# Comparative Analysis of Vitamin A, Vitamin C, Vitamin E, and selected B vitamins in human milk for different storage conditions.

## Introduction

It is reported that as of January of 2003, 60.7% of women worked outside the home.[1] Breastfeeding women working outside the home commonly utilize a breast pumping device to provide milk for their infants. Human milk storage for use later in infant feeding is on the increase as a result of the economic activities of breastfeeding mothers. Providing support for women inclined to continue providing milk for their infants should not be undervalued when evaluating health-promoting activities.

One reason for providing expressed milk to an infant is the anti-oxidant properties of human milk in the form of vitamins. It is known that fresh human milk has a higher antioxidant capacity than infant formula.[2] Expressed breast milk is usually stored frozen for consumption at a later time. It is known that changes occur in human milk stored either via refrigeration or frozen. In one study the total vitamin C levels decreased on average by one-third in the refrigerator, short-term or after one month of freezing, with a wide variation of nutrients between individuals (6 to 76% and 3 to 100%, respectively). After 2 months of freezing, the average decrease was two-thirds with a range of 7 to 100%.[3] Many nutrients, including vitamins, are susceptible to oxidation. Turoli, et al. reported that breast milk is subject to a strong peroxidation either at room temperature or at -20 degrees C.[4]

The oxidation of food products is well documented. Changes in product color, odor, and taste are the most easily detected effects of oxidation in foods.[5] Nutrient loss, rancidity, and microbial spoilage are all a part of milk degradation as well. This degradation, over time, is caused in part, by the presence of oxygen, and oxidation of the food components.[6] Currently, human milk is stored in containers with headspace. Removal of headspace oxygen should minimize the oxidative effects in stored breastmilk, prolong shelf life, and improve nutritional quality. The purpose of this study was to analyze and compare any differences in vitamin A, E, C, B1, B2, and B3 in milk frozen in containers with ambient air in the headspace versus vacuum sealed storage containers.

**Procedure**

Fresh, mature, human milk donated by eight women with healthy infants and typical American diets was used for analysis. Infant formula from powder and liquid was also analyzed. Baseline analysis of each sample was completed within 24 hours of collection for Vitamin A, C, E, B1, B2, and B3. Each sample was divided into two sets, set S(tandard) and V(acuum) with aliquots of 1 ounces each: i.e., Day 10, Day 20, and Day 40. A 20 ml sample was removed from each aliquot and tested for the nutrients of interest. Half the samples were then vacuum sealed and placed in the freezer at -20 degrees C. The other half of the samples were sealed with ambient air and placed the in freezer at -20 degrees C. After 10 days, one aliquot from each group was removed for analysis. At 20 days and 40 days the process was repeated.

Vitamin A and E content were analyzed using high-performance liquid chromatography (HPLC) and described by Chavez-Servin, et, al.[7] Prior to testing, the samples were briefly immersed in warm water (40$^{o}$C) to thaw them, then mixed using a vortex to provide a homogenous sample. One ml of the sample was transferred to a centrifuge tube and 3 mls of ethanol were added. The samples were mechanically shaken and 1 ml of hexane was added, then schaken for another minute. After resting the samples for 5 minutes, 3 mls of saturated NaCl was added to aid separation. The mixture was shaken by inversion. The samples were centrifuged for 5 minutes at 3000 rpm at room temperature. The hexane phase was recovered and directly filtered through a .22 um nylon filter and collected in a 1 ml amber glass vial. Twenty ul was injected into the HPLC system.

Vitamin C content was analyzed using HPLC described by Romeu-Nadal, et al.[8] The samples were protected from light by wrapping tubes and flasks with aluminum foil and preparing the samples in a darkened room. Three hundred ul of milk mixed with 300ul of 0.56% meta-phosphoric acid solution were added to the same centrifuge and filtration tube, which was shaken for 30 seconds and centrifuged at 10$^{o}$C (10 minutes, 3000 X g). Ascorbic acid was identified by comparing the retention time of the sample peak with that of the ascorbic standard at 254 nm. Quantification was carried out using external standardization.

Vitamin B1, B2, and B3 were analyzed using HPLC as described by Albala-Hurtado, et al.[9] The samples were protected from light by wrapping tubes and flasks with aluminum foil and preparing the samples in a darkened room. 10.5 grams of sample were weighed into a 50 ml centrifuge tube. 1 g of TCA solid and a magnetic stirring bar were added. The mixture was stirred for 10 min over a magnetic stirring plate, then centrifuged for 10 minutes at 1250 g to separate the two phases. Three ml of 4% TCA was added to the solid residue obtained and mixed for 10 minutes and then centrifuged. The solid-phase was discarded. The two acid extracts were combined in a 10 ml volumetric flask and the volume was filled with 4% TCA. Acid extracts were filtered through a .45um filter and injected into the HPLC system.

This process was repeated for fresh milk stored under refrigeration for 48 hours, with measurement of nutrients occurring at 12, 24, and 48 hour intervals.

Descriptive statistics were calculated and nutrient mean values were compared using paired t-tests. Statistical significance was determined when $p \leq .01$.

**Results**

The baseline values for each sample and nutrient are listed in Table 1. These values are highly variable, as would be expected in human milk. The infant formula nutrient values are generally higher than the human milk values. The range for vitamin A values was 197 mcg/L in human milk to a high of 1277 mcg/L in infant formula. For vitamin E, the lowest level was 0.879 mg/ml to a high level of 4.672 mg/ml. The Vitamin C levels ranged from 32 mg/ml to a high of 87 mg/ml. The range for vitamin $B_1$ was 0.032 mg/ml to 0.676 mg/ml.

The range for vitamin $B_2$ was 0.156 mg/ml to 1.015 mg/ml. The range for vitamin $B_3$ was 1.25 mg/ml to 7.10 mg/ml. The baseline values and the final values for each group are listed in Table 2.

Table 1. Baseline values by nutrient and sample.

| | A (mcg/L) | E (mg/L) | C (mg/L) | $B_1$ (mg/L) | $B_2$ (mg/L) | $B_3$ (mg/L) |
|---|---|---|---|---|---|---|
| HM1 | 478 | 2.201 | 51 | 0.123 | 0.348 | 3.29 |
| HM2 | 764 | 3.252 | 87 | 0.185 | 0.511 | 5.33 |
| HM3 | 197 | 1.632 | 32 | 0.091 | 0.221 | 1.25 |
| HM4 | 308 | 4.485 | 89 | 0.082 | 0.456 | 2.61 |
| HM5 | 604 | 2.865 | 68 | 0.103 | 0.198 | 4.28 |
| HM6 | 236 | 1.683 | 44 | 0.032 | 0.156 | 1.86 |
| HM7 | 289 | 0.879 | 44 | 0.054 | 0.237 | 2.04 |
| HM8 | 404 | 1.768 | 50 | 0.096 | 0.396 | 1.94 |
| IF1 | 607 | 3.251 | 61 | 0.676 | 1.015 | 7.10 |
| IF2 | 1277 | 4.672 | 81 | 0.554 | 0.945 | 6.76 |
| Average | 516 | 2.669 | 61 | 0.200 | 0.448 | 3.65 |

Due to the high variability of the values for human milk the mean values were used for comparison. The decrease in levels of vitamin A, E, C, B1, B2, and B3 was higher in the S group than in the V group and was statistically significantly different. The mean values for the human milk samples are depicted in Table 3.

Table 3. Mean values of vitamins in human milk.

| Vitamin | Baseline | 10 days | | 20 days | | 40 days | |
|---|---|---|---|---|---|---|---|
| | | S Group | V Group | S Group | V Group | S Group | V Group |
| A IU/ml | 1.366 | 1.254* | 1.334 | 1.199 | 1.386 | 1.137* | 1.361 |
| E mcg/ml | 2.346 | 2.222* | 2.243 | 2.159* | 2.243 | 2.040* | 2.182 |
| C mg/ml | 0.058 | 0.041 | 0.052 | 0.034 | 0.047 | 0.018 | 0.041 |
| B1 mcg/ml | 0.096 | 0.073* | 0.088 | 0.064* | 0.080 | 0.045* | 0.067* |
| B2 mcg/ml | 0.315 | 0.281# | 0.300 | 0.255# | 0.286 | 0.219# | 0.261# |
| B3 mcg/ml | 0.283 | 0.249* | 0.267 | 0.223* | 0.246 | 0.195* | 0.229 |

*Statistically significantly below baseline at $p \leq 0.01$.

#Statistically significantly below baseline at $p \leq 0.001$.

For the samples that were refrigerated, the nutrient levels were higher in the V group than in the S group. Each time-point measurement indicated consistent decreases in the level of all nutrients in the S group compared to slight decrease in the V group. While the differences were not statistically different, there was practical meaning of the differences. A larger sample size may have yielded significant differences.

**Discussion**

All of the nutrient levels dropped for the duration of the time in storage in both groups but consistently less in the V group. For example, at baseline, most subjects had adequate levels of vitamin A and C at baseline to meet the RDI for their infant. However, by the end of the 40 days storage time not all samples contained the RDI for vitamin C of 50 mg/day for infants of less than 1 year of age. In particular, vitamin C content from the S group was reduced to nondetectable levels in four of the human milk samples by the last measurement, while the V group all had. Nutrients were better preserved by vacuum sealing, providing nutrient levels at of near the RDI for infants under the age of 1 year.

Women around the world are storing their milk frozen for times of separation from their infants.[10] Providing a storage system that preserves the nutrients in frozen human milk is critical for the long-term health of those infants fed with frozen milk.

Bibligraphy


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Table 2. Values at Baseline and 40 days.

| | A (mcg/L) | | | E (mg/L) | | | C (mg/L) | | | $B_1$ (mg/L) | | | $B_2$ (mg/L) | | | $B_3$ (mg/L) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Baseline | S40 | V40 | Baseline | S40 | V40 | Baseline | S40 | V40 | Baseline | S40 | V40 | Baseline | S40 | V40 | Baseline | S40 | V40 |
| HM1 | 478 | 337 | 591 | 2.201 | 1.372 | 1.932 | 51 | 0 | 21 | 0.123 | 0.035 | 0.062 | 0.348 | 0.204 | 0.236 | 3.29 | 1.32 | 1.68 |
| HM2 | 764 | 602 | 737 | 3.252 | 2.799 | 2.901 | 87 | 28 | 68 | 0.185 | 0.119 | 0.147 | 0.511 | 0.362 | 0.425 | 5.33 | 4.09 | 4.86 |
| HM3 | 197 | 179 | 185 | 1.632 | 1.480 | 1.501 | 32 | 0 | 20 | 0.091 | 0.000 | 0.068 | 0.221 | 0.065 | 0.186 | 1.25 | 0.54 | 0.95 |
| HM4 | 308 | 267 | 297 | 4.485 | 4.201 | 4.398 | 89 | 46 | 71 | 0.082 | 0.048 | 0.068 | 0.456 | 0.389 | 0.401 | 2.61 | 2.01 | 2.02 |
| HM5 | 604 | 512 | 587 | 2.865 | 2.611 | 2.811 | 68 | 32 | 56 | 0.103 | 0.059 | 0.082 | 0.198 | 0.132 | 0.169 | 4.28 | 3.68 | 4.01 |
| HM6 | 236 | 219 | 212 | 1.683 | 1.599 | 1.536 | 44 | 0 | 28 | 0.032 | 0.000 | 0.000 | 0.156 | 0.111 | 0.109 | 1.86 | 1.21 | 1.49 |
| HM7 | 289 | 272 | 277 | 0.879 | 0.738 | 0.777 | 44 | 0 | 30 | 0.054 | 0.020 | 0.041 | 0.237 | 0.145 | 0.200 | 2.04 | 1.31 | 1.79 |
| HM8 | 404 | 341 | 383 | 1.768 | 1.517 | 1.602 | 50 | 36 | 31 | 0.096 | 0.075 | 0.068 | 0.396 | 0.342 | 0.359 | 1.94 | 1.41 | 1.52 |
| IF1 | 607 | 507 | 487 | 3.251 | 2.266 | 2.358 | 61 | 489 | 41 | 0.676 | 0.476 | 0.496 | 1.015 | 0.821 | 0.901 | 7.10 | 6.489 | 6.564 |
| IF2 | 1277 | 964 | 1038 | 4.672 | 3.001 | 3.581 | 81 | 0 | 54 | 0.554 | 0.359 | 0.486 | 0.945 | 0.699 | 0.703 | 6.76 | 5.680 | 5.897 |
| Average | 516 | 420 | 479 | 2.669 | 2.158 | 2.340 | 61 | 63 | 42 | 0.200 | 0.119 | 0.152 | 0.448 | 0.327 | 0.369 | 3.65 | 2.7739 | 3.0781 |