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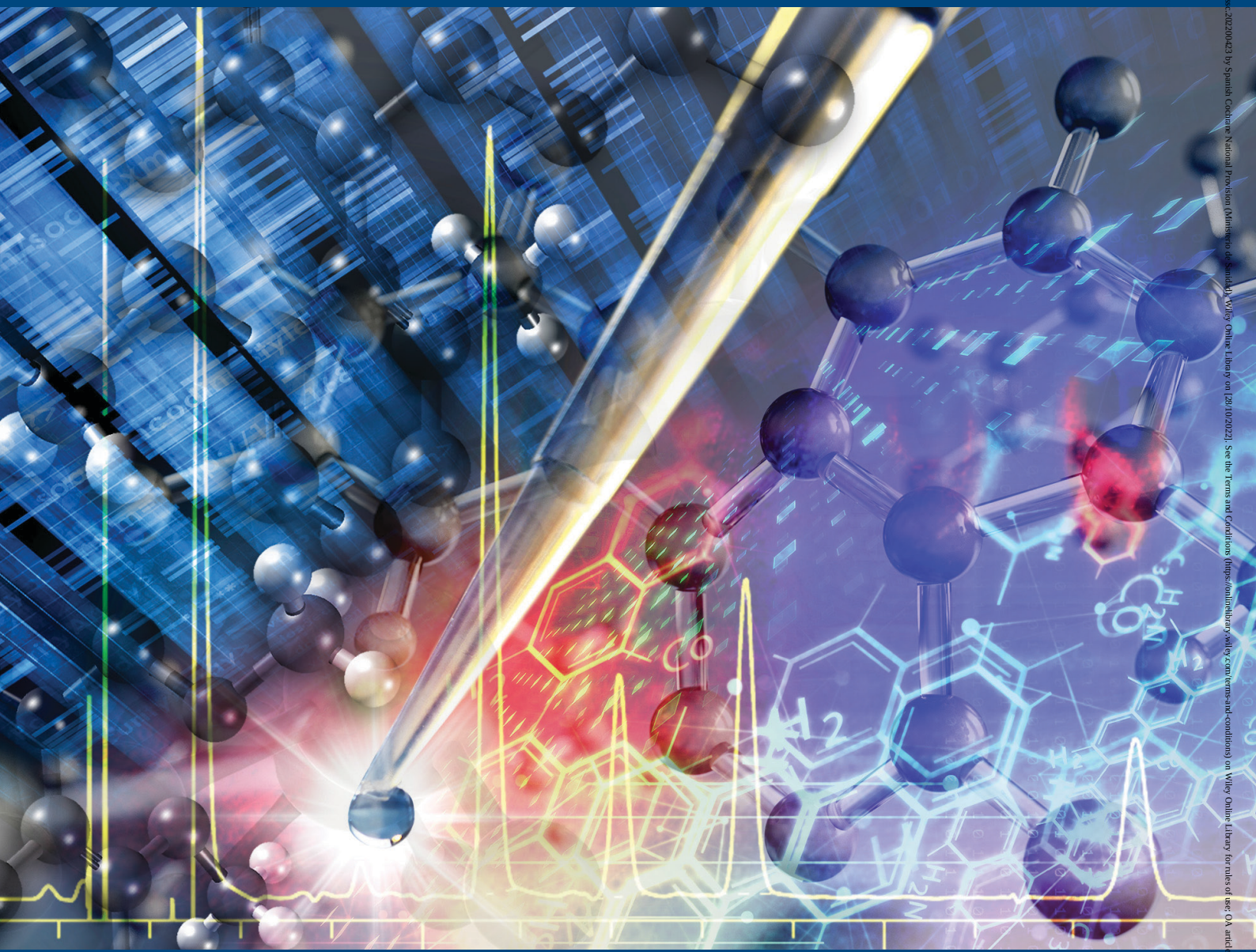

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RESEARCH ARTICLE

Separation and characterization of bovine milk proteins by capillary electrophoresis-mass spectrometry

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Protein profiling of major bovine milk proteins (i.e., whey and casein proteins) is of great interest in food science and technology. This complex set of protein proteoforms may vary with breed, genetics, lactation stage, health, and nutritional status of the animal. Current routine methods for bovine milk protein profiling at the intact level are typically based on capillary electrophoresis-ultraviolet, which does not allow confirming unequivocally the identity of the separated proteins. As an alternative, in this study, we describe for the first time a novel and simple capillary electrophoresis-mass spectrometry method in positive electrospray ionization mode. Under the optimized conditions, capillary electrophoresis-mass spectrometry allowed the separation and identification at the intact level of major bovine milk whey and casein proteins in less than 15 min. Furthermore, high-resolution mass spectrometry confirmed its importance in the reliable characterization of bovine milk protein proteoforms, especially those with slight molecular mass differences, such as β-casein A1 and A2, which are relevant to unequivocally identify milk with specific β-casein compositions (e.g., A2A2 milk, which is widely known as A2 milk). This differentiation was not possible by matrix-assisted laser desorption/ionization mass spectrometry, which provided rapidly and easily a rich but less accurate fingerprint of bovine milk proteins due to the lower mass resolution.

KEYWORDS
capillary electrophoresis, food analysis, mass spectrometry, milk proteins, proteoforms

1 | INTRODUCTION

Bovine milk is an important source of proteins in human nutrition. Caseins (CNs) and whey proteins account for 80% (m/m) and 20% (m/m) of the total protein content in bovine milk (3%–3.5% [m/m] protein), respectively [1]. Both conform to a complex set of protein variants or proteoforms [2], which in addition to the nutritional importance, may have an impact on the technological, digestive,

Article Related Abbreviations: αLA, α-lactalbumin; α-s0-CN-B, α-S0-casein B; α-s1-CN-B, α-S1-casein B; BCM-7, β-casomorphin 7; βCN-A1, β-casein A1; βCN-A2, β-casein A2; βLG-A, β-lactoglobulin A; βLG-B, β-lactoglobulin B; DTT, dithiothreitol; EIE, extracted ion electropherogram; HAc, acetic acid; HEC, hydroxyethyl cellulose; HFor, formic acid; HPC, hydroxypropyl cellulose; κCN-A, κ-casein A; κCN-B, κ-casein B; L_T, total length; MHEC, methylhydroxyethylcellulose; M_r, molecular mass (i.e., relative molecular mass); SA, sinapinic acid; UHT, ultra-high temperature.

and bioactive properties of milk and dairy products [3, 4]. On the one hand, whey proteins are the group of milk proteins that remain soluble after precipitation of CNs at pH 4.6, including mainly β-lactoglobulins, α-lactalbumin, serum albumin, immunoglobulins, and proteose-peptone fragments [3]. On the other hand, CNs are organized as micelles and submicelles in four different types (α-s1, α-s2, β, and κ), and βCNs account for more than one-third, with 13 genetic variants identified to date (A1, A2, A3, B, C, D, E, F, G, H1, H2, I, and G) [3, 4]. Indeed, bovine milk genetic variants vary with breed, but more widely the complex set of proteoforms may also change with lactation stage, health and nutritional status of the animal, and type of technological treatments [3–5]. Therefore, protein profiling of bovine milk proteins is of great interest in food science and technology.

Different methods have been described for the analysis at the intact level of bovine milk proteins [6, 7], including gel electrophoresis, isoelectric focusing, LC, and CE. Current routine methods are based on CE-UV [1, 8–12] because due to the excellent resolving power, it provides a more detailed protein profile than LC-UV. The most typically applied CE-UV methods share several common features [1, 8, 9]. Prior to the analysis, casein micelles are typically disrupted under reducing and denaturing conditions provided by dithiothreitol (DTT) and urea. Later, the addition of urea to the BGE avoids micelle reassembling during the electrophoretic separation, which is typically performed at a low pH value (i.e., 2.5–3.0). Furthermore, other additives (i.e. hydrophilic polymers) and the use of hydrophilic coated or deactivated capillaries are necessary to prevent protein adsorption on the inner capillary wall. In such conditions, the electroosmotic flow is practically totally suppressed, and increasing temperature until 45°C helps decreasing the BGE viscosity and migration times while improving protein peak shape [1, 8, 9]. These CE-UV methods are simple to implement but do not allow confirming unequivocally the identity of the proteins in the complex electrophoretic profiles, which are mainly tentatively identified by analyzing purified individual protein standards and spiking experiments. This limitation is overcome by using MS detection.

Different MS-based techniques have been applied to bovine milk protein profiling [6], including MALDI-MS [13–15] and LC-MS [16]. However, to the best of our knowledge, CE-MS has been focused only on the target analysis of specific bovine proteins, such as the whey proteins α-lactalbumin (αLA) and β-lactoglobulin (βLG), to investigate milk adulteration [17]. Therefore, this study is aimed to describe for the first time a CE-MS method to obtain reliable global fingerprints from bovine milk proteins. To obtain high mass accuracies and resolution, high-resolution MS detection is necessary. The results with the novel CE-MS method, are compared with those that can be obtained by CE-UV, which does not allow an accurate identification, or without prior separation by MALDI-MS, which is typically applied at low mass resolution. CE-MS with high-resolution MS demonstrates a great potential to rapidly obtain accurate fingerprints at the intact level of bovine milk proteins, which may be relevant in quality control and authentication of milk and dairy products, as well as in the development of novel foodstuff.

2 | MATERIALS AND METHODS

2.1 | Chemicals and samples

All the chemicals used in the preparation of buffers and solutions were of analytical reagent grade. propan-2-ol (≥99.9%), methanol (≥99.9%), acetone (99.8%), acetic acid (HAc, glacial), formic acid (HFor, 99.0%), TFA (99.0%), hydrochloric acid (37% [v/v]), sinapinic acid (SA, ≥99.0%), sodium hydroxide (≥99.0%, pellets), potassium hydroxide (≥95.0%, pellets), sodium citrate dihydrate (≥99.0%), citric acid (99.5%), urea (99.0%–100.5%), DL-DTT (97%), hydroxyethyl cellulose (HEC, relative molecular mass (M_r) of 250 000), hydroxypropyl cellulose (HPC, 100 000 M_r), β-casein (βCN, ≥98%), β-lactoglobulin (βLG, ≥90%), α-casein (αCN, ≥70%), k-casein (κCN, ≥70%), β-lactoglobulin A (βLG-A, ≥90%), and β-lactoglobulin B (βLG-B, ≥90%) from bovine milk were supplied by Merck (Darmstadt, Germany). ACN (LC-MS grade) and water (LC-MS grade) were provided by PanReac Applichem (Barcelona, Spain). Conventional ultra-high temperature (UHT) skim (ten different brands) and semi-skim milk (1 brand), as well as pasteurized semi-skim milk that was supposed to have a higher content of β-casein A2 (βCN-A2) (one brand), were produced in Spain and acquired in a local market of Barcelona.

2.2 | Reduction buffer, background electrolyte, and sheath liquid solutions

The CE-UV BGE was prepared as described by N. De Jong et al. [1], substituting methylhydroxyethylcellulose (30 000 M_r) with HEC (250 000 M_r). Sodium citrate dihydrate 10 mM (147 mg) and HEC 0.05% (m/v) (25 mg) were mixed with 37.5 mL of 8 M urea in a 50 mL volumetric flask (i.e. Final urea concentration was 6 M). Then, the pH was adjusted to 2.5 with 2.5 M citric acid solution and the volume was made up with water. The reduction

buffer for sample preparation did not contain HEC and was prepared in a similar way, but with DTT (38 mg), less amount of sodium citrate dihydrate (73 mg), and adjusting the pH to 8.0 with sodium hydroxide solution. The CE-MS BGE contained 2 M HAc (pH 2.2) [18, 19]. The sheath liquid solution consisted of a hydroorganic mixture of 60:40 (v/v) propan-2-ol:water with 0.05% (v/v) of HFor. Both BGEs were passed through 0.20 μm nylon filters (Macherey-Nagel, Düren, Germany) and the sheath liquid was degassed for 10 min by sonication before the analyses. pH measurements were made with a Crison 2002 potentiometer and a Crison electrode 52-03 (Crison Instruments, Barcelona, Spain).

2.3 | Sample preparation

Semi-skim milk was defatted as follows: an aliquot of 1.5 mL was centrifuged at 14 500 × g and 4°C for 30 min in a cooled Rotanta 460 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), and a 500 μL volume was collected from the middle of the tube. Centrifugation was repeated for 15 min and 300 μL of skim milk was collected from the bottom of the tube for further sample pretreatment.

Skim milk (1 mL) was diluted with reduction buffer (5 mL) and incubated for 1 h at room temperature. For CE-UV analysis, the resulting solution from protein extraction was filtered through 0.20 μm nylon filters. The filtered solution was further desalted using MF-Millipore membrane filters (Merck) before MALDI-MS analysis. Briefly, 10 μL of sample solution and 2 μL of ACN were deposited onto the membrane filter and dialyzed with water for 45 min at room temperature. For CE-MS analysis, the resulting solution from protein extraction was filtered through 3000 M_r cutoff cellulose acetate centrifugal filters (Amicon Ultra-0.5; Millipore, Bedford, MA, USA) as described in our previous studies [20]. All centrifugation steps were performed at 11 000 × g and 25°C in the cooled Rotanta 460 centrifuge. First, the filter was washed with 500 μL of water for 15 min and the filtrate was discarded. Then, 500 μL of the sample was centrifuged for 10 min. The filtration residue was washed two times for 10 min with 100 μL of water, and the last time for 10 min with 50 μL of water. The residue was recovered by inverting the upper reservoir in a vial and spinning once more at a reduced centrifugal force (2 min at 300 x g). Finally, water was added to a final volume of 100 μL. The desalted sample was mixed with CE-MS BGE 1:1 (v/v) and the supernatant collected after centrifugation (11 000 × g, 2 min and 25°C) was analyzed. All desalted samples were stored at -20°C when not in use.

2.4 | Capillary electrophoresis (CE)

All CE experiments were performed in hydrophilic capillaries coated with HPC. HPC coating was prepared in 50 or 75 μm id × 365 μm od fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) by stabilization of the coating layer at high temperature. The preparation method was adapted from the literature [21, 22]. A 5% (m/v) solution of HPC was prepared by sonicating and heating the mixture for 1 h at 40°C. The solution was kept under magnetic agitation at room temperature overnight to eliminate air bubbles. Solvents and solutions were flushed at 930 mbar in the CE-UV instrument (see below). At room temperature, a piece of 100 cm of fused silica capillary was flushed with methanol (10 min), 1 M KOH (10 min), water (10 min), 1 M HCl (10 min) and 5% (m/v) HPC solution (1 h). A kitasato connected to an N_2 source and closed with a rubber cup blocked with a clamp was used to flush N_2 through the capillary at 1.5 bar. Before capping the kitasato, a vial empty was placed inside to hold the inlet end of the capillary. The outlet end was passed through the rubber cup using a needle as a guide to avoiding clogging and breakage. Most of the capillary was standing outside of the kitasato to be placed inside of a home convection oven (Moulinex OX4448 Optimo, Groupe SEB, Lyon, France), which was conveniently modified with two small holes drilled in the metal casing, in the opposite side to the electric controls. The outlet capillary end was passed through one of the holes to introduce the capillary inside the oven. Then, the capillary was coiled and placed in the oven tray. Finally, the outlet end was passed through the other hole to leave it outside of the oven in a waste vial (filled always with water to prevent clogging). N_2 was flushed for 60 min or until bubbles were visibly exiting the outlet end (the outlet end was cut if clogged [0.5 cm]). After 10 min bubbling up, the oven temperature was set to 140°C for 60 min, maintaining the N_2 flow. The temperature was externally monitored during the whole preparation with a K-type thermocouple thermometer (Proster PST095; Shenzen, China). Temperatures above 160°C should be prevented to avoid polymer degradation. Once the coating from one of the ends was completed, the capillary was reversed, flushed with 5% (m/v) HPC solution (1 h), and the N_2 flushing and thermal stabilization procedure was repeated to coat from the other end. The capillary parts that were outside the oven were cut, and the capillary was cut to the desired total length (L_T). Under optimized conditions 50 μm id × 58 cm L_T and 75 μm id × 58 cm L_T capillaries were used in CE-UV and CE-MS, respectively. Polyimide external coating was removed from the capillaries using a scalpel to prepare the UV window at 8.5 cm from the outlet end or 2 cm of

the outlet end for CE-MS, to prevent HPC internal coating damage.

2.4.1 | CE-ultraviolet (CE-UV)

CE-UV experiments were conducted at 45°C in a 7100 CE (Agilent Technologies, Waldbronn, Germany) with an ultraviolet absorption diode-array detector (UV spectra were acquired between 200 and 400 nm). All capillary flushes were performed at 930 mbar. Capillaries were flushed with water (2 min), 1 M HCl (2 min), water (2 min), 1 M NaOH (2 min), water (2 min), and BGE (2 min). The capillary was finally equilibrated by applying 30 kV of separation voltage for 20 min (normal polarity, cathode in the outlet). Samples were injected at 50 mbar for 10 s. Between runs, capillaries were conditioned by rinsing with 1 M NaOH (2 min), water (2 min), and BGE (2 min). The capillary was filled with water for short (e.g., hours) and long storage (e.g., overnight) to prevent coating damage and avoid salt buildup in the capillary, prepunchers, and electrodes. Both ends of the capillary were submerged in vials with water during the storage.

2.4.2 | CE-Mass spectrometry (CE-MS)

CE-MS experiments were performed at 25°C in a 7100 CE coupled with an orthogonal G1603A sheath-flow interface to a 6220 oaTOF LC/MS spectrometer (Agilent Technologies). The sheath liquid was delivered at a flow rate of 3.3 μL/min by a KD Scientific 100 series infusion pump (Holliston, MA, USA). The mass spectrometer was operated under optimum conditions in positive ESI mode using the following parameters: capillary voltage 4000 V, drying gas temperature 300°C, drying gas flow rate 4 L/min, nebulizer gas 7 psig, fragmentor voltage 325 V, skimmer voltage 80 V, and OCT 1 RF Vpp voltage 300 V. Data were collected in profile at 1 spectrum/s between 100 and 3200 *m/z*. ChemStation C.01.06 software (Agilent Technologies) was used for CE control and data acquisition and was run in combination with MassHunter B.04.00 workstation software (Agilent Technologies) for control, data acquisition, and analysis of the mass spectrometer.

All capillary flushes were performed at 930 mbar. Samples were injected at 50 mbar for 10 s, sandwiched between two plugs of water (20 s at 50 mbar) to prevent protein precipitation and capillary irreversible blockage. Analyses were carried out under normal polarity. A separation voltage of 15 kV was employed for the electrophoretic separations. Between runs, the capillary was flushed with water (3 min) and BGE (3 min). The capillary was filled with water for long storage (e.g. overnight). The inlet end of the capillary was submerged in a vial with water during the storage.

2.4.3 | MALDI-mass spectrometry (MALDI-MS)

MALDI-MS mass spectra were obtained using a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems, Waltham, MA, USA) equipped with a nitrogen laser (355 nm) and a microchannel plate detector (MCP). Laser intensity was set to 7900 (100%). Mass spectra were acquired over a range of 5000–30 000 *m/z* using the mid-mass positive mode. Data acquisition and data processing were performed using the 4000 Series Explorer and Data Explorer software (Applied Biosystems). Sample-matrix mixtures were freshly prepared before the analysis by depositing onto the stainless steel MALDI plate 1 μL of SA in 99:1 (v/v) acetone:water (final concentration 27 mg/mL), 1 μL of sample solution (allow drying for 15 min) and 1 μL of SA in 50:50 (v/v) ACN:water with 0.1% (v/v) of TFA (final concentration 10 mg/mL) [23].

3 | RESULTS AND DISCUSSION

3.1 | CE-ultraviolet (CE-UV)

Current CE-UV methods applied for the analysis of bovine milk proteins were established almost 30 years ago and some of the reagents are no longer straightforward available. This is the case of the method based on a citrate BGE at pH 2.5 and 6 M urea described by N. de Jong et al [1, 8], which requires the addition of 0.05% (m/v) of methylhydroxyethylcellulose (30 000 M_r) and the use of a commercial hydrophilic coated capillary (Supelco CElect Pl), or a fused-silica deactivated capillary (BGB Analytik OV-1701-OH), in the modification subsequently proposed [8, 9]. For this reason, we introduced some changes in the original method, including the addition of 0.05% (m/v) of HEC (250 000 M_r) and the use of a lab-made hydrophilic HPC coated capillary. Figure 1 shows the CE-UV electropherogram obtained for a UHT skim milk sample, which was very similar to those obtained for other 9 different commercial milk brands (data not shown). As can be observed, under these novel conditions a characteristic protein fingerprint can be obtained in less than 45 min. Spiking experiments with protein standards and comparison with the original method allowed the tentative assignment of the peaks corresponding to αLA, βLG-A, βLG-B, α-S1-casein B (α-s1-CN-B), α-S0-casein B (α-s0-CN-B), κ-casein A (κCN-A), κ-casein B (κCN-B), β-casein A1 (βCN-A1), and βCN-A2. Results were repeatable in terms

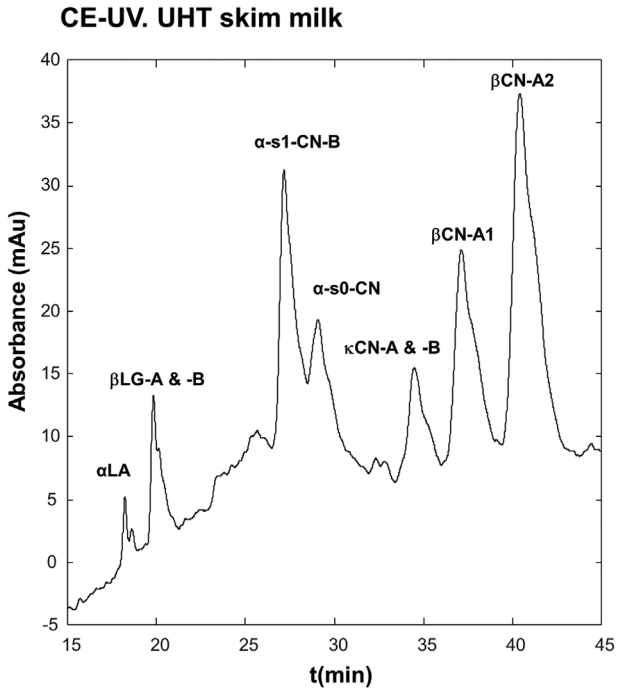

FIGURE 1 CE-UV electropherogram at 214 nm for an ultra-high temperature (UHT) skim milk sample. Hydroxypropyl cellulose (HPC) coated capillary (58 cm L_T × 50 μm id × 365 μm od, 49.5 cm effective length). BGE: sodium citrate 10 mM, 0.05% (m/v) hydroxyethyl cellulose (HEC), 6 M urea, adjusted to pH 2.5 with citric acid. Sample injection: 10 s at 50 mbar. Temperature 45°C and voltage 30 kV

of migration times and peak areas with relative standard deviation in percentage (%RSD, $n = 3$) of: 1.0 and 14.6% (αLA), 1.1 and 4.4% (βLG-A + βLG-B), 1.7 and 9.4% (α-s1-CN-B + α-s0-CN-B), 2.5 and 8.0% (κCN-A + κCN-B), 2.8 and 14.6% (βCN-A1) and 3.1 and 13.9% (βCN-A2), respectively. Unfortunately, the typical BGEs applied for protein profiling of milk by CE-UV, as they present non-volatile salts and additives, are not compatible with on-line ESI-MS detection, which is required for more reliable identification of the separated proteins. Another major drawback of these methods is that the presence of urea in the BGE requires intensive instrument maintenance, to clean the unavoidable salt deposits formed during sequences of analyses.

3.2 | CE-mass spectrometry

Peptides and proteins are typically analyzed by CE-MS at low pH values with BGEs containing HAc or HFor to maximize ionization efficiency, hence sensitivity, with on-line ESI-MS detection in positive ion mode [7, 24, 25]. Intact proteins are usually analyzed with 1 M and 2 M HAc BGEs, as we have shown for barley hordeins [23], transthyretin [20], or erythropoietin and its analogs [19]. However, to the best of our knowledge, these conditions have not been described yet for the analysis of milk proteins. Before CE-MS, it was necessary to desalt and preconcentrate by centrifugal filtration with 3000 M_r cutoff filters the solution resulting from the treatment of milk with reduction buffer. Preliminary experiments with 50 μm id HPC coated capillaries showed improved sensitivity with a 2 M HAc and results were worse at higher and lower HAc concentrations, hence this concentration was selected for milk protein profiling. Results were promising, but even taking the precaution of precipitating part of the proteins in the desalted samples by diluting 1:1 (v/v) with BGE and injecting between two plugs of water, protein precipitation inside the capillary resulted in irreversible capillary blockage after a couple of analyses. This issue was completely solved by using a 75 μm id HPC coated capillary. Figure 2 shows the extracted ion electropherograms (EIEs) of the bovine proteins detected by CE-MS in a UHT skim milk sample, which migrated in less than 15 min and agreed with those observed by CE-UV. Under these conditions, results were even more repeatable than by CE-UV in terms of migration times and peak areas with %RSD (n = 3) of: 2.8% and 2.0% (βCN-A2), 2.8% and 3.6% (βCN-A1), 2.8% and 2.7% (κCN-A), 3.2% and 3.4% (κCN-B), 2.7% and 1.3% (α-s1-CN-B), 3.5% and 3.6% (α-s0-CN-B), 2.4% and 3.6% (βLG-B), 2.7% and 3.9% (βLG-A), 2.4% and 0.3% (αLA), 2.6% and 0.2% (βLG-B with S-S) and 2.7% and 0.2% (βLG-A with S-S), respectively. As an example of the information available for protein identification, Figure 3 shows the experimental and deconvoluted mass spectra obtained for βCN-A1 and βCN-A2. In the experimental mass spectrum (Figure 3A) appear the typical clusters of multiply-charged molecular ions from proteins, which are overlapped for the different proteoforms detected. After deconvolution (Figure 3B), the M_r of these proteoforms is obtained. In this specific case, in addition to βCN-A1 and βCN-A2, which differ only in one amino acid (i.e. His67 vs Pro67, respectively, $\Delta M_r = 40$), proteoforms with lactose adducts from the Maillard reaction were also detected, which are related to heat treatment (i.e. UHT in this case) and storage of milk [16]. Table 1 shows the experimental M_r values for all the detected proteoforms, excluding those from the Maillard reaction. As high-resolution MS measurements were performed, mass accuracy was excellent with ΔM_r of less than 0.7 units between the experimental and theoretical M_r values. The combination of the electrophoretic separation and mass accuracy allowed the reliable characterization of proteoforms with slight molecular mass differences, as shown before in Figures 2 and 3 for βCN-A1 and βCN-A2, which are relevant to unequivocally identify milk with specific βCN compositions, such as novel A2A2 milk (i.e., A2 milk) [4]. βCN-A1 and βCN-A2 are the most common

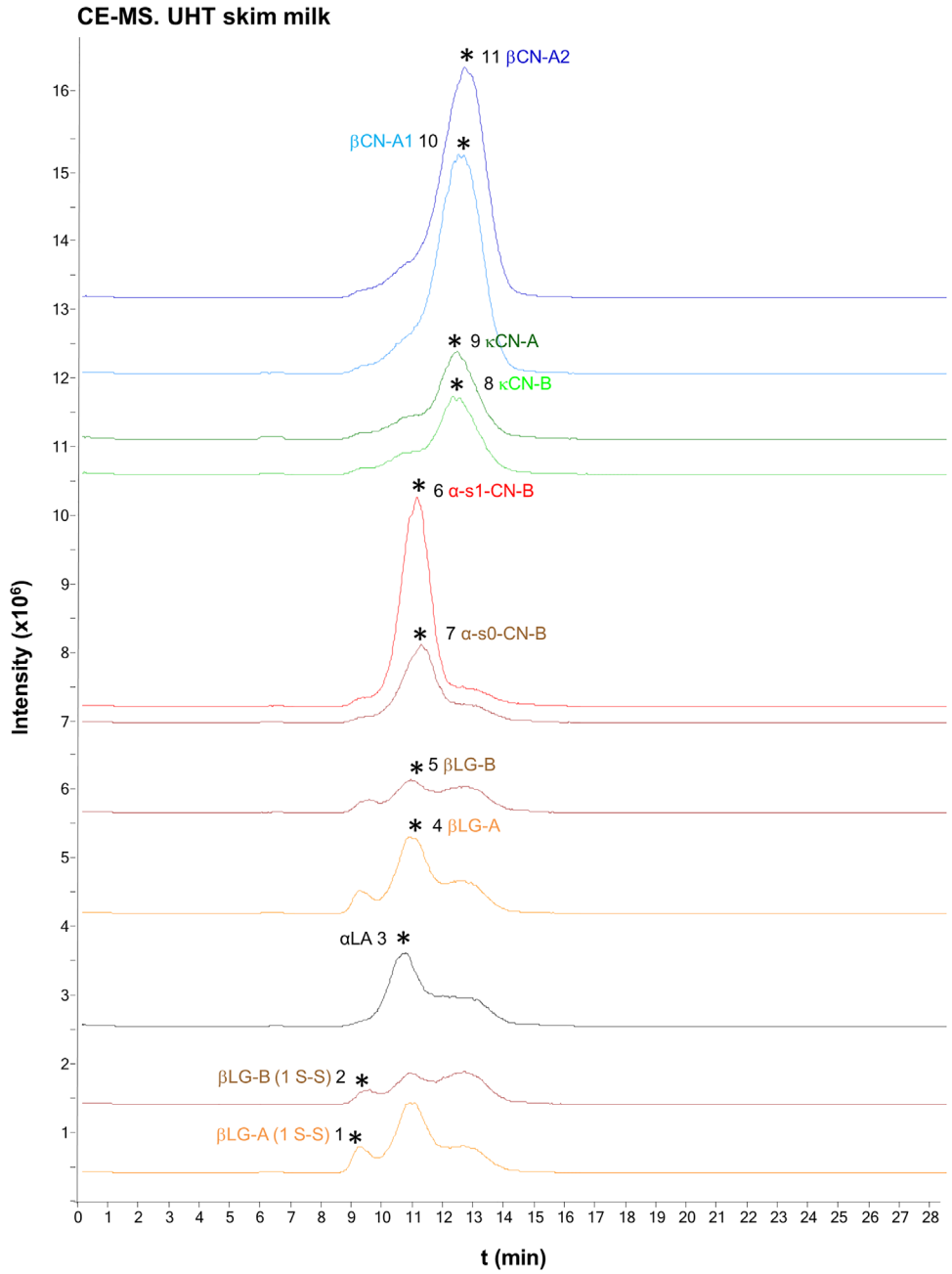

FIGURE 2 Extracted ion electropherograms (EIEs) of the bovine milk proteins detected by CE-MS in an ultra-high temperature (UHT) skim milk sample. Detection of the different proteins in the peaks labeled with an asterisk was confirmed by obtaining the experimental relative molecular mass (M_r) after deconvolution of the mass spectra (experimental M_r values are shown in Table 1). The summed intensity of the *m/z* values of the 5 most intense molecular ions in the mass spectra was considered for each EIE. Hydroxypropyl cellulose (HPC) coated capillary (58 cm × 75 μm id × 365 μm od). BGE: HAc 2 M (pH 2.2). Sample injection: 10 s at 50 mbar (sandwiched between two plugs of water of 20 s at 50 mbar). Temperature 25°C and voltage 15 kV

βCN variants, and A1A2 milk is the most widely commercially available [4]. Fermentation and gastrointestinal digestion of milk proteins yield bioactive peptides, such as β-casomorphin 7 (BCM-7), which is mainly produced from βCN-A1 and βCN-B and has been related, among other health issues, to milk allergy and intolerance [26]. This is the reason why there is interest in such novel A2 milk, which contains only βCN-A2. As an example, supplementary Figure S-1 shows the EIEs and deconvoluted mass spectra of βCN-A1 and βCN-A2 for two semi-skim milk samples, namely a conventional UHT milk and a pasteurized milk that was supposed to have a higher content of βCN-A2. Indeed, a higher relative abundance of βCN-A2 could be observed in the EIEs of the non-conventional

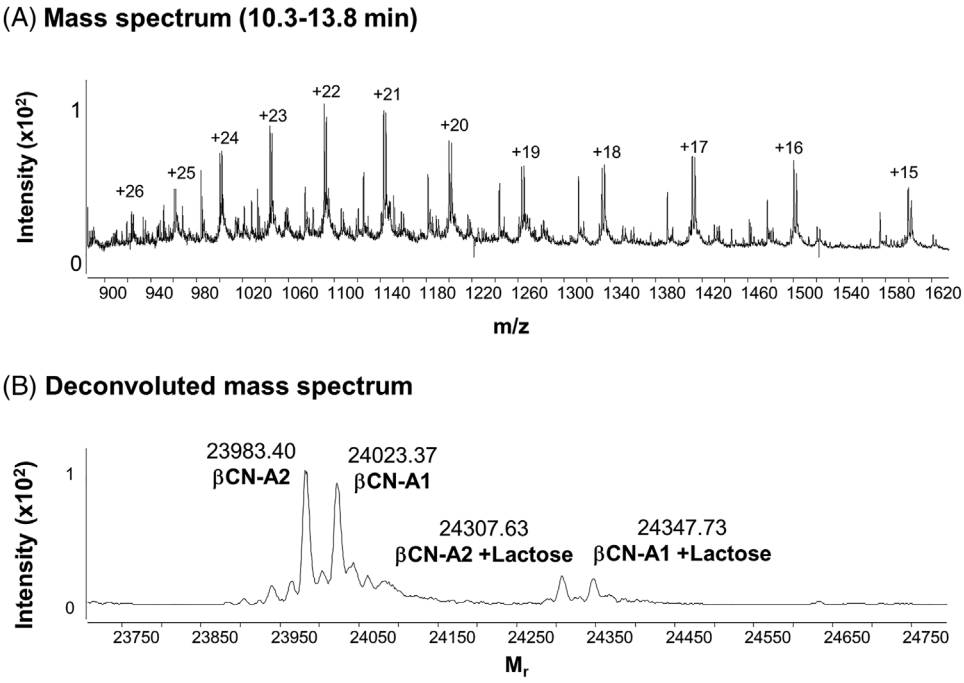

FIGURE 3 a) Experimental and b) deconvoluted mass spectra obtained by CE-MS for the time window of the β-casein A1 (βCN-A1) and β-casein A2 (βCN-A2) peaks in the extracted ion electropherograms (EIEs) of Figure 2

TABLE 1 Mass accuracy in relative molecular mass (M_r) determination for the detected bovine milk proteins by CE-MS

Bovine protein	Theoretical M_r[a]	Experimental M_r	ΔM_r
βLG-A (with S-S)	18 365.13	18 365.29	−0.16
βLG-B (with S-S)	18 279.04	18 279.12	−0.08
βLG-A	18 367.15	18 367.40	−0.25
βLG-B	18 281.06	18 281.04	0.02
αLA	14 185.96	14 185.27	0.69
α-s1-CN-B	23 614.48	23 614.59	−0.11
α-s0-CN-B	23 694.46	23 694.61	−0.15
κCN-A	19 037.18	19 037.75	−0.57
κCN-B	19 005.23	19 005.95	−072
βCN-A1	24 022.97	24 023.37	−0.40
βCN-A2	23 982.95	23 983.40	−0.45
γ2-βCN (106-209-βCN)[b]	11 823.81	11 823.79	0.02

[a]Average molecular mass was calculated by retrieving information from the UniProtKB database (http://www.uniprot.org) using mMass open source software (version 5.5.0, http://www.mmass.org/).

[b]Detected βCN fragment is not shown in the EIEs of CE-MS analysis (Figure 2).

pasteurized milk, but less than expected. Furthermore, lactose adducts were almost undetectable because milk proteins were less prone to Maillard reaction during pasteurization. In addition to βCN-A1 and βCN-A2, among the detected proteins by CE-MS, we find αLA, βLG-A, βLG-B, α-s1-CN-B, α-s0-CN-B, κCN-A, and κCN-B, as well as non-reduced βLG-A and βLG-B or the γ2-β-casein fragment. Different βCN fragments, including γ-βCN and proteose peptone fragments, are supposed to be generated from the proteolysis of βCN by plasmin [16]. Overall, the proposed CE-MS method with high-resolution MS detection allows obtaining a very accurate and reliable fingerprint at the intact level of major bovine milk proteins in less than 15 min, in contrast to CE-UV that lacks molecular mass confirmation. Compared to a recent LC-MS method with high-resolution MS presented for bovine milk protein profiling [16], CE-MS provides the benefits of a rapid and solventless complementary separation at microscale over an RP LC-MS method working with a conventional size column at 0.5 mL·min^{-1} in UHPLC.

3.3 | MALDI-MASS SPECTROMETRY (MALDI-MS)

MALDI-MS was explored as a complement to rapidly and easily obtaining MS-based fingerprints from bovine milk proteins. Figure 4 shows the MALDI-MS mass spectrum for a UHT skim milk sample and Table 2 the experimental M_r calculated from the *m/z* values of the detected singly-charged molecular ions. As expected, Table 2 shows that mass accuracy in the identification was lower than by

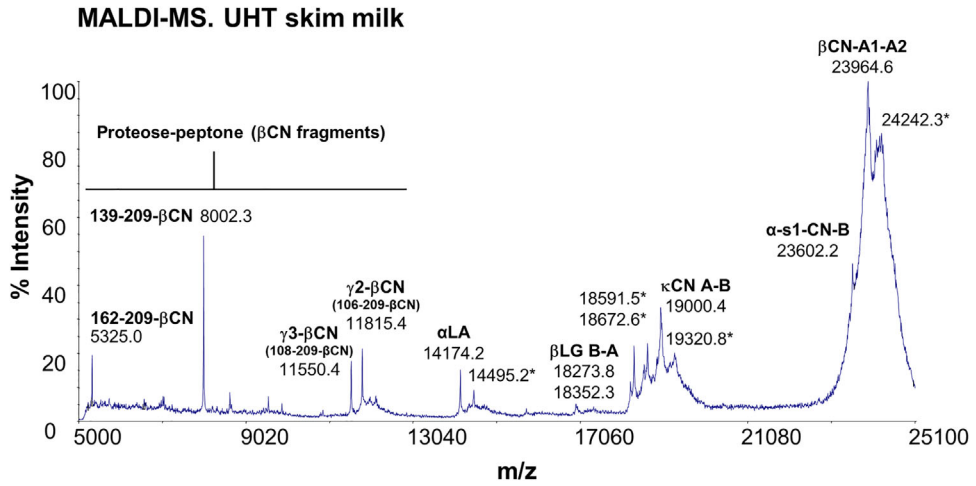

FIGURE 4 MALDI-MS mass spectrum for an ultra-high temperature (UHT) skim milk sample (acquired over a range of 5000–30 000 *m/z* using the mid-mass positive mode. The mass spectrum is shown in the *m/z* range where peaks were detected). Protein *m/z* values labeled with an asterisk correspond to lactose adducts from the Maillard reaction

TABLE 2 Mass accuracy in relative molecular mass (M_r) determination for the detected bovine milk proteins by MALDI-MS

Bovine protein	Theoretical M_r[a]	Experimental M_r	ΔM_r
βLG-A	18 367.2	18 351.3	15.9
βLG-B	18 281.1	18 272.8	8.3
αLA	14 186.0	14 173.2	12.8
α-s1-CN-B[b]	23 614.5	23 601.2	13.3
α-s0-CN-B[b]	23 694.5	23 601.2	93.3
κCN-A[c]	19 037.2	18 999.4	37.8
κCN-B[c]	19 005.2	18 999.4	5.8
βCN-A1[d]	24 023.0	23 963.6	59.4
βCN-A2[d]	23 983.0	23 963.6	19.4
γ2-βCN (106-209-βCN)	11 823.8	11 814.4	9.4
γ3-βCN (108-209-βCN)	11 558.5	11 549.4	9.1
139-209-βCN	8006.5	8001.3	5.2
162-209-βCN	5327.3	5324.0	3.3

[a]Average molecular mass was calculated by retrieving information from the UniProtKB database (http://www.uniprot.org) using mMass open source software (version 5.5.0, http://www.mmass.org/).

[b,c,d]Mass accuracy and resolution were not enough to resolve both proteoforms.

CE-MS with high-resolution MS, and it was not possible to differentiate between closely related variants, which presented similar M_r values, such as α-s1-CN-B and α-s0-CN-B, κCN-A and κCN-B, or βCN-A1 and βCN-A2. In addition to the major proteins, different βCN fragments were clearly detected (Table 2 and Figure 4), as well as lactose adducts from the Maillard reaction (Figure 4). Therefore, the mass spectrum of Figure 4 shows a rich but less accurate protein fingerprint due to the lower mass resolution. Then, MALDI-MS may find an application to solve certain analytical problems dealing with milk proteins, as long as the solution does not require resolving closely related protein variants. For example, current studies dealing with milk protein profiling by MALDI-MS [13–15], typically use the obtained mass spectra as global fingerprints for milk classification and differentiation, and even little importance is given to the identification of the detected proteins.

4 | CONCLUDING REMARKS

In this study, we have proposed a simple CE-MS method to separate and characterize at the intact level major bovine milk proteins in less than 15 min, as an alternative to CE-UV that lacks molecular mass confirmation. The method uses lab-made 75 μm id capillaries permanently coated with HPC and a BGE of 2 M HAc to prevent protein adsorption and achieve the best repeatability and sensitivity in positive ESI-MS. High-resolution MS was demonstrated to be necessary to accurately and reliably identify proteoforms with slight molecular mass differences, including βCN-A1 and -A2. This fine differentiation was not possible at lower mass resolution by MALDI-MS, which provided very informative but less accurate fingerprints of bovine milk proteins. The presented CE-MS method constitutes an excellent and complementary novel tool for quality control and authentication of bovine milk and dairy products, as well as for the development of innovative foodstuff. More widely, it may be applied to the analysis of milk from other species.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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