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Determination of a lectin protein allergen in food by on-line aptamer affinity solid-phase extraction capillary electrophoresis-mass spectrometry

María Vergara-Barberán [a,b], Ernesto Francisco Simó-Alfonso [b], José Manuel Herrero-Martínez [b], Fernando Benavente [a,*]

[a] Department of Chemical Engineering and Analytical Chemistry, Institute for Research on Nutrition and Food Safety (INSA·UB), University of Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain

[b] Department of Analytical Chemistry, University of Valencia, Doctor Moliner 50, 46100 Burjassot, Spain

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ABSTRACT

An aptamer affinity sorbent was prepared for clean-up, preconcentration, separation and characterization of a food allergen protein by on-line aptamer affinity solid-phase extraction capillary electrophoresis-mass spectrometry (AA-SPE-CE-MS). SPE microcartridges were packed with a sorbent based on magnetic bead particles modified with an aptamer against the target lectin protein concanavalin A (Con A). After optimization of several parameters of the SPE-CE-MS method, the sample (ca. 30 μL) was loaded in separation background electrolyte (BGE, 2 M acetic acid pH 2.2), while the retained protein was eluted with 100 mM NH_4OH (pH 11.2) (ca. 100 nL). The developed method was linear between 0.5 and 20 $mg \cdot L^{-1}$ and the limit of detection (LOD) was 0.25 $mg \cdot L^{-1}$, which was 100 times lower than by CE-MS. The repeatability of the method was satisfactory, with relative standard deviations (RSD) for migration times and peak areas below 1.9 and 8.1%, respectively. In addition, the microcartridges could be reused more than 25 analyses without significant loss of extraction efficiency. Finally, the applicability and versatility of the developed method were demonstrated by analyzing low levels of Con A in different food matrices (i.e. white beans, as well as chickpea, lentils, and wheat flours), leading to satisfactory results, with recoveries between 87 and 115%.

1. Introduction

Aptamers have been defined as a new generation of affinity ligands for different bioanalytical applications, such as diagnosis, imaging or biosensing, amongst others [1–4]. The most common aptamers consist of single-stranded oligonucleotides that are typically selected by an iterative *in vitro* process of selective isolation known as systematic evolution of ligands by exponential enrichment (SELEX) [5,6]. The main benefits of aptamers are their cost-effective and reproducible production, high thermal and chemical stability, low immunogenicity, reusability, and easy modification in the 3′ or 5′ end to assist an appropriate covalent immobilization on solid supports [7–12]. Also, the high affinity and selectivity of aptamers make them a potential alternative to antibodies [5,6]. In the last years, the application of aptamers to isolate a wide range of compounds has been widely demonstrated, from small molecules to biomacromolecules, such as proteins, or even biological entities (e.g. cells) [1–4,7–12].

Due to their well-known features, aptamer-based sorbents have been applied in sample preparation by solid-phase extraction (SPE) or related techniques before liquid chromatography (LC) analysis, off-line or coupled on-line to minimize sample handling and increase analytical throughput [3,7,8,12]. As an alternative to the complex instrumental set-ups operated with valves required for such on-line coupling with LC, it has been recently described valve-free on-line aptamer affinity solid-phase extraction capillary electrophoresis with laser-induced fluorescence (AA-SPE-CE-LIF) [9] or mass spectrometry detection (AA-SPE-CE-MS) [10,11]. In valve-free AA-SPE-CE-MS (hereinafter referred to as AA-SPE-CE-MS), a microcartridge containing an AA sorbent against the compound of interest is integrated near the inlet of the separation capillary to work in unidirectional configuration [10,11,13]. This means that the large volume of sample (ca. 100 μL) for the selective clean-up and preconcentration is loaded in the same direction as later it is injected the small volume of eluent (ca. 25–50 nL) and performed the electrophoretic separation and detection [10,11,13].

The use of MS detection for AA-SPE-CE-MS is especially convenient, because it allows an accurate identification of the targeted compound,

* Corresponding author.
E-mail address: fbenavente@ub.edu (F. Benavente).

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hence preventing false positives or erroneous quantifications of typical biosensors or bioassays [14,15]. In addition, AA sorbents demonstrated to be highly compatible with the acidic background electrolytes (BGEs) required for optimum detection sensitivity in positive electrospray ionization (ESI+) mode [10,11], in contrast to the neutral BGEs required to avoid antibody denaturation [16,17]. So far, AA-SPE-CE-MS has been proved for the direct analysis in blood of α-synuclein, which is a Parkinson's disease protein biomarker, and limits of detection (LODs) were enhanced 100 times compared to CE-MS [10]. More recently, it has been shown that AA-SPE-CE-MS can be fully integrated with on-line immobilized enzyme microreactors (IMERs) for the indirect analysis of α-synuclein from specific surrogate tryptic peptides by AA-SPE-IMER-CE-MS, and LODs were enhanced 10 times compared to AA-SPE-CE-MS [11]. The potential of both approaches for the analysis of protein biomarkers in biological fluids is of great interest. However, further studies are needed for a better understanding and expand their applicability in different fields, such as in food allergen protein analysis, where sensitivity and accuracy are also mandatory [18,19].

In this study, AA-SPE-CE-MS is described for the direct analysis of the allergenic protein concanavalin A (Con A) at the intact level in several food matrices. Con A belongs to a group of leguminous lectins present in different fruits and vegetables, which are responsible of many allergic responses [19–21]. Recently, an aptamer against Con A was selected by SELEX, characterized, and applied in an enzyme-linked aptamer based bioassay to the detection of Con A in different agri-food products [21]. Here, this aptamer was used to prepare an AA sorbent and develop a method that integrates clean-up, preconcentration, separation, characterization, and quantification of Con A by AA-SPE-CE-MS. After method optimization and validation with standards, the practical applicability of the developed method was evaluated by analyzing the target protein in several food matrices at the typical health-based intake limits established as reference doses for allergenic proteins [22].

2. Experimental

2.1. Reagents and materials

Acetic acid (HAc) (glacial), ammonium hydroxide (NH_4OH) (25%), formic acid (HFor) (99.0%), potassium chloride (99.0%), potassium dihydrogenphosphate (≥99.0%), sodium chloride (≥99.5%), and sodium hydrogenphosphate (≥99.0%) were supplied by Merck (Darmstadt, Germany). Acetonitrile (LC-MS) and ethanol (96%) were purchased from Panreac AppliChem (Barcelona, Spain). Ammonium acetate (NH_4Ac) (≥99.9%), bovine serum albumin (BSA), and Tween® 20 were provided by Sigma-Aldrich (Steinheim, Germany). Propan-2-ol (LC-MS grade) was obtained from Scharlau (Barcelona, Spain). Con A and water (LC-MS grade) were purchased from Fisher Scientific (Loughborough, UK).

The amino modified single-stranded DNA-aptamer against ConA (5′–NH_2-$(CH_2)_6$-CGAGTAACGCTGTCTCTTCCGAATCGGGGGAAGGCG-GAGGG-3′, 41-mer, molecular mass (M_r) = 12,951 [21]) purified by HPLC was provided by Integrated DNA Technologies (Coralville, IA, USA). Magnetic beads (MBs) LOABeads™ AffiAmino of 45–165 µm diameter were supplied by Lab on a Bead (Uppsala, Sweden). Fused-silica capillaries (75 and 250 µm inner diameter (i.d.) × 375 µm outer diameter (o.d.)) were obtained from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Background electrolyte, sheath liquid, protein standards and food samples

The optimized BGE for CE-MS and AA-SPE-CE-MS was a 2 M HAc (pH 2.2) solution. The sheath liquid for MS detection consisted of a hydro-organic mixture of 60:40 (v/v) propan-2-ol:water with 0.25% (v/v) of HFor, and it was delivered at 3.3 $\mu L \cdot min^{-1}$ by a KD Scientific 100 series infusion pump (Holliston, MA, USA). All solutions were degassed for 10 min by sonication and filtered through a 0.20 µm nylon filter (Micron Separations Inc, Westborough, MA, USA) before use.

Con A standard protein purified from jack beans (*Canavalia ensiformis*) and jack beans were provided by Sigma-Aldrich. A 1 $mg \cdot mL^{-1}$ stock solution of Con A standard was prepared in water, then it was aliquoted and stored in the freezer. Aliquots were defrosted before use and working standard solutions were prepared by dilution in water. Working standard solutions were stored in the fridge when not in use.

White beans and flours from chickpea, lentil, and wheat were purchased from a local market in Barcelona.

2.3. Con A extraction from food samples

Jack and white beans samples were milled in a mixer grinder to obtain a fine powder. Two grams of milled jack or white beans or flour from chickpea, lentil, or wheat, were dissolved in 20 mL of extraction buffer (10 mM Na_2HPO_4 containing 0.5 M NaCl, pH 7.6) [21,23]. The resulting suspension was magnetically stirred at room temperature overnight and centrifuged at 10,000 × *g* for 10 min at 4 °C. After that, an aliquot of the supernatant (2 mL) was incubated at 70 °C for 2 h in order to deplete non-thermostable proteins [23]. The mixture was centrifuged at 10,000 × *g* for 10 min at 4 °C and the resulting supernatant was filtered through a 0.22 µm polyvinylidene difluoride centrifugal filter (Ultrafree-MC, Millipore, Bedford, MA, USA). Then, 100 µL of the filtered extract was spiked with a proper volume of 100 $mg \cdot L^{-1}$ Con A standard solution to obtain a final concentration of 5 mg Con A per 100 g of food sample (excepting jack bean extracts, which were not spiked but just 25-fold diluted). Finally, low M_r compounds were removed with 3,000 M_r cut-off (MWCO) cellulose acetate centrifugal filters (Amicon Ultra-0.5, Millipore) [16,17]. Briefly, the sample was centrifuged at 25 °C for 10 min at 10,000 × g, and the residue was washed three times with 50 µL of water under the same conditions. In order to recover the final residue, the upper reservoir was inverted in a vial and spun at a reduced centrifugal force (2 min at 300 × g). Then, sufficient water was added to adjust the final volume to 100 µL before AA-SPE-CE-MS analysis.

2.4. Apparatus

pH measurements were made with a Crison 2002 potentiometer and a Crison electrode 52–03 (Crison Instruments, Barcelona, Spain). Centrifugal filtration was carried out in a cooled Rotanta 460 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). Incubations were carried out in a TS-100 thermoshaker (Biosan, Riga, Latvian Republic).

2.5. Preparation of AA sorbent

The MBs were functionalized according to our previous study [11]. Briefly, a 200 µL aliquot of MBs solution was vortexed and the supernatant was removed by magnetic separation (20 µL of sedimented MBs). The MBs were washed three times with 200 µL of phosphate-buffered saline (PBS) (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) with 0.1% Tween 20 (PBS-T) and the supernatants were removed using a neodymium cube magnet (12 mm, N48, Lab on a Bead), being the resulting MBs resuspended in the same volume of PBS-T. Ten µL of activation buffer was added to the MBs suspension and the mixture was incubated for 15 min at room temperature. The supernatant was magnetically removed, the MBs were washed with 200 µL of PBS-T, and resuspended with 150 µL of PBS-T. Then, 50 µL of a 100 µM Con A aptamer solution in water was added to the MBs suspension, being the mixture incubated with moderate shaking for 40 min at room temperature. After removing the supernatant, the AA-MBs were successively washed three times with 200 µL of PBS and resuspended in the same volume of PBS. The residual reactive groups on AA-MBs were blocked adding 200 µL of blocking buffer (5% (m/v) BSA in PBS-T) and the mixture was incubated while moderately shaking for 2 h at 37 °C.

Finally, the supernatant was removed and the AA-MBs were subsequently washed three times with 200 μL of PBS. The AA-MBs were used immediately or stored in PBS with 20% (v/v) ethanol at 4 °C when not in use.

2.6. CE-MS analysis

CE-MS experiments were carried out in a 7100 CE coupled with an orthogonal G1603A sheath-flow interface to a 6220 oa-TOF LC/MS spectrometer (Agilent Technologies, Waldbronn, Germany). CE and TOF mass spectrometer control, data acquisition and processing were performed with the ChemStation and MassHunter softwares (Agilent Technologies). The mass spectrometer parameters were: capillary voltage 4000 V, drying gas temperature 300 °C, drying gas flow rate 4 $L{\cdot}min^{-1}$, nebulizer gas 7 psi, fragmentor voltage 325 V, skimmer voltage 80 V, OCT 1 RF Vpp voltage 300 V. Data were collected in profile at 1 spectrum/s between 100 and 3200 *m/z*, with the mass range set to high resolution mode (4 GHz).

Separations were performed at 25 °C in a 60 cm total length (L_T) × 75 μm i.d. × 365 μm o.d. capillary. All capillaries were rinsed flushing at 930 mbar. New capillaries were conditioned with 1 M NaOH (15 min), water (15 min), and BGE (30 min), flushing off-line to avoid the unnecessary contamination of the mass spectrometer. Samples were hydrodynamically injected at 50 mbar for 10 s (ca. 54 nL, estimated using the Hagen–Poiseuille equation [24]). A voltage of +20 kV (normal polarity, cathode in the outlet) and 50 mbar of pressure were applied during separation, as later in AA-SPE-CE-MS. The autosampler was kept at 12 °C using an external water bath (Minichiller 300, Peter Huber Kältemaschinenbau AG, Offenburg, Germany). Between runs, the capillary was equilibrated flushing with water (2 min) and BGE (2 min), whereas between working days, capillaries were reconditioned flushing off-line with 1 M NaOH (5 min), water (5 min), and BGE (5 min).

2.7. AA-SPE-CE-MS

For AA-SPE-CE-MS, fritless particle-packed microcartridges were prepared as described in our previous works [10,11,13]. Microcartridge preparation was easy and reproducible, since the average size of the sorbent particles was larger than the inner diameter of the separation capillary. The microcartridge (0.7 cm L_T × 250 μm i.d. × 365 μm o.d. capillary) was filled by vacuum with the AA-MB based sorbent and connected with plastic sleeves between two capillary fragments (7.5 cm L_T × 75 μm i.d. × 365 μm o.d. (inlet) and 52.5 cm L_T × 75 μm i.d. × 365 μm o.d. (outlet)), which were conditioned before cutting, as a conventional CE-MS capillary.

All experiments and capillary flushes were performed at 25 °C and 930 mbar. Under the optimized conditions, AA-SPE-CE-MS capillaries were conditioned flushing with BGE for 5 min. Samples were loaded at 930 mbar for 5 min (ca. 30 μL [24]), followed by a flush with BGE for 1 min to eliminate non retained molecules and fill the capillary before the electrophoretic separation. During these steps, to prevent the entrance of contaminants into the mass spectrometer, the nebulizer gas and the ESI capillary voltage were switched off. Then, both were switched on and a small volume of eluent consisting of 100 mM NH_4OH (pH 11.2) was injected at 100 mbar for 20 s (ca. 100 nL [24]). For a rapid and repeatable protein elution, the small plug of eluent was pushed with BGE at 50 mbar for 100 s, before applying the separation voltage (+20 kV) and a small pressure (50 mbar), which was necessary to compensate the microcartridge counter-pressure. Between consecutive runs, to avoid carry-over, the capillary was flushed for 1 min with water, eluent was injected at 50 mbar for 40 s, and the capillary was flushed again for 1 min with water.

2.8. Quality parameters

To determine the quality parameters, migration times and peak areas were obtained from the extracted ion electropherograms (EIEs) of the detected Con A proteoforms (i.e. full length α-chain, β-chain and γ-chain fragments, Table 1), using the *m/z* of the most abundant molecular ions (ions with charges from +20 to +27 for the α-chain, from +14 to +10 for the β-chain and from +9 to +13 for the γ- chain). The LOD (defined as S/N = 3) was obtained by analyzing low-concentration Con A standard solutions. The linear range was established by analyzing standard solutions of Con A at concentrations ranging from 0.5 to 20 $mg{\cdot}L^{-1}$. Repeatability was evaluated as the relative standard deviation (RSD) of migration time and peak area. The microcartridge lifetime was investigated by repeatedly injecting a 5 $mg{\cdot}L^{-1}$ Con A standard solution and protein extracts from food samples. The microcartridge was discarded when a significant loss of extraction efficiency was detected, which meant that the peak area of Con A in the EIE decreased more than 25% compared to the mean value of the first three analyses with the microcartridge under consideration [10].

3. Results and discussion

3.1. CE-MS

In CE-MS, a volatile BGE with low conductivity should be employed to avoid salt precipitation in the ionization source, electrical arcing and contamination of the mass spectrometer. Intact proteins are typically analyzed at low pH values to ensure an appropriate ionization efficiency and BGEs containing HAc at concentrations from 0.1 to 2 M combined with sheath liquids of propan-2-ol/water with HFor have demonstrated an excellent performance in sheath-flow CE-MS [10,11,16,25,26]. For this reason, BGEs composed by HAc (0.1, 1 and 2 M with pH values 2.8, 2.3 and 2.2, respectively) combined with sheath liquids of 60:40 (*v/v*) propan-2-ol/water with 0.05 or 0.25% (*v/v*) of HFor were evaluated for the analysis of Con A. The best results in terms of sensitivity were achieved with a BGE of 2 M HAc and a sheath liquid of 60:40 (*v/v*) propan-2-ol/water with 0.25% (*v/v*) of HFor, applying +20 kV and 50 mbar during separation. Fig. 1 (A) shows, under these CE-MS conditions, the extracted ion electropherograms (EIEs) of the detected Con A proteoforms for a 100 $mg{\cdot}L^{-1}$ standard solution, while the mass spectrum and the deconvoluted mass spectrum in the time window corresponding to the protein peaks are illustrated in Fig. 1 (C) and (D), respectively. As observed, Con A was detected as three proteoforms with different relative abundances, the full length α-chain (M_r experimental (M_r exp), 25597.91) and its derived β-chain (M_r exp 12935.95) and γ-chain (M_r

Table 1
Relative abundance, theoretical and experimental M_r for the detected Con A proteoforms in a 100 $mg{\cdot}L^{-1}$ Con A standard solution by CE-MS.

Detected Con A proteoforms	Relative abundance[a)] (%)	Theoretical average M_r	Deconvoluted experimental	
			M_r	$E_r^{b)}$ (ppm)
Full length α-chain	45	25598.19	25597.91	−11
β-chain fragment	38	12936.36	12935.95	32
γ-chain fragment	17	12679.85	12680.82	77

a) The relative abundance was calculated from the EIEs by diving the individual peak areas by the total sum of the three peak areas (n = 3).
b) Relative error (E_r) was calculated in ppm as: (M_r exp – M_r theo)/M_r theo × 10^6 (exp = experimental and theo = theoretical). M_r exp was obtained as an average of three replicates (n = 3).

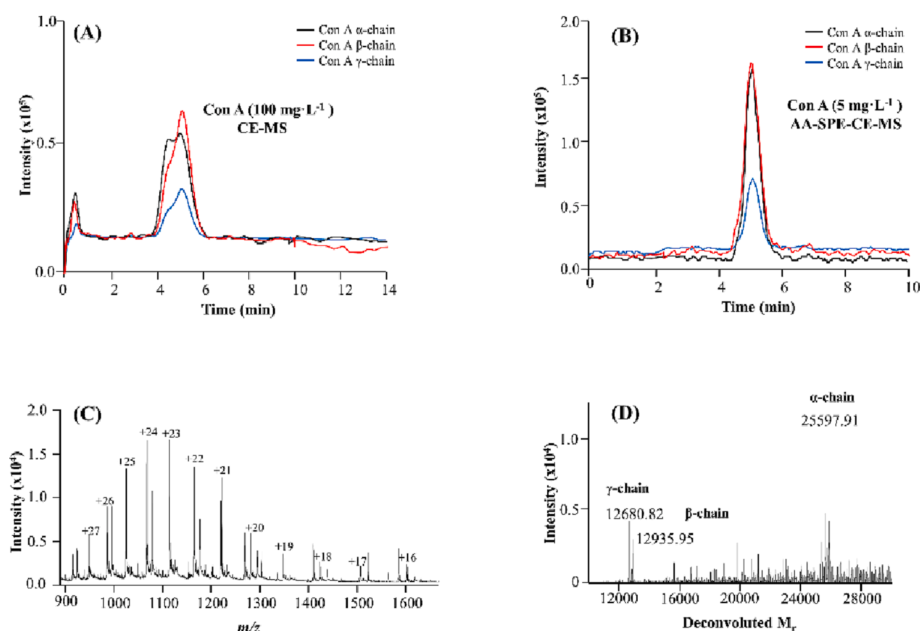

Fig. 1. (A and B) Extracted ion electropherograms (EIEs) for the CE-MS and AA-SPE-CE-MS analyses of 100 and 5 mg·L^{-1} Con A standard solutions, respectively; (C) mass spectrum in the time window of Con A peaks (ca. 4–6 min by CE-MS. Charges correspond to the ConA α-chain molecular ions), and (D) deconvoluted mass spectrum (by CE-MS).

exp 12680.82) fragments (Fig. 1 (D)). These results agree to other studies related to the analysis of Con A [27,28] and other legume lectins such as *Canavalia brasiliensis* [29]. The theorical average M_r of the three proteoforms of Con A and the relative error (E_r) for the experimental deconvoluted M_r are indicated in Table 1. As observed, mass accuracy was satisfactory ($E_r < 77$ ppm). In order to confirm that Con A appeared in a real food sample as the same mixture of proteoforms with similar relative abundances found in the purified Con A standard, a 25-fold dilution of the extract prepared from commercial jack beans, which present Con A at high concentration (ca. 1.2 mg·kg^{-1}), was subjected to CE-MS analysis (Supplementary material, Fig. S1).

To evaluate the repeatability of the CE-MS method, consecutive analyses of a 100 mg·L^{-1} Con A standard solution were conducted and good RSD values in terms of migration time and peak area were achieved (RSD (n = 3) were comprised between 1.4 and 1.6% and 4.2 to 4.6%, respectively). LODs for the three proteoforms were determined considering their relative abundances (Table 1), and were 11.0, 10.0 and 4.0 mg·L^{-1} for α, β, and γ-chains, respectively (i.e. 25 mg·L^{-1} for total Con A). These LOD values were lower than those previously obtained for the CE-MS analysis of other lectins, such as *Urtica dioica agglutinin* lectin [30].

3.2. AA-SPE-CE-MS

The AA sorbent for AA-SPE-CE-MS was prepared using an aptamer with good selectivity and high affinity against Con A [21]. In our previous works [10,11], an acidic BGE containing 0.1 M HAc (pH 2.8) and a basic eluent of 0.1 M NH_4OH (pH 11.2) were used in AA-SPE-CE-MS. Taking as a reference these conditions and the 2 M HAc BGE with a sheath liquid of 60:40 (*v/v*) propan-2-ol/water with 0.25% (*v/v*) of HFor, optimized for Con A analysis by CE-MS, BGEs containing HAc at different concentrations (0.1, 1 and 2 M) were investigated for the analysis of Con A by AA-SPE-CE-MS. The highest sensitivity for the three Con A proteoforms was achieved using the BGE of 2 M HAc (pH 2.2), which was selected for further experiments. The volume of the eluent

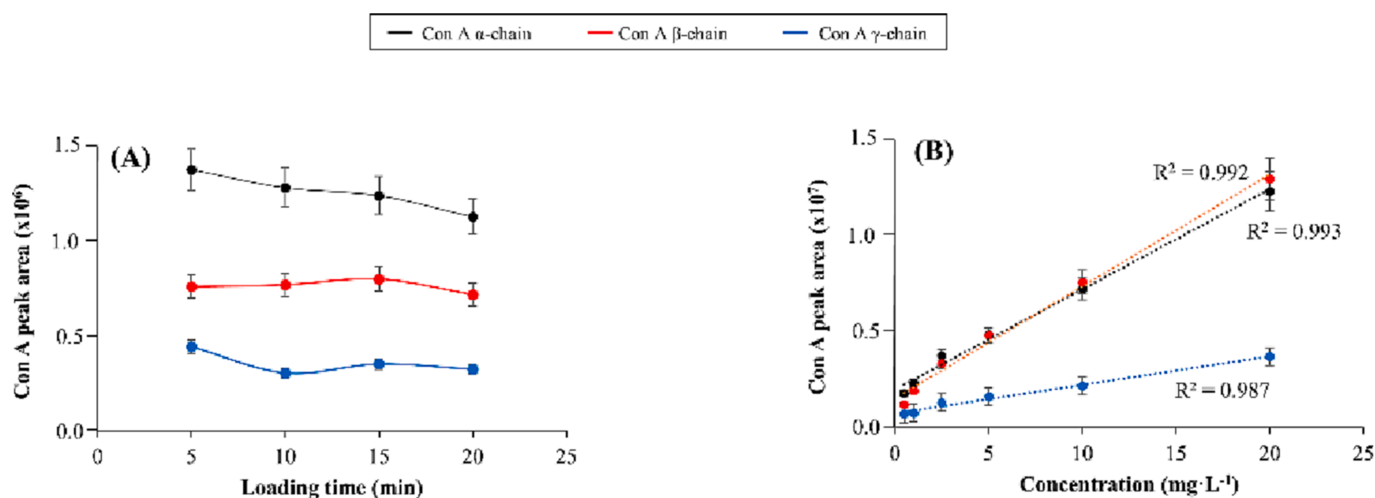

Fig. 2. (A) Effect of the loading time at 930 mbar on the peak area of the detected Con A proteoforms. (1 mg·L^{-1} Con A standard solution in water, using the optimized elution and separation conditions). (B) Calibration curve for the three Con A proteoforms under the final optimized conditions (see section 2.7).

plug was set at 20 s since longer times provided broad peaks jointly to a decrease in peak areas. In addition, to avoid carry over, the capillary was washed between injections with water, a small plug of eluent, and again water.

Under the selected conditions, the sample loading time was optimized introducing at 930 mbar, from 5 to 20 min, 1 mg·L^{-1} Con A standard solutions prepared in water. As illustrated in Fig. 2 (A), the maximum peak area for the three Con A proteoforms was observed when the loading time was 5 min. Longer loading times gave rise to a decrease in the Con A peak areas since at this point the sample breakthrough volume was surpassed, being the protein washed away. Consequently, a sample loading time of 5 min was selected to reduce the analysis time, while achieving the best protein recoveries.

Fig. 1 (B) shows the analysis of a 5 mg·L^{-1} Con A standard solution by AA-SPE-CE-MS under the optimized conditions. As observed, the Con A proteoforms migrated at a similar time compared to CE-MS, due to the small pressure applied during separation to overcome the microcartridge counter-pressure. In addition, the AA sorbent was able to recognize the three Con A proteoforms with no special preference, as their relative abundances were identical to those previously obtained by CE-MS (Table 1). Therefore, AA-SPE-CE-MS provided the possibility of accurately identifying and quantifying the different Con A proteoforms, which could present different allergenic properties.

Linearity, LODs and repeatability of the AA-SPE-CE-MS method were evaluated under the optimized conditions with Con A standard solutions. A satisfactory linear relationship between peak area and concentration of the three proteoforms was achieved from 0.5 to 20 mg·L^{-1} of Con A ($R^2 < 0.987$) (Fig. 2 (B)). LODs for the three proteoforms were determined as by CE-MS, and were 110, 100, and 40.0 µg·L^{-1} for α, β, and γ-chains, respectively (i.e. 250 µg L^{-1} for total Con A) (Supplementary Fig. S2). These values were 100 times lower than those obtained by CE-MS. Repeatability of migration times and peak areas were evaluated by consecutive analyses of a 5 mg·L^{-1} Con A standard solution. The RSD values of retention times and peak areas ranged from 1.4 to 1.9 and 3.4 to 8.1%, respectively. In addition, the microcartridges could be reused until 25 analyses (at 5 mg·L^{-1}) without significant loss of extraction efficiency. These figures of merit indicated the good performance of the AA-SPE-CE-MS method.

3.3. Analysis of Con A in food samples

To demonstrate the applicability of the developed AA-SPE-CE-MS method, Con A was analyzed in different food matrices, which could be contaminated or contain similar allergenic lectins [31–33]. Concretely, white beans as well as chickpea, lentil, and wheat flours were included in this study.

Preliminary experiments were performed extracting the proteins from the selected food matrices with a buffer containing 10 mM Na_2HPO_4 and 0.5 M NaCl (pH 7.6) [21,23] and spiking the extract at 5 mg·L^{-1} of Con A (equivalent to 5 mg Con A per 100 g of food sample) [22]. However, the Con A proteoforms were not detected by AA-SPE-CE-MS, due to the presence of other highly abundant proteins. Therefore, to remove some of these proteins and considering the thermal stability of Con A [23], a thermoenrichment pretreatment at 70 °C followed by filtration was applied to the blank and spiked protein extracts. After this pretreatment, the three Con A proteoforms were properly detected by AA-SPE-CE-MS in white beans and chickpea, lentil, and wheat flour spiked extracts but not in the blanks (Fig. 3), while maintaining the relative abundance profile observed previously for Con A in standard solutions (Fig. 1 (B)). This fact demonstrated the high affinity and selectivity of the aptamer, which was able to recognize the target analyte even in a wide variety of complex matrices. In addition, Con A recoveries were satisfactory for all food matrices, with values comprised from 87 to 115% (Table 2), RSD for migration times and peak areas were below 3 and 16 %, respectively (Table 2), and microcartridges could be reused until 10 analyses with no significant decrease on extraction efficiency. Indeed, repeatability and lifetime were slightly worse compared to standards, but in line with the complexity of the sample matrices. Overall, these results confirmed the potential of the proposed method to purify, preconcentrate, separate and characterize Con A in food samples.

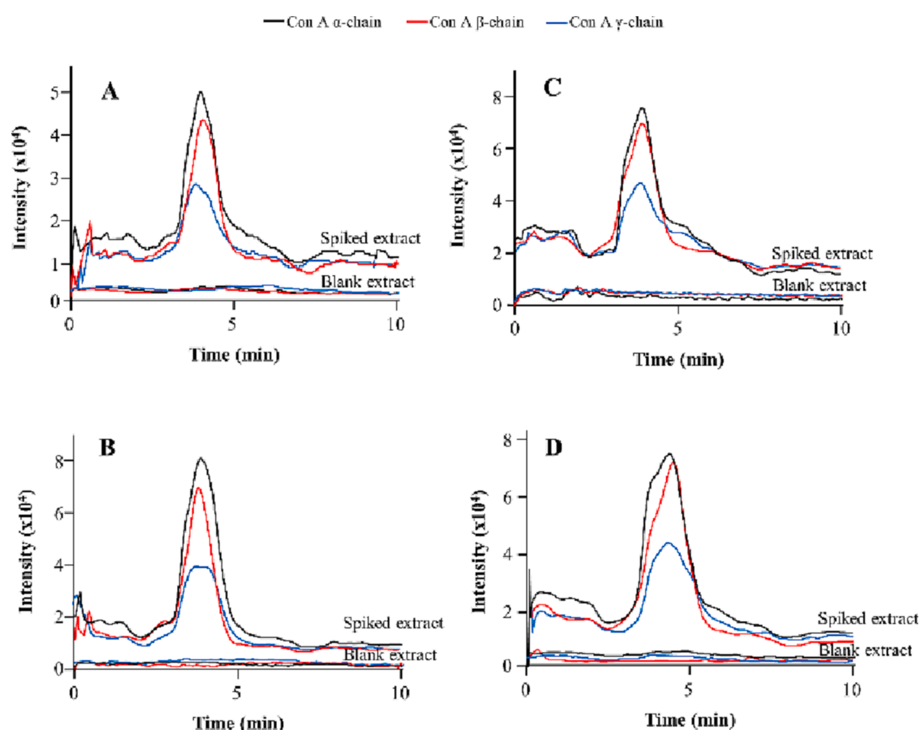

Fig. 3. EIEs for the AA-SPE-CE-MS analyses of different food matrices (blank extracts and spiked extracts with 5 mg·L^{-1} Con A (5 mg Con A per 100 g of food sample)): (A) white beans, (B) chickpea flour, (C) lentil flour, and (D) wheat flour.

Table 2
Recovery study, migration time and peak area RSD values (n = 3) of Con A proteoforms for the analysis of spiked food samples by AA-SPE-CE-MS.

Sample	Con A proteoform	Recovery (%) ± s	Migration time RSD (%)	Peak area RSD (%)
White bean	α-chain	87 ± 4	1.5	6.4
	β-chain	107 ± 3	2.2	9.2
	γ-chain	94 ± 8	1.7	11
Lentil flour	α-chain	101 ± 3	1.4	8.3
	β-chain	115 ± 6	2.0	11
	γ-chain	103 ± 4	1.8	12
Chickpea flour	α-chain	86 ± 2	1.4	8.4
	β-chain	114 ± 5	2.3	10
	γ-chain f	114 ± 4	1.7	16
Wheat flour	α-chain	97 ± 7	1.4	8.6
	β-chain	107 ± 5	3.0	10
	γ-chain	114 ± 6	1.6	11

4. Conclusions

An AA-SPE-CE-MS method has been developed for clean-up, preconcentration, separation, characterization, and quantification of the proteoforms of the allergenic lectin protein Con A. The microcartridge preparation was easy and reproducible (~3 €/microcartridge). Analysis of standard Con A under the optimized conditions showed good repeatability (below 1.9 and 8.1% RSD for migration times and peak areas), and reusability was established at 25 analyses. The method was linear from 0.5 to 20 mg·L^{-1} and LOD was 0.25 mg·L^{-1} for total Con A (100 times lower than by CE-MS, 25 mg·L^{-1}). The applicability of the method was demonstrated by analyzing Con A (5 mg per 100 g of food sample) at the typical health-based intake limits established as reference doses for allergenic proteins in food. For the analysis of food protein extracts, an off-line thermal pretreatment and filtration were required to eliminate the most abundant proteins before AA-SPE-CE-MS. In the future, the developed method could be easily adapted for the confirmatory analysis of other allergenic proteins using appropriate aptamers. AA-SPE-CE-MS is a fully integrated microscale approach that provides a green, inexpensive, and instrumentally simple alternative to LC-MS approaches for the direct analysis of protein biomarkers. Furthermore, it allows an accurate identification and quantification at low levels of the targeted protein in complex samples, preventing false positives or erroneous quantifications of non-MS based biosensors or bioassays. Moreover, it enables detailed information about the different proteoforms of the targeted protein, which could be relevant to reveal those especially allergenic. This may help to improve the understanding of the mechanisms involved in allergenicity, while upgrading food security programs and promoting the development of novel safer foods.

CRediT authorship contribution statement

María Vergara-Barberán: Methodology, Validation, Investigation, Writing – review & editing, Writing – original draft. **Ernesto Francisco Simó-Alfonso:** Writing – review & editing, Funding acquisition, Supervision. **José Manuel Herrero-Martínez:** Conceptualization, Investigation, Writing – review & editing, Funding acquisition, Supervision. **Fernando Benavente:** Conceptualization, Investigation, Writing – review & editing, Funding acquisition, Supervision.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2023.108746.

References

[1] T. Mairal, V. Cengiz Özalp, P. Lozano Sánchez, M. Mir, I. Katakis, C.K. O'Sullivan, Aptamers: Molecular tools for analytical applications, Anal. Bioanal. Chem. 390 (2008) 989–1007, https://doi.org/10.1007/s00216-007-1346-4.

[2] M. Witt, J.-G. Walter, F. Stahl, Aptamer microarrays-current status and future prospects, Microarrays 4 (2015) 115–132, https://doi.org/10.3390/microarrays4020115.

[3] V. Pichon, F. Brothier, A. Combès, Aptamer-based-sorbents for sample treatment - A review, Anal. Bioanal. Chem. 407 (2015) 681-698, https://doi.org/10.1007/s00216-014-8129-5.

[4] P. Röthlisberger, C. Gasse, M. Hollenstein, Nucleic acid aptamers: Emerging applications in medical imaging, nanotechnology, neurosciences, and drug delivery, Int. J. Mol. Sci. 18 (2017) 2430, https://doi.org/10.3390/ijms18112430.

[5] M. Darmostuk, S. Rimpelova, H. Gbelcova, T. Ruml, Current approaches in SELEX : An update to aptamer selection technology, Biotechnol. Adv. 33 (2015) 1141–1161, https://doi.org/10.1016/j.biotechadv.2015.02.008.

[6] Z. Zhuo, Y. Yu, M. Wang, J. Li, Z. Zhang, J. Liu, X. Wu, A. Lu, G. Zhang, B. Zhang, Recent advances in SELEX technology and aptamer applications in biomedicine, Int. J. Mol. Sci. 18 (2017) 2142, https://doi.org/10.3390/ijms18102142.

[7] F. Brothier, V. Pichon, Miniaturized DNA aptamer-based monolithic sorbent for selective extraction of a target analyte coupled on-line to nanoLC, Anal. Bioanal. Chem. 406 (2014) 7875–7886, https://doi.org/10.1007/s00216-014-8256-z.

[8] F. Du, M.N. Alam, J. Pawliszyn, Aptamer-functionalized solid phase microextraction-liquid chromatography/tandem mass spectrometry for selective enrichment and determination of thrombin, Anal. Chim. Acta 845 (2014) 45–52, https://doi.org/10.1016/j.aca.2014.08.018.

[9] A. Marechal, F. Jarrosson, J. Randon, V. Dugas, C. Demesmay, In-line coupling of an aptamer based miniaturized monolithic affinity preconcentration unit with capillary electrophoresis and laser induced fluorescence detection, J. Chromatogr. A 1406 (2015) 109–117, https://doi.org/10.1016/j.chroma.2015.05.073.

[10] R. Pero-Gascon, F. Benavente, Z. Minic, M.V. Berezovski, V. Sanz-Nebot, On-line Aptamer Affinity Solid-phase extraction capillary electrophoresis-mass spectrometry for the analysis of blood α-synuclein, Anal. Chem. 92 (1) (2020) 1525–1533.

[11] H. Salim, R. Pero-Gascon, E. Giménez, F. Benavente, On-line coupling of aptamer affinity solid-phase extraction and immobilized enzyme microreactor capillary electrophoresis-mass spectrometry for the sensitive targeted bottom-up analysis of protein biomarkers, Anal. Chem. 94 (2022) 6948–6956, https://doi.org/10.1021/acs.analchem.1c03800.

[12] M. Vergara-Barberán, M.J. Lerma-García, E.F. Simó-Alfonso, M. García-Hernández, M.E. Martín, A. García-Sacristán, J.M. Herrero-Martínez, Selection and characterization of DNA aptamers for highly selective recognition of the major allergen of olive pollen Ole e 1, Anal. Chim. Acta 1192 (2022) 339334, https://doi.org/10.1016/j.aca.2021.339334.

[13] L. Pont, R. Pero-Gascon, E. Gimenez, V. Sanz-Nebot, F. Benavente, A critical retrospective and prospective review of designs and materials in in-line solid-phase extraction capillary electrophoresis, Anal. Chim. Acta 1079 (2019) 1–19, https://doi.org/10.1016/j.aca.2019.05.022.

[14] Q. Wang, Y. Lei, X. Lu, G. Wang, Q. Du, X. Guo, Y. Xing, G. Zhang, D. Wang, Urea-mediated dissociation alleviate the false-positive Treponema pallidum-specific antibodies detected by ELISA, PLoS ONE 14 (2019) 1-9, https://doi.org/ 10.1371/journal.pone.0212893.

[15] A. Latiano, F. Tavano, A. Panza, O. Palmieri, G.A. Niro, N. Andriulli, T. Latiano, G. Corritore, D. Gioffreda, A. Gentile, R. Fontana, M. Guerra, G. Biscaglia, F. Bossa, M. Carella, G. Miscio, L. di Mauro, False-positive results of SARS-CoV-2 IgM/IgG antibody tests in sera stored before the 2020 pandemic in Italy, Int. J. Infect. Dis. 104 (2021) 159-163, https://doi.org/10.1016/j.ijid.2020.12.067.

[16] R. Pero-Gascon, L. Pont, F. Benavente, J. Barbosa, V. Sanz-Nebot, Analysis of serum transthyretin by on-line immunoaffinity solid-phase extraction capillary electrophoresis mass spectrometry using magnetic beads, Electrophoresis 37 (2016) 1220–1231, https://doi.org/10.1002/elps.201500495.

[17] L. Pont, F. Benavente, J. Barbosa, V. Sanz-Nebot, On-line immunoaffinity solid-phase extraction capillary electrophoresis mass spectrometry using Fab́antibody fragments for the analysis of serum transthyretin, Talanta 170 (2017) 224–232, https://doi.org/10.1016/j.talanta.2017.03.104.

[18] L. Monaci, R. Pilolli, E. de Angelis, J.F. Crespo, N. Novak, B. Cabanillas, Food allergens: Classification, molecular properties, characterization, and detection in food sources, in: Advances in Food and Nutrition Research, Academic Press Inc. (2020) 113-146, https://doi.org/10.1016/bs.afnr.2020.03.001.

[19] M. López-Pedrouso, J.M. Lorenzo, M. Gagaoua, D. Franco, Current trends in proteomic advances for food allergen analysis, Biology 9 (2020) 1–13, https://doi.org/10.3390/biology9090247.

[20] A. Barre, E.J.M. van Damme, M. Simplicien, H. Benoist, P. Rougé, Are dietary lectins relevant allergens in plant food allergy? Foods 9 (2020) 1724, https://doi.org/10.3390/foods9121724.

[21] R. Ahirwar, P. Nahar, Screening and identification of a DNA aptamer to concanavalin A and its application in food analysis, J. Agric. Food Chem. 63 (2015) 4104–4111, https://doi.org/10.1021/acs.jafc.5b00784.

[22] C.B. Madsen, M.W. van den Dungen, S. Cochrane, G.F. Houben, R.C. Knibb, A.C. Knulst, R.W. Crevel, Can we define a level of protection for allergic consumers that everyone can accept?. Regul. Toxicol. Pharmacol. 117 (2020) 104751, https://doi.org/10.1016/j.yrtph.2020.104751.

[23] B. Jiang, X. Wang, L. Wang, X. Lv, D. Li, C. Liu, Z. Feng, Two-step isolation, purification, and characterization of lectin from Zihua snap bean (*Phaseolus vulgaris*) seeds, Polymers 11 (2019) 785, https://doi.org/10.3390/polym11050785.

[24] H.H. Lauer, G.P. Rozing, (Eds), High performance capillary electrophoresis, 2nd Edition, Agilent Technologies, Waldbronn, Germany, (2014) 60-61, https://doi.org/10.1371/journal.pone.0016148.

[25] E. Giménez, F. Benavente, J. Barbosa, V. Sanz-Nebot, Analysis of intact erythropoietin and novel erythropoiesis-stimulating protein by capillary electrophoresis-electrospray-ion trap mass spectrometry, Electrophoresis 29 (2008) 2161–2170, https://doi.org/10.1002/elps.200700788.

[26] L. Pont, I. Compte, V. Sanz-Nebot, J. Barbosa, F. Benavente, Analysis of hordeins in barley grain and malt by capillary electrophoresis-mass spectrometry, Food Anal. Methods 13 (2020) 325–336, https://doi.org/10.1007/s12161-019-01648-8.

[27] J.L. Wang, B.A. Cunningham, G.M. Edelman, Unusual fragments in the subunit structure of concanavalin A, Proc. Natl. Acad. Sci. 68 (1971) 1130–1134, https://doi.org/10.1073/pnas.68.6.1130.

[28] A.B. Edmundson, K.R. Ely, D.A. Sly, F.A. Westholm, D.A. Powers, I.E. Leiner, Isolation and characterization of concanavalin A polypeptide chains, Biochemistry 10 (1971) 3554–3559, https://doi.org/10.1021/bi00795a010.

[29] W.M. Bezerra, C.P. Carvalho, R.D.A. Moreira, T.B. Grangeiro, Establishment of a heterologous system for the expression of *Canavalia brasiliensis* lectin: a model for the study of protein splicing, Genet. Mol. Res. 5 (2006) 216–223.

[30] M. Ganzera, D. Piereder, S. Sturm, C. Erdelmeier, H. Stuppner, *Urtica dioica* agglutinin: Separation, identification, and quantitation of individual isolectins by capillary electrophoresis and capillary electrophoresis-mass spectrometry, Electrophoresis. 26 (2005) 1724–1731, https://doi.org/10.1002/elps.200410369.

[31] R.K. Gupta, K. Gupta, A. Sharma, M. Das, I.A. Ansari, P.D. Dwivedi, Health risks and benefits of chickpea (*Cicer arietinum*) consumption, J. Agric. Food Chem. 65 (2017) 6–22, https://doi.org/10.1021/acs.jafc.6b02629.

[32] C. Cuadrado, G. Hajos, C. Burbano, M.M. Pedrosa, G. Ayet, M. Muzquiz, A. Pusztai, E. Gelencser, Effect of natural fermentation on the lectin of lentils measured by immunological methods, Food Agric. Immunol. 14 (1) (2002) 41–49.

[33] F. Brouns, G. van Rooy, P. Shewry, S. Rustgi, D. Jonkers, Adverse reactions to wheat or wheat components, Compr. Rev. Food Sci. Food Saf. 18 (2019) 1437–1452, https://doi.org/10.1111/1541-4337.12475.

Supplementary material

Determination of a lectin protein allergen in food by on-line aptamer affinity solid-phase extraction capillary electrophoresis-mass spectrometry

María Vergara-Barberán[1,2], Ernesto Francisco Simó-Alfonso[2], José Manuel Herrero-Martínez[2], Fernando Benavente*[1]

[1]*Department of Chemical Engineering and Analytical Chemistry, Institute for Research on Nutrition and Food Safety (INSA •UB), University of Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain*

[2]*Department of Analytical Chemistry, University of Valencia, Doctor Moliner 50, 46100 Burjassot, Spain*

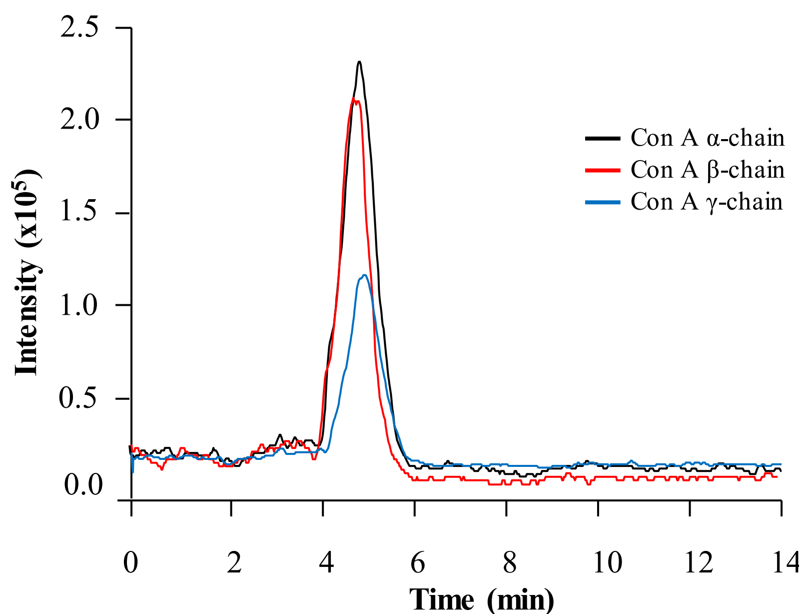

Fig. S1. Extracted ion electropherograms (EIEs) for the CE-MS analysis of a 25-fold dilution of the extract prepared from commercial jack beans under the optimized conditions (a 3 mg L^{-1} of total Con A extract solution was diluted with water 1:25 (v/v), BGE of 2 M HAc and sheath liquid of 60:40 (*v/v*) propan-2-ol/water with 0.25% (*v/v*) of HFor, +20 kV and 50 mbar during separation).

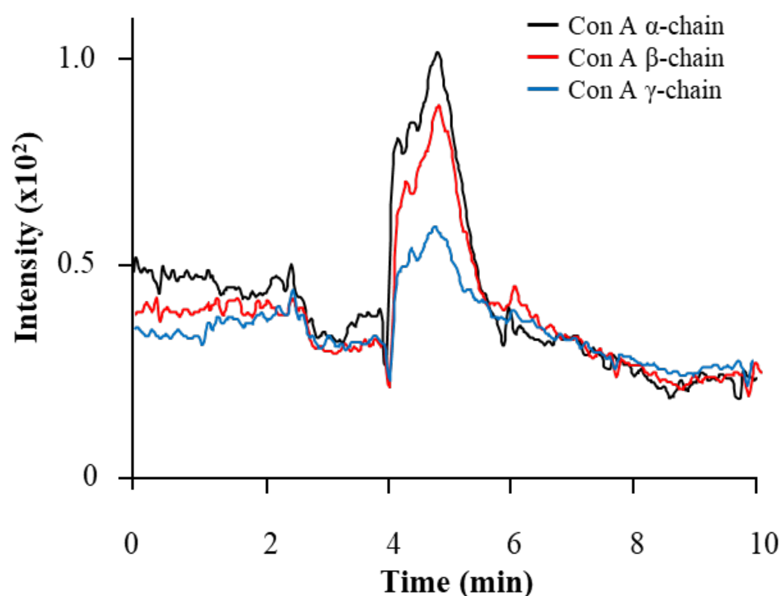

Fig. S2. Extracted ion electropherograms (EIEs) for the AA-SPE-CE-MS analysis of a 250 $\mu g \cdot L^{-1}$ Con A standard solution in water, under the optimized conditions (see section 2.7).