

# Stability of the Trolox Equivalent Antioxidant Capacity (TEAC) of Human Milk Frozen at $-20^{\circ}\text{C}$ for 6 Months

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**Abstract** The practice of storing human breast milk (HM) for later use has substantially increased over the last few decades because it allows lactating mothers to continue breastfeeding despite lifestyle changes. Several studies have focused on the safety and nutritional value of milk during the period from the time of storage to that of consumption. To date, few studies have investigated the effect of the storage duration on the antioxidant capacities of stored HM. This study aimed to evaluate the impact of different storage times on the Trolox equivalent antioxidant capacity (TEAC) of frozen HM ( $-20^{\circ}\text{C}$ ). HM samples from 186 healthy women were collected from women who had term deliveries and a lactating duration between 1 and 24 months. The TEAC values of the frozen HM stored for 1, 2, 4, and 6 months were determined using a working 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) solution. The mean TEAC level of an HM sample, which was frozen and stored at  $-20^{\circ}\text{C}$  for 1 month ( $1.86 \pm 0.82$  mM), was compared to those of samples stored for different lengths of time. No significant difference in the mean TEAC values was observed after frozen storage for 2 months ( $1.88 \pm 0.86$  mM), 4 months ( $1.96 \pm 0.93$  mM), and 6 months ( $1.91 \pm 1.01$  mM) ( $p > 0.05$ ). These results suggest that in cases in which HM needs to be stored for more than 1 month, the storage of frozen HM at  $-20^{\circ}\text{C}$  appears to be the optimal condition for preserving the antioxidant capacity of HM for up to 6 months.

**Keywords:** antioxidant, breast milk, frozen milk, human milk, Trolox equivalent antioxidant capacity

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## 1. Introduction

Newborns are susceptible to the conditions associated with the rapid shift from an intrauterine to a relatively hyperoxic extrauterine environment, and this shift might increase the risk of an excessive production of reactive oxygen species (ROS), which can result in oxidative damage to proteins, lipids, and DNA and thus potentially in cell dysfunction or death [1,2]. The antioxidant defense mechanisms of the body commonly prevent the production of ROS or neutralize these species. However, when the production of ROS exceeds the capacity of the body's antioxidant defenses to detoxify these species, a condition known as oxidative stress occurs, and this condition might be involved in the pathogenesis of neonatal diseases, which might include oxidative injuries such as bronchopulmonary dysplasia, retinopathy of prematurity, necrotizing enterocolitis, and intraventricular hemorrhage [2,3,4].

Human milk (HM) provides essential nutrients for neonates and has antioxidant properties that aid their

ability to cope with increased oxidative stress [2,5]. Various methods and tools have been used to measure antioxidant properties, and these include the investigation of component levels with antioxidant properties and direct antioxidant activity. However, the complete list of active antioxidant components in HM is unknown [6]. The antioxidative enzymes in HM that have been frequently analyzed in previous studies are superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), coenzyme Q10 (Co-Q10), lactoperoxidase (LPO), lactoferrin (LF), and ceruloplasmin (CP) [1,2,6,7,8]. Nonenzymatic antioxidative agents, including vitamins E, A, and C, have also been examined in a previous study [9].

Several studies have reported that many determinants influence the antioxidant components in HM, and these determinants include maternal factors (e.g., age, nutritional status, dietary intake, and financial level), infant factors (e.g., gestational age, age, and sex), and physiological factors (e.g., lactation stage and diurnal variation) [10-15]. In addition to these factors, the storage temperature and duration reportedly affect the antioxidant activity of HM. Păduraru et al [16]. compared the total

antioxidant status (TAS) of fresh, refrigerated (+4 °C), and frozen (−20 °C) milk samples, and a slight reduction in TAS was found after 72 h of refrigeration. The same study found a significant decrease in total antioxidants in a sample frozen for 1 week, and freezing for 12 weeks decreased the total antioxidant level by more than 50%. Hanna et al. [17] showed that refrigeration (4°C) and freezing (−20°C) significantly decreased the antioxidant activity of preterm and term HM and that freezing resulted in a greater decrease than refrigeration. These researchers also reported that storage for 7 days resulted in a lower antioxidant activity than storage for 2 days, and this finding was obtained with both refrigeration (4°C) and freezing (−20°C). Taking all these findings into consideration, it is reasonable to conclude that the storage conditions affect the antioxidant level of stored HM.

The storage of HM for later use has become an alternative that allows mothers whose lifestyles have changed to continue breastfeeding [18]. According to the storage guidelines for HM detailed by many health authorities, HM should be administered to infants within 6-12 months of freezing (−18°C or colder) [19,20], and this recommendation is mainly based on the safety and nutritional values of stored milk [18]. Most of the previous studies evaluated the impact of storage by comparing stored and fresh HM and found that the total antioxidant levels in refrigerated or frozen samples were lower than those in fresh samples [16,17,21]. The impact of different storage durations on the Trolox equivalent antioxidant capacity (TEAC) of frozen HM has not yet been clearly described. This study aimed to fill this knowledge gap by investigating whether the TEAC levels in frozen HM stored at −20 °C for 2, 4, and 6 months differed from those in samples stored for 1 month.

## 2. Methods

### 2.1. Study Design and Sample Characteristics

This study included 186 mature milk samples from participants recruited from January 2019 to April 2019 through study posters posted in the well-baby clinic and the lactation rooms of four hospitals in Chiang Mai City, Thailand, as well as in a Facebook parenting group. The interested mothers contacted our research assistants via telephone and answered a set of questions corresponding to the inclusion and exclusion criteria. The exclusion criteria included any underlying disease in the mother or her offspring, illiteracy in the Thai language, inability of the mother to travel to our lactation room on her own, and dietary restrictions (carbohydrate-restricted diet, vegan diet, or vegetarian diet). All eligible participants were requested to set an appointment for milk collection.

The participants completed a self-report questionnaire to provide baseline information, including the maternal age, child's birthdate, gestational age, birth order, parental status, and mode of delivery. Paper-based questionnaires in the Thai language were used for this data collection. The weight and height of each participant were measured and used to calculate the patient's current BMI. The participants' BMIs were classified into four different groups in accordance with the Asia-Pacific obesity

classification: underweight (<18.5 kg/m<sup>2</sup>), normal weight (18.5–22.9 kg/m<sup>2</sup>), overweight (23–24.9 kg/m<sup>2</sup>), and obese (>25 kg/m<sup>2</sup>) [22]. Before providing any information and breast milk samples, all the participants signed informed consent forms.

### 2.2. Breastmilk Collection

The participants were required to provide milk samples in the lactation room at Maharaj Nakorn Chiang Mai Hospital, Nakornping Hospital, Health Promotion Hospital Region One, and Lampang Hospital. HM was expressed using Lactina Electric Selection pumps (Medela®, Switzerland) under the same conditions between 8:00 a.m. and 12:00 p.m. to minimize possible circadian influences [23] and to ensure the uniformity of the samples. The pump was left on for approximately 15 min or until no further milk could be expressed for at least 5 min. The milk samples were divided into four portions, placed into 1.5-mL microcentrifuge tubes and frozen at −20 °C until further analysis.

### 2.3. Trolox Equivalent Antioxidant Capacity (TEAC) Analysis

The TEAC of HM was determined using a working 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) solution, which was prepared by mixing two equal volumes of 0.768 g of ABTS® (AppliChem, A1088,0005) and 0.132 g of K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> (VWR Chemical, 26915.291). The mixture was incubated at room temperature (RT) for 12 h, and the working ABTS solution was prepared by a 50-fold dilution of the incubated solution in deionized H<sub>2</sub>O. Twenty microliters of each HM sample was mixed with 2 mL of the working ABTS solution. The reaction was allowed to continue for exactly 6 min at RT, and the absorbance at 734 nm was read using a Genesys™ 20 instrument (Thermo Scientific, USA). The TEAC of each HM sample was calculated using a Trolox (Sigma, 238813) standard curve with a concentration range of 0 to 5 mM and is reported as the millimolar (mM) Trolox equivalence.

### 2.4. Statistical Analysis

A descriptive analysis was performed based on various characteristics of the study population, including the marital age, maternal education, marital status, maternal BMI, parity, breastfeeding frequency per day, months of lactation, type of delivery, and infant weight. Continuous variables were analyzed using the Kolmogorov–Smirnov test to verify the normality of their distribution, and the data are expressed as the means, standard deviations, and 25th and 75th percentiles. To compare the TEAC levels, the mean values obtained after storage for 1, 2, 4, and 6 months were used to assess the level of significant difference using paired-sample *t*-tests. Spearman's correlation coefficient and the Mann–Whitney U-test were used to analyze the correlation between maternal characteristics and the TEAC after frozen storage at −20 °C for 1 month. All analyses in this study were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL., USA), and the significance level was set to  $p < 0.05$ .

## 2.5. Ethical Consideration

Approval for this study was obtained from the Research Ethics Committee, Faculty of Medicine, Chiang Mai University (No. 158/2018). Written informed consent was obtained from all the participants. This study complied with the principles set forth in the Declaration of Helsinki (1964) and all of its subsequent amendments.

## 3. Results

### 3.1. Characteristics of the Study Population

Milk samples were provided by 186 breastfeeding mothers who had given birth to full-term infants. The baseline characteristics of the participants and their offspring are summarized in Table 1. The mean ( $\pm$  standard deviation (SD)) values for the age, months of lactation and breastfeeding times per day of the participants were  $32.01 \pm 4.56$  years,  $11.17 \pm 5.99$  months, and  $7.06 \pm 4.10$ ,

respectively. The study found that close to half of the participants had a normal BMI at the point of assessment (51.1%), the majority were educated to a graduate degree level (69.9%), and almost all lactating women (98.4%) were married. The characteristics of the pregnancies were primiparas (62.9%) and vaginal delivery (61.8%) (Table 2).

### 3.2. TEAC of Frozen HM Stored for 1, 2, 4, and 6 Months

Table 3 compares the TEAC levels of frozen HM samples stored at  $-20^\circ\text{C}$  for different durations with those of samples frozen for 1 month ( $1.86 \pm 0.82$  mM). No significant difference in the mean values of TEAC was observed after the samples were frozen for 2 months ( $1.88 \pm 0.86$  mM), 4 months ( $1.96 \pm 0.93$  mM), and 6 months ( $1.91 \pm 1.10$  mM) ( $p > 0.05$ ). No significant differences were observed at each frozen storage time point ( $p > 0.05$ ). The TEAC levels of HM stored for 1, 2, 4, and 6 months are shown in Figure 1.

Table 1. Characteristics of the study population: continuous variables

Variable	Mean $\pm$ SD	Median (25 <sup>th</sup> , 75 <sup>th</sup> percentile)	Minimum–Maximum
Maternal age, years	$32.01 \pm 4.56$	32.00 (29.00, 35.25)	22.00–42.00
Months of lactation	$11.17 \pm 5.99$	12.00 (6.00, 16.00)	1.00–23.00
Breastfeeding, times per day ( $n = 157$ )	$7.06 \pm 4.10$	6.00 (4.00, 8.50)	1.00–25.00
Infant weight at birth (boys), kg ( $n = 99$ )	$3.16 \pm 0.40$	3.20 (2.90, 3.40)	2.30–4.30
Infant weight at birth (girls), kg ( $n = 87$ )	$3.04 \pm 0.36$	3.00 (2.80, 3.30)	2.30–3.80

SD, standard deviation; kg, kilogram.

Table 2. Characteristics of the study population: qualitative variables

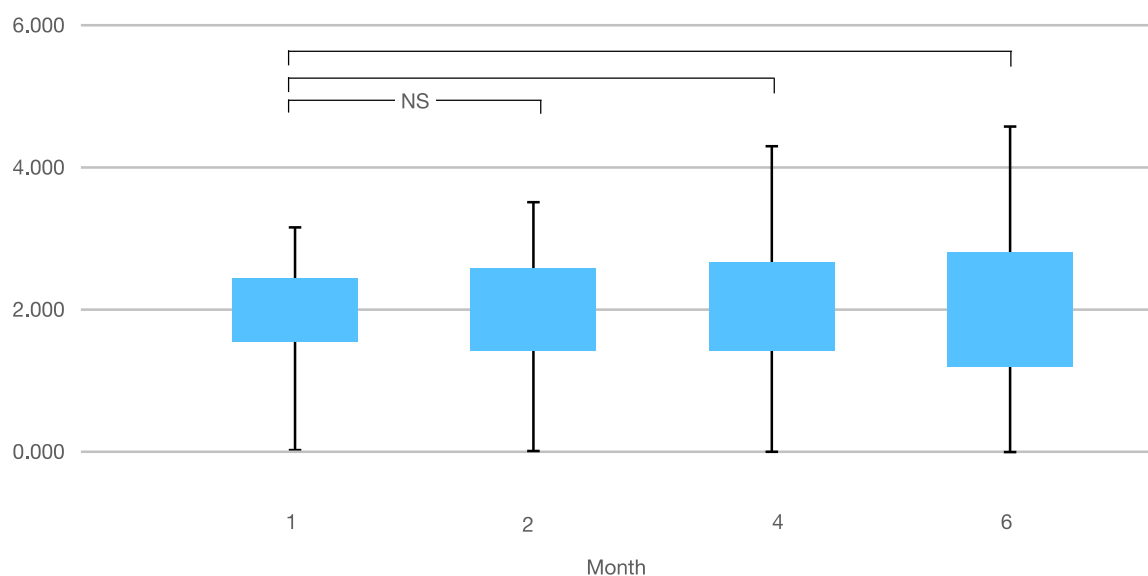
Variable	<i>n</i>	%
<b>Maternal BMI</b>		
under	22	11.8
normal	95	51.1
over	30	16.1
obese	39	21.0
<b>Maternal education</b>		
undergraduate	56	30.1
graduate degree	130	69.9
<b>Marital status</b>		
married	183	98.4
single	3	1.6
<b>Parity</b>		
primiparous	117	62.9
multiparous	69	37.1
<b>Type of delivery</b>		
vaginal	115	61.8
cesarean section	71	38.2

BMI, body mass index.

Table 3. Trolox equivalent antioxidant capacity (TEAC) of human milk (HM) frozen at  $-20^\circ\text{C}$  for 1, 2, 4, and 6 months

	TEAC of Frozen HM				<i>p</i> -Value
	1 month (F1) <sup>a</sup> ( <i>N</i> = 186)	2 months (F2) <sup>b</sup> ( <i>N</i> = 186)	4 months (F4) <sup>c</sup> ( <i>N</i> = 186)	6 months (F6) <sup>d</sup> ( <i>N</i> = 185)	
Mean $\pm$ SD	$1.86 \pm 0.82$	$1.88 \pm 0.86$	$1.96 \pm 0.93$	$1.91 \pm 1.10$	ab = 0.75
(95% CI)	(1.74–1.98)	(1.75–2.00)	(1.83–2.10)	(1.75–2.07)	ac = 0.06
Minimum–maximum	0–4.06	0–4.63	0–4.68	0–5.13	ad = 0.47
Median	1.87	1.77	1.93	1.97	bc = 0.09
25th–75th percentiles	1.45–2.37	1.33–2.40	1.38–2.53	1.15–2.57	cd = 0.47

All the data are expressed as the means  $\pm$  SDs, minimum–maximum values, medians, and 25th–75th percentiles. The analysis was performed using a paired-sample *t*-test. Significant differences between groups are indicated by  $p < 0.05$ . CI = confidence interval; TEAC = Trolox equivalent antioxidant capacity (mM, Trolox equivalent).



**Figure 1.** Boxplot illustrating the change in the TEAC of HM after storage at  $-20^{\circ}\text{C}$  for 1, 2, 4, and 6 months (NS = no significant difference,  $p > 0.05$ . The mean, standard deviation, minimum, and maximum values are included in the plot.)

**Table 4.** Correlation between maternal characteristics and Trolox equivalent antioxidant capacity (TEAC) of frozen HM stored at  $-20^{\circ}\text{C}$  for 1 month

Variables	Correlation coefficient <sup>†</sup>	Mean rank <sup>‡</sup>	<i>p</i> -value
Maternal age, years	0.05		0.54
Maternal BMI, $\text{kg}/\text{m}^2$	0.02		0.75
Breastfeeding frequency per day	-0.98		0.22
Months of lactation	-0.09		0.20
Parity			
multipara ( $n = 117$ )		96.12	
primipara ( $n = 68$ )		87.63	0.30

The analyses were performed using <sup>†</sup> Spearman's correlation coefficient and <sup>‡</sup> the Mann-Whitney U-test.

### 3.3. Correlation between Maternal Characteristics and TEAC after Frozen Storage at $-20^{\circ}\text{C}$ for 1 Month

Table 4 shows the Spearman's correlation coefficients for maternal characteristics and TEAC in frozen HM stored at  $-20^{\circ}\text{C}$  for 1 month. Although the TEAC level was positively associated with the maternal age ( $r = 0.05$ ) and the maternal BMI ( $r = 0.02$ ) was inversely associated with the breastfeeding frequency per day ( $r = -0.98$ ) and months of lactation ( $r = -0.09$ ), no statistically significant differences were found among these variables ( $p > 0.05$ ). The Mann-Whitney U-test also suggested no significant differences between multipara and primipara mothers ( $p = 0.30$ ) and the TEAC levels.

## 4. Discussion

This report presents the first analysis of TEAC level in longitudinal samples of frozen HM. The mean TEAC level of a frozen HM sample stored at  $-20^{\circ}\text{C}$  for one month was compared with that in samples stored for 2, 4 and 6 months. We observed no significant differences in the TEAC levels of HM at each time point and found a small insignificant increase in the TEAC of our HM

samples after the longer storage period. The increase in the TEAC level over time could be explained by the slow process of milk protein hydrolysis, which generates antioxidant peptides via unknown enzymatic reactions [24,25]. This result was consistent with the results from an observational study, which found that glutathione peroxidase (GHSPx) and selenium glutathione peroxidase (SeGHSPx) in HM increased during the 12-week study period [7]. Furthermore, an increase in antioxidant activity during freezing was observed in the food industrial freezing process, and this increase could be affected by an increase in the release of bioactive compounds and might be related to the formation of ice crystals within the cell matrix that modify the permeability of cell membranes, which leads to tissue damage, during the freezing and thawing process [26]. However, the antioxidant components of breast milk, such as GPx [27,28] and vitamin C [29], tend to decrease with increases in the storage duration. The mechanism underlying the changes in the TEAC observed in our HM samples during the longer storage period is unclear because we determined the TEAC values using the ABTS test, which reflects the activity of antioxidant components without identifying the details of each active antioxidant component in HM [30]. Establishing comparisons between our study and existing studies is challenging due to the high variability in the



methods used to evaluate the antioxidant capacity between various investigators. For example, many researchers have used a direct measurement of the antioxidant activity [16,21,31-35], whereas some have analyzed the enzymes that play a role in antioxidant defense [27,28].

Temperature is likely an important factor affecting our results. Previous studies have reported that different magnitudes of temperature affect the antioxidant components in HM after storage and observed that a lower freezing temperature could preserve the more significant antioxidant activity of HM. Silvestre et al. [28] compared the effects of two temperatures ( $-20^{\circ}\text{C}$  and  $-80^{\circ}\text{C}$ ) on the GPx activity of term HM ( $n=10$ ). The freezing of HM at  $-20^{\circ}\text{C}$  induced an abrupt decrease in GPx activity, whereas freezing at  $-80^{\circ}\text{C}$  did not induce any marked changes in enzyme activity during the first 30 days. This study indicated that the magnitude of the reduction in antioxidant properties is correlated with temperature. Lower decreases in the vitamin C levels in HM were observed after storage for 12 months at  $-80^{\circ}\text{C}$  than after storage at  $-20^{\circ}\text{C}$  [36]. Sari et al. [21] compared TAC in fresh HM and HM stored at  $-80^{\circ}\text{C}$  for 2 months and observed no significant difference in the TAC level between fresh and stored colostrum. Similar results were observed in studies by Akdag et al. [31], who compared the TAC levels of fresh HM and HM stored at  $-80^{\circ}\text{C}$  for 3 months. These data suggest that the decreases in the antioxidant capacity of HM observed during storage depends on the temperature. We stored samples in a deep freezer ( $-20^{\circ}\text{C}$ ) based on the assumption that this temperature would be sufficient to preserve the TAC of HM for at least 6 months.

The second purpose of this study was to investigate the relationship between the TEAC levels of HM and maternal factors. We found no significant correlations between the TEAC levels of HM and the maternal age, maternal BMI, breastfeeding frequency per day, months of lactation, or parity. This finding ensures that the TEAC of HM stored in a freezer at  $-20^{\circ}\text{C}$  for 1 month or longer periods up to 6 months was not significantly different. The correlation between the TEAC of HM and maternal characteristics has been repeatedly investigated, particularly regarding its relationship with lactation duration. Previous studies [10,21,37] provide evidence suggesting that the antioxidant activity and antioxidant substances in HM tend to decrease from colostrum to mature milk. A previous longitudinal [37] study collected milk samples from 115 healthy women with full-term infants, and the findings indicated that the total antioxidant levels of mature milk at 6 months postpartum were significantly lower than those found in colostrum ( $p<0.05$ ). Based on limited research, the highest levels of antioxidant components and TAC are present in colostrum, and these levels then decrease during early lactation. However, most of the previous studies described TAC in breast milk from women at 6 months postpartum. The present study provides the first description of the TAC in breast milk from women up to 2 years postpartum and indicates that the duration of lactation is not associated with the TAC in mature milk.

Studies focusing on the relationship between the antioxidant activity in HM and other maternal factors,

including the maternal BMI, maternal age, breastfeeding frequency per day, and parity, are less frequently described in the literature, the results are controversial, and no conclusions can be drawn. With regard to the maternal BMI, our data were consistent with those obtained by Ahmed et al. [9], who observed no relationship between the maternal BMI and TAC in HM. Our results were also similar to previous findings that demonstrated that the maternal age and parity [9,13] do not affect either the antioxidant activity or antioxidant substances. In addition to the above-mentioned factors, previous studies have explored many maternal factors that might influence the antioxidant activity and the level of antioxidant compounds in HM, including the maternal diet, route of delivery, nutritional status, use of tobacco, and financial level. Infant factors (birth weight, pre-mature milk and term milk) and physiological factors (diurnal variation) are also thought to influence the antioxidant capacity and antioxidant substances in HM [9,11,38,39]. We minimized the variability of the samples by including only mature milk from lactating mothers who had given birth at full term and reduced the influence of different maternal characteristics by using a longitudinal study design.

However, this study has some limitations that should be noted. First, our participants are likely to be of higher socioeconomic status than the general population because the recruitment process was limited to populations in urban areas and populations with internet access. Therefore, those who were informed about the study were more likely to be of higher socioeconomic status than individuals in the general population. The application of the findings of this study to the general population will require further investigation. Second, we determined the effect of the duration of storage at  $-20^{\circ}\text{C}$ . The findings of our study might not be transferable to domestic freezers and refrigerators that vary between  $-18^{\circ}\text{C}$  and  $-3^{\circ}\text{C}$  [32]. To apply the results to a more common situation, the impact of freezing at different storage temperatures should be evaluated in future research.

## 5. Conclusions

In our study, we investigated whether the TEAC levels of frozen HM stored at  $-20^{\circ}\text{C}$  for 2, 4, and 6 months differed from those of HM stored for 1 month. No significant differences in the TEAC levels of HM were observed at each time point. These results suggest that in cases where HM needs to be stored for more than 1 month, the storage of frozen HM at  $-20^{\circ}\text{C}$  appears to be the optimal condition for preserving the antioxidant capacity of HM for up to 6 months.

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## Abbreviations

BMI: body mass index; DNA: deoxyribonucleic acid; HM: human milk; ROS: reactive oxygen species; TAC: total antioxidant capacity; TEAC: Trolox equivalent antioxidant capacity.

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