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Enhanced capillary electrophoresis-mass spectrometry with deactivated fused-silica capillaries for bovine milk protein profiling and A2 milk authentication

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ABSTRACT

Profiling intact bovine milk proteins, including casein and whey proteins, is fundamental in nutrition and food technology. Routine analysis is typically performed using capillary electrophoresis with ultraviolet detection (CE-UV), a well-established technique that, however, does not allow unequivocal identification of the separated proteins. To overcome this limitation, we previously developed a capillary electrophoresis-mass spectrometry (CE-MS) method that enabled both separation and confident identification of major bovine milk proteins. This approach, nevertheless, relied on the preparation of lab-made hydroxypropyl cellulose (HPC)-coated capillaries to prevent protein adsorption on the capillary walls, a procedure that is laborious, time-consuming, and suffers from limited reproducibility between batches unless tightly controlled.

In this study, we present for the first time a CE-MS method employing commercially available OV-1701-OH deactivated fused-silica capillaries. This advancement streamlines the workflow and improves repeatability, peak resolution, and capillary lifetime. The enhanced CE-MS method, together with the routine CE-UV approach, was applied to the analysis of milk samples from cows carrying the β-casein (β-CN) A1A1, A1A2, and A2A2 genotypes, as well as to quantify low-level contamination of A2A2 milk with A1A1 milk. Both methods demonstrated strong potential for comprehensive profiling of bovine casein and whey proteins, providing rapid and reliable tools for the authentication of the highly valued A2A2 milk, commonly referred to as A2. Importantly, accurate mass high-resolution MS proved essential for the precise characterization of β-CN A1 and A2 proteoforms, enabling unambiguous identification of milk based on its specific β-CN composition.

1. Introduction

The characterization of milk proteins, particularly those in bovine milk, is crucial in food science and the dairy industry, as it provides key information related to product quality, authenticity, and potential health implications [1–3]. Bovine milk proteins are broadly categorized into caseins (CNs) and whey proteins, with CNs, primarily α-S1-, α-S2-, β-, and κ-CN, accounting for approximately 80% of the total protein content [3,4]. Among them, β-CN is notable for its genetic polymorphism, especially the A1 and A2 proteoforms, which differ by a single amino acid at position 67: histidine in A1 and proline in A2 [3–6]. This subtle substitution influences both the structural conformation of the proteoforms and their digestive behavior. Specifically, digestion of β-CN A1 generates β-casomorphin-7 (βCM-7), an opioid-like peptide associated with inflammatory responses, whereas β-CN A2 does not readily release this peptide sequence, making it a potentially healthier and more desirable alternative [3,5]. In response to growing consumer interest in A2 milk, the common term for milk from cows carrying β-CN A2A2 genotypes, there is an increasing demand for sensitive and reliable analytical methods capable of distinguishing these closely related proteoforms.

Several analytical techniques have been employed for the separation

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of intact proteins in bovine milk [1–3], with electrophoretic methods demonstrating superior performance compared to other approaches [3]. Traditional gel-based electrophoretic methods, such as isoelectric focusing (IEF) and urea-polyacrylamide gel electrophoresis (urea--PAGE), separate proteins based on their isoelectric point and charge-to-size ratio, respectively [7–9]. While effective, these techniques often require complex and time-consuming sample preparation, and suffer from limited throughput, reproducibility issues, and reduced quantitative precision. As a faster and more efficient alternative, capillary electrophoresis with ultraviolet detection (CE-UV), specifically in its capillary zone electrophoresis mode, is widely used for the analysis of bovine milk proteins [10–13]. This technique separates proteins according to their electrophoretic mobility under controlled pH conditions, which reflects their charge-to-size ratios. In addition, it offers excellent performance in terms of sensitivity, reproducibility, separation resolution, and total analysis time, while requiring minimal consumption of sample, solvents, and reagents [10–13]. Prior to analysis, CN micelles must be disrupted via reduction and denaturation, typically using dithiothreitol and urea [10]. To prevent micelle reformation during separation, urea is also incorporated into the background electrolyte (BGE), and analyses are conducted under acidic conditions (pH 2.5-3.0) [10–13]. Despite its advantages, CE-UV lacks the ability to unequivocally identify the separated proteoforms. To overcome this limitation, CE can be coupled to mass spectrometry (CE-MS), enabling simultaneous separation and accurate molecular mass (Mr) determination [13]. However, adapting a CE-UV method to CE-MS involves significant technical challenges, especially when non-volatile components like urea are present in the BGE. As in liquid-chromatography-mass spectrometry (LC-MS), the substances can interfere with electrospray ionization (ESI), reduce sensitivity, and pose a risk of long-term contamination of the mass spectrometer [13–15].

To address these challenges, we previously developed a CE-MS method that enabled characterization at the intact level of major bovine milk proteins in under 15 min [13]. This approach employed lab-made capillaries permanently coated with hydroxypropyl cellulose (HPC) to minimize protein adsorption and improve reproducibility. A volatile BGE composed of 2 M acetic acid (AcOH) was employed to ensure compatibility with positive ESI mode and to enhance run-to-run reproducibility [13]. Using an accurate mass high-resolution mass spectrometer, this CE-MS method successfully distinguished closely related proteoforms such as β-CN A1 and A2, demonstrating enhanced performance compared to lower-resolution MS-based techniques, such as matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [13,16].

However, the use of lab-made coated capillaries presents several drawbacks. The process of coating the capillaries is labor-intensive and time-consuming, requiring meticulous preparation and handling to ensure consistency [17–19]. Therefore, reproducibility between batches can be inconsistent, especially when strict protocols are not followed, leading to potential variations in performance. Additionally, the capillary coatings tend to degrade over time or with prolonged use, compromising the reliability and sensitivity of the analysis [20]. This degradation not only affects analytical performance but also requires frequent replacement of the capillaries, further increasing operational costs and downtime. These issues negatively impact the long-term robustness and reliability of the method, making it less suitable for routine or high-throughput analyses. Moreover, commercially coated capillaries are either not readily available or tend to be very expensive, limiting the broader adoption of this method, particularly in resource-limited or non-specialist laboratories. As a result, these limitations drive continuous research into novel coated capillaries, hindering their widespread adoption in routine analytical practice [17–19].

In this study, we propose an alternative CE-MS method for bovine milk protein profiling that retains the advantages of high-resolution separation and MS compatibility while simplifying the operation. This method retains 2 M AcOH as a volatile BGE, ensuring compatibility with positive ESI mode and providing stable, reproducible separations [13], but replaces lab-made HPC-coated capillaries with commercially available OV-1701-OH deactivated fused-silica capillaries, previously proposed only for bovine milk protein profiling by CE-UV [12]. OV-1701-OH is a silanol-terminated, relatively polar polydimethylsiloxane with 7% cyanopropyl and 5% phenyl substitution, typically used in gas chromatography (GC) to deactivate fused silica columns and related components through polymer bonding and crosslinking, thereby enhancing inertness toward polar compounds and compatibility with aqueous matrices [21]. These deactivated capillaries are readily available in CE-compatible inner diameters (IDs) at costs similar to uncoated capillaries and have been shown excellent performance under typical conditions used for bovine milk protein profiling by CE-UV [12]. In addition to eliminating the need for complex capillary coating procedures, the CE-MS method implemented with deactivated capillaries provides several other advantages over our previous approach [13]. It improves repeatability, peak resolution, and capillary lifetime. It also enables the identification of β-CN A1 and A2 proteoforms, facilitates reliable discrimination of milk from different β-CN genotypes (A1A1, A1A2, and A2A2), and allows quantification of low-level A1A1 contamination in A2A2 milk. These features make the method particularly valuable for routine milk analysis, offering robust support for dairy product authentication, quality control, and genotype verification.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of analytical reagent grade or higher. Propan-2-ol (≥99.9%), AcOH (glacial), formic acid (HFor, 99.0%), Tris(hydroxymethyl)aminomethane (Tris, 99.8%), Titriplex® EDTA (ethylenediaminetetraacetic acid disodium salt dihydrate ≥99.5%), 3-morpholinopropanesulfonic acid (MOPS≥99.5%), sodium citrate dihydrate (≥99.0%), citric acid (99.5%), urea (99.0-100.5%), hydroxypropylmethylcellulose (HPMC 10000 Mr, Sigma-Aldrich, Darmstadt, Germany), lactic acid (≥98.0%), and DL-dithiothreitol (DTT, 97.0%) were supplied by Merck (Darmstadt, Germany). Water (LC-MS grade) was obtained from PanReac Applichem (Barcelona, Spain). Milk samples from cows carrying the β-CN A1A1, A1A2, and A2A2 genotypes were obtained through the Center for Innovation, Research, and Transfer in Food Technology (CIRTTA, Autonomous University of Barcelona, UAB). The genetic information of individual cows was provided by CONAFE (Confederation of Spanish Friesian Associations, Valdemoro, Madrid, Spain). Raw whole milk was collected during the morning milking of selected Friesian cows at a local farm (La Cavalleria, Manlleu, Barcelona, Spain), then immediately cooled at 4 °C and transported to CIRTTA, where fat was separated by centrifugation. In addition, commercially available skimmed A1A2 milk treated by ultra-high temperature (UHT) processing was purchased from a local retailer.

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

The reduction buffer used for sample preparation was prepared as described in previous studies [11,12], except that methylhydroxyethylcellulose (MHEC 30000 Mr) was changed by HPMC (15000 Mr). Specifically, the reduction buffer was composed of 505 mg of Tris, 620 mg of Titriplex® EDTA, 220 mg of MOPS, and 64 mg of DTT, all dissolved in 25 mL of a 10 M urea solution with 0.083 % (m/v) HPMC and subsequently diluted with water at a 1.5:1 (v/v) ratio. For CE-UV analysis, the BGE at pH 3.0 was prepared by dissolving 148 mg of sodium citrate dihydrate and 1 g of citric acid in 10 mL of water and 15 mL of 10 M urea with 0.083 % (m/v) HPMC [11,12]. For CE-MS, the BGE was 2 M AcOH at pH 2.2, as previously reported with lab-made HPC-coated capillaries [13]. The sheath liquid was prepared as described in the same study, namely a mixture of propan-2-ol and water

(60:40 v/v) with 0.05% (v/v) HFor. This solution was delivered at a flow rate of 3.3 μL/min using a KD Scientific 100 series infusion pump (Holliston, MA, USA). Both the sheath liquid and BGEs were degassed for 10 min using ultrasonic agitation before use.

2.3. Sample preparation

Three hundred μL of skim milk from either a commercial A1A2 sample, pure individual A1A1, A1A2, or A2A2 samples, or A2A2 samples contaminated with A1A1 at ratios of 99:1, 95:5, 90:10 and 85:15 (v/v) (prepared using the individual samples), were mixed with 1 mL of diluted reduction buffer [11,12]. The mixture was incubated at room temperature for 1 h to facilitate CN micelle disruption and protein extraction. Then, 20 μL of 18% (v/v) lactic acid was added to adjust the pH to 8.5. The obtained solution was filtered, first through a 0.20 μm nylon filter (Macherey-Nagel, Düren, Germany) and subsequently through a 3000 Mr cutoff cellulose acetate centrifugal filter (Amicon Ultra-0.5, Millipore, Bedford, MA, USA), as described in our previous study [13]. All centrifugation steps were performed at 13000×*g* and 25 °C. Before the filtration of the sample, the centrifugal filter was pre-conditioned by washing with 500 μL of water for 15 min, after which the filtrate was discarded. Next, 500 μL of the sample was loaded onto the filter and centrifuged for 10 min. The filtration residue was washed twice with 100 μL of water for 10 min each, followed by a final wash with 50 μL of water for another 10 min. To recover the concentrated residue, the upper reservoir of the filter was inverted into a vial and spun at a reduced centrifugal force (300×*g* for 2 min). The recovered sample (~75 μL) was reconstituted in water to a final volume of 100 μL. For CE-MS analysis, the desalted sample was mixed with BGE (2 M AcOH, pH 2.2) in a 1:1 (v/v) ratio. This mixture was centrifuged at 13000×*g* for 2 min at 25 °C to remove any remaining particulates, and the supernatant was collected for analysis. Desalted samples were kept at −20 °C until further use.

2.4. CE

The main CE experiments were conducted using commercially available OV-1701-OH deactivated fused-silica capillaries (58 cm total length (L_T) × 50 μm inner diameter (ID) × 375 μm outer diameter (OD)) from BGB Analytik AG (TSP-050375-P-10, Böckten, Switzerland). Lab-made HPC-coated capillaries (58 cm L_T × 75 μm ID × 365 μm OD) were prepared as described in our previous work [13].

2.4.1. CE-UV

CE-UV experiments were performed at 25 °C using a 7100 CE system from Agilent Technologies (Waldbronn, Germany) equipped with a diode-array detector (DAD). UV spectra were recorded in the range of 200-400 nm, with detection carried out at 214 nm. Prior to analysis, the capillaries were conditioned by flushing with the BGE at 930 mbar for 20 min. Final equilibration was achieved by applying a separation voltage of +25 kV for 15 min under normal polarity (cathode at the outlet). The sample injection was performed hydrodynamically at 50 mbar for 15 s. To maintain repeatability and prevent carryover, capillaries were rinsed (at 930 mbar) between runs with water (5 min) and BGE (10 min). CE control, data acquisition, and analysis were performed using ChemStation C.01.06 software (Agilent Technologies).

2.4.2. CE-MS

CE-MS experiments were conducted at 25 °C using a 7100 CE system coupled to a 6220 oa-TOF LC/MS spectrometer (Agilent Technologies, Waldbronn, Germany) via an orthogonal G1603A sheath-flow interface. The mass spectrometer operated in positive ESI mode under optimized conditions: capillary voltage, 4000 V; drying gas temperature, 300 °C; drying gas flow rate, 4 L/min; nebulizer gas pressure, 7 psig; fragmentor voltage, 325 V; skimmer voltage, 80 V; and OCT 1 RF Vpp, 250 V. Data were acquired in profile mode at a rate of 1 spectrum per second over an *m/z* range of 100-3200. CE control was performed using ChemStation C.01.06 software (Agilent Technologies), in combination with MassHunter Workstation software version B.06.00 (Agilent Technologies) for MS control, data acquisition, and data analysis. Prior to CE-MS analyses, the capillaries were conditioned by flushing with the BGE (2 M AcOH at pH 2.2) at 930 mbar for 20 min. Samples were injected hydrodynamically at 50 mbar for 15 s. Electrophoretic separations were carried out under normal polarity using a separation voltage of +30 kV, with a constant pressure of 25 mbar to ensure repeatability (current intensity, I ~ 21 μA). Between runs, the capillary was rinsed (at 930 mbar) with water for 3 min followed by BGE for 3 min. Electrophoretic separations using lab-made HPC-coated capillaries were conducted at 25 °C with the same BGE. Samples were injected 10 s at 50 mbar, and a voltage of +15 kV was applied without pressure (I ~ 12 μA), as previously described in our work [13].

3. Results and discussion

3.1. CE-UV

The first CE-UV method for the analysis of bovine milk proteins was established more than three decades ago [10]. In the original conditions proposed by de Jong et al., a citrate reduction buffer at pH 8.0 containing DTT and urea was used to disrupt, reduce, and denature CN micelles and recover the proteins from milk samples. The same components were then used in the BGE at pH 2.5 supplemented with MHEC as a dynamic coating, and combined with a capillary permanently coated with a hydrophilic polymer (e.g. CElect P1) to further minimize protein adsorption during CE-UV separations at 45 °C [10]. Recio et al. later proposed substantial modifications to the reduction buffer composition to further optimize the protein profiles [11]. These conditions were subsequently adopted by Heck et al., who replaced CElect p1 capillaries to OV-1701-OH deactivated capillaries, most likely due to the discontinuation of CElect p1 [12]. In the present study, this method was applied using HPMC instead of MHEC in de BGE. HPMC had previously been proposed as a suitable alternative to MHEC by de Jong et al. [10], and was subsequently adopted by other authors when MHEC was not available [22,23]. As shown in Fig. 1A–C, the established method provided a fast and straightforward approach for protein profiling of individual bovine milk samples carrying the A1A1, A2A2, and A1A2 genotypes. The resulting profiles were comparable to those obtained in the method of Heck et al. [12] and our previous work [13]. In this last case, due to the unavailability of the original materials, we adapted de Jong's method by employing a lab-made HPC-coated capillary and hydroxyethyl cellulose (HEC, 250 000 Mr) as the dynamic coating agent in the BGE. Tentative identification of the main protein peaks was achieved by comparison with the method of Heck et al. [12]. Peaks were assigned to α-lactalbumin (α-LA), β-lactoglobulin A and B (β-LG A-B), α-S1-casein B (α-S1-CN B), α-S0-casein B (α-S0-CN B), κ-casein A and B (κ-CN A-B), β-CN A1, and β-CN A2. The method demonstrated good repeatability in terms of migration times and peak areas, with relative standard deviations (%RSD, A1A2 milk, $n = 3$) as follows: 0.7% and 3.8% for α-LA; 1.6% and 2.2% for β-LG A + β-LG B; 0.6% and 3.0% for α-S1-CN B; 0.6% and 1.4% for α-S0-CN B, 0.5% and 5.3% for κ-CN A + κ-CN B; 0.5% and 2.8% for β-CN A1, and 0.5% and 1.6% for β-CN A2. These values were comparable to those using previously reported CE-UV methods for bovine milk protein profiling [10–13,22,23]. Importantly, the current method provided excellent separation of closely related protein variants, notably between α-S1-CN B and α-S0-CN B, as well as between β-CN A1 and β-CN A2, achieving baseline resolution in both cases. As shown in Fig. 1A–C, the distinctive β-CN peaks enabled clear and rapid differentiation of individual A1A1, A2A2, and A1A2 genotypes, respectively.

To further assess the method's efficiency in detecting low levels of A1A1 contamination in A2A2 bovine milk, Fig. 2 displays the CE-UV electropherograms obtained for an A2A2 sample spiked with (A) 15%,

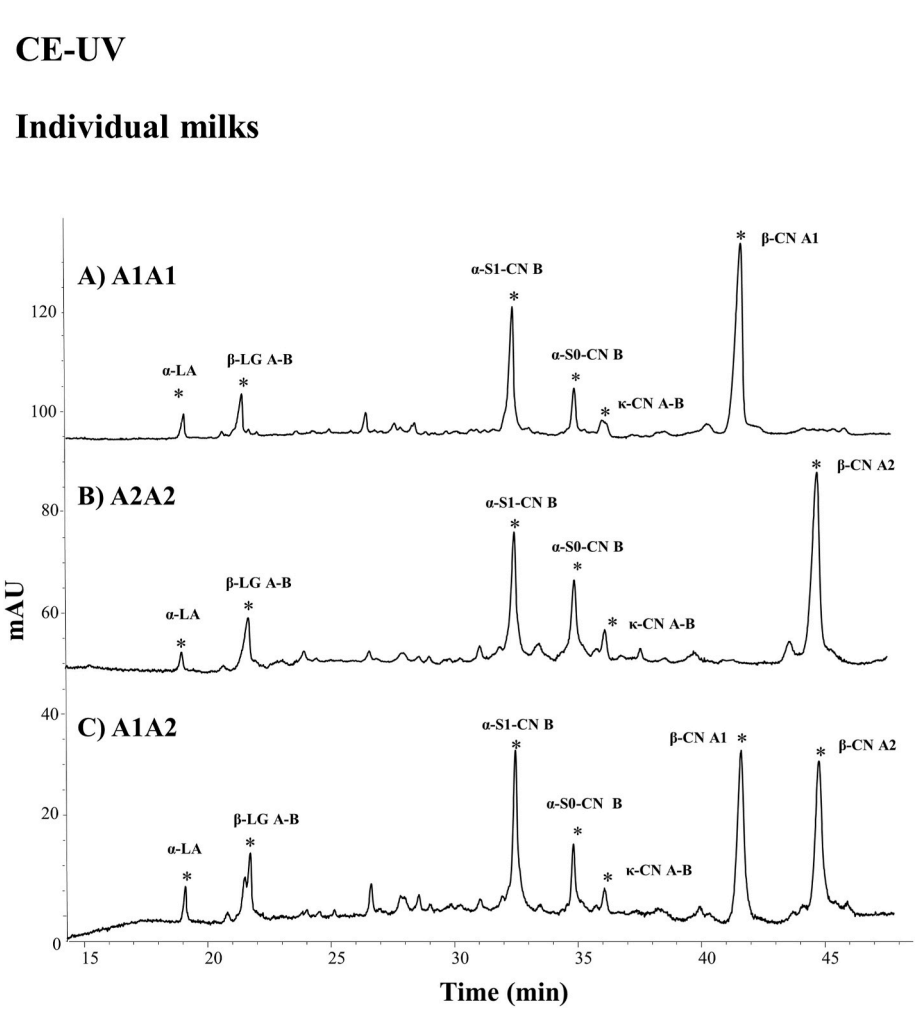


Fig. 1. CE-UV electropherograms obtained at 214 nm using the method described by Recio et al. [11] with some modifications (see the experimental section) for the analysis of individual bovine milk samples with different β-CN genotypes: A) A1A1, B) A2A2, and C) A1A2. The detected casein and whey protein peaks are labelled with an asterisk, namely α-lactalbumin (α-LA), β-lactoglobulin A and B (β-LG A-B), α-S1-casein B (α-S1-CN B), α-S0-casein B (α-S0-CN B), κ-casein A and B (κ-CN A-B), β-casein A1 (β-CN A1), and β-caseinA2 (β-CN A2).

(B) 10%, (C) 5%, and (D) 1% (v/v) of A1A1 milk. In all cases, the β-CN A1 peak was detectable, with a gradual decrease in signal intensity corresponding to the decreasing levels of contamination. Linearity was observed across the 2.5%-15% (v/v) contamination range ($y = 4.25x+294.89$, $R^2 = 0.9981$), and the limit of detection (LOD) would be slightly below 1% (v/v) A1A1 contamination in A2A2 milk (Fig. 2-D). These results demonstrate the method's capability to detect low amounts of β-CN A1, underscoring its potential as a fast, simple, and effective tool for routine screening and preliminary authentication of A2 milk. However, for reliable identification and accurate quantification of the detected proteoforms, ESI-MS detection using an accurate mass, high-resolution mass spectrometer was required.

3.2. CE-MS

The lab-made HPC-coated capillaries proposed in our previous CE-MS method [13] enabled separation and characterization at the intact level of major bovine milk proteins with good sensitivity and repeatability. However, the coating procedure was labor-intensive, time--consuming, and prone to limited batch-to-batch reproducibility unless carefully controlled by an experienced operator. In contrast, OV-1701-OH deactivated capillaries are commercially available at a reasonable cost, offering a practical advantage for resource-limited laboratories and enhancing the robustness of CE-MS methods for routine milk protein profiling. Further encouraged by their excellent performance in CE-UV, we evaluated here for the first time their applicability in CE-MS, using a 2 M AcOH BGE to minimize protein adsorption and ensure optimal repeatability and sensitivity in positive ESI mode, as in our previous study [13].

Fig. 3A and B shows the extracted ion electropherograms (EIEs) for the main proteins identified by CE-MS with deactivated capillaries in a commercial skimmed milk A1A2 sample using (A) lab-made HPC-coated capillaries from our previous study [13] and (B) OV-1701-OH deactivated fused-silica capillaries (both with a 2 M AcOH BGE). These EIEs were generated by summing the detected protonated ions for each protein, whose charge states and calculated exact Mr are listed in Table 1. Protein identification was confirmed unequivocally by deconvoluting the mass spectra of the peaks detected in the EIEs, yielding experimental accurate Mr values. Typical mass errors (ΔMr) for the identification were <2.5 mass units (data not shown). Repeatability was assessed for migration times and peak areas of the identified proteins. The %RSD values (n = 3) for migration times and peak areas were as follows: 0.1% and 1.3% (β-CNA2), 0.8% and 2.3% (β-CNA1), 0.9% and 2.6% (κ-CNA), 0.5% and 0.4% (κ-CNB), 0.8% and 0.8% (α-s1-CN-B), 0.9% and 0.2% (α-s0-CN-B), 1.3% and 2.7% (β-LGB), 1.1% and 2.3% (β-LGA), 0.5% and 1.6% (α-LA). These values were slightly lower than those obtained by CE-MS with HPC-coated capillaries (%RSD values ranged from 2.4% to 3.5% and from 2.6 to 3.9% for migration times and peak areas, respectively [13]). This finding suggested a modest but consistent improvement in repeatability, likely attributable to the

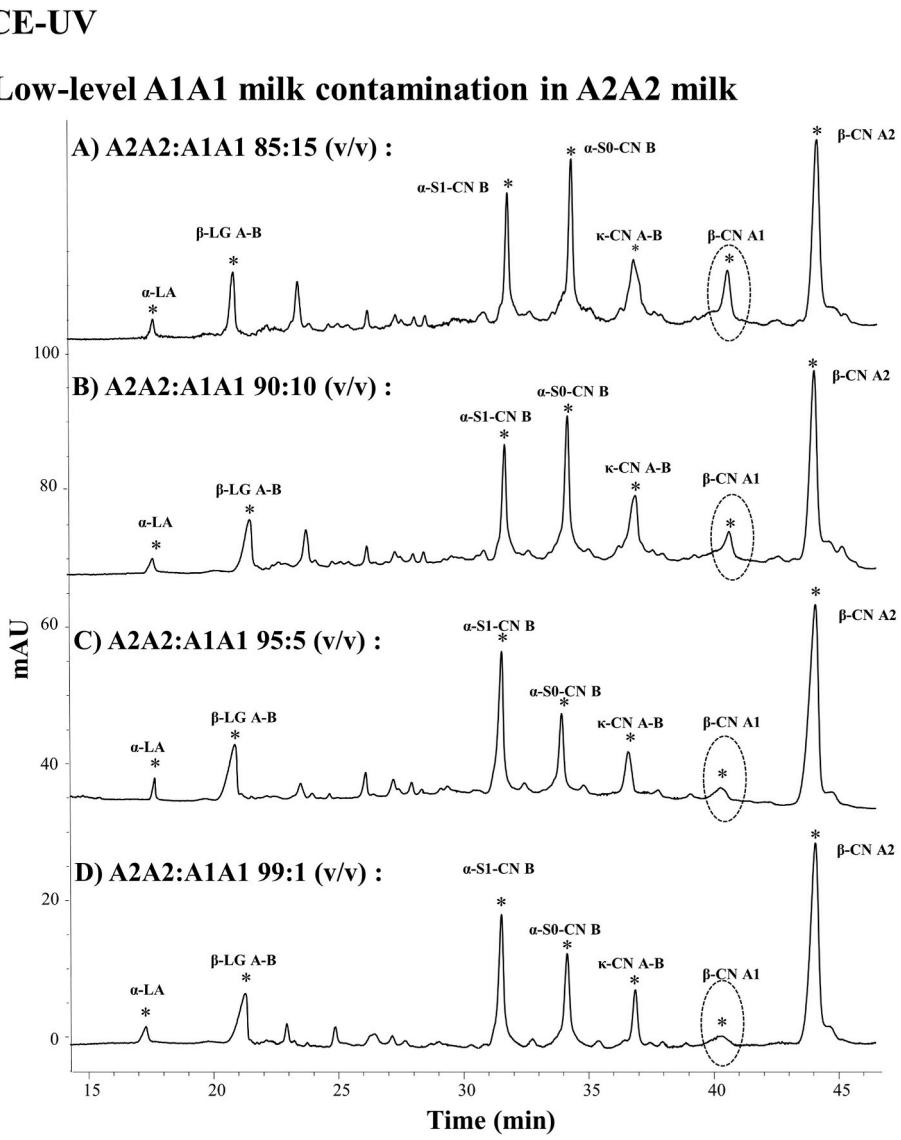


Fig. 2. CE-UV electropherograms obtained at 214 nm using the method described by Recio et al. [11] with some modifications (see the experimental section) for an A2A2 bovine milk sample contaminated with A1A1 milk at the following levels: (A) 15%, (B) 10%, (C) 5%, and (D) 1% (v/v). The detected casein and whey protein peaks are labelled with an asterisk, namely α-lactalbumin (α-LA), β-lactoglobulin A and B (β-LG A-B), α-S1-casein B (α-S1-CN B), α-S0-casein B (α-S0-CN B), κ-casein A and B (κ-CN A-B), β-casein A1 (β-CN A1), and β-casein A2 (β-CN A2). The β-CN A1 peaks are highlighted with circles.

well-documented inertness of mid-polarity polydimethylsiloxane OV-1701-OH coatings toward polar analytes and their favorable compatibility with aqueous matrices [21].

An additional advantage of the deactivated capillaries was the reduction in analysis time, facilitated by operating at higher separation voltage in a capillary of a smaller ID (30 kV and 50 μm ID vs 15 kV and 75 μm ID). This was further enhanced by applying 25 mbar of pressure during separation in a capillary that, due to the chemical composition of the coating, is expected to exhibit a very small but detectable EOF, in contrast to the near-zero EOF characteristic of HPC-coated capillaries. While HPC-coated capillaries required ~15 min to obtain the protein profile, the deactivated capillaries completed the analysis in ~10 min, representing a modest gain in throughput. Importantly, this reduction in analysis time did not compromise peak width or resolution. On the contrary, the protein peaks were noticeably narrower, reflecting enhanced resolution. This improvement could be attributed to the 50 μm ID of the deactivated capillaries, compared to the 75 μm ID required to prepare HPC-coated capillaries to avoid clogging and current instability. Additionally, the deactivated capillaries exhibited significantly improved durability, probably because performing the separations at room temperature prevented short-term acidic and aqueous hydrolysis of the covalently-bonded and crosslinked polydimethylsiloxane OV-1701-OH coating [21]. More than 50 analyses were conducted without noticeable performance degradation, and no ion suppression or mass spectrometer contamination was observed. However, the capillary was discarded after this number of analyses because the %RSD values for β-CNA2 peak areas increased to approximately 15%, compared with 1.3% observed for the first three analyses of a commercial skimmed milk A1A2 sample (see Supplementary Fig. S1). In contrast, HPC-coated capillaries typically allowed only ~15 analyses. This extended lifetime is particularly relevant given the complexity of milk protein samples.

Although the deactivated capillaries offered clear advantages, they, like the HPC-coated capillaries, were unable to baseline resolve closely related proteoforms by electrophoresis. This limitation is common in CE-MS compared with CE-UV, largely due to the constraints imposed by the use of acidic, low-conductivity, and volatile BGEs, as well as the need to apply pressure during separation to ensure optimal repeatability and sensitivity in CE-MS operated in positive ESI mode. Nonetheless, the

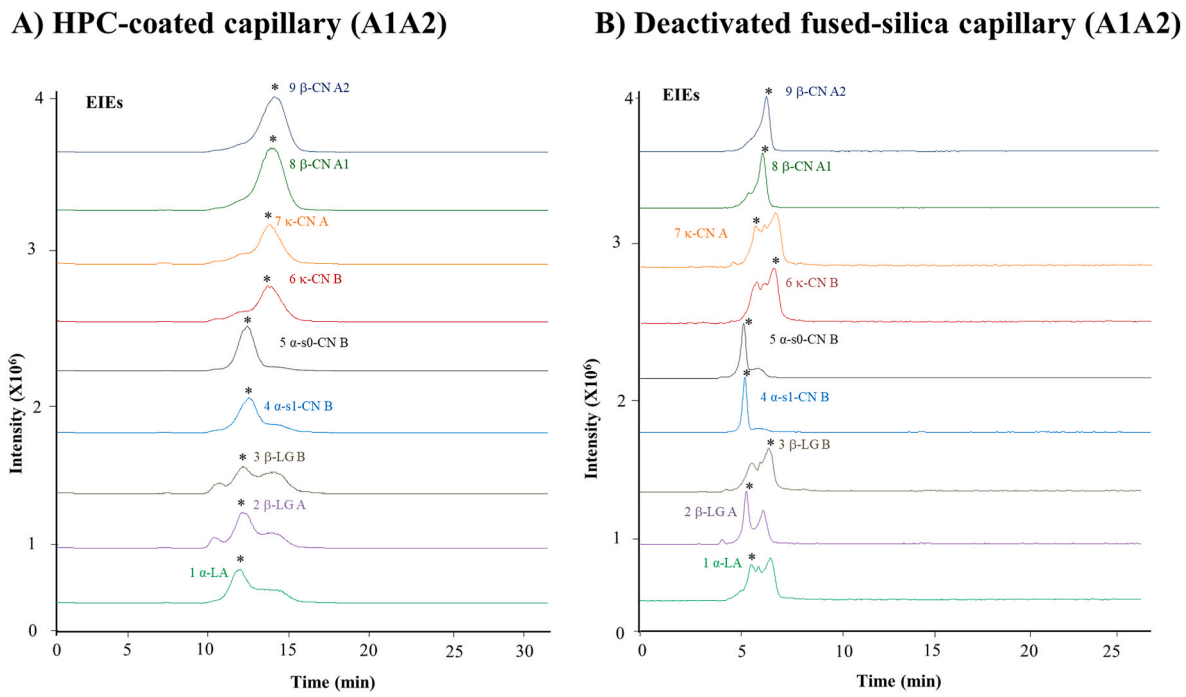


Fig. 3. Extracted ion electropherograms (EIEs) of the proteins identified by CE-MS in a commercial skimmed A1A2 milk using (A) a lab-made HPC-coated capillary (58 cm LT × 75 μm ID × 365 μm OD) and (B) a commercial OV-1701-OH deactivated fused-silica capillary (58 cm LT × 50 μm ID × 375 μm OD). These EIEs were generated by summing the detected protonated ions for each protein, whose charge states and calculated exact Mr are listed in Table 1. Protein identification was confirmed unequivocally by deconvoluting the mass spectra of the peaks detected in the EIEs to obtain their experimental accurate Mr values. Typical mass errors (ΔMr) for identification were <2.5 mass units. The detected casein and whey protein peaks are labelled with an asterisk, namely α-lactalbumin (α-LA), β-lactoglobulin A and B (β-LG A-B), α-S1-casein B (α-S1-CN B), α-S0-casein B (α-S0-CN B), κ-casein A and B (κ-CN A-B), β-casein A1 (β-CN A1), and β-casein A2 (β-CN A2).

Table 1
Average exact molecular masses (Mr) and ion charge state ranges for the bovine milk proteins identified by the CE-MS method.

Protein	Exact Mr	Ion charge states
β-CN A2	23982.95	19-24
β-CN A1	24022.97	19-24
κ-CN A	19037.18	15-20
κ-CN B	19005.23	15-20
α-S0-CN B	23694.46	17-22
α-S1-CN B	23614.48	17-22
β-LG B	18281.06	11-16
β-LG A	18367.15	11-16
α-LA	14185.96	11-16

high mass accuracy and resolution of the TOF mass spectrometer in the CE-MS setup enabled the characterization of individual proteoforms with minimal Mr differences, including β-CN variants in bovine samples carrying A1A2 genotypes (Fig. 3-B). To assess the sensitivity of the proposed CE-MS method in detecting low levels of A1A1 contamination in A2A2 bovine milk, Fig. 4A and B presents the combined EIEs for β-CN A2 and β-CN A1 in a pure A2A2 milk sample and the same sample after being spiked with 5% (v/v) of A1A1 milk, respectively. In the EIE of the contaminated sample (Fig. 4B (i)), a distinct small slope appears for β-CN A1, which notably overlaps with the β-CN A2 peak. Fig. 4A and B (ii) and (iii) depict the raw and deconvoluted mass spectra, respectively, corresponding to the time window where the protein peaks were observed in the EIEs. In the deconvoluted spectrum of the pure A2A2 sample (Fig. 4A (iii)), a major signal for the protonated ion and minor signals corresponding to sodium and potassium adducts of β-CN A2 were detected. The inset tables in Fig. 4A and B (iii) show that the deconvoluted Mr of the (β-CN A2 + K) adduct closely resembled that of β-CN A1, making their distinction by MS alone unfeasible. Due to the limited electrophoretic resolution (Fig. 4B (i)) and the overlapping signal from (β-CN A2 + K) ions, accurately quantifying low levels of β-CN A1 via EIE integration was challenging. To overcome this, the ratio of peak areas from the deconvoluted mass spectra, corresponding to [(β-CN A2 + K) + β-CN A1] and β-CN A2, was calculated for a pure A2A2 sample and A2A2 samples spiked with 1%, 5%, 10%, and 15% (v/v) of A1A1 milk. This approach revealed a linear response ($R^2 = 0.99$, Supplementary Fig. S2) within the 1% to 15% (v/v) contamination range, enabling sensitive and reliable quantification of low-level A1A1 milk contamination in A2A2 milk. The LOD would be slightly below the limit of quantification (LOQ) of 1% v/v, as the contribution of β-CN A1 to the overall peak area is minimal compared to that of the coexisting (β-CN A2 + K) adduct. Consequently, CE-MS performed slightly better than CE-UV for quantification of contamination (LOQs were 1% (v/v) and 2.5% (v/v), respectively), while both methods showed similar LODs (slightly below 1% (v/v) in each case). Therefore, CE-UV may be considered an excellent alternative for routine screening and preliminary authentication of A2 milk, whereas CE-MS using an accurate mass, high-resolution mass spectrometer provides the most reliable identification and accurate quantification at the lowest contamination levels. The low LOQ, achieved by CE-MS is comparable to the LOQ recently reported for a competitive immunoassay with highly specific monoclonal antibodies claimed to discriminate between βCN A1 and A2 proteoforms with minimal cross-reactivity [24]. Nevertheless, this approach lacks direct MS confirmation of the detected proteoforms and relies critically on the specificity of monoclonal antibodies for both identification and quantification of proteoforms that differ by only a single amino acid, an issue that remains controversial [3]. Similarly, PCR-based genotyping on DNA extracted from milk somatic cells is highly sensitive and selective, but it does not directly confirm which proteoforms are actually present in the milk and suffers from quantitative limitations [2,9].

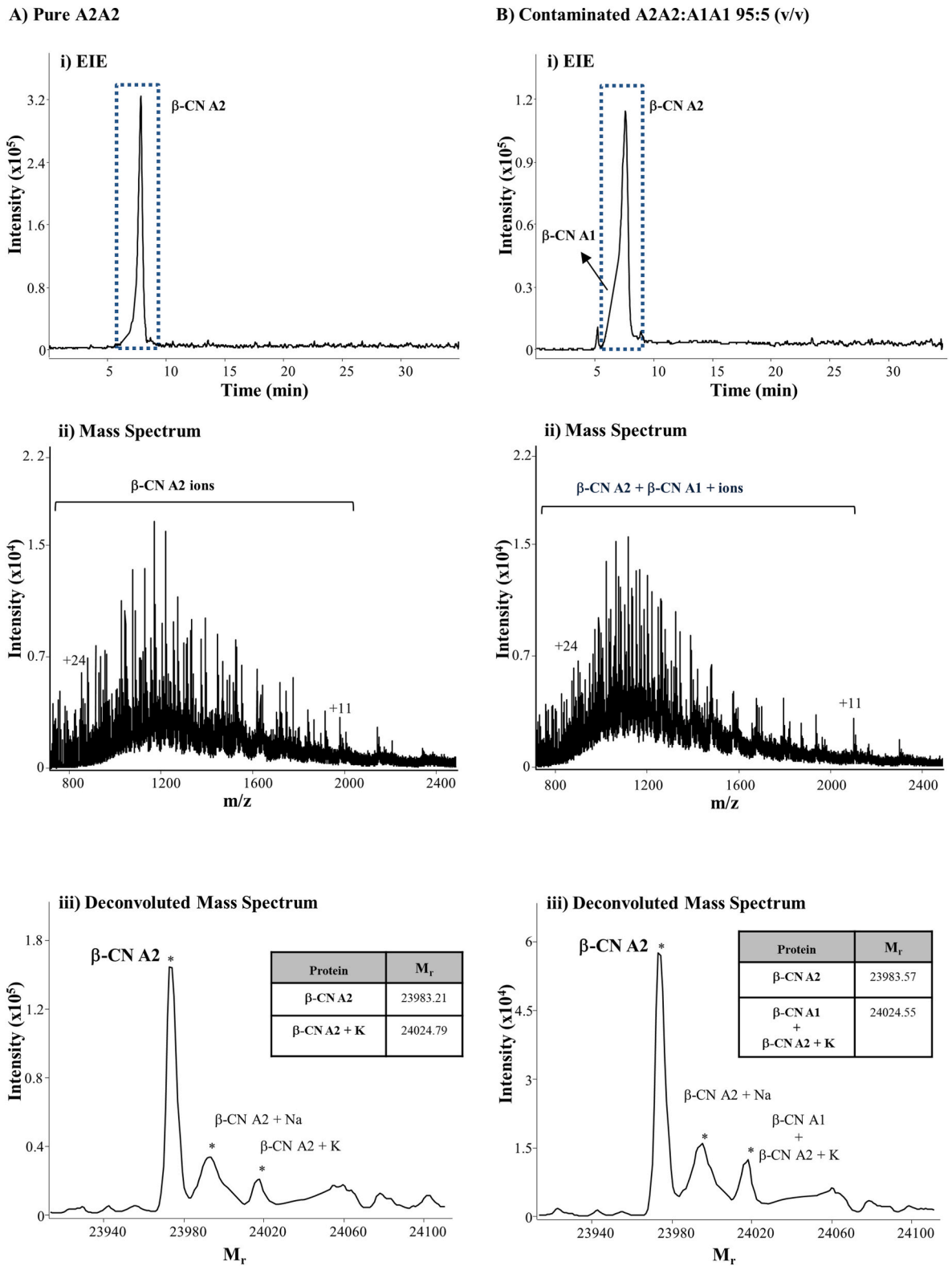


Fig. 4. CE-MS analysis using OV-1701-OH deactivated fused-silica capillaries of (A) an A2A2 bovine milk sample and (B) the same milk sample contaminated with 5% (v/v) of A1A1 milk. (i) Extracted ion electropherograms (EIEs) combining β-CN A1 and β-CN A2, (ii) raw mass spectra for the indicated time window in the EIEs, and (iii) deconvoluted mass spectra (inset tables show the experimental accurate Mr for the most relevant proteoforms to quantify A1A1 contamination in A2A2 milk).

4. Conclusions

This study demonstrates the effective integration of CE-UV and CE-MS strategies for the analysis of intact bovine milk proteins, using commercially available OV-1701-OH deactivated capillaries. A CE-UV method was established for a fast and straightforward protein profiling, successfully differentiating β-CN A1A1, A2A2, and A1A2 genotypes, and detecting low-level contamination of A2A2 milk with A1A1 milk, supporting its potential for high-throughput screening and preliminary authentication of A2 milk.

In parallel, a novel CE-MS method was developed using the same deactivated capillaries as an alternative to the previously reported approach based on lab-made HPC-coated capillaries. Compared to the coated capillaries, the deactivated capillaries provided comparable or improved performance across several key metrics: narrower peaks (improved resolution), shorter analysis time (10 vs. 15 min), higher repeatability, and significantly enhanced capillary lifetime (>50 analyses vs. ~15 analyses). While electrophoretic resolution of closely related proteoforms remained limited, the high mass accuracy of the TOF mass spectrometer combined with spectral deconvolution enabled reliable identification and quantification of β-CN variants, even in samples with low-level A1A1 contamination (down to 1% (v/v)).

In summary, the proposed CE-MS workflow using deactivated capillaries offers a robust, reproducible, and accessible platform for high-throughput milk protein analysis. By eliminating the need for preparation of labor-intensive capillary coatings without sacrificing performance, this method is well suited for routine applications in dairy protein analysis from different species, quality control, and the authentication of A2 milk. Beyond bovine milk, it can also be readily extended to the characterization of milk from other species.

CRediT authorship contribution statement

Tahereh Tehrani: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **Laura Pont:** Writing – review & editing, Supervision, Investigation. **Bibiana Juan:** Writing – review & editing, Methodology, Investigation. **Antonio-José Trujillo:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Fernando Benavente:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2026.129604.

Data availability

Data will we made available on reasonable request.

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