On-line protein digestion by immobilized enzyme microreactor capillary electrophoresis-mass spectrometry

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ABSTRACT

In this study, we present the use of microreactors packed with immobilized trypsin particles for the rapid and efficient bottom-up analysis of proteins by on-line immobilized enzyme microreactor capillary electrophoresis mass spectrometry (IMER-CE-MS). The results obtained digesting β-lactoglogulin (β-LG) off-line with free trypsin in solution and with immobilized trypsin particles were taken as a reference for the optimization of the on-line protein digestion. Under the optimized conditions, on-line digestion, separation and characterization of the protein digests were possible in less than 30 min. The limit of detection for complete sequence coverage was around 10 μg mL^{-1} (~500 μM) of β-LG, the repeatability was comparable to the off-line digestion methods and the microreactor could be reused until thirty times. The good performance of IMER-CE-MS was also demonstrated for several other proteins as α-casein (α-CSN), β-casein (β-CSN), and κ-casein (κ-CSN), as well as for a complex protein mixture (an *Escherichia coli* whole cell lysate).

1. Introduction

A huge number of studies have been developed to monitor the functions, interactions, location, and regulation of proteins due to their essential role in most biological processes and their relationship with many diseases [1]. In proteomics research, peptide mapping is essential for characterization of the different proteoforms of a certain protein, including their primary amino acid sequence and their co-translational and post-translational modifications [2,3]. The bottom-up analysis of proteins by mass spectrometry (MS) requires the enzymatic digestion of proteins into peptides. Trypsin is the most typically used proteolytic enzyme due to its specificity, effectiveness, and low price. This enzyme cleaves proteins at the C-terminal side of lysine and arginine residues, resulting in peptides of small relative molecular mass (M_r) usually between 500 and 3000 [3]. These peptides are in general detected in MS as singly, doubly or triply charged ions in positive electrospray ionization mode (ESI+) [4], which facilitates the most sensitive detection, the fragmentation by MS/MS and the interpretation of the mass spectra.

Traditionally, the enzymatic digestion with trypsin has been performed in a homogeneous solution after mixing the free enzyme and the protein solutions. The pH, the temperature, the enzyme-to-protein ratio and the reaction time can be optimized to obtain the maximum digestion yield. However, in general, very long times (e.g. 18 h) are recommended for an appropriate digestion, before separation and characterization by liquid chromatography mass spectrometry (LC-MS) or capillary electrophoresis mass spectrometry (CE-MS) [5]. Immobilized enzymes have been alternatively explored to decrease the sample volume and the total digestion times, minimize the

sample handling, improve the digestion yields, as well as to stabilize the enzyme, avoid its autoproteolysis, simplify its recovery and make it reusable [6]. The possibility of using an immobilized enzyme microreactor (IMER) coupled on-line to LC-MS or CE-MS has interesting advantages such as further reducing the sample handling and the required sample volumes while increasing the analysis throughput [7–10]. Furthermore, IMER-CE-MS has the additional advantage over the on-line approaches based on LC-MS that does not require the use of complex instrumental set-ups with valves [9,10].

In the last 25 years, a limited number of scientific articles about the enzymatic digestion of proteins by IMER-CE have been published, probably due to the instrumental and methodological difficulties of the on-line coupling and the limited applicability of the methods without on-line MS detection [7]**.** Most of the applications were based on open tubular microreactors with immobilized trypsin on the inner capillary surface [11–19] and only a few were reported with monolithic [20,21], particle packed [8,22,23] or sol-gel [24,25] microreactors. Since the pioneering studies of Amankwa et al. [11–13], the use of open tubular microreactors has been proposed by other research groups [14–19]. However, now is widely accepted that digestion yields with these microreactors are in general low because of the scarce interaction between the protein and the immobilized trypsin within the very short time of the on-line digestion [7,8]. Monolithic, sol-gel and particle packed supports with immobilized trypsin were developed to increment the digestion yield due to the extended active surface areas and the reduced distances between the protein and the immobilized enzyme [8,20–25]. Nowadays, preparation of monoliths with immobilized trypsin requires rather challenging, specialized and long and tedious synthesis procedures to obtain reproducible results [7]. In contrast, a wide range of activated particle supports ready to immobilize trypsin or with immobilized

trypsin are commercially available [8,22,23]. These immobilized trypsin particles can be simply and reproducibly packed in the on-line microreactors for IMER-CE-MS. Unfortunately, the number of studies describing completely on-line IMER-CE-MS are rather scarce, and all of them are using open tubular IMERs [14,17,18]. Recently, Liu *et al.* described a novel method with packed IMERs using commercial silica particles with immobilized trypsin for the on-line digestion of proteins by IMER-CE with UV and fluorescence detection, and fractions were collected to be analyzed by MS [8]. In this study, we use commercial cellulose resin particles with immobilized trypsin for the packed IMERs and we develop a completely on-line IMER-CE-MS method for the bottom-up analysis of standard proteins (e.g. β-lactoglobulin (β-LG), α-casein (α-CSN), β-casein (β-CSN) and κ-casein, (κ-CSN)) and complex protein mixtures (e.g. an *Escherichia coli* whole cell lysate). The performance of the on-line protein digestion is compared with the off-line digestion using free trypsin and immobilized trypsin to demonstrate the potential of the novel method, and expand the applicability of IMER-CE-MS.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals used in the preparation of the background electrolyte (BGE) and the rest of solutions were of analytical reagent grade or better. Water (LC-MS grade), ammonium acetate (NH_4Ac, ≥99.9%), ammonium bicarbonate (LC-MS grade), DL-dithiothreitol (DTT, ≥99.0%) and iodoacetamide (IAA, ≥98.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid (HAc, glacial), formic acid (HFor, 99.0%), ammonia (25%) and sodium hydroxide (≥99.0%) were supplied by Merck (Darmstadt, Germany). Iodoacetamide (IAA, ≥98.0%) was provided by Fluka (Buchs,

Switzerland). Acetonitrile (≥99.9%) was purchased from Panreac (Castellar del Vallès, Spain). 2-propanol (≥99.9%) was supplied by Scharlau (Sentmenat, Spain).

Free trypsin (*sequencing grade modified trypsin*) and particles with immobilized trypsin (*immobilized trypsin*) were provided by Promega (Madison, Wisconsin, USA). β-LG (~90%, PAGE), α-CSN (>70%), β-CSN (>90%) and κ-CSN (≥80%) were purchased from Sigma-Aldrich. An *Escherichia coli* cell lysate (*E. coli* positive control whole cell lysate – expressing 6X His tag protein) was supplied by Abcam (Cambridge, UK).

2.2. Electrolyte solutions, sheath liquid, protein standards and samples.

The BGE for the CE-MS separation was a solution of 50 mM HAc : 50 mM HFor (pH 2.3) and was filtered through a 0.22 µm nylon filter (MSI, Westboro, MA, USA). The sheath liquid solution consisted of a mixture of 60:40 (v/v) 2-propanol:water with 0.05% (v/v) of HFor. The sheath liquid and the BGE were degassed for 10 min by sonication before use.

An aqueous standard solution (2000 µg mL^{-1}) of each model protein (β-LG, α-CSN, β-CSN and κ-CSN) was prepared and aliquoted into 50 µL portions. These aliquots (100 µg protein each) were dried at 37 ºC and stored in a freezer at -20 ºC when not in use.

The *E. coli* cell lysate was a lyophilized pellet that contained 250 µg of protein and was dissolved in 125 µL of 50 mM NH_4HCO_3 (2000 µg mL^{-1} of protein). Excipients of low M_r were removed by passage through a 10,000 M_r cut-off cellulose acetate centrifugal filters (Millipore Microcon Ultracel PL, Merck). The sample (25 µL) was centrifuged at 25 ºC for 7 min at 11,300 x g and the residue was washed three times for 7 min in the

same way, with 50 µL of digestion buffer. Under the optimized conditions, the digestion buffer was 50 mM NH_4HCO_3, pH = 7.9 for the off-line digestions and 10 mM NH_4HCO_3, pH = 7.9 for the on-line digestion. The final residue was recovered by placing the filter upside down in a new vial and centrifuging once more at a reduced centrifugal force (2 min at 700 x g). Sufficient digestion buffer was added to the vial to adjust the final volume to 50 µL (1000 µg mL^{-1}). Filtered samples were stored in the fridge at 4 ºC when not in use.

2.3. Apparatus and procedures

pH measurements were made with a Crison 2002 potentiometer and a Crison electrode 52-03 (Crison Instruments, Barcelona, Spain). Protein stock aliquots were dried in a Savant™ SPD111V SpeedVac™ (Thermo Fisher Scientific, Waltham, MA, USA). Centrifugal filtration was carried out in a Mikro 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). Agitation was performed with a Vortex Genius 3 (Ika®, Staufen, Germany). Digestion mixtures were incubated in a Thermo-Shaker TS-100 (Biosan, Riga, Latvia) at controlled temperature.

All CE-MS experiments were performed in a 7100 CE system coupled with an orthogonal G1603 sheath-flow interface to a 6220 oa-TOF LC/MS spectrometer (Agilent Technologies, Waldbronn, Germany). The sheath liquid was 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 was operated under optimized conditions in positive mode using the following parameters: capillary voltage 4000 V, drying temperature 200 ºC, drying gas flow rate 2.9 L min^{-1}, nebulizer gas 7 psig, fragmentor voltage 190 V, skimmer voltage 60 V, OCT 1 RF Vpp voltage 300 V. Chemstation and MassHunter

softwares (Agilent Technologies) were used for CE and TOF mass spectrometer control, data acquisition and integration. 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).

2.4. Off-line digestion with free and immobilized trypsin and CE-MS

The off-line digestion with trypsin in solution was conducted following the procedures described elsewhere [5,26]. A dried stock aliquot of β-LG was reconstituted with 100 µL of digestion buffer (50 mM NH_4HCO_3 under the optimised conditions) and was reduced adding 2.5 µL of 0.5 M DTT in digestion buffer (1000 µg mL^{-1} of β-LG, i.e. ~50 µM). The mixture was incubated with agitation at 56 ºC for 30 min and let it cool down to room temperature. Then, the reduced protein was alkylated adding 7 µL of a 0.73 M IAA solution in digestion buffer (30 min at room temperature with agitation in the dark). To remove the excess of DDT and IAA, the mixture was filtered using 10,000 M_r cut-off centrifugal filters at 25 ºC. First, the filters were washed with 100 µL of the digestion buffer and centrifuged for 10 min at 11,300 x g. Then, the sample was added and centrifuged (10 min at 11,300 x g). Finally, the sample was washed three times with 100 µL of digestion buffer (10 min at 11,300 x g). The final residue was recovered by placing the filter upside down in a new vial and centrifuging at a reduced centrifugal force (2 min at 700 x g). Sufficient digestion buffer was added to the vial to adjust the final volume to 100 µL (1000 µg mL^{-1} of β-LG). Trypsin (2.5 µL at 1000 µg mL^{-1}) was added to the protein sample and the mixture was vortexed and incubated with agitation at 37 ºC for 18 h. The reaction was stopped by heating the mixture for 10 min at 100 ºC. The protein digests were dried at 37 ºC and stored in a freezer at -20 ºC when not in use. Before the digestion, the *E. coli* cell lysate solution in digestion buffer (50 µL, 1000 µg

mL^{-1} of protein) was reduced and alkylated as β-LG adding 1.25 µL of 0.5 M DTT in digestion buffer and 3.5 µL of IAA 0.73 M.

The off-line enzymatic digestion procedure with immobilized trypsin was performed following the manufacturer instructions [27]. β-LG (1000 µg mL^{-1}) was reduced and alkylated as explained above in digestion buffer (10 mM NH_4HCO_3 under the optimized conditions) and dried at 37 ºC before use. First, 5 µL of digestion buffer were added to the dried reduced and alkylated protein. After that, 16 µL of acetonitrile and 19 µL of digestion buffer were added to prepare the final protein sample. A spin column was placed in a 1.5 mL centrifuge tube and 200 µL of the suspension with the immobilized trypsin resin were added. Then, the liquid was separated from the resin by centrifugation at 25 ºC, and the resin was washed three times with 200 µL of the digestion buffer (5 s at 300 x g in all cases). The spin column was placed in a clean 1.5 mL tube, the protein sample was added, and it was incubated without agitation at room temperature for 30 minutes. Then, 100 µL of the digestion buffer were added to recover the peptides by centrifugation at 25 ºC (5 s at 300 x g). This last step was repeated three times. The protein digests were dried at 37 ºC and stored in a freezer at -20 ºC when not in use. The dried protein digests were reconstituted with 100 µL of digestion buffer before the analysis (1000 µg mL^{-1} of β-LG). To study the reusability of the immobilized trypsin resin, the spin column was washed four times with 100 µL of the digestion buffer between digestions to prevent carry-over effects. Otherwise, the resin was discarded.

Fused silica capillaries (72 cm total length (L_T) x 75 µm internal diameter (id) x 365 µm outer diameter (od)) were supplied by Polymicro Technologies (Phoenix, AZ, USA) and

were activated flushing at 930 mbar with water (5 min), 1 M NaOH (15 min), water (15 min) and BGE (50 mM HAc : 50 mM HFor, pH = 2.3, 10 min). Activation was performed off-line to avoid mass spectrometer contamination. Before CE-MS analysis, capillaries were conditioned flushing with water (1 min), 1 M HAc (3 min), water (1 min) and BGE (5 min). Samples were hydrodynamically injected at 50 mbar for 15 s (~80 nL with the Hagen-Poiseuille equation [28]) and the separation was carried out at 25 ºC or 37 ºC applying 25 kV during 30 min. The analyses were performed in triplicate.

2.5. On-line IMER-CE-MS

The on-line enzymatic digestion was performed in an IMER (0.7 cm L_T x 250 µm id x 365 µm od fused silica capillary) that was inserted at 7.5 cm of the beginning of an activated separation capillary (72 cm L_T x 75 µm id x 365 µm od fused silica capillary) [29]. The IMER preparation was very simple. First, a polymeric frit was placed at the beginning of the microreactor and this side was connected to a 7.5 cm separation capillary using a plastic sleeve. Second, the microreactor was filled with the immobilized enzyme resin applying vacuum during 10 s. The packing was checked under an optical microscope (100x) and the procedure was repeated until the microcartridge was completely packed. Then, another polymeric frit was introduced at the end of the microreactor that was finally connected to a 64.5 cm separation capillary using another plastic sleeve. The IMER-CE capillary was checked for abnormal flow restriction, flushing with water and BGE with a syringe, and applying the separation voltage for 15 min (25 kV).

Under the optimized conditions, IMER-CE capillaries were conditioned flushing with BGE (50 mM HAc : 50 mM HFor, pH = 2.3) for 2 min. Two plugs of digestion buffer (10 mM NH_4HCO_3 under the optimized conditions) were injected at 50 mbar for 8 s (~40 nL, i.e. ~1 cm) before and after the protein sample, which was injected at 50 mbar for 15 s (~80 nL, i.e. ~2 cm). Then, the BGE was introduced at 5 mbar for 600 s (~325 nL, i.e. ~7 cm) to ensure that the sample plug passed through the microreactor giving enough time for the protein digestion. Finally, 25 kV were applied for the separation. Between the analyses, the capillary was flushed with BGE (5 min) and water (5 min). The analyses were performed in triplicate.

β-LG, α-CSN, β-CSN and κ-CSN (1000 μg mL^{-1} all; i.e. ~50, ~40, ~40 and ~50 μM, respectively) and the *E. coli* cell lysate (1000 μg mL^{-1} of protein) were analyzed by on-line IMER-CE-MS. Before the on-line digestion, β-LG, κ-CSN and the *E. coli* cell lysate were reduced and alkylated as explained in Section 2.4. α-CSN and β-CSN dried stock aliquots were reconstituted directly with the digestion buffer, because no disulfide bonds were present.

3. Results and discussion

3.1. Off-line digestion with trypsin in solution and CE-MS

Protein digestion with trypsin has been traditionally conducted in solution using free trypsin and long digestion times (≥18 h) at 37 ºC and pH around 8 to ensure efficient and reproducible digestions [3,5,26]. The use of a digestion buffer of 50 mM NH_4HCO_3, at pH 7.9 has been widely described in the literature [5]. In this study, as an alternative, we also tested digestion buffers of 10 mM NH_4HCO_3 and 10 mM NH_4Ac at pH 7.9, because low ionic strength buffers are more recommendable for the analysis of

protein digests by CE-MS and IMER-CE-MS. Furthermore, we also evaluated the influence on peptide sensitivity of the separation temperature in CE-MS and on the digestion yield of the digestion time, because later the on-line digestion and the separation by IMER-CE-MS were performed at the same temperature, and digestion was produced in a very short time.

Table 1 shows the sequence and the mass-to-charge (m/z) ratio of the molecular ions of the peptides detected by CE-MS for β-LG (1000 μg mL^{-1}). β-LG was chosen as a model protein due to its low M_r (~19,900) and structural simplicity, even though the presence of disulfide bonds that needed reduction and alkylation for an efficient digestion. At this protein concentration, all the expected peptides after tryptic digestion were detected by CE-MS (sequence coverage was 100%, calculated considering the number of amino acids detected from the peptide sequence). Table 1 also shows the relative peak area and the migration time values, as well as the percentages of relative standard deviation (%RSD, n=3), for the CE-MS separation at 37 ºC, with a 50 mM NH_4HCO_3 digestion buffer and 18 h of digestion time. As can be observed, repeatability was good, ranging between 0.1-8.0% and 1.6-1.8% for peak areas and migration times, respectively. These values agreed with the values obtained by CE-MS for peptide digests in previous studies [30]. To easily compare the results with the different digestion buffers, separation temperatures and digestion times, Figure 1 shows two bar graphs with the total sum of the peak areas for the detected tryptic peptides in the different conditions. As can be observed in Figure 1-A, at 18 h of digestion, sensitivity was higher when separation by CE-MS was conducted at 37 ºC. At higher separation temperatures, the peaks were narrower and the migration times were shorter due the lower viscosity of the BGE, improving ionization efficiency and peak areas. In addition, peak areas were higher with

NH_4HCO_3 digestion buffers, due to the higher digestion yields compared to the NH_4Ac digestion buffer. Figures 1-C and 1-D show the total ion electropherogram (TIE) and extracted ion electropherograms (EIEs) for the CE-MS separation at 37 ºC of the tryptic digest of β-LG with a 50 mM NH_4HCO_3 digestion buffer (1000 μg mL^{-1} of protein digested at 37 ºC during 18 h), which allowed the highest digestion yield and detection sensitivity (Figure 1-A). Under these optimized conditions, total separation times were shorter than 9 minutes (Figure 1-D). Regarding the digestion time, Figure 1-B shows that the peak areas significantly decreased when it was reduced to 30 min, which is the time recommended by the manufacturer for the off-line digestion with immobilized trypsin particles, as shown in the next section. The digestion yield decreased because the interaction time between the enzyme and the protein was shorter. However, it is worth mentioning that at this protein concentration, digestion yield with 30 min of digestion was enough to have total sequence coverage, as with 18 h of digestion.

3.2. Off-line digestion with immobilized trypsin and CE-MS

The off-line digestion with immobilized trypsin was first carried out using commercial particles and the digestion buffer, temperature and time recommended by the manufacturer (50 mM NH_4HCO_3 pH 7.9, 25 ºC and 30 min) [27]. Under these digestion conditions, Table 1 shows that the sequence coverage was total as in the off-line digestion with trypsin in solution (1000 μg mL^{-1} of β-LG analyzed by CE-MS at 37 ºC in both cases). In addition, the repeatability of peak areas and migration times was also similar, with %RSD ranging between 0.6-14% and 0.4-1.3%, respectively. Figure 2-A shows a bar graph with the total sum of the peak areas for the detected tryptic peptides using the different digestion buffers and separation temperatures in CE-MS. Again, sensitivity was higher when CE-MS was conducted at 37 ºC. However now, in

contrast to the off-line digestion with trypsin in solution, peak areas were slightly higher with 10 mM NH_4HCO_3 or NH_4Ac digestion buffers. The reusability of the immobilized trypsin particles was also evaluated performing three consecutive digestions with the different digestion buffers. As can be observed in Figure 2-B, the digestion yield was similar between consecutive digestions for the 10 and 50 mM NH_4HCO_3 digestion buffers, but significantly decreased for the 10 mM NH_4Ac after the first digestion, probably because of the enzyme degradation with the 10 mM NH_4Ac digestion buffer. Therefore, the use of NH_4HCO_3 digestion buffers allowed the highest digestion yields, detection sensitivities and made the enzyme reusable. Figures 2-C and 2-D show the TIE and EIEs for the CE-MS separation of the tryptic digest of β-LG at 37 ºC with a 10 mM NH_4HCO_3 digestion buffer (1000 μg mL^{-1} of protein digested at 25 ºC during 30 min). Under these optimized conditions, total separation times were again shorter than 9 minutes (Figures 2-C and D), but digestion yields with immobilized trypsin particles were lower than with free trypsin in solution (compare the total sum of the values of the peak areas for the detected tryptic peptides under the optimized conditions, which are labelled with an asterisk in Figures 1 A-B and 2 A-B). Taking into account the information provided by the enzyme manufacturer, the main explanation could be that the quality of the immobilized trypsin was lower than the quality of the free trypsin, which was a *sequencing grade modified trypsin*. Nevertheless, the use of immobilized trypsin substantially reduced digestion times while allowing the enzyme reuse. This was an essential feature to develop the on-line IMER-CE-MS method, which allowed reducing the manual sample handling and increasing the analysis throughput.

3.3. On-line digestion by IMER-CE-MS

The typical digestion buffer of 50 mM NH_4HCO_3 pH 7.9 was rapidly discarded for IMER-CE-MS after some preliminary studies because of current instability and breakdowns during the electrophoretic runs. This was probably due to CO_2 bubble formation during the on-line mixing of the digestion buffer and the acidic BGE (50 mM HAc : 50 mM HFor, pH 2.3). This issue was not found with the 10 mM NH_4HCO_3 and 10 mM NH_4Ac digestion buffers. Figure 3-A shows that the digestion yield and detection sensitivity for β-LG (1000 μg L^{-1}) at 25 ºC were higher with the 10 mM NH_4HCO_3 digestion buffer than with the 10 mM NH_4Ac digestion buffer, and results were further improved at 37 ºC. This temperature is well-known to be the optimum for trypsin digestion, despite the manufacturer of the immobilized trypsin particles recommended a digestion temperature of 25 ºC as was indicated in the previous section. Furthermore, as we explained before, separations at 37 ºC were faster and sensitivity was higher. Once selected these conditions, we evaluated the influence of the contact time between the β-LG and the immobilized trypsin. The protein solution (50 mbar, 15 s, ~80 nL) was injected between two plugs of digestion buffer (50 mbar, 8s, ~40 nL) and the sandwich was pushed through the microreactor with BGE (~325 nL) at different velocities (5 mbar, 600 s; 15 mbar, 200 s and 25 mbar, 120 s). As can be observed in Figure 3-B repeatability was higher when the protein solution passed through the microreactor at the smallest velocity (5 mbar, 600 s), probably because the contact time between the protein and the immobilized enzyme was longer. Figures 3-C and 3-D show the TIE and EIEs for the analysis of β-LG (1000 μg mL^{-1}) by IMER-CE-MS at 37 ºC with the 10 mM NH_4HCO_3 digestion buffer and the on-line digestion at the smallest velocity. Under these optimized conditions, separations were improved with regard to the off-line digestions because the total separation times were slightly longer (<13 min) than by CE-MS (<9 min, Figures 1 C-D and 2 C-D) probably because of the

backpressure promoted by the presence of the microreactor. Furthermore, digestion yields were comparable, but slightly lower, than in the off-line digestion with immobilized trypsin (compare the total sum of the values of the peak areas for the detected tryptic peptides under the optimized conditions, which are labelled with an asterisk in Figures 2 A-B and 3 A-B). This was probably due to the reduced contact time between the protein and the enzyme and the lower amount of protein. Digestion velocity could not be further reduced, because 5 mbar was the smallest pressure that can be applied by the CE instrument. With regard to the amount of protein, the injected volume of protein solution was increased, from 80 nL (50 mbar, 15 s) to 160 nL (50 mbar, 30 s) but results did not significantly improved. Under the optimized conditions, Table 1 shows that %RSD for peak areas and migration times ranged between 2.2-15 and 4.8-7.6%, respectively. Therefore, repeatabilities in peak areas and migration times were only slightly lower than for the off-line digestions (Table 1). With regard to the sequence coverage, it was found to be total until a concentration of around 10 μg mL^{-1} of β-LG, which was considered as the limit of detection (at 5 μg mL^{-1} of protein the coverage decreased to 80%). The reusability of a microreactor was tested studying the variation in peak area and migration time of the tryptic peptides through consecutive protein digestions and analysis of β-LG (1000 μg mL^{-1}). As can be observed in Figure S-1A, the microreactor could be reused for more than 30 consecutive times without a significant decrease in the values and the repeatability of the total sum of peak areas of the detected tryptic peptides (average %RSD values for 5, 10, 20, and 30 consecutive digestions are inserted in the graphics of Figure S-1). However, average migration times and their %RSD values increased through consecutive analyses because of gradual repacking of the immobilized trypsin particles (Figure S-1B). Therefore, a maximum of ten analyses per microreactor would be a good compromise taking into account the

overall performance of the system. No carry-over effect was observed when blank samples were analyzed between consecutive digestions.

To validate the IMER-CE-MS method, α-CSN (a mixture of α-CSN1 and α-CSN2), β-CSN and κ-CSN were also analyzed (1000 μg mL^{-1} of protein), and results in terms of repeatability and sequence coverage were similar as for β-LG (1000 μg mL^{-1} of protein). Supplementary Tables S-1, S-2, S-3 and S-4 show the %RSD (n=3) for peak areas of the peptides detected by IMER-CE-MS for the different proteins. At this protein concentration, sequence coverages were 89%, 79%, 88% and 66% for α-CSN1, α-CSN2, β-CSN and κ-CSN, respectively. In α-CSN, the sequence coverage for α-CSN2 was lower because this proteoform was at lower abundance in the mixture. Among all the proteins, the sequence coverage was the lowest for κ-CSN because a high M_r peptide (53 amino acids long) was not detected. Supplementary Figures S-2 A, B and C show the TIE and EIEs for the IMER-CE-MS analysis of α-CSN1, β-CSN and κ-CSN, respectively, and total separation times were shorter than 15 min in all cases.

In order to demonstrate the feasibility of IMER-CE-MS for the analysis of complex real samples an *E. coli* cell lysate (1000 μg mL^{-1}) was analyzed under the optimized conditions by off-line digestion with free trypsin and CE-MS and by on-line IMER-CE-MS. Supplementary Figure S-2 D and E show the TIEs for the off-line and the on-line digestions, respectively. In both cases, a similar electrophoretic profile was obtained but it was difficult to appreciate electrophoretic peaks due to the tryptic peptides because of the low abundance of the different proteins in the cell lysate. The studied cell lysate was specifically prepared as a quality control for purification of His-tagged proteins, which contained an overexpressed protein with six additional His amino acids in the C-

terminal position (6x-His, M_r ~14,200). Taking into account the western blot provided by the manufacturer and the LC-MS/MS bottom-up analysis study of Wright *et al.* [31], the 6x-His protein was identified as 6x-His human α-lactalbumin. As Wright *et al.* [31], it was also possible to identify other high abundant proteins in the cell lysate with the off-line and the on-line digestions. Table 2 shows the sequence of the detected peptides, the sequence coverage and the identified proteins in both cases. As can be observed, sequence coverages were only slightly lower with the on-line digestion, due to the shorter digestion times and the lower amount of protein, and in both cases 6x-His human α-lactalbumin, myoglobin C, cathepsin D, creatine kinase-MM and antithrombin III were identified. The number of identified peptides and the sequence coverages were also very similar to those found by Wright *et al.* by LC-MS/MS [31], indicating the good performance of the on-line digestion by IMER-CE-MS compared to the traditional off-line digestion methods and the potential for the high-throughput bottom-up analysis of complex protein mixtures in proteomics research.

4. Conclusions

We have developed and validated an IMER-CE-MS method for the on-line enzymatic digestion, separation and characterization of proteins. Under the optimized conditions, using 10 mM NH_4HCO_3 pH 7.9 as the digestion buffer, a very low protein sample flow through the microreactor, a separation BGE of 50 mM HAc : 50 mM HFor, pH 2.3, and a temperature of digestion and separation of 37 ºC, proteins were analyzed in less than 30 min (including all the steps). Furthermore, the sequence coverage for β-LG was complete until a concentration of 10 µg mL^{-1} of protein and the microreactor could be reused until 10 times with optimum performance, without decreasing repeatability or observing carry-over. Results were comparable to the off-line digestion with free or

immobilized trypsin and CE-MS, despite a slightly smaller digestion yield was observed due to the shorter digestion times and the lower amount of protein. The good performance of the IMER-CE-MS method was also demonstrated for α-CSN1, α-CSN2, β-CSN and κ-CSN, as well as for an *E. coli* cell lysate, confirming the great potential of this approach to reduce the protein sample volume and the digestion times, while minimizing the sample handling and reusing the microreactors. These features are critical to perform rapid, reliable and high-throughput analysis of complex protein mixtures in proteomics research, with trypsin or any other proteolytic enzyme, in bottom-up or middle-down approaches using hybrid mass spectrometers.

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The authors declare no competing interests.

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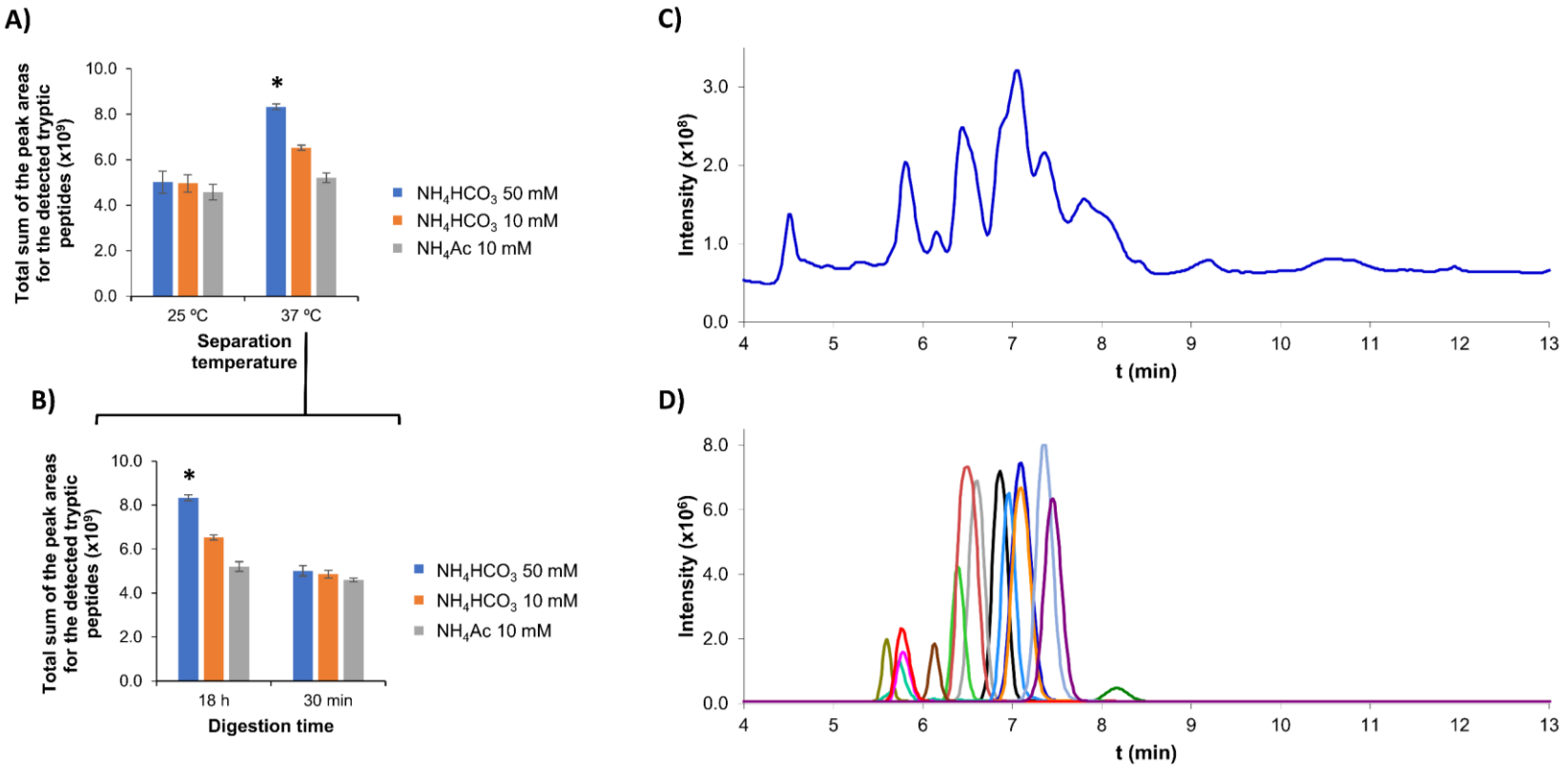

Figure 1. Off-line protein digestion of β-LG (1000 μg mL^{-1}) with free trypsin at 37 ºC and CE-MS analysis. Comparison of the total sum of peak areas for the detected tryptic peptides **(A)** with different separation temperatures and digestion buffers (18 h of digestion time) and **(B)** with different digestion times and digestion buffers (37 ºC of separation temperature). **(C)** Total ion electropherogram (TIE) and **(D)** extracted ion electropherograms (EIEs) for 18 h of digestion time and CE-MS analysis at 37 ºC using 50 mM NH_4HCO_3 as the digestion buffer. Optimized conditions are indicated in **(A)** and **(B)** with an asterisk.

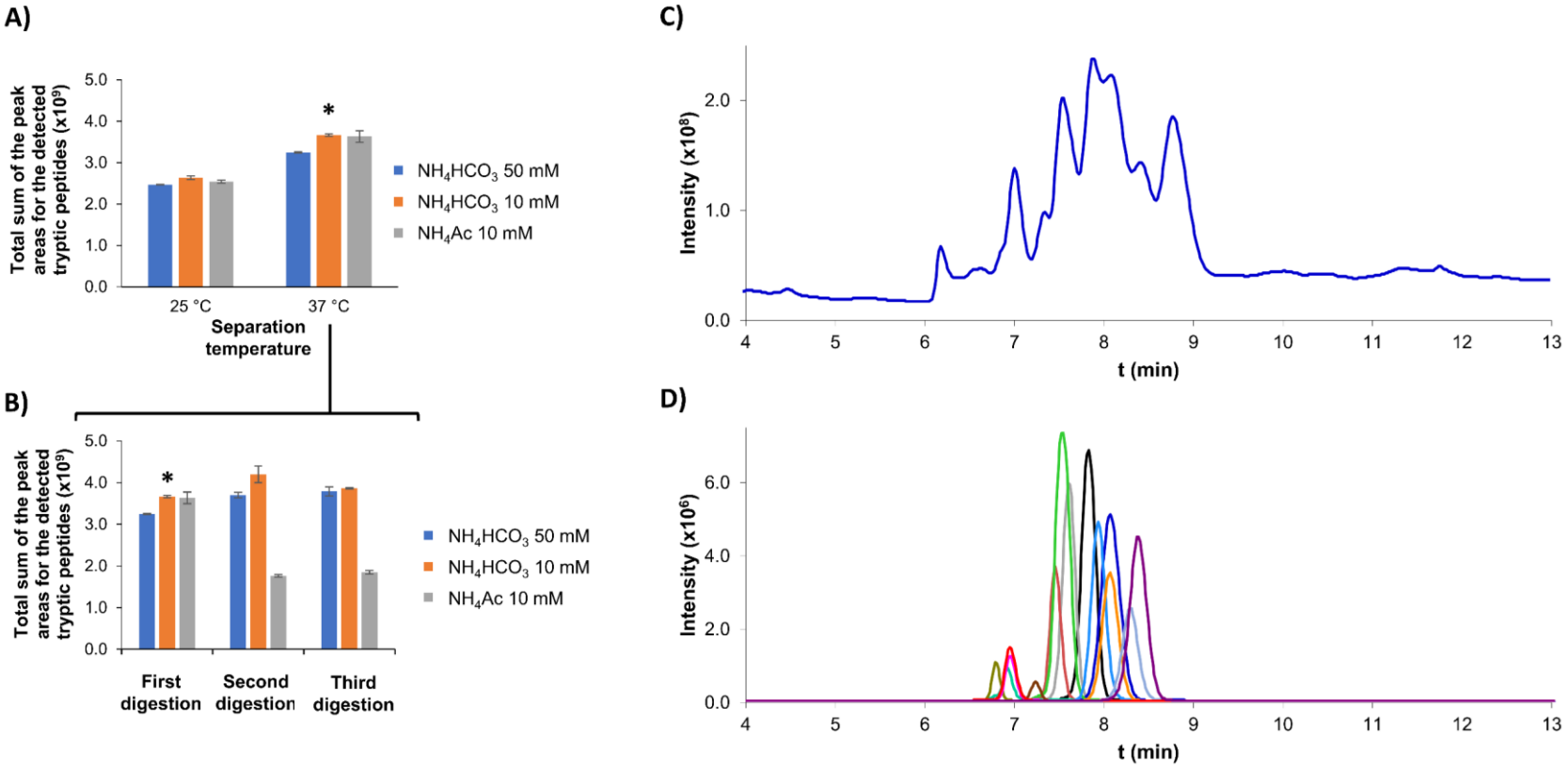

Figure 2. Off-line protein digestion of β-LG (1000 µg mL^{-1}) with immobilized trypsin performed during 30 min at 25º C and CE-MS analysis. Comparison of the total sum of peak areas for the detected tryptic peptides **(A)** with different separation temperatures and digestion buffers and **(B)** after consecutives enzymatic digestions with different digestion buffers (37 ºC of separation temperature). **(C)** TIE and **(D)** EIEs for the CE-MS analysis at 37 ºC using 10 mM NH_4HCO_3 as the digestion buffer. Optimized conditions are indicated in **(A)** and **(B)** with an asterisk.

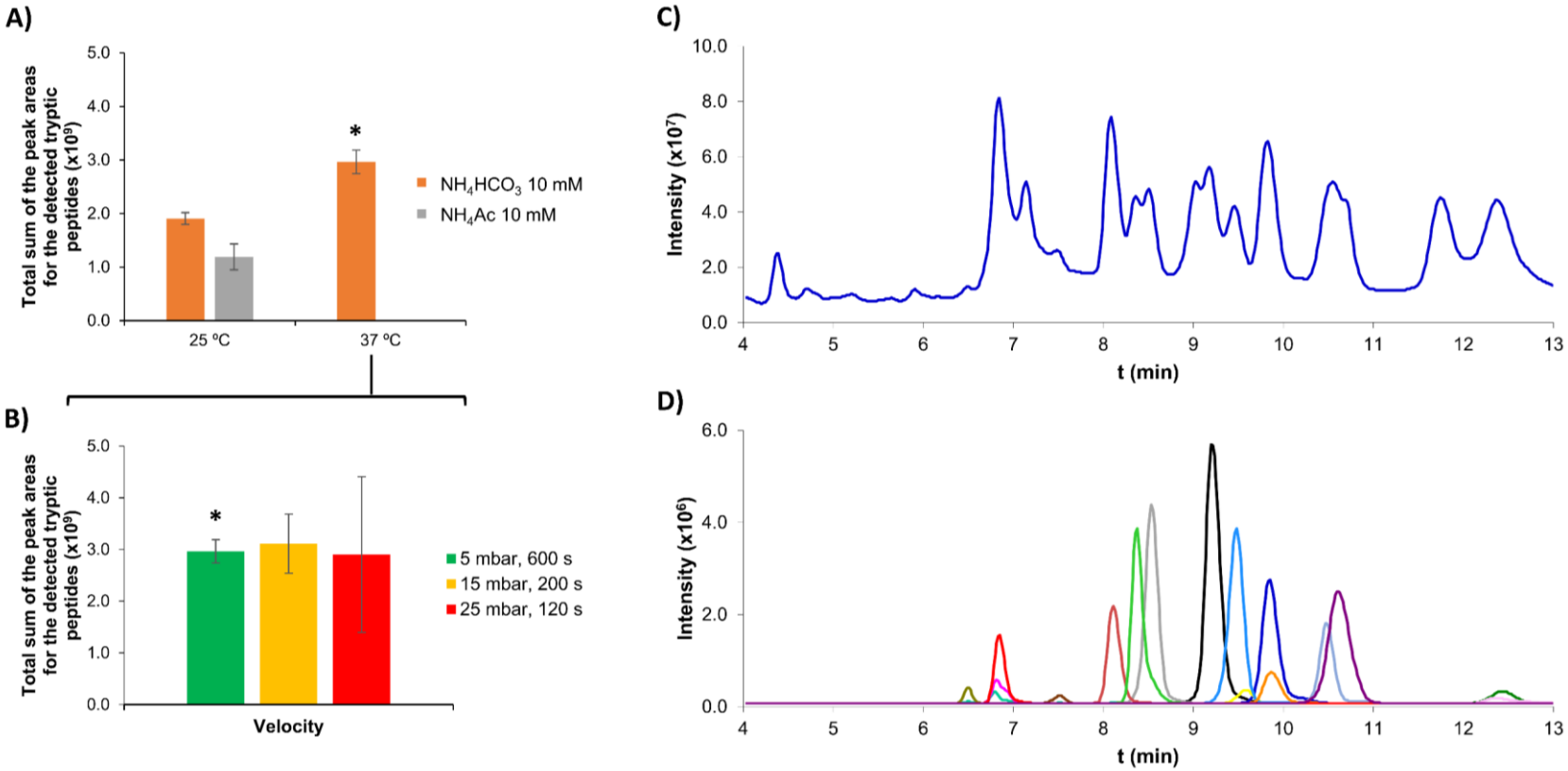

Figure 3. On-line IMER-CE-MS protein digestion and analysis of β-LG (1000 μg mL^{-1}) with immobilized trypsin. Comparison of the total sum of peak areas for the detected tryptic peptides **(A)** with different digestion-separation temperatures and digestion buffers (with 5 mbar, 600 s of contact time) and **(B)** with different contact times between the protein and the enzyme of the microreactor (37 ºC of digestion-separation temperatures). **(C)** TIE and **(D)** EIEs for the on-line IMER-CE-MS digestion-separation at 37 ºC using 10 mM NH_4HCO_3 as the digestion buffer. Optimized conditions are indicated in **(A)** and **(B)** with an asterisk.

Table 1. Digestion of β-LG (1000 μg mL^{-1}). Relative peak areas, migration times and percentage of relative standard deviations (%RSD) of the peptides detected by the off-line digestion with free and immobilized trypsin in solution and CE-MS and by the on-line digestion with immobilized trypsin in IMER-CE-MS. The analyses were performed in triplicate under the optimized conditions in all cases. Single amino acids were not taken into account.

Peptide sequence		$[M+nH]^{n+}$		Off-line digestion with trypsin in solution and CE-MS				Off-line digestion with immobilized trypsin and CE-MS				On-line digestion with immobilized trypsin by IMER-CE-MS			
				Relative peak area [a)]		Migration time (min)		Relative peak area [a)]		Migration time (min)		Relative peak area [a)]		Migration time (min)	
		m/z	n	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
[1-8]	**LIVTQTMK**	467.2758	2	0.101	1.8	6.9	1.8	0.137	0.6	7.8	0.7	0.181	4.3	9.6	6.1
[9-14]	**GLDIQK**	337.1979	2	0.102	1.0	6.6	1.8	0.112	0.9	7.6	0.5	0.137	2.2	8.9	5.7
[15-40]	**VAGTWYSLAMAASDISLLDAQSAPLR**	903.1306	3	0.009	1.6	8.1	1.8	0.0003	14	8.9	0.7	0.018	10	13.3	7.5
[41-60]	**VYVEELKPTPEGDLEILLQK**	771.7582	3	0.135	0.6	7.1	1.8	0.140	1.8	8.1	0.7	0.106	8.5	10.3	6.3
[61-69]	**WENGECAQK**	561.2379	2	0.002	4.9	7.0	1.7	0.007	12	8.0	0.7	0.010	15	10.0	6.2
[71-75]	**IIAEK**	287.1842	2	0.071	0.7	6.4	1.8	0.084	3.4	7.5	0.6	0.085	4.3	8.4	5.6
[76-77]	**TK**	248.1605	1	0.015	8.0	5.4	1.7	0.012	5.8	6.8	0.4	0.006	11	6.6	4.8
[78-83]	**IPAVFK**	337.7157	2	0.089	0.8	6.5	1.7	0.102	1.4	7.5	0.6	0.067	6.6	8.7	5.7
[84-91]	**IDALNENK**	458.7406	2	0.085	0.8	6.9	1.7	0.099	2.0	8.0	0.7	0.122	6.1	9.9	6.1
[92-100]	**VLVLDTDYK**	533.2953	2	0.115	0.9	7.1	1.8	0.088	1.6	8.1	0.7	0.028	4.2	10.3	6.3
[102-124]	**YLLFCMENSAEPEQSLACQCLVR**	940.0941	3	0.002	7.3	8.1	1.8	0.000	9.8	8.9	1.3	0.009	7.8	13.3	7.6
[125-135]	**TPEVDDEALEK**	623.2962	2	0.098	0.1	7.3	1.8	0.047	3.7	8.3	0.6	0.047	6.0	11.0	6.5
[136-138]	**FDK**	409.2082	1	0.016	6.5	6.1	1.8	0.006	4.3	7.2	0.6	0.005	9.9	7.7	5.2
[139-141]	**ALK**	331.2340	1	0.018	4.4	5.8	1.6	0.018	4.1	6.9	0.6	0.015	7.1	7.0	5.0
[142-148]	**ALPMHIR**	419.2421	2	0.043	4.2	5.8	1.8	0.038	4.9	6.9	0.6	0.048	10	7.0	5.0
[149-162]	**LSFNPTQLEEQCHI**	858.4068	2	0.100	2.7	7.4	1.7	0.108	5.5	8.4	0.8	0.116	3.6	11.2	6.7

a) The relative peak areas were obtained dividing the raw peak area of the peptide by the total sum of raw peak areas of the detected peptides.

Table 2. Sequence of detected peptides, sequence coverage and identified proteins for the off-line digestion with free trypsin and CE-MS analysis and for the on-line digestion and analysis by IMER-CE-MS of the *E. Coli* cell lysate (1000 µg mL^{-1}). Single amino acids were not taken into account.

Identified protein	Off-line digestion with free trypsin and CE-MS			On-line IMER-CE-MS digestion		
	Detected peptides		Coverage	Detected peptides		Coverage
α-lactalbumin M_r ~14,200	[2-5] QFTK	[109-114] ALCTEK	8%	[2-5] QFTK	[109-114] ALCTEK	8%
Myoglobin C M_r ~17,000	[43-45] FDK	[48-50] HLK	4%	[43-45] FDK	[48-50] HLK	4%
Cathepsin D M_r ~38,000	[110-112] VER	[278-281] LGGK	17%	[110-112] VER		7%
	[113-120] QVFGEATK	[282-284] GYK		[246-249] ELQK		
	[131-141] FDGILGMAYPR	[285-293] LSPEDYTLK		[282-284] GYK		
	[190-192] YYK	[294-299] VSQAGK		[285-293] LSPEDYTLK		
	[246-249] ELQK	[340-347] VGFAEAAR		[294-299] VSQAGK		
Creatine kinase-MM M_r ~43,000	[1-9] MPFGNTHNK	[149-151] GER	21%	[26-32] HNNHMAK	[267-292] AGHPFMWNQHLGYVLTCPSNLGTGLR	17%
	[26-32] HNNHMAK	[173-177] YYPLK		[42-43] LR	[315-316] LR	
	[42-43] LR	[267-292] AGHPFMWNQHLGYVLTCPSNLGTGLR		[44-45] DK	[317-319] LQK	
	[44-45] DK	[315-316] LR		[131-132] VR	[359-365] LMVEMEK	
	[131-132] VR	[317-319] LQK		[133-135] TGR	[367-369] LEK	
	[133-135] TGR	[359-365] LMVEMEK		[136-138] SIK		
	[136-138] SIK	[367-369] LEK		[149-151] GER		
Antithrombin III M_r ~49,200	[14-24] DIPMNPMCIYR	[184-188] AAINK	19%	[48-53] VWELSK		7%
	[48-53] VWELSK	[223-226] GLWK		[54-57] ANSR		
	[54-57] ANSR	[237-241] ELFYK		[140-145] LVSANR		
	[140-145] LVSANR	[263-275] VAEGTQVLELPFK		[146-150] LFGDK		
	[146-150] LFGDK	[351-359] LPGIVAGR		[223-226] GLWK		
	[177-183] ENAEQSR	[394-399] SLNPNR		[394-399] SLNPNR		

Supporting Information

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Table S-1. On-line IMER-CE-MS digestion and analysis at 37 ºC of α-CSN1 (1000 µg mL^{-1} of α-CSN). 33

Table S-2. On-line IMER-CE-MS digestion and analysis at 37 ºC of α-CSN2 (1000 µg mL^{-1} of α-CSN). 34

Table S-3. On-line IMER-CE-MS digestion and analysis at 37 ºC of β -CSN (1000 µg mL^{-1}). 35

Table S-4. On-line IMER-CE-MS digestion and analysis at 37 ºC of κ-CSN (1000 µg mL^{-1}). 36

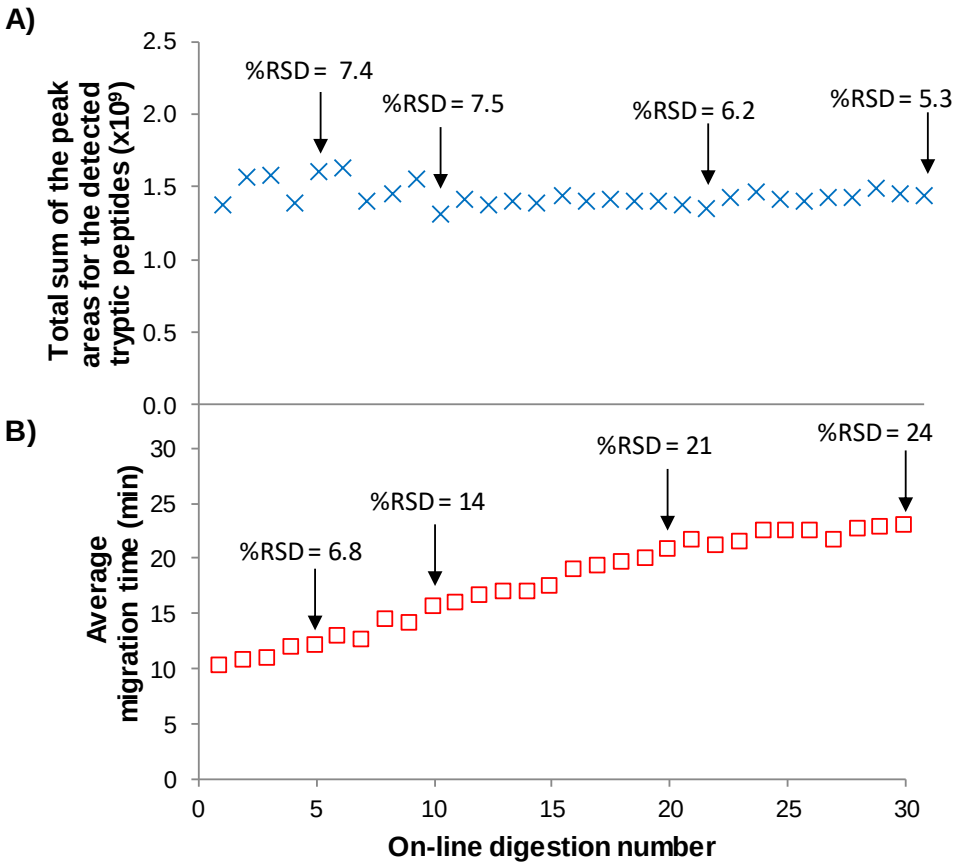

Figure S-1. Reusability of a microreactor for consecutive protein digestion and analysis of β-LG (1000 µg mL^{-1}) under the optimized conditions (37 ºC of digestion-separation temperatures and 5 mbar, 600 s of contact time**). (A)** Total sum of peak areas, and **(B)** average migration time for the detected tryptic peptides. Average %RSD values for the analysis n= 5, 10, 20 and 30 appear as insets.

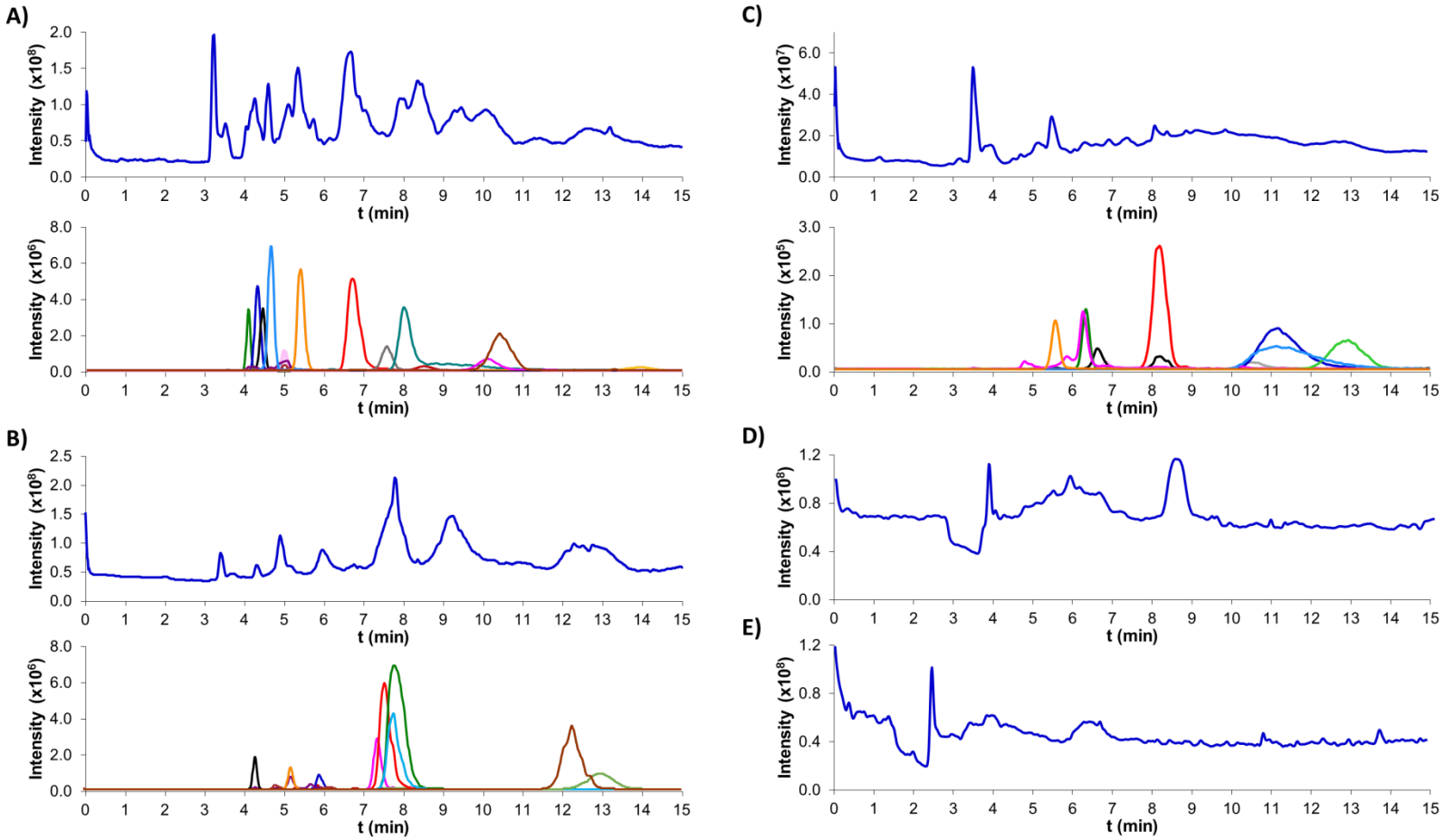

Figure S-2. Total ion electropherograms (TIEs) and extracted ion electropherograms (EIEs) for the on-line IMER-CE-MS digestion and analysis at 37 ºC of **(A)** α-CSN1, **(B)** β-CSN and **(C)** κ-CSN (1000 µg mL $^{-1}$ all). TIEs of an *E. coli* cell lysate (1000 µg mL $^{-1}$) **(D)** for the off-line digestion with free trypsin and CE-MS separation at 37 ºC and **(E)** for the on-line IMER-CE-MS digestion-separation at 37 ºC.

Table S-1. Relative peak areas and percentage of relative standard deviations (%RSD, n=3) of the peptides detected by the on-line IMER-CE-MS digestion and analysis at 37 ºC of α-CSN1 (1000 µg mL^{-1} of α-CSN). Single amino acids were not taken into account.

Peptide sequence		$[M+nH]^{n+}$		Relative peak area [a]	
		m/z	n	Mean	% RSD
[1-3]	RPK	200.6373	2	0.029	9.9
[4-7]	HPIK	247.6582	2	0.093	5.4
[8-22]	HQGLPQEVLNENLLR	587.3202	3	0.203	1.7
[23-34]	FFVAPFPEVFGK	462.2486	3	0.013	4.4
[35-36]	EK	276.1554	1	0.014	1.2
[37-42]	VNELSK	385.1785	2	0.004	4.5
[43-58]	DIGSESTEDQAMEDIK	964.3497	2	0.017	3.5
[59-79]	QMEAESISSSEEIVPNSVEQK	-	-	-	-
[80-83]	HIQK	263.1611	2	0.093	5.9
[84-90]	EDVPSER	416.1961	2	0.076	2.6
[91-100]	YLGYLEQLLR	634.3562	2	0.148	5.0
[101-102]	LK	260.1969	1	0.004	4.3
[104-105]	YK	310.1761	1	0.013	4.1
[106-119]	VPQLEIVPNSAEER	830.9010	2	0.033	6.3
[120-124]	LHSMK	308.1681	2	0.093	4.5
[125-132]	EGIHAQQK	304.1633	3	0.069	2.7
[133-151]	EPMIGVNQELAYFYPELFR	772.7175	3	0.029	2.6
[152-193]	QFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEK	1572.7295	3	0.001	4.2
[194-199]	TTMPLW	748.3698	1	0.068	2.4

a) The relative peak areas were obtained dividing the raw peak area of the peptide by the total sum of raw peak areas of the detected peptides.

Table S-2. Relative peak areas and percentage of relative standard deviations (%RSD, n=3) of the peptides detected by the on-line IMER-CE-MS digestion and analysis at 37 ºC of α-CSN2 (1000 µg mL^{-1} of α-CSN). Single amino acids were not taken into account.

Peptide sequence		$[M+nH]^{n+}$		Relative peak area [a]	
		m/z	n	Mean	% RSD
[2-21]	**NTMEHVSSSEESIISQETYK**	873.6402	3	0.003	8.2
[22-24]	**QEK**	404.2140	1	0.004	9.9
[25-32]	**NMAINPSK**	477.7096	2	0.001	35
[33-41]	**ENLCSTFCK**	522.7284	2	0.002	2.7
[42-45]	**EVVR**	251.6531	2	0.028	5.3
[46-70]	**NANEEEYSIGSSSEESAEVATEEVK**	-	-	-	-
[71-76]	**ITVDDK**	345.6873	2	0.250	1.7
[77-80]	**HYQK**	288.1507	2	0.034	4.9
[81-91]	**ALNEINQFYQK**	684.3516	2	0.050	1.7
[92-113]	**FPQYLQYLYQGPIVLNPWDQVK**	903.8077	3	0.007	4.1
[115-125]	**NAVPITPTLNR**	598.3436	2	0.058	4.8
[126-136]	**EQLSTSEENSK**	-	-	-	-
[138-149]	**VDMESTEVFTK**	733.8100	2	0.028	5.4
[151-152]	**TK**	248.1605	1	0.023	2.0
[153-158]	**LTEEEK**	748.3723	1	0.165	0.6
[159-160]	**NR**	-	-	-	-
[161-165]	**LNFLK**	317.7001	2	0.077	4.0
[167-170]	**ISQR**	-	-	-	-
[171-173]	**YQK**	438.2347	1	0.007	5.2
[174-181]	**FALPQYLK**	490.2845	2	0.100	1.2
[182-188]	**TVYQHQK**	452.2381	2	0.019	6.4
[189-197]	**AMKPWIQPK**	366.8762	3	0.065	1.9
[198-199]	**TK**	248.1605	1	0.023	2.3
[200-205]	**VIPYVR**	373.7319	2	0.012	2.7
[206-207]	**YL**	295.1652	1	0.044	1.0

a) The relative peak areas were obtained dividing the raw peak area of the peptide by the total sum of raw peak areas of the detected peptides.

Table S-3. Relative peak areas and percentage of relative standard deviations (%RSD, n=3) of the peptides detected by the on-line IMER-CE-MS digestion and analysis at 37 ºC of β-CSN (1000 µg mL^{-1}). Single amino acids were not taken into account.

Peptide sequence		$[M+nH]^{n+}$		Relative peak area [a)]	
		m/z	n	Mean	%RSD
[2-25]	ELEELNVPGEIVESLSSSEESITR	-	-	-	-
[26-28]	INK	187.6238	3	0.014	2.1
[30-32]	IEK	389.2395	2	0.004	3.9
[33-48]	FQSEEQQQTEDELQDK	687.9481	1	0.005	3.6
[49-97]	IHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK	1772.9564	2	0.000	22
[98-99]	VK	246.1812	1	0.016	3.0
[100-105]	EAMAPK	323.6654	2	0.078	2.6
[106-107]	HK	284.1717	2	0.012	10
[108-113]	EMPFPK	374.6888	2	0.176	1.0
[114-169]	YPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK	2120.4239	1	0.000	30
[170-176]	VLPVPQK	390.7528	3	0.338	1.4
[177-183]	AVPYPQR	415.7299	2	0.146	0.3
[184-202]	DMPIQAFLLYQEPVLGPVR	729.3945	1	0.090	9.1
[203-209]	GPFPIIV	742.4498	1	0.121	2.8

a) The relative peak areas were obtained dividing the raw peak area of the peptide by the total sum of raw peak areas of the detected peptides.

Table S-4. Relative peak areas and percentage of relative standard deviation (%RSD, n=3) of the peptides detected by the on-line IMER-CE-MS digestion and analysis at 37 ºC of κ-CSN (1000 μg mL^{-1}). Single amino acids were not taken into account.

Peptide sequence		$[M+nH]^{n+}$		Relative peak area [a]	
		m/z	n	Mean	%RSD
[1-10]	**QEQNQEQPIR**	635.3130	2	0.007	66
[11-13]	**CEK**	436.1646	2	0.009	22
[14-16]	**DER**	419.1885	3	0.028	25
[17-21]	**FFSDK**	322.1582	3	0.293	19
[22-24]	**IAK**	331.2340	2	0.063	2.8
[25-34]	**YIPIQYVLSR**	626.3587	1	0.219	15
[35-68]	**YPSYGLNYYQQKPVALINNQFLPYPYYAKPAAVR**	1337.3599	2	0.002	69
[69-86]	**SPAQILQWQVLSNTVPAK**	660.7023	1	0.130	16
[87-97]	**SCQAQPTTMAR**	625.7740	2	0.191	6.9
[98-111]	**HPHPHLSFMAIPPK**	536.9542	2	0.058	6.6
[113-116]	**NQDK**	-	-	-	-
[117-169]	**IPTINTIASGEPTSTPTTEAVESTVATLEDSPEVIESPPEINTVQVTSTAV**	-	-	-	-

a) The relative peak areas were obtained dividing the raw peak area of the peptide by the total sum of raw peak areas of the detected peptides.