ANALYSIS OF MILK INTACT PROTEINS USING LC/MS

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ABSTRACT

Cow's milk is a complex fluid whose proteome displays a diverse set of proteins of high abundance such as caseins (CNs) and medium to low abundance whey proteins such as beta-lactoglobulin (bLG), alpha-lactabumin (aLA), lactoferrin, immunoglobulins, glycoproteins, peptide hormones and enzymes. CNs represent 80% of cow's milk proteins and possess important nutritional and functional properties, whilst bLG (largely unknown function) and aLA (involved in lactose biosynthesis) represents approximately 10 and 4%, respectively. In addition, a variety of post-translational modifications of these proteins introduces further complexity to the composition of the milk proteome. As genetic variants of CNs and whey proteins influence many properties of milk that are essential to the dairy industry, the development of robust analytical methods for the separation and quantification of variants of milk proteins within single protein fractions is of interest.

This work aims at establishing a robust LC-MS-based method to study intact major proteins from cow's skim milk. The effects of column temperature, mobile phase composition, and gradient conditions were evaluated to improve protein separation prior to analysis using ESI-Qq-TOF MS (data not shown). High resolution spectra subsequently allowed accurate mass determination and top-down sequencing (TDS) of major proteins. This LC-MS workflow enables a high-throughput profiling of abundant proteins of cow's milk with minimal sample preparation.

MATERIALS AND METHODS

Sample preparation

The milk proteins standards (aCN C6780 70%pure, bCN C6905 98% pure, kCN C040670% pure, aLA type I L538585% pure, bLG L3908 90% pure, and BSA A7906 98% pure) were purchased from Sigma. 10 or 5 mg of each standard was resuspended in 0.5mL milliQ H_2O and 0.5mL Solubilisation Buffer (SB: 0.1M Bis-Tris buffer, 6M Guanidine-HCl, 5.37mM sodium citrate tribasic dihydrate, and 20mM DTT). Standards were vortexed for 1min and incubated at room temperature for 50min. Serial dilutions were performed in SB to assess LOD and LOQ.

Bulk milk aliquots from Jersey and Holstein cows were collected on farms in Victoria, Australia and were analysed in duplicates. Full cream milk was skimmed by centrifugation at 4500rpm for 30min at 4°C. A 0.5mL aliquot of skim milk was diluted in 0.5mL SB. Milk samples were vortexed for 1min and incubated at room temperature for 50min. A 20uL of 50% acetic acid/H_2O was added and homogenised to lower the pH.

HPLC separation

HPLC instrument: UHPLC Agilent 1290 Infinity Binary LC
Separation column: Phenomenex Aeris™ 3.6 µm WIDEPORE XB-C8 200 Å, LC Column 150 x 2.1 mm. RP core-shell silica.
Injection volume: 3µL
DAD signals: 214 and 280 nm
HPLC duration: 40min
Temperatures: 75°C
Flow rates: 200µL/min
Mobile phases: A: 0.02%TFA/0.1%FA/H_2O;
B: 0.02%TFA/0.1%FA/ACN
Solvent gradient: starting conditions 20%B, ramping to 28%B in 2.5min, ramping to 40%B in 27.5min, ramping to 99%B in 1min, isocratic at 99%B for 4min, ramping to 20%B in 0.1min, equilibration at 20%B for 9.9 min.

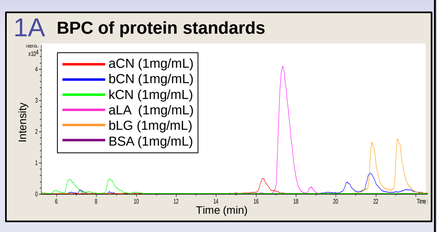
MS analysis

MS instrument: Bruker maXis classic ESI-Qq-TOF (40,000 resolution) with ESI source
Ion polarity: positive
Capillary voltage: 4500V
Time segments: 2.5min (Na-formate infusion) + 37.5min (HPLC).
Mass range: 600-3000 m/z with 15000 summations
Collision energy for MS/MS fragmentation (ISCID): 90eV
Post-processing: Bruker DataAnalysis (Maximum Entropy and SNAP algorithms for protein deconvolution) and SequenceEditor/Biotools (for TDS based on MS/MS data)

RESULTS (1)

Ionisation, LOD and LOQ of milk protein standards

The LC-MS analysis of milk protein standards showed little co-elution **(Fig. 1A)**. The ionisation efficiency varied across standards **(Fig. 1A)**, yet it was not concentration-dependent **(Fig. 1B)**. The limit of detection (LOD) ranged from 0.28 (bLG) to 1.35 (ACN) mg/mL and the limit of quantitation (LOQ) ranged from 0.86 (bLG) to 4.09 (aCN) mg/mL **(Fig. 1B)**.



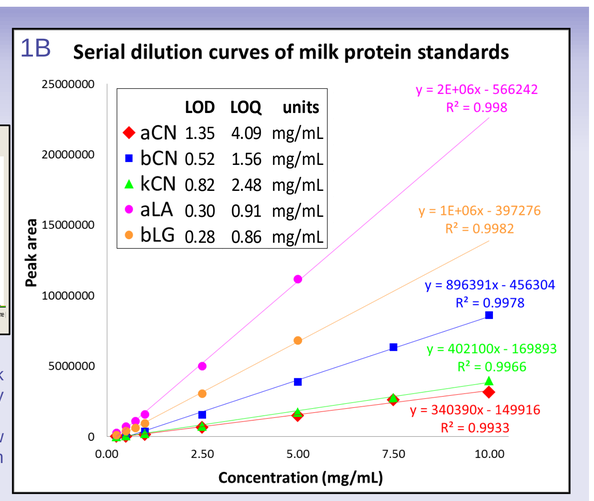


Figure 1:
(A) Base Peak Chromatograms (BPCs) of milk standards at the same concentration (1mg/mL) vary in ionisation efficiency (aLA>bLG>aCN=bCN>kCN).
(B) Serial dilution curves of milk standards show linear responses (R^2>0.993) indicating that ionisation is not concentration-dependent.

RESULTS (2)

Deconvolution and quantitation of standards and milk proteins

Mass spectrum were isotopically resolved **(Fig. 2A)** which allowed for accurate monoisotopic mass determination by deconvolution followed by SNAP annotation of the standards **(Fig. 2A,B)** and milk proteins. Each standards eluted several peaks **(Fig. 3A)** which were successfully deconvoluted into variants and quantified **(Fig. 3B)**. Milk samples were reproducible and produce 14 peaks **(Fig. 3C)** whose abundance varied across breeds **(Fig. 3D)** .

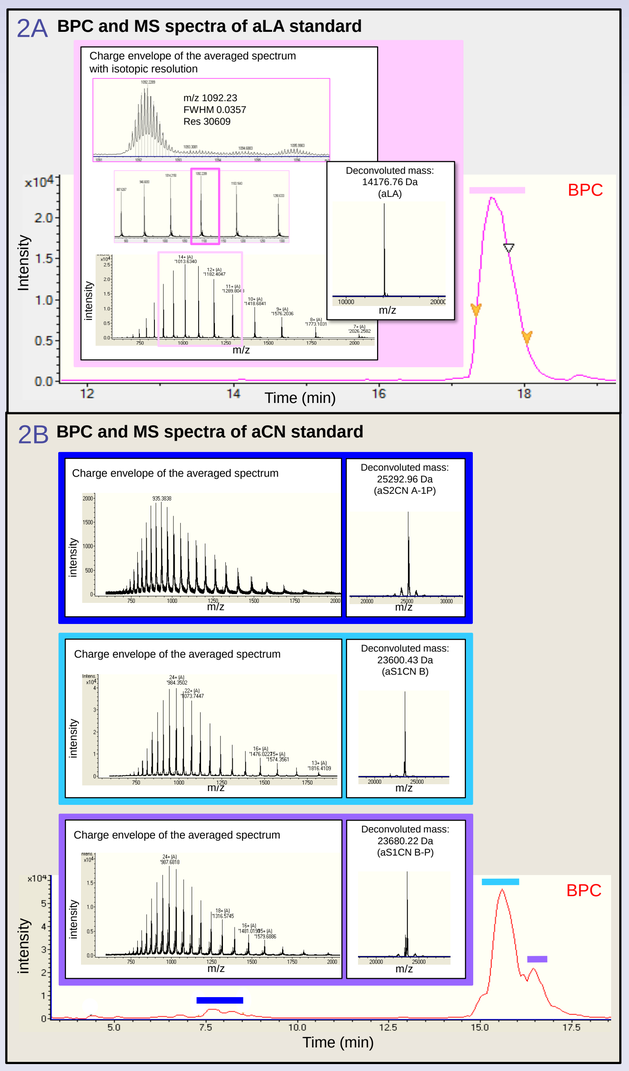


Figure 2: Illustration of mass spectrum deconvolution to determine accurate mass on aLA (A) and aCN elution peaks (B). Isotopic resolution was obtained for all the standards except for BSA (not shown).

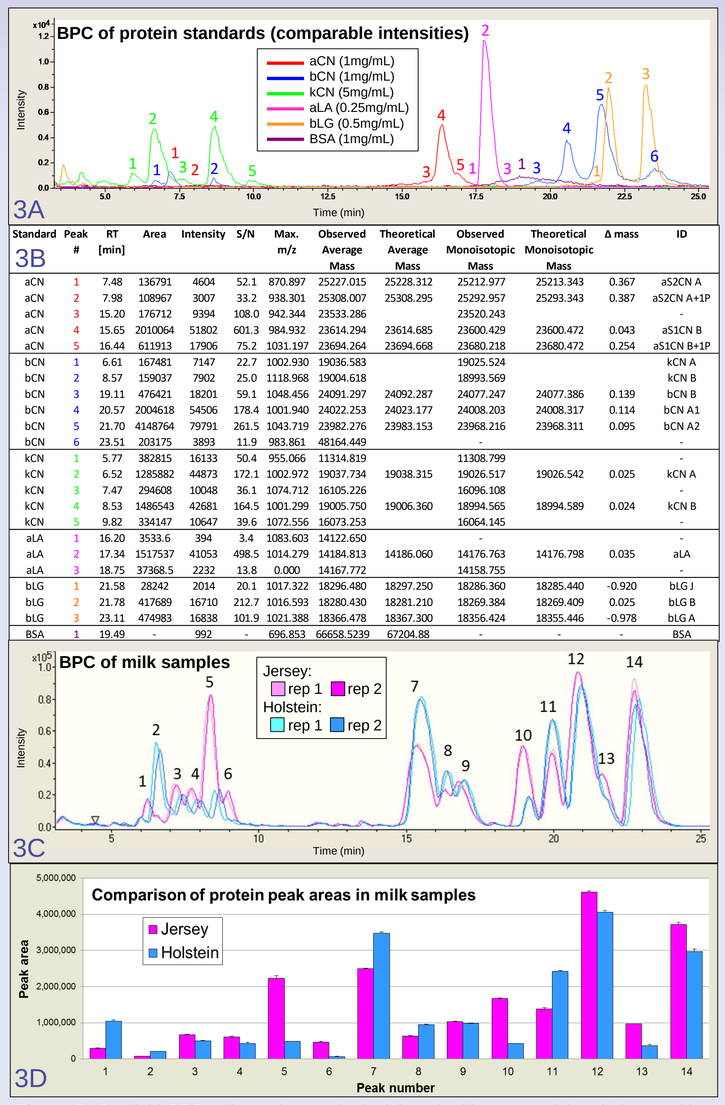


Figure 3: Overlaid BPCs of protein standards (A) and table summarising their quantities and deconvoluted masses (B). Overlaid BPCs of milk sample duplicates (C). Protein quantitation is based on peak areas and shows variation across breed (D).

RESULTS (3)

Top-Down Sequencing

MS/MS fragmentation using in-source cell-induced-collision (ISCID) was successfully obtained on standards, such as aCN **(Fig. 4A)** and milk proteins. Using a-, y-, and b-ion series, sequence tags were produced at both C- and N-termini using BioTools software of SNAP annotated spectra, as illustrated for aCN **(Fig. 4B, C)**.

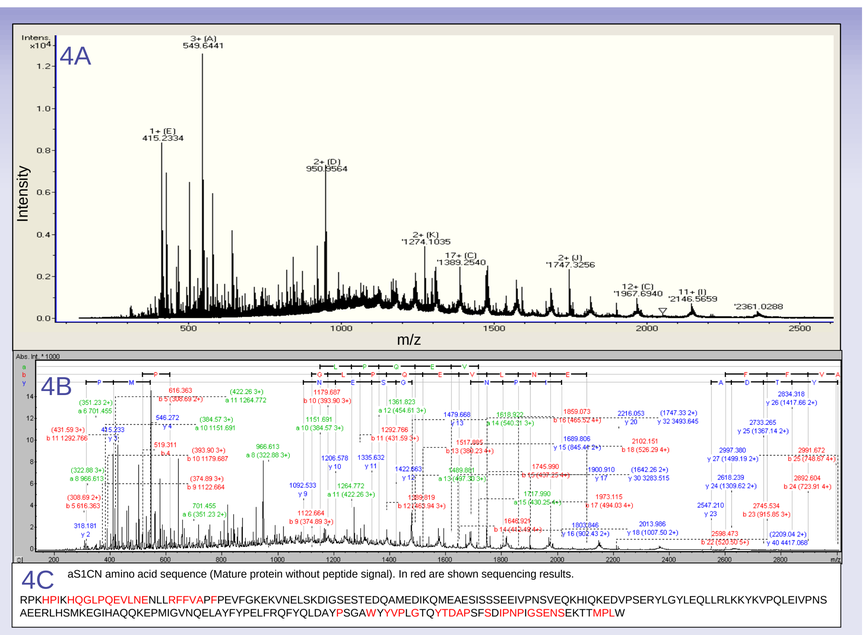


Figure 4: MS/MS analysis of aS1CN. (A) Fragmentation pattern. (B) Identified peptide sequence tags; a-ions are in green, b-ions are in red, and y-ions are in blue. (C) Amino acid sequence of aS1CN with identified peptide sequence tags highlighted in red.

CONCLUSIONS

In this study, we were able to perform quantitative MS and MS/MS analyses of proteins from Sigma standards and cow's milk samples. MS spectra were of high resolution and accurate protein masses were obtained, thus identifying milk protein isoforms. Top-down sequencing results achieved partial amino acid coverage of C- and N-termini for protein isoform characterisation. This method will be applied in a high-throughput fashion to hundreds of milk samples in order to better understand the dynamic nature of the milk proteome, with potential applications for milk quality and processing (e.g. cheese, health).

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