

Determination of whey proteins in different types of milk

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Abstract

Protein analysis is very important both in terms of milk protein allergy, and of milk and dairy product adulteration (β -lactoglobulin may be an important marker in the detection of milk adulteration). The aim of this study was to detect major whey proteins α -lactalbumin and β -lactoglobulin and their genetic variants by reversed-phase high-performance liquid chromatography. Milk samples from cows ($n = 40$), goats ($n = 40$) and sheep ($n = 40$) were collected at two farms and milk bars in the Czech Republic from April to June 2010. The concentration of α -lactalbumin was higher in goat's milk ($1.27 \pm 0.05 \text{ g}\cdot\text{l}^{-1}$, $P < 0.001$) and cow's milk ($1.16 \pm 0.02 \text{ g}\cdot\text{l}^{-1}$, $P = 0.0037$) compared to sheep's milk ($0.95 \pm 0.06 \text{ g}\cdot\text{l}^{-1}$); however, concentration of α -lactalbumin in goat's milk and cow's milk did not differ significantly ($P < 0.05$). Goat's milk contained less β -lactoglobulin ($3.07 \pm 0.08 \text{ g}\cdot\text{l}^{-1}$) compared to cow's milk ($4.10 \pm 0.04 \text{ g}\cdot\text{l}^{-1}$, $P < 0.001$) or sheep's milk ($5.97 \pm 0.24 \text{ g}\cdot\text{l}^{-1}$, $P < 0.001$). A highly significant positive correlation ($r = 0.8686$; $P < 0.001$) was found between fraction A and B of β -lactoglobulin in sheep's milk, whereas in cow's milk there was a negative correlation ($r = -0.3010$; $P = 0.0296$). This study summarizes actual information of the whey protein content in different types of milk which may be relevant in assessing their allergenic potential.

α -lactalbumin, β -lactoglobulin, cow, goat, sheep, RP-HPLC

Major whey proteins α -lactalbumin (LA) and β -lactoglobulin (LG) are strongly correlated with the nutritional value and the functional properties (i.e. gelling, film-forming, foaming and emulsifying) (Moatsou et al. 2005). However, lactoglobulins can also cause development of allergy to cow's milk, mainly affecting children (Monaci et al. 2006). The most allergenic whey protein is β -lactoglobulin, which constitutes 50% of whey proteins. Prevention of allergies to cow's milk is based on total elimination of cow's milk from a diet. Nevertheless, some studies (Skripak et al. 2008; Passalacqua et al. 2012) indicate that administration of gradually increasing doses of the allergen leads in children to induction of tolerance to the substance that originally caused the allergic reaction. It is therefore important, especially for people suffering from allergy to milk protein, to know the content of particular proteins in food (Monaci et al. 2006; Passalacqua et al. 2012). The content of whey proteins and identification of individual fractions are also important because of milk adulteration, especially in case of replacement of goat's or sheep's milk by cow's milk. The content of whey proteins differs in different milk types. In some cases, we can also identify the presence of different genetic variants which can help reveal adulteration (Veloso et al. 2002; Karoui and Baerdemaeker 2007). Genetic variants may influence the production of milk and its nutritional and technological properties (Formaggioni et al. 1999; Kuczyńska et al. 2012). In cow's milk, the most common genetic variant of α -lactalbumin is the B variant and the major genetic variant of β -lactoglobulin are A and B (Farrell et al. 2004). Whey proteins are used to indicate the method of

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heat treatment, which is important in connection with new technologies (ESL Milk – Extended Shelf Life Milk).

The aim of this study was to determine the content of major whey proteins and identify their genetic variants in cow's, goat's and sheep's milk. The whey protein profile needs to be updated because of the reduction of dairy cattle number and changing breed profile.

Materials and Methods

Samples

A total of 120 samples of milk collected from cows, goats and sheep on two farms and eleven milk bars in the Czech Republic were analyzed. Cow's milk ($n = 40$) from Holstein and Czech Fleckvieh breeds were collected from eleven milk bars from April to June 2010. Goat's milk ($n = 40$) was collected from White Shorthaired goats on a goat farm in the Southern Moravian Region of Czech Republic from May to June 2010. Samples ($n = 40$) of sheep's milk were collected from a sheep farm in the Zlín Region from May to June 2010. The sheep were mainly Lacaupe (87.5%), and the minor breeds were Improved Wallachian and East Friesian sheep. The samples were stored at $-18\text{ }^{\circ}\text{C}$ until analysis.

Sample preparation

Prior to HPLC analysis the milk was thawed. Raw milk samples were defatted by centrifuging at $3,000\text{ g}$ for 15 min. The supernatant was precipitated by the addition of 10% acetic acid (Penta, Czech Republic) until reaching a pH of 4.6 (López-Fandiño et al. 1993) and filtered through a $0.22\text{ }\mu\text{m}$ -pore nylon filter into vials, and then analyzed.

Standards of α -lactalbumin ($\geq 85\%$) and β -lactoglobulin ($\geq 90\%$) from bovine milk (Sigma Aldrich, USA) were weighed (10 mg) and dissolved in mobile phase A in 10 ml volumetric flasks.

Validation and optimization of RP-HPLC

Optimization of HPLC analysis was performed using standard solutions of α -lactalbumin and β -lactoglobulin. Calibration curve for the α -lactalbumin was designed over a concentration range of $0.404\text{--}1.571\text{ mg}\cdot\text{ml}^{-1}$ ($y = 0.5835x - 0.215$; $R^2 = 0.989$). Calibration curve for the β -lactoglobulin was designed over a concentration range of $0.406\text{--}1.133\text{ mg}\cdot\text{ml}^{-1}$ ($y = 0.3635x + 0.076$; $R^2 = 0.9752$). The method's sensitivity was detected using a slope of calibration line.

The repeatability of the procedure was determined from the results of multiple measurements per sample ($n = 7$) and determined as RSD 2.53% for α -lactalbumin and RSD 2.40% for β -lactoglobulin. The repeatability of retention times was determined from the results of multiple measurements per sample ($n = 12$) and determined as RSD 1.02% for α -lactalbumin and RSD 0.33% for β -lactoglobulin. The limit of detection was determined as 3 S/N (signal/noise ratio) $0.0045\text{ mg}\cdot\text{ml}^{-1}$ for α -lactalbumin and β -lactoglobulin. The limit of quantification (determined as 10 S/N) was $0.015\text{ mg}\cdot\text{ml}^{-1}$ for α -lactalbumin and β -lactoglobulin. Evaluation was performed using an external standard and quantification was performed using timed groups (Ruprichová et al. 2011).

RP-HPLC analysis

Samples of milk were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) and repeated $\times 2$ per sample. Separation of whey proteins was performed by liquid chromatograph Alliance 2695 with PDA 2996 detector (Waters, USA) and XBridge TM C18, $150 \times 3.0\text{ mm}$, $3.5\text{ }\mu\text{m}$ column (Waters, Ireland). Column temperature for whey protein detection was $40\text{ }^{\circ}\text{C}$, run time was 35 min. Injection volumes were $10\text{ }\mu\text{l}$ for whey proteins. Mobile phase A consisted of water/acetonitrile (Merck, Germany)/trifluoroacetic acid (TFA) (Sigma Aldrich, USA) at a ratio of 95/5/0.1 (v/v/v) and mobile phase B contained water/acetonitrile/TFA (5/95/0.1, v/v/v). Gradient elution and mobile phase flow rate of $0.4\text{ ml}\cdot\text{min}^{-1}$ were applied. The detection was performed at 205 nm.

Collection and evaluation of data were performed by the Empower 2 software (Waters, USA).

Statistics

Basic statistical characteristics (mean, standard deviation, maximum value, minimum value) were computed using Microsoft Excel. The results were analyzed using the statistical package Unistat 5.1. (Unistat Ltd., London, UK). For all variables tested in both experiments, normality was checked using a Shapiro-Wilk test (Zar 1999), and homogeneity of variances among groups was tested using a Bartlett-Box test (Zar 1999). Data were subjected to one-way ANOVA with the type of milk as the main effect with three levels (cow, goat, sheep), and subsequently to Tukey-HSD test (Zar 1999) for multiple comparisons to assess the significance of differences between all possible pairs of groups. To assess correlations in the study, Pearson's correlation coefficients between LG-A and LG-B were calculated. A P -value < 0.05 was considered significant.

Results

Table 1 shows the concentration of whey proteins and also the frequencies of individual β -lactoglobulin genetic variations in cow's and sheep's milk, which were revealed in the chromatogram (Figs 1, 2). The concentrations of α -lactalbumin were $1.27 \pm 0.05 \text{ g}\cdot\text{l}^{-1}$ in goat's milk, $1.16 \pm 0.02 \text{ g}\cdot\text{l}^{-1}$ in cow's milk, and $0.95 \pm 0.06 \text{ g}\cdot\text{l}^{-1}$ in sheep's milk. The highest content of β -lactoglobulin was found in sheep's milk at $5.97 \pm 0.24 \text{ g}\cdot\text{l}^{-1}$. The concentration of β -lactoglobulin was $3.07 \pm 0.08 \text{ g}\cdot\text{l}^{-1}$ in goat's milk and $4.10 \pm 0.04 \text{ g}\cdot\text{l}^{-1}$ in cow's milk. Fig. 3 shows the profile of α -lactalbumin and β -lactoglobulin depending on the concentration of different types of milk.

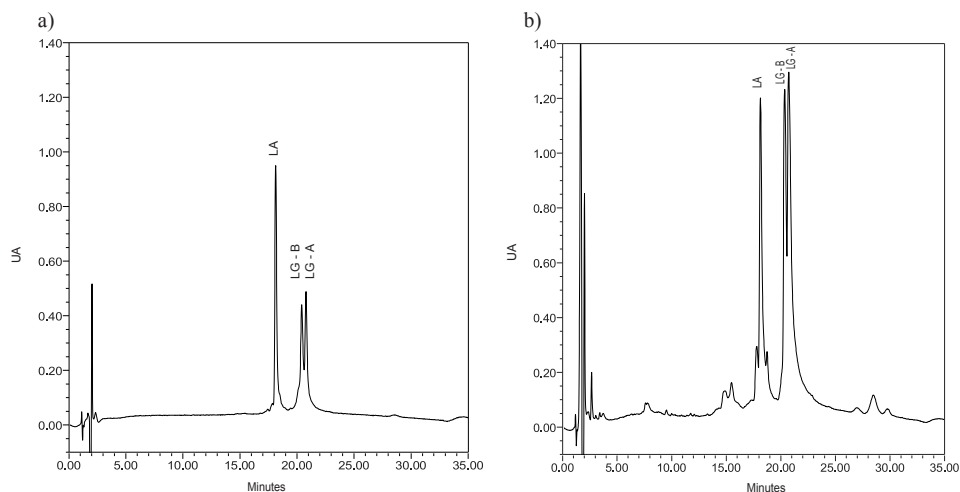


Fig. 1. Chromatogram of α -lactalbumin (LA) and β -lactoglobulin (LG) of cow's milk

a) standard, b) samples

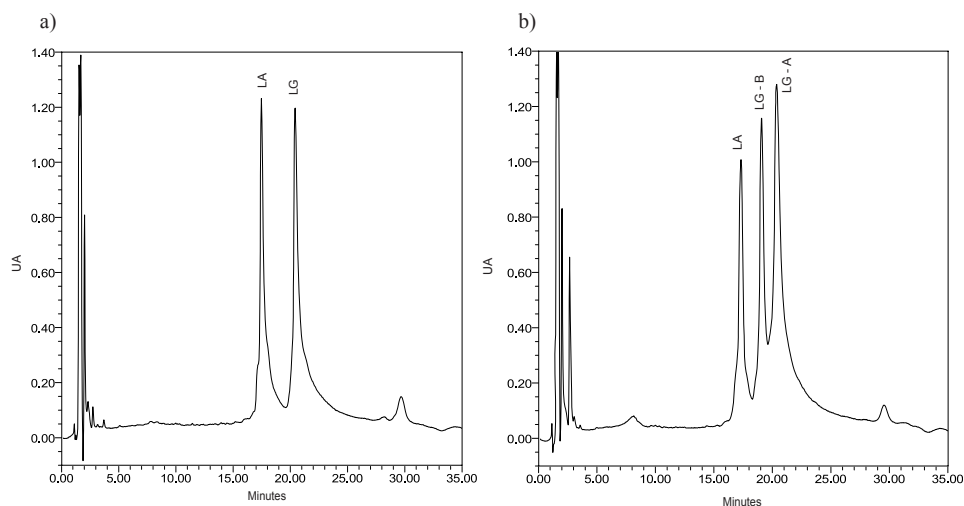


Fig. 2. Chromatogram of α -lactalbumin (LA) and β -lactoglobulin (LG) in goat's milk (a) and in sheep's milk (b)

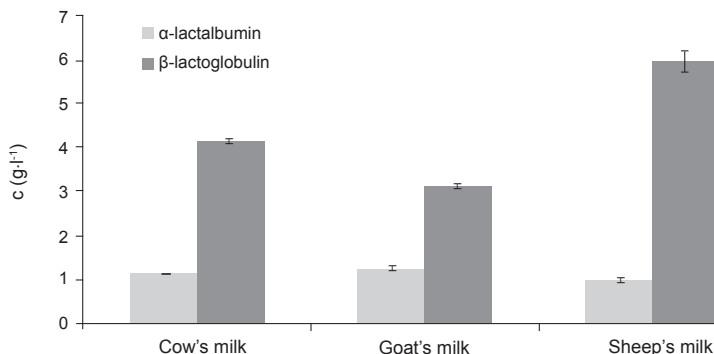


Fig. 3. Concentration (c) of α -lactalbumin and β -lactoglobulin in different types of milk

Table 1. Concentration of whey proteins in different types of milk.

(g·l ⁻¹)	Cow's milk				Goat's milk		Sheep's milk			
	LA	LG-B	LG-A	LG*	LA	LG	LA	LG-B	LG-A	LG*
Mean	1.16	1.19	2.87	4.10	1.27	3.07	0.95	1.80	4.13	5.97
Min	0.99	1.03	2.45	3.61	0.65	1.74	0.44	0.95	2.36	3.43
Max	1.33	1.43	3.40	4.53	1.87	3.78	1.58	2.91	6.37	9.25
SD	0.10	0.11	0.26	0.25	0.34	0.49	0.38	0.55	1.00	1.54
SEM	0.02	0.02	0.04	0.04	0.05	0.08	0.06	0.09	0.16	0.24

LA – α -lactalbumin, LG-B – β -lactoglobulin-B, LG-A – β -lactoglobulin A, * LG was evaluation summarization of peaks LG-A and LG-B, SD – Standard deviation, SEM – Standard error of the mean

The LA content in goat's milk differed with a high significance ($P < 0.001$) from LA in sheep's milk. Similarly, LA in cow's milk differed with a high significance ($P = 0.0037$) from LA in sheep's milk. In contrast, the LA content in goat's milk did not differ significantly ($P < 0.05$) from LA in cow's milk. When evaluating LG concentrations, highly significant differences were found between goat's milk and sheep's milk ($P < 0.001$). Similarly, LG in cow's milk differed with a high significance ($P < 0.001$) from goat's milk. The difference between the LG content in goat's milk and sheep's milk was also highly significant ($P < 0.001$). Study of the dependence between the LG-A and LG-B content in sheep's milk revealed highly significant positive correlation ($r = 0.8686$; $P < 0.001$), whereas LG-A significantly negatively correlated ($r = -0.3010$; $P = 0.0296$) with LG-B in cow's milk.

Discussion

The RP-HPLC method is suitable for determination of whey protein contents in cow's, goat's and sheep's milk and detection of their individual genetic variants. In previous studies, the content of α -lactalbumin and β -lactoglobulin in cow's milk was the following: 1–1.5 g·l⁻¹ and 3–4 g·l⁻¹ (Monaci et al. 2006), 0.6–1.7 g·l⁻¹ and 2–4 g·l⁻¹ (Farrell et al. 2004), respectively. Sztankóová (2006) detected α -lactalbumin and β -lactoglobulin in cow's milk (1.05 g·l⁻¹ and 3.83 g·l⁻¹), goat's milk (1.31 g·l⁻¹ and 3.33 g·l⁻¹) and sheep's milk (1.16 g·l⁻¹ and 6.58 g·l⁻¹), respectively. These values are comparable with data from our study.

The contents and genetic variants of fractions of different types of milk are different. Three genetic variants of α -lactalbumin (A, B, C) and nine genetic variants of β -lactoglobulin (A, B, C, D, E, F, H, I, J) have been identified in cow's milk (Ng-Kwai-Hang 2003). Dziuba et al. (2010) and Formaggioni et al. (1999) reported that more variants G and W of β -lactoglobulin were found. Variant B of α -lactalbumin and variants A and B of β -lactoglobulin are major genetic variants (Farrell et al. 2004). Our results confirmed this fact.

Moioli et al. (1998) reported that α -lactalbumin and β -lactoglobulin in goat's milk had protein variants A and B and their DNA polymorphisms do not exist. In our study, we found only one variant of β -lactoglobulin in goats of the White Shorthaired breed.

There are three protein variants of β -lactoglobulin in sheep's milk: A, B and C and the same DNA polymorphisms (Moioli et al. 1998). Our chromatogram of sheep's milk in β -lactoglobulin A and B revealed two peaks. Major part of sheep's milk samples in our study originated from the Lacaune breed, minor part came from Improved Wallachian and East Friesian sheep breeds. Amigo et al. (2000) stated in their work that in sheep of the Laucane breed, Improved Wallachian sheep and East Friesian sheep, A and B genetic variants were observed like in our study. Alpha-lactalbumin has two genetic variants (A and B) but DNA polymorphisms do not exist (Moioli et al. 1998). The α -lactalbumin B variant is rare and was identified only in some breeds (Amigo et al. 2000). In our study, we did not identify α -lactalbumin B fraction in sheep of any breed tested (Lacaune, Improved Wallachian and East Friesian).

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