ELSEVIER

Research Communications

Major carotenoids in mature human milk: Longitudinal and diurnal patterns

Joan G. Jackson, Eric L. Lien, Sharon J. White,* Nicholas J. Bruns,† and Charles F. Kuhlman

*Wyeth Nutritionals International, Nutritional Research, and *Clinical Nutrition, Radnor, PA USA; and †Nicholas J. Bruns, Quest International, 5115 Sedge Blvd., Hoffman Estates, IL USA*

The present study was undertaken to quantitate the major carotenoids in human milk, monitoring their variance among fore, mid, and hind mature milk samples; during morning, midday, and evening, and among different weeks of lactation. Twenty-three mothers, 6 weeks to 16 weeks postpartum, participated in the study.

Reversed-phase high performance liquid chromatography (RP-HPLC) was used to identify and quantitate the major carotenoids found in human milk, including lutein/zeaxanthin, beta cryptoxanthin, lycopene, alpha carotene, and beta carotene. To determine the validity of analyzing carotenoids in frozen milk, freshly expressed and subsequently frozen milk samples were analyzed for four mothers. Freezing did not alter carotenoid concentrations.

Wide variations in carotenoid concentrations existed within and between the mothers. Major carotenoid totals ranged from 29 nM to 493 nM. Complete breast expressions at three periods within a day and at specified weekly intervals allowed the accurate determination of diurnal as well as longitudinal carotenoid concentrations. Diurnal evaluation of carotenoid concentrations suggested a peak at midday and highest longitudinal concentrations were at 10 to 12 weeks. Neither of these trends was statistically significant. The analysis of fore, mid, and hind milk carotenoid concentrations demonstrated that hind milk was significantly higher than fore or mid milk ($P < 0.05$*).* (J. Nutr. Biochem. 9:2–7, 1998) © *Elsevier Science Inc. 1998*

Keywords: carotenoids; mature human milk; diurnal, longitudinal; within-feed; HPLC, within-between person

Introduction

Human milk is the preferred source of nourishment for infants because it contains a variety of nutrients not universally found in infant formula or bovine milk. Among the nutrients contained in human milk are carotenoids, which may offer enhanced protection to the infant against infection.[1,2] Beta carotene and some of the other carotenoids are important because they are precursors of vitamin A, (required for normal growth and eyesight as well as resistance to infection) may enhance immune functions, impart antioxidant action, and demonstrate anticancer properties.[3–5] The major carotenoids include beta carotene, alpha carotene, lycopene, beta cryptoxanthin, and lutein/zeaxanthin, all of which are supplied to the infant during breast feeding.[6,7] Beta carotene, comprising approximately 25% of the total carotenoid content in human milk, has the highest vitamin A activity of all known carotenoids and is a potent antioxidant.[4,8,9]

Serum concentrations of carotenoids in breast fed infants increase significantly starting 2 days after birth, whereas carotenoid concentrations in many formula fed infants decrease because of a lack of carotenoid fortification in most infant formulas.[1] A few infant formulas are fortified with beta carotene, thereby increasing the serum beta carotene concentrations in formula-fed infants, although

Address correspondence and reprint requests to Joan Jackson, Wyeth Nutritionals International, 145 King of Prussia Road, Nutritional Research, Radnor, Pennsylvania, 19087.
Received May 6, 1997; accepted August 28, 1997.

655 Avenue of the Americas, New York, NY 10010

0955-2863/98/$19.00
PII S0955-2863(97)00132-0

total carotenoid concentrations are higher in breast fed infants.[10] Undernourished children often have serum concentrations of vitamin A and carotenoids much lower than found in well nourished children and often have serious health problems.[11] One could speculate that these children would benefit from a consistent diet rich in carotenoids.

Many previous studies quantitating carotenoids in human milk have used pooled milk from several donors or partial emptying of the breasts.[12] Complete emptying of the breasts is the preferable method for obtaining representative samplings of human milk used for the analysis of lipid-soluble compounds such as carotenoids. The lipid content of human milk is known to vary within a feed and diurnally.[13–16] Neville et al reported that lipid concentrations increased two- to three-fold between fore and hind milk.[14] This within feed variation of lipids was also observed by Hall, who cited that concentrations increased three fold.[13] Hall also reported diurnal variations in lipid concentrations, being lowest at 6 AM and highest at 2 PM with a 2.5-fold increase. It is conceivable that variations in carotenoid levels within a feed and diurnally could be related to the variations in lipid concentrations.

The present study analyzed full breast expressions of mature milk both diurnally and longitudinally. Expressions were collected in 30 mL fractions, which allowed the examination of fore, mid, and hind milk. We examined the validity of analyzing frozen milk samples by comparing carotenoid concentrations in freshly expressed milk and subsequently frozen samples.

Method and materials

Subjects

Subjects were recruited through a local breast-feeding support group. All participants were healthy adults, either primipara or multipara women. All delivered full-term infants and were lactating for at least 6 weeks. Informed consent was obtained from all participants and procedures followed were in accord with standard collection techniques recommended by breast pump manufacturers and used by many hospitals. Subjects were provided with individualized instructions regarding the study and use of the breast pump. Because a full breast expression was required for the study, each participant was counseled regarding pumping and freezing of their milk.

Sample collection

An Egnell breast pump and a sterile accessory kit were assembled in each mother's home. They were instructed to extract one full expression using the pump at least 1 hr after the previous nursing. Alphabetically labeled polypropylene tubes were filled consecutively, each with approximately 30 mL of milk, to separate fore, mid, and hind milk. Expression continued until the milk flow diminished. The tubes were placed in the home freezer until collected by the investigator within 1 week. The investigator packed the frozen milk tubes in dry ice for transport to the analyzing laboratory where they were placed in a −70°C freezer until analyzed.

Human milk from 23 mothers was examined to determine individual and total major carotenoids for this study. Our goals were to determine: (1) diurnal variations; (2) longitudinal variations; (3) within-feed variations in fore, mid, and hind milk; and (4) concentrations of individual carotenoids as well as totals. Complete expressions were obtained from each mother. When the number of individual sample tubes for a complete expression exceeded three, the milk was pooled into three fractions to determine fore, mid, and hind concentrations. In cases where a mother's full expression consisted of only two tubes, she was excluded from the within feed study.

For the diurnal variation, milk samples were collected at AM, midday, and PM from seven mothers. The AM and PM samples were expressed 12 ± 1 hr apart, and the midday sample was expressed 6 ± 1 hr after the AM expressions. The stages of lactation ranged from 9 to 16 weeks. Seven mothers were in the diurnal study.

The longitudinal variation study included nine mothers with samples expressed at 6 to 8, 10 to 12, and 14 to 16 weeks postpartum. Three of these mothers were also in the diurnal study. Each mother's collection represented three full expressions in a 12-hr period. For the within feed study (fore, mid, and hind milk), 12 of the 16 subjects in the diurnal and longitudinal studies were used. Four of the sixteen subjects had expression volumes that we felt were too low to be included in this portion of our work.

Full expressions from an additional seven subjects were collected. Milk from four of these subjects was collected on site in the morning and analyzed before freezing and later after freezing for 2 weeks. The milk from the final three subjects represented a single complete expression or pooled collections.

Reagents

All trans beta carotene, alpha carotene, lycopene, and lutein were obtained from Sigma Chemicals (St. Louis, MO, USA). Zeaxanthin and beta cryptoxanthin were obtained from Indofine Chemicals (Sommerville, NJ USA). Acetonitrile, methanol, hexane, and tetrahydrofuran (THF), stabilized with butylated hydroxy toluene (BHT) were Omnisolv grade high purity and obtained from E.M. Science (Gibbstown, NJ USA).

Sample and standard analyses

Reference solutions of beta carotene, alpha carotene, lycopene, and beta cryptoxanthin were analyzed by UV spectroscopy at 450 nm to determine concentration. Purity of references was checked by HPLC to ensure there was only one peak, because analyses by UV spectroscopy give total absorbance at a given wavelength. Analytical data was corrected for purity measurements. The HPLC carotenoid reference solution contained lutein, beta cryptoxanthin, lycopene, alpha carotene and beta carotene at concentrations 22.6, 14.3, 22.7, 9.3, and 35.1 nM respectively.

The entire analysis was performed under yellow lights. Additionally, low actinic glassware or aluminum foil was used to protect the carotenoids from light. All glassware had screw caps or stoppers to minimize contact with the air. The procedure developed for carotenoid analyses used an ethanolic saponification at 45°C for 2 hr (unpublished data). Saponification at 45°C has been shown to cause a 23% loss of lycopene and losses of alpha carotene as well.[7] The present study was designed to analyze all of the carotenoids simultaneously while attempting to minimize any potential losses from sample preparation.

Frozen milk was thawed overnight in the refrigerator at 5°C. The following morning, the samples were allowed to warm to room temperature and stirred with a mechanical stirrer until mixed (about 10 min). Saponification was performed by adding 1.5 mL of 50% (wt/vol) potassium hydroxide and 2.5 mL absolute ethanol to 2 mL of milk in a corex centrifuge tube, blanketing the contents with nitrogen and placing in an oscillating water bath at 45°C for 2 hr. The carotenoids were extracted from the saponified matrix using 3 mL hexane with 0.025% (wt/vol) BHT added, three times. The combined hexane extracts were then evaporated under nitro-

gen. The resulting residue was dissolved in the HPLC mobile-phase solvent, diluted to 5 mL, and analyzed by RP-HPLC.

HPLC

A Waters Nova-Pak C18 column, 3.9 mm × 300 mm, 4 μm, was used for the chromatographic separation. No guard column was used because previous experience had shown that loss of carotenoids can occur on the guard column. The isocratic mobile phase solvent was Acetonitrile/Methanol/THF (50:45:5 by vol). RP-HPLC was performed using a Waters Baseline HPLC system. Waters 600E system controller and 712 WISP were controlled by a Waters Baseline Data System. A Laboratory Data Control (LDC) Analytical SM4000 UV detector equipped with a second order filter to remove UV interferences was used for detection. A wavelength of 450 nm was used to monitor and quantitate the carotenoids using peak areas. The injection size was 200 μL and the pump speed was 2.5 mL/min. Data acquisition was completed in 20 min.

Reproducibility of injection, linearity, and recovery of beta carotene

A reference standard solution was serially injected six times to determine reproducibility of injection areas. The relative standard deviation was 1.56%.

A three-point calibration curve was generated using the concentration ranges typically found in the mature human milk matrix. Beta carotene was the only carotenoid calibrated at all three concentrations because of very limited quantities of alpha carotene, lutein, lycopene, beta cryptoxanthin, and zeaxanthin. The beta carotene concentrations used were 9.3 nM, 37.2 nM, and 372 nM. The beta carotene response was linear throughout the calibration range. It was assumed that the other carotenoids would be linear within the same ranges. The external standard method was used for quantitation.

A pooled human milk sample was used to validate the methodology. This pooled sample was analyzed in triplicate on two successive days to determine reproducibility. On the third and fourth days, triplicate aliquots of the pooled sample were spiked with a commercial infant formula containing a known amount of beta carotene, at one and two times the innate concentration of beta carotene in the pooled human milk sample to determine recovery. The overall mean recovery of beta carotene in mature human milk was 97.5%. It was assumed that the other carotenoids would have a similar recovery.

Statistical analysis

The maximum likelihood Box-Cox transformation, which maximizes the normality, the homogeneity of variance, and the goodness of fit of the data, suggests that a single carotenoid should be analyzed separately by square roots, whereas total carotenoids should be analyzed simultaneously by logarithms. Because of the gain in power and interpretability of doing simultaneous analyses, most analyses were performed on natural log nM concentration. In this scale, means are known as geometric means and standard deviations are approximate coefficients of variation. Differences in mean carotenoid concentrations with respect to diurnal and longitudinal variations were analyzed together using SAS PROC MIXED by a heterogeneous pure error variance random effects model. All of the useful *P*-values are the results of CONTRAST statements. Differences in mean carotenoid concentrations with respect to fore, mid and hind milk were also analyzed using SAS PROC MIXED by a heterogeneous pure error variance random effects model. Here the useful *P*-values are contained in the tests of fixed effects. Within and between person variability was estimated by Restricted Maximum Likelihood (REML) variance component procedure. The variance of the diurnal and longitudinal samples were not significantly different ($P > 0.05$) for each carotenoid, so the within and among day variances were pooled in the within mother variance component. Because the pure assay error variances were estimated in a separate set of analyses, they were not removed from the within mother variance components.

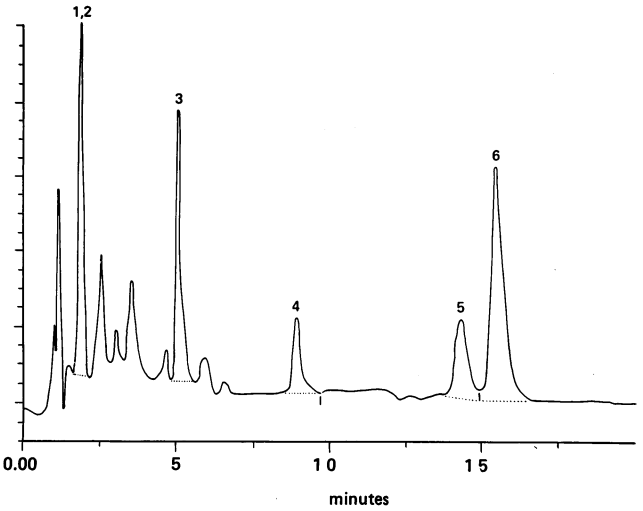

Figure 1 HPLC chromatogram of mature human milk. RP HPLC chromatogram of human milk analyzed as described in Method and materials. 1,2 = lutein/zeaxanthin, 3 = beta cryptoxanthin, 4 = lycopene, 5 = alpha carotene, and 6 = beta carotene.

Results and discussion

This is the only published report examining total major carotenoid concentrations in human milk diurnally and longitudinally, the effects of freezing on carotenoids in fresh milk and concentrations of fore, mid, and hind human milk using modern technology. Reversed-phase HPLC was used to analyze the carotenoids. It allowed the simultaneous quantitation of lutein/zeaxanthin, beta cryptoxanthin, lycopene, alpha carotene, and beta carotene. Because of the short retention time and structural similarity, lutein and zeaxanthin coeluted in this system. A chromatogram of a mature human milk sample is presented in *Figure 1*. The major carotenoid peaks (labeled 3, 4, 5, 6) are well separated with the exception of lutein and zeaxanthin (peaks 1 and 2). Mean lutein/zeaxanthin, lycopene and beta carotene accounted for the greatest concentrations, although the mother in *Figure 1* had a beta cryptoxanthin level much higher than the mean.

Because the objective of this work was the quantitation of the major carotenoids, the amount of *cis* isomers in the samples was not quantitated. It can be assumed that *cis* isomers of lycopene and beta carotene were present in human milk in very minute quantities. Our sample preparation procedure would not be expected to have generated significant quantities of *cis* carotenoids. Examination of the chromatograms showed some *cis* carotenoids, but they did not coelute with the major carotenoids.

The method developed for the analysis of carotenoids used a saponification time of two hours at 45°C. In a

Table 1 Effect of freezing and thawing on human milk carotenoid concentration

Carotenoids	Day 1 Mean (nM) before freezing	Day 14 Mean (nM) after freezing
Lutein/zeaxanthin	91	91
Standard deviation	19	22
Beta cryptoxanthin	26	18
Standard deviation	13	11
Lycopene	65	70
Standard deviation	47	75
Alpha carotene	11	11
Standard deviation	9	7
Beta carotene	49	48
Standard deviation	25	25
Total carotenoids	270	269
Standard deviation	75	108

Human milk carotenoid analyses were performed in duplicate immediately after expression. The remaining milk was frozen at −70°C and reanalyzed in fourteen days.
$n = 4$.

methods development study (unpublished data) aliquots of pooled milk were saponified for 1, 2, and 24 hr. Alpha carotene and beta carotene analyses were slightly higher at 2 hr than at 1 hr. Lycopene degradation was very evident at 45°C and 24 hr. Approximately 15% degradation of the lycopene peak was noted at 2 hr, but 2 hr of saponification was optimal for analyzing all carotenoids simultaneously.

Sample preparation and HPLC analyses were completed within the same day. Extracted milk samples dissolved in mobile phase showed lycopene, alpha, and beta carotene peak degradation with a 15% reduction in peak areas after 12 hr, at ambient temperature (unpublished data). Therefore, samples were extracted and analyzed in groups of six or eight eliminating prolonged exposure to the mobile phase solvent while awaiting HPLC analysis.

All samples were stored at −70°C (except those analyzed the same day of collection) until analyzed and all analyses were completed within 1 year of collections. There was some concern about the stability of carotenoids after freezing, so full breast expressions were collected on site from four mothers whose infants' ages ranged from 7 to 14 weeks. Duplicate aliquots of each mother's milk were extracted and analyzed immediately for carotenoids. An additional aliquot of milk was frozen at −70°C and the analyses were repeated in 14 days. This allowed the determination of major carotenoid concentrations in fresh milk, assay reproducibility, and the effect of freezing and thawing on analytical results. Total major carotenoid concentrations ranged from 196 nM to 373 nM for fresh milk and 176 nM to 424 nM after freezing for 14 days. Beta cryptoxanthin analyses were lower after freezing for all mothers, the decrease ranging from 17% to 43%. Therefore, it is possible that loss of beta cryptoxanthin could occur during freezing. The other major carotenoids did not show a consistent pattern of losses after freezing. Mean carotenoid concentrations before and after freezing are illustrated in *Table 1*. One subject's lycopene results after freezing were 47% higher than the initial analyses. Although this observation was unexpected, it could not be discarded on a statistical basis.

Within feed variations of fore, mid, and hind milk carotenoid concentrations for 12 mothers were studied. Fore, mid, and hind milk total carotenoid concentrations ranged from 100 to 378 nM for fore milk, 120 to 357 nM for mid milk, and 193 to 642 nM for hind milk. There were significant differences in the mean carotenoid concentrations of fore, mid and hind milk ($P < 0.05$). Hind milk had 25% greater total carotenoid concentrations than mid or fore milk, while mid, and fore milk were not significantly different from one another. The percent changes of carotenoid concentrations in fore, mid, and hind milk, using a 95% confidence interval, are shown in *Figure 2*. The five carotenoids behaved parallelly ($P = 0.42$) on a natural logarithmic scale with respect to fore, mid and hind milk concentrations. Increasing carotenoid concentrations during the course of a feeding could be a reflection of increasing total lipid concentrations, which have been shown to increase as much as threefold during the course of a feeding.[13] Fore, mid and hind carotenoid concentrations in mature human milk have not been reported previously.

Diurnal variations in major carotenoids were determined for seven mothers whose infants ranged in ages from 9 to 16 weeks. *Figure 3* shows diurnal variations for three of the mothers for the sake of brevity, representing mothers with low, mid, and high total carotenoid concentrations. The total carotenoid concentrations ranges were 33 to 493 nM in the

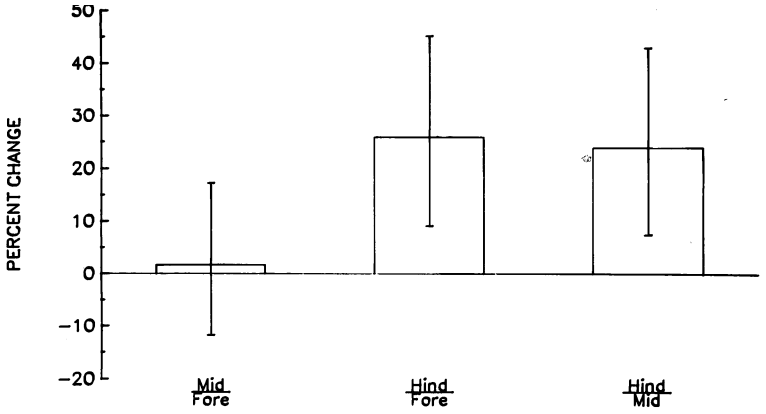

Figure 2 Comparison of fore, mid, and hind milk concentrations. This figure demonstrates a significant difference when comparing hind milk with fore and mid milk concentrations ($P < 0.05$). The percent change between fore and hind milk ranged from 9% to 45%, with a mean of 26%; 95% confidence interval; $n = 12$.

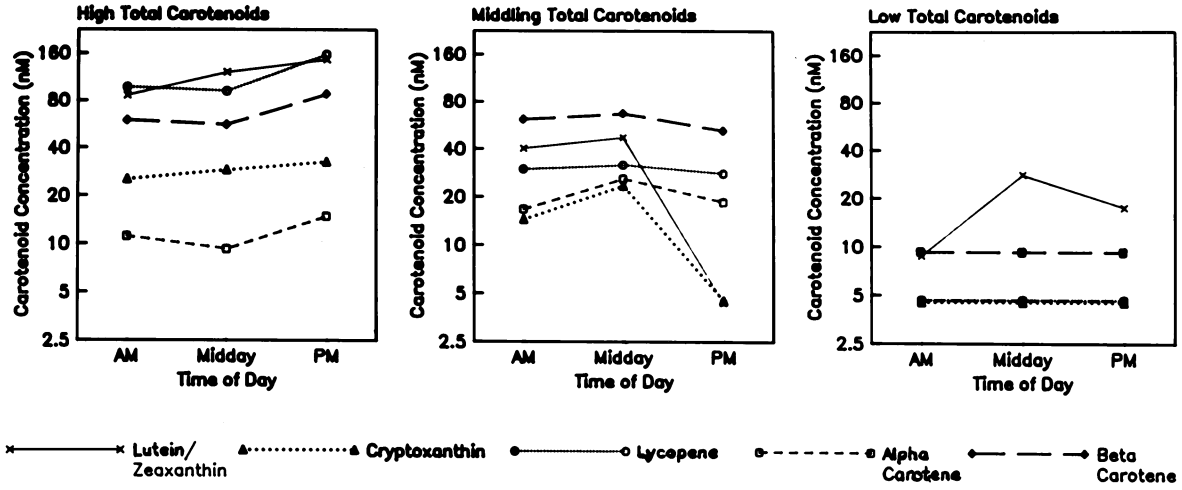

Figure 3 Diurnal variations in carotenoids by tertiles. The overall trend was higher carotenoid levels at midday, although individual carotenoids did not demonstrate the same trend in all mothers ($P > 0.05$). x = lutein/zeaxanthin, Δ = beta cryptoxanthin, ○ = lycopene, □ = alpha carotene, and ◇ = beta carotene; $n = 7$, only three are illustrated.

morning, 52 to 419 nM for midday, and 42 to 438 nM in the evening. Although there was a trend toward higher total carotenoid concentrations at midday, there were no significant differences ($P > 0.05$). Diurnal fluctuations of lipids have been known to occur and could possibly account for the rise in total carotenoid concentrations.[14]

Nine mothers were included in the longitudinal study. Mature milk from 6 to 16 weeks was examined. Wide variations existed among the mothers. Concentrations of total carotenoids ranged from 85 to 366 nM at 6 to 8 weeks, 73-420 nM at 10 to 12 weeks, and 62-362 nM at 14 to 16 weeks. Three mothers representative of this study at low, mid, and total carotenoid concentration ranges are illustrated in *Figure 4*. No significant longitudinal differences were found ($P > .05$).

Full breast expressions of mature milk from 23 mothers were examined. *Table 2* depicts geometric means of individual carotenoid concentrations for these mothers. Major carotenoid concentrations found in human milk were in general agreement with the findings by Giuliano et al., although lutein/zeaxanthin concentrations were not reported in this previous publication.[17] The five carotenoids in all the mothers studied were all positively correlated with each other, having a correlation of +0.52. Individual values for the major carotenoids showed substantial variability within the same subject on the same day and at different weeks. Wide variations also existed between the subjects. The between subject variability of beta carotene was the largest and accounted for 79% of the total beta carotene variability. Lutein/zeaxanthin had the least between subject variability. Most of the variability among carotenoid concentrations is attributable to subject-to-subject variability and not to diurnal or longitudinal variations. The within and between subject variability of alpha carotene and beta carotene in this study were similar to findings by Giuliano et al.[17] However, the within and between subject variabilities of

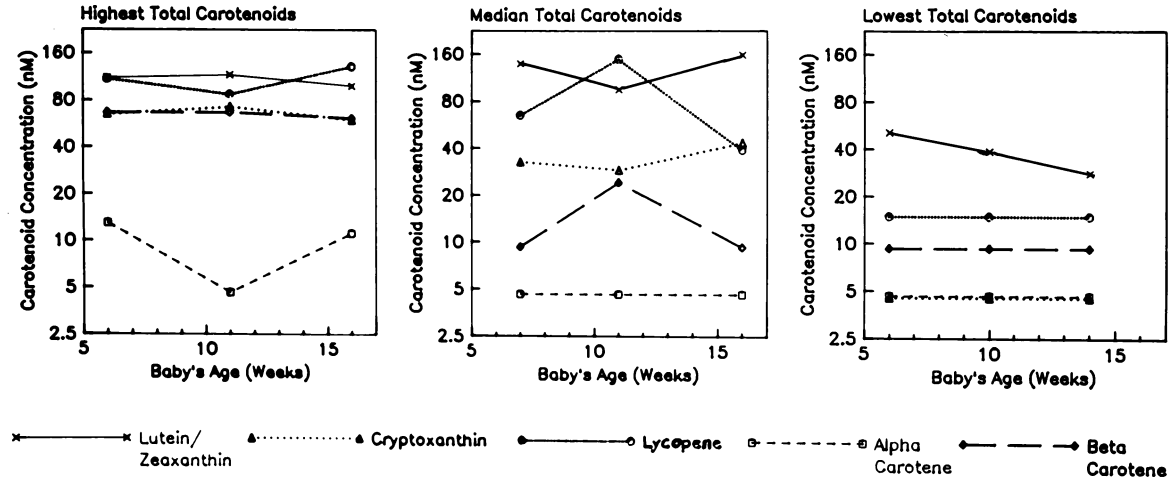

Figure 4 Longitudinal variations in carotenoids by tertiles. Graphic representation of three typical mothers in the longitudinal study depicting six carotenoid concentrations at three postpartum periods. There was a trend of higher carotenoid concentrations at 10 to 12 weeks. x = lutein/zeaxanthin, △ = beta cryptoxanthin, ○ = lycopene, □ = alpha carotene, and ◇ beta carotene; $n = 9$, only three are illustrated.

Table 2 Mean carotenoid concentrations for 23 mothers

	Lutein-zeaxanthin	Cryptoxanthin	Lycopene	Alpha carotene	Beta carotene	Total carotenoids
Geometric mean (nM)	68	24	46	8	37	**207**
% CV	63	80	109	91	96	62

$n = 23$.
Mean carotenoid concentrations of 23 mothers. Wide variations in concentrations existed between mothers resulting in high % coefficient of variations.

beta cryptoxanthin and lycopene in our study were greater than the values found by Giuliano et al. A weighted average of all five carotenoids demonstrated that 62% of the variability is attributable to between mother differences.

Carotenoids are important dietary components, being associated with improved immune response, cancer protection and antioxidant properties.[3] These compounds are provitamins for retinol with beta carotene having the highest vitamin A activity.[8] Beta carotene, alpha carotene, lycopene, beta cryptoxanthin, and lutein/zeaxanthin are the carotenoids typically found in human milk.[6,17] Although breast feeding is the method of choice for infant nutrition, many infants are fed commercial infant formulas. Only a limited number of infant formulas contain added beta carotene.

Conclusion

Our findings demonstrate that total carotenoid concentrations in mature human milk seem to remain fairly constant diurnally and longitudinally. Longitudinal carotenoid concentrations in human milk have not been reported previously. Consequently, this study is significant in that it gives a more complete carotenoid profile of human milk than has been reported previously. Variations in carotenoid concentrations existed within and between the mothers; however, variations between mothers were much wider than within mothers. Guiliano et al. reported similar intra- and inter-variability in carotenoid concentrations in a similarly sized population.[17] Hind milk had significantly higher concentrations of total carotenoids than fore or mid milk, but fore and mid milk did not vary significantly from one another. This finding was not unexpected because of within feed variations known to occur in lipid levels. Carotenoids in breast milk may impart many benefits to the infant. Formula-fed infants may benefit from formula fortified with beta carotene and possibly other carotenoids.

Acknowledgments

The authors thank Dr. Ken Goldberg for statistical analysis of all the data and Judy Seibert for her expert secretarial assistance.

References

1 Ostrea E., Balun J., Winkler R., and Porter T. (1986). Influence of breast feeding on the restoration of the low serum concentration of vitamin E and beta carotene in the newborn infant. *Am. J. Obstet. Gynecol.* **154,** 1014–1017
2 Goldman A., Goldblum R., and Hanson L. (1990). Anti inflammatory systems in human milk. *Adv. Exp. Med. Biol.* **262,** 69–76
3 Bendich A. (1988). A role for carotenoids in immune function. *Clin. Nutr.* **7,** 113–117
4 Burton G.W. (1989). Antioxidant actions of carotenoids (Review). *J. Nutr.* **119,** 109–111
5 Olson J. (1989). Provitamin A function of carotenoids: the conversion of beta-carotene into vitamin *Am. J. Nutr.* 105–108
6 Patton S., Canfield L., Huston G., Ferris A., and Jensen R. (1990). Carotenoids of human colostrum. *Lipids.* **25,** 159–165
7 Giuliano A., Neilson E., Kelly B., and Canfield L. (1992). Simultaneous quantitation and separation of carotenoids and retinol in human milk by high performance liquid chromatography. *Methods Enzymol.* **213,** 391–399
8 Bauernfeind J. (1972). Carotenoid vitamin A precursors and analogs in foods and feeds. *J. Agricul. Food Chem.* **20,** 456–473
9 Merck Index. (1989). *An encyclopedia of chemicals, drugs and biologicals.* Merck and Company, Rahway, NJ USA
10 Johnson L., Norkus E., Abbasi S., Gerdes J.S., and Buhtani V.K. (1994). Contribution of beta carotene (β-C) from β-C enriched formulae to individual and total serum carotenoids in term infants. *Pediatric Res.* **35,** (abstr. No. 1869).
11 Rankin J., Green N., Tremper W., Stacewitcz-Sapuntzakis M., Bowen P., and Ndiaye M. (1993). Undernutrition and vitamin A deficiency in the Department of Linguere, Louza Region of Senegal. *Am. J. Clin. Nutr.* **58,** 91–97
12 Kim Y., English C., Reich P., Gerber L., and Simpson K. (1990). Vitamin A and carotenoids in human milk. *J. Agric. Food Chem.* **38,** 1930–1933
13 Hall B. (1979). Uniformity of human milk. *Am. J. Clin. Nutr.* **32,** 304–312
14 Neville M., Keller R., Seacat J., Casey C., Allen J., and Archer P. (1984). Studies on human lactation. I. Within-feed and between breast variation in selected components of human milk. *Am. J. Clin. Nutr.* **40,** 635–646
15 Freed L., Neville M., Hamosh P., and Hamosh M. (1986) Diurnal and within-feed variations of lipase activity and triglyceride content in human milk. *J. Pedia. Gastro. Nutr.* **5,** 938–942
16 Lammi-Keefe C., Ferris A., and Jensen R. (1990). Changes in human milk at 0600, 1000, 1400, 1800, and 2000 hours. *J. Pedia. Gastro. Nutr.* **11,** 83–88
17 Giuliano A., Neilson E., Yap Hui-Han, Baier M., and Canfield L. (1994). Quantitation of and inter/intra individual variability of major carotenoids of mature human milk. *J. Nutr. Biochem.* **5,** 551–556