Journal of Food Composition and Analysis 123 (2023) 105629

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

# Changes during lactation in the mineral element content of mare milk produced in semi-extensive rural farms

A. Blanco-Doval, L.J.R. Barron, N. Aldai *

*Lactiker Research Group, Department of Pharmacy and Food Sciences, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

ARTICLE INFO

*Keywords:*
Macrominerals
Trace elements
Equid milk
Basque mountain horse breed
Grazing
Lactation stage

ABSTRACT

This study characterizes, for the first time, the content of individual mineral elements in mare milk from Basque Mountain Horse breed, as well as changes that occur during lactation and among semi-extensive rural farms. Individual milk samples from eighteen mares belonging to three commercial farms were collected along six months of lactation, and the contents of eleven mineral elements in milk were determined using a triple quadrupole inductively coupled plasma mass spectrometer (ICP-MS). Basque Mountain Horse milk contained calcium (Ca; 958 ± 248 μg/g milk), potassium (K; 581 ± 117 μg/g milk), phosphorus (P; 454 ± 160 μg/g milk), sodium (Na; 141 ± 38 μg/g milk), magnesium (Mg; 64.1 ± 15.7 μg/g milk), zinc (Zn; 2.50 ± 0.88 μg/g milk) and copper (Cu; 0.343 ± 0.179 μg/g milk) amounts similar to those reported in other horse breeds. Conversely, low sulphur (S; 156 ± 42 μg/g milk) and chlorine (Cl; 49.3 ± 33.4 μg/g milk), and high iron (Fe; 2.43 ± 2.44 μg/g milk) contents were found. Mn was only quantifiable in 12% of the samples. The content of all macrominerals, except Na and Cl, decreased at the end of lactation, while that of trace elements (Zn, Cu and Fe) either increased or fluctuated. In addition, the content of Ca, P, S and Na differed significantly among semi-extensive rural farms, probably due to differences in feeding management. From a multivariate perspective, the mineral element composition of the milk made it possible to differentiate samples from early, mid and late lactation stage.

## 1. Introduction

Minerals are inorganic compounds that play a key role in the functioning of human organism. Calcium (Ca), chlorine (Cl), cobalt (Co), copper (Cu), iodine (I), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), phosphorous (P), potassium (K), selenium (Se), sodium (Na), and zinc (Zn) are known to be essential for human physiological functions (Hunt and Nielsen, 2009); besides, sulphur (S) is detrimental for the synthesis of several metabolic intermediates (Nimni et al., 2007). Overall, mineral elements conform structural tissues and take part in numerous metabolic reactions, so deficiencies can lead to serious health disorders. Their bioavailability depends on many factors, and even with an adequate consumption of minerals, deficiencies may appear due to an inefficient absorption (European Food Safety Authority, 2017).

Mammalian milk, particularly cow milk, and dairy products, are the main food source of bioavailable Ca, a good source of P, and a moderate source of K, Na, Mg and Cl. Conversely, dairy products are poor in trace elements such as Fe, Cu, Mn and Zn. Despite the low concentration of Zn in milk and dairy products, these are one of the main sources of this trace element for children (Fantuz et al., 2016; Hunt and Nielsen, 2009). Significant differences in the concentrations of mineral elements have been reported among mammalian species (Claeys et al., 2014). Moreover, the bioavailability of mineral elements varies according to their distribution among milk fractions as well as the presence of other compounds such as ascorbate, citrate, lactose, or caseinophosphopeptides (Bouhallab and Bouglé, 2004; Fantuz et al., 2016; Zamberlin et al., 2012).

The studies carried out to date on different breeds showed that equid milk is chemically different to that from other mammalian species, but more similar to human milk. Compared to ruminant milk, mare milk showed lower fat, protein, ash and dry matter contents (0.3–4%, 1–3%, 0.3–0.5% and 9–12%, respectively), but it was richer in lactose (6–7%) (Claeys et al., 2014; Uniacke-Lowe et al., 2010). Despite its lower macronutrient content, some authors have reported that mare milk is rich in other nutritionally significant and bioactive compounds which may be responsible for different functional properties (Doreau and Martin-Rosset, 2002; Sheng & Fang, 2009), yet knowledge on this topic

* Corresponding author.
*E-mail address:* noelia.aldai@ehu.eus (N. Aldai).

https://doi.org/10.1016/j.jfca.2023.105629
Received 22 June 2023; Received in revised form 31 July 2023; Accepted 20 August 2023
Available online 24 August 2023
0889-1575/

remains scarce. In essence, considering the essential action of mineral elements in human, these (or some of these) could contribute to mare milk functionality. However, very limited data is available concerning mare milk, very especially about the content of some of them such as S, Fe, Mn and Cl. Previous studies have reported that, in general, the content of mineral elements in mare milk was not significantly affected by the dietary intake of these, with exception of Se and I (Fantuz et al., 2016; Hunt and Nielsen, 2009). On the contrary, lactation stage has shown to affect the mineral element content of mare milk, but only a few scientific works have monitored the lactation period (Grace et al., 1999; Schryver et al., 1986b; Smolders et al., 1990; Summer et al., 2004; Ullrey et al., 1966, 1974). To the best of our knowledge, only three studies were performed under grazing conditions (Grace et al., 1999; Ullrey et al., 1966, 1974). Exploring changes in the chemical composition of milk along lactation – and potential interactions with animal feeding – could lead to a better understanding of the factors that affect equine milk quality while providing tools for optimising the production.

Therefore, the aim of this study was to characterize the composition of macrominerals and trace elements, and their concentration changes over six months of lactation in mare milk from Basque Mountain Horse breed reared in semi-extensive rural farms. In this respect, it should be noted that these mares had never been milked before. In addition, it was also investigated whether differences in animal management among farms had any impact on the mineral element content of mare milk. Mare milk from this breed has never been analysed from a scientific perspective before and, considering that different horse breeds have shown variations in milk mineral element composition (Sheng and Fang, 2009), the characterisation of milk from Basque Mountain Horse is of great interest.

## 2. Materials and methods

### 2.1. Experimental design and milk sampling

Eighteen lactating mares from three different commercial equine farms (six mares *per* farm; from now on, farm I, farm II and farm III) located in the same region of Spain (Araba) were milked. All mares belonged to Basque Mountain Horse breed, and averaged ( ± standard deviation) 9.53 ± 3.76 years old and 5.68 ± 3.56 parturitions. Mares were milked only in the sampling days. Parturitions were not synchronized, and happened within a range of 39 days from late March to early May 2021; therefore, lactation time of each mare was established according to parturition day. Individual milk samples were collected during the lactation period, which was from May 4th until October 13th, 2021. During the first half of the lactation (until end July), samples were collected every 7 days, whereas during the second half (from early August to mid-October), collections happened every 14 days. In total, 304 milk samples were obtained (farm I, n = 75; farm II, n = 125; farm III, n = 104).

Table 1 shows the feeding management followed by the three commercial farms during the lactation period. In farm I, mares grazed on pastures only during May and they were fed with a mixture (15–20 kg *per* mare) of alfalfa, silage, hay, fruits and potatoes afterwards. Farms II and III kept mares on pasture all along the lactation period, and from the beginning of July, onwards, when grass availability was scarce, farm II supplemented mares with hay (4–5 kg *per* mare) and farm III with grass and alfalfa silage (9–10 kg *per* mare). All the three farms provided a mixture (2–3 kg *per* mare) of cereals and alfalfa or pea in the milking parlour in order to ease and get the animals accustomed to the milking process. Farm I performed this accustoming management only at the sampling days, whereas farms II and III got the mares to the milking parlour and repeated the process every day during the lactation period. All farms supplemented mare feeding with salt blocks.

Mares were milked as completely as possible using either automatic or manual milking. Farm I used a portable milking machine (model Canarias-1, Ultramilk, Socuéllanos, Spain) fitted with two sheep teatcups, whereas farms II and III used manual milking. In the three farms, milking happened in the presence of foal after suckling deprivation of 2–3 h. After milk sampling at the farm, these were transported in portable coolers to the laboratory and subsequently subsampled and frozen at − 80 °C until analysis. In order to avoid degradation of compounds, milk samples were thawed at 25 °C for 60 min in a water bath for the analysis of different compounds, including mineral elements in this study.

### 2.2. Chemicals

Nitric acid ($HNO_3$; 68–70%, v/v) was purchased from Fisher Chemical (Madrid, Spain) and redistilled in the laboratory for purification; hydrogen peroxide ($H_2O_2$; 30–32%, v/v) was Optima™ grade and purchased from Fisher Chemical; CMS-5 standard solution (in $HNO_3$; 5% v/v) containing 10 μg/mL each of the mineral elements Ca, Mg, Na, K, Fe, Cu, Zn and Mn was purchased from Inorganic Ventures (Christiansburg, USA); standard solutions (1000 μg/mL in water) of S, P and rhodium (Rh) were purchased from SCP Science (Quebec, Canada); and ultrapure (99.9999%) oxygen, hydrogen and helium were purchased by Air Liquide (Madrid, Spain). Millipore Milli-Q grade water (18.2 MΩ/cm) was used for dilutions.

### 2.3. Sample preparation and mineral element analysis

For digestion of milk samples, 5 mL of 65% (v/v) $HNO_3$ and 1 mL $H_2O_2$ were added to 0.20 ± 0.01 mg of liquid milk, and the mixture was submitted to a Speedwave-four microwave assisted digester (Berghof Products + Instruments, Berlin, Germany) at a maximum of 190 °C and 80% power. Digested samples were diluted in Milli-Q water, and

**Table 1**
Feeding management used by each commercial farm during the lactation period of mares (from May to October).

| | Grazing regime | | | | Supplements* | | |
|---|---|---|---|---|---|---|---|
| Farm | Pasture type | Quantity | Time period | Meadow size | Type | Quantity (*per* mare) | Time period |
| I | Vetch, ryegrass, oat | *Ad libitum* | May | 2–3 ha | Alfalfa (20%), silage (20%), hay (20%), fruits (20%), potato (20%) | Daily ration of 15–20 kg of mixture | June-October |
| | | | | | Alfalfa, oat and corn | 2–3 kg of mixture | During the milking process |
| II | Alfalfa, clover, ryegrass, *Festuca spp.*, orchard grass, dandelion | *Ad libitum* | May-October | 28 ha | Hay | Daily ration of 4–5 kg | July-October |
| | | | | | Oat (60%), wheat (20%) and pea (20%) | 2–3 kg of mixture | During the milking process |
| III | Alfalfa, clover, ryegrass, *Festuca spp.*, orchard grass, dandelion | *Ad libitum* | May-October | 28 ha | Grass and alfalfa silage | Daily ration of 9–10 kg | July-October |
| | | | | | Oat (60%), wheat (20%) and pea (20%) | 2–3 kg of mixture | During the milking process |

* Percentages and weights of feeding ingredients are approximate.

analysed in an 8900 triple quadrupole inductively coupled plasma mass spectrometer (ICP-MS; Agilent, Santa Clara, USA). The ICP-MS system was equipped with a MicroMist concentric nebulizer and a Fassel-type torch with an internal diameter of 2.5 mm. Plasma was generated with argon gas as follows: 1550 W radio frequency power, 1.2 V radio frequency matching, 15 L/min plasma gas flow, 1.05 L/min nebulizer gas (argon) flow, 0.9 L/min auxiliary gas (argon) flow. An MS/MS scan was used for P and S with oxygen as cell gas (flow 30%), and for Ca with hydrogen as cell gas (flow 4.7 mL/min). For the rest of the elements, a single Quad scan was performed using helium at a flow-rate of 4.6 mL/min. Rh was used as internal standard, and for mineral element quantification, Na, Mg, K, Ca, P, S, Mn, Fe, Cu and Zn standard solutions were analysed by triplicate in a range greater than 0.05 and less than 2400 ng/mL. These concentrations were prepared from commercial standard solutions using 1% (v/v) $HNO_3$ in Milli-Q grade water. Table 2 shows the analytical parameters evaluated for method validation. Linearity was assessed by calculating the determination coefficient ($R^2$) of the calibration curves adopting between five and eight calibration levels depending on each mineral element. Precision was estimated as the percent relative standard deviation (RSD) for six replicates of standard solutions at the lowest and highest concentration for each mineral element (Table 2). In the same way, accuracy was evaluated in terms of recovery by analysing six replicates of mineral element standard solutions at these concentrations. Limits of quantification (LOQ) were determined, for each element, by analysing ten replicates of the lowest concentration of the mineral element standard solution that obtained an RSD value less than 20%. LOQ values were expressed as µg/g referring to the wet weight of the milk sample, considering the dilution step occurred in the sample preparation. Data in Table 2 showed a high linearity with $R^2$-values higher than or equal to 0.9940 for all the mineral elements studied, as well as a good precision with RSD values less than 8.50% except for S at high concentration. Recovery values (accuracy) ranged between 95.39% and 105.13% for all mineral element and concentration, which was an acceptable value for macromineral and trace element quantification. Although LOQ values for macromineral elements were provided in mare milk, these elements were far above their LOQ values. On the other hand, those limits were of interest for those trace elements ranging between 0.650 and 0.044 µg/g milk (for Fe and Mn, respectively).

The amount of Cl in the milk samples was estimated by using the semi-quantitative mode of ICP-MS. This method is based on predefined default values, and the approximate concentration of the mineral element is calculated from the counts *per* second measured by ICP-MS (Krzciuk et al., 2016).

### 2.4. Statistical analysis

Software version 28.0 of IBM-SPSS Statistics (IBM, New York, USA) was used to perform statistical data treatment. Three significant figures were used to express the mineral element content of the milk samples. Mineral element content data were log transformed, and normality, homoscedasticity and residual randomness were checked. Outliers (values higher or lower than 3 times the interquartile range) checked by box-plots were excluded from the data set. In order to make more comprehensible the study of the changes in the mineral element content of mare milk samples during lactation, the data was pooled in several lactation ranges of 14 days with the earliest lactation range corresponding to weeks 3 and 4, and the last range to weeks 25 and 26. Data (excluding Mn) were analysed using the Linear Mixed Model (LMM) procedure of analysis of variance including individual animal as subject, farm as fixed factor and lactation time as repeated measure factor. The interaction term between farm and lactation time was also included in the model. The parameters of the LMM were estimated using the Restricted Maximum Likelihood Method and the compound symmetry matrix was selected for the repeated measures covariance structure following the Akaike information criterion. Least square means of dependent variables for the levels of the fixed factor (farm) were compared using the Tukey's test. In addition, a Stepwise Discriminant Analysis (SDA) was applied to the mineral element composition (excluding Mn and Cl contents) of the milk samples from the commercial farms to classify milks according to mare lactation stage. For this purpose, milk samples were labelled as early lactation (from week 3–10), mid lactation (from week 11 to week 18) or late lactation (from week 19–26). Significance level was declared at $P \leq 0.05$.

## 3. Results

In mare milk samples analysed from all commercial farms over lactation, Ca, K and P were the major mineral elements with an average ( ± standard deviation) content of 958 ± 248, 581 ± 117 and 454 ± 160 µg/g milk, respectively, and an average Ca:P ratio of 2.26 ± 0.60. Among other macrominerals, an average of 156 ± 42 µg S, 141 ± 38 µg Na, 64.1 ± 15.7 µg Mg and 49.3 ± 33.4 µg Cl *per* g of milk were found (Table 3). Except Mn, the trace elements were found above the LOQ in most milk samples collected during the lactation period. The average content of trace elements was of 2.50 ± 0.88 µg Zn, 2.43 ± 2.44 µg Fe and 0.343 ± 0.179 µg Cu *per* g of milk. Mn was only present above the LOQ in 35 out of the 304 samples analysed, with an average of 0.118 ± 0.230 µg/g milk. Milk samples containing quantifiable amounts of Mn were from different animals and were randomly distributed along lactation and among farms; therefore, no further statistical data treatment was performed for Mn.

Table 3 shows the content of all the macrominerals and trace elements by farm, as well as the effect of the factors lactation time and farm. Farm effect was significant ($P \leq 0.05$) for Ca, P, S and Na contents in mare milk samples. For these minerals, milk samples from farm I (mares grazing only during the first month of lactation) contained higher amounts than those from farm II and III (mares grazing along lactation and supplemented after July with hay or silage, respectively). On the other hand, lactation time significantly ($P \leq 0.05$) influenced the

**Table 2**
Validation parameters of the ICP-MS analytical method used to quantify macrominerals and trace elements in mare milk samples.

| Mineral element | Calibration range (ng/mL) | $R^2$ | LOQ (µg/g milk) | RSD (%) | | Recovery (%) | |
|---|---|---|---|---|---|---|---|
| | | | | LConc | HConc | LConc | HConc |
| Ca | 43.00 – 2147.00 | 0.9999 | 26.875 | 2.12 | 0.11 | 95.76 | 101.86 |
| K | 12.36 – 2074.91 | 0.9999 | 7.725 | 2.89 | 3.86 | 99.29 | 98.82 |
| P | 1.54 – 2336.11 | 1.0000 | 0.963 | 2.86 | 7.79 | 103.66 | 104.84 |
| S | 1.40 – 2074.91 | 0.9999 | 0.875 | 11.20 | 8.29 | 103.11 | 97.42 |
| Na | 12.36 – 746.49 | 0.9940 | 7.725 | 3.83 | 5.70 | 103.37 | 104.51 |
| Mg | 0.37 – 372.70 | 0.9998 | 0.231 | 5.46 | 6.05 | 95.39 | 98.65 |
| Zn | 0.70 – 372.70 | 0.9999 | 0.438 | 7.73 | 4.85 | 103.01 | 99.22 |
| Fe | 1.04 – 372.70 | 0.9998 | 0.650 | 3.01 | 4.00 | 105.13 | 100.40 |
| Cu | 0.13 – 175.14 | 0.9999 | 0.081 | 5.88 | 5.81 | 98.57 | 100.93 |
| Mn | 0.07 – 57.80 | 1.0000 | 0.044 | 1.53 | 5.43 | 97.25 | 102.37 |

RSD, relative standard deviation; R2, determination coefficient; LOQ, limit of quantification; LConc; lowest concentration; HConc, highest concentration.

**Table 3**
Average concentration (µg/g milk) and range (minimum-maximum) of macrominerals and trace elements in mare milk samples from the commercial farms over the lactation period (from May to October), and statistical significance (*P*-value) of the principal factors (farm and lactation) and their interaction. The total number of milk samples collected in commercial farms were 75, 125 and 104 for farm I, II and III, respectively.

| | Farm | | | | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Mineralelement | I | n | II | n | III | n | SEM | Farm | Lactation | Farm * Lactation |
| Ca | 104·10$^{1\ a}$<br>(654–169·10$^{1}$) | 75 | 928$^{b}$<br>(504–153·10$^{1}$) | 125 | 936$^{a,b}$<br>(503–146·10$^{1}$) | 104 | 18 | 0.025 | < 0.001 | 0.857 |
| K | 582<br>(448–856) | 75 | 560<br>(385–755) | 125 | 604<br>(414–103·10$^{1}$) | 104 | 9 | 0.461 | < 0.001 | 0.104 |
| P | 502$^{a}$<br>(201–837) | 75 | 431$^{b}$<br>(193–781) | 125 | 447$^{a,b}$<br>(202–806) | 104 | 12 | 0.013 | < 0.001 | 0.471 |
| S | 165$^{a}$<br>(96.7–287) | 75 | 153$^{b}$<br>(86.6–259) | 125 | 154$^{a,b}$<br>(90.1–253) | 104 | 3 | 0.015 | < 0.001 | 0.493 |
| Na | 179$^{a}$<br>(111–304) | 75 | 134$^{b}$<br>(89.6–180) | 125 | 123$^{b}$<br>(69.0–198) | 104 | 4 | < 0.001 | < 0.001 | 0.005 |
| Mg | 65.4<br>(38.2–118) | 75 | 61.6<br>(29.2–114) | 125 | 66.2<br>(41.5–103) | 104 | 1.1 | 0.493 | < 0.001 | 0.013 |
| Cl | 41.2<br>(16.5–143) | 75 | 52.3<br>(18.1–202) | 125 | 51.5<br>(19.6–262) | 104 | 2.4 | 0.193 | < 0.001 | 0.895 |
| Zn | 2.26<br>(1.15–4.06) | 75 | 2.67<br>(1.18–7.43) | 125 | 2.47<br>(1.21–5.13) | 103 | 0.06 | 0.061 | < 0.001 | 0.491 |
| Fe | 2.75<br>(0.709–24.5) | 73 | 2.31<br>(0.707–8.97) | 118 | 2.32<br>(0.651–18.0) | 100 | 0.18 | 0.427 | 0.006 | 0.035 |
| Cu | 0.329<br>(0.120–1.06) | 70 | 0.327<br>(0.101–1.03) | 121 | 0.372<br>(0.134–0.956) | 98 | 0.013 | 0.056 | < 0.001 | 0.080 |
| Mn | 0.0665<br>(0.0477–0.0903) | 5 | 0.0735<br>(0.0450–0.127) | 20 | 0.200<br>(0.0441–1.39) | 15 | 0.040 | | | |
| Ca:P | 2.24<br>(1.29–3.54) | – | 2.30<br>(1.14–4.45) | — | 2.24<br>(1.00–3.55) | — | 0.04 | 0.794 | < 0.001 | 0.707 |

$^{a,b}$ Different letter superscripts indicate significant differences ($P \leq 0.05$) among farms.
n, number of milk samples from each farm in which mineral elements were found to be above the limit of quantification (see Table 2); SEM, standard error of the mean.

concentrations of all the minerals and the Ca:P ratio. Figs. 1 and 2 depict the changes in the mineral element content of milk samples from each farm. Contents of Ca, K, P, S and Mg showed a downward trend throughout lactation (Fig. 1). Contents of Mg and K steadily decreased during lactation. Moreover, the evolution of Mg content differed among farms (significant interaction, Table 3): in farm I and farm III, average Mg content continuously decreased except for one time point in farm I (weeks 17–18), whereas the decrease in farm II was discontinuous (Fig. 1). The evolution of Ca, P and S contents in milk samples along lactation followed similar patterns in all farms, with a slight reduction during the first weeks of lactation, peaking at mid-lactation (Ca: at weeks 15–16; P and S: at weeks 13–14), and noticeably decreasing afterwards. In farm I, the peaking time of Ca and S appears one or two weeks delayed. On the other hand, Na contents slightly increased (2.77% on average from weeks 3–4 to weeks 25–26) and Cl contents fluctuated considerably in all farms during lactation (Fig. 1). However, Na content changed differently in milk from the different farms with significant ($P \leq 0.05$) interaction between farm and lactation time (Table 3). In fact, Na content increased during the second half of lactation in farms I and III, remaining more constant in farm II.

The content of the trace elements either increased (Cu and Zn) or fluctuated (Fe) along the lactation period (Fig. 2). Both Cu and Zn contents remained more or less constant until they increased from weeks 9–12 until weeks 15–20, with a later decreasing trend. The content of Fe did not show a clear pattern during lactation apart from final contents being overall lower than initial ones. An interaction between farm and lactation stage was seen in Fe concentration as well (Table 3), since the evolution along lactation appears one or two weeks delayed in farm III compared to farms I and II. On average, Ca:P ratio decreased from 2.22 to 1.68 during the first half of lactation, and then increased for the second half, achieving values as high as 2.78 (Fig. 2).

## 4. Discussion

According to the results obtained (Table 3), the major mineral element in Basque Mountain Horse breed milk was Ca, followed by K and P in approximately half the amount of Ca (as confirmed by the Ca:P ratio). Average macromineral content found in this study falls within the range reported in literature for other equine breeds: 261–1380 µg Ca, 341–701 µg K, 152–884 µg P, 107–220 µg Na, and 29–104 µg Mg *per* g or mL raw milk (Alipour et al., 2023; Anderson, 1991; Bilandžić et al., 2014; Csapó-Kiss et al., 1995; Grace et al., 1999; Schryver et al., 1986a, b; Smolders et al., 1990; Summer et al., 2004; Ullrey et al., 1966) for mare milk samples collected at 14 or more days of lactation. The content of S was on average lower in Basque Mountain Horse breed milk than in pasture-fed Thoroughbred breed milk reported by Grace et al. (1999), who found 196–230 µg S/mL milk. This could be a consequence of lower protein content found in Basque Mountain Horse breed milk samples in the present study (1.84 vs. 2.10 g protein/100 g milk, on average). In fact, proteins, particularly the sulphur containing amino acids methionine and cysteine, are the main source of dietary S (Nimni et al., 2007). In addition, Cl content in the milk samples of the present study was 3–4 times lower than that reported by Summer et al. (2004) in Haflinger breed milk (150–198 µg Cl/g milk). Changes in the chlorine content in bovine and human milks are known to be mainly due to pathological processes or microbial contamination, as well as to lactation stage, but not to dietary factors (Chen et al., 2018; European Food Safety Authority, 2019; Gaucheron, 2005). However, being involved – together with lactose, Na and K – in the balancing of the osmotic pressure of milk, Cl content has been also correlated with the content of lactose and/or Na in bovine milk (Bijl et al., 2013).

Regarding trace elements, Zn and Cu contents (Table 3) were in accordance to those found in the literature (1.1–4.1 µg Zn and 0.07–0.64 µg Cu *per* g or mL milk), whereas Fe contents in milk samples from Basque Mountain Horse breed were higher than those reported in other equine breeds (0.18–1.30 µg/g or mL) (Alipour et al., 2023; Anderson, 1992; Bilandžić et al., 2014; Csapó-Kiss et al., 1995; Grace et al., 1999; Schryver et al., 1986a, b; Ullrey et al., 1974). Reports on Mn concentration vary from less than 0.01 µg/g milk up to 0.05 µg/g milk (Alipour et al., 2023; Anderson, 1992; Bilandžić et al., 2014; Csapó-Kiss et al., 1995). In the present work, Mn contents lower than 0.0438 µg/g milk were below the LOQ established in the ICP-MS methodology

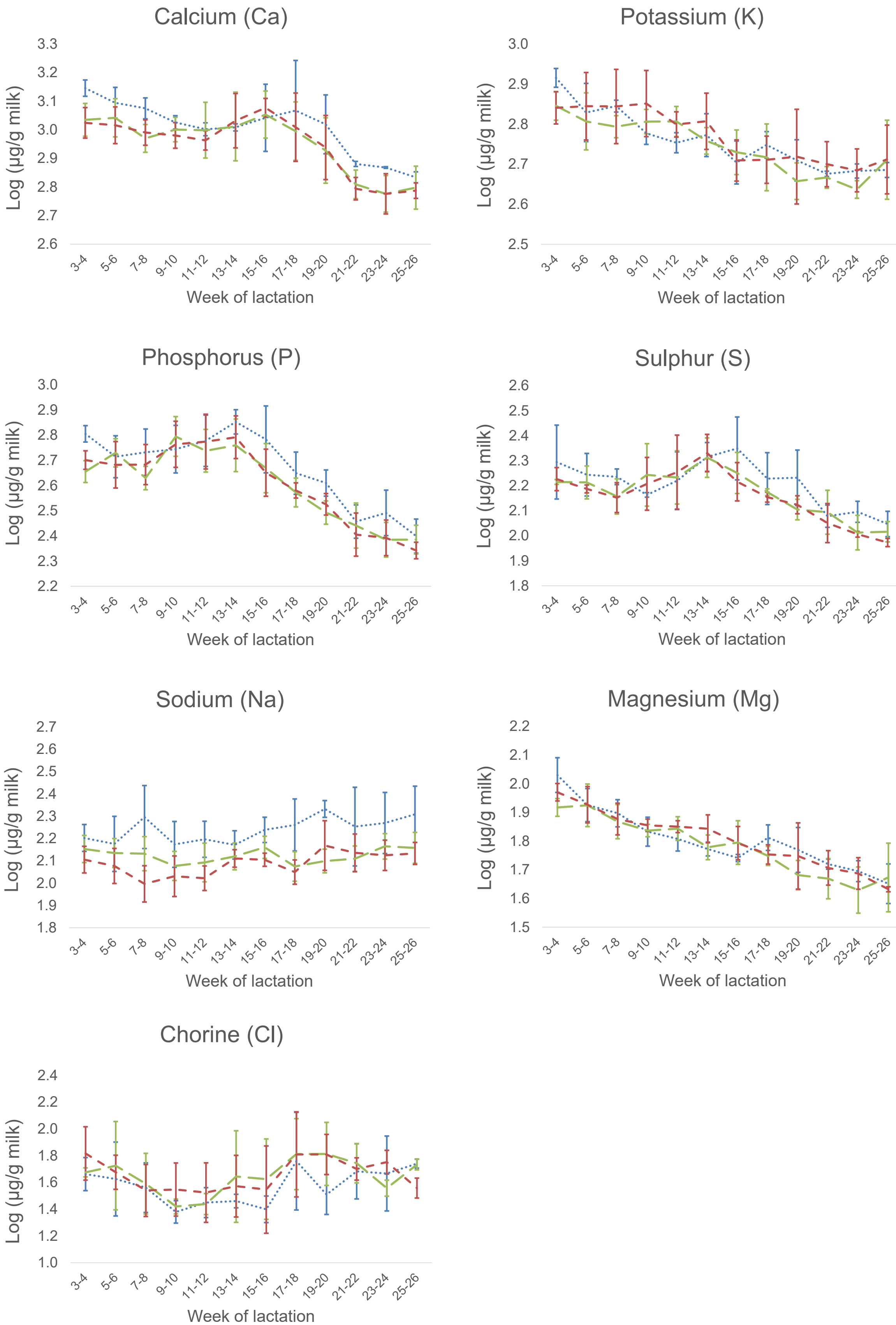


**Fig. 1.** Changes in macromineral content of mare milk samples from semi-extensive rural farms during lactation. ····, farm I; — —, farm II; - - -, farm III.

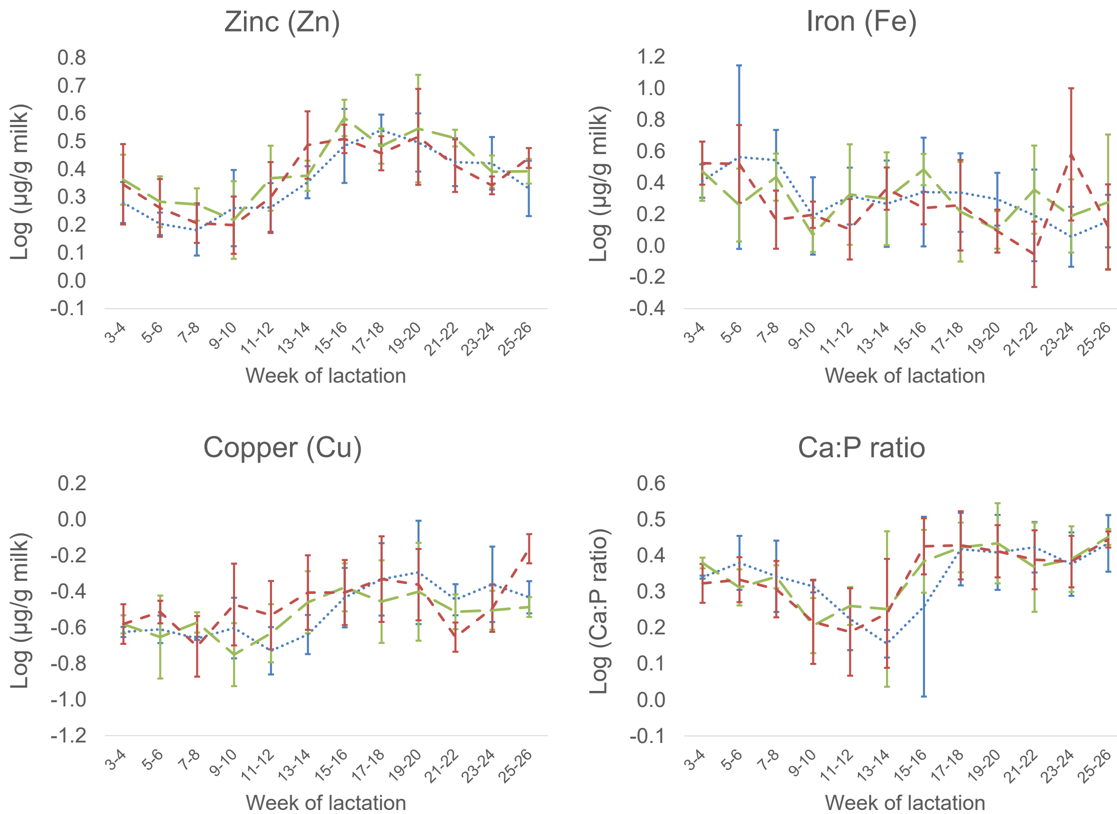


**Fig. 2.** Changes in trace element content and Ca:P ratio of mare milk samples from semi-extensive rural farms during lactation. , farm I; , farm II; , farm III.

(Table 2). In consequence, comparison with literature data was difficult. Anyway, both human and animal milks have been considered a poor source of Mn (Fantuz et al., 2016).

Results (Table 3, Figs. 1 and 2) confirmed the significant ($P \leq 0.05$) effect of lactation time on the content of mare milk mineral elements previously reported by other authors (Grace et al., 1999; Summer et al., 2004). According to literature, Ca, P, Mg, Na, K, Cu, Fe and Zn contents decrease from week 3 to mid- (Schryver et al., 1986b; Smolders et al., 1990; Ullrey et al., 1966, 1974) or late-lactation (Martuzzi et al., 2004; Summer et al., 2004). Conversely, Grace et al. (1999) did not find significant differences in S, Cu and Fe contents after the 3rd week of lactation. Higher mineral element contents within the initial stages of lactation are inversely related to lactose content (which increases throughout lactation; Fantuz et al., 2016; Uniacke-Lowe et al., 2010). This negative correlation between mineral elements and lactose contents has also been observed in milk from other mammalian species, and has been described as responsible for osmotic regulation. In this sense, mainly $Na^+$, $K^+$ and $Cl^-$ ions, and Ca and P associated to caseins, control milk volume in early lactation, and this role is taken up by lactose afterwards (Kobeni et al., 2020; Shennan & Peaker, 2000).

Overall, the evolution of mineral elements over the lactation period slightly differed among mare milk studies. The most variable macrominerals seemed to be Na and K since the content of both fluctuate along lactation (as the Na content in the present study; Fig. 1), but differently among studies (Grace et al., 1999; Schryver et al., 1986b; Summer et al., 2004). In the case of K content, present results resemble those reported by Ullrey et al. (1966), with a more consistent decrease and less fluctuation during lactation (Fig. 1). Similar to the present study, Summer et al. (2004) found that Cl content fluctuated without specific pattern. In the present research, mineral element changes along lactation were, in general, similar to those obtained by Grace et al. (1999) in pasture-fed mare milk, with higher content fluctuations, more prominent decreases after mid-lactation (particularly in Ca and P), and low content of Na and Zn found around weeks 7–9, also reported by Ullrey et al. (1974) for Zn. Interestingly, the peaking of some mineral elements in Basque Mountain Horse breed milk at mid-lactation has not been previously described in other mare milk studies.

From a multivariate perspective, the results of the SDA applied to the mineral element composition of mare milk samples collected in commercial farms made it possible to classify more than 86% of the samples into their lactation stage (early-, mid- and late-lactation). The milk sample distribution of Fig. 3 displayed the two canonical discriminant functions obtained. As depicted, the three lactation stages were considerably discriminated along the function 1 axis, whereas function 2 mainly contributed to discriminate between mid-lactation from the other two lactation stages (early- and late-). This multivariate approach confirmed the previously mentioned results about the individual mineral element content changes throughout lactation period. Despite of inter-farm variability, principally related to feeding differences among semi-extensive rural farms, the SDA was able to differentiate mare milk samples from early-, mid- and late-lactation.

The Ca:P ratio can affect both Ca and P bioaccessibility and bioavailability in humans. This ratio ranges between 1.9 and 2.4 in human milk, and is generally lower in cow, goat and ewe milk (Bass & Chan, 2006; Bonjour, 2011), yet there is high variability among studies. The Ca:P ratio obtained in the present study was, overall, higher than that previously reported in mare milk in other equine breeds (Alipour et al., 2023; Schryver et al., 1986b; Smolders et al., 1990; Summer et al., 2004) probably due to slightly higher Ca and slightly lower P concentrations. Other authors also found average Ca contents 1.4–1.9 times higher than P contents in mare milk (Anderson, 1991; Martuzzi et al., 2004; Schryver et al., 1986a). In accordance to the present study (Fig. 2), literature showed a decrease in the Ca:P ratio from early- to mid-lactation in mare milk (Schryver et al., 1986b; Smolders et al., 1990; Summer et al., 2004) and a posterior increase after the 16th week (Summer et al., 2004).

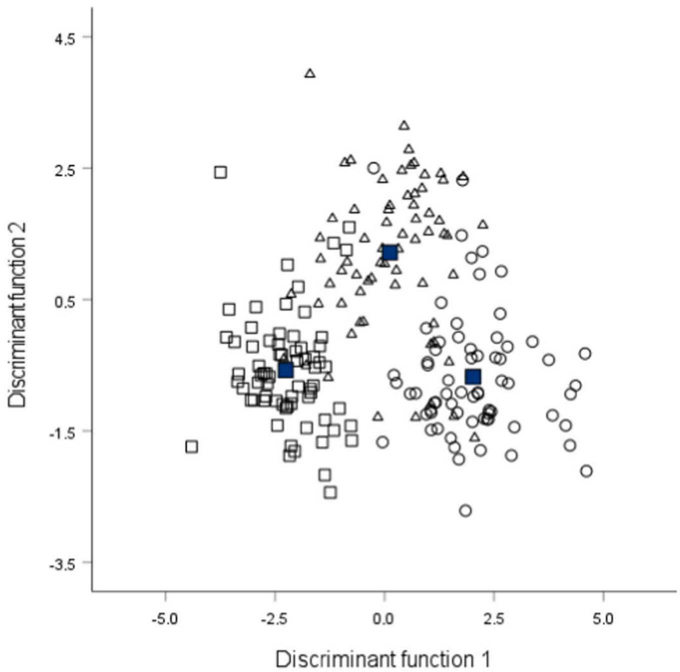


**Fig. 3.** Distribution of mare milk samples collected during early- (○), mid- (Δ), and late- (□) lactation stages according to Stepwise Discriminant Analysis performed using the mineral element composition (■, group centroid). Canonical functions 1 and 2 explained 85.7% and 14.3% of the total variance, respectively.

Other factors (apart from lactation) affecting mare milk mineral element composition are poorly understood and, to our knowledge, there is no previous data analysing the effect of management system. Actually, the understanding of this effect might be relevant when considering mare milk production for human consumption, specifically from a commercial perspective. Results from this study showed that mineral element composition of mare milk coming from different semi-extensive rural farms was generally similar, although Ca, P, S and Na contents significantly ($P \leq 0.05$) varied (Table 3). These variations could be related to differences associated to the management practices – including feeding – followed by each farm (Table 1). Except for Se and I, it is known that dietary mineral intake is unlikely to affect milk mineral element composition (Asai et al., 1995; Fantuz et al., 2016; Grace et al., 1999; Hunt & Nielsen, 2009; Kavazis et al., 2002). However, grazing could contribute to mineral element changes in milk. In a study by Soyeurt et al. (2022), they were able to discriminate between grass-fed and non-grass-fed cows according to protein, predicted Ca and, in a lower extent, predicted P and Mg contents in milk. Another study found that protein (and casein), Ca, P, Na, Zn, Cu, Mn and Se contents significantly varied among milks from cows fed concentrates and grazing on different pastures (Gulati et al., 2018). Grazing activity (low, moderate or high) also affected protein, Ca and P contents in cow milk (Stergiadis et al., 2021). In this regard, the results of the present study showed significantly ($P \leq 0.05$) higher contents of Ca, P and S (this last one related to proteins) in milk samples from low grazing mares (farm I) than in those samples from mares that grazed for a longer time and were supplemented with hay (farm II). Significantly higher Na contents were also found in milk samples from low grazing (farm I) than in those from long-term grazing mares (farms II and III). Furthermore, the peaks of concentration found in Ca, P and S at mid-lactation could be related to changes in grass availability after July.

Other factors such as age of the animal (Alipour et al., 2023; Asai et al., 1995), number of parturitions or health status of the mammary gland (Linn, 1988) could also have had a significant effect on the content of specific mineral elements in milk, but scientific literature on this topic remains very scarce. Since the present study was performed in commercial farms, other management practices apart from feeding could not be controlled. Consequently, correlation of the variability in milk mineral element content among farms with specific factors was not possible.

As previously indicated, milk is a great source of some essential elements described in the present work, yet not every milk type contributes equally to fulfil human daily mineral requirements. Compared to other animal species, mare milk is not the richest milk in terms of mineral element concentrations. Despite this, its mineral element composition is very similar to that of donkey (also an equid) and human milks, containing, overall, less mineral elements than other mammalian species (Claeys et al., 2014; Fantuz et al., 2016; Garhwal et al., 2023). On the other hand, mineral bioavailability of milk differs among mammalian species and can be affected by various factors. Therefore, not only mineral element content but also their bioavailability affects the nutritional value of the product, so the contribution of each type of milk to the recommended daily mineral intake should be carefully considered. Further research should address the bioavailability of mineral elements present in mare milk in order to properly evaluate the contribution of this food product to human health.

## 5. Conclusions

Milk from Basque Mountain Horse breed mares contains similar average mineral element concentrations compared to those described in the scientific literature in other equine breeds, except for lower S and Cl, and higher Fe contents. The macromineral (Ca, K, P, S, Na, Mg and Cl) and trace element (Zn, Fe and Cu) contents studied, as well as the Ca:P ratio, were significantly influenced by lactation time. Overall, the content of all macrominerals except Na and Cl decreased, Na, Zn and Cu increased, and Cl and Fe fluctuated throughout lactation. In addition, the management system used in farm affected Ca, P, S and Na concentrations in milk. This could be related to differences in the feeding management of mares in semi-extensive rural farms. These findings can contribute to the promotion of mare milk as a diversification option for the current equine meat production system performed in semi-extensive rural farms, and overall, to livestock grazing system sustainability.

## CRediT authorship contribution statement

**Ana Blanco-Doval:** Methodology, Formal analysis, Investigation, Writing – original draft. **Luis Javier R. Barron:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Noelia Aldai:** Conceptualization, Methodology, Formal analysis, Writing – review & editing.

## Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from noelia.aldai@ehu.eus.

## Data Availability

Data will be made available on request.

## Acknowledgements

The Department of Education of the Basque Government for the predoctoral fellowship of A. Blanco-Doval. This work was supported by the Basque Government through Biotasma (Elkartek, 2019), Behor Esne (Cooperation, 2020) and IT944-16 and IT1568-22 projects. Authors thank the commercial farms that participated in the study, and also the SGIker – Advanced Research Facilities of the University of the Basque Country (UPV/EHU) for assisting in sample analysis.

## References


Alipour, A., Mohajeri, F.A., Javdan, G., Pourramezani, F., Fallahzadeh, H., Sadrabad, E. K., 2023. Concentration of mineral and heavy metals in raw mare (horse) milk consumed in Yazd, Iran: a risk assessment study. Vet. Med. Sci. 9, 1592–1598.

Anderson, R.R., 1991. Comparison of minerals in milk of four species. Comp. Biochem. Physiol. Part A: Physiol. 100, 1045–1048.

Anderson, R.R., 1992. Comparison of trace elements in milk of four species. J. Dairy Sci. 75, 3050–3055.

Asai, Y., Katsuki, R., Matsui, A., Nanbo, Y., 1995. Effects of rations and age on mineral concentrations of Throughbred mare's colostrum. J. Equine Sci. 6, 21–24.

Bass, J.K., Chan, G.M., 2006. Calcium nutrition and metabolism during infancy. Nutrition 22, 1057–1066.

Bijl, E., van Valenberg, H., Huppertz, T., van Hooijdonk, A., 2013. Protein, casein, and micellar salts in milk: Current content and historical perspectives. J. Dairy Sci. 96, 5455–5464.

Bilandžić, N., Sedak, M., Đokić, M., Kolanović, B.S., Varenina, I., Božić, Đ., Končurat, A., 2014. Differences of the essential mineral element levels in the milk of Croatian Coldblood horse and Littoral-Dinaric donkey. Mljekarstvo 64, 12–18.

Bonjour, J.P., 2011. Calcium and phosphate: A duet of ions playing for bone health. J. Am. Coll. Nutr. 30, 438S–448S.

Bouhallab, S., Bouglé, D., 2004. Biopeptides of milk: Caseinophosphopeptides and mineral bioavailability. Reprod. Nutr. Dev. 44, 493–498.

Chen, X., Chu, B., Xi, H., Xu, J., Lai, L., Peng, H., Deng, D., Huang, G., 2018. Determination of chlorine ions in raw milk by pulsed amperometric detection in a flow injection system. J. Dairy Sci. 101, 9647–9658.

Claeys, W.L., Verraes, C., Cardoen, S., De Block, J., Huyghebaert, A., Raes, K., Dewettinck, K., Herman, L., 2014. Consumption of raw or heated milk from different species: An evaluation of the nutritional and potential health benefits. Food Control 42, 188–201.

Csapó-Kiss, Z., Stefler, J., Martin, T.G., Makray, S., Csapó, J., 1995. Composition of mares' colostrum and milk. Protein content, amino acid composition and contents of macro- and micro-elements. *Int. Dairy J.* 5, 403–415.

Doreau, M., & Martin-Rosset, W. (2002). Dairy animals: Horse. In H. Roginski (Ed.), Encyclopedia of dairy sciences (pp. 630–637). Massachusetts, MA, USA: Academic Press.

European Food Safety Authority, 2017. Dietary reference values for nutrients: summary report. EFSA Support. Publ. 14. Article e15121E.

European Food Safety Authority, 2019. Dietary reference values for chloride. EFSA J. 17. Article e05779.

Fantuz, F., Salimei, E., Papademas, P., 2016. Macro- and micronutrients in non-cow milk and products and their impact on human health. In: Tsakalidou, E., Papadimitriou, K. (Eds.), Non-Bovine milk and Milk Products. Academic Press, Massachusetts, MA, USA, pp. 209–261.

Gaucheron, F., 2005. The minerals of milk. Reprod. Nutr. Dev. 45, 473–483.

Garhwal, R., Bhardwaj, A., Sangwan, K., Mehra, R., Pal, Y., Nayan, V., Iquebal, M.A., Jaiswal, S., Kumar, H., 2023. Milk from Halari donkey breed: nutritional analysis, vitamins, minerals, and amino acids profiling. Foods 12, 853.

Grace, N.D., Pearce, S.G., Firth, E.C., Fennessy, P.F., 1999. Concentrations of macro-and micro-elements in the milk of pasture-fed thoroughbred mares. Aust. Vet. J. 77, 177–180.

Gulati, A., Galvin, N., Lewis, E., Hennessy, D., O'Donovan, M., McManus, J.J., Fenelon, M.A., Guinee, T.P., 2018. Outdoor grazing of dairy cows on pasture versus indoor feeding on total mixed ration: Effects on gross composition and mineral content of milk during lactation. J. Dairy Sci. 101, 2710–2723.

Hunt, C.D., & Nielsen, F.H.. (2009). Nutritional aspects of minerals in bovine and human milks. In P. McSweeney, & P. Fox (Eds.), Advanced dairy chemistry, Vol. 3: Lactose, water, salts and minor constituents (3rd ed) (pp. 391–456). New York, NY, USA: Springer.

Kavazis, A.N., Kivipelto, J., Ott, E.A., 2002. Supplementation of broodmares with copper, zinc, iron, manganese, cobalt, iodine, and selenium. J. Equine Vet. Sci. 22, 460–464.

Kobeni, S., Osthoff, G., Madende, M., Hugo, A., Marabini, L., 2020. The dynamic changes of African elephant milk composition over lactation. Animals 10, 948 (Article).

Krzciuk, K., 2016. Intelligent analysis of samples by semiquantitative inductively coupled plasma-mass spectrometry (ICP-MS) technique: A review. Crit. Rev. Anal. Chem. 46, 284–290.

Linn, J.G., 1988. Factors affecting the composition of milk from dairy cows. In National Research Council (Ed.), Designing foods (pp). Animal Product Options in the Marketplace. National Academies Press (US), Washington DC, CO, USA, pp. 224–241 (pp).

Martuzzi, F., Summer, A., Formaggioni, P., Mariani, P., 2004. Milk of Italian saddle and haflinger nursing mares: physico–chemical characteristics, nitrogen composition and mineral elements at the end of lactation. Ital. J. Anim. Sci. 3, 293–299.

Nimni, M.E., Han, B., Cordoba, F., 2007. Are we getting enough sulfur in our diet? Nutr. Metab. 4, 24 (Article).

Schryver, H.F., Oftedal, O.T., Williams, J., Cymbaluk, N.F., Antczak, D., Hintz, H.F., 1986a. A comparison of the mineral composition of milk of domestic and captive wild equids (Equus przewalski, E. zebra, E. burchelli, E. caballus, E. asinus). Comp. Biochem. Physiol. Part A: Physiol. 85, 233–235.

Schryver, H.F., Oftedal, O.T., Williams, J., Soderholm, L.V., Hintz, H.F., 1986b. Lactation in the horse: the mineral composition of mare milk. J. Nutr. 116, 2142–2147.

Sheng, Q., Fang, X., 2009. Bioactive components in mare milk. In: Park, Y.W. (Ed.), Bioactive Components in Milk and Dairy Products. Wiley-Blackwell, Iowa, IA, USA, pp. 195–213.

Shennan, D.B., Peaker, M., 2000. Transport of milk constituents by the mammary gland. Physiol. Rev. 80, 925–951.

Smolders, E.A.A., van der Veen, N.G., van Polanen, A., 1990. Composition of horse milk during the suckling period. Livest. Prod. Sci. 25, 163–171.

Soyeurt, H., Gerards, C., Nickmilder, C., Bindelle, J., Franceschini, S., Dehareng, F., Veselko, D., Bertozzi, C., Gengler, N., Marvuglia, A., Bayram, A., Tedde, A., 2022. Prediction of indirect indicators of a grass-based diet by milk Fourier transform mid-infrared spectroscopy to assess the feeding typologies of dairy farms. Animals 12, 2663 (Article).

Stergiadis, S., Qin, N., Faludi, G., Beauclercq, S., Pitt, J., Desnica, N., Pétursdóttir, A.H., Newton, E.E., Angelidis, A.E., Givens, I., Humphries, D.J., Gunnlaugsdóttir, H., Juniper, D.T., 2021. Mineral concentrations in bovine milk from farms with contrasting grazing management. Foods 10, 2733 (Article).

Summer, A., Sabbioni, A., Formaggioni, P., Mariani, P., 2004. Trend in ash and mineral element content of milk from Haflinger nursing mares throughout six lactation months. Livest. Prod. Sci. 88, 55–62.

Ullrey, D.E., Ely, W.T., Covert, R.L., 1974. Iron, zinc and copper in mare's milk. J. Anim. Sci. 38, 1276–1277.

Ullrey, D.E., Struthers, R.D., Hendricks, D.G., Brent, B.E., 1966. Composition of mare's milk. J. Anim. Sci. 25, 217–222.

Uniacke-Lowe, T., Huppertz, T., Fox, P.F., 2010. Equine milk proteins: Chemistry, structure and nutritional significance. Int. Dairy J. 20, 609–629.

Zamberlin, Š., Antunac, N., Havranek, J.J., Samaržija, D., 2012. Mineral elements in milk and dairy products. Mljekarstvo 62, 111–125.