Microchemical Journal 219 (2025) 116141

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Development of a trifluoroacetic acid-free liquid chromatography-mass spectrometry method for protein profiling of bovine milk and authentication of A2 milk

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ARTICLE INFO

Keywords:
β-casein
Fingerprinting
Formic acid
Liquid chromatography
Mass spectrometry
Milk proteins

ABSTRACT

Milk proteins, particularly caseins and whey proteins, are essential to the nutritional and functional properties of milk. However, understanding their detailed composition and genetic variations requires precise and reliable analytical techniques. In this study, we present a novel method for protein profiling of bovine milk using liquid chromatography with ultraviolet absorption and mass spectrometry detection (LC-UV and LC-MS, respectively). Initially, a reversed-phase LC-UV method was developed, using a wide-pore C4 column and acetonitrile: water mobile phases with 0.1 % (*v*/v) formic acid (HFor), replacing the commonly used ion-pairing agent trifluoroacetic acid (TFA). TFA is not recommended in best practices for LC-MS, as it interferes with on-line electrospray ionization (ESI) and contaminate the mass spectrometer due to its ion-suppression effects. By adopting HFor in LC-UV, the total analysis time was reduced by 10 min, and separation was slightly affected. Moreover, the TFA-free method was fully compatible with LC-MS, demonstrating consistent performance with low relative standard deviations (%RSDs) in retention time and peak area across extended sequences of analyses, with no mass spectrometer contamination. LC-MS analysis was performed using an accurate mass, high-resolution time-of-flight (TOF) mass spectrometer, enabling accurate identification and quantification of key bovine milk proteins from whey and casein fractions, including proteoforms with subtle structural differences, such as β-casein (β-CN) A1 and A2. This allowed differentiation between various β-casein genotypes (A1A1, A1A2, and A2A2) and the quantification of low levels of contamination of A2A2 milk with A1A1 milk. These findings highlight the method's potential for high-throughput, reliable, and efficient dairy research, quality control, and authentication, without compromising the mass spectrometer performance.

1. Introduction

Proteins, particularly caseins and whey proteins, play a crucial role in the nutritional and technofunctional properties of bovine milk, influencing its physical and chemical characteristics, as well as its implications for human health and dairy innovation [1–3]. However, a comprehensive understanding of their composition and genetic variants requires precise and reliable analytical techniques.

Various separation techniques have been proposed for the profiling of intact proteins in bovine milk [2,3]. These include slab gel electrophoresis [4–6], capillary electrophoresis (CE) [7–10], and liquid chromatography (LC) [11–19]. Currently, CE with ultraviolet absorption detection (CE-UV) is one of the preferred techniques for routine analysis. Specifically, CE-UV operating in capillary zone electrophoresis (CZE) mode offers excellent performance in terms of sensitivity, repeatability, separation resolution, and total analysis time, while requiring minimal consumption of sample, solvents, and reagents [7–9]. Due to the broader acceptance and accessibility of chromatographic instrumentation, reversed-phase LC-UV has been proposed as an alternative to CE-UV [11–17]. The hydrophobicity-based separation

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https://doi.org/10.1016/j.microc.2025.116141
Received 6 August 2025; Received in revised form 5 November 2025; Accepted 9 November 2025
Available online 10 November 2025

mechanism in reversed-phase LC is widely recognized as complementary to the charge-to-hydrodynamic radius separation principle in CZE [20]. Typical LC-UV methods for bovine milk protein profiling employ wide-pore (e.g. 300 Å) C4, C8 or C18 columns (3.5–5 μm particle size) and acetonitrile:water mobile-phase gradients with 0.1 % (*v*/v) of trifluoroacetic acid (TFA) as an ion-pairing agent [11–17]. TFA is a strong acid that fully dissociates at low pH, forming ion pairs with protonated basic residues in proteins and protonating acidic ones, thereby increasing protein hydrophobicity and improving retention on reversed-phase stationary phases. Additionally, the protonation of acidic residues also enhances hydrophobic interactions, while suppression of surface silanols decreases undesired ionic interactions with the support [21,22]. These combined effects influence retention time, peak efficiency, and resolution. While these TFA-based LC-UV methods offer good analytical performance, CE-UV continues to provide superior resolution for certain proteoforms [3]. Consequently, there is growing interest in further enhancing the performance and applicability of chromatographic techniques for milk protein analysis.

An effective strategy to improve both CE-UV and LC-UV methods is the on-line coupling to electrospray ionization-mass spectrometry (ESI-MS) detection, which enables molecular mass (M_r) confirmation, detailed structural characterization, and accurate quantification [10,17–19]. However, the transition from UV to ESI-MS detection is not straightforward, as it requires careful optimization of background electrolytes and mobile phases to ensure compatibility with ESI-MS, particularly regarding volatility, low conductivity, and the absence of ion-suppressing agents [3,23]. We recently described a CE-MS method for bovine milk protein profiling that meets all these requirements [10]. In contrast, currently available LC-MS methods for this application still rely on TFA as a mobile phase ion-pairing agent [17–19]. TFA, however, is a strong ion suppressor that significantly reduces protein ionization efficiency and contributes to mass spectrometer contamination, thereby compromising analytical robustness and long-term instrument performance [22,23].

To overcome this limitation and align with best practices in LC-MS, we developed a novel TFA-free method for bovine milk protein profiling. In this approach, formic acid (HFor) is used in the mobile phase instead of TFA, providing a widely accepted alternative for reversed-phase LC-MS analysis of proteins and peptides [22,23]. The LC-MS analysis was conducted using an accurate mass, high-resolution time-of-flight (TOF) mass spectrometer, enabling the reliable identification and quantification of key bovine milk proteins from whey and casein fractions, including proteoforms with subtle structural differences [1,3,24,25]. Among these, the β-casein (β-CN) A1 and A2 genetic variants are particularly significant and have garnered considerable attention from the scientific community, largely due to growing consumer interest in A2A2 milk, commonly referred to as A2 [3,24,25]. These two β-CN variants differ by a single amino acid at position 67 (histidine in A1 and proline in A2). During digestion, β-CN A1 releases β-casomorphin-7 (BCM-7), a peptide linked to inflammatory and opioid-like effects. In contrast, β-CN A2 does not produce BCM-7, supporting its perception as a potentially healthier alternative [3,24,25]. Accurate differentiation of β-CN A1 and β-CN A2 requires reliable β-CN profiling techniques, which are essential for addressing milk intolerance concerns, as well as for supporting product differentiation, labeling, and fraud prevention [3,24,25]. To the best of our knowledge, the authentication of A2 milk currently relies on voluntary certification schemes based on herd genotyping and milk traceability, as no regulatory framework presently defines analytical procedures for verifying the β-CN composition of marketed dairy products. The method developed in this study fills an analytical gap by demonstrating excellent performance for comprehensive bovine milk protein profiling, enabling differentiation of milk from various β-CN genotypes (A1A1, A1A2, and A2A2) and quantification of low levels of contamination of A2A2 milk with A1A1 milk. It constitutes a valuable addition to the current milk protein analytical toolbox, offering a TFA-free LC-MS method for high-throughput, reliable, and efficient applications in dairy research, quality control, and authentication.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used in the preparation of buffers and solutions were of analytical reagent grade or better. HFor (99.0 %), TFA (99.0 %), sodium citrate dihydrate (≥99.0 %), urea (99.0–100.5 %), DL-dithiothreitol (DTT, 97 %), acetonitrile (ACN, LC and LC-MS grade), water (LC-MS grade), α-lactalbumin (α-LA, ≥90 %), and β-lactoglobulin (β-LG A-B, ≥90 %) from bovine milk were provided by Merck (Darmsatdt, Germany). Water was purified using a Milli-Q water purification system (Millipore, Molsheim, France). Samples of A1A1, A1A2, and A2A2 bovine milk were kindly provided by the Center for Innovation, Research, and Transfer in Food Technology (CIRTTA, Autonomous University of Barcelona, UAB). Raw whole milk was collected from morning milking of selected individual Friesian cows with the appropriate β-CN genotypes at a local dairy farm (La Cavalleria, Manlleu, Barcelona, Spain). Milk was cooled at 4 °C and transported to CIRTTA, where fat was removed by centrifugation. Commercial skim ultra-high temperature (UHT) A1A2 milk was purchased from a local supermarket.

2.2. Apparatus and procedures

pH measurements were performed using a Crison 2002 potentiometer, with a Crison electrode 52-03 (Crison Instruments, Barcelona, Spain). Centrifugal filtration at 25 °C was carried out in a cooled Rotanta 460 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany).

2.2.1. Sample preparation

Bovine milk contains 3 %–3.5 % (m/m) protein, of which caseins and whey proteins account for 80 % (m/m) and 20 % (m/m), respectively [7]. A schematic workflow diagram of the sample preparation procedure for the efficient and reproducible extraction of proteins is shown in Supplementary Fig. S1.

One mL of skim milk from a commercial A1A2 sample, from pure individual A1A1, A1A2, and A2A2 samples, or from intentionally contaminated A2A2:A1A1 mixtures (at ratios of 99:1, 95:5, 90:10, and 85:15 (*v*/v) prepared using the individual samples), was mixed with 5 mL of a reducing and denaturing buffer [7,10,26]. This buffer was prepared by dissolving dithiothreitol (DTT, 38 mg) and sodium citrate dihydrate (73 mg), in 37.5 mL of 8 M urea, and bringing the solution to a final volume of 50 mL with water in a volumetric flask. The mixture was incubated at room temperature for 1 h to facilitate casein micelle disruption and protein extraction. The resulting solution was sequentially filtered, first through a 0.20 μm nylon filter (Macherey-Nagel, Düren, Germany), and subsequently through a 3000 M_r cutoff (MWCO) cellulose acetate centrifugal filter (Amicon Ultra-0.5, Millipore, Bedford, MA, USA), as described in our previous study [10]. All centrifugation steps were performed at 13000 ×*g* and 25 °C. Specifically, prior to sample filtration, 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 clean vial and centrifuged at a reduced force (300 ×*g* for 2 min). The recovered sample (~75 μL) was then adjusted to a final volume of 100 μL by adding a solution of 27:73 *v*/v ACN:water with 0.1 % (*v*/v) TFA or HFor. After centrifugation at 13000 ×*g* for 2 min at 25 °C, the supernatant was collected for analysis. Samples were stored at −20 °C if not analyzed immediately.

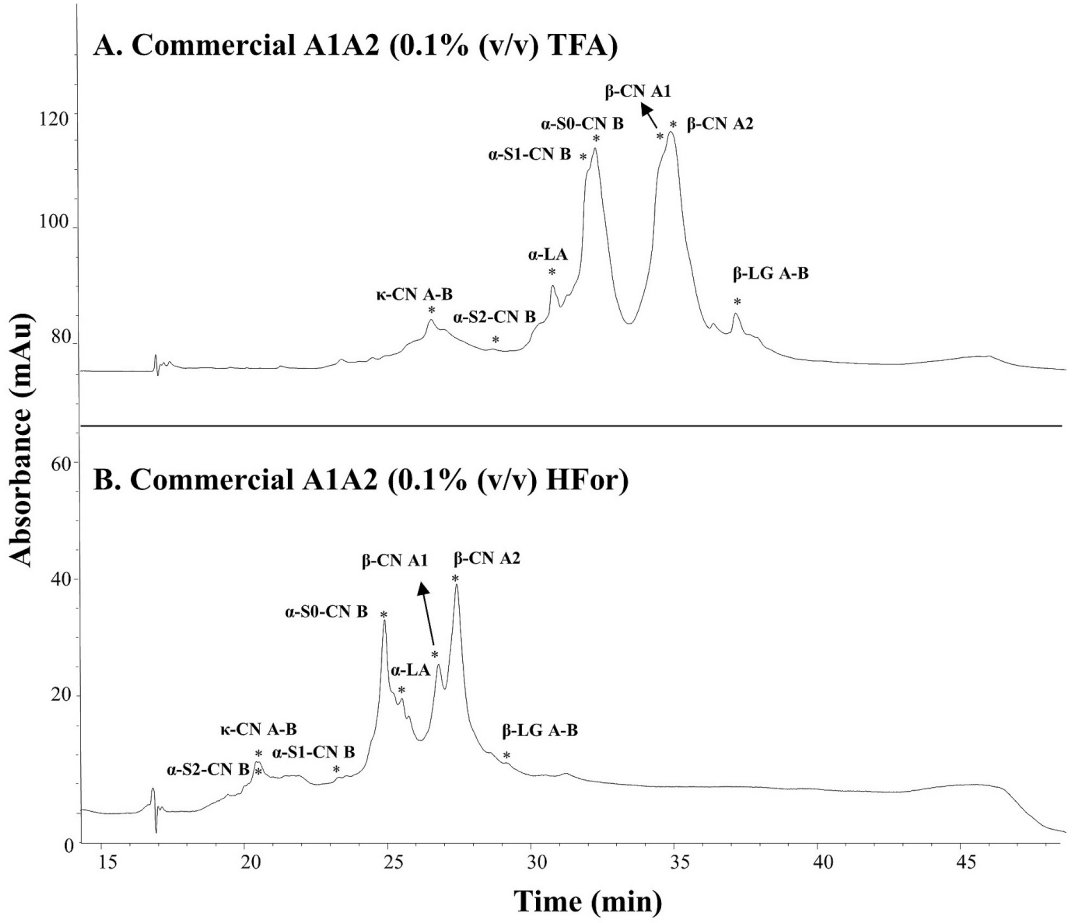

Fig. 1. Reversed-phase C4 LC-UV chromatograms at 200 nm for the analysis of a commercial A1A2 bovine milk sample using acetonitrile:water mobile gradients with A) 0.1 % (*v*/v) TFA and B) 0.1 % (v/v) HFor. The main casein and whey proteins were tentatively identified in the peaks labeled with an asterisk reviewing previous literature on TFA-based methods, analyzing spiked samples with standards (α-LA and β-LG A-B), and considering the subsequent LC-MS results obtained using the HFor-based method. β-casein A1 (β-CN A1), β-casein A2 (β-CN A2), α-lactalbumin (α-LA), β-lactoglobulin A and B (β-LG A-B), α-S0-casein B (α-S0-CN B), α-S1-casein B (α-S1-CN B), α-S2-casein B (α-S2-CN B), and κ-casein A and B (κ-CN A-B).

2.2.2. LC

2.2.2.1. LC-UV. The LC-UV analyses were conducted using an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany), equipped with an autosampler (1260 Infinity, G1329B), a binary pump (G1312A), and a UV-DAD detector (G1315B). Instrument control, data acquisition, and processing were managed using Chemstation LC3D software (Agilent Technologies). Chromatographic separation was performed using a reversed-phase bioZen™ WidePore C4 column (Phenomenex, Torrance, CA, USA) with a 2.6 μm particle size, 400 Å pore size, 150 mm length, and 4.6 mm inner diameter. This column, featuring core-shell particles with a butyl stationary phase, was selected for its wide pore size, which is recommended for improved resolution of high M_r compounds such as proteins. Additionally, the stationary phase's stability allows for the use of low-pH mobile phases containing TFA or HFor. The mobile phases consisted of (A) water with 0.1 % (*v*/v) HFor or TFA and (B) ACN with 0.1 % (v/v) HFor or TFA. Both were degassed by sonication before use (10 min). Chromatographic separation was achieved at room temperature using a linear gradient at a flow rate of 0.35 mL/min, with an injection volume of 0.5 μL. The gradient program of solvent B was as follows: from 27 % to 32 % over 2 min, from 32 % to 43 % over 7 min, from 43 % to 45 % over 7 min, from 45 % to 50 % over 5 min, and finally returning to 27 % for re-equilibration over 5 min. This gradient was adapted from the method described by Bobe et al. [12]. Our method allowed for the detection of the characteristic global fingerprint of the milk protein extract within a total runtime of 30–40 min. The UV signal was recorded at 200 nm at a rate of 2.5 Hz.

2.2.2.2. LC-MS. LC-MS analyses were performed at room temperature using the same LC equipment, sample injection volumes, and linear gradient composition, using water and ACN both with 0.1 % (*v*/v) HFor as mobile phases A and B, respectively. The LC instrument was coupled to a 6220 oaTOF LC/MS spectrometer (Agilent Technologies) with an orthogonal ESI interface. The mass spectrometer was operated under optimum conditions in positive ESI mode using the following parameters: drying gas temperature 350 °C, drying gas flow rate 8 $L{\cdot}min^{-1}$, nebulizer gas 40 psig, fragmentor voltage 325 V, skimmer voltage 80 V, OCT 1 RF Vpp voltage 250 V. Data were collected in profile at 1 $spectrum{\cdot}s^{-1}$ between 100 and 3200 *m/z*. Instrument control, data acquisition, and processing were performed using ChemStation C.01.06 and MassHunter B.06.01 software (Agilent Technologies).

3. Results and discussion

3.1. LC-UV

Current LC-UV methods for the analysis of bovine milk proteins commonly use reversed-phase columns and ACN:water mobile phases with TFA as the ion-pairing agent to improve protein retention and separation [11–17]. However, TFA is not recommended in best practices for LC-MS, as it can cause ion suppression in on-line ESI-MS, reducing detection sensitivity and contaminating the mass spectrometer for

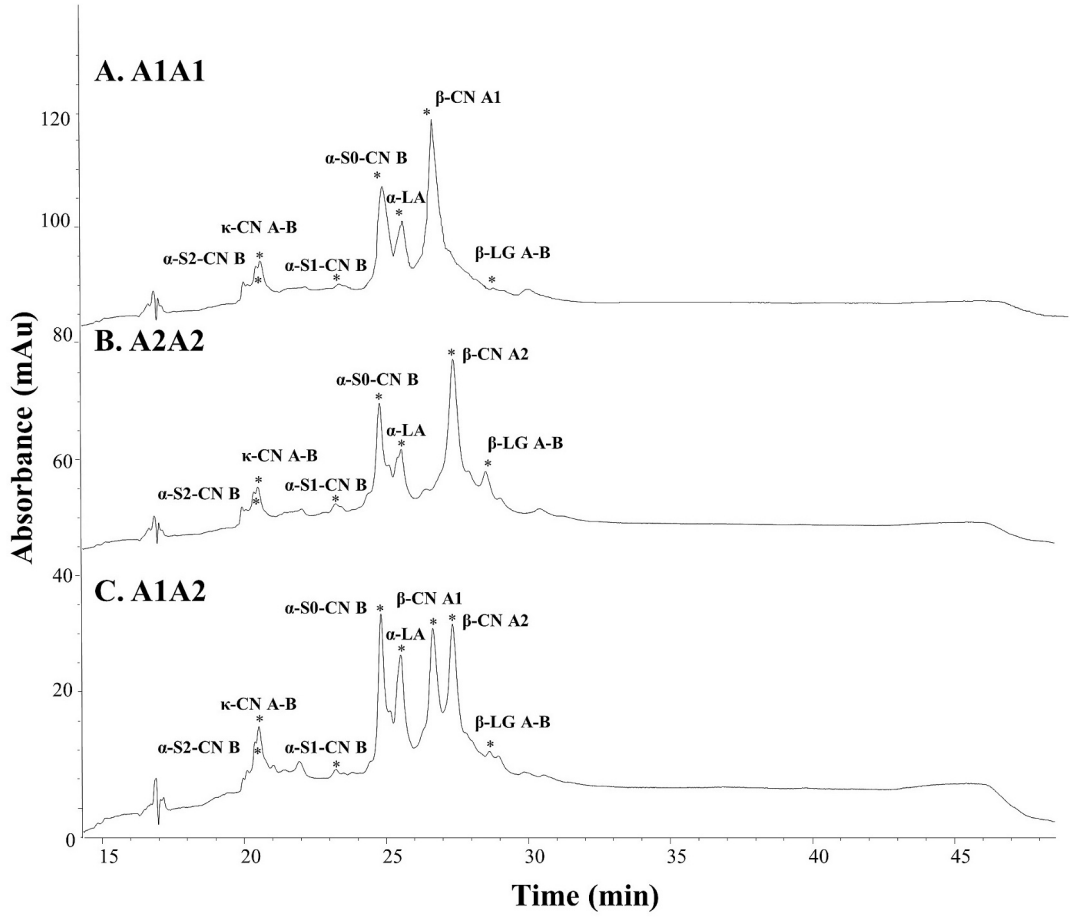

Fig. 2. Reversed-phase C4 LC-UV chromatograms at 200 nm for the analysis of individual bovine milk samples using an acetonitrile:water mobile phase gradient with 0.1 % (v/v) HFor. A) A1A1, B) A2A2, and C) A1A2. The main casein and whey proteins were tentatively identified in the peaks labeled with an asterisk reviewing previous literature on TFA-based methods, analyzing spiked samples with standards (α-LA and β-LG A-B), and considering the subsequent LC-MS results obtained using the HFor-based method. β-casein A1 (β-CN A1), β-casein A2 (β-CN A2), α-lactalbumin (α-LA), β-lactoglobulin A and B (β-LG A-B), α-S0-casein B (α-S0-CN B), α-S1-casein B (α-S1-CN B), α-S2-casein B (α-S2-CN B), and κ-casein A and B (κ-CN A-B).

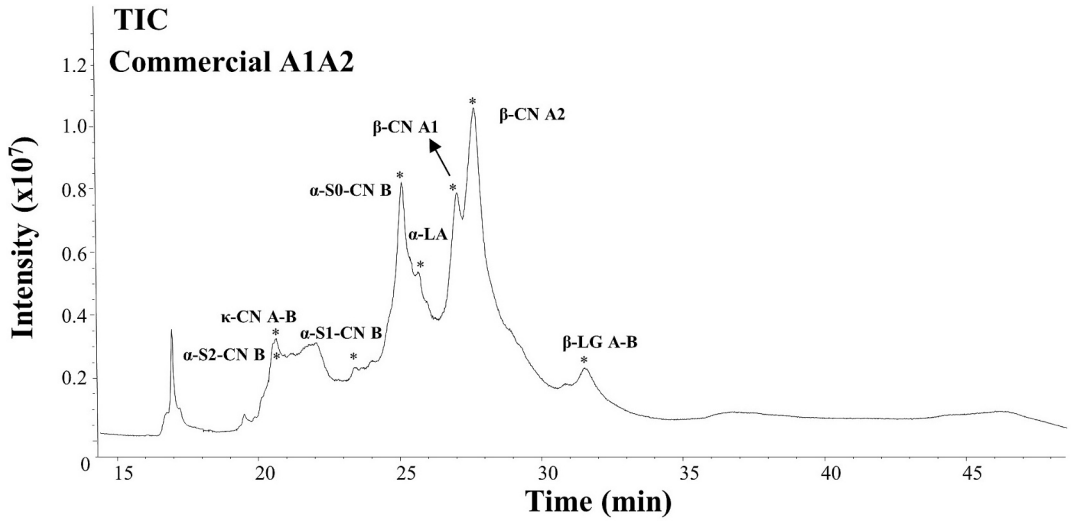

Fig. 3. Reversed-phase C4 LC-MS total ion chromatogram (TIC) obtained for the analysis of a commercial A1A2 bovine milk sample using an acetonitrile:water mobile phase gradient with 0.1 % (v/v) HFor. The main casein and whey proteins were identified in the peaks marked with an asterisk (*) based on the extracted ion chromatograms (EICs) shown in Fig. 4. β-casein A1 (β-CN A1), β-casein A2 (β-CN A2), α-lactalbumin (α-LA), β-lactoglobulin A and B (β-LG A-B), α-S0-casein B (α-S0-CN B), α-S1-casein B (α-S1-CN B), α-S2-casein B (α-S2-CN B), and κ-casein A and B (κ-CN A-B).

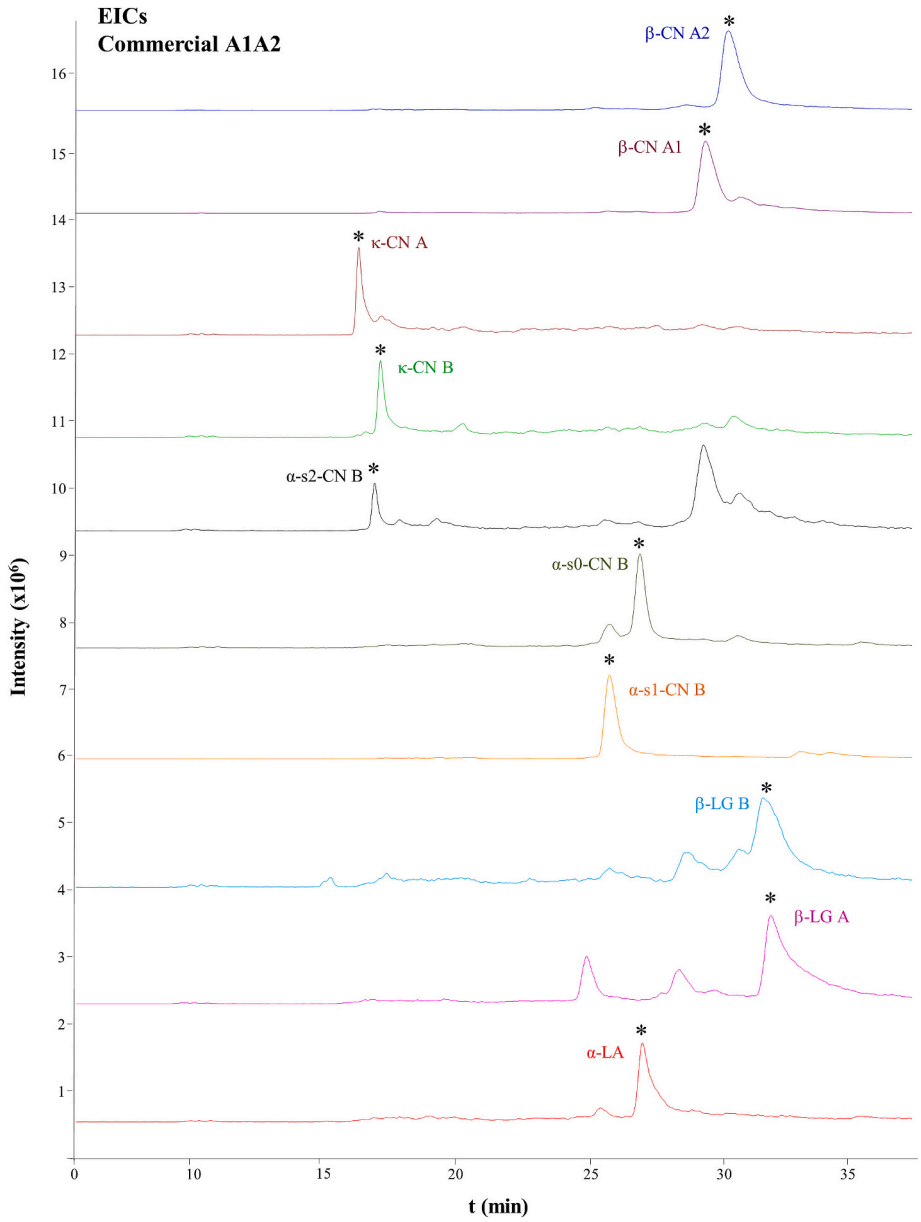

Fig. 4. Reversed-phase C4 LC-MS extracted ion chromatograms (EICs) for the main casein and whey proteins in the analysis of a commercial A1A2 bovine milk sample using an acetonitrile:water mobile phase gradient with 0.1 % (v/v) HFor. β-casein A1 (β-CN A1), β-casein A2 (β-CN A2), α-lactalbumin (α-LA), β-lactoglobulin A (β-LG A), β-lactoglobulin B (β-LG B), α-S0-casein B (α-S0-CN B), α-S1-casein B (α-S1-CN B), α-S2-casein B (α-S2-CN B), κ-casein A (κ-CN A), and κ-casein B (κ-CN B).

extended periods [23]. Despite this widely acknowledged limitation, to the best of our knowledge, currently described reversed-phase LC-MS methods for bovine milk protein analysis still employ ACN:water mobile phases with TFA [17–19]. This practice limits their widespread application, as analysts must use them at their own risk. These limitations prompted the development of a TFA-free LC-UV method using an alternative ion-pairing agent that is potentially compatible with on-line ESI-MS. Therefore, we replaced 0.1 % (*v*/v) TFA with 0.1 % (v/v) HFor, a widely accepted alternative for protein and peptide analysis in reversed-phase LC-MS. Fig. 1 compares LC-UV chromatograms at 200 nm of a commercial A1 A2 bovine milk sample analyzed using the same reversed-phase column, flow rate, and linear gradient of an ACN:water mobile phase containing 0.1 % (*v*/v) TFA (Fig. 1A) or 0.1 % (v/v) HFor (Fig. 1B). Bovine milk proteins were tentatively assigned to the different peaks of the complex profile reviewing previous literature on TFA-based methods [11–17], analyzing spiked samples with standards (α-LA and β-LG A-B), and considering the subsequent LC-MS results obtained using the HFor-based method. As observed, the HFor-based method yielded shorter retention times, as HFor is a weaker ion-pairing agent than TFA, resulting in less protein retention on the stationary phase (30 vs 40 min total analysis time, respectively) [21,22]. Regarding separation, the protein elution order remained the same excepting for α-LA and α-S2-casein (α-S2-CN) (see Fig. 1). In general, no major differences were observed in peak shape and resolution, except for β-CN A1 and β-CN A2 proteoforms, which were partially resolved. The repeatability of the TFA-free LC-UV method was evaluated by examining retention time and peak area of the peak corresponding to β-CN A1 + β-CN A2 in three replicates of the commercial A1A2 bovine milk sample. The %RSD values ($n = 3$) for retention times and peak areas were 0.5 and 1.5 %, respectively. These values indicated good repeatability, consistent with other established TFA-based LC-UV bovine milk protein profiling methods [11–17]. Fig. 2 illustrates that the developed TFA-free LC-UV method effectively distinguished between individual A1A1, A2A2, and A1A2 bovine milk samples. By adopting HFor in the mobile phase, we

Table 1
Average exact molecular mass (M_r), ion charge state range, repeatability of retention time and peak area for the proteins detected in a commercial A1A2 bovine milk sample using the TFA-free LC-MS method.

	Proteins	Exact M_r	Ion charge states	Repeatability (%RSD, $n = 3$)	
				Retention time	Peak area
Casein proteins	β-CN A2	23,982.95	19–24	0.01	0.92
	β-CN A1	24,022.97	19–24	0.31	1.27
	κ-CN A	19,037.18	9–14	0.95	0.03
	κ-CN B	19,005.23	9–14	0.13	0.07
	α-S0-CN B	23,694.46	17–22	0.16	0.42
	α-S1-CN B	23,614.48	17–22	0.37	0.99
	α-S2-CN B	25,228.10	19–24	0.17	0.02
Whey proteins	β-LG B	18,281.06	11–16	0.14	1.17
	β-LG A	18,367.15	11–16	0.57	0.79
	α-LA	14,185.96	11–16	0.34	1.09

not only developed a fully-compatible method with LC-MS, but we also achieved partial resolution of β-CN A1 and A2 proteoforms and enabled the potential detection of A2A2 milk contamination or adulteration with A1A1 or A1A2 milks. However, for reliable identification and quantification of the detected proteoforms, on-line ESI-MS detection using an accurate mass, high-resolution mass spectrometer was required.

3.2. LC-MS

The developed TFA-free LC-UV method was directly transferred to LC-MS using an accurate mass, high-resolution TOF mass spectrometer. Fig. 3 presents the total ion chromatogram (TIC) of a commercial A1A2 bovine milk sample. Proteins were assigned to the different peaks within the complex profile based on the extracted ion chromatograms (EICs) shown in Fig. 4. These EICs were generated by summing the contributions of the detected protonated molecular ions for each protein, whose charge states and calculated exact M_r values are listed in Table 1. Protein identification was confirmed unequivocally by deconvoluting the mass spectra of the peaks detected in the EICs of Fig. 4 to obtain their experimental accurate M_r values (typical mass errors (ΔM_r) for identification were lower than 1.75 mass units (data not shown). The information from Figs. 3 and 4, allowed the tentative identification of the protein peaks in the LC-UV profiles shown in Figs. 1B and 2. In LC-MS retention times were slightly increased (total analysis time: 35 min) due to the longer post-column path to the detector. This total analysis time was also longer than that of the TFA-based reversed-phase ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) method with a C4 column and an accurate mass, high-resolution Orbitrap mass spectrometer proposed by Fuerer et al. (total analysis time: 20 min) [19]. However, this UHPLC-MS method provided lower separation resolution between closely related proteoforms, such as β-CN A1 and A2. Compared to our previous CE-MS method for bovine milk protein profiling [10], which stood out for its reduced use of solvents, reagents, and sample volumes, the developed LC-MS method was simpler to implement. Unlike CE-MS using hydroxypropyl cellulose-coated capillaries, the present method could be performed on widely available LC-MS instrumentation and did not require lab-made separation columns, which are time-consuming to prepare and susceptible to batch variability. Additionally, it achieved significantly improved separation resolution between proteoforms, including β-CN A1 and A2, with only a modest increase in total analysis time (35 min versus 15 min). Overall, the developed LC-MS method proved to be well-suited for cost-effective, high-throughput, reliable, and efficient protein profiling of bovine milk, eliminating the drawbacks associated with TFA usage. Repeatability was assessed by evaluating retention times and peak areas of the detected proteins in three replicates of the commercial A1A2 bovine milk sample. The %RSD values ($n = 3$) for retention times and peak areas ranged between 0.01 and 0.95 % and 0.02–1.27 %, respectively, as shown in Table 1 These values demonstrated good repeatability, consistent with UV detection results. Although the method was developed for bovine milk, it could be readily adapted to milk from other species, such as goat or sheep milk, which share similar major protein families but differ in proteoform composition, lipid content, mineral balance, and viscosity. Minor adjustments in sample preparation and chromatographic conditions would likely ensure comparable analytical performance, as demonstrated in previous inter-species LC studies of milk [27,28].

To evaluate the sensitivity of the developed LC-MS method in detecting low levels of β-CN A1 contamination in A2A2 bovine milk, Fig. 5A and B show the combined EICs corresponding to β-CN A2 and β-CN A1 for (A) a pure A2A2 sample, and (B) the same sample after contamination with 5 % (*v*/v) of A1A1 bovine milk. As observed in the EIC of the contaminated sample (Fig. 5B (i)), a distinct peak corresponding to β-CN A1 appeared, significantly overlapping with the β-CN A2 peak. Fig. 5A and B (ii) and (iii) display the raw and deconvoluted mass spectra, respectively, corresponding to the time window containing the detected protein peaks in the EICs of Fig. 5A and B (i). In the deconvoluted mass spectrum of the pure A2A2 sample (Fig. 5A (iii)), small amounts of sodium and potassium adduct ions were found for β-CN A2. The inset tables in Figs. 5A ad B (iii) show that the deconvoluted M_r values of the (β-CN A2 + K) adduct closely matched that of β-CN A1, making them indistinguishable by MS. Indeed, the limited chromatographic resolution (Fig. 5B (i)), combined with the presence of (β-CN A2 + K) ions, hindered the accurate quantification of low levels of β-CN A1 based on EIC integration. To address this, the ratio of peak areas integrated from the deconvoluted mass spectra for [(β-CN A2 + K) + β-CN A1] and β-CN A2 was calculated using a pure A2A2 sample and A2A2 samples contaminated with 1 %, 5 %, 10 %, and 15 % (*v*/v) of A1A1 milk. Linearity was then assessed, revealing a linear relationship within the range of 1 % to 15 % (v/v) of A1A1 milk ($R^2 = 0.998$, Supplementary Fig. S2), which enabled sensitive and accurate quantification of low-level contamination (limit of quantification (LOQ) was 1 % (v/v) of A1A1 milk). The limit of detection (LOD) would be slightly below this LOQ, when the increase in peak area from β-CN A1 compared to the present (β-CN A2 + K) adduct would be detectable but minimal (Supplementary Fig. S2). For accurate quantification of higher proportions of A1A1 milk, linearity should be evaluated over an extended concentration range. However, no issues are expected given the demonstrated excellent selectivity of accurate mass, high resolution MS detection.

4. Conclusion

In this study, we developed a TFA-free reversed-phase LC-UV and LC-MS method to analyze bovine milk proteins with high efficiency and reliability. By replacing TFA with 0.1 % (*v*/v) HFor in ACN:water mobile phases, we established a method fully compatible with on-line ESI-MS, avoiding the ion suppression and long-term instrument contamination that TFA typically causes. The HFor-based LC-UV method allowed obtaining global profiles of major casein and whey proteins, with excellent repeatability. Notably, partial separation of β-CN A1 and β-CN A2 proteins enabled discrimination of A1A1, A2A2, and A1A2 milk. When transferred to accurate mass, high-resolution LC-MS, the method successfully confirmed protein identities and enabled extended sequences of repeatable analyses. The method also proved to be sensitive to quantify low levels of A2A2 milk contamination with A1A1 milk (<1 % (v/v)), but the use of deconvoluted mass spectra is recommended for a straightforward an accurate quantification. This novel TFA-free LC-MS method offers a simple, reliable, and high-throughput solution for profiling bovine milk proteins. It is well suited for dairy research, routine quality control, authenticity testing, and supporting A2 milk

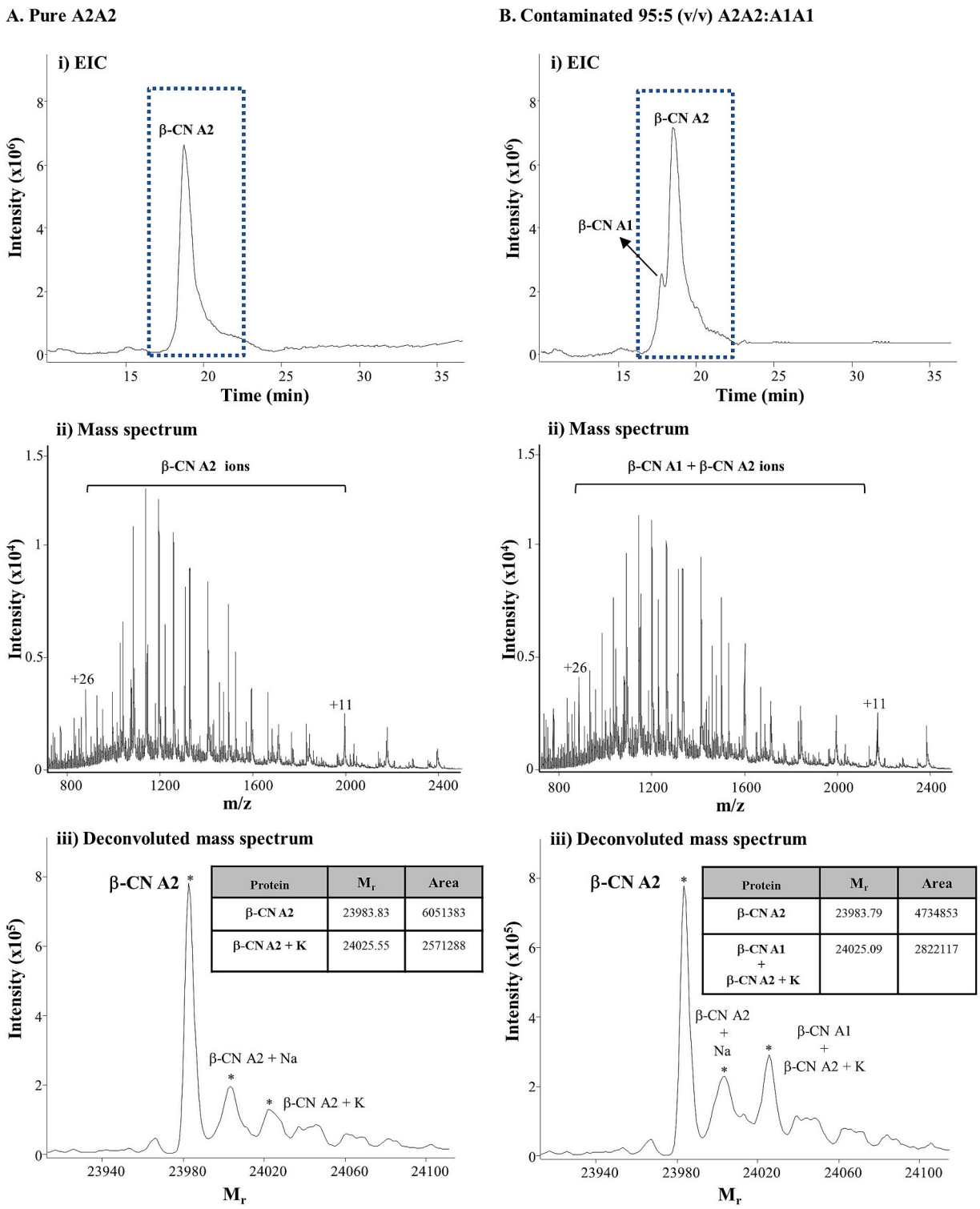

Fig. 5. Reversed-phase C4 LC-MS analysis using an acetonitrile:water mobile phase gradient with 0.1 % (v/v) HFor of A) a pure individual A2A2 bovine milk and B) the same milk contaminated with 5 % (v/v) individual A1A1 milk. (i) Extracted ion chromatograms (EICs) combining β-CN A1 and β-CN A2, (ii) raw mass spectra for the indicated time window in the EICs, and (iii) deconvoluted mass spectra (inset tables show the experimental accurate M_r and peak are values for the most relevant proteoforms).

product claims without contaminating the mass spectrometer with TFA. Additionally, it can be applied to the characterization of milk from other species.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2025.116141.

CRediT authorship contribution statement

Tahereh Tehrani: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **Laura Pont:** Writing – review & editing, Supervision, Investigation, Conceptualization. **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.

Acknowledgments

This research was supported by grants PID2021-127137OB-I00 and PID2019-110752RB-100 funded by MCIN/AEI/10.13039/501100011033 and by "ERDF A way of making Europe". The Bioanalysis group of the University of Barcelona (UB) is part of the INSA-UB Maria de Maeztu Unit of Excellence (Grant CEX2021-001234-M) funded by funded by MICIN/AEI/FEDER, UE.

Data availability

Data will be made available on reasonable request

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