*IZVORNI ZNANSTVENI RAD / ORIGINAL SCIENTIFIC PAPER*

# Changes in some quality properties of frozen mare milk during 6 months of storage

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## Abstract

*The aim of this study was to determine how storage in the frozen state affects the quality of mare milk. Therefore, physicochemical properties such as pH, total dissolved solids (TDS), electrical conductivity (EC), particle size distribution and near-infrared (NIR) spectroscopy, as well as changes in antioxidant activity and the total phenol content were determined during 6 months of storage at -18°C. All of the determined parameters were compared to those in raw mare milk prior to freezing. According to the obtained results, frozen storage caused certain changes in mare milk quality, including increase in pH, electrical conductivity and TDS values, changes in protein structure recorded by NIR spectroscopy and SDS PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis), as well as changes in particle size distribution. There was also a continuous decrease in the antioxidant activity and the total phenol content of frozen mare milk throughout the 6 months of frozen storage.*
*Thus, after 6 months of frozen storage, a decrease in the DPPH value of approximately 59%, in the FRAP value of approximately 26% and in the TPC of approximately 70% was observed, respectively.*
*In conclusion, freezing and frozen storage affected the quality of mare milk, which might have negative consequences on its therapeutic value.*

*Keywords: mare milk, quality, storage, freezing, antioxidant activity*

## Introduction

Mare milk and its products have been popular in the Asian region since ancient times (Outram et al., 2009). Until the mid-20th century, numerous sanatoriums were established in the former SSSR that used mare milk for therapeutic purposes. In the last two decades, with the increase in global connectivity accompanied by a growing interest in functional food, there has been a rise in demand and production of mare milk in other parts of the world. Therefore, specialized dairy farms for breeding milking mares can now be found in Europe, including countries such as Germany, France, Hungary, and Belgium. Mare milk production is seasonal, occurring during spring and summer, with the lactation period typically lasting between five and eight months (Božanić et al., 2018). Consequently, fresh mare milk is not available throughout the entire year. The popularity of mare milk consumption relies on its well-known therapeutic properties coming from a unique nutrient profile characterised by the presence of approximately 40 bioactive components in optimal ratios for utilization in the human body. Some of therapeutic properties of mare milk include beneficial effects on the human immune and nervous systems, cardiovascular disease, gastrointestinal diseases and diabetes, liver detoxification and regeneration, as well as hypoallergenic properties that favour consumption of mare milk in people with milk protein intolerance or in infants as a substitute for breast milk (Brezovečki et al., 2014; Jastrzębska et al., 2017; Czyżak-Runowska et al., 2018). There are also other benefits of mare milk, like the presence of specific health-promoting compounds with potent antibacterial, antiviral, and anti-inflammatory effects coming from lactoferrin (Lf) and lysozyme, which are present in significantly higher amounts compared to cow milk (Claeys et al., 2014; Pieszka et al., 2016; Miraglia et al., 2020). However, most of these specific bioactive components are sensitive to thermal treatments and tend to lose their activity. For that reason, as well as due to its seasonal nature, mare milk is consumed raw and is usually stored in a frozen state. The aim of this study was to examine changes in the quality of mare milk in terms of physicochemical properties and antioxidant activity during six months of frozen storage, and compare those to raw milk.

## Materials and methods

For the purpose of this study, mare milk donated by the family farm Zoran Rebić (Veliko Trojstvo, Croatia) was used. Portions of milk (50 mL) were transferred into six plastic Falcon tubes, frozen, and stored at -18°C for 6 months.

During this storage period, every month a sample of frozen mare milk was thawed to room temperature and subjected to analyses as described below. The same analyses were performed on raw milk before freezing.

The active acidity of milk samples was measured by a pH meter (WTW-ProfiLine pH 3110, Xylem Analytics, Germany), while electrical conductivity (EC) and the total dissolved solids (TDS) were determined by a conductivity meter (SevenCompact, Mettler Toledo, Switzerland).

Antioxidant activity was determined by Ferric Reducing Antioxidant Power (FRAP) method according to the procedure described by Benzie and Strain (1999) for preparation of all chemicals and reagents and following the protocol described by Sangsopha et al. (2019) for absorbance reading. The standard curve was obtained using a 2 mM Trolox (Sigma-Aldrich, St. Louis, MO, USA) stock solution, whereby Trolox concentrations were 25, 50, 75, 100, 125, 250, 500, 750, 1000 and 1500 µM. FRAP results were expressed as µmol TE/L.

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay was used according to Alyaqoubi et al. (2014), and the results were expressed as % of the remaining radical scavenging activity.

The total phenols content (TPC) was determined according to Singleton et al. (1999) with some modifications, and the standard curve was plotted using 500 mg/L gallic acid (Sigma-Aldrich, St. Louis, MO, USA) as a stock solution. The results were expressed as mg of gallic acid equivalent per litre (mg GAE/L).

Particle size distribution was determined in raw and frozen mare milk after 6 months of storage, using the Zetasizer UltraNano ZS device (Malvern Panalytical Ltd, UK), with installed software Zetasizer Ultra-Pro ZS Xplorer. Each sample was recorded in triplicate and the mean value was calculated.

Near infrared spectroscopy (NIRs) was conducted in the range of 904–1699 nm, using the NIR spectrophotometer (NIR-128-1.7-USB/6.25/50µm, Control Development Inc., USA) with installed Control Development software Spec32 (Control Development Inc., USA) and a halogen light source. All samples were diluted with distilled water (1:10) prior to NIR measurements. Each sample was recorded in triplicate and the mean value of the spectra was further used.

All previously mentioned analyses were performed in triplicate, and the observed results were expressed as mean values ± SD.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in raw and frozen mare milk after 6 months of storage, as previously described by Leboš Pavunc et al. (2012) with slight modification. 15 µL of each milk sample were resuspended in 2 x concentrated Laemmli buffer {1.25 mL of 1 M Tris-HCl (pH=6.8) (Carlo Erba, Italy), 4 mL of SDS [10% (w/v)] (Sigma-Aldrich, USA), 2 mL glycerol [100% (v/v)] (Alkaloid, Macedonia), 0.5 mL 0.5 M EDTA (Sigma-Aldrich, USA), 4 mg bromophenol blue (Sigma-Aldrich, USA), 0.2 mL β-mercaptoethanol (Sigma-Aldrich, USA)}. Electrophoretic separation was performed in an electrophoresis chamber at a constant voltage of 150 V using ProSieve QuadColor Protein Marker as standard.

## Results and discussion

Acidity (pH), TDS and electrical conductivity of raw and frozen mare milk samples

Results regarding the changes in acidity, TDS and EC of mare milk over a six-month period are presented in Figure 1. Initial pH of raw mare milk was on average 6.96 and EC 1507.7 mS/cm, which corresponded well to data previously published by other authors (Brezovački et al., 2014; Claeys et al., 2014; Cais-Sokolińska et al., 2017). It could be observed that all of the measured parameters increased during the storage period when compared to the initial values measured in raw milk samples prior to freezing. Similar trends were already observed by Mariani et al. (2001), where the pH value of mare milk was determined to be 6.6 four days postpartum, 6.9 twenty days postpartum, and 7.1 after 180 days postpartum, respectively. The obtained increase in the pH value of mare milk, is most probably related to the dissociation of acidic salts such phosphates, nitrates, which results in releasing H+ ions.

Along with the observed increase in pH, TDS and EC values also increased, confirming the assumption that during the storage of mare milk, salt dissociation occurred, obviously resulting in the release of multiple ions. Since EC of milk mostly depends on salts, including serum ions, a corresponding increase in electrical conductivity was recorded in mare milk over the 6-month storage period.

Particle size distribution, NIR spectroscopy and SDS PAGE of raw and

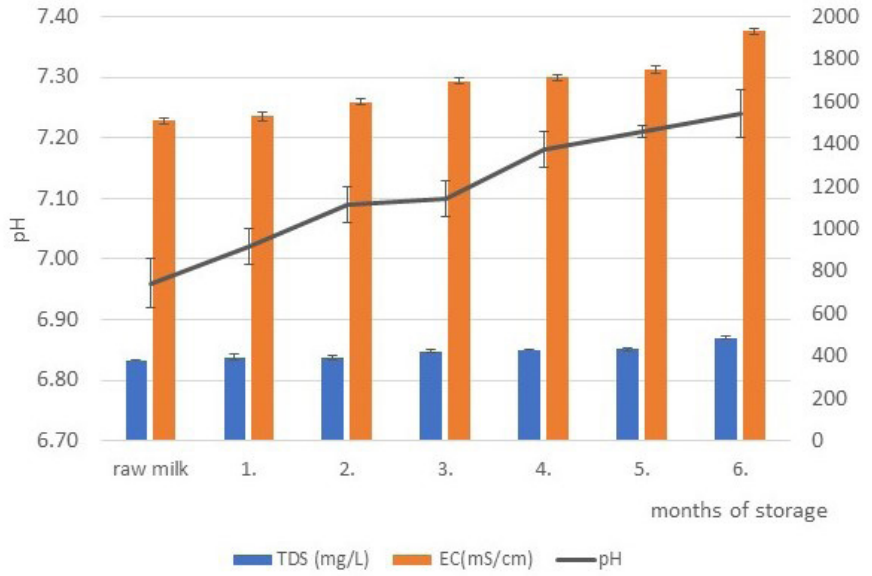


*Figure 1. Changes in acidity (pH), total dissolved solids (TDS, mg/L) and electrical conductivity (EC, mS/cm) in frozen mare milk samples during 6 months of storage at -18°C compared to raw mare milk*

frozen mare milk

Determination of particle size distribution belongs to the group of optical methods based on the scattering of a laser beam as it passes through a water suspension of sample particles. Particle size distribution of raw and frozen mare milk was determined based on the intensity of the occurrence (%) of particles of a certain size in the analysed sample. The obtained results are presented in Figure 2, and indicate that there was a decrease in the volume intensity (%) of particles with size diameter between 100 and 1000 nm, as well as between 4000 and 6000 nm during the 6-month period of storage at -18°C.

According to Samaržija (2016), particles in the size range of 2000–6000 nm are defined as a colloidal emulsion, including fat globules. Particles above 200 nm were described as a colloidal suspension, including casein-calcium phosphate, while particles in the size range of 1–200 nm correspond to whey proteins. Particles smaller than 1 nm are classified as a true solution, including lactose, salts, and other substances. Goff and Sahagian (1996) previously found that casein micelles lose their stability during frozen storage, which can result in the eventual formation of flocculates or aggregates, and their dispersion depends on the thawing method. Taking into consideration this classification and the obtained results, during frozen storage of mare milk obviously a dissociation of casein micelles occurred. Also, as Gaber et al. (2020) recently concluded, frozen samples of micellar casein formed aggregates of different mass median diameters in the range from 1 to 10 μm, and the average size was dependent on the temperature and days of storage. They also found that the aggregates increased significantly in size in the thawed samples throughout the storage. Lactose has also been reported as a factor influencing casein stability during frozen storage due to its crystallisation. Since mare milk contains higher amounts of lactose compared to cow milk (Claeys et al., 2014), this also probably affected the extent to which casein micelles were destabilized and formed aggregates. From our results (Figure 2) it is also evident that milk fat globules decreased in size after 6 months of frozen storage. According to Zhang et al. (2022) freezing of human milk at -18°C and thawing at 45°C could deteriorate the degree of initial lipolysis and increase the aggregation of human milk fat globules (HMFG) and proteins. Knowing that mare milk is by its composition and properties very similar to human milk (Claeys et al., 2014), the particles observed in the size range between approximately 3.500 and 6.400 nm might be related to the formation of such aggregates (Figure 2).

Structural changes of mare milk were additionally determined using NIR spectroscopy by recording spectra in the wavelength range from 904 nm to 1699 nm (Figure 3). NIR spectroscopy is based on the absorption of electromagnetic radiation in the range from 780 nm to 2500 nm, where overtone bands and combination bands occur, with the most prominent being C-H, O-H, S-H, and N-H bonds, i.e., hydrogen bonds, which exhibit high non-coincidence and high vibration frequencies (Ozaki et al., 2007).

According to the NIR spectra shown in Figure 3, it is evident that there was an increase in the absorbance in the entire range of the recording wavelength after 6 months of frozen storage. Considering the previously discussed results related to the observed increase in pH values and electrical conductivity (Figure 1), as well as changes in particle size distribution (Figure 2) during the storage of mare milk, the obtained NIR spectra indicated certain structural changes that probably occurred in protein fractions of the frozen samples. Such presumptions are possible since during protein denaturation, non-covalent bonds in the secondary and tertiary structure of the protein rupture, form a charge on the side chains of the protein structure, leading to changes in the electrical conductivity and pH value of the entire system (Časek, 2010).

SDS-PAGE is a separation method commonly used in the food industry for detecting milk adulteration, as well as for determining the degree of denaturation of protein fractions present in milk, which occurs during milk processing at different temperatures. Figure 4 contains the results of SDS-PAGE of raw mare milk (Figure 4a) and frozen mare milk stored for 6 months (Figure 4b). According to the obtained results (Figure 4a) and the available data from past studies that focused on protein profile of mare milk, raw mare milk contains lower molecular weight (Mw) fractions between 17 and 26 kDa, which usually correspond to glycosylated α-LA (17 kDa; Lisak Jakopović et al., 2016), β-Lg (18.4 kDa; Dupont et al., 2018), glycosylated α-lysozime (21.5 kDa; Girardet et al., 2004), α- (23 kDa) and β – (24 kDa) casein (Dupont et al., 2018). Another group of visible bands was detected in the Mw area between 55 and approximately 80 kDa (Figure 4a), corresponding to the serum albumin fraction at 66 kDa and Lf at 76.1 kDa (Inglinstad et al., 2010).

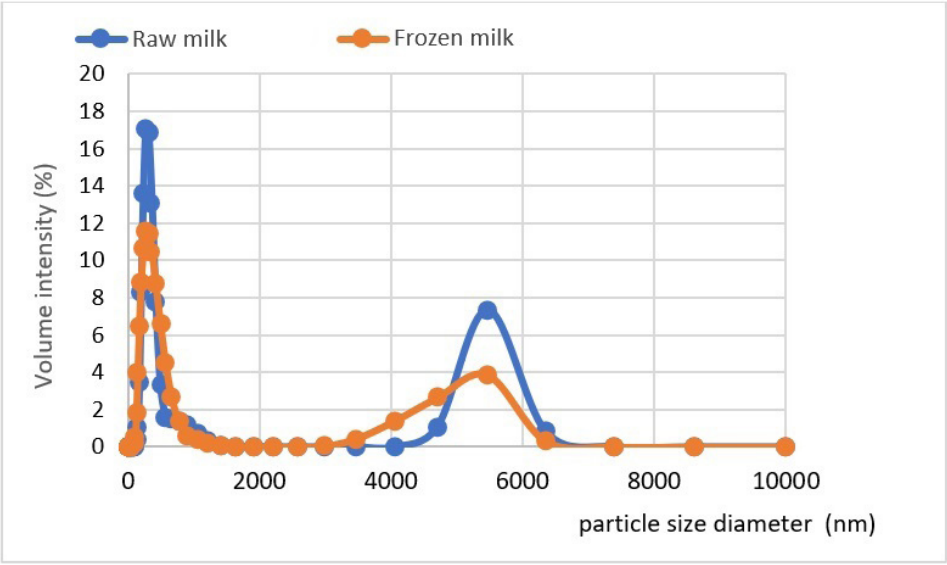


*Figure 2. Particle size distribution for the sample of fresh mare milk and for the sample of mare milk stored for 6 months in frozen form*

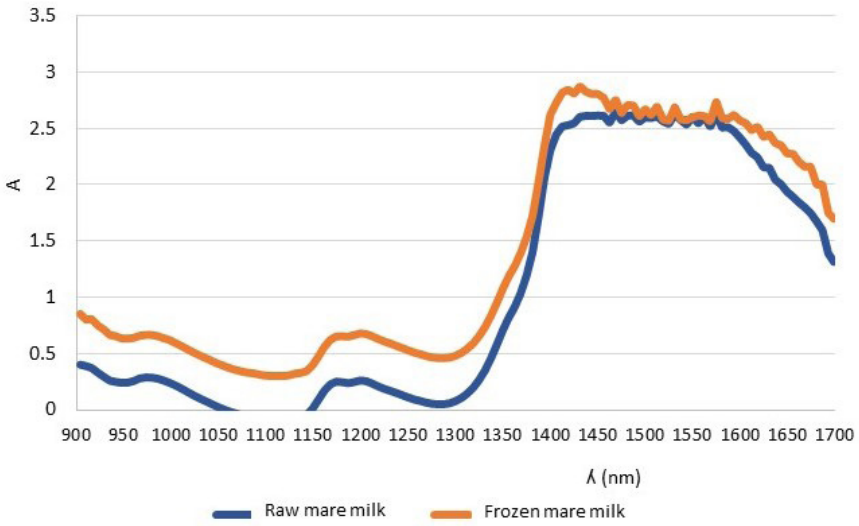


*Figure 3. Raw NIR spectra recorded of samples of raw and frozen mare milk stored for 6 months*

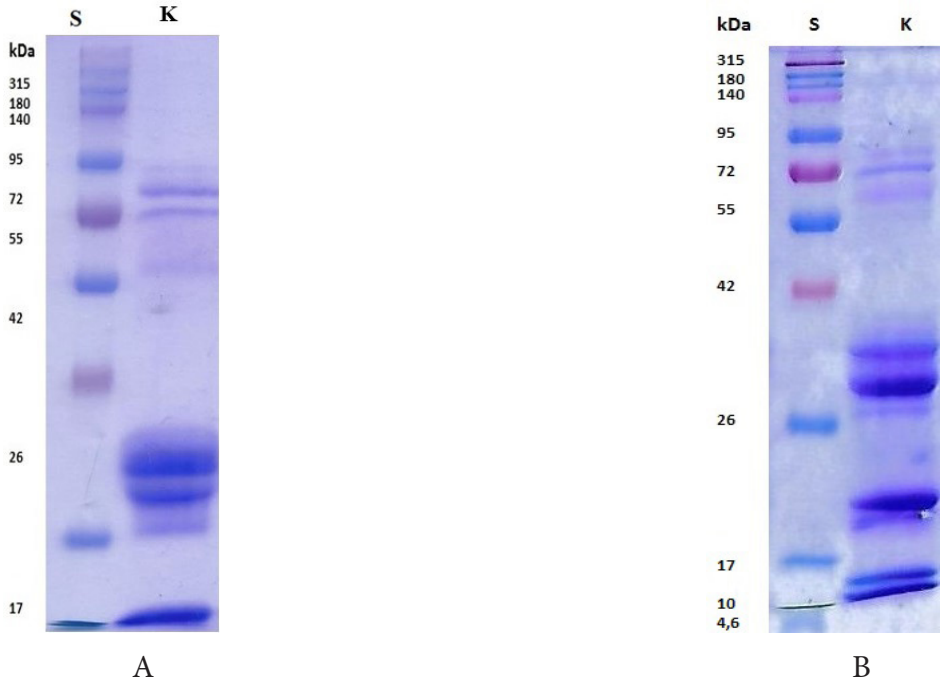


*Figure 4. SDS-PAGE of raw milk (a) and milk stored for 6 months in frozen form (b)*

*S - standard, K - mare milk

According to Figure 4b, storage for 6 months at -18°C resulted in some changes in the lower Mw area, most probably due to destabilization of casein fractions and the formation of aggregates, which was previously found by Gaber et al. (2020), who reported that freezing of casein concentrates at temperatures ≤ -20°C caused formation of aggregates. These results correspond well to the result of the particle size distribution presented in Figure 2. Rutigliano et al. (2018) investigated changes in β-casein of buffalo milk that was stored in frozen state for 104 weeks. Besides the observed decrease in β-casein fraction, they also found a remaining enzymatic activity at - 20°C up to 24 weeks of storage. This finding could be a reason for occurrence of bands between 17 and 26 kDa in Figure 4b, indicating formation of polypeptides by enzymatic action on casein fractions. The obtained results align with the results of NIR spectroscopy, which also indicated structural changes that might be associated to structural changes in proteins during the storage of mare milk for 6 months in frozen form.

## *Antioxidant activity of raw and frozen mare milk*

Antioxidants are chemical compounds that can prevent or slow down cell damage by donating electrons and neutralizing free radicals. Mare milk contains various compounds such as whey proteins, casein, β-carotene, vitamins A, E, and C, lactoferrin, α-tocopherol, selenium, zinc, and sulphur-containing amino acids with proven antioxidant properties (Usta and Yilmaz-Ersan, 2013). However, various recent studies indicated that antioxidant activity of mare milk is mainly associated with the high contents of some whey protein fractions, vitamin C, the non-protein nitrogen (NPN) and bioactive peptides (Pieszka et al., 2016; Waili et al., 2021; Stobiecka et al., 2022). In comparison to cow milk, mare milk contains much higher amounts of whey protein fractions lactoferrin and lysozime (Claeys et al., 2014). These have been associated to antimicrobial action, preventive action against lipid oxidation (Tong et al., 2006), binding pro-oxidant iron ions

and reducing the conversion of hydrogen peroxide to hydroxyl radicals (Khan et al., 2019) and promoting the increase in the concentration of glutathione peroxidase, another important antioxidant in milk. Mare milk also contains a significant amount of water-soluble vitamin C, which neutralizes superoxide radicals, iron and nitrogen oxides, and inhibits the degradation of vitamin B2. Among fat-soluble vitamins mare milk contains considerable amounts of vitamin E (Claeys et al., 2014) that has been demonstrated to protect polyunsaturated fatty acids, suppress the activity of the proteolytic enzyme plasmin and to generally prevent the generation of free radicals.

There are numerous methods that can be used to determine antioxidant activity of milk and dairy products, among which DPPH, FRAP and ABTS assay are most often used (Stobiecka et al., 2022). The DPPH method measures the ability of the analysed sample to scavenge radicals by the DPPH antioxidant. Unlike other free radicals, DPPH does not dimerize and remains stable due to the electron delocalization across the entire molecule, which causes occurrence of a purple colour in an ethanol solution and can be read at 520 nm. The advantage of this method is that DPPH reacts with the antioxidant as a whole, and a sufficiently long period allows DPPH to react slowly even with weaker antioxidants. Besides, it can be used to determine lipophilic and hydrophilic antioxidants, as well as water and organic solvents (Pregiban, 2017). The FRAP method is based on the reduction reaction of Fe3+ ions - TPTZ (iron(III)-2,4,6-tri(2-pyridyl)-s-triazine) to Fe2+ - TPTZ in the presence of an antioxidant. As a result of this reaction, the formation of an intensely blue-coloured solution occurs which is read at 550 nm (Pregiban, 2017).

Figure 5 presents the results of measuring antioxidant activity by the DPPH and FRAP methods of raw and frozen raw milk samples during 6 months of storage. According to the obtained results, it is evident that the antioxidant activity of mare milk samples after freezing and during 6 months of frozen storage decreased, regardless of the applied method. Such results align with previously discussed structural changes in whey proteins (Figures 3 and 4) as one of the main contributors to antioxidant properties of mare milk. Thus, the DPPH value decreased for approximately 59% 6 months of frozen storage, while the FRAP value decreased approximately 26%. Abarshi et al. (2021) observed similar trends using the DPPH and FRAP methods for analysing refrigerated mare milk at 4ºC during a 3-week storage. They suggested that the observed decrease in antioxidant activity might be associated to the loss or breakdown of certain components in the composition of mare milk, as well as the inactivation of bioactive peptides. Hanna et al. (2004) obtained similar results for human breast milk, whereby antioxidant activity at refrigeration and freezing temperatures significantly decreased. Freezing -20°C resulted in a larger decrease than refrigeration at 4°C, while the lowest values of antioxidant activity were determined for frozen milk stored for seven days. However, the authors could not draw a clear conclusion on the specific components that underwent changes, which caused the observed decrease in antioxidant activity. Păduraru et al. (2018) also found that freezing of human breast milk significantly decreased its total antioxidant status, most probably due to mare milk and human breast milk being very similar by their chemical composition (Claeys et al., 2014), so the results of our study correspond to previous findings on antioxidant activity of frozen human breast milk.

Similar to the values of antioxidant activity, the concentration of total phenols also decreased by approximately 70% throughout the 6 months of frozen storage (Figure 5). Some previous studies confirmed that those phenolic compounds were directly related to antioxidant activity and positively correlated to the FRAP method results (Alyaquobi et al., 2014; Zor et al., 2022). In line with that, the decrease in the total phenol concentration might be related not only to degradation of phenolic compounds, but also to the loss in antioxidant activity of some other components like Lf, lysozyme, unsaturated fatty acids and vitamin C, which have been found to react with the Folin Ciocalteau reagent (Everette et al., 2010).

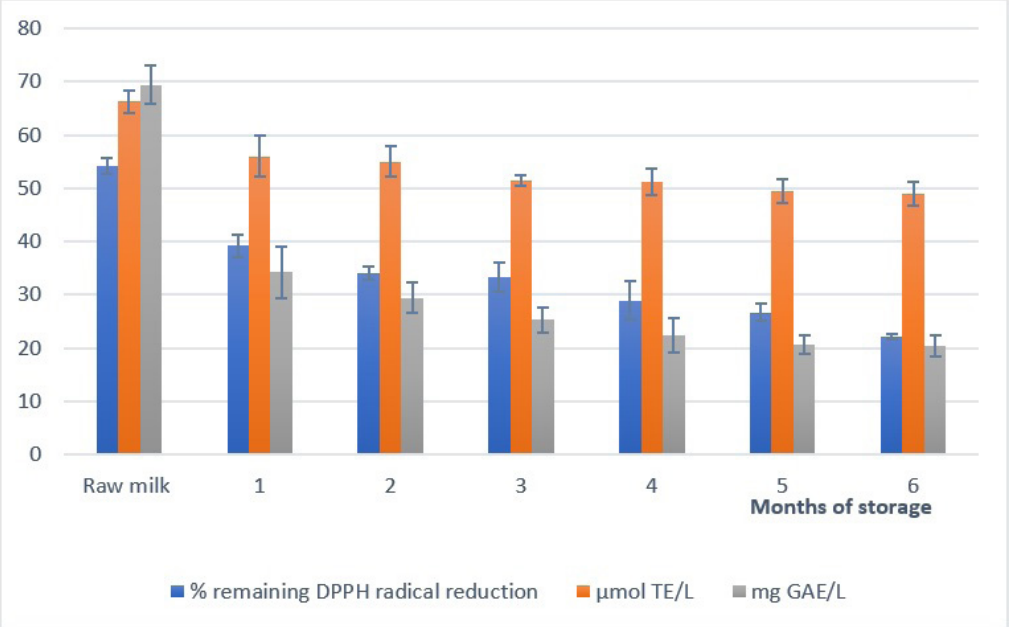


*Figure 5. Antioxidant activity determined by the DPPH (% remaining DPPH radical reduction) and FRAP (µmol TE/L) methods and the total phenol concentration (mg GAE/L) in samples of raw and frozen mare milk stored for 6 months*

## Conclusions

During the storage of mare milk for 6 months in a frozen state, an increase in pH value and the concentration of total dissolved solids occurred, resulting in an increase in the electrical conductivity of mare milk. This might indicate the dissociation of salts and denaturation of proteins present in mare milk, which could be confirmed by the analysis of particle size distribution, by the obtained NIR spectra and by the performed SDS-PAGE analysis. The determination of the antioxidant activity of raw and frozen mare milk showed a significant decrease in the values obtained by the DPPH and the FRAP assay throughout the storage period of 6 months. In line with that, freezing and storage in frozen state also cause a significant decrease in the total phenol concentration determined by the Folin Ciocalteau reagent. Taking into account all the obtained results, it can be concluded that the storage of mare milk in a frozen state at -18°C for 6 months led to a certain decrease in quality, and consequently, a reduction in the nutritional and health value of mare milk. However further studies are need in order to gain more knowledge on the type of compounds that are degraded.

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