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Investigation of Lactation Period and Technological Treatments on Mineral Composition and IR-Profiles of Donkey Milk by Chemometrics

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Featured Application: Multi-platform analysis of donkey milk.

Abstract: Donkey milk represents an efficient substitute for human milk in infants' diets being unlikely to cause allergic reactions. In this study, different donkey milks were collected at two lactation times (T_0 and T_1), subjected to freezing–thawing and freeze-drying, and analyzed by Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES) and ATR-FT-IR. The data collected on freeze–thaw (FT-) and reconstituted (R-)milks were investigated by ANOVA–Simultaneous Component Analysis (ASCA) and Principal Component Analysis (PCA). The following concentrations (μg/mL) for FT and R-milks, respectively, at T_0, were found: Ca: 712 ± 71, 600 ± 72; Fe: 0.7 ± 0.3, 0.1 ± 0.1; K: 595 ± 49, 551 ± 59; Mg: 75 ± 5, 67 ± 4; Na: 117 ± 16, 114 ± 16; P: 403 ± 30, 404 ± 38; Zn: 1.6 ± 0.2, 1.6 ± 0.3. At T_1, the concentrations (μg/mL for FT and R-milks, respectively) were: Ca: 692 ± 60, 583 ± 43; Fe: 0.13 ± 0.02, 0.13 ± 0.03; K: 641 ± 71, 574 ± 61; Mg: 72 ± 4, 63 ± 1; Na: 116 ± 9, 109 ± 8; P: 412 ± 30, 405 ± 24; Zn: 1.6 ± 0.3, 1.6 ± 0.3. ASCA demonstrated the treatment has a substantial effect, and PCA revealed that the largest quantities of metals, specifically Fe, Mg, and Ca for T_0 and K, P, and Na for T_1, are present in the FT-milk samples. The IR spectra of FT- and R-milks revealed no macroscopic changes among them or between lactation periods, indicating this technique may not suitably capture variability in lactation or conservation processes in donkey milk. Despite the relatively small sample size, this study offers insight on the mineral composition changes in donkey milk and emphasizes the significance of milk preprocessing and the lactation period on it.

Keywords: donkey milk; freezing–thawing; freeze-drying; ICP-OES; mineral composition; FT-IR; chemometrics; explorative analysis; PCA; ASCA

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1. Introduction

In recent years, donkey milk has gained appeal as a healthy alternative for a newborn's diet [1,2] due to its resemblance to human milk and lack of risk of allergic reactions.

The beneficial effects associated with its intake have been known since ancient times, and it is considered an effective supplement to counteract the effects of various pathologies [3–7]. A number of researchers [8–11] have proven that the composition of human and donkey milk is similar under several aspects, and that it can provide to infants a number of benefits from microbiological [12] and anti-inflammatory points of view [13,14]. In fact, it has been demonstrated that it is a suitable medium for *L. rhamnosus* probiotic strains, with all the derived benefits, and it prompts the production of anti-inflammatory cytokines [15]. In addition, positive aspects on neural functioning have been highlighted [16]. In the

literature, only few nutritional drawbacks have been pointed out in donkey milk, such as low caloric, lipid, and iron intake; consequently, it is important to include it in a balanced diet, or to fortify it [1]. In general, this food is recommended for neonate nutrition; therefore, analytical methods to ensure its genuineness have been proposed [17]. Numerous chemical and biochemical studies have been conducted on the components of donkey milk, especially with regards to proteins, such as caseins, whey protein, fats, and vitamins [18–21]. This milk also contains a rich bouquet of minerals; nevertheless, little attention has been given to the elemental composition of this food. Fantuz et al. quantified Ca, P, S, Mg, K, and Na in whole, skimmed, ultracentrifuged, and ultrafiltered donkey milk, in order to understand how the different elements distribute in the diverse fractions (fats, caseins, whey proteins, and aqueous phase), and they have even tried to correlate elemental concentrations in milk with those in the animal feed [22–24]. A similar study has been conducted by Malacarne et al. [25], who characterized different components of whole donkey milk during lactation (second-ninth month). Among the others, they exploited Atomic Absorption Spectrometry to quantify mineral elements such as: Ca, Mg, Na, K, Zn, Fe, and Cu, and a colorimetric method for P, concluding that the mineral content varies significantly with the lactation period. A further interesting study on this regard is the one from Astolfi and collaborators, who compared the elemental composition of milks from different mammals (among the others, donkey) with plant-based milks and concluded that the levels of toxic elements in all specimens do not endanger consumers [26].

In the literature, sensory analysis trials of donkey milks, aiming at inspecting consumer acceptance, have been discussed [27,28]. For example, Malissova et al. [29] investigated the sensory characteristics of 30 samples of donkey milk from Greece and Cyprus. Panelists estimated the perceived intensities of different attributes: appearance, taste, scent, flavor, aftertaste, and texture in a standardized sensory test form (with a 5-point hedonic scale). The outcome revealed donkey milk is an excellent culinary option since it presents an appreciated sweet taste because of its considerable lactose content.

Despite the scientific attention that donkey milk has aroused in recent years, low focus has been given on how conservative processes can change its properties. It is known that freeze–thaw cycles can alter milk's composition. Di Donato et al. investigated frozen and thawed (FT) donkey milk, in order to determine whether, despite the process, it was still possible to detect adulterations with cow milk. Ma et al. [14] evaluated the effect of freezing and thawing cycles on goat milk and they observed a considerable reduction in fats and perceived viscosity, higher proteins' oxidation, and alterations on their secondary structure, and, so far, to the best of our knowledge, the same has not been studied on donkey milk. Similarly, several works have focused on the effect of freeze-drying processes on milk from different mammals [30,31], but no attention was given to donkeys. In light of this, the present work aims at studying donkey milk subjected to two different preprocessing processes: freezing–thawing and freeze-drying. Freeze–thaw (FT-) and reconstituted (R-) milks were analyzed by Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES) to quantify their mineral composition, and by attenuated total reflection–Fourier transform infrared (ATR-FT-IR) spectroscopy. Data obtained by these platforms were then explored by analysis of variance–simultaneous component analysis (ASCA) and Principal Component Analysis (PCA).

2. Materials and Methods

2.1. Donkey Milk Samples

The bulk raw donkey milk samples were gathered in July 2019 (T_0) from the early-day milking of Ragusana and Amiantina breed pluriparous jennies (50–160 days after foaling) and in October 2019 (T_1, 130–240 days from foaling). Donkeys were raised in accordance with Regulations EC n. 853/2004 in a recognized farm called "Ciucolandia" in Capestrano (AQ), Abruzzo Region, Italy. The farm used normal agricultural practices based on natural pasture merged with ad libitum polyphite hay and a 1 kg/head per day concentrate integration. The jennies were machine milked daily, and the foals were manually taken

from the females three hours before the milking. The milk specimens have been acquired in duplicate, transferred to the laboratory in a refrigerated bag (+4 °C), and stored in a refrigerator at −18 °C. Before analysis or freeze-drying, the samples were defrosted at room temperature and homogenized by sonication for 30 min using a Branson 1510 sonicator. Based on the yield of the freeze-drying process, ranging between 8.09 and 9.27%, reconstituted donkey milk (R-milk) samples were prepared from the freeze-dried samples, by adding the required amount of water (typically 10 mL to 0.8–0.9 g of freeze-dried milk) to have the same moisture content as the non-processed sample.

2.2. Chemicals

Analytical calibration curves were built using six standard solutions (by dilution in 50 and 100 mL polymethylpentene (PMP) volumetric flasks) and a blank made of ultrapure water. The multi-element TraceCERT® standard solution for ICP (Fluka Analytical, Sigma Aldrich) contained Fe and Zn (both at 100 mg/L) and the mono-element TraceCERT® certified standards for AAS contained Ca, K, Mg, Na, and P. The samples were digested using suprapure nitric acid (65% w/w) from Merck KGaA (Darmstadt, Germany) and hydrogen peroxide solution (30% w/w) for ultra-trace analysis from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Freeze-Drying

The freeze-drying process was performed in the Department of Industrial and Information Engineering and Economics of L'Aquila University (L'Aquila, Italy) by a Flexy-Dry MP lyophilizer (ALT, American Laboratory Trading, San Diego, CT, USA). The apparatus was set up to perform initial freezing at −45 °C for 1 h, primary drying at 30 °C and 0.10 mbar pressures for 48 h, and secondary drying at 5 °C for 3 h at the same pressure [32]. The dish, milk, and condenser temperatures were all continuously monitored during the procedure.

2.4. ATR-FTIR Measurements

Using a PerkinElmer Spectrum Two TM (PerkinElmer, Waltham, MA, USA) FT-IR spectrometer supplied with a PerkinElmer Universal Attenuated Total Reflectance (uATR) device (single bounce diamond crystal) and a deuterated triglycine sulfate (DTGS) detector, infrared spectra of donkey milk samples were obtained. The spectral range under inspection ranged from 4000 cm^{-1} to 400 cm^{-1} (1 cm^{-1} nominal resolution).

The background was gathered and updated around every ten samples. Using soft tissues and methanol, any potential sample residue on the uATR diamond was eliminated. Cleaning operations were carried out prior to each measurement. FT- and R-milks were examined by pouring a few drops of the sample (using a glass pipette) into the device designed to contain liquids on the diamond.

2.5. Microwave-Assisted Digestion

Amounts of 2.5 mL volumes of FT- or R- donkey milk were divided into Teflon vials and digested in an Ethos One (Milestone, Bergamo, Italy) microwave oven after being mixed with 1 mL of hydrogen peroxide and 5 mL of nitric acid.

After precisely homogenizing the freeze-dried donkey milk samples, mineralization was carried out on 180 mg aliquots, which were then dispersed in 2 mL of water. Subsequently, equivalent amounts of hydrogen peroxide and nitric acid were added to the samples. The digestion process involved heating the samples at a constant power of 1500 W. The temperature was raised to 120 °C within 5 min and maintained for 3 min. Then, the temperature was further increased to 200 °C in 8 min and held at this level for 15 min. The resulting solutions were diluted with ultrapure water into 25 mL PMP volumetric flasks after cooling at room temperature. Fortified samples were prepared and consumed in the same manner as unfortified samples to verify the accuracy of the analytical procedure. The same donkey milk lot was used to collect aliquots meant to act as both genuine and

enhanced samples in recovery evaluation. Recovery experiments were carried out individually for macro- (Ca, Na, P, K, and Mg) and micro- (Fe and Zn) elements, accounting for the sample amount and various dilutions utilized in the measurements. The recovery tests were carried out using spiking levels similar to the naturally occurring quantities of the identified components in donkey milk (as revealed by preliminary analysis).

2.6. Multi-Elemental Analysis by ICP-OES

The analysis of seven elements of interest (Ca, Fe, K, Mg, Na, P, and Zn) was carried out using an Iris Intrepid ER/S Thermo-Elemental (ThermoScientific, Waltham, MA, USA) ICP-OES spectrometer equipped with an Echelle grating optical system and a charge injection device (CID) solid-state detector on the digested samples (after transfer into polypropylene vials). A Timberline II (ThermoScientific) autosampler collected the samples with a flow rate of 1.85 mL/min, controlled by a peristaltic pump. Each sample was conveyed in a concentric pneumatic nebulizer, where it was nebulized (nebulizer gas flow of 0.6 mL/min) into the argon plasma, connected to a cyclonic spray chamber and eventually analyzed in a radial torch-reading configuration using the operating conditions recommended by the manufacturer, i.e., radiofrequency power of 1.200 kW, coolant gas flow of 12 L min^{-1}, and auxiliary gas flow of 0.5 L min^{-1}. Ultra-High Purity 5.0 Grade Argon (99.999%) was used in the spectrometric analysis. The emission lines with the best signal-to-noise ratio and fewest spectral interferences were chosen, and the emission intensity underwent a manual background correction. The measurements on standards and digested samples were performed in four replicates and results averaged. A 200 µg/L Yttrium solution as the Internal Standard (line 324.228 nm) was used to monitor matrix effects, possible non-spectral interferences, and instrumentation drift. Cleanliness of the introduction system and an absence of memory effects were controlled by the analysis of one standard solution (0.06 µg/mL for Fe and Zn, 10 µg/mL for Mg, 20 µg/mL for K, and 30 µg/mL for Ca, Na, and P) followed by a blank every six samples.

2.7. Statistical Analysis

2.7.1. ANOVA–Simultaneous Component Analysis

As the name suggests, ANOVA–Simultaneous Component Analysis (ASCA) [33] combines Analysis of Variance (ANOVA) [34] and Simultaneous Component Analysis (SCA) [35] with the aim of exploring data. Taking into account two different effects (in the present case, the conservation process (α) and lactation time (β)) ASCA begins breaking down the data matrix (**X**) as follows:

$$\mathbf{X} = \mathbf{X}_{\alpha} + \mathbf{X}_{\beta} + \mathbf{X}_{\alpha\beta} + \mathbf{X}_{E} \quad (1)$$

where $\mathbf{X}_{\alpha}$ and $\mathbf{X}_{\beta}$ are the matrices of the effects, $\mathbf{X}_{\alpha\beta}$ is the interaction matrix, and $\mathbf{X}_{E}$ contains the unexplained variability.

After the decomposition, SCA is applied on the individual matrices, in order to obtain a more detailed distribution of the variance among the various levels associated with variables α and β. Eventually, similarly to PCA, the ultimate result of the analysis can be visualized using scores plots, which enable the depiction of patterns across the studied individuals.

2.7.2. Principal Component Analysis

Principal Component Analysis (PCA) [36] was applied to evaluate the influence of lactation time and/or technological processes on the donkey milk samples. PCA enables the representation of multivariate data in a low-dimensional space of Principal Components (PCs) that are uncorrelated because they are orthogonal to one another. They can be described as the linear combination of initial variables that explains disparate informative components. Equation (2) describes how the initial data matrix **X** was transformed:

$$\mathbf{X} = \mathbf{T}\mathbf{P}^{\mathbf{T}} + \mathbf{E} \quad (2)$$

The new directions are defined by the loading matrix **P**, whereas samples' coordinates are given by the scores matrix **T**. **E** represents the residuals matrix. Objects and loadings can be projected onto the compressed PC sub-space to display multivariate information; this offers an easy-to-understand graphical visualization of trends within the data samples (score plot), variables (loading plot), or both (bi-plot). PCA was performed using in-house routines in the MATLAB environment (R2015b; The Mathworks, Natick, MA, USA).

3. Results and Discussion

3.1. Validation of Multi-Elemental ICP-OES Analysis of Donkey Milk

The elemental analysis of donkey milk samples was proven according to the EURACHEM guidelines [37]. Table 1 summarizes the figures of merit related to this methodology.

Table 1. Detection wavelength (λ) of the analyzed elements, determination coefficient (R^2) of calibration curves and limit of detection (LOD) and quantification (LOQ), mean recovery (R) observed in the analysis of the enriched samples (n = 6), and relative standard deviation (RSD).

Element	λ (nm)	R^2	LOD (mg/L)	LOQ (mg/L)	R (%)	RSD (%)
Ca	315.887	0.9999	1.93	6.43	99	6
K	766.491	0.9998	4.13	13.78	101	2
Mg	280.271	0.9997	1.27	4.24	100	2
P	213.618	0.9999	2.59	8.63	100	5
Na	588.995	0.9999	0.82	2.73	105	1
Fe	259.940	0.9983	0.04	0.12	95	19
Zn	206.200	0.9997	0.02	0.06	91	4

For every investigated element, linearity and sensitivity were obtained using analytical calibration curves as the Determination Coefficient (R^2), and Limit of Detection (LOD) and Quantification (LOQ), respectively. The observed R^2 values are greater than 0.9995, except for Fe (R^2 = 0.9983), indicating a good linearity of the relationship between the analytical response and the concentration for all the elements in the working range investigated. LOD and LOQ values were both determined according to the Background Equivalent Concentration 0BEC0 concept. The relations $LOD = 3 \cdot RSD_{blank} \cdot BEC/100$ and $LOQ = 10 \cdot RSD_{blank} \cdot BEC/100$ were used, where RSD_{blank} is the Relative Standard Deviation (n = 10) of the blank and BEC is equivalent to the concentration producing twice the intensity of the background. The observed LOQ values (from 0.06 to 13.78 mg/L) are largely below the mean native levels of each target element in donkey milk samples (see below), except Fe, which in some samples presents a content comparable to the corresponding LOQ value. The sensitivity is generally sufficient to ensure a reliable determination of the detected elements in the donkey milk samples. Trueness was evaluated by a spike recovery analysis. In order to assess the mean recoveries (R (%)) of the target elements, six authentic samples and six fortified ones were examined (Table 1). Relative Standard Deviation (RSD (%)), which was calculated using data from six procedural replicates, was used to measure the precision of the ICP-OES method. Comparison of the two parameters mentioned above with the permitted values listed in the specialized literature [38,39] proves that the seven investigated elements are assessable with reasonable accuracy and precision. In fact, as depicted in Table 1, the macro-elements Ca, K, P, Mg, and Na show R (%) values from 99 to 105% which fall within the recovery benchmark ranges of 90−105% or 97–107% for 100 ppm and 1000 ppm concentration levels, respectively. On the other hand, the RSD% threshold values according to the AOAC and Horwitz criteria are, respectively, 5.3 and 8% for 100 ppm concentration levels and 5.7 and 3.7% for 1000 ppm. It follows that precision is good for K, Mg, and Na, and acceptable for Ca and P. Both recoveries of Fe (95%) and Zn (91%), whose expected concentrations in the real samples are close to 100 ppb and 1 ppm, respectively, fall within the acceptable range (80–110%), as well as RSD% of Zn (4%) which is largely below the AOAC threshold and Horwitz threshold values (11 and 16%, respectively). It must be noted that a greater RSD% value (19%) is observed in the analysis

of Fe, although this value is below the Horwitz reference value (22.6%) and only slightly higher than the AOAC threshold (15%).

3.2. Multi-Elemental ICP-OES Analysis of Donkey Milk Samples

Data were collected as described in Section 2.6. In Figure 1, a first overview of the ICP-OES profiles is given; mean concentrations element by element are reported in Table A1 in Appendix A. In the plots, the average content of Ca, Fe, K, Mg, Na, P, and Zn for freeze–thaw milk (FT-) and reconstituted (R-) milk at T_0 and T_1 are shown (bars represent the standard deviation among samples). These values are in strong agreement with the concentrations reported by Paksoy et al. [40], Potortì et al. [41], and Fantuz et al. [9] in Turkish and Italian donkey milks, respectively.

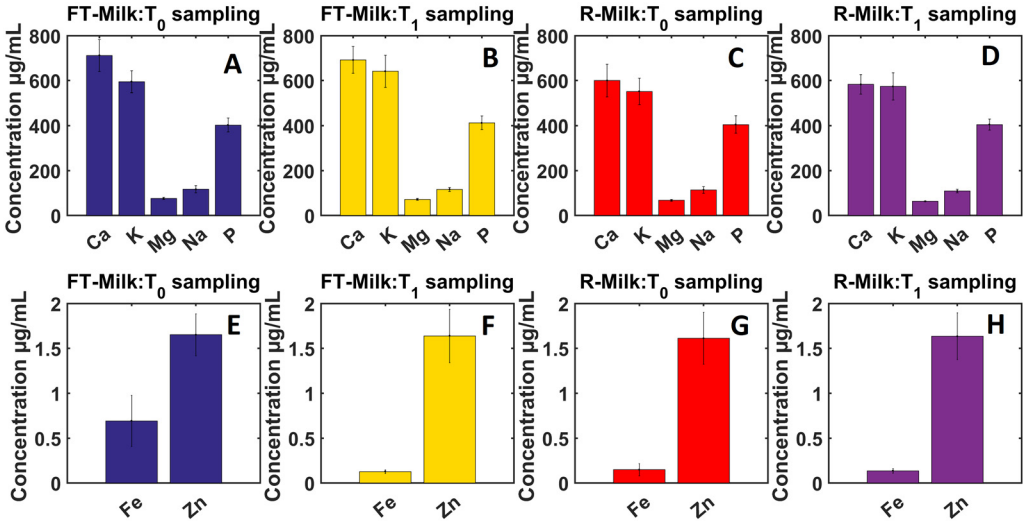

Figure 1. Bar plot of the average concentrations of the diverse quantified elements with standard deviations. (**A**) Average concentration (µg/mL) of Ca, K, Mg, Na, and P in FT-milk collected at T_0; (**B**) Average concentration (µg/mL) of Ca, K, Mg, Na, and P in FT-milk collected at T_1; (**C**) Average concentration (µg/mL) of Ca, K, Mg, Na, and P in R-milk collected at T_0; (**D**) Average concentration (µg/mL) of Ca, K, Mg, Na, and P in R-milk collected at T_1; (**E**) Average concentration (µg/mL) of Fe and Zn in FT-milk collected at T_0; (**F**) Average concentration (µg/mL) of Fe and Zn in FT-milk collected at T_1; (**G**) Average concentration (µg/mL) of Fe and Zn in R-milk collected at T_0; (**H**) Average concentration (µg/mL) of Fe and Zn in R-milk collected at T_1.

Figure 1 shows that, regardless of sampling time, the composition of reconstituted milk appears less rich than FT-milk. Considering sampling, as far as FT-milk is concerned, the differences between T_0 and T_1 are mainly associated to the concentration of K and Fe.

On the contrary, for R-milk, the (already lower) concentrations remain fairly constant between the two sampling times.

The analysis has revealed that R-milk generally exhibits a lower concentration of elements compared to FT samples. This observation aligns with findings from other researchers, such as Singhal et al. [42], who also observed a similar aspect, and they imputed it to oxidation and the disintegration of the mineral compounds, as previously proposed by Kiharason et al. [43] and by Barrett and Lloyd [44].

ASCA was used to evaluate whether the conservation process and the lactation period are significant effects. The model was calculated on auto-scaled data, and both variables appeared significant ($p < 0.001$).

A more insightful and engaging interpretation of the data can be accomplished by examining the PCA biplot (Figure 2). The PCA model was computed by extracting four Principal Components (PCs), accounting for a total explained variance of 90%. In Figure 2, it is evident that FT- and R-milk samples tend to separate along the first principal component.

FT-samples (dark blue and yellow diamonds) predominantly cluster with positive PC1 scores, while the majority of reconstituted individuals (red and purple dots) exhibit negative PC1 scores.

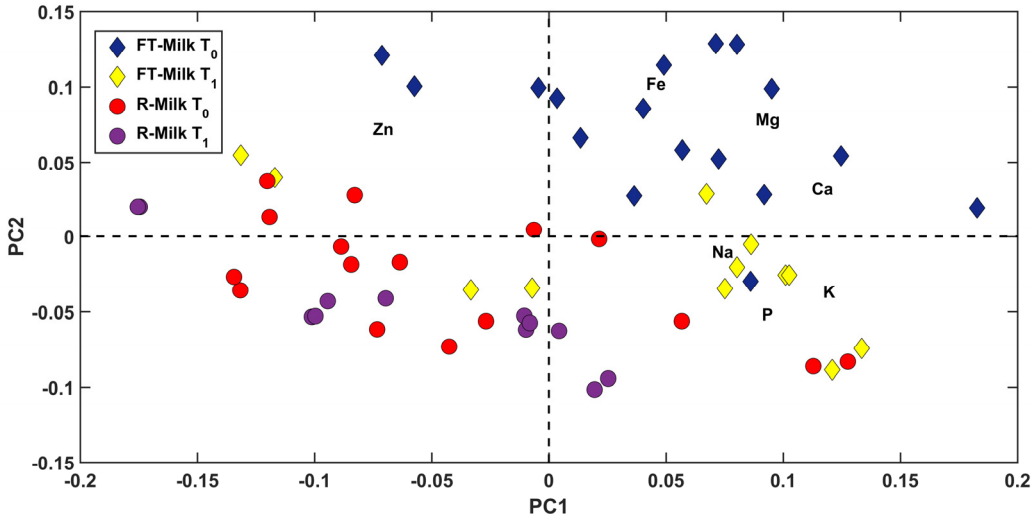

Figure 2. Biplot associated to the PCA model calculated on ICP-OES data. Legend: Blue Diamonds: FT-Milk at T_0; Yellow Diamonds: FT-Milk at T_1; Red Dots: R-Milk at T_0; Purple Dots: R-Milk at T_1.

In agreement with what has been previously observed, for the reconstituted milk, no difference with respect to T_0 and T_1 can be appreciated. Conversely, FT-milk is prone to form two groups according to the sampling time. As expected, the biplot shows that the highest concentrations of metals are present in the FT-milk samples, in particular Fe, Mg, and Ca for T_0, and K, P, and Na for T_1. The behavior of Zn is opposite to the other elements and peculiar. This is probably due to the fact that the concentration of this metal is, on average, the same in FT- and R-milk, but two R-milk and one FT-milk samples have slightly higher concentrations of Zn.

3.3. ATR-FT-IR Analysis of Donkey Milk Samples

Several aliquots of the samples were subjected to ATR-FT-IR analysis as described in Section 2.4. The mean spectra collected on FT- and R-milks at the two sampling times are shown in Figures 3A and 3B, respectively. In the average spectra, it is possible to observe some common peaks representative of the main constituents of milk. The main ones are the broad band at approximately 3327 cm^{-1} ascribable to hydroxy group, OH, or NH stretching of water, carbohydrates, and proteins. This latter family of constitutes can also be attributed to the peaks at 1638 cm^{-1} and 1548 cm^{-1}, associated with the absorbance of C=O (amide I), NH (amide I and II), and CN (amide II) groups. The presence of long-chain fatty acids can be spotted in the range of 2900 cm^{-1}–1700 cm^{-1} where the stretching of CH in methyl and methylene takes place. Eventually, peaks in the fingerprint region can be associated with the CO stretching of carbohydrates.

Comparing the two spectra associated to FT-milk, at T_1, it is possible to appreciate an increase of the intensities of the peaks at 2924 cm^{-1}, 2854 cm^{-1}, and 1743 cm^{-1}, which can be attributed to fats. This observation is completely in line with the metabolic factors; in fact, the lipid component of milk decreases in quantity in the first weeks of lactation (T_0) until it reaches a minimum. Afterwards, the amount of milk decreases, while the concentration of fats increases (T_1). To a lesser extent, the same trend can be also noticed in R-milks.

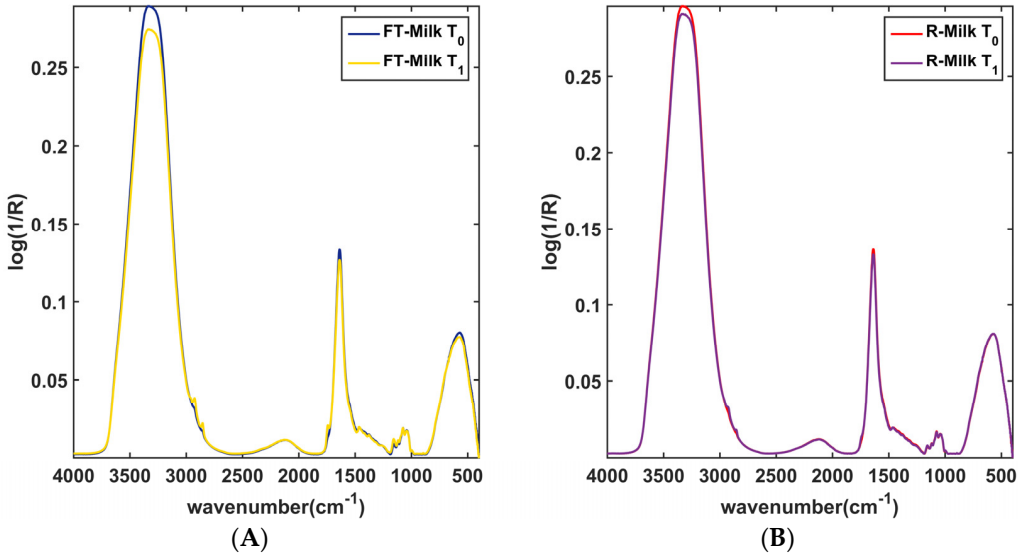

Figure 3. Average ATR-FT-IR spectra of (**A**) FT- and (**B**) R-milk at the two lactation times (T_0 and T_1). Legend: Blue Line: FT-Milk at T_0; Yellow Line: FT-Milk at T_1; Red Line: R-Milk at T_0; Purple Line: R-Milk at T_1.

A slight difference among the spectra collected on FT-milk at T_0 and T_1 can be observed also on the peak at around 1638 cm^{-1}, ascribable to proteins. In order to further investigate this aspect, proteins quantification has been carried out using the Bradford method [10]. In FT-milk, total proteins constitute the 1.26% $\pm$ 0.07 of the milk; in particular, lysozyme is 0.81 $\pm$ 0.15 mg/mL, α-lactalbumin is 3.34 $\pm$ 0.08 mg/mL, and β-lactalbumin is 2.04 $\pm$ 0.18 mg/mL. In R-milk, total proteins constitute the 1.15% $\pm$ 0.04 of the milk; lysozyme is 0.90 $\pm$ 0.14 mg/mL, α-lactalbumin is 2.08 $\pm$ 0.14 mg/mL, and β-lactalbumin is 1.86 $\pm$ 0.08 mg/mL.

IR signals were also analyzed by PCA. However, this did not show clear differences neither for the technological process nor for sampling. ASCA was also applied to confirm this outcome, and it did. Both sampling time and technological process were investigated as factors, and neither of them were considered significant. In light of this, it is possible to conclude that, from the IR point of view, no statistically significant differences can be observed on FT- and R-milks.

4. Conclusions

This study investigated the mineral composition and IR profiles of donkey milk, focusing on two different types of preprocessing: freezing–thawing and freeze-drying. The exploratory analysis using ASCA and PCA revealed significant differences in the mineral concentrations depending on the milk preprocessing and lactation time. FT-milk samples had the highest metal concentrations, with Fe, Mg, and Ca being more prominent at T_0, and K, P, and Na showing higher levels at T_1.

However, the IR spectra of both freezing–thawing and freeze-dried milks did not show macroscopic differences between them or among different lactation times. This indicates that IR spectroscopy may not be suitable for capturing variability related to lactation periods or changes during the conservation process.

The study provides valuable insights into mineral composition changes in donkey milk and highlights the importance of milk preprocessing and lactation periods in affecting mineral concentrations.

The study's limitations, including the small sample size and specific circumstances of milk collection and processing, should be acknowledged. Further research is needed to delve deeper into these issues and validate the findings. Nonetheless, this study represents a significant step toward a better understanding of the mineral content and IR profiles of donkey milk and sets the stage for future investigations in this area.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Mean concentrations (µg/mL) of the investigated elements in freeze–thaw milk (FT-) and reconstituted (R-) milks are reported in Table A1.

Table A1. Mean concentrations (µg/mL) of the investigated elements in freeze–thaw milk (FT-) and reconstituted (R-) milks.

	FT-Milk		R-Milk	
Element	**T_0**	**T_1**	**T_0**	**T_1**
Ca	712 ± 71	692 ± 60	600 ± 72	583 ± 43
Fe	0.7 ± 0.3	0.13 ± 0.02	0.1 ± 0.1	0.13 ± 0.03
K	595 ± 49	641 ± 71	551 ± 59	574 ± 61
Mg	75 ± 5	72 ± 4	67 ± 4	63 ± 1
Na	117 ± 16	116 ± 9	114 ± 16	109 ± 8
P	403 ± 30	412 ± 30	404 ± 38	405 ± 24
Zn	1.6 ± 0.2	1.6 ± 0.3	1.6 ± 0.3	1.6 ± 0.3

References

1. Souroullas, K.; Aspri, M.; Papademas, P. Donkey milk as a supplement in infant formula: Benefits and technological challenges. *Food Res. Int.* **2018**, *109*, 416–425. [CrossRef]
2. Lajnaf, R.; Feki, S.; Ben Ameur, S.; Attia, H.; Kammoun, T.; Ayadi, M.A.; Masmoudi, H. Cow's milk alternatives for children with cow's milk protein allergy—Review of health benefits and risks of allergic reaction. *Int. Dairy J.* **2023**, *141*, 105624. [CrossRef]
3. Li, Y.; Fan, Y.; Shaikh, A.S.; Wang, Z.; Wang, D.; Tan, H. Dezhou donkey (Equus asinus) milk a potential treatment strategy for type 2 diabetes. *J. Ethnopharmacol.* **2020**, *246*, 112221. [CrossRef]
4. Akca, C.; Vatan, O.; Yilmaz, D.; Huriyet, H.; Cinkilic, N.; Cavas, T. In vitro cytotoxic and genotoxic effects of donkey milk on lung cancer and normal cells lines. *Czech J. Food Sci.* **2019**, *37*, 29–35. [CrossRef]
5. Martini, M.; Altomonte, I.; Tricò, D.; Lapenta, R.; Salari, F. Current Knowledge on Functionality and Potential Therapeutic Uses of Donkey Milk. *Animals* **2021**, *11*, 1382. [CrossRef]
6. Trinchese, G.; Cimmino, F.; Cavaliere, G.; Rosati, L.; Catapano, A.; Sorriento, D.; Murru, E.; Bernardo, L.; Pagani, L.; Bergamo, P.; et al. Heart Mitochondrial Metabolic Flexibility and Redox Status Are Improved by Donkey and Human Milk Intake. *Antioxidants* **2021**, *10*, 1807. [CrossRef]
7. Hassan, Z.M.; Manyelo, T.G.; Nemukondeni, N.; Sebola, A.N.; Selaledi, L.; Mabelebele, M. The Possibility of Including Donkey Meat and Milk in the Food Chain: A Southern African Scenario. *Animals* **2022**, *12*, 1073. [CrossRef] [PubMed]
8. Guo, H.Y.; Pang, K.; Zhang, X.Y.; Zhao, L.; Chen, S.W.; Dong, M.L.; Ren, F.Z. Composition, Physiochemical Properties, Nitrogen Fraction Distribution, and Amino Acid Profile of Donkey Milk. *J. Dairy Sci.* **2007**, *90*, 1635–1643. [CrossRef] [PubMed]
9. Salimei, E.; Fantuz, F.; Coppola, R.; Chiofalo, B.; Polidori, P.; Varisco, G. Composition and characteristics of ass's milk. *Anim. Res.* **2004**, *53*, 67–78. [CrossRef]

10. Vincenzetti, S.; Polidori, P.; Mariani, P.; Cammertoni, N.; Fantuz, F.; Vita, A. Donkey's milk protein fractions characterization. *Food Chem.* **2008**, *106*, 640–649. [CrossRef]
11. Deepa, P.R.; Divya Dharshini, C.S.; Dev, B.S.; Jayan, J.; Harisankaran, P.S.; Rajan, N.S.; Karthik, S.; Nandhana, J.P.; Athulya, K.G.; Pran, M.; et al. Donkey milk: Chemical make-up, biochemical features, nutritional worth, and possible human health benefits-Current state of scientific knowledge. *J. Exp. Biol. Agric. Sci.* **2023**, *11*, 251–263. [CrossRef]
12. Coppola, R.; Salimei, E.; Succi, M.; Sorrentino, E.; Nanni, M.; Ranieri, P.; Blanes, R.B.; Grazia, L. Behaviour of *Lactobacillus rhamnosus* strains in ass's milk. *Ann. Microbiol.* **2002**, *52*, 55–60.
13. Derdak, R.; Sakoui, S.; Pop, O.L.; Muresan, C.I.; Vodnar, D.C.; Addoum, B.; Vulturar, R.; Chis, A.; Suharoschi, R.; Soukri, A.; et al. Insights on health and food applications of equus asinus (Donkey) milk bioactive proteins and peptides—An overview. *Foods* **2020**, *9*, 1302. [CrossRef] [PubMed]
14. Ma, Y.; Li, J.; Huang, Y.; Liu, X.; Dou, N.; Zhang, X.; Hou, J.; Ma, J. Physicochemical stability and in vitro digestibility of goat milk affected by freeze-thaw cycles. *Food Chem.* **2023**, *404*, 134646. [CrossRef] [PubMed]
15. Jirillo, F.; Magrone, T. Anti-inflammatory and anti-allergic properties of donkey's and goat's milk. *Endocr. Metab. Immune Disord. Drug Targets* **2014**, *14*, 27–37. [CrossRef]
16. de Oliveira Moura Araújo, E.; Gavioli, E.C.; Holanda, V.A.D.; da Silva, V.C.; Oliveira Nunes Messias, T.B.; Dutra, L.M.G.; de Oliveira, M.C.; Ramos do Egypto Queiroga, R.D.C.; Guerra, G.C.B.; Soares, J.K.B. Repeated donkey milk consumption reduces anxiety-like behaviors and brain oxidative damage to lipids in mice. *Behav. Brain Res.* **2023**, *449*, 114477. [CrossRef]
17. Di Donato, F.; Biancolillo, A.; Ferretti, A.; D'Archivio, A.A.; Marini, F. Near infrared spectroscopy coupled to chemometrics for the authentication of donkey milk. *J. Food Compos. Anal.* **2023**, *115*, 105017. [CrossRef]
18. Živkov Baloš, M.; Ljubojević Pelić, D.; Jakšić, S.; Lazić, S. Donkey Milk: An Overview of its Chemical Composition and Main Nutritional Properties or Human Health Benefit Properties. *J. Equine Vet. Sci.* **2023**, *121*, 104225. [CrossRef]
19. Cimmino, F.; Catapano, A.; Villano, I.; Di Maio, G.; Petrella, L.; Traina, G.; Pizzella, A.; Tudisco, R.; Cavaliere, G. Invited review: Human, cow, and donkey milk comparison: Focus on metabolic effects. *J. Dairy Sci.* **2023**, *106*, 3072–3085. [CrossRef]
20. Kayili, H.M.; Barlas, N.; Demirhan, D.B.; Yaman, M.E.; Atakay, M.; Güler, Ü.; Kara, M.; Tekgunduz, K.S.; Salih, B. Utilizing linkage-specific ethyl-esterification approach to perform in-depth analysis of sialylated N-glycans present on milk whey glycoproteins. *Food Chem.* **2023**, *421*, 136166. [CrossRef]
21. Di Salvo, E.; Conte, F.; Casciaro, M.; Gangemi, S.; Cicero, N. Bioactive natural products in donkey and camel milk: A perspective review. *Nat. Prod. Res.* **2023**, *37*, 2098–2112. [CrossRef]
22. Fantuz, F.; Ferraro, S.; Todini, L.; Cimarelli, L.; Fatica, A.; Marcantoni, F.; Salimei, E. Distribution of calcium, phosphorus, sulfur, magnesium, potassium, and sodium in major fractions of donkey milk. *J. Dairy Sci.* **2020**, *103*, 8741–8749. [CrossRef]
23. Fantuz, F.; Ferraro, S.; Todini, L.; Mariani, P.; Piloni, R.; Salimei, E. Essential trace elements in milk and blood serum of lactating donkeys as affected by lactation stage and dietary supplementation with trace elements. *Animal* **2013**, *7*, 1893–1899. [CrossRef]
24. Fantuz, F.; Ferraro, S.; Todini, L.; Spurio, R.; Fatica, A.; Marcantoni, F.; Salimei, E. Distribution of selected trace elements in the major fractions of donkey milk. *J. Dairy Sci.* **2022**, *105*, 6422–6430. [CrossRef]
25. Malacarne, M.; Criscione, A.; Franceschi, P.; Bordonaro, S.; Formaggioni, P.; Marletta, D.; Summer, A. New insights into chemical and mineral composition of donkey milk throughout nine months of lactation. *Animals* **2019**, *9*, 1161. [CrossRef]
26. Astolfi, M.L.; Marconi, E.; Protano, C.; Canepari, S. Comparative elemental analysis of dairy milk and plant-based milk alternatives. *Food Control* **2020**, *116*, 107327. [CrossRef]
27. Šarić, L.Ć.; Gubić, J.M.; Šarić, B.M.; Mandić, A.I.; Jovanov, P.T.; Plavšić, D.V.; Okanović, D.G. Domestic Balkan donkeys' milk: Microbiological, chemical and sensory properties. In *CEFood 2012—Proceedings of 6th Central European Congress on Food*; University of Novi Sad: Novi Sad, Serbia, 2012; pp. 359–363. Available online: https://www.scopus.com/inward/record.uri?eid=2-s2.0-84926040156&partnerID=40&md5=53d321312e5cac1af05d189b74f370f3 (accessed on 4 August 2023).
28. Nayak, C.M.; Ramachandra, C.T.; Nidoni, U.; Hiregoudar, S.; Ram, J.; Naik, N. Physico-chemical composition, minerals, vitamins, amino acids, fatty acid profile and sensory evaluation of donkey milk from Indian small grey breed. *J. Food Sci. Technol.* **2020**, *57*, 2967–2974. [CrossRef] [PubMed]
29. Malissiova, E.; Arsenos, G.; Papademas, P.; Fletouris, D.; Manouras, A.; Aspri, M.; Nikolopoulou, A.; Giannopoulou, A.; Arvanitoyannis, I.S. Assessment of donkey milk chemical, microbiological and sensory attributes in Greece and Cyprus. *Int. J. Dairy Technol.* **2016**, *69*, 143–146. [CrossRef]
30. Nanou, E.; Stefas, D.; Couris, S. Milk's inorganic content analysis via laser induced breakdown spectroscopy. *Food Chem.* **2023**, *407*, 135169. [CrossRef] [PubMed]
31. Harizi, N.; Madureira, J.; Zouari, A.; Ayadi, M.A.; Cabo Verde, S.; Boudhrioua, N. Effects of Spray Drying, Freeze Drying and Gamma Irradiation on the Antioxidant Activities of Camel and Cow Milk Fractions. *Processes* **2023**, *11*, 897. [CrossRef]
32. Ibrahim, A.H.; Khalifa, S. Effect of freeze-drying on camel's milk nutritional properties. *Int. Food Res. J.* **2015**, *22*, 1438–1445.
33. Jansen, J.J.; Hoefsloot, H.C.J.; Van der Greef, J.; Timmerman, M.E.; Westerhuis, J.; Smilde, A.K. ASCA: Analysis of multivariate data obtained from an experimental design. *J. Chemom.* **2005**, *19*, 469–481. [CrossRef]
34. Searle, S.R. *Linear Models*, 1st ed.; John Wiley & Sons, Inc.: New York, NY, USA, 1971.
35. Ten Berge, J.M.F.; Kiers, H.A.L.; Van der Stel, V. Simultaneous component analysis. *Stat. Appl.* **1992**, *4*, 377–392.
36. Jolliffe, I. Principal Component Analysis. In *Encyclopedia of Statistics in Behavioral Science*; American Cancer Society: New York, NY, USA, 2005; ISBN 9780470013199.

37. Eurachem. Eurachem Guide: The Fitness for Purpose of Analytical Methods—A Laboratory Guide to Method Validation and Related Topics. 2014. Available online: www.eurachem.org (accessed on 20 April 2023).
38. Huber, L. *Validation and Qualification in Analytical Laboratories*; CRC Press: Boca Raton, FL, USA, 2007.
39. Gustavo González, A.; Ángeles Herrador, M.A. Practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. *TrAC-Trends Anal. Chem.* **2007**, *26*, 227–238. [CrossRef]
40. Paksoy, N.; Dinç, H.; Altun, S.K. Evaluation of levels of Essential Elements and Heavy Metals in Milks of Dairy Donkeys, Goats and Sheep in Turkey. *Pak. J. Zool.* **2018**, *50*, 1097–1105. [CrossRef]
41. Potortì, A.G.; Di Bella, G.; Lo Turco, V.; Rando, R.; Dugo, G. Non-toxic and potentially toxic elements in Italian donkey milk by ICP-MS and multivariate analysis. *J. Food Compos. Anal.* **2013**, *31*, 161–172. [CrossRef]
42. Singhal, P.; Satya, S.; Naik, S.N. Effect of different drying techniques on the nutritional, antioxidant and cyanogenic profile of bamboo shoots. *Appl. Food Res.* **2022**, *2*, 100036. [CrossRef]
43. Kiharason, J.W.; Isutsa, D.K.; Ngoda, P.N. Effect of drying method on nutrient integrity of selected components of pumpkin (*Cucurbita moschata* Duch.) fruit flour. *ARPN J. Agric. Biol. Sci.* **2017**, *12*, 110–116.
44. Barrett, D.M.; Lloyd, B. Advanced preservation methods and nutrient retention in fruits and vegetables. *J. Sci. Food Agric.* **2012**, *92*, 7–22. [CrossRef] [PubMed]