the separation capillary by flushing with NaOH 1 M (15 min) and water (15 min). Perform the activation outside the CE-MS interface in order to avoid contamination of the mass spectrometer. Cut the separation capillary into two pieces of 7.5 and 64.5 cm (inlet and outlet separation capillary, respectively). Flush them manually with water.
- Microcartridge body: Cut a 0.7 cm L_T x 250 μm id x 360 μm od capillary. Flush it manually with water. A shorter or longer L_T can be used to prevent excessive counter-pressure or to increase the amount of packed sorbent, respectively.
- Adapted needle: Use a 40 mm L_T × 0.8 mm od hypodermic metal needle to prepare a needle adapted to fit 360 μm od capillaries. Sand the beveled tip of the needle with small grit sandpaper and wash the resulting flat-tip needle with water. Connect a 0.5 cm L_T of a Tygon® plastic tube (250 μm id, orange-blue retaining stops) to the needle tip. Use the adapted needle in combination with a 5 mL disposable polypropylene syringe to flush manually 360 μm od capillaries. While connecting a capillary, be careful not to push it too much inside the plastic connector to avoid contact between the flat-tip metal needle and the capillary. All capillaries need to be easily pulled out later.

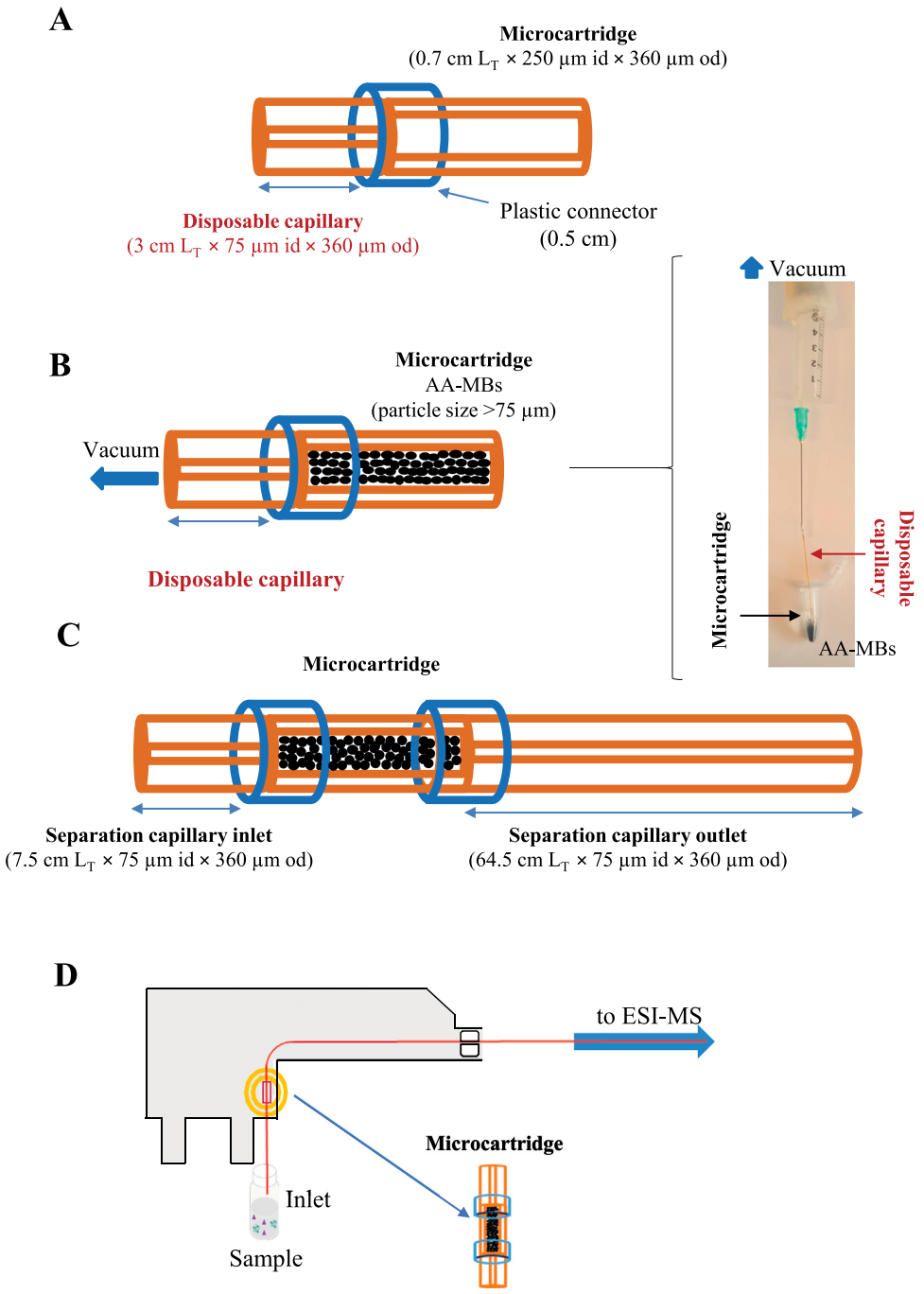

Fig. 1. Construction procedure of a fritless AA-SPE-CE microcartridge using AA-MBs.

The construction of the AA microcartridge is depicted in Fig. 1. All the steps must be controlled under a stereomicroscope at 100x magnification to ensure the best reproducibility.

- Cut a 0.5 cm L_T piece of the Tygon® plastic tube. Push carefully the microcartridge body until it is 0.25 cm inside the plastic tube. Connect to the microcartridge body a disposable 3 cm L_T x 75 μm id x 360 μm od capillary using the plastic tube (Fig. 1A). Push carefully the disposable capillary inside the plastic connector until no dead volume between both capillaries is observed. Flush manually with water from the free end of the disposable capillary.
- Prepare a device to connect the adapted needle to the vacuum system. Cut 1 cm from the bottom of the body of a 5 mL disposable polypropylene syringe and fit it in an appropriate plastic vacuum hose. Attach the adapted needle connected to the disposable capillary-microcartridge body piece to the syringe tip. Pack the microcartridge by vacuum with the AA-MBs sorbent until it is completely filled (Fig. 1B). Disconnect the adapted needle from the vacuum and use a soft paper towel to eliminate the sorbent from the outside of the microcartridge.
- Connect the outlet end of the microcartridge body to the 64.5 cm piece of the outlet separation capillary, using a 0.5 cm L_T piece of the Tygon® plastic tube. Pull the disposable capillary carefully out of the connection with the microcartridge body inlet. Then, connect the 7.5 cm piece of inlet separation capillary (Fig. 1C).
- Check that the microcartridge is completely filled with the AA-MBs sorbent under the microscope and that there is no dead volume between both capillary connections. Flush manually the AA-SPE-CE

capillary from the inlet with water to check the system for abnormal flow restriction and to ensure an appropriate packing. Discard the AA-SPE-CE capillary if water is not flowing or if you observe sorbent leakage.
- Install the AA-SPE-CE capillary in the cartridge cassette as shown schematically for the typical cartridge cassette model fitting in a CE instruments of Agilent Technologies (Waldbronn, Germany) CE instruments (Fig. 1D). In this case, the best position to fit the microcartridge near the inlet of the separation capillary is at 7.5 cm from the inlet. At this position the microcartridge is completely inside the cartridge cassette, which is thermostated by air.
- According to our experience, the removal of 2 cm of the nonconductive polyimide coating (burning with a lighter) from the outlet end of the AA-SPE-CE capillary allows an appropriate capillary tip for a stable spray and a proper mixture between the BGE and the sheath liquid. Install very carefully the capillary outlet in the CE-MS sheathflow interface (Agilent Technologies). Consider that a proper capillary protrusion from the metal electrode tip (i.e., 0.1 cm) minimizes the mixing void volume between the BGE and the sheath liquid, allowing the best sensitivity and repeatability in CE-MS. Note that the AA-SPE-CE capillary can be alternatively installed first in the CE-MS sheathflow interface and the tip prepared before the final adjustment to prevent erosion or breakage during the installation.

2.4. AA-SPE-CE-MS method

The AA-SPE-CE-MS method detailed below is described to be performed on a 7100CE coupled with an orthogonal G1603A sheathflow interface to a 6220 oa-TOF LC/MS spectrometer (Agilent Technologies). ChemStation and MassHunter softwares (Agilent Technologies) are used for the CE and TOF mass spectrometer control, data acquisition, and processing. The sheath liquid is delivered at a flow rate of 3.3 μL·min^{-1} by a KD Scientific 100 series infusion pump (Holliston, MA, USA). The TOF mass spectrometer is operated in positive electrospray ionization (ESI) mode, and the optimized parameters are: capillary voltage 4000 V, drying gas temperature 300 ºC, drying gas flow rate 4 L·min^{-1}, nebulizer gas 7 psig, fragmentor voltage 325 V, skimmer voltage 80 V, OCT 1 RF Vpp voltage 300 V. Data are collected in profile at 1 spectrum/s between 100 and 3200 *m/z*, with the mass range set to high resolution mode (4 GHz) [35].

The AA-SPE-CE-MS method is described below:

- Flush the AA-SPE-CE-MS capillary with BGE at 930 mbar for 2 min for an appropriate AA sorbent and separation capillary conditioning.
- For sample loading, flush diluted standard solutions (generally dissolved in water or PBS, depending on the application) or real samples at 930 mbar for a time comprised between 2.5 and 20 min (30–120 μL, respectively, estimated using the Hagen-Poiseuille equation [36]). Note that the sample loading time should be optimized (see Section 3.1). To avoid protein degradation, the autosampler should be kept at low temperatures (e.g., 12 °C) using an external water bath (e.g., Minichiller 300, Peter Huber Kältemaschinenbau AG, Offenburg, Germany).
- Remove non-retained molecules and equilibrate the capillary before the electrophoretic separation flushing with BGE at 930 mbar for 2 min.
- It is worth mentioning that the conditioning, sample loading, and washing steps must be performed with the nebulizer gas and the ESI capillary voltage switched off to prevent the entrance of contaminants into the mass spectrometer.
- Inject the eluent at 50 mbar for a time comprised between 10 and 40 s (50–200 nL, respectively [36]). Note that the eluent injection time should also be optimized (see Section 3.1).
- For a rapid and repeatable protein elution before the electrophoretic separation, push the small plug of eluent with BGE at 50 mbar for 100 s (this guarantees the passage of the eluent through the microcartridge containing the AA sorbent).
- Switch on the nebulizer gas and the ESI capillary voltage and apply an appropriate separation voltage to detect the target protein in a reasonable time and with a good peak shape (e.g., 15 kV (~25 μA) for a 2 M acetic acid BGE [26,27]). Note that electrophoretic currents lower than 50 μA are recommended to prevent electrical arcs between the electrode tip of the CE-MS interface and the mass spectrometer entrance. In any case, the Ohm's law must be fulfilled [36].
- During the electrophoretic separation, a small pressure (usually 25 mbar) can be applied to compensate for the microcartridge counter-pressure.
- Between consecutive runs, to avoid carryover, flush the AA-SPE-CE capillary with water for 1 min, inject the eluent at 50 mbar for 40 s, and flush again with water for 1 min. Blank analyses must be done between consecutive analyses to confirm the absence of carryover. If necessary, the elution and postconditioning steps can be reoptimized for the specific application.

3. Development of AA-SPE-CE-MS applications

3.1. Optimization of the AA-SPE-CE-MS method

Some conditions of the AA-SPE-CE-MS method must be optimized to maximize the extraction selectivity and efficiency, while allowing the best electrophoretic separation and MS limits of detection. The optimization experiments must be performed in triplicate with protein standard solutions at a concentration at least ten times higher than the LOD. These solutions are generally prepared in water and PBS, but BGE can be also investigated to improve protein stability or retention in certain applications.

BGE and sheath liquid composition: Volatile acidic BGEs and sheath liquids are preferred for the analysis of intact proteins by CE-MS and AA-SPE-CE-MS in positive ESI mode, as they maximize protein ionization and enhance the sensitivity of the analyses. In case of analyzing proteins with acidic isoelectric points (pIs) (such as α-syn, pI=4.74; Con A, pI=5.4, and β-LG, pI=4.9), BGEs composed of acetic acid (between 0.1 and 2 M, pH 2.2–2.9) or 50 mM acetic acid:50 mM formic acid (pH 2.3) can be used together with sheath liquids consisting of 60:40 (v/v) propan-2-ol:water mixtures with 0.05 or 0.25% (v/v) of formic acid [24–27,31]. Specifically, optimum sensitivity was observed with a 2 M acetic acid BGE in combination with a sheath liquid of 0.25% (v/v) of formic acid for Con A and β-LG [26,27] and with a 0.1 M acetic acid BGE with a sheath liquid of 0.05% (v/v) of formic acid for α-syn [24,25,31].

Eluent composition and time: Using acidic BGEs, the basic volatile eluent composed of 100 mM NH_4OH (pH 11.2) is typically used for protein elution [24–27,31]. The addition to this eluent of organic solvents (e. g., methanol or acetonitrile) can also be explored to improve the disruption of the aptamer-protein interaction [24,34,37]. However, according to our experience with the analysis of α-syn, Con A, and β-LG, electropherograms and mass spectra are extremely poor with these hydroorganic mixtures, probably due to sorbent deterioration during the elution [24]. A higher concentration than 100 mM of NH_4OH in the aqueous eluent can also be tested (0.5 or 1 M) [24], despite a 100 mM concentration is recommended to prevent aptamer denaturation and to expand the sorbent lifetime.

Elution time must be optimized by injecting the eluent (typically at 50 mbar), for example, for 10, 20, and 40 s (50, 100, and 200 nL [36]). Select the optimum elution time as the best compromise between peak area, peak shape, and repeatability. In case of α-syn, Con A, and β-LG, longer elution times than 20 s at 50 mbar lead to broader peaks and smaller preconcentration factors [24,26,27].

Sample loading time: Loading time must be optimized by introducing the standard protein solution (usually flushing at 930 mbar) at different sample loading times (usually between 2.5 and 20 min, i.e., 30–120 μL [36]). If the protein concentration is not enough to saturate the AA sor-

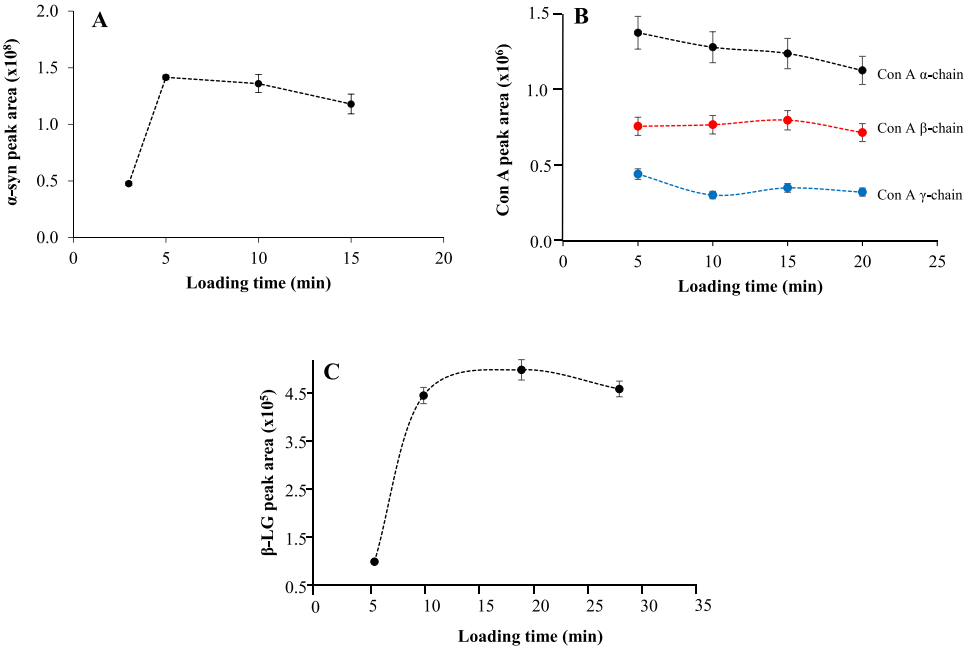

Fig. 2. Effect of the loading time at 930 mbar on the peak area of the eluted α-syn [24] (A), Con A proteoforms [26] (B), and β-LG proteoforms [27] (C) using the optimized elution and separation conditions and introducing 1 μg·mL^{-1} of α-syn, 1 μg·mL^{-1} Con A and 5 μg·mL^{-1} β-LG standard solutions. Reproduced with permission.

Table 1
Quality parameters for the analysis of α-syn, and Con A[a] and β-LG by AA-SPE-CE-MS.

Protein	Migration time RSD (%)[b]	Peak area RSD (%)[b]	CE-MS LOD (μg·mL^{-1})	AA-SPE-CE-MS LOD (μg·mL^{-1})	Linearity range (μg·mL^{-1})	Microcartridge lifetime (analyses) [b]	Refs.
α-syn	2.1	5.4	20	0.20	0.5–10	20	[24]
Con A	1.9	8.1	25	0.25	0.5–20	25	[26]
β-LG	0.5	2.4	10	0.05	0.1–20	25	[27]

[a] Con A concentration is given as the sum of the three Con A proteoforms.
[b] Established by analyzing 1, 5, and 5 μg·mL^{-1} of α-syn, Con A, and β-LG standard solutions, respectively.

bent, this investigation allows estimating the loaded sample volume for which the protein washed away promotes a decrease in the final retained protein (i.e., the sample breakthrough volume) [35]. Fig. 2 presents representative graphs of the peak area of the eluted protein versus loading times at 930 mbar for α-syn, Con A, and β-LG [24,26,27]. As it is shown, maximum peak areas for α-syn and the three Con A proteoforms, are obtained when the loading time is 5 min, whereas for β-LG 10 min are necessary. In all cases, longer loading times exceed the sample breakthrough volume, resulting in lower protein recoveries.

3.2. Method validation

The optimized AA-SPE-CE-MS method is typically validated with protein standard solutions in terms of repeatability, LOD, linearity, and microcartridge lifetime. Repeatability ($n \geq 3$) is evaluated at the protein concentration of the optimization as the percent relative standard deviation (%RSD) of migration times (t_m) and peak areas, which are obtained from the extracted ion electropherograms (EIEs) of the most abundant molecular ions detected for the target protein. Linearity range ($R^2 > 0.99$) is established from the limit of quantification (LOQ) to the limit of linearity (LOL), by analyzing standard solutions at different concentrations. This sequence of experiments also allows estimating the LOD, as the lowest concentration giving a S/N higher than 3. The microcartridge lifetime is evaluated by repeatedly analyzing a protein standard solution (usually at a concentration in the middle of the linearity range). It is worth mentioning that the microcartridge should be discarded when the protein peak areas in the EIE decrease more than 25% compared to the mean value of the first three analyses. At this point, we recommend to completely replace the AA-SPE-CE capillary but reuse of the inlet and outlet separation capillary pieces can be alternatively explored. Note that the microcartridge lifetime is typically reduced by approximately half when analyzing complex matrices (e.g., biological fluids and food) due the presence of interfering compounds, e.g., salts and highly abundant proteins, which can saturate the small amount of AA sorbent contained in the microcartridge, among other undesired effects (see Section 3.3). Fig. 3 shows a comparison between the EIEs obtained for 100 and 1 μg·mL^{-1} of α-syn, 100 and 5 μg·mL^{-1} of Con A, and 50 and 1 μg·mL^{-1} of β-LG standards under the optimized conditions by CE-MS and AA-SPE-CE-MS, respectively [24,26,27]. As observed, in all cases the AA-SPE-CE-MS method allows a significant preconcentration of the target proteins. In some applications, the small pressure applied during the separation in AA-SPE-CE-MS can result in a time shift of the protein peaks as observed in Fig. 3A for α-syn. In addition, Con A and β-LG are detected as a mixture of proteoforms with different M_r. Table 1 shows the quality parameters for the analysis of α-syn, Con A, and β-LG by AA-SPE-CE-MS [24,26,27]. As can be observed, repeatability in t_m and peak area is good with %RSD values in all cases lower than 2.1 and 8.1%, respectively. LODs are between 100 and 200 times lower than by CE-MS, until detecting 0.20, 0.25, and 0.05 μg·mL^{-1} of α-syn, Con A, and β-LG, respectively. Linearity ranges are over two orders of magnitude (0.5–10, 0.5–20, and 0.1–20 μg·mL^{-1}), and microcartridge lifetime is around 20 analyses in the worst case. Overall, the method performance is the best for β-LG, probably because of the aptamer characteristics and the protein suitability for CE-MS analysis (e.g., lower protein capillary adsorption, better peak shape, and higher ionization efficiency compared to α-syn and Con A).

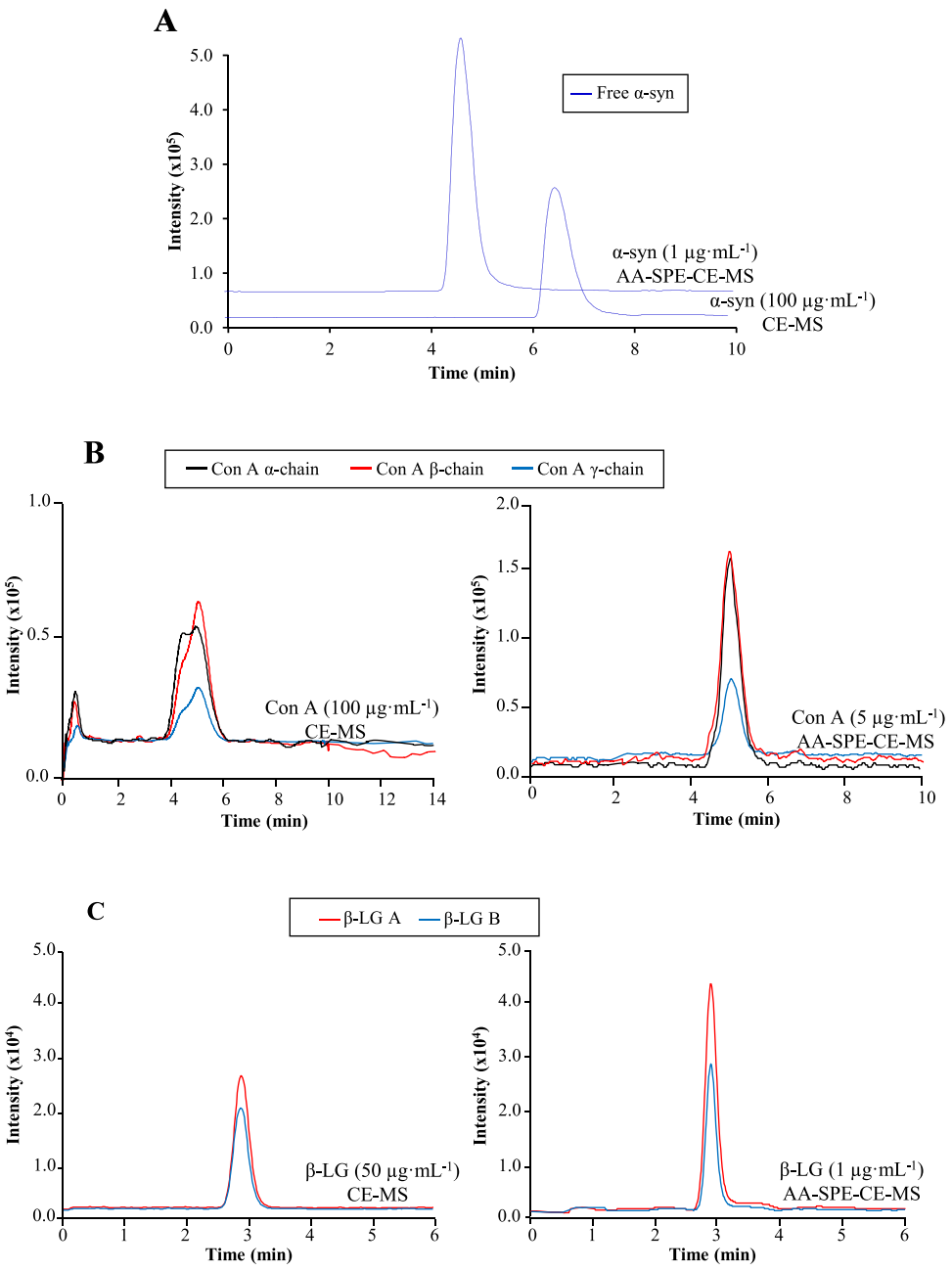

Fig. 3. Extracted ion electropherograms (EIEs) for 100 and 1 µg·mL^{-1} *α*-syn standard [24] (A), 100 and 5 µg·mL^{-1} Con A standard [26] (B), and 50 and 1 µg·mL^{-1} *β*-LG standard [27] (C) by CE-MS and AA-SPE-CE-MS, respectively, under the optimized conditions. Reproduced with permission.

3.3. Analysis of protein biomarkers in biological fluids and food

The AA-SPE-CE-MS methods described above have been successfully applied to the analysis of *α*-syn, Con A, and *β*-LG in biological fluids and food [24,26,27].

When analyzing intact proteins in such complex matrices, targeted extraction efficiency of AA sorbents, as with other highly selective SPE sorbents, can be compromised due to non-selective retention of interferents and matrix effects. To overcome this limitation and ensure an appropriate AA extraction, sample clean-up prior analysis by AA-SPE-CE-MS is essential. A wide range of procedures have been described for this purpose, including filtration with M_r cut-off low-protein binding centrifugal filters, evaporation, protein precipitation for the removal of highly abundant proteins (e.g., albumin in serum), liquid-liquid extraction, and solid-liquid extraction, among others [9]. However, the simplest pretreatments are recommended to avoid excessive sample handling, which should be carefully optimized for each specific application. For instance, *α*-syn can be purified from red blood cells (RBCs) lysates by thermo-enrichment before the analysis by AA-SPE-CE-MS [24]. This pretreatment allows depletion of non-thermostable abundant proteins (e.g., hemoglobin and carbonic anhydrase), thus removing interfering proteins that saturate the microcartridge, damage the capillary inner surface, or hinder the ionization of the minor abundant target protein (i.e., *α*-syn). The analysis by AA-SPE-CE-MS of thermo-enriched RBCs lysates reveals that N-acetylated *α*-syn is the main proteoform of *α*-syn in blood (blue line, Fig. 4A), as well as that no other minor *α*-syn proteoforms related with Parkinson's disease can be detected in patient samples. As can be observed, N-acetylated *α*-syn can be differenciated from the recombinant *α*-syn standard (red line, Fig. 4A), due to the excellent mass accuracy and resolution of the mass spectrometer. In addition, it is also worth highlighting that N-acetylated *α*-syn is separated from ubiquitin, which is

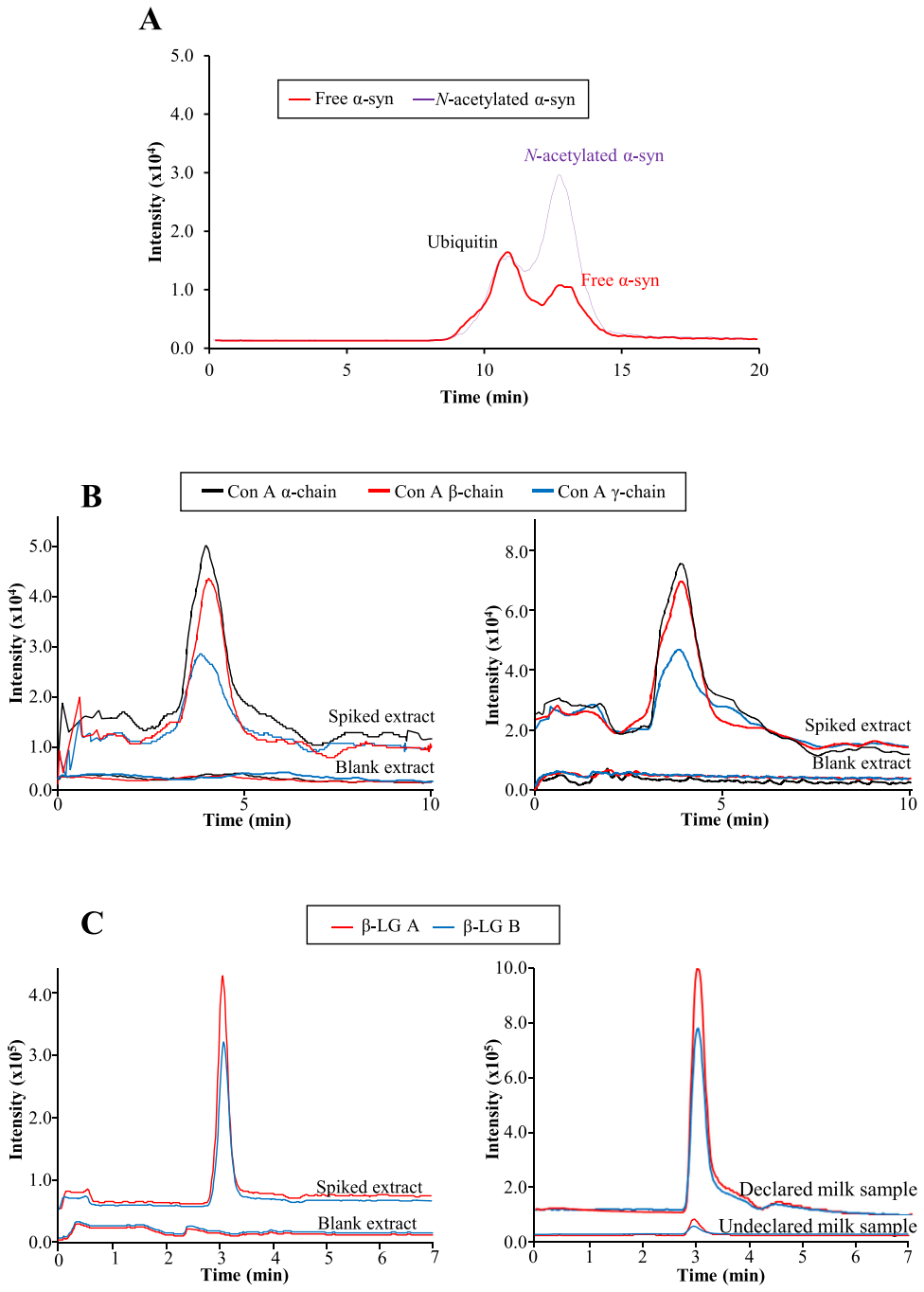

Fig. 4. Extracted ion electropherograms (EIEs) for the AA-SPE-CE-MS analysis of a thermoenriched red blood cells (RBCs) lysate sample spiked with 0.3 $\mu g \cdot mL^{-1}$ of free α-syn standard [24] (A); a blank extract and a spiked extract with 5 $mg \cdot mL^{-1}$ of Con A for white bean and lentil flour (left and right, respectively) [26] (B); a blank extract and a spiked extract with 0.1 mg β-LG per g of sample (100% cocoa dark chocolate) (left) and white bread samples with declared and undeclared milk (right) [27] (C). Reproduced with permission.

another protein coextracted from the thermo-enriched sample by non-specific adsorption on the AA sorbent. However, in some cases, the remaining sample matrix components in the pretreated samples preclude an appropriate analysis by AA-SPE-CE-MS. This is the case of the analysis of Con A in different food matrices (i.e., white beans, as well as chickpea, lentils, and wheat flours), where the thermo-enriched samples need to be filtered through 3000 M_r cut-off centrifugal filters [26]. The analysis by AA-SPE-CE-MS of thermo-enriched and filtered samples allows the accurate determination of three Con A proteoforms at the low concentration levels typically established as reference doses for allergenic proteins in food (Fig. 4B). For β-LG analysis in food samples (a 100% cocoa dark chocolate, a hydrolyzed hypoallergenic infant formula, and different types of white bread with declared and undeclared milk), a pretreatment consisting of a casein precipitation is required to obtain β-LG as part of the whey fraction [27]. The analysis by AA-SPE-CE-MS of the resulting extract, filtered through 10,000 M_r cut-off centrifugal filters, allows the detection of β-LG proteoforms at the threshold concentration that can cause food allergy symptoms, including in white bread samples with undeclared milk (Fig. 4C).

3.4. Troubleshooting

Table 2 includes several troubles that can occur when implementing AA-SPE-CE-MS in protein analysis, as well as possible sources and solutions.

Table 2
AA-SPE-CE-MS troubleshooting.

Problem	Possible sources	Possible solutions
MS SIGNAL/ CE PEAKS		
No or unstable MS signal	-Capillary outlet tip	-Check that the capillary protrudes 0.1 cm from the metal electrode tip of the CE-MS sheathflow interface -Check removal of 2 cm of the nonconductive polyimide external capillary coating -Clean carefully with ethanol after burning the coating with a lighter
	-Sheath liquid flow issues	-Check that the sheath liquid tube and the connectors are not blocked -Check that the infusion pump works properly -Check for sheath liquid leaks due to improper connections
	-Insufficient degasification of the sheath liquid or the BGE	-Degas the sheath liquid and the BGE at least 10 min in an ultrasonic bath before use
	-Nebulizer gas issues	-Check the N_2 gas pressure in the external manometer and in the mass spectrometer software
	-Inappropriate ion source and MS analysis conditions	-Check MS conditions in the mass spectrometer software: ESI interface and ionization mode, ion source parameters, and the rest of MS method parameters
MS sensitivity loss	-MS ion source contamination	-Clean the ion source as recommended by the mass spectrometer manufacturer
	-MS ion transfer contamination	-Clean or change the ion transfer as recommended by the mass spectrometer manufacturer
	-AA-microcartridge or separation capillary deterioration	-Replace the AA-SPE-CE-MS capillary -Avoid loading highly concentrated protein standard solutions -Improve the sample pretreatment to avoid too short microcartridge lifetimes, damage of the separation capillary, or ion suppression
	-BGE or sheath liquid deterioration or contamination	-Prepare new solutions -Refresh the separation BGE vial (i.e., where voltage is applied) after each analysis
	-Sample instability or degradation	-Check the stability of the protein standard solution in the selected solvent -Check the sample preparation (regarding protein stability, solubility, etc.) -Check that the CE autosampler is properly thermostated
Sudden drops or spikes in the MS baseline	-Ion suppression, high salt levels, bubbles, etc.	-Degas the eluent at least 10 min in an ultrasonic bath before use -Check the sample pretreatment and, if necessary, include a desalting step to remove salts and interfering compounds
Carry-over	-Sorbent saturation	-Load a smaller volume of sample -Dilute the sample
	-Inadequate washing conditions	-Optimize the washing solution composition -Optimize the washing time
	-Inadequate elution conditions	-Optimize the eluent composition -Optimize the eluent time
	-Inadequate postconditioning	-Optimize the postconditioning step
Poor repeatability in migration times or peak areas, tailed and wide peaks, resolution loss. etc.	-Excessive heating during the separation due to Joule effect	-Check that the Ohm's law is fulfilled -Check that the cooling system of the CE instrument is working properly -Optimize the BGE and eluent compositions
	-BGE deterioration or contamination	-Prepare new solution -Refresh the separation BGE vial (i.e., where voltage is applied) after each analysis
	-Protein adsorption in the inner capillary walls	-Improve the capillary conditioning between analyses -Improve the sample pretreatment -Use a permanently coated capillary (lab-made or commercial, e.g., hydroxypropylcellulose HPC, polyvinyl alcohol PVA, etc.)
	-Sorbent saturation	-Load a smaller volume of sample -Dilute the sample
	-Uncomplete packing of the AA microcartridge	-Check that the microcartridge is full of AA sorbent before the first use
	-Inadequate elution conditions	-Optimize the eluent composition -Optimize the eluent time
	-AA-microcartridge or separation capillary deterioration	-Replace the AA-SPE-CE-MS capillary -Avoid loading highly concentrated protein standard solutions -Improve the sample pretreatment to avoid too short microcartridge lifetimes, damage of the separation capillary, or ion suppression
	-Carry-over from previous runs	-Avoid carry-over
	-Sample instability and/or degradation	-Check the stability of the protein standard solution in the selected solvent -Check the sample preparation (regarding protein stability, solubility, etc.) -Check that the CE autosampler is properly thermostated
Additional peaks	-Carry-over from previous runs	-Avoid carry-over
	-Presence of contaminants/interferents in BGE, sheath liquid, and/or sample solutions	-Use high-quality reagents and solvents -Store appropriately standards, samples, and solutions -Check the sample preparation -Prepare new solutions
CE CURRENT		
No or unstable CE current	-AA-SPE-CE capillary assembly problems	-Check that there is no dead volume between the AA-SPE-CE capillary connections
	-Air bubbles in the system	-Flush the capillary with the BGE -Degas the sheath liquid, the BGE, and the eluent at least 10 min in an ultrasonic bath before use
	-Capillary outlet tip	-Check that the capillary protrudes 0.1 cm from the metal electrode tip of the CE-MS sheathflow interface -Check removal of 2 cm of the nonconductive polyimide external capillary coating -Clean carefully with ethanol after burning the coating with a lighter

(*continued on next page*)

Table 2 (*continued*)

Problem	Possible sources	Possible solutions
	-AA-microcartridge or separation capillary deterioration	-Replace the AA-SPE-CE-MS capillary -Avoid loading highly concentrated protein standard solutions -Improve the sample pretreatment to avoid too short microcartridge lifetimes, damage of the separation capillary, or ion suppression
	-Excessive backpressure or clogging due to the AA microcartridge	-Replace the AA microcartridge -Pack a shorter microcartridge (e.g., 0.5 cm)
	-Excessive heating during the separation due to Joule effect	-Check that the Ohm's law is fulfilled -Check that the cooling system of the CE instrument is working properly -Optimize the BGE and eluent compositions
	-BGE deterioration or contamination	-Prepare new solution -Refresh the separation BGE vial (i.e., where voltage is applied) after each analysis
	-High protein concentration and/or salt levels after elution	-Dilute the sample -Improve the sample pretreatment

4. Conclusions

This tutorial describes a valve-free AA-SPE-CE-MS method for purification, preconcentration, separation, detection, and characterization of intact protein biomarkers using as illustrative examples the analysis in blood of the Parkinson's biomarker α-syn and in food of the allergenic proteins Con A and β-LG. The preparation of aptamer affinity sorbents using amino-modified aptamers, the construction of fritless microcartridges, the most critical steps to optimize an AA-SPE-CE-MS method, and a troubleshooting guide for non-expert users are included. Under optimized conditions, the performance of the AA-SPE-CE-MS method for the described applications in terms of repeatability, linearity, LODs, and microcartridge reusability is very satisfactory. For the analysis of the target proteins in biological fluids and food matrices, very simple sample pretreatments based on thermoenrichment and M_r cut-off centrifugal filtration are proposed, which in the future may be minimized, while globally improving the method performance, by developing novel AA sorbents with enhanced affinity, selectivity, and active surface area. It is presented a clear and simple guide for the successful application of AA-SPE-CE-MS beyond the specific cases of study, as a platform to explore other analytes, complex samples, and analytical strategies. The latter is what we have already done developing on-line aptamer affinity solid-phase extraction and immobilized enzyme microreactor capillary electrophoresis-mass spectrometry (AA-SPE-IMER-CE-MS) for the analysis of protein biomarkers at the peptide map level [25] or on-line aptamer affinity solid-phase extraction direct mass spectrometry (AA-SPE-MS) for the analysis of intact protein biomarkers with the typical SPE microcartridges and instrumental set-ups necessary for AA-SPE-CE-MS [31]. In any of these cases, the possibility of accurately identifying and quantifying low levels of the target proteins in complex samples, represents a unique advantage over non-MS based biosensors and bioassays using any kind of high selectivity affinity probe, including aptamers.

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 request.

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