

Article

B-Vitamins Determination in Donkey Milk

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Abstract: Background: Donkey milk is used in infant nutrition for the treatment of children affected by cow's milk protein allergy, thanks to its hypo-allergenicity, mainly due to its protein profile, which is very close to that of human milk. Furthermore, donkey milk is characterized by the presence of a fair amount of bioactive proteins, that may have a positive impact on human health. Despite the presence of numerous articles that describe the chemical composition and nutraceutical properties of donkey milk, few works have been performed to determine its vitamin profile; therefore, the purpose of this work is to give further information on the B-vitamins profile of donkey milk. Methods: B-vitamins have been determined through a reversed-phase liquid chromatography analysis. Results: The results indicated that donkey milk (DM) contains thiamine $0.661 \pm 0.26 \mu\text{M}$, riboflavin $0.168 \pm 0.04 \mu\text{M}$, nicotinic acid $18.75 \pm 1.92 \mu\text{M}$, pyridoxine $5.38 \pm 3.3 \mu\text{M}$, and folic acid $0.83 \pm 0.08 \mu\text{M}$. Conclusions: Donkey milk contains a high quantity of nicotinic acid, known for its lipid-lowering effect, and a good amount of vitamin B6 and folic acid, the latter very important for children's growth. To notice, vitamin B6 has been determined for the first time in donkey milk.

Keywords: donkey milk; B-vitamins; RP-HPLC

1. Introduction

Vitamins are essential compounds for human health, necessary in small quantities that must be introduced with the diet. These important compounds are involved in several functions; some of them are part of the molecular structure of many coenzymes necessary in different enzymatic reactions that occur in metabolic processes [1]. In bovine milk, vitamins A, K, and E, are the most abundant among the fat-soluble ones. Vitamin A is found in bovine milk ($0.35\text{--}3.14 \mu\text{M}$) and derivatives, mainly as retinyl ester, and its content is influenced by various factors, such as feeding, lactation period, climatic conditions, and livestock breed [2,3].

In bovine milk, vitamin E is present as α -tocopherol (mean content: $0.46\text{--}4.64 \mu\text{M}$), and its presence is influenced by livestock breed. It was reported in the literature that feeding the bovine with rapeseed led to an increased amount in the milk of monoenoic fatty acids and vitamin E [2,4]. The vitamin K content in the bovine milk is very low; it ranges from 0.007 to $0.015 \mu\text{M}$, and depends on the amount of fat, but its content shows a seasonal variation linked to the feeding and lactation period [2].

Among water-soluble vitamins, and in particular B-vitamins, Rutten and co-workers [5] found that vitamin B12 content in Dutch Holstein Friesian cow's milk varied from $0.73 \times 10^{-3} \mu\text{M}$ to $93 \times 10^{-3} \mu\text{M}$, with a mean concentration of $3.23 \times 10^{-3} \mu\text{M}$. These authors established a correlation between animals' genetic variation and vitamin B12 content; they suggested that the genotype of the cow influences the microbial population in the rumen which, in turn, affects the vitamin B12 content that is synthesized by

these microorganisms [5]. In other works [6,7], it was found that the vitamin content of vitamin B12 in bovine milk is higher in colostrum than in mature milk, and that this value depends on the animal diet. These authors found a vitamin B12 content of $2.8 \times 10^{-3} \mu\text{M}$ at day 11 of lactation, and $2.4 \times 10^{-3} \mu\text{M}$, at day 39 of lactation.

The folate (vitamin B9) content in bovine milk can show wide variations due to factors such as cattle nutrition, the presence of antioxidant vitamins, and the presence of oxygen [2]. In bovine milk, folate concentration has the highest concentration in the colostrum ($0.99 \mu\text{M}$), and then decreases during the lactation period: at day 11 of lactation, the concentration is $0.21 \mu\text{M}$, whereas at 39 days of lactation, it is $0.18 \mu\text{M}$ [6].

Riboflavin (vitamin B2) content in bovine milk ranges from 2.12 to $6.91 \mu\text{M}$; in pasteurized whole bovine milk, the amount of this vitamin is around $4.8 \mu\text{M}$ [2,8]. Furthermore, Poulsen and co-workers found that organic dairy cows milk shows a seasonal variation on riboflavin content that reaches the highest value during the wintertime [8]. Thiamine (vitamin B1), niacin (vitamin B3), and vitamin B6 content in bovine milk ranged from 0.59 – $2.37 \mu\text{M}$, 2.43 – $16.24 \mu\text{M}$, and 1.0 – $11.23 \mu\text{M}$, respectively [2,3,9]. Therefore, it is interesting to note that one cup of bovine milk supplies up to 62% of vitamin B12, 47% of riboflavin, and 23% of pantothenic acid RDI [3].

Donkey milk (DM) is used in infant nutrition in treating children affected by cow's milk protein allergy (CMPA), thanks to its hypo-allergenic properties, mainly due to the low total casein content and the protein profile, which is very close to that of human milk [10–12]. Besides, DM is characterized by the presence of a fair amount of some bioactive proteins, such as lysozyme, lactoferrin and lactoperoxidase, that have a positive impact on human health [13–16]. DM lactose content is similar to that of human milk. Lactose is involved in bone mineralization, since it stimulates the intestinal absorption of calcium in the infant. The fat content is much lower than that observed in human and bovine milk [17]. The Ca/P ratio in donkey milk is lower compared to bovine milk, but higher if compared to human milk [18]. Although in the literature several articles describe the chemical composition and nutraceutical properties of donkey milk, few works have been performed to determine the vitamin profile of this milk. In a previous work, it was found that the vitamin C content in DM is 0.32 mM [19], very close to the value detected in human milk and in donkey milk from Indian small grey breed (0.28 mM) [20,21], but higher than the one determined in bovine milk (28.4 – $170.3 \mu\text{M}$) [22]. Vitamin C is an antioxidant agent that acts as a free radical scavenger. In another work, the vitamin D content in DM was determined, the concentration resulted to be $0.042 \mu\text{M}$ for the vitamin D₂, and $0.016 \mu\text{M}$ for the vitamin D₃. These values were higher if compared to those found in human milk and bovine milk ($0.008 \mu\text{M}$) [23]. The same authors showed that the vitamin D content has a seasonal variation, since it is more concentrated in summer compared to winter, probably because of the greater sun exposure of the animals. Furthermore, it was found that this vitamin is not thermostable, and this fact led to a significant reduction of this vitamin in the thermally treated DM [23]. In a very recent study, the physicochemical composition, minerals, vitamins, amino acids, and the fatty acid profile of donkey milk from Indian small grey breed have been determined [21]. These authors found that vitamins A, B12 and B2 were below the limit of quantification, and determined the content of vitamin B1 ($2.9 \mu\text{M}$), niacin ($100 \mu\text{M}$), vitamin E ($3.4 \mu\text{M}$), and vitamin C (0.28 mM).

With the present work, we would give further information on the vitamin content in donkey milk from the Amiata breed, especially as regards the B-vitamins. At this purpose, a reversed-phase liquid chromatography (RP-HPLC) method has been used for the determination of thiamine (vitamin B1), riboflavin (vitamin B2), nicotinic acid (niacin, vitamin B3), nicotinamide (niacinamide), pyridoxine hydrochloride (vitamin B6), folic acid (vitamin B9) and cyanocobalamin (vitamin B12).

2. Materials and Methods

2.1. Chemicals

All reagents and solvents were HPLC or LC–MS grade. Vitamin standard (thiamine hydrochloride, vitamin B1; riboflavin, vitamin B2; nicotinic acid, niacin; nicotinamide; pyridoxine hydrochloride, vitamin B6; folic acid, vitamin B9; cyanocobalamin, vitamin B12), trichloroacetic acid (TCA), glacial acetic acid, methanol and octane sulphonic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water was prepared using a Milli-Q water system equipped with a 0.22 µm point-of-use membrane filter cartridge (Millipore-Waters, Milford, MA, USA).

2.2. Donkey Milk Samples Preparation

The experimental protocol was conducted in compliance with the requirements of the Animal Ethics Committee of the University of Camerino. Bulk raw DM samples (around 50 mL) were collected during routine herd management (machine milking once per day) from 10 asses, belonging to Amiata breed at midstage of lactation (around 120–140 days after parturition), reared in a local farm (Mamma Asina, Colmurano, MC, Italy) that produces DM for human consumption according to the EU Regulation 853/2004. After milking, DM was transported in a refrigerated bag (+4 °C) to the laboratories, to be processed and analyzed. The samples were protected from light by wrapping tubes and flasks with aluminum foil. Before being analyzed for the vitamin content, the milk samples were processed according to the method described by Albalá-Hurtado and co-workers [24]. The vitamin extraction procedure from DM samples was performed in a darkened room. Briefly, to 10.5 g of sample, 1 g of TCA was added, and the mixture was stirred for 10 min over a magnetic stirring plate. Subsequently, the mixture was centrifuged for 10 min at 1250 g, and the first supernatant was recovered. To the precipitate, 3 mL of 4% TCA was added, mixed for 10 min, and then centrifuged again (same conditions as above). After centrifugation, the supernatant was recovered and combined with the first supernatant. These two acid extracts were brought to 10 mL with 4% TCA, and filtered through a 0.45 µm filter (Whatman, Maidstone, UK), before being subjected to the RP-HPLC analysis. Three determinations were performed by adding a known amount of thiamine (31 µM) to the DM sample before the TCA treatment, for the recovery calculation.

2.3. Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) Analysis

The chromatographic courses were performed according to Albalá-Hurtado and co-workers [24], using an HPLC Agilent 1260 Infinity LC System (Agilent Technologies), consisting of a 1260 Infinity Quaternary Pump, 1260 Infinity Multisampler, 1260 Infinity Multicolumn Thermostat, and 1260 Infinity Diode Array Detector. Data were acquired by the OpenLab Software (Agilent technologies). The column was a C18 HiQSil HS, 5 µm, 4.6 mm i.d., 250 mm (Kya Tech Corporation, Tokyo, Japan). The mobile phase was: methanol (85:15), containing 0.5% triethylamine, 2.4% glacial acetic acid, and 5 mM octane sulfonic acid (pH 3.6). The analyses were carried out isocratically at 25 °C, with a flow rate of 0.9 mL/min; the total run was 40 min. The injection volume was 100 µL. The eluent was detected at 254 and 270 nm. The analyses were performed in quintuplicate.

Standard solutions of vitamin B1 (0.1; 0.3; 0.5; 0.75; 1.5; 2.0; 3.1; 6.2; 9.3; 15.5; 18.7 µM), vitamin B2 (0.5; 0.75; 1.0; 1.5; 2.0; 5.0; 7.5; 10; 15 µM), nicotinamide (0.3; 0.5; 0.75; 1.0; 1.5; 2.0; 3.0; 5.0; 7.5 µM), nicotinic acid (0.75; 1.0; 2.0; 3.0; 5.0; 7.5; 10.0; 15.0; 20.0 µM), vitamin B6 (0.75; 1.0; 2.0; 3.0; 5.0; 7.5; 10.0; 15.0; 20.0 µM) and folic acid (0.3; 0.5; 0.75; 1.0; 1.5; 2.0; 3.0; 5.0; 7.5 µM) were prepared in ultra-pure water. A 100 µL solution of each standard was separately loaded on the RP-HPLC column. The area of each standard peak was measured using the valley-to-valley integration mode, and quantification was achieved by a calibration curve obtained relating the concentration (µM) of each standard loaded in the column to the respective peak area. The quantity of vitamin B1, B2, nicotinamide, nicotinic acid, vitamin B6, folic acid in donkey milk was determined using the calibration curve. The recovery (%)

was calculated by the following equation: $\text{Recovery (\%)} = \frac{C_t - C_s}{C_a} \times 100$ (C_t : concentration obtained from the fortified sample; C_s : concentration obtained from the blank; C_a : sample concentration added).

3. Results and Discussion

B-Vitamins Determination in Donkey Milk by RP-HPLC

Vitamins are essential for life, and their deficiency induces specific diseases. B-vitamins deficiency can be caused by (a) low-quality diet in terms of micronutrient supply; (b) intestinal disorders that can lead to B-vitamin malabsorption; (c) Crohn's disease (vitamin B3); (d) pernicious anemia (vitamin B12); (e) chronic alcoholism (vitamins B1, B2, B3); (f) age; worm infections (vitamins B1 and B6) [25].

In this work, some B-vitamins have been determined in the milk of the donkey from the Amiata breed. Table 1 shows retention times, and the equation of the calibration lines for each vitamin standard, with the respective correlation coefficient (R^2) values, obtained after the RP-HPLC analysis. It should be noted that the R^2 is equal to 0.99 for all vitamin standards, indicating the very good linearity of the method. The obtained recovery for thiamine was 95%.

Table 1. Retention time (RT) and equation of the calibration lines for each vitamin standard together with the respective R^2 values.

Vitamin Standard	RT (min)	Equation	R^2
Thiamine (vitamin B1)	24.2–24.6	$y = 33.53x - 3.8732$	0.992
Riboflavin (vitamin B2)	19.4–19.9	$y = 121.82x - 0.9437$	0.999
Nicotinic acid	4.27	$y = 15.323 - 0.3843$	0.999
Nicotinamide	5.8–6.0	$y = 10.343x + 3.4316$	0.995
Pyridoxine (vitamin B6)	8.14	$y = 11.605x - 3.3578$	0.999
Folic acid	12.2–13.4	$y = 110.71x - 15.488$	0.999
Cyanocobalamin (vitamin B12)	27.5	$y = 48.13x + 0.6341$	0.997

Figure 1 shows a typical chromatogram obtained for donkey milk after the RP-HPLC analysis. The concentrations of vitamins B1 and B2, nicotinamide, nicotinic acid, vitamin B6 and folic acid, determined by the RP-HPLC method, are shown in Table 2 and values are compared with those found in the literature for human [20,26], bovine, [2,3,27], mare [28,29] and goat milk [30,31].

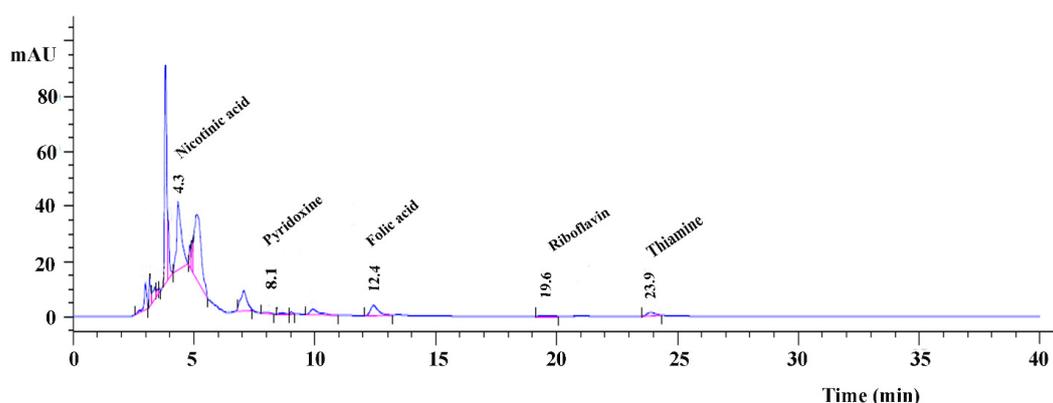


Figure 1. Chromatographic profile of B-vitamins from donkey milk obtained after RP-HPLC analysis (Column: C18 HiQSil HS, 5 μm , 4.6 mm i.d, 250 mm, connected to an Agilent 1260 Infinity LC System, Agilent Technologies).

Table 2. Content of B-vitamins in donkey milk (DM) and comparison with human milk (HM), bovine milk (BM), mare milk (MM) and goat milk (GM).

Vitamin	DM (μM)	HM (μM) *	BM (μM) §	MM (μM) °	GM (μM) **
Thiamine (vitamin B1)	0.661 \pm 0.26	0.12	0.59–2.37	0.68–1.38	1.6
Riboflavin (vitamin B2)	0.168 \pm 0.04	0.079	2.12–6.91	0.076–1.28	4.5
Nicotinic acid (niacin)	18.75 \pm 1.92	4.64	2.43–16.24	0.57–1.14	22.5
Nicotinamide	n.d.	-	-	-	-
Pyridoxine (vitamin B6)	5.38 \pm 3.35	0.480	1.00–11.23	0.49–3.6	2.7
Folic acid (vitamin B9)	0.83 \pm 0.082	0.365	0.022–0.226	0.29 $\times 10^{-3}$	0.020
Cyanocobalamin	n.d.	-	3.3 $\times 10^{-3}$	0.22 $\times 10^{-3}$	0.51 $\times 10^{-3}$

* From: [20,26]; § From: [2,3,27]; ° From: [28,29]; ** From: [30,31]; n.d.: not detected.

Thiamine concentration in DM from the Amiata breed is 0.661 μM , a value close to that reported for the bovine milk [2,3] and mare's milk [28]; about 6-fold higher to that the value observed in the human milk [20] but lower to the value (2.9 μM) found by Nayak and co-worker [21] in DM from Indian small grey breed, and lower than the value found in goat milk (1.6 μM) [30,31]. The difference in vitamin content between one breed and another may be due to genetic factors (animal breed), but also it can be possible to find differences within the same breed, and this may be caused by several factors, such as feeding, lactation period, season, climatic conditions [3]. Thiamine is the precursor of the thiamine pyrophosphate (TPP), a cofactor required for the proper functioning of four enzymes involved in the metabolism: pyruvate dehydrogenase complex (convert pyruvate in acetyl-CoA), α -ketoglutarate dehydrogenase complex (an enzyme of the tricarboxylic acid cycle), transketolase (an enzyme of the pentose phosphate shunt), and branched-chain α -ketoacid dehydrogenase complex (an enzyme of the branched-chain amino acids metabolism). In light of this, a thiamine deficiency can cause alteration in brain functions, neurotransmitters, inflammation, and oxidative stress. Besides, thiamine deficiency can cause cardiovascular system impairment, due to the pyruvate accumulation that leads to increased lactic acid production and consequently increased ventricular filling pressures and oxygen consumption [32]. Furthermore, thiamine deficiency can cause disorders in alcoholic patients due to brain damage known as acute Wernicke's encephalopathy and Wernicke–Korsakoff syndrome [33].

In this work, we found a riboflavin (vitamin B2) concentration in donkey's milk of 0.168 μM , about double of the value found in the human milk [20], but much lower concerning the riboflavin concentration found in the bovine milk and in the goat milk (Table 2) [2,3,27,31]. In mare's milk, this vitamin showed a large variability (0.076–1.28 μM) [28]. Riboflavin is the precursor of the cofactors flavin adenine mononucleotide (FMN), and flavin adenine dinucleotide (FAD), involved in the biological oxidation-reduction reaction. Furthermore, riboflavin is involved in the metabolism of folate, vitamin B12, and vitamin B6. A deficiency of riboflavin could occur in the case of dietary deprivation or stress, causing several clinical abnormalities, such as growth retardation, renal damage, anaemia, skin lesions, and nervous system impairment [34]. Riboflavin is particularly photosensitive: this is the reason why milk should be protected from light. Vitamin B2, on the other hand, does not undergo variations in the case of temperature treatment, and it is stable, even at high temperatures, as in the case of UHT milk [35].

The concentration of nicotinic acid in DM is 18.75 μM , a value higher with respect to human, bovine and mare's milk [2,3,20,26,27,29], but very close to the value found in the goat milk (22.5 μM) [31]. Nicotinic acid or niacin is a member of the B3 vitamin group showing lipid-lowering effects, since it suppresses the release of free fatty acids from adipocytes, reducing their levels in plasma and consequently reducing triglycerides and VLDL formation. It has been shown that nicotinic acid alters the expression of genes involved in the lipid metabolism, such as the diacylglycerol O-acyltransferase, an enzyme involved in the triglyceride synthesis [36].

This molecule is used as a drug in the treatment of lipid dysmetabolism, since it can help to reduce the progression of atherosclerotic cardiovascular disease [36,37]. Recently, other authors showed

that the systemic administration in mice of nicotinic acid induces robust sleep and hypothermic responses [38].

Vitamin B6 (pyridoxine) concentration in DM is 5.38 μM (Table 2), ten-fold higher, concerning the human and mare's milk [20,26,28], but close to that found in the bovine milk [2,3,27], and goat milk [31]. Navrátilová and co-workers [28] found that in mare's milk there are large variations in pyridoxine values most probably linked to the lactation period. This vitamin is involved in several metabolic processes, such as amino acid metabolism, lipid metabolism and gluconeogenesis, and is involved in the proper development and functioning of the nervous system and the regulation of the immune response. The deficiency of vitamin B6 is frequent, especially among the elderly and in women of childbearing age, causing an impairment of the immune responses [39].

Very important in milk is the presence of folic acid also known as vitamin B9. Milk represents a fundamental source of folic acid in children up to 1 year of age. The concentration of folic acid in donkey milk (0.83 μM) is higher with respect to the other kind of milk taken into consideration in Table 2. This is a significant result, since folic acid is involved in the nucleic acid synthesis, accelerates cell division, and consequently is important for the growth and development of the fetus [40]. Considering this, folic acid deficiency can cause several diseases, such as neural tube defects in new-born, megaloblastic anemia, cardiovascular disease, cancer, and Alzheimer's disease [41]. In untreated milk, native folate is essentially linked to FBP (folate binding protein). However, heat pasteurization and UHT treatments cause the loss of folic acid and a strong decrease in the concentration of FBPs and their binding capacity with this vitamin [42]. The value of vitamin B9 found in this work resulted in being higher with respect to that found by other authors in donkey milk ($3.6 \times 10^{-3} \mu\text{M}$) [43]. Finally, cyanocobalamin (vitamin B12) and nicotinamide were not detected in donkey milk. Nayak and co-workers [21] found that the level of vitamin B12 in the Indian small grey DM was below the limit of quantification, whereas in bovine milk vitamin B12 concentration is reported as $3.3 \times 10^{-3} \mu\text{M}$ [3,5]. The absence of this vitamin in donkey milk, compared to cow's milk could be explained by the different digestive systems between these two species, considering that vitamin B12 is produced by the microorganisms of the digestive tract. It will be interesting to study in depth the metabolism of vitamin B12 in donkey.

4. Conclusions

This work confirms, once again, the excellent nutraceutical properties of donkey milk, which, in addition to being a good source of bioactive proteins, is also an excellent source of vitamins. This milk contains valid amounts of nicotinic acid, which is known for its lipid-lowering effect, a good amount of vitamin B6, and appreciable quantities of folic acid, the latter very important for children's growth. Finally, it should be noted that the concentration of vitamin B6 has been determined for the first time in donkey milk. Previous works showed that donkey milk has hypoallergenic properties [44], possesses proteins with antimicrobial activity [10], has a considerable amount of vitamin C [19], vitamin D [23], nucleosides and nucleotides with sleep-inducing properties [45]. This study has demonstrated the presence of essential vitamins in donkey milk and confirmed that this milk is suitable for feeding children affected by CMPA that cannot be breastfed, but also that it can be appropriated for the elder people.

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