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Assessment of betaine content in commercial cow and goat milk

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Abstract

Betaine is a cytoplasmic osmolyte and a methyl group donor in many biochemical pathways. It exhibits numerous beneficial biological effects and has shown health benefits against some common chronic metabolic and degenerative diseases. In humans, it is obtained mostly from the diet, but it can also be endogenously synthesised by choline oxidation. Although betaine is a valuable human nutrient, information concerning its concentration in milk is still limited. Therefore, the aim of the present work was to quantify and compare the betaine content in commercial cow and goat milk. The betaine content was estimated using a simple isocratic HPLC-UV method following derivatisation with 4-bromophenacyl bromide. The sample pre-treatment included deproteinisation with 0.3% trifluoroacetic acid in acetonitrile. Betaine concentrations in cow milk ranged from 5.56 mg/L in milk with 0.5% fat, to 8.14 mg/L in milk with 2.8% fat. A positive but not significant relationship between fat and the betaine content in milk (r = 0.43) was observed. The average betaine concentration in commercial cow milk (7.21 mg/L) was in line with the results of previous studies. On the other hand, the average level of betaine in commercial goat milk was three times greater (22.82 mg/L). As far as the authors are aware, there are no published results of the betaine content in goat milk, and the present work is the first in this field. The present work demonstrated that the application of a simple and efficient sample pre-treatment and the HPLC-UV method for betaine determination allows for its successful quantification in milk.

Introduction

Betaine (2-(trimethylazaniumyl)acetate) is an essential cytoplasmic osmolyte. This small molecule helps maintain intercellular osmolarity, and protects cells against environmental stress. Its osmoprotective property can be attributed to its dipolar zwitterion characteristics and high water-solubility. It is accumulated in various cells including renal medullary cells, and responsible for the preservation of osmotic equilibrium. Betaine provides control of the osmotic pressure inside intestinal epithelial cells consequently facilitating the secretion of digestive enzymes (Eklund *et al.,* 2005; Ueland, 2011). When used as a dietary supplement, low-molecular-weight bioprotectant betaine reduces the activity of membrane-bound ATPases, and improves efficiency in dairy, meat, and egg production from heat-stressed animals (Fedotova, 2019; Le *et al.,* 2020).

Based on the chemical structure, betaine is a trimethyl derivative of the essential amino acid glycine. Thus, it can also be called glycine betaine. It is one of the key methyl-group donors that take part in one-carbon metabolism (Sun *et al.,* 2016). Methyl groups are considered essential for many biochemical pathways and numerous cellular functions such as DNA methylation, phosphatidylcholine, and protein synthesis (Obeid, 2013).

Besides those, betaine has many other physiological effects. In the methionine-homocysteine cycle, it provides a methyl group for the conversion of homocysteine into methionine. Thus, betaine supplementation in clinical practice leads to the reduction of the homocysteine level, whose elevated concentration is considered a risk factor for cardiovascular diseases (Shai *et al.,* 2004). This compound is also studied as an antioxidant in agriculture and human health. In the methionine-homocysteine cycle, methionine is converted into S-adenosylmethionine (SAM), and through the regulation of this cycle, betaine increases the levels of non-enzymatic antioxidants, SAM, and methionine.

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The three methyl groups of betaine were found to significantly contribute to its antioxidant activity (Zhang *et al.,* 2016). Apart from antioxidant properties, betaine reduces pain responses in a dose-dependent manner. Its antinociceptive effect is mediated through opioidergic and GABA receptors (Hassanpour *et al.,* 2020). Betaine supplementation could have a beneficial impact by reducing the total body fat mass and body fat percentage, and might improve body composition in overweight and obese subjects (Gao *et al.,* 2019).

Choline, betaine, methionine, myoinositol, and carnitine are known as lipotropic compounds whose action stimulates lipid metabolism. These compounds proved to be useful in limiting excess triglyceride deposits within the liver, which could lead to liver steatosis or fatty liver. Non-alcoholic hepatic steatosis is associated with obesity, type 2 diabetes, metabolic syndrome, and dyslipidaemia (Fardet and Chardigny, 2013). Animal studies have demonstrated that betaine reduces elevated hepatic levels of lipids, homocysteine, and apoptosis in alcohol-fed mice, possibly by decreasing oxidative stress (Ji and Kaplowitz, 2003). Non-alcoholic fatty liver disease (NAFLD), the most common liver disorder, has a worldwide prevalence of 25%. Non-alcoholic hepatic steatosis is a subtype (stage) of this disorder (Cotter and Rinella, 2020). Studies show that betaine deficiency is associated with NAFLD severity. Chen *et al.* (2021) examined NAFLD models *in vivo* and *in vitro*, and confirmed that betaine contributes to the treatment by inhibiting lipoxygenase, and improving fatty acid oxidation.

Betaine is widely used as a feed additive for food-producing animals. There is growing evidence that it could have a positive impact on animal performance due to its lipotropic and growth-promoting effects (Eklund *et al.,* 2005). In the rumen, betaine is converted into acetate by microorganisms, and acetate is the major substrate for milk fat synthesis (Peterson *et al.,* 2012). Betaine supplementation has a beneficial effect on lactation performance. Wang *et al.* (2010) reported that betaine supplementation of lactating dairy cow diets increased growth, as well as milk yield, and fat percentage.

In humans, betaine is obtained mostly from the diet. It can also be endogenously synthesised by a two-step oxidation of another methyl-donating compound choline in the liver. Betaine was primarily isolated from sugar beet, and high levels of this native compound were also determined in other members of the beet family (silver beet, 910 mg/kg). Foods rich in betaine are grains (wheat bran or germ) and vegetables (spinach, 740 mg/kg), and some animal products like shellfish (clams, 2.500 mg/kg) (Craig, 2004; Lever and Slow, 2010). Nowadays, the main source of betaine in the Western diet is probably cereal-based food such as bread and pasta. The betaine content in flour and pasta amounts to 730 and 820 mg/kg, respectively. On the other hand, in cow milk, a much lower content of betaine (less than 10 mg/kg) is obtained (de Zwart *et al.,* 2003). So far, there has been little information about the content of betaine in cow milk, and to our knowledge, there are no available data about its content in goat milk.

The health benefits of betaine are numerous. This nutrient is responsible for the health of the liver, cellular replication, and detoxification reactions. However, studies of the betaine content in commonly consumed foods such as milk, as well as the improvement of its quantification methods are rare. The complexity of the milk matrix is challenging, relating to the presence of numerous compounds with very different physical and chemical properties. Moreover, the expected concentrations of betaine in the examined samples are low. Thus, the choice of the appropriate sample preparation procedure, as well as optimal chromatographic conditions, is of great importance for achieving high selectivity and sensitivity of determination. As a result, it was necessary to make some modifications to the HPLC method based on ion paired chromatography and UV detection of betaine in human plasma and urine, previously used by Laryea *et al.* (1998). The present work demonstrated the evaluation and comparison of betaine content in commercial cow and goat milk which differ in their fat contents available on the Serbian market.

Materials and methods

Chemicals

Betaine hydrochloride, choline chloride, 18-crown-6, 4-bromophenacyl bromide, and sodium dihydrogenphosphate were of analytical grade, and purchased from Acros Organics (Geel, Belgium). Methanol and acetonitrile of HPLC purity were purchased from J. T. Baker (Deventer, Netherlands). All other chemicals were of the highest analytical grade, and purchased from reputable suppliers.

Preparation of standard solutions and calibration curve

Betaine in milk was determined by using an external standard method. Standard stock solution (0.10 g/L) of betaine was prepared by dissolving the required amount of betaine hydrochloride in

deionised water. Working standard solutions of betaine (in the concentration range from 1 to 25 mg/L) were prepared by diluting an appropriate volume of stock solution with deionised water. The range of the calibration curve was chosen to cover the expected concentrations of betaine in analysed samples.

Equipment

Chromatography was performed on an Agilent Technologies 1200 Series high-performance liquid chromatography (HPLC) system (Agilent, Santa Clara, CA, USA) with diode-array detector (DAD) SL (Agilent Technologies Inc.). A Spherisorb SCX column, 5 µm, 4.6 × 150 mm (Waters, USA) was used as the stationary phase.

Sample collection

Commercial ultra-high temperature (UHT) cow and goat milk samples from modern milk and dairy processing companies in Serbia were purchased from local shops in Niš (Serbia) in September 2019: 12 UHT cow milk samples from three local manufacturers, and three UHT goat milk samples from three local manufacturers. Each sample was analysed at least in triplicate.

Fat content

The milk fat content was determined following the Gerber method (Kleyn *et al.,* 2001).

Preparation of derivatising reagent

Samples were derivatised following the method described by Laryea *et al.* (1998). Briefly, 66 mg (2.5 mmol) of 18-crown-6, and 1.390 mg (50 mmol) of 4-bromophenacyl bromide were dissolved in 100 mL of acetonitrile for the preparation of the derivatising reagent.

Milk deproteinisation

The mixture of 0.3% trifluoroacetic acid (TFA) in acetonitrile (ACN) was used as a deproteinisation agent. It was added to milk at a ratio of 1:1 (v/v). Next, 200 µL of milk was transferred into Eppendorf tube, and mixed with 200 µL of freshly prepared deproteinisation agents. The solution was centrifuged at 4,000 rpm for 10 min (Choma *et al.,* 2012).

Derivatisation

Following centrifugation, 100 µL of the supernatant was added to 100 µL of 100 mmol/L NaH_2PO_4 in 1.5 mL tubes, and the solution was mixed. Then, 800 µL of the derivatising solution was added with constant mixing. The tube was vortexed, heated at 80°C for 60 min, and allowed to cool to room temperature for 10 min (Laryea *et al.,* 1998). Prior to the analysis, the derivatised sample was again vortexed and centrifuged at 4,000 rpm for 10 min. Finally, the obtained supernatant containing 4-bromophenacyl esters of betaine was filtered through an Econofilter 25/0.45 µm RC (Agilent Technologies), and injected into the HPLC system.

Chromatographic conditions

The column temperature was maintained at room temperature, and the mobile phase consisted of 90% solvent A (methanol containing 30 mmol/L choline) and 10% solvent B (water) at isocratic mode. The flow rate was kept at 1.0 mL/min, and the UV detector was set at 260 nm. The sample injection volume and the total time of analysis were 40 µL and 12 min, respectively, and the concentration of betaine was quantified by comparing it to an external standard. Each sample was analysed in triplicate within the same run, and the average peak area was used for calculating the betaine content. The mean betaine concentration was assessed based on mg/L.

Statistical analysis

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

Results and discussion

Optimisation of the sample preparation

The prerequisite for chromatography of foods of animal origin, such as milk, is the appropriate sample preparation. Milk is an emulsion of fat globules within a solution of dissolved carbohydrates and protein aggregates with minerals, and deproteinisation was proven to be mandatory in the sample pre-treatment. Deproteinisation can be achieved using various procedures, commonly using the addition of organic solvents (ethanol, methanol, ACN) or strong acids (TCA or TFA) (Choma *et al.,* 2012). Based on the results of Choma *et al.* (2012), the procedure of milk deproteinisation with a mixture of strong organic acid in ACN was adopted. Different

volumes of milk and ACN, and various concentrations of TFA were tested. The goal was to achieve maximum protein precipitation without major dilution or acidification of the sample. The procedures with insufficient protein precipitation (which was indicated by the visual turbidity of the sample) were discarded. To obtain a clear supernatant, a deproteinisation mixture composed of 0.3% TFA in ACN was found to be the most optimal. This mixture was added to milk sample at a volume ratio of 1:1.

Optimisation of HPLC chromatographic conditions

Betaine is a quaternary ammonium compound possessing a permanent positive charge on a fully methylated nitrogen atom. As it is charged at low pH, ion exchange columns are commonly used for its separation (Kalsoom *et al.,* 2016). The 4-bromophenacyl esters of betaine also carry a positive charge at low pH, therefore, a strong cation exchange (SCX) column for HPLC separation was selected for the analysis.

Since methanol shortened the retention time and gave a peak of betaine without tailing, it was used instead of ACN as an eluent in the method of Laryea *et al.* (1998). The chromatograms of betaine standard and cow milk are presented in Figures 1a and 1b, respectively. The correspondent chromatograms for betaine standard and goat milk are shown in Figures 2a and 2b, respectively.

The retention time of the 4-bromophenacyl ester of betaine was 9.38 min. To efficiently separate betaine 4-bromophenacyl ester from other compounds present in the deproteinised milk sample, a mixture consisting of 30 mmol/L of choline in 900 mL/L of methanol was selected as the most efficient. The betaine peak was completely resolved from the strong absorbing compound eluted at 7.9 min, and from the compounds eluted between 8.2 and 8.8 min in the milk sample (Figure 1b). All of the aforementioned enabled the selective determination of betaine in complex milk samples without any interference.

This quaternary amine has a low molar absorptivity in the UV region, thus it is necessary to use a derivatisation reagent before its HPLC analysis with UV detection (de Zwart *et al.,* 2003). The optimal conditions for betaine derivatisation were examined at different temperatures (60, 70, 80, and 90°C) in periods from 30 to 90 min. Based on the obtained results, the derivatisation should be conducted at 80°C for 60 min. The reaction conditions chosen were in line with the procedures described by Laryea *et al.* (1998).

Method validation

Validation parameters are needed to assess the method, especially since betaine was determined after derivatisation by 4-bromophenacyl bromide. Milk is a highly complex mixture of macromolecules, numerous small organic and inorganic molecules, and ions. Therefore, a possible influence of these species on the derivatisation reaction should be examined. The analytical parameters of the method for the determination of betaine in milk are summarised in Table 1.

The excellent linearity of the method with a high correlation coefficient (R^2 = 0.9998) was established for the selected concentration ranging from 1 to 25 mg/L. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated by multiplying the standard deviation of the noise by 3 and 10, respectively. The obtained LOD and LOQ were significantly lower than the expected betaine concentrations in milk. The repeatability of injection was < 2%, and the relative standard deviation (RSD) of the method was 1.80%. Assay recovery was carried out by spiking samples of cow milk (2.8%

Table 1. Validation results for betaine determination in milk.

Parameter	Value
Calibration range (mg/L)	1 - 25
Number of point	6
Calibration curve	$y = 14.82x - 0.8491$
Correlation coefficient (R^2)	0.9998
Limit of detection (LOD) (mg/L)	0.35
Limit of quantification (LOQ) (mg/L)	1.17
Recovery (%)	97.7
Repeatability (n = 6, RSD) (%)	1.80
Precision (n = 6, RSD) (%)	4.30

fat) from three different producers with a known amount (5.86 mg/L) of betaine hydrochloride. The mean recovery percentage was 97.7%, thus indicating good accuracy of the method. The precision of the betaine determination, expressed as RSD, was obtained from the analysis of six replicates, which resulted in 4.30%. All these parameters confirmed that the applied method was sensitive, accurate, and precise. The derivatisation reaction was quantitative and selective enough, so there were no side reactions with other compounds in milk that could interfere with the determination of betaine. This suggested that the modified HPLC method (Laryea *et al.,* 1998) is appropriate for the determination of betaine in milk.

Betaine content in milk samples

The chromatograms of the commercial cow and goat milk samples are shown in Figures 1b and 2b, respectively. In the analysed samples, the peak of betaine at the retention time of 9.38 ± 0.01 min was determined. The betaine peak area and height significantly increased in the chromatogram of goat milk (Figure 2b) as compared to the betaine peak in cow milk (Figure 1b).

The results of the betaine content in cow milk are given in Table 2. Betaine concentrations varied from 5.56 mg/L in milk with 0.5% fat to 8.14 mg/L in milk with 2.8% fat content, and the fat content in milk ranged from 0.5 to 3.5%. A considerable betaine concentration (8.13 mg/L) was also determined in whole milk with 3.5% fat (producer 1).

The results of the betaine concentration in goat milk are shown in Table 3. A high level of betaine (23.95 mg/L) was determined in goat milk with the highest fat content of 3.2% (producer 3). Moreover, a considerable amount of betaine was found in a milk sample (3% fat) (producer 1) with the lowest measured content (20.61 mg/L). A positive but not significant relationship ($p > 0.05$) between fat and the betaine content in commercial cow and goat milk ($r = 0.43$) was determined. Based on Tables 2 and 3, the precision of the betaine content determination, expressed as the relative standard deviation (RSD%), was between 2.18 to 6.17%. RSD for the fat content varied from 0.63 to 6.00%. The average betaine content in goat milk (22.82 mg/L) was more than three times greater as compared to the average content in cow milk (7.21 mg/L), and these results contribute to the evaluation of goat milk as highly nutritious food. However, we can also classify that

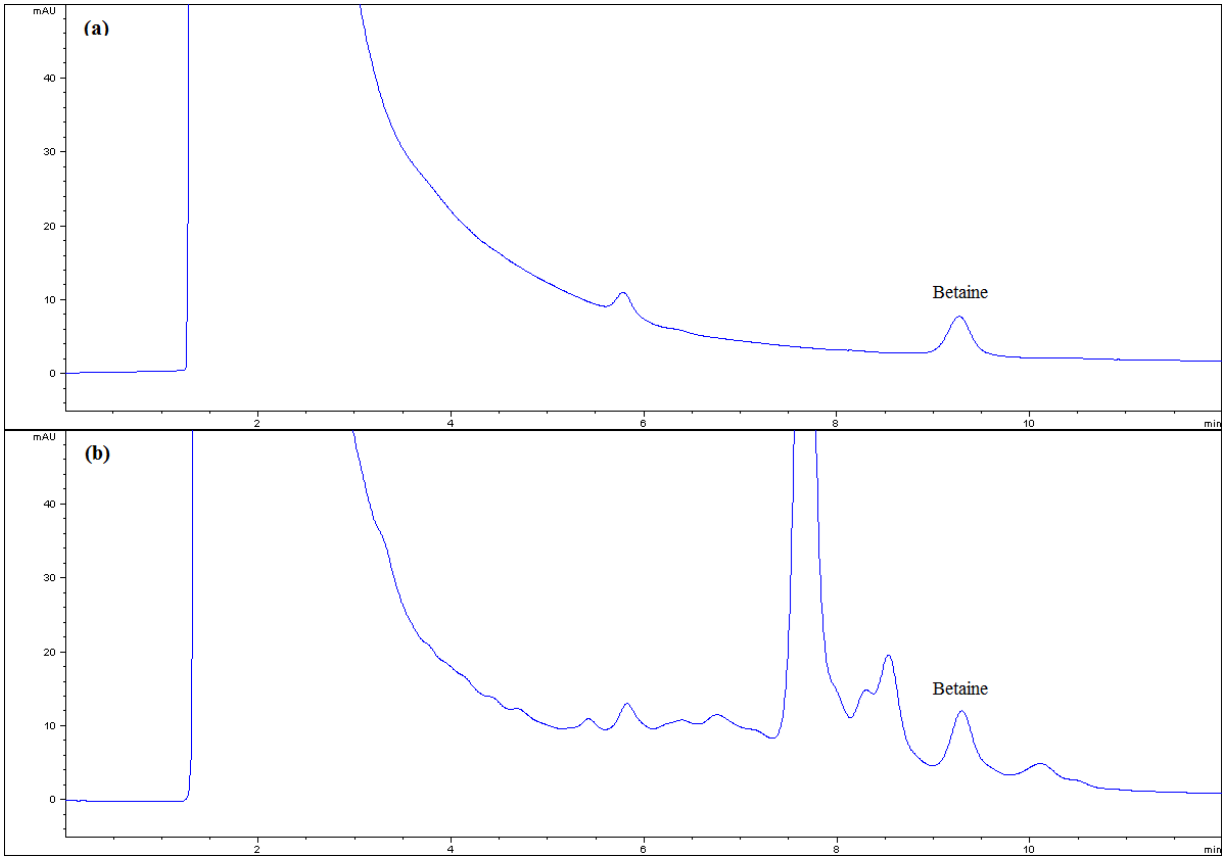

Figure 1. HPLC chromatograms of (a) standard solution of betaine hydrochloride (5.86 mg/L), and (b) betaine (7.85 mg/L) in cow milk sample.

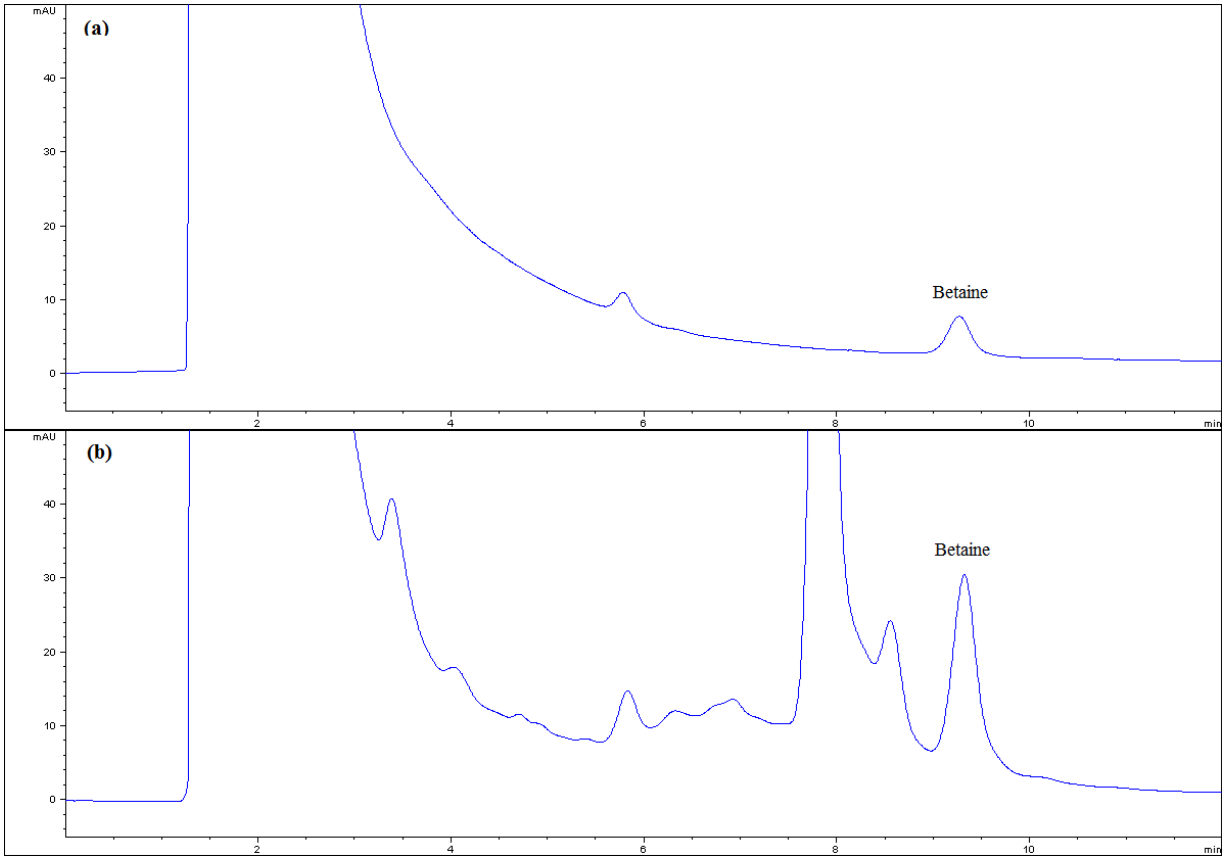

Figure 2. HPLC chromatograms of (a) standard solution of betaine hydrochloride (5.86 mg/L), and (b) betaine (23.90 mg/L) in goat milk sample.

Table 2. Betaine content in cow milk.

Producer	Fat content (%)	RSD (%)	Concentration of betaine (mg/L)	RSD (%)
1	0.9 ± 0.04^{e}	4.44	7.85 ± 0.35^{ac}	4.46
	1.5 ± 0.05^{d}	3.33	7.58 ± 0.32^{aej}	4.22
	2.8 ± 0.07^{c}	2.50	6.81 ± 0.32^{eh}	4.70
	3.5 ± 0.06^{a}	1.71	8.13 ± 0.26^{af}	3.20
2	0.6 ± 0.03^{f}	5.00	6.41 ± 0.14^{ghij}	2.18
	1.6 ± 0.08^{d}	5.00	7.78 ± 0.48^{ad}	6.17
	2.8 ± 0.07^{c}	2.50	8.14 ± 0.22^{af}	2.70
	3.2 ± 0.03^{b}	0.94	7.52 ± 0.18^{cdef}	2.40
3	0.5 ± 0.03^{f}	6.00	5.56 ± 0.32^{j}	5.75
	1.5 ± 0.04^{d}	2.67	6.91 ± 0.21^{ei}	3.04
	2.8 ± 0.06^{c}	2.14	7.17 ± 0.29^{cdeg}	4.04
	3.2 ± 0.06^{b}	1.88	6.71 ± 0.27^{fge}	4.02

Data are mean ± SD of triplicates (n = 3). Means with the same letter within the same column are not significantly different at (p > 0.05) via one-way ANOVA.

both cow and goat milk as foods which contain moderate to small levels of betaine (range 5 - 150 mg/kg) (Slow *et al.,* 2005).

Results for the betaine concentration in commercial cow milk which are similar to the results obtained in the present work were presented in the database released by the U.S. Department of Agriculture (USDA, 2008) for choline and betaine

Table 3. Betaine content in goat milk.

Producer	Fat content (%)	RSD (%)	Concentration of betaine (mg/L)	RSD (%)
1	3.0 ± 0.03[b]	1.00	20.61 ± 0.75[b]	3.64
2	3.0 ± 0.04[b]	1.33	23.90 ± 0.57[a]	2.38
3	3.2 ± 0.02[a]	0.63	23.95 ± 0.89[a]	3.72

Data are mean ± SD of triplicates (n = 3). Means with the same letter within the same column are not significantly different at (p > 0.05) via one-way ANOVA.

contents. The betaine content was 6, 9, and 6 mg/kg in low fat (1% milk fat), reduced-fat (2% milk fat), and whole cow milk (3.25% milk fat), respectively. Furthermore, according to Slow *et al.* (2005), the betaine content of New Zealand homogenised cow milk was 7 mg/kg. Sakamoto *et al.* (2001) compared betaine concentrations of human breast and cow milk originated from Japan. The average content in colostrum and transitional breast milk during one week of postpartum hospitalisation of mothers was 4.3 ± 0.9 mg/L. Also, a slightly higher betaine content (6.4 ± 0.3 mg/L) was obtained in cow milk.

The recommended daily intake of betaine has not yet been established (Craig, 2004). Studies indicated that the dietary intake of this nutrient was between 100 and 300 mg/day (Lever and Slow, 2010). de Zwart *et al.* (2003) found that the mean betaine intake was 298 mg/day. It decreased with age, and men had higher intakes than females. In a study conducted by Yonemori *et al.* (2013), the mean intake of betaine varied between sexes, too. It was 154 mg/day in men and 128 mg/day in women. Furthermore, according to Lever and Slow (2010), the concentration of betaine in plasma is also highly individual. In women, it was 20 - 60 μmol/L and in men was 25 - 75 μmol/L.

Milk has a valued role in healthy human nutrition and development throughout life, particularly in childhood. It contains essential nutritional, immunological, and biologically active components. According to the food-based dietary guidelines in the European Region (WHO, 2003), there are no recommendations for milk or dairy consumption. Many countries have developed national guidelines, and those recommendations on milk and dairy products vary significantly. Most recommend at least one serving of milk per day [a glass of yogurt or milk (250 mL)], whereas some recommend up to three servings per day. The high nutritional value and benefits of goat milk have been known for a long time. Although cow milk is the most commonly used animal milk, goat milk provides better digestibility and fat profiles. This feeding resource has proteins of high biological value, and higher mineral and vitamin contents. Higher digestive potential is due to smaller fat globules present in goat milk, while the specific chemical properties of proteins help reduce its allergenic potential. Also, the composition of goat milk is much closer to human milk in comparison to other sources of milk (Verruck *et al.,* 2019; Chen *et al.,* 2020). All of these favour the use of goat milk in the human diet over cow milk. As far as we know, there are no published data on betaine concentration in goat milk. From this standpoint, we believe that our work contributes to the study of the functional benefits of goat milk as an unexplored food matrix.

Long *et al.* (2021) suggested that increasing dietary betaine intake may help maintain or improve skeletal muscle mass (SMM) during aging. Higher dietary betaine intake was significantly associated with a reduction in SMM loss in middle-aged adults (41 - 60 years) over a three-year study. Dietary betaine intake was determined according to the food composition data obtained from the U.S. Department of Agriculture Database (USDA, 2008). Given that the number of older people in the total population is rapidly growing, particular attention should be given to valuable nutrients, such as betaine, in their healthy eating patterns.

The importance of choline and betaine is evident for experimental studies of normal physiology and in their apparent pathogenetic contribution to common chronic metabolic and degenerative diseases (Ueland, 2011). Thus, in the prevention of cancers, dietary factors have long been regarded as significant, among which choline and betaine are likely to be essential and protective nutrients. A recent epidemiologic study has demonstrated that an increment in diet intake of 100 mg/day of choline and betaine helped reduce cancer incidence by 11% (Sun *et al.,* 2016). Further, in the management of diabetes mellitus and metabolic syndrome, dietary factors have long been considered crucial as well. The supply of betaine affects lipid metabolism, and it can be insufficient in those

patients. As a result, they commonly have elevated plasma homocysteine and dyslipidaemia. Betaine deficiency may contribute to their health problems (Lever and Slow, 2010). With a slight change in dietary habits, the health of this growing population of patients could probably be improved. Therefore, regular consumption of goat milk may provide higher concentrations of lipotropic compound betaine, and consequently improve the health status of patients. To provide for healthy aging, the nutrition of older adults should include more of this type of milk. Also, bearing in mind of betaine multifunctionality, there is a lack of studies that cover a possible application of this valuable nutrient in different food resources, which would be included as part of the usual diet and in the prevention of diseases.

Conclusion

Derivatisation with 4-bromophenacyl bromide has proven to be an effective way for the selective and sensitive determination of betaine by HPLC in milk samples without complex sample preparation. Sample pre-treatment included only the deproteinisation step. The proposed method for the assessment of betaine in milk met the requirements for sensitivity, accuracy, and precision. The average betaine content in commercial cow milk (7.21 mg/L) is in line with the results of previous studies. The average content of betaine in commercial goat milk was three times greater (22.82 mg/L), and we did not find literature data on the betaine content in this type of milk. A positive but not significant relationship between fat and the betaine content in milk was found. In support of the growing consumer interest in goat milk related to its nutritional value, the significant content of betaine in this food source could also be emphasised, since this molecule expresses numerous beneficial biological effects. Betaine has shown health benefits against some common diseases and disorders such as fatty liver disease, metabolic syndrome, diabetes, cardiovascular disease, and cancer. Due to its multifunctionality, it would be of great interest to study betaine content in various other dietary sources.

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