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Research article

Quantitative LC-MS/MS analysis of high-value milk proteins in Danish Holstein cows

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ABSTRACT

High-value milk proteins, which can be obtained by optimized fractionation procedures, are ideal ingredients in many food applications. Thus, a simple and robust analytical method is required for the identification and quantification of these individual milk proteins. Here, we present a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method using multiple reaction monitoring (MRM) to simultaneously detect and measure target peptides of two major milk proteins, α-lactalbumin (α-LA) and β-casein (β-CN), in raw milk samples from 662 Danish Holstein cows. The MRM quantification of α-LA and β-CN was achieved with limit of detection (LOD) of 0.14 and 0.16 g/L, respectively and reproducibility of the assay <15%. By this newly established MRM-based method, the concentration of α-LA and β-CN in an individual cow's milk ranged from 0.5 to 1.9 (average 1.1) g/L, and from 7.5 to 23.4 (average 15) g/L, respectively. There was no significant effect of parity, whereas significantly increasing concentrations of α-LA and β-CN were observed through lactation ($P < 0.001$). This shows a considerable biological variation of these two ingredient milk proteins, providing potential varying outputs of fractionation in the dairy streams.

1. Introduction

High-value milk proteins, including bioactive proteins or proteins with specific functionality, are a growing market for specialized milk additives and ingredients. These high-value proteins can be of benefit to human health or have other specific functionalities in food applications. For example, α-lactalbumin (α-LA), which comprises 20–25% of the total whey protein in cow's milk (Farrell et al., 2004), is added to infant formula in order to balance the composition towards human milk. Further, α-LA assists the absorption of minerals, as well as having antimicrobial and antitumor activities (Yadav et al., 2015). Furthermore, β-casein (β-CN), which accounts for one-third of the protein in bovine milk, is involved in the transport and absorption of important nutrients (Kamiński et al., 2007). β-CN is a great source of bioactive peptides, showing physiologically beneficial effects such as immune modulation, mineral binding, opioid agonism, thrombin inhibition, antioxidant capacity, and reduction of blood pressure through angiotensin 1-converting enzyme (ACE) inhibition (Silva and Malcata, 2005; Korhonen and Pihlanto, 2006).

The development of robust and reliable quantitative methods for these bioactive and/or functional proteins benefits both research and industrial applications. Bonfatti, Di Martino, Cecchinato, Vicario and Carnier (2010) used reversed-phase HPLC to measure relative and absolute contents of the major proteins in milk from Simmental cattle. The average content of α-LA and β-CN was reported to be 1.25 and 12.99 g/L, respectively (Bonfatti et al., 2010). This HPLC or UV-based approach could be restricted in quantifying co-eluting or low-abundance proteins; as well, it greatly relies on the purity of protein standards. In other words, quantitative protein analysis using MS-based quantification of target peptides is considered a better approach due to its high sensitivity and specificity, allowing both identification and quantification of multiple peptides in a complex sample such as milk. Multiple or selected reaction monitoring (MRM or SRM) has been applied to the quantification of lactosylated milk proteins, mostly at relative concentration (Le et al., 2013). Nevertheless, there have been a number of studies published on absolute quantification of either milk bioactive peptides (Asledottir et al., 2017; Asledottir et al., 2018), human milk proteins (Chen et al., 2016) or cow's milk proteins in processed dairy products (Lutter et al., 2011). Recently, the application of MRM for the absolute and simultaneous quantification of twenty bovine milk proteins was reported (Bär et al., 2019). In this work, quantitative levels of these proteins were determined

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from twelve individual raw cow's milk samples, in addition to one raw cream, one Emmenthal cheese and one sweet whey sample.

The aim of the current study was to develop an LC-MRM-MS method for the specific quantification of α-LA and β-CN in raw milk and apply this method to a large number of milk samples from 662 individual Danish Holstein cows to ascertain natural biological variation. The overall aim of the present project is to provide knowledge of possible variation in the content of high value proteins in cow's milk in relation to its fractionation and possible outputs therefrom, process control and yield calculations, thereby ensuring the optimal value proposition of milk in the global ingredient market.

2. Materials and methods

2.1. Chemicals and reagents

α-LA standard (≥85% purity) from bovine milk, modified porcine trypsin (proteomics grade), iodoacetamide, dithioerythritol (DTE), and triethylammonium bicarbonate (TEAB) buffer (1M) were obtained from Sigma-Aldrich Inc. (Steinheim, Germany). LC-MS grade acetonitrile (ACN) and formic acid (FA) were obtained from Merck (Damstadt, Germany) and Honeywell Fluka (Roskilde, Denmark), respectively. β-CN standard (>90% purity) was purified from bovine milk according to Petrat-Melin et al. (2015). The purity relative to total protein was determined by LC/ESI-MS. The peptide standards (LDQWLC[CAM]EK, VGINYWLAHK, VLPVPQK and AVPYPQR) and isotope labelled peptides as internal standards (ISTD) (LDQWLC[CAM]EK*, VGINYWLAHK*, VLPVPQK* and AVPYPQR* with the asterisk representing isotope labelled C13 and N15 at K or R) were purchased from Thermo Fisher Scientific (Biopolymers, Ulm, Germany) as AQUA Ultimate. Purity of these peptides was above 97% as in the certificates.

2.2. Milk samples

Morning milk samples were collected from 663 Danish Holstein cows in 21 herds as described by Poulsen et al. (2012) and Gebreyesus et al. (2017). All milk-collecting procedures followed the protocols approved by the National Guidelines for Animal Experimentation and the Danish Animal Experimental Ethics Committee. All sampling was restricted to routine on-farm procedures that did not cause any inconvenience or stress to the animals, and hence, no specific permission was required.

At sampling, cows were at different stages of lactation (days 4–877 in milk) and parities (1–6): P1 = parity 1 (n = 262), P2 = parity 2 (n = 243), P3 = parity 3 (n = 141), P4 = parity 4, 5, and 6 (n = 16). Immediately after sampling, milk samples were aliquoted and skimmed by centrifugation for 30 min at 2,643 × *g* at 4 °C. Skim milk samples were stored at -40 °C until further analysis.

2.3. In-solution tryptic digestion

For α-LA and β-CN standards: Approximately 1 mg of protein was dissolved in 1 mL of 40 mM TEAB, pH 8, to obtain a 1 mg/mL protein solution. Addition of 1.5 μL of a reducing agent (20 mg/mL DTE in 40 mM TEAB, pH 8) to 20 μL of the protein solution was followed by incubation for 30 min at 60 °C. Protein thiol groups from the reduced solution were then alkylated with iodoacetamide: 1.5 μL of 50 mg/mL iodoacetamide in 40 mM TEAB, pH 8, was added to the reduced solution, followed by incubation for 30 min at 37 °C in the dark. The solution was digested with 20 μL of trypsin (10 μg/mL in 40 mM TEAB, pH 8) and incubated at 37 °C for 16 h. The digests were acidified by 7.5% formic acid (FA) to obtain a final concentration of 1% FA before LC-MS/MS analysis.

For skimmed milk samples: 0.6 μL of skim milk was dissolved in 19.4 μL of 40 mM TEAB, pH 8. The reduction, alkylation, digestion and acidification steps were the same as described for α-LA and β-CN standards.

2.4. Full scan LC-MS/MS analysis

The acidified digests of α-LA and β-CN standards were centrifuged at 10,000 × *g* at 10 °C for 5 min, then diluted 5-fold in 5% ACN and 0.1% FA. The diluted digests were loaded into an Aeris Peptide C18 column of dimensions 250 mm × 2.1 mm, with a particle size of 3.6 μm (Phenomenex, Torrance, CA, USA) of an Agilent LC 1200 series directly connected to an HCT Ultra ion trap (Bruker Daltonics, DE). The LC separation was achieved using a mobile phase of LC-MS grade water with 0.1% FA (A) and 90% ACN with 0.1% formic acid (B). Initial mobile phase conditions were 2% B, which was held for 5 min before increasing to 40% over 80 min. The flow rate was 200 μL/min, the column was maintained at 40 °C and the injection volume was 10 μL. The mass scan for MS mode was from 400 to 1800 *m/z* and MS/MS mode was from 200 to 1200 *m/z*. Spectra were collected and analysed by Data Analysis and Biotools software (Bruker Daltonics, DE). The data were searched against an in-house Mascot database, particularly modified for bovine milk proteins and including genetic variants of the major milk proteins (Rauh et al., 2014), with the following settings: enzyme (trypsin), missed cleavage (2), variable modification (oxidation of M) and fixed modification (carbamidomethylation of C). MS mass tolerance was 0.1%, and an MS/MS mass tolerance was 0.5 Da. Peptide hits above the Mascot score significance threshold (equivalent to $p < 0.05$) were accepted.

2.5. Multiple reaction monitoring (MRM)

The acidified digests were centrifuged at 10,000 × g at 10 °C for 5 min and diluted 50-fold in 5% ACN and 0.1% FA. A mixture of ISTD with the concentration of 25 fmol/μL were spiked into the calibration standard solutions (12.5, 25, 50, 75, 125 and 250 fmol/μL) and the diluted digests. The solutions were then analyzed on a 1260 Infinity LC system (Agilent Technologies, Waldbronn, DE) coupled to a 6460 Triple Quad (QQQ) MS (Agilent Technologies, Waldbronn, DE). Peptides generated from the tryptic digestion were separated on a Zorbax Eclipse Plus C18 column (2.1 mm × 50 mm, 1.8 μm, Agilent) at 45 °C. The mobile phases contained (A) 0.1 % FA in Milli-Q water and (B) 90 % ACN in Milli-Q water with 0.1 % FA at a flow rate of 550 μL/min. The gradient was as follows: 0–40 % B over 18 min and increasing to 80 % B over 5 min. The injection volume was 10 μL. The 6460 QQQ was operated in MRM mode with optimal dwell time, fragmentor (F) and collision energy (CE). A list of Q1/Q3 masses of the four peptides from α-LA and β-CN (Table 1) was submitted for data acquisition. Quantification was based on the calibration curve with x axis as the standard concentration and y axis as the ratio of peak area of the analyte to the ISTD; this was performed by MassHunter Quantitative Analysis software (Agilent Technologies, Waldbronn, DE). The samples were measured in duplicate.

2.6. Method validation

The developed MRM method was tested for linearity, sensitivity, precision, accuracy, matrix effect, recovery and reproducibility. The linearity was determined by constructing a calibration curve using six different concentrations of peptide standards. Residual standard deviation (RSD) of the response and the slope of the calibration curve was used to estimate limit of detection (LOD) and limit of quantification (LOQ) of the method as Eqs. (1) and (2):

$$\mathrm{LOD} = 3.3(\mathrm{RSD}/\mathrm{Slope}) \quad \text{(eq.1)}$$

$$\mathrm{LOQ} = 10(\mathrm{RSD}/\mathrm{Slope}) \quad \text{(eq.2)}$$

Standards at estimated LOD and LOQ levels were checked for the signal-to-noise ratio about 3 and 10, respectively. Precision (%CV) and accuracy (%bias of theoretical value) of the method was assessed by analysing 6 calibration standards within a day (intra-day) or in 6 consecutive days (inter-day). The matrix effect (ion suppression/enhancement) was determined for each peptide by comparison of the MS

Table 1. Selected peptide sequences, their respective peptide transitions and the optimal fragmentor (F) and collision energy (CE).

Protein	Peptide sequence	M	Charge	Q1	Q3	F (V)	CE (eV)
α-LA	LDQWLC[CAM]EK[a]	1090.5	2	546.2	735.4 (y5)	105	13
	LDQWLC[CAM]EK[a]	1090.5	2	546.2	863.5 (y6)	105	13
	LDQWLC[CAM]EK[a]	1090.5	2	546.2	978.5 (y7)	105	13
	VGINYWLAHK	1199.6	3	400.9	468.0 (y4)	95	10
	VGINYWLAHK	1199.6	3	400.9	654.4 (y5)	95	10
	VGINYWLAHK	1199.6	3	400.9	817.6 (y6)	95	10
β-CN	VLPVPQK	779.5	2	390.8	372.2 (y3)	75	12
	VLPVPQK	779.5	2	390.8	471.3 (y4)	75	12
	VLPVPQK	779.5	2	390.8	568.3 (y5)	75	12
	AVPYPQR[b]	829.4	2	415.7	400.2 (y3)	75	12
	AVPYPQR[b]	829.4	2	415.7	563.3 (y4)	75	12
	AVPYPQR[b]	829.4	2	415.7	660.4 (y5)	75	12

[CAM]: carbamidomethylation.
a,b: peptides used for protein quantification.

response of a spiked blank sample (final solvent mix ready for injection, i.e. 5% ACN and 0.1% FA) to a spiked and non-spiked milk sample digest, according to the following Eq. (3):

$$\text{Matrix effect } (\%) = \left(\frac{\text{Spiked digest - Nonspiked digest}}{\text{Spiked blank}} - 1\right) \text{x } 100 \quad \text{(eq.3)}$$

The samples were spiked with standard peptide mix at concentration 50 fmol/μL. The response was calculated as the peak area of the quantitative ion monitored for each peptide.

The recovery of the peptides (digestion efficiency) was studied by spiking a pooled milk sample (spiked before) and the pooled milk sample digest (spiked after) with ISTD mix at concentration 50 fmol/μL. The spiked before samples were analysed after trypsin digestion. The peptide recovery was calculated by the following Eq. (4):

$$\text{Peptide recovery } (\%) = \frac{\text{Peak area of ISTD spiked before}}{\text{Peak area of ISTD spiked after}} \text{ x } 100 \quad \text{(eq.4)}$$

In addition, "pre-digestion recovery" was determined by spiking α-LA and β-CN standards to TEAB buffer at concentration 1 mg/mL; the spiked solution was digested with trypsin before analysis. The recovery of the proteins (pre-digestion recovery) was studied by the ratio of MRM-measured protein to the spiked amount (e.g., 1 mg/mL) of protein standards before digestion.

The reproducibility of the assay was assessed on 10 individual skimmed milk samples which were digested on three separate days. The mean of the resulting peak areas for each peptide across triplicate analyses was used to calculate the percent coefficient of variation (%CV), hence reproducibility. The completion of trypsin digestion was also determined by monitoring target peptides at various digestion time points (4 and 16 h) and trypsin-to-protein ratios (1:20, 1:50 and 1:100).

2.7. Statistical analysis of the data

For effect of parity and days in milk (DIM), the following Eq. (5) was used in the analysis:

$$Y_{ijk} = \mu + \text{parity}_i + \text{DIM}_j + e_{ijk} \quad \text{(eq.5)}$$

Where Y_{ik} is the phenotype of animal k; μ is the overall mean of the trait and parity is a fixed effect (i = 1, 2, 3, 4) and DIM is a covariate of days in milk (d4 to d450, excluding DIM >450 days, n = 16). Correlation between α-LA and β-CN was determined using Pearson correlation tests. All tests were performed in R (version 3.5.0).

3. Results and discussion

3.1. Selection of target peptides and MRM transitions

The protein quantification of α-LA and β-CN in milk samples was based on quantitative LC-MRM-MS analysis of their target peptides. Target peptides of α-LA and β-CN were selected based on full scan MS/MS experimental data obtained from the ion trap. This step was done to check the signal intensity of precursor ions (Q1) and product ions (Q3) to assure maximal selectivity and sensitivity for the performance of an MRM experiment by a triple quadrupole (QQQ) MS.

Based on this initial full scan MS/MS screening experiment, a total of four target peptides from α-LA and β-CN (two for each protein) were selected (Figures 1A and 2A). Each chosen peptide fulfilled the criteria of being unique to its protein origin and having a relatively high MS signal response. These peptides have no methionine and contain 7-10 amino acid residues in their sequences. As can be seen, the two peptides LDQWLC[CAM]EK with *m/z* 546.2 ($[M+2H]^{+2}$) and VGINYWLAHK with *m/z* 600.8 ($[M+2H]^{+2}$) and *m/z* 400.9 ($[M+3H]^{+3}$) selected for α-LA display the most intense signals in the MS spectrum (Figure 1A). The peak with *m/z* 693.5 also presents an intense signal but was identified as pyroCys at the N-terminus of CEVFR, which could not be selected due to its short length and instability. As the triply-charged peptide (*m/z* 400.9) gave higher signal response than the doubly-charged (*m/z* 600.8) (Figure 1A), *m/z* 400.9 was selected for the final MRM transition list (Table 1).

In contrast, the two peptides representing β-CN, VLPVPQK with *m/z* 390.8 ($[M+2H]^{+2}$) and AVPYPQR with *m/z* 415.7 ($[M+2H]^{+2}$), did not display the highest MS signal intensity (Figure 2A), but are unique for β-CN; these peptides were also selected for the quantification of β-CN in the study of Lutter et al. (2011). Although one of the peptides selected for β-CN, VLPVPQK, may be subjected to genetic variation (e.g., in genetic variants A1 and G, the "Q" is replaced with an "E") as reviewed in UniProt, the 1 Da difference in mass between VLPVPQK and VLPVPEK peptides (0.5 Da difference for their doubly charged ions) does not affect the results. Because QQQ is known as a mass spectrometer with low resolution mass/charge (the usual resolution is 0.7–1 Da), it is unable to differentiate peptides with "Q" converted to "E" and vice versa. Undoubtedly, both peptides, if present, would be quantified by the QQQ. The frequency

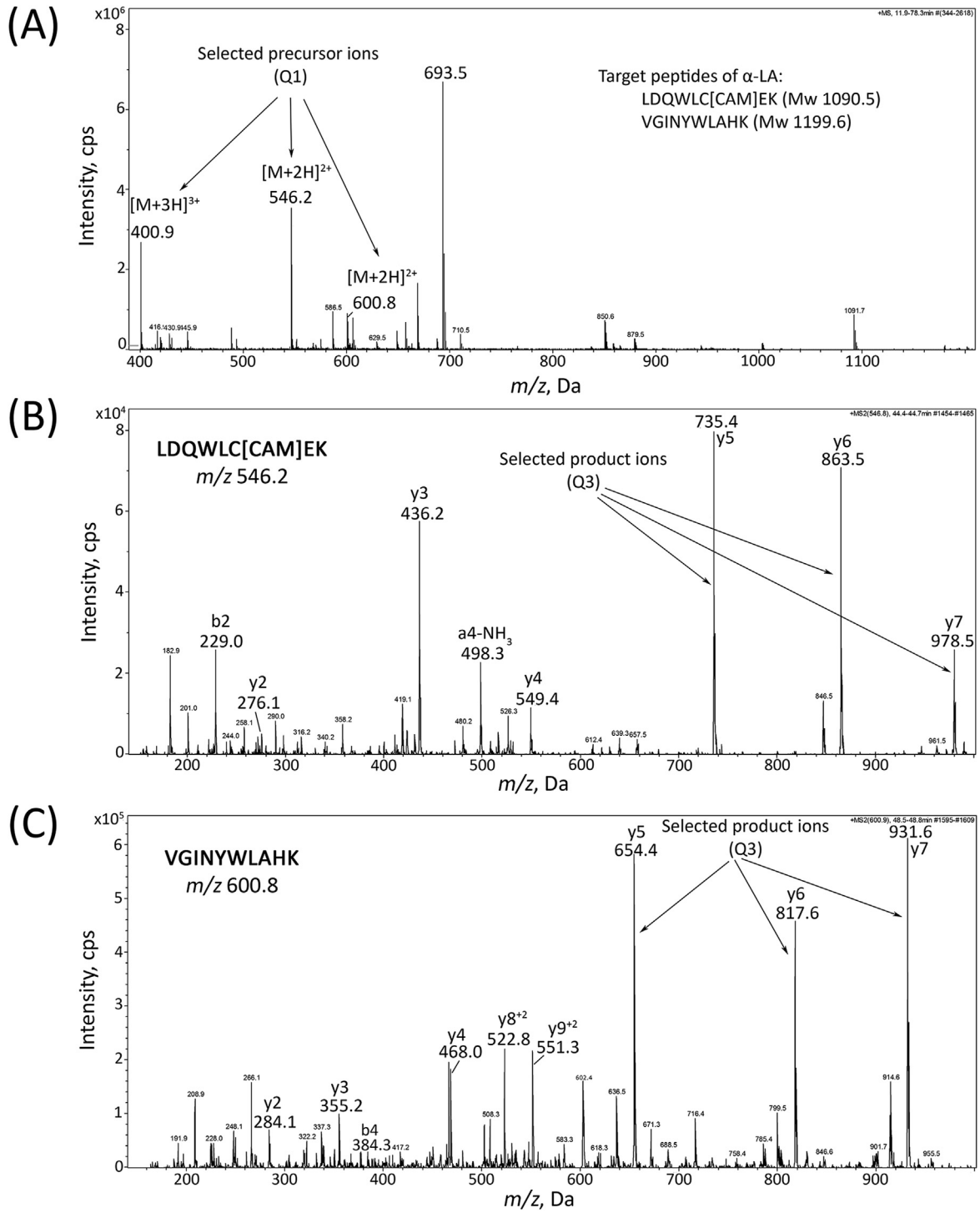

Figure 1. Selection of precursor ions (Q1) and product ions (Q3) for MRM quantification of α-LA. (A) MS of precursor ions of two target peptides from α-LA tryptic digest. (B–C) Product-ion tandem MS of precursor ions at (B) *m/z* 546.2 and (C) *m/z* 600.8.

of this genetic variation in different breeds is, however, not known and to the best of the authors' knowledge has not been reported in Danish Holstein.

Similar to the precursor ion (Q1) selection, product ions (Q3) were chosen based on their signal intensities and, preferably, y ions and Q3 has higher *m/z* than Q1, as a greater proportion of the original peptide is represented; thus, highly specified transitions can be obtained (Mead et al., 2009). However, in some cases, Q3 *m/z* lower than Q1 *m/z* is selected, if only a few reliable and specific fragment candidates can be found. The fragment with highest sensitivity was chosen as the quantitative ion, while the others were used as the qualitative ions. Figures 1B-C and 2B–C show the fragmentation patterns of target peptides obtained from trypsin-digested α-LA and β-CN. It can be observed that the most intense ions are y5, y6 and y7, corresponding to *m/z* 735.4, 863.5 and 978.5, for the peptide LDQWLC[CAM]EK with *m/z* 546.2 from α-LA (Figure 1B). The other peptide VGINYWLAHK with *m/z* 600.8 also generated the most intense fragments as y5, y6 and y7, corresponding to *m/z* 654.4, 817.6 and 931.6 (Figure 1C) or, with *m/z* 400.9, the most intense fragments reported as y4, y5 and y6, corresponding to *m/z* 468.0, 654.4, 817.6 (Table 1). These Q3 ions have higher *m/z* than their Q1 and

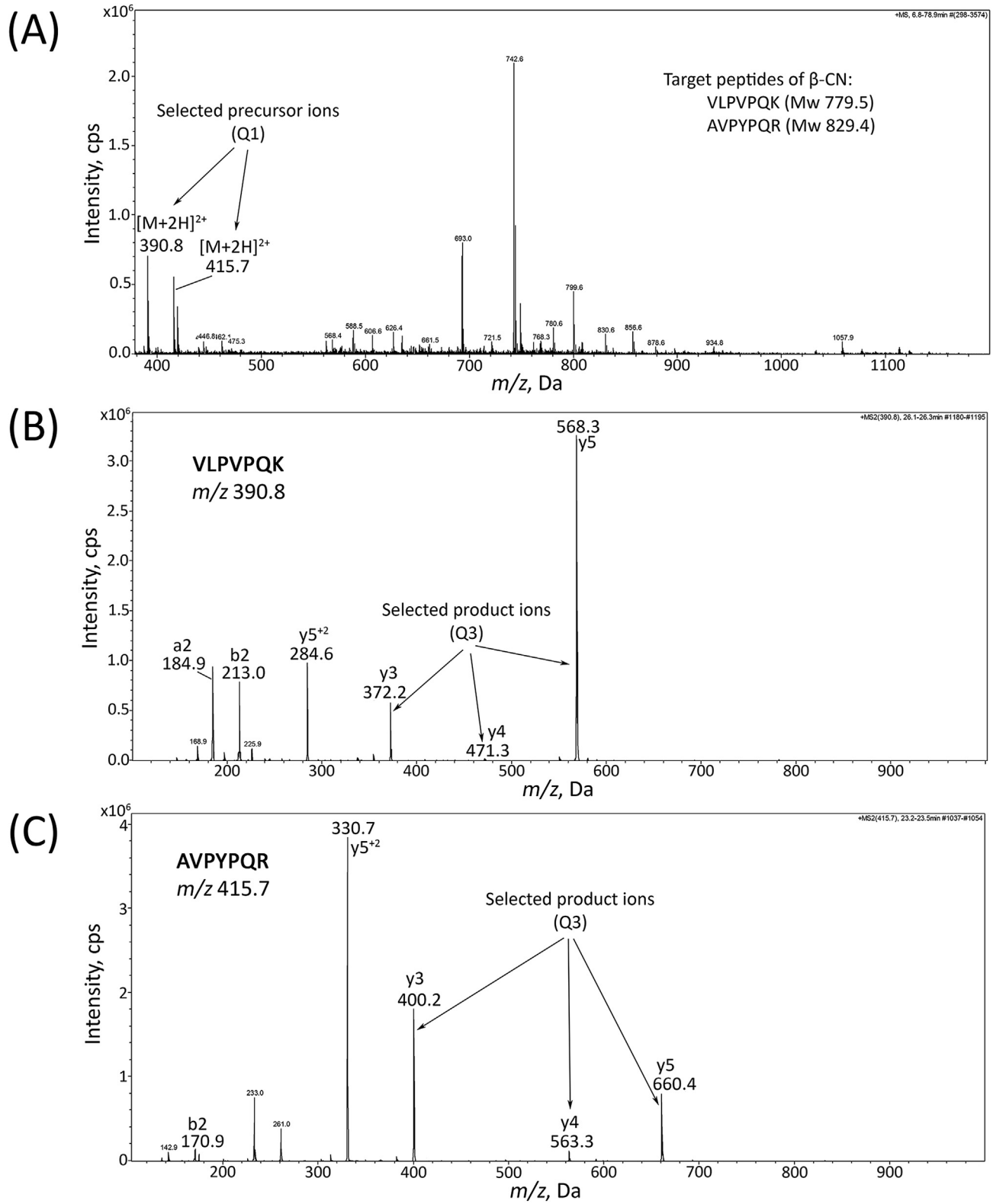

Figure 2. Selection of precursor ions (Q1) and product ions (Q3) for MRM quantification of β-CN. (A) MS of precursor ions of two target peptides from β-CN tryptic digest. (B–C) Product-ion tandem MS of precursor ions at (B) *m/z* 390.8 and (C) *m/z* 415.7.

both fulfilled the selection requirements for MRM transitions. Similarly, product ions y3, y4 and y5 are shown with highest sensitivities in both peptides VLPVPQK with *m/z* 390.8 and AVPYPQR with *m/z* 415.7 from β-CN, corresponding to *m/z* 372.2, 471.3, and 568.3 and 400.2, 563.3, and 660.4, respectively (Figures 2B and 2C). Although the *m/z* of y3 ions in both peptides were slightly lower than their precursor ions, they were present as the most intense peaks in QQQ, and therefore were selected as the quantitative ions.

Hence, the target peptides and their transitions (Q1/Q3 pairs) corresponding with their optimal F and CE values are shown in Table 1. The developed MRM method was then validated for linearity, matrix effect,

Table 2. Linearity, LOD, LOQ, matrix effect and recovery of target peptides of α-LA and β-CN.

Analyte	Linearity (R^2)	LOD (fmol/μL)	LOQ (fmol/μL)	Matrix effect (%)	Pre-digestion recovery/Digestion efficiency (%)
α-LA LDQWLCEK 546.4 → 735.4	0.998	2.4	7.4	4.1	104.5/106.5
α-LA VGINYWLAHK 400.9 → 468.0	0.995	6.3	19.0	0.7	109.4/105.3
β-CN VLPVPQK 390.8 → 372.2	0.999	3.8	11.4	10.5	119.5/115.5
β-CN AVPYPQR 415.7 → 400.2	0.998	1.6	4.7	7.6	106.7/110.8

sensitivity, precision, accuracy, recovery and reproducibility before being applied to the analysis of 662 milk samples.

3.2. Linearity, matrix effect and sensitivity

A linear calibration curve was obtained for each peptide by plotting the ratio of peak area of peptide/peak area of ISTD (y axis) against the peptide standard concentration (x axis). As can be seen in Table 2, a linear relationship was obtained for all four peptides in the concentration range 12.5–250 fmol/μL ($R^2 > 0.99$). In addition, a good linearity was presented for these peptides in milk digest matrix ($R^2 = 0.99$) (results not shown). As the milk digest shows only limited matrix effect (<11%) (Table 2) and the use of ISTD can reduce the matrix effect if present, calibration standards can be prepared in 5% ACN and 0.1% FA.

The sensitivity of the method was evaluated by LOD and LOQ which are the lowest concentration of analyte that can be reliably detected and quantified by an analytical instrument, typically differentiated from the background level S/N = 3 and 10, respectively. The LOD and LOQ were reported for each peptide as fmol/μL (Table 2). The LODs and LOQs for LDQWLC[CAM]EK were 2.4 and 7.4 fmol/μL (0.14 and 0.44 g/L of α-LA), for VGINYWLAHK were 6.3 and 19 fmol/μL (0.37 and 1.12 g/L of α-LA), for VLPVPQK were 3.8 and 11.4 fmol/μL (0.37 and 1.12 g/L of β-CN), and for AVPYPQR were 1.6 and 4.7 fmol/μL (0.16 and 0.46 g/L of β-CN), respectively.

3.3. Precision, accuracy, recovery and reproducibility

Intra- and inter-day precision (% CV) and accuracy (% bias) was shown in Table 3. Precision is required to be within 15% RSD and bias is

Table 3. Intra-day and inter-day precision and accuracy of developed MRM method.

Target concentration (fmol/μL)	Intra-day precision (% CV, n = 6)	Intra-day accuracy (% bias, n = 6)	Inter-day precision (% CV, n = 6)	Inter-day accuracy (% bias, n = 6)
α-LA LDQWLC[CAM]EK				
12.5	5.64	6.47	5.33	1.42
25	3.21	2.02	2.80	-2.39
50	2.90	-2.84	2.69	0.40
75	1.99	-2.82	3.04	-1.07
125	1.92	-0.47	3.09	1.23
250	1.71	1.59	2.76	-0.21
α-LA VGINYWLAHK				
12.5	5.97	-0.76	6.54	11.03
25	5.84	1.49	9.76	-9.29
50	4.72	-2.66	6.08	-1.01
75	2.72	-1.28	6.59	-0.30
125	4.12	0.86	3.23	2.14
250	1.59	-0.14	6.72	2.52
β-CN VLPVPQK				
12.5	1.48	3.25	1.23	10.91
25	1.63	0.02	1.91	2.03
50	1.05	-2.20	1.05	0.72
75	1.18	-1.85	1.31	-2.17
125	0.89	0.52	0.99	-1.44
250	1.01	0.62	0.91	0.48
β-CN AVPYPQR				
12.5	2.30	0.90	2.60	-4.02
25	1.70	0.25	1.66	4.56
50	2.10	-0.85	2.53	1.25
75	1.53	-1.62	1.75	-2.65
125	1.68	0.63	1.11	-2.86
250	2.13	0.27	1.91	0.82

Table 4. Reproducibility (% CV) of tryptic digestion of α-LA and β-CN in 10 Danish Holstein milk samples.

Sample ID	α-LA		β-CN	
	LDQWLC[CAM]EK	VGINYWLAHK	VLPVPQK	AVPYPQR
1	13.6	2.2	6.8	5.1
2	13.9	13.1	2.8	6.4
3	9.4	8.0	5.9	5.6
4	9.4	14.4	5.4	8.7
5	9.6	8.5	3.0	6.8
6	8.6	9.1	6.3	6.7
7	8.3	9.4	10.5	9.0
8	4.6	11.4	7.0	7.4
9	6.4	13.0	13.2	9.9
10	6.7	9.4	7.6	8.5
Mean value (%)	9.1	9.8	6.9	7.4

accepted within ±15% of the accepted true value (Tiwari and Tiwari, 2010). The bias values for intra- and inter-day studies were from -2.84 to 6.47% for LDQWLC[CAM]EK, -9.29 to 11.03% for VGINYWLAHK, -2.20 to 10.91% for VLPVPQK, and -4.02 to 4.56 for AVPYPQR (Table 3). The CV of intra- and inter-day precision ranged from 0.89 to 9.76%, with the highest range for VGINYWLAHK. The CV of the other three peptides was <6%. These values are within acceptable range (<15%) for peptide analysis by LC-MS/MS.

Overall (total) recovery of the analytical method is the combined recoveries of the protein during pre-digestion treatment (pre-digestion recovery), and of the target peptides from enzymatic digestion (digestion efficiency) (Jenkins et al., 2015). Pre-digestion recovery was carried out in the spiked mixture of α-LA and β-CN standards before digestion; the recoveries of α-LA and β-CN were 104.5–109.4% and 106.7–119.5%, respectively (Table 2). For digestion efficiency, ISTD was spiked into milk samples and milk digests; the recoveries of four peptides ranged from

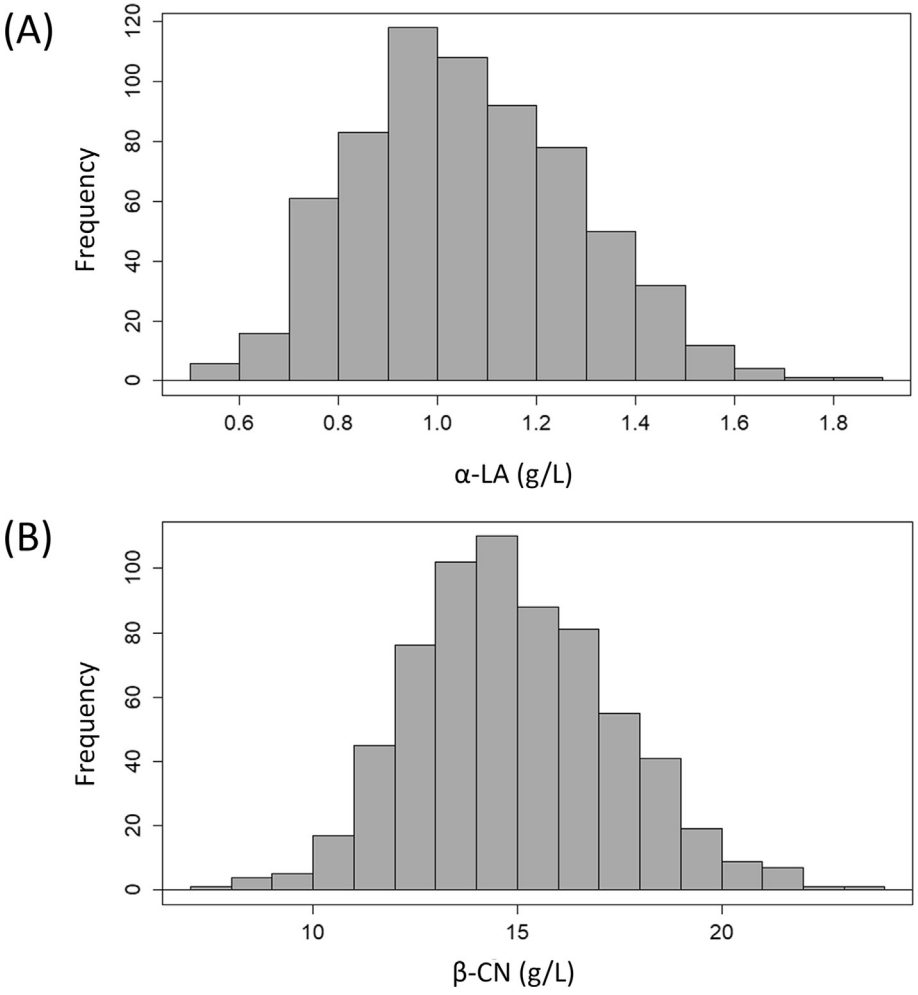

Figure 3. Distribution of (A) α-LA and (B) β-CN content (g/L) in the 662 Danish Holstein milk samples.

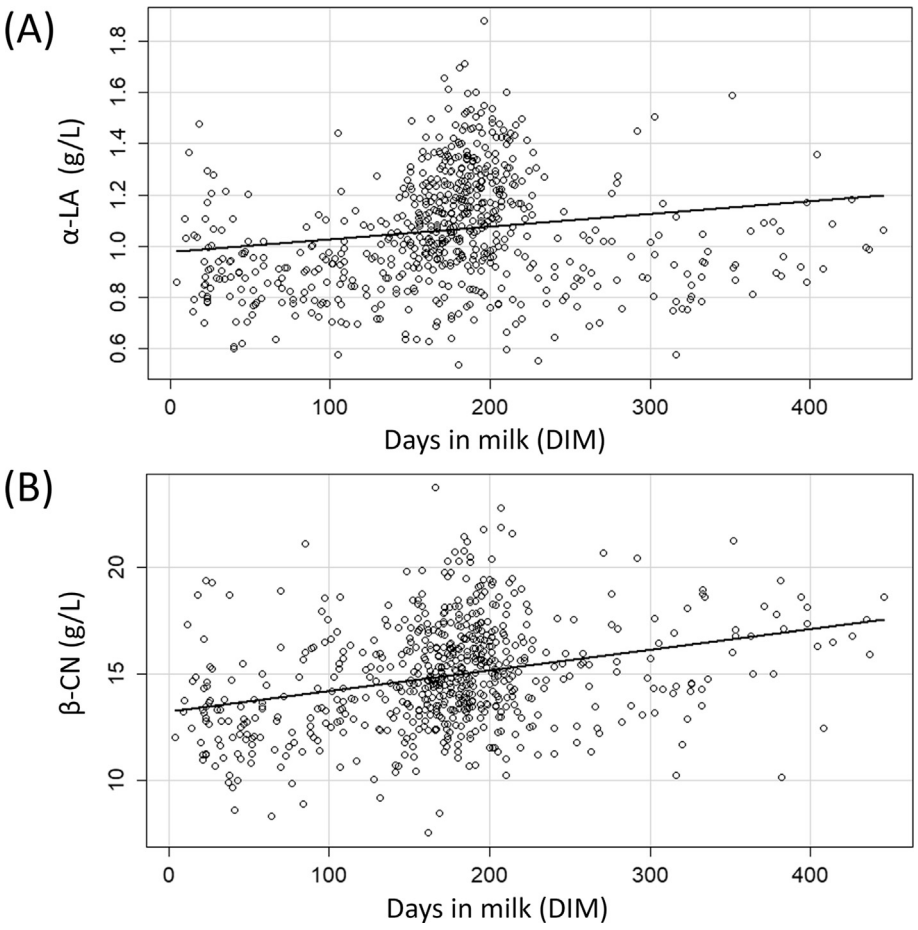

Figure 4. Correlation of (A) α-LA and (B) β-CN content (g/L) in the 662 Danish Holstein milk samples with the number of days in milk (DIM).

105.3 to 115.5% (Table 2). These data suggest that the trypsin efficiently digested α-LA and β-CN within the digestion time of our study and provided reproducible recoveries. Moreover, the completion of trypsin digestion was obtained after 4 h, with the ratio 1:100 of trypsin-to-protein (results not shown); however, a 16-h digestion was chosen in this study for an overnight incubation.

Reproducibility of the analytical method (e.g., in trypsin digestion) is critical in MS-based quantification. The variation in digestibility was tested on ten different milk samples digested on three separate days. The two selected peptides LDQWLC[CAM]EK and VGINYWLAHK from α-LA had average RSD values of 9.1% (4.6–13.9%) and 9.8% (2.2–14.4%), respectively, while β-CN peptides VLPVPQK and AVPYPQR had average RSD values of 6.9% (2.8–13.2%) and 7.4% (5.1–9.9%), respectively (Table 4). This could be explained by the variation of the enzyme activity in each trypsin batch. Laboratory conditions and human error could also cause some variation in the trypsin digestion, particularly in a complex matrix like milk. However, all values are acceptable (<15%) for the reproducibility of analytical methods (Tiwari and Tiwari, 2010). Due to

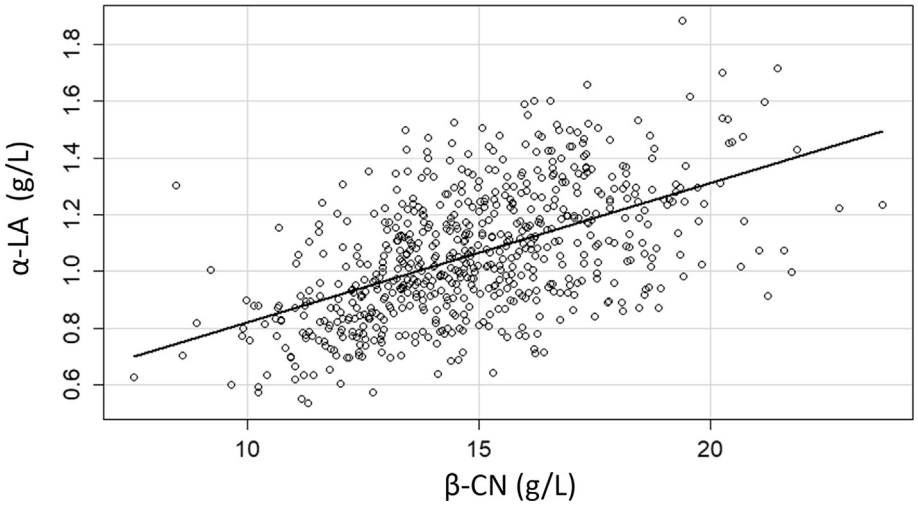

Figure 5. Correlation between α-LA and β-CN content (g/L) in the 662 Danish Holstein milk samples ($r = 0.54$, $P < 0.001$).

the large number of samples (n = 662), some digests could not be run on the same day as the milk was digested, and those digest solutions were stored at -80 °C for up to a week; the peptides were shown to be stable in this storage condition. The peptides also remained their stability at 10 °C (LC auto-sample's temperature) for up to 24 h.

3.4. Application of method to individual cow's milk samples

Based on the linear standard curve, the concentration of four peptides were quantified, hence α-LA and β-CN concentration. The purpose of having at least two peptides per protein for MRM quantification is to obtain a parallel comparison between them. Theoretically, the protein concentration results from its two peptides should be the same. In the case of β-CN, the two peptides showed a good match; the β-CN content can be reported as either from peptide VLPVPQK or AVPYPQR. The peptide AVPYPQR was chosen to report the quantification result of β-CN for its irrelevance to genetic variation. However, the peptide VGINYWLAHK of α-LA displayed unexpectedly non-specific binding to the plastic tubes and degraded over the time of analysis. Although there was a relatively good correlation between the two selected peptides LDQWLC[CAM]EK and VGINYWLAHK for the concentration of α-LA, the quantification of α-LA was, therefore, based on the peptide LDQWLC[CAM]EK.

The validated method was successfully employed for the determination of α-LA and β-CN in the 662 Danish Holstein cows' individual milk samples. The level of α-LA determined by LC-MRM-MS varied from 0.5 to 1.9, with an average of 1.1 g/L. Levels of β-CN varied from 7.5 to 23.7, with an average of 15 g/L (Figure 3). Significantly increasing concentrations of α-LA and β-CN were observed through lactation ($P < 0.001$), whereas there was no significant effect of parity (data not shown). Despite being significant, Pearson correlation coefficients between DIM (below 450 DIM) and α-LA and β-CN, respectively, were low ($r = 0.17$ and 0.30, respectively) (Figure 4). Moreover, α-LA and β-CN levels were significantly ($P < 0.001$) positively correlated ($r = 0.54$, Figure 5), which may relate to overall protein content. Although our study and Bonfatti et al. (2010)'s study used two different approaches to determine the concentration of major proteins in cow's milk (e.g., protein versus peptide standards and HPLC versus LC-MRM-MS), similar levels of α-LA and β-CN were obtained. They reported the content of α-LA and β-CN in 2167 milk samples from Simmental cows to vary from 0.53 to 2.1 g/L and 5.99–22.83 g/L, respectively, using reversed-phase HPLC.

One of the main advantages of LC-MRM-MS is that it can resolve close or co-eluting peaks of similar proteins and/or impurities, due to its high selectivity. In addition, the LC-MRM-MS method could include >100 peptides in one run, meaning >25 proteins (2 target peptides plus 2 ISTD per protein) can be quantified within a short time, e.g., <30 min compared to 45 min up to 1h using traditional HPLC methods. Thus, the method established could be used to simultaneously quantify various protein fractions in bovine milk samples ranging from major proteins such as casein and whey proteins to minor ones (e.g., milk fat globule membrane proteins) as has been recently reported in the study of Bär et al. (2019) in which 20 different milk proteins were quantified in raw milk, raw cream, cheese and whey. MRM with its great sensitivity, specificity and speed can overcome the main drawbacks of traditional HPLC (e.g., low sensitivity, overlapping peaks) or two-dimensional gel electrophoresis (e.g., difficult automation, poor dynamic range, limited sensitivity). Although ELISA or Western blot techniques have been applied for the quantification of individual proteins, specific antibodies are required. Thus, MRM quantification capabilities allow high throughput and multiplex analyses which are restricted in ELISA and Western blot methods. In addition, the MRM technique is solely based on peptide standards which can be readily synthesized with high quality and at reasonable cost; this allows the quantification of less soluble or low concentration proteins due to the lack of individual certified milk protein standards.

4. Conclusion

This is the first time an MRM-based method has been used to determine the absolute concentration of α-LA and β-CN in a large number of bovine milk samples. This MRM approach is based on specific peptides selected for each protein; therefore, a good target peptide is the main focus. The reasonable cost of peptides compared to purified proteins, together with good accuracy and sensitivity, makes the developed method highly applicable to milk proteins. Similar MRM approaches can be used with all proteins in milk. The levels of α-LA and β-CN varied greatly from 0.5 to 1.9 g/L and 7.5–23.7 g/L, respectively. The content of the two proteins increased with increasing days in milk. These high-value proteins showed a large variation among the 662 Danish Holstein cows analyzed in this study; this indicates considerable natural variability in the composition of the milk used for fractionation and points to the possible optimal selection of animals used for producing these potential ingredients.

Declarations

Author contribution statement

Thao T. Le: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nina A. Poulsen: Analyzed and interpreted the data; Wrote the paper.

Gitte H. Kristiansen: Performed the experiments.

Lotte B. Larsen: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

Asledottir, T., Le, T.T., Petrat-Melin, B., Devold, T.G., Larsen, L.B., Vegarud, G.E., 2017. Identification of bioactive peptides and quantification of b-casomorphin-7 from bovine b-casein A1, A2 and I after ex vivo gastrointestinal digestion. Int. Dairy J. 71, 98–106.

Asledottir, T., Le, T.T., Poulsen, N.A., Devold, T.G., Larsen, L.B., Vegarud, G.E., 2018. Release of β-casomorphin-7 from bovine milk of different β-casein variants after ex vivo gastrointestinal digestion. Int. Dairy J. 81, 8–11.

Bär, C., Mathis, D., Neuhaus, P., Dürr, D., Bisig, W., Egger, L., Portmann, R., 2019. Protein profile of dairy products: simultaneous quantification of twenty bovine milk proteins. Int. Dairy J. 97, 167–175.

Bonfatti, V., Di Martino, G., Cecchinato, A., Vicario, D., Carnier, P., 2010. Effects of beta-kappa-casein (CSN2-CSN3) haplotypes and beta-lactoglobulin (BLG) genotypes on milk production traits and detailed protein composition of individual milk of Simmental cows. Int. Dairy Sci. 93, 3797–3808.

Chen, Q., Zhang, J., Ke, X., Lai, S., Li, D., Yang, J., Mo, W., Ren, Y., 2016. Simultaneous quantification of α-lactalbumin and β-casein in human milk using ultra-performance liquid chromatography with tandem mass spectrometry based on their signature peptides and winged isotope internal standards. Biochim. Biophys. Acta 1864, 1122–1127.

Farrell, H.M., Jimenez-Flores, R., Bleck, G.T., Brown, E.M., Butler, J.E., Creamer, L.K., Hicks, C.L., Hollar, C.M., Ng-Kwai-Hang, K.F., Swaisgood, H.E., 2004. Nomenclature of the proteins of cows' milk - sixth revision. J. Dairy Sci. 87, 1641–1674.

Gebreyesus, G., Lund, M.S., Buitenhuis, B., Bovenhuis, H., Poulsen, N.A., Janss, L.G., 2017. Modeling heterogeneous (co)variances from adjacent-SNP groups improves genomic prediction for milk protein composition traits. Genet. Sel. Evol. 49, 89.

Jenkins, R., Duggan, J.X., Aubry, A.F., Zeng, J., Lee, J.W., Cojocaru, L., Dufield, D., Garofolo, F., Kaur, S., Schultz, G.A., Xu, K., Yang, Z., Yu, J., Zhang, Y.J., Vazvaei, F., 2015. Recommendations for validation of LC-MS/MS bioanalytical methods for protein biotherapeutics. J. Am. Assoc. Pharmaceut. Sci. 17, 1–16.

Kamiński, S., Cieslińska, A., Kostyra, E.J., 2007. Polymorphism of bovine beta-casein and its potential effect on human health. J. Appl. Genet. 48, 189–198.

Korhonen, H., Pihlanto, A., 2006. Bioactive peptides: production and functionality. Int. Dairy J. 16, 945–960.

Le, T.T., Deeth, H.C., Bhandari, B., Alewood, P.F., Holland, J.W., 2013. Quantification of lactosylation of whey proteins in stored milk powder using multiple reaction monitoring. Food Chem. 141, 1203–1210.

Lutter, P., Parisod, V., Weymuth, H., 2011. Development and validation of a method for the quantification of milk proteins in food products based on liquid chromatography with mass spectrometric detection. J. AOAC Int. 94, 1043–1059.

Mead, J.A., Bianco, L., Ottone, V., Barton, C., Kay, R.G., Lilley, K.S., Bond, N.J., Bessant, C., 2009. MRMaid, the web-based tool for designing multiple reaction monitoring (MRM) transitions. Mol. Cell. Proteomics 8, 696–705.

Petrat-Melin, B., Andersen, P., Rasmussen, J.T., Poulsen, N.A., Larsen, L.B., Young, J.F., 2015. *In vitro* digestion of purified beta-casein variants A^1, A^2, B, and I: effects on antioxidant and angiotensin-converting enzyme inhibitory capacity. J. Dairy Sci. 98, 15–26.

Poulsen, N.A., Gustavsson, F., Glantz, M., Paulsson, M., Larsen, L.B., Larsen, M.K., 2012. The influence of feed and herd on fatty acid composition in 3 dairy breeds (Danish Holstein, Danish Jersey, and Swedish Red). J. Dairy Sci. 95, 6362–6371.

Rauh, V.M., Johansen, L.B., Ipsen, R., Paulsson, M., Larsen, L.B., Hammershøj, M., 2014. Plasmin activity in UHT milk: relationship between proteolysis, age gelation, and bitterness. J. Agric. Food Chem. 62, 6852–6860.

Silva, S.V., Malcata, F.X., 2005. Caseins as source of bioactive peptides. Int. Dairy J. 15, 1–15.

Tiwari, G., Tiwari, R., 2010. Bioanalytical method validation: an updated review. Pharmaceut. Methods 1, 25–38.

Yadav, J.S.S., Yan, S., Pilli, S., Kumar, L., Tyagi, R.D., Surampalli, R.Y., 2015. Cheese whey: a potential resource to transform to bioprotein, functional/nutritional proteins and bioactive peptides. Biotechnol. Adv. 33, 754–756.