Detection and Quantification of α_{S1}-, α_{S2}-, β-, κ-casein, α-lactalbumin, β-lactoglobulin and Lactoferrin in Bovine Milk by Reverse-Phase High-Performance Liquid Chromatography

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Summary

Bovine milk proteins has been widely studied because of the strong association and relationship with composition and technological properties of milk. Cow's milk quality is very important, above all in such countries like Italy, where about 70% of whole milk production is used in cheese-making industry. A reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed to identify and quantify rapidly the most common genetic variants of bovine milk proteins, included lactoferrin. A reverse-phase analytical column C8 (Aeris WIDEPORE XB-C8, Phenomenex, 3,6 µm, 300Å, 250 x 2,1 I.D.) was used for the analysis. All the most common casein (CN) and whey protein genetic variants were detected and separated simultaneously in less than 20 min; purified bovine milk protein genetic variants were employed in calibration. A linear relationship (R^2 >0.99%) between concentration and peak areas of individual milk protein variants was observed.

Key words

bovine casein, whey protein, lactoferrin, genetic variants, HPLC

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Aim

Several methods were employed to analyze milk protein fractions, such as electrophoretic techniques (Veloso et al., 2002), proteomic approaches (Jensen et al., 2012), isoelectric focusing (IEF) (Strange et al., 1992), or HPLC chromatography coupled with mass spectrometry (Bonizzi et al., 2009; Mollè et al., 2009). Previous investigations focused on the separation of bovine milk protein fraction, overlooking the quantification of single milk protein genetic variants with few exceptions (Bonfatti et al., 2008); furthermore very few studies focused on other minor components, like lactoferrin (Palmano et al., 2001), and generally are time consuming. The aim of this study was to develop an RP-HPLC method able to identify and quantify the single genetic variants of protein fractions, included some minor components such as lactoferrin, improving the run time analysis of chromatograms, even so ensuring good separation of protein fractions and high resolution. Validation of the method includes testing linearity.

Material and methods

The present study is part of a larger project aimed at the study of relationships between quality and technological traits of milk of Brown Swiss cows (Bittante, 2012; Bittante et al., 2011a, b, and 2013; Cecchinato et al., 2009, 2011, 2012a, b; Cipolat et al., 2012; Macciotta et al., 2012; Maurmayr et al., 2011). Guanidine hydrochloride (GdnHcl) (lot G-4505, purity >99%), Bis-tris Buffer (lot B-9754, >98%), Trifluoroacetic acid (lot T-6508,>99%), sodium citrate (lot 71498, >99%) DL-Dithiothreitol (lot 43817, >99%) and purified major protein from bovine milk were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) : κ-CN (lot C-0406, >80%), α-CN (lot C-6780, >70%), β-CN (lot C-6905, >90%), α-lactalbumin (lot L-5385 type I, ~85%), β-lactoglobulin B variant (lot L-8005, >90%), β-lactoglobulin A variant (lot L-7880, >90%) and lactoferrin (lot L-9507, >85%). Ultra pure water (Milli-Q System, >18.2 MΩ cm) was obtained in the laboratory. Individual and bulk bovine milk was collected directly in dairy herds. Preservative (Bronopol, 2-bromo-2nitropropan-1,3-diol) was added to raw milk samples to prevent microbial growth and 2 aliquots for each sample containing 1 ml of milk were frozen at -20°C during milk collection and transferred at -80°C in the laboratory since the HPLC analysis was performed. Milk samples were prepared following the method of Bobe et al. (1998). No preliminary separation or precipitation procedures of the casein fraction was required. The HPLC equipment consisted of an Agilent 1260 Series chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump (Agilent 1260 Series, G1311B). A Diode Array Detector (Agilent 1260 Series, DAD VL+, G1315C) was used. The equipment was controlled by the Agilent Chemstation for Lc System software which sets solvent gradient, data acquisition and data processing. Separation was performed on a reversed-phase analytical column C8 (Aeris WIDEPORE XB-C8, Phenomenex) with a large pore core-shell packing (3,6 µm, 300Å, 250 x 2,1 I.D.). A Security Guard ULTRA Cartridge System (product No. AJ0-8785, Phenomenex) was used as pre-column (UHPLC WIDEPORE C8, 2,1 mm I.D.). Sample vials were kept at low constant temperature (4°C) and injected via an auto-sampler (Agilent 1100 Series, G1313A). After comparing different chromatographic conditions, the followed was adopted to optimize analytical quality and time required: a) gradient elution was carried out with a mixture of two solvents: solvent A consisted of 94.9% water, 5.0% acetonitrile and 0,1% trifluoroacetic acid and Solvent B consisted of 0,1% TFA in acetonitrile; b) separation of bovine protein fraction was performed with the following program: linear gradient from 20 to 29 % B in 0.5 min, from 29 to 33% B in 5.5 min, from 33 to 36% B in 6 min, from 36 to 45% B in 6 min and return linearly to the starting condition in 1 min; c) the column was re-equilibrated under starting conditions for 3 min, before inject the following sample and the total analysis time per sample was 22 min; d) the flow rate was 0.5 ml/min; e) the column temperature was kept at 70°C; f) the detection was made at a wavelength of 214 nm; and g) the injection volume consisted of 2 µl. Concerning standard solutions, single-fraction mother solutions were prepared by dissolving, respectively, 5 mg of purified α-CN, 2,5 mg of purified κ-CN, 4 mg of purified β-CN, 1.5 mg of purified lactoferrin, 1 mg of purified α-lactalbumin, 2 mg of both purified β-lactoglobulin A and B variants in 0.75 ml of GndHcl solution; then, a set of five decreasing concentration solutions was obtained by each single mother solution by applying the dilution scheme reported in table 1. The resulting standard solutions were analyzed in order to construct the α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein,

Table 1. Concentration of the standard casein fractions

Milk protein fraction:	Concentration mg/mL				
	A	B	C	D	E
κ-CN	1.05	1.40	1.87	2.50	3.33
α-CN[1]	2.10	2.81	3.75	5.00	6.67
Lactoferrin	1.68	2.25	3.00	4.00	5.33
β-CN	0.63	0.84	1.12	1.50	2.00
α-lactalbumin	0.42	0.56	0.75	1.00	1.33
β-Lg A or B	0.84	1.12	1.50	2.00	2.67

[1] for quantification it was applied a 4:1 proportion between αs_1 and α_{S2} fractions

Table 2. Parameters of linear regression equation for individual milk protein fractions[1]

Milk protein fraction:	Average	SD	a	b	RSD[b]	R^2
κ-CN A	763	399	-126	583	50	0.989
κ-CN B	665	289	24	420	42	0.989
α_{S2}-CN 1	658	347	-109	252	52	0.998
α_{S2}-CN 2	624	377	-217	276	33	0.998
α_{S1}-CN B	4517	2722	-1553	1992	204	0.994
α_{S1}-CN C	1873	1107	-593	809	96	0.993
Lactoferrin	3446	1594	-101	3886	91	0.997
β-CN B	513	321	-205	293	26	0.999
β-CN A[1]	2425	1329	-555	1216	36	0.999
β-CN A[2]	4469	2031	-79	1855	107	0.999
α-La	2146	1167	-461	4280	76	0.997
β-Lg A	1887	1206	-799	2190	137	0.989
β-Lg B	2682	1679	-1073	3053	72	0.998

[1] Separated solutions of purified protein genetic variants at different concentration in duplicate; [2] Residual standard deviation

lactoferrin, α-lactalbumin, β-lactoglobulin A and B variants calibration curves. Since α_{S1} and α_{S2}-casein are not available as single proteins, the corresponding values were calculated from the α-casein applying the 4:1 proportion known for cow milk (Bonizzi et al., 2009). Before calibration, linearity was tested by running the same sample at the five dilution points in triplicate. Areas under peaks of the chromatogram were used to validate the method. Concerning validation procedure, 30 individual milk samples were analyzed and every sample was run two times repeating the analysis of the same sequence (sample injection=2 µl). The external standard method was used for the calibration of the chromatographic system for the protein quantification. Calibration curves were computed for each protein genetic variant by applying simple linear regression of the peak area on the amount injected, at decreasing injection volume (table 2).

Results and discussion

All major peaks and chromatograms are reported in Figure 1. HPLC conditions were optimized for mobile phase conditions, gradient, flow rate and temperature. Retention times of the major eluted peaks coincided with the retention times of the major milk proteins present in standards. It was ascertained that proteins eluted following this order: κ-CN, α_{S2}-CN, α_{S1}-CN, lactoferrin, α-Lactalbumin, β-CN and β-Lactoglobulin. The identification of peaks of genetic variants was confirmed comparing them with commercial standards which consisted of purified genetic variants (just for β-Lactoglobulin variants exhibits a high frequency in the Finnish Ayrshire (Ikonen et al., 1996), but not in Brown Swiss (Bittante et al. 2012) and is rather common Holstein Friesian breeds (Caroli et al., 2009). The genetic variants of this protein fraction significantly affect also on cheese yield and quality (Alipanah and Kalashnikova, 2007; Caroli et al., 2009; Bonfatti et al., 2011). Between κ-$CN_{A/E}$ and κ-CN_B peaks eluted α_{S2}-CN, which consisted of two major peaks; multiple peaks and shoulders of α_{S2}-CN are probably caused by the partial separation of many phosphorylated forms of α_{S2}-CN (Bordin et al., 2001). Separation of αS1-Casein variant B and C was enough feasible with the current method, because the height of the inflection point between the two peaks is respectively the fifth part of the first peak B variant and the second part of the second peak C variant. In the current analysis it was not found an animal carrying α_{S1}-CN A or α_{S1}-CN D variants. Separation of lactoferrin was well resolved, retention time and peak area is the same for the sample of unknown amount and the standard used for calibration curves, although peak area of lactoferrin is very small because of the very low amount of this iron-binding protein in bovine milk (20-200 mg/L) (Farrell et al., 2004; Plate et al., 2006). α-Lactalbumin eluted after α_{S1}-CN and showed monomorphic peak in variant B in all samples analyzed. Concerning β-CN, B, A^1 and A^2 variants eluted after α-Lactalbumin and all peaks were identified and well resolved. The C variant coeluted with A^1 variant and it was not detectable with the current method; the A^3 variant, which eluted close to the A^2 variant was detected with the current method. There is

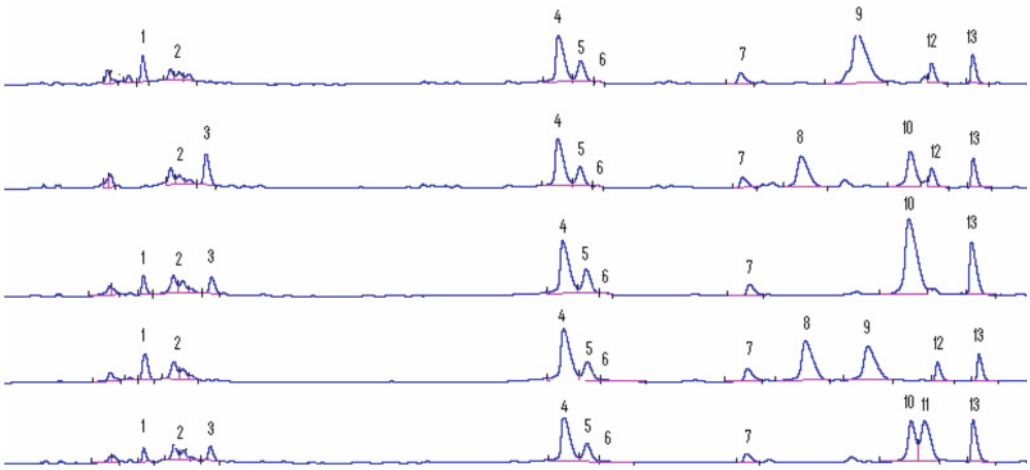

Figure 1. Chromatograms relative to raw milk from individual cows (samples 1-5) obtained using the optimized elution condition described in materials and method section (no 1: k-CN A; no 2: α_{s2}-CN ; no 3: k-CN B; no 4: α_{s1}-CN B; no 5: α_{s1}-CN C; no 6: Lactoferrin; no 7: α-La; no 8: β-CN B; no 9: β-CN A^1; no 10: β-CN A^2; no 11: β-CN A^3; no 12: β-Lg B; no 13: β-Lg A).

are available, A and B respectively) or comparing them with chromatograms of individual milk samples of DNA-genotyped animals. The κ-CN eluted as three distinct peaks which consisted of glycosylated and unglycosylated forms of κ-CN_A and κ-CN_B. Chromatograms with a different κ-CN genetic variants were well resolved , variant A and B are evident, although the infrequent variant κ-CN_E coeluted with the A variant using the current method; κ-CN_E variant is a uncommon variant which another variant that eluted between A^1 and A^2 variant which is not named because of their absence in milk samples of DNA-genotyped animals that were used for the identification and detection of the major milk protein genetic variants during the validation procedure of the method. For β-Lactoglobulin, variant B eluted before variant A; this last one has got a peak followed by a main one and the proportions of the area between these two peaks can be considered as an indicator of proteolysis

(Bordin et al., 2001). Comparing the injections of standards, BSA eluted between lactoferrin and α-lactalbumin, and Igs eluted at the end of the chromatographic run, after β-lactoglobulin. It was not possible to detect and quantify both BSA and Igs during the analysis of milk samples, although peaks were better resolved and visible analyzing colostrums; however colostrum seems to need a different preparation or a different method development to detect other proteins, such as BSA and Igs, which are in low proportion in milk, but in high level at the beginning of the lactation (Farrell et al., 2004). Concerning quantification, calibration curves have been derived from parameters of simple linear regression computed for whole protein fraction by using commercial standards. Considering all the five-point calibration setting used for each standard at five different dilutions, the relation between peak area and injected amount of protein variant was linear ($R^2 > 0.99\%$). Concerning milk sample, CN content was nearly 84% of total protein. Within caseins, α_s-CN was 42% of the total casein fraction, whereas k-CN was 19% and β-CN was 37% of total casein fraction, whereas the β-lactoglobulin was 84% of the total whey protein. The CN content of samples as a proportion of total protein content in our study was greater than the casein index reported by other studies because non-protein nitrogen, proteose-peptones and minor constituents of whey protein cannot be quantified with this method.

Conclusions

A RP-HPLC method was developed to identify and quantify the most common milk protein genetic variants of cows. The method guarantied the detection and the quantification of all major milk protein fractions, included some minor components like lactoferrin, and their main genetic variants in one fast run with good resolution. It allows a run time equal or lower than halved if compared to methods proposed earlier. In conclusion this method can be applied to analysis of raw individual and bulk milk samples; preparation procedure is very easy and fast and the total analysis time per sample is short (22 min) considering the amount of information that could be collected: concentration of different protein fractions of milk, separately for each main genetic variants, and thus also genotype of animals for the gene codifying for protein fractions can be derived. This methodology can favor new understanding on the complex relationships among protein fractions and variants, milk quality, milk coagulation, cheese yield and quality.

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