**Original article**

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# BETAINE CONTENT IN RAW COW AND SHEEP MILK

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Betaine (trimethylglycine) exists at a physiological pH value in a zwitterionic form. It acts as a methyl group donor, an osmolyte, and a lipotropic agent. Although this micronutrient is a valuable ingredient of a healthy diet, there is limited data on its content in various foods.

The aim of this study was to determine the betaine content in raw, unprocessed cow and sheep milk from household farms in southeastern Serbia. The content of fat and protein in raw cow milk was (4.20 ± 0.38%) and (3.25 ± 0.12%), respectively. Furthermore, the content of fat and protein in raw sheep milk was (6.67 ± 0.33%) and (5.58 ± 0.16%), respectively. The content of betaine in raw cow and sheep milk was (7.51 ± 0.66 mg/l) and (15.68 ± 3.52 mg/l), respectively.

Given the importance of betaine as a significant micronutrient, its twice as high content as in cow milk contributes to the high nutritional value of sheep milk.

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***Key words:*** *betaine, cow milk, sheep milk, HPLC method*

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## Introduction

Betaine (trimethylglycine) consists of three methyl groups attached to the nitrogen atom of the amino acid glycine. At a physiological pH value, this small, highly water-soluble molecule exists in a zwitterionic form with positively charged quaternary ammonium and negatively charged carboxyl functional groups (Figure 1) (1).

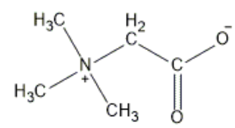

**Figure 1.** Chemical structure of betaine (2)

Betaine was first isolated in the nineteenth century from sugar beets (*Beta vulgaris*) and hence the name betaine (3). It could be synthesized/transported and accumulated in bacteria, fungi, animals, and plants (4). In mammals, betaine can be synthesized endogenously by two-step oxidation of choline in the liver or readily absorbed through dietary intake obtained from the diet (5, 6). Choline and its metabolite betaine, both quaternary ammonium compounds, actively participate in one-carbon (7) and lipid metabolism (8).

The amount of betaine is considerably more abundant in the kidneys and liver than in other mammalian organs. Its main role in the kidneys is osmoprotectant in the cells of the medulla. As a vital intracellular osmolyte, betaine regulates cell volume by countering changes in extracellular tonicity and stabilizing macromolecules against a variety of physiological perturbations. Its concentration in blood plasma is about 0.1 mmol/l, derived from the diet and choline metabolism (9). According to Obeid (10), plasma concentrations of betaine are highly individual. Women have lower plasma concentrations of betaine than men, whereas younger adults show lower concentrations than older ones.

Another biological function of betaine is that it is one of the essential methyl-group donors included in many biochemical pathways (11). Betaine and micronutrients such as folate, choline, vitamin B12, and other B vitamins contribute to DNA methylation as methyl donors and cofactors. The status of these nutrients correlates with DNA methylation and represents potential preventive and therapeutic targets in pathological conditions such as cancers, in which atypical DNA methylation is frequently presented (12).

As a direct methyl-group donor, betaine provides a methyl group for the conversion of homocysteine into methionine, catalyzed by betaine-homocysteine methyltransferase (BHMT), increasing the plasma and tissue concentrations of methionine while reducing homocysteine levels (3). Betaine lowers plasma levels of total homocysteine (tHcy), a possible risk factor in several age-related pathologies such as cardiovascular disease, osteoporosis, dementia, stroke, cancer, and mortality. Betaine has been used clinically to lower plasma homocysteine levels in pediatric and adult patients and has proven to be safe and efficient (13).

The effects of betaine on blood and tissue lipids have been documented in animal models, suggesting that betaine supplementation has a useful impact on obesity-related health risk factors. Although it is a lipophobic compound, betaine has physiological interactions with lipids. The intensive animal industry uses additional betaine in animal food to produce leaner meat with lower fat content and decrease obesity. In a study with patients with acute coronary syndrome, betaine and lipid concentrations in plasma suggested that low plasma betaine concentrations correlated with an unfavourable lipid profile (10, 14). Higher betaine intake was also related to atherosclerosis and fatty liver (15). The hepatoprotective effects of betaine were examined in many experimental animal models of liver diseases, including alcoholic liver disease and bile acid-induced liver injury, with different mechanisms involved. Promising therapeutic effects of betaine supplementation on non-alcoholic fatty liver disease (NAFLD) have also been investigated and reported in both clinical and experimental studies (16).

Betaine is a significant methyl-group donor, osmolyte, and lipotropic agent. Although this micronutrient is a valuable ingredient of a healthy diet, there is still only limited data on its content in various foods. As far as the authors are aware, there are no published results on the betaine content in sheep milk, with this study being the first with this finding.

**The aim**

The aim of this study was to determine the betaine content in raw, unprocessed cow and sheep milk from household farms in southeastern Serbia. The previously developed HPLC method for betaine determination in commercial milk was used for its quantification. The content of macronutrients - fat and protein in samples was also measured. It was examined whether there was a statistically significant difference in the level of analyzed compounds in milk samples.

**Materials and methods**

*Sample collection*

Raw cow and sheep milk samples were collected from household farms in southeastern Serbia in July 2019. The cow farms were located in Pasi Poljana (a suburb of the city of Niš) (F1), Aleksinac municipality (F2), and Lipovica (a village in Leskovac municipality) (F3), whereas sheep farms were located in Bela Palanka (F4 and F5) and Gadžin Han (F6) municipalities.

The ruminants were healthy, bread on the same farm, and manually milked. Samples were taken from the farm bulk tank and milk cans and put into clean plastic sampling flasks of 100 ml, cooled at a temperature of 4 $^{O}$C, and transported to the laboratory. Commercial ultra-high temperature (UHT) processed cow and goat milk, produced by dairy companies in Serbia, was purchased from local shops in Niš in July 2019. The flasks with raw milk and UHT milk carton packaging were thoroughly shaken for 2 min before the analysis to ensure the homogeneity of the samples. Each milk sample was analyzed at least in triplicate.

*Determination of milk fat content*

Milk fat content was determined using the UV spectrophotometric method according to Forcato et al. (17). An aliquot of milk (30 µl) was added to 3 ml of absolute ethanol (Zorka-Pharma, Šabac, Serbia, 99.5%), and all vials were hermetically capped and stored at −20 $^{O}$C for 1 hour. This procedure enables the precipitation of proteins and hydrophobic peptides that interfere with UV measurement. The samples were centrifuged (15 min at 13,000 rpm) and allowed to reach room temperature. The aliquots of the supernatants were directly transferred to a quartz cuvette 1 cm path length and the absorbance was measured by the Evolution 60 UV/Vis scanning spectrophotometer (Thermo Scientific, USA) at 208 nm. A blind probe was absolute ethanol. The calibration curve for milk fat content quantification was performed using a series of specimens with increasing fat content by adding proper amounts of cow milk cream (20% fat content) to 0.5% fat cow milk.

*Determination of milk protein content*

The protein content in the milk samples was determined according to the procedure prescribed by Lowry et al. (18) and later modified by Polberger and Lönnerdal (19). Proteins first react with alkaline cupric sulfate and sodium-potassium tartrate to form a complex. The produced copper(1+) ions then reduce phosphomolybdic-phosphotungstic acid, Folin-Ciocalteu (FC) phenol reagent, and result in a complex with intense blue color. After 30 min incubation at room temperature, the absorbances of the samples were measured with a spectrophotometer at 750 nm. Bovine serum albumin (Serva Feinbiochemica, Heidelberg, Germany) was used as the protein standard for calibrating absorbance values. A commercially available FC reagent (2.0 mol/l) (Merck, Germany) was applied.

*Determination of content of betaine*

The betaine content in milk samples was estimated using the isocratic HPLC method that we previously developed and used for betaine quantification in commercial cow and goat milk (20). The sample pretreatment included deproteinization with 0.3% trifluoroacetic acid in acetonitrile. As a derivatization agent, 4-bromophenol bromide was used. Chromatography was performed on the Agilent Technologies 1200 Series high-performance liquid chromatography (HPLC) system (Agilent, Santa Clara, CA, USA) with a diode-array detector (DAD) SL (Agilent Technologies Inc.) and stationary phase Spherisorb SCX column (5 μm, 4.6 × 150 mm) (Waters, USA).

**Statistical analysis**

All examinations for each sample were done in triplicate, and the data were expressed as the mean value ± standard deviation (SD). The one-way analysis of variance (ANOVA) was used for testing significant differences between the mean, followed by Tukey's honest significant difference (HSD) post hoc comparison. The correlation was analyzed using the Pearson correlation coefficient, and the level of significance was set at $p < 0.05$. IBM Corp. SPSS Statistics 21.0 statistical software was applied for data analyses (21).

**Results**

*Betaine content in raw cow milk*

Table 1 shows the content of fat, protein, and betaine in raw cow milk.

The fat content in raw cow milk ranged from 3.86 (F3) to 4.69% (F1), and the average value was (4.20 ± 0.38%). There was a significant difference in fat content among the samples ($p < 0.05$). A statistically significant difference (95%) was found between the protein content in sample F3 with the lowest protein content (3.11%) and other tested milk. The average protein content in raw cow milk was (3.25 ± 0.12%). Sample F3 had the highest betaine content (8.29 mg/l), whereas the lowest content was determined in sample F1 (7.07 mg/l). There was a statistically significant difference in betaine content between those samples ($p < 0.05$). The average betaine content was (7.51 ± 0.66 mg/l).

*Betaine in raw sheep milk*

The content of fat, protein, and betaine in raw sheep milk is presented in Table 2.

Sample F4 had the highest fat content (7.03%), and a statistically significant difference (95%) was found between the fat content in samples F4 and F6, with 7.03 and 6.34% fat, respectively. The average fat content in raw sheep milk was (6.67 ± 0.33%). There was no statistically considerable difference between the protein content in the samples of raw sheep milk ($p > 0.05$), and the average value for this compound was (5.58 ± 0.16%). The betaine content ranged from 12.25 mg/l (F4) to 20.13 mg/l (F6), and there was a significant difference in the betaine content between the samples ($p < 0.05$). The average betaine content in raw sheep milk was (15.68 ± 3.52 mg/l).

**Table 1.** Content of fat, protein, and betaine in raw cow milk

| Farm | Fat (%) | RSD (%) | Protein (%) | RSD (%) | Betaine (mg/l) | RSD (%) |
|---|---|---|---|---|---|---|
| F1 | 4.69 ± 0.04a* | 0.85 | 3.36 ± 0.08a | 2.38 | 7.07 ± 0.33b | 4.67 |
| F2 | 4.04 ± 0.08b | 1.98 | 3.28 ± 0.04a | 1.22 | 7.16 ± 0.40ab | 5.59 |
| F3 | 3.86 ± 0.07c | 1.81 | 3.11 ± 0.05b | 1.61 | 8.29 ± 0.27a | 3.26 |
| Average | 4.20 ± 0.38 | | 3.25 ± 0.12 | | 7.51 ± 0.66 | |

* Data are shown as mean ± standard deviation of three replicates. Values in the column followed by different lowercase letters are significantly different according to Tukey's test ($p < 0.05$).

**Table 2.** Content of fat, protein, and betaine in raw sheep milk

| Farm | Fat (%) | RSD (%) | Protein (%) | RSD (%) | Betaine (mg/l) | RSD (%) |
|---|---|---|---|---|---|---|
| F4 | 7.03 ± 0.20a* | 2.84 | 5.67 ± 0.14a | 2.47 | 12.25 ± 0.47c | 3.84 |
| F5 | 6.65 ± 0.09ab | 1.35 | 5.64 ± 0.12a | 2.13 | 14.67 ± 0.63b | 4.29 |
| F6 | 6.34 ± 0.16b | 2.52 | 5.43 ± 0.14a | 2.57 | 20.13 ± 0.35a | 1.74 |
| Average | 6.67 ± 0.33 | | 5.58 ± 0.16 | | 15.68 ± 3.52 | |

* Values in the column followed by different lowercase letters are significantly different according to Tukey's test ($p < 0.05$)

The HPLC chromatograms of betaine in standard, raw cow, and raw sheep milk are presented in Figure 2 (A, B, and C, respectively). The peak of betaine content was determined in the milk samples at the retention time of (9.38 ± 0.01 min). Also, the peak area and height significantly increased in the chromatogram of raw sheep milk (Figure 2C) compared to the corresponding peak in raw cow milk (Figure 2B).

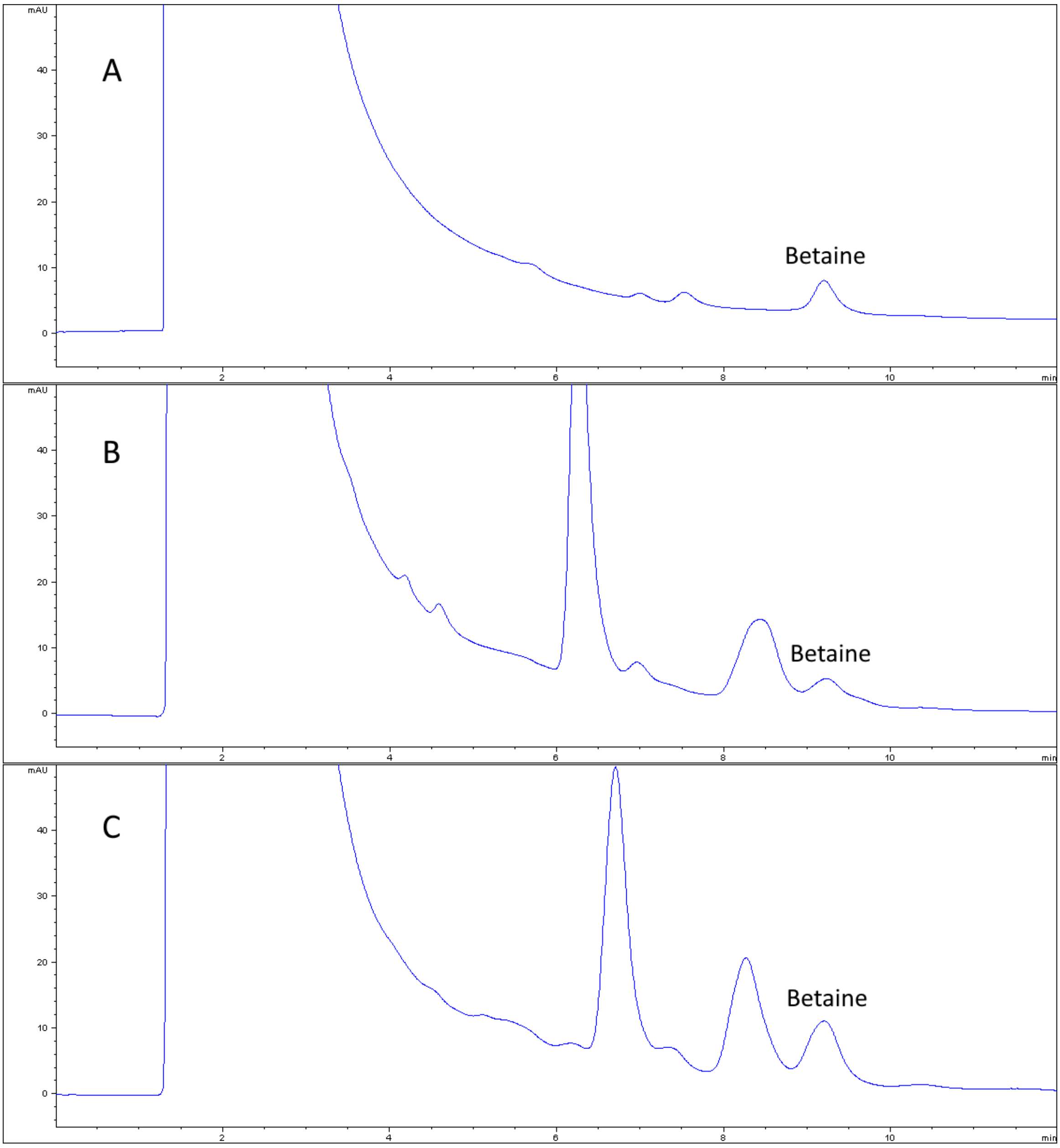


**Figure 2.** HPLC chromatograms of (A) standard solution of betaine hydrochloride (5.86 mg/l), (B) betaine (7.16 mg/l) in F2 raw cow milk, and (C) betaine (14.67 mg/l) in F5 raw sheep milk.

*Betaine in commercial milk*

Table 3 shows the content of fat, protein, and betaine in commercial milk.

There was a statistically significant difference between the samples in the analyzed parameters (fat, protein, and betaine) ($p < 0.05$).

The precision of quantification of the compound expressed as the relative standard deviation (RSD) for fat and protein varied from (0.85 to 2.84%) and (0.60 to 2.57%), respectively. RSD for betaine ranged between 0.57 and 5.59% (Tables 1, 2, and 3).

*Correlation matrix*

The correlation between the fat, protein, and betaine content for all analyzed milk samples is shown in Table 4.

The betaine and fat and betaine and protein content in the samples (r = 0.790 and 0.857, respectively) correlated highly and significantly. Also, the correlation between the fat and protein content was highly significant (r = 0.977).

**Table 3.** Content of fat, protein, and betaine in commercial milk

| Milk | Fat (%) | RSD (%) | Protein (%) | RSD (%) | Betaine (mg/l) | RSD (%) |
|---|---|---|---|---|---|---|
| Cow | 3.20 ± 0.03b* | 0.94 | 3.00 ± 0.05b | 1.67 | 7.04 ± 0.04b | 0.57 |
| Sheep | 6.00 ± 0.07a | 1.17 | 5.00 ± 0.03a | 0.60 | 14.35 ± 0.34a | 2.37 |

* Values in the column followed by different lowercase letters are significantly different according to Tukey's test ($p < 0.05$)

**Table 4.** Pearson correlation coefficients (r) between fat, protein, and betaine content

| | Fat | Protein |
|---|---|---|
| Protein | 0.977** | |
| Betaine | 0.790* | 0.857* |

* Correlation is significant at the 0.05 level
** Correlation is significant at the 0.01 level

**Discussion**

Given that betaine can readily and quickly be metabolized from choline, it is considered a non-essential nutrient. There are currently no recommended betaine dietary intake values, and the average daily intake varies considerably (3). Choline and betaine are found in many different types of food. Animal-based food, including red meat, poultry, milk, and eggs, is the leading source of choline, whereas betaine is found in grain products, cruciferous vegetables, beets, and seafood. In a study of dietary intake of these nutritions conducted by Cho et al. (15), the mean choline and betaine intake was (313 ± 61 mg/day) and (208 ± 90 mg/day), respectively. The betaine intake was slightly higher in women (216 mg/day) than in men (200 mg/day). Yonemori et al. (22) studied dietary choline and betaine intakes in the adult multiethnic population. The resulting mean intake estimated for betaine varied by gender and amounted to 154 mg/day in men and 128 mg/day in women. The main betaine intake was from grains (58.4%), followed by dark-green vegetables (12.6%) and alcohol (7.3%; beer mostly), and was similar among ethnic groups. The obtained intake of betaine from milk was 1.3% due to foods of animal origin, such as cow milk being a scarce source of betaine. In the average New Zealand diet, the mean betaine intake was (298 ± 4 mg/day). Men exhibited higher betaine intake values than women, which decreased with age. Betaine intake is dominated by grain products (67% of the total intake), with bread contributing approximately one-third of the total betaine intake. Significant non-grain contributors to betaine intake include tea and beer, while dairy products contribute only 1% (23). On the other hand, daily intake of milk varies much among regions, with an estimated average value of approximately (200-240 g/day) in Western Europe and North America, (130-300 g/day) in Latin America, (100-200 g/day in Africa), and (20-150 g/day) in Asia (24).

Milk contains a wide range of relevant nutrients and therefore plays a significant role in assisting both children and adults in meeting their nutrient requirements. From the earliest times, ruminant milk and milk products have formed an integral component of human diet. More than 95% of dairy products consumed in developed countries originate from bovine milk (25). After cow milk, goat and sheep milk are the two most produced and consumed types of ruminant milk. Cow milk dominates in the total milk production in Serbia (96.84%), whereas goat (2.20%) and sheep milk (0.96%) constitute a smaller share (26). Even though researchers are primarily focused on bovine milk, milk obtained from small ruminants, including goats and sheep, remains an appealing area of study (27). Milk composition varies depending on the species (cow, goat, sheep), with fat, protein, lactose, and mineral compounds being the major milk components (28). The average and SD values for fat and protein in raw cow milk obtained from household farms in southeastern Serbia were (4.20 ± 0.38%) and (3.25 ± 0.12%), respectively (Table 1), which is in line with the results presented in the literature. According to Roy et al. (28), the fat and protein range in cow milk was (3.3-5.4%) and (3.0-3.9%), respectively. The average fat and protein content in raw sheep milk was (6.67 ± 0.33%) and (5.58 ± 0.16%), respectively (Table 2). Similar results were found in a study by Ferro et al. (29), in which the average milk fat value amounted to (6.9 ± 1%), i.e., (5.4 ± 0.4%) for milk protein. In addition, a close range to our study, (5.0-9.0%) of the fat and (4.5-7.0%) of the protein content in sheep milk, was obtained by Roy et al. (28).

Sheep milk contains higher total solids (protein and fat) and more necessary nutrients than goat and cow milk. The beneficial effects of this kind of milk originate from a high content of fatty acids, immunoglobulins, proteins, hormones, vitamins, and minerals. Compared to cow milk, it has more than twice as much Vitamin C, double or triple other essential vitamins, especially B vitamins which are significant in the functioning of the nervous system. In addition, among animal milk, only buffalo milk has more fat content than sheep milk (30). Sheep milk has high concentrations of fat globules, which are smaller than in cow milk. Their size and dispersion make it easier to digest and confer greater milk consistency. Sheep milk also has the highest linoleic acid content of all ruminant milk due to sheep milk being effective in preventing obesity and type 2 diabetes. Also, it has more than twice as much high-quality protein (casein and whey proteins) as cow milk. Many bioactive peptides in sheep milk have proven antiviral, antibacterial, and anti-inflammatory properties (31, 32).

The average content of betaine in raw cow and sheep milk was (7.51 ± 0.66 mg/l) and (15.68 ± 3.52 mg/l), respectively (Tables 1 and 2). The betaine content in commercial (UHT) cow and sheep milk was (7.04 ± 0.04 mg/l) and (14.35 ± 0.34 mg/l), respectively (Table 3). The result obtained for (UHT) cow milk (7.04 mg/l) was in line with the average betaine concentration in commercial cow milk (7.21 mg/l) that we determined in our previous study (20). Zeisel et al. (33) reported a slightly higher betaine content (8.40 mg/kg) in 2% fat cow milk. In both raw and processed forms, sheep milk contains more than twice as much betaine compared to cow milk. A high and significant correlation was determined between the betaine and fat ($r = 0.790$) and the betaine and protein ($r = 0.857$) content in milk, while fat and protein correlate highly significantly ($r = 0.977$) (Table 4). Comparing betaine levels in raw and pasteurized (UHT) cow milk (7.51 and 7.04 mg/l) and sheep milk (15.68 mg/l and 14.35 mg/l), a lower betaine value was measured in processed milk (Tables 1, 2, and 3). We could not attribute a decrease in the betaine content in commercial milk types compared to raw to the pasteurization because the same samples did not process from a raw to a commercial form. Yet, according to Zeisel et al. (33), given that betaine is a small, highly water-soluble molecule, losses during some food processing and cooking were not unexpected.

Although cow milk is the most abundant type of milk (with 83% of global production), the use of milk from other animals has increased in recent years. Sheep milk is processed manly into cheese, yoghurt, and other dairy products. A notable advantage is that most dairy sheep production systems are environmentally friendly and play a significant role in developing rural communities (34).

## Conclusion

Currently, sheep milk is considered a delicacy in many countries, including Serbia. It is known to have high concentrations of fat and proteins, and this was confirmed by the results obtained from the analysis of raw sheep milk samples from southeastern Serbia. The high fat content in sheep milk highlights that this type of milk is a valuable energy source. In addition, bearing in mind the importance of betaine as an influential micronutrient, the high nutritional value of this foodstuff provides twice more betaine than cow milk. The current study is the first with the betaine content in sheep milk and is valuable for the estimation of this compound with a wide range of actions.

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# SADRŽAJ BETAINA U SIROVOM KRAVLJEM I OVČIJEM MLEKU

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Betain (trimetilglicin) postoji na fiziološkoj pH vrednosti u cviterjonskom obliku. Deluje kao donor metil grupa, osmolit i lipotropni agens. Iako je ovaj mikronutrijent vredan sastojak zdrave ishrane, još uvek postoje samo ograničeni podaci o njegovom sadržaju u različitim namirnicama. Cilj ovog istraživanja bio je da se utvrdi sadržaj betaina u sirovom, neprerađenom kravljem i ovčijem mleku sa gazdinstava u Jugoistočnoj Srbiji. Sadržaj masti i proteina u sirovom kravljem mleku iznosio je 4,20% ± 0,38% i 3,25% ± 0,12%, respektivno. Zatim, sadržaj masti i proteina u sirovom ovčijem mleku bio je 6,67% ± 0,33% i 5,58% ± 0,16%, respektivno. Sadržaj betaina u sirovom kravljem i ovčijem mleku iznosio je 7,51 mg/l ± 0,66 mg/l i 15,68 mg/l ± 3,52 mg/l, respektivno. S obzirom na značaj betaina, kao važnog mikronutrijenta, njegov dvostruko veći sadržaj, u ovčijem nego u kravljem mleku, doprinosi visokoj nutritivnoj vrednosti ovčijeg mleka.

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***Ključne reči:*** *betain, kravlje mleko, ovčije mleko, HPLC metoda*