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Assessment of phytochemical content in human milk during different stages of lactation

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ABSTRACT

Objective: The present study reports the presence of several carotenoids and flavonoids in human milk samples.
Methods: Samples were collected from 17 women who delivered healthy term babies (≥37 wk of gestation) at 1-, 4-, and 13-wk postpartum intervals.
Results: Epicatechin (63.7–828.5 nmol/L), epicatechin gallate (55.7–645.6 nmol/L), epigallocatechin gallate (215.1–2364.7 nmol/L), naringenin (64.1–722.0 nmol/L), kaempferol (7.8–71.4 nmol/L), hesperetin (74.8–1603.1 nmol/L), and quercetin (32.5–108.6 nmol/L) were present in human milk samples with high inter-/intraindividual variability. With the exception of kaempferol, the mean flavonoid content in human milk was not statistically different among lactation stages. In contrast, carotenoids α-carotene (59.0–23.2 nmol/L), β-carotene (164.3–88.0 nmol/L), α-cryptoxanthin (30.6–13.5 nmol/L), β-cryptoxanthin (57.4–24.8 nmol/L), zeaxanthin (46.3–21.4 nmol/L), lutein (121.2–56.4 nmol/L), and lycopene (119.9–49.5 nmol/L) significantly decreased from weeks 1 to 13 of lactation.
Conclusion: The observed differences in the relative concentrations of the two phytochemical classes in human milk may be a result of several factors, including dietary exposure, stability in the milk matrix, efficiency of absorption/metabolism, and transfer from plasma to human milk. These data support the notion that flavonoids, as with carotenoids, are dietary phytochemicals present in human milk and potentially available to breast-fed infants.

Introduction

Phytochemicals are secondary plant metabolites believed to impart health benefits, including protection against oxidative stress and inflammation, and may decrease the risk of chronic and degenerative diseases such as cancer, obesity, diabetes, and neurodegenerative disorders [1,2]. Two classes of phytochemicals, the flavonoids and carotenoids, have received significant attention in recent years because of their proposed nutritional and health-promoting functions in humans. Carotenoids are a family of hydrophobic pigments abundant in algae and plants. Although the provitamin A activity of β-carotene and β-cryptoxanthin is well documented, non-provitamin A carotenoids including lutein, zeaxanthin, and lycopene have been increasingly studied for their biological activities, including antioxidant activity, cardiovascular protection, eye health, and skin health [3,4]. Flavonoids comprise a class of phytochemicals that broadly includes flavonols, isoflavones, flavan-3-ols, and flavanones. These polyphenols have demonstrated biological activities, including antioxidant and anti-inflammatory activities, consistent with the promotion of vascular health, bone health, and cognitive function [5–7]. With the potential for a nutritional and health-promoting role, interest in the content and variability of these phytochemicals from foods has grown significantly.

For infants, human milk represents the primary and preferred source of nutrition. Previous studies have reported the composition of compounds in human milk that provide benefits beyond basic nutrition through stages of lactation [8]. Furthermore, the carotenoid content of human milk has been the subject of many studies, with the general conclusion that human milk content remains proportional to the mother's diet and correlates well to

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plasma carotenoid levels [9–12]. Although these data support the notion that carotenoids are present in infant diets, little is known about the content of plant-derived flavonoids in human milk. Franke et al. [13–15] characterized isoflavones in mothers consuming soy; however, little information is available on the natural levels of flavonoids in human milk, including catechins and flavonols, commonly found in fruits and vegetables. More recently, Besle et al. [16] identified flavonoids, including quercetin, luteolin, and apigenin, in the milk of cows fed hay, maize, and rye grass silage.

Considering the potential biological activity of plant-derived carotenoids and flavonoids, additional insight into the phytochemical profiles of human milk and their variation during lactation is required to better understand their potential exposure to and function in breast-fed infants. The objective of the present study was to characterize the profiles of epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate, naringenin, kaempferol, hesperetin, quercetin, lutein, zeaxanthin, α/β-cryptoxanthin, α/β-carotene, and lycopene (Fig. 1A, B) in human milk samples collected at different stages of lactation.

Materials and methods

Chemicals and standards

L-Ascorbic acid, Na_2-ethylenediaminetetraacetic acid, pepsin (P7000), NaOH, KOH, β-glucuronidase (G0751), formic acid, 2,6-di-tert-butyl-4-methylphenol, ethyl gallate, and β-apo-8-carotenal were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents including ethyl acetate, methanol, isopropyl alcohol, acetone, petroleum ether, and HCl were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). Flavonoid standards including epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate, naringenin, kaempferol, hesperetin, and quercetin dihydrate standards were purchased from Sigma-Aldrich. For carotenoids, zeaxanthin was purchased from Chromadex (Irvine, CA, USA). The β-cryptoxanthin, β-carotene, lycopene, and lutein standards were obtained from Sigma-Aldrich.

Human milk samples

Human milk samples (2-mL aliquots) were provided by the Cincinnati Children's Hospital Medical Center and is a subset of the Cincinnati cohort described by Woo et al. [17]. Analysis of human milk samples was completed with approval from the Purdue University institutional review board for human subject research. Samples were collected from 17 women (Table 1) who delivered healthy term babies (≥37 wk of gestation) at 1-, 4-, and 13-wk postpartum intervals. The time points and sample size of 17 were based on the availability of samples from individual mothers at each time point. This sample size is similar to another pilot investigation on human milk carotenoid composition [9]. Because this was a preliminary investigation, diets were not controlled or recorded and health records/clinical characteristics were not obtained. All women lived within 25 miles of the Cincinnati Children's Hospital Medical Center. On collection days, nurses visited the women from 10:00 to 13:00 h. The entire content of one breast was emptied using an electric pump. Milk samples were then aliquoted, coded, and frozen and stored at −70°C. The researchers were blinded to the sample identity and interval of each sample until the analysis was completed. All sample transfers and carotenoid extractions were completed under amber lights to minimize photo-oxidative reactions.

Flavonoid extraction

Owing to the absence of information on the flavonoid content of human milk and the limited sample size available for analysis (2.0 mL), the decision was made to deconjugate the potential metabolites (glucuronides and sulfate derivatives) to aglycones using β-glucuronidase/sulfatase treatment in an effort to simplify the separation and quantification of the major flavonoid classes present in human milk. An aliquot of human milk (1 mL) was defatted with two 3-mL aliquots of hexane. After the removal of lipids, 50 μL of L-ascorbic acid 2.7 mmol/L and Na_2-ethylenediaminetetraacetic acid 2.2 mmol/L in water was added followed by 6 mL of pepsin 40 mg/mL in 0.1 N HCl. Samples were incubated in a shaking water bath for 15 min at 37°C with mild agitation. After incubation, the pH of the mixture was adjusted to 4.5 with 1.0 N NaOH, 3.85 kU of β-glucuronidase with sulfatase contaminant was added, and the samples were incubated for an additional 45 min at 37°C in a shaking water bath with mild agitation. After enzymatic deconjugation, flavonoids were extracted three times with a 3-mL aliquot of ethyl acetate. The ethyl acetate layers were collected, combined, and dried under vacuum. The dried extracts were dissolved in 200 μL of mobile phase A before analysis.

Carotenoid extraction

An aliquot (0.75 mL) of human milk sample was saponified with 0.3 mL of 30% methanolic KOH for 15 min at ambient temperature. Carotenoids were then extracted with 3:1 petroleum ether with 0.1% 2,6-di-tert-butyl-4-methylphenol:acetone a total of three times. The ether layers were collected combined and the solvent was removed under vacuum. The dried extracts were resolubilized in 150 μL of 1:1 ethyl acetate and methanol before analysis.

Method validation

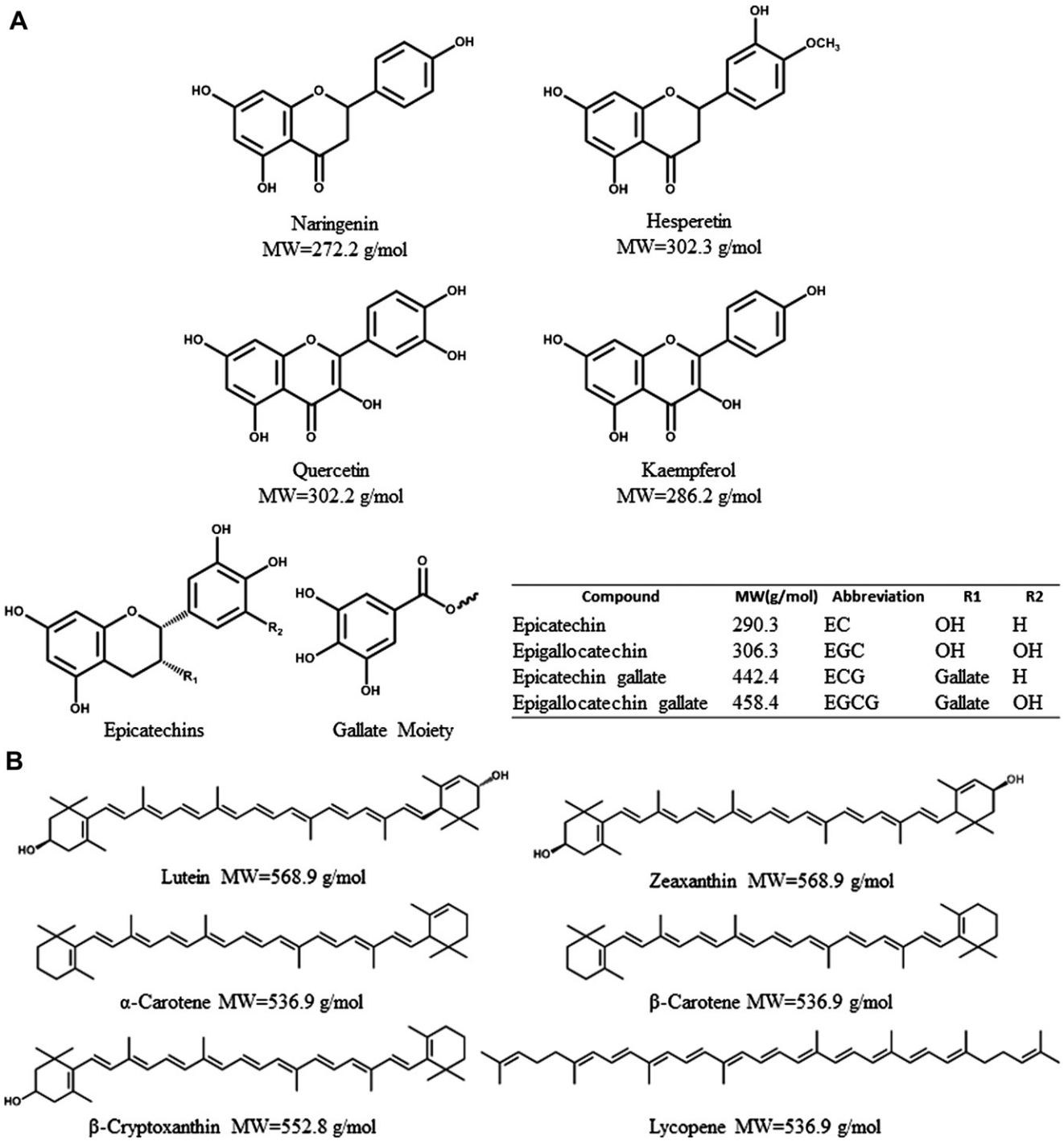
The efficiencies of the flavonoid and carotenoid extractions were evaluated by spiking freshly thawed human milk with ethyl gallate (in H_2O) and β-apo-8-carotenal (in ethanol) for final concentrations of 200 and 120 nmol/L, respectively. Then, spike addition samples were extracted as described previously. The average extraction efficiencies for the triplicate experiments between the flavonoid and carotenoid extractions were 88.2 ± 2.3% and 75.4 ± 2.6%, respectively. In addition, intraday and interday variations in extraction were evaluated for each analyte by a single operator who repeatedly extracted and analyzed triplicate samples of a pooled human milk sample on 3 consecutive days (Table 2). Epigallocatechin gallate and epigallocatechin were not detected in the pooled sample; thus, the coefficients of variation could not be calculated by this method. To approximate the coefficients of variation, green tea extract containing epigallocatechin gallate, epigallocatechin, and other catechins was spiked into a separate aliquot of pooled human milk and extracted by the same operator in triplicate on 3 separate days. The limits of detection (LODs) for individual flavonoids and carotenoids were determined by serial dilutions prepared for each standard from a stock solutions of 1.0 to 10 μmol/L. An LOD was defined as a response three times the peak-to-peak noise level and the results were expressed as the minimum detectable amount in nanomoles per liter of human milk (Table 2).

Flavonoid analysis by liquid chromatography–mass spectrometry

Flavonoid analysis was completed using a Waters 2695 Separations Module (Waters, Milford, MA, USA) equipped with a Waters Xterra Reversed Phase C18 column (3.5 μm, 2.1 × 100 mm). The column and sample temperatures were set to 40°C and 8°C, respectively. An elution gradient was used with a constant 0.30-mL/min flow rate and mobile phases A (0.4% formic acid in water), B (0.4% formic acid, 4% isopropyl alcohol in methanol), and C (methanol). The initial conditions were set at 98:2 (A/B) followed by a linear gradient to 55:42:3 (A/B/C) at 15 min, 20:80 (A/B) at 30 min, and a reset to 98:2 (A/B) at 35 min. After elution, the column effluent was split 1:1 before its introduction by electrospray ionization in negative ion mode into a Waters Micromass ZQ2000 mass spectroscopy detector. The mass spectrometer conditions were set as follows: capillary voltage 3.5 kV, cone voltage 35 V, extractor voltage 3 V, radio frequency lens 0.5 V; the source and desolvation temperatures were set to 150°C and 250°C, respectively, and the desolvation and cone gas flow were set to 250 and 60 L/h of nitrogen, respectively. The mass spectrometer was set to detect two groups of single ion responses (SIRs) at various periods. The two groups of SIRs had dwell times of 0.2 s, interchannel delays of 0.01 s, interscan delays of 0.01 s, and spans of 0.2. The first set of SIRs included 289, 305, 441, and 457 mass/charge signals for 0 to 18 min and targeted epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate, respectively. The second set of SIRs included 271, 285, 301, and 301 mass/charge signals for 16 to 35 min and targeted naringenin, kaempferol, hesperetin, and quercetin, respectively. The quantitation of each flavonoid was accomplished using calibration curves constructed from serial dilutions of authentic flavonoid standard stock solutions. Standard solutions were subsequently injected on to the liquid chromatography–mass spectrometry to produce the calibration curves for each compound at its corresponding mass/charge signals and elution times.

Carotenoid analysis by liquid chromatography–diode array detector

Carotenoid analysis was completed by liquid chromatography–diode array detector using a gradient elution as described by Kean et al. [18]. Separations were achieved on an HP 1090 device (Hewlett Packard, Palo Alto, CA, USA) equipped with a Waters YMC Carotenoid C30 column (2.0 × 150 mm) and a guard column (2.0 × 50 mm). The detection of carotenoids was accomplished using an HP 79880A diode array detector scanning 250 to 600 nm at a 1.2-nm resolution. The quantitation of lutein, zeaxanthin, α/β-cryptoxanthin, and α/β-carotene was accomplished using the response at 450 nm, whereas the lycopene quantitation was based on the response at 470 nm. Major carotenoids, including



Compound	MW(g/mol)	Abbreviation	R1	R2
Epicatechin	290.3	EC	OH	H
Epigallocatechin	306.3	EGC	OH	OH
Epicatechin gallate	442.4	ECG	Gallate	H
Epigallocatechin gallate	458.4	EGCG	Gallate	OH

Fig. 1. Structure of (A) major flavonoid and (B) carotenoid compounds commonly found in fruits and vegetables and detected in human milk. MW, molecular weight.

all-transversions, lutein, zeaxanthin, β-cryptoxanthin, and α/β-carotene and lycopene were identified in human milk based on the comparison of retention times and inline electronic absorption spectra with those of authentic standards. Owing to a lack of an authentic standard for α-cryptoxanthin, this carotenoid was tentatively identified based on the elution order before β-cryptoxanthin and by inline electronic absorption spectra [19,20].

Lipid analysis

The lipid content of human milk was determined using the creamatocrit method as described by Lucas et al. [21]. An aliquot of freshly thawed human milk was drawn into a 100-μL capillary tube, sealed with clay, placed in a Clay Adams Readacrit Centrifuge (Parsippany NJ, USA), and centrifuged at 3000 rpm for 15 min. The ratio of lipid length to column length was used to estimate the lipid percentage of the milk samples as previously described. The conversion of milk lipid percentage to grams of lipid per liter was accomplished using this equation: lipid (g/L) = (creamatocrit [%] − 0.59)/0.146.

Data analysis

The flavonoid and carotenoid contents of human milk are expressed as mean ± standard error of the mean. Only samples in which the compounds were detected at quantifiable levels were used in the calculation of the mean and standard error of the mean. Data were analyzed using StatView 5.0 (SAS Institute,

Table 1
Demographic data for donor mothers and infants ($n = 17$)

Age (y)	31.7 ± 4.2
Weight (kg)	66.7 ± 8.6
Length (cm)	164 ± 5.0
No. of pregnancies	2.25 ± 0.77
Birth weight (g)	3490.4 ± 342
Birth height (cm)	51.0 ± 1.6

Data are presented as mean ± SD.

Cary, NC, USA). Group differences were determined by analysis of variance and the Fisher protected least significant differences post hoc test ($\alpha = 0.05$).

Results

Flavonoid content of human milk

The separation and detection of flavonoid aglycones from a representative sample of human milk are shown in Figure 2. SIR chromatograms showed the presence of epicatechin, epicatechin gallate, epigallocatechin gallate, naringenin, kaempferol, hesperetin, and quercetin as common flavonoids in the human milk samples assayed in this study. The retention times of authentic standards and SIR signals (selected m/z) were used in combination to identify the flavonoids in the samples.

Overall, the presence and concentration of the flavonoids in the human milk samples encompassed a wide range (Table 3). The LODs for naringenin, epicatechin gallate, kaempferol, and quercetin in the milk were determined at 10.2, 6.2, 0.4, and 8.2 nmol/L, respectively. Epicatechin, epigallocatechin gallate, epigallocatechin, and hesperetin had higher LODs at 13.5, 50.3, 31.4, and 22.2 nmol/L, respectively. The difference in LODs likely influenced the number of samples in which individual flavonoids were detected. For instance, quercetin and kaempferol were detected in all 51 samples, whereas epigallocatechin gallate was detected in only eight samples.

The flavonoid profile was compared in milk collected at 1, 4, and 13 wk postpartum. Changes in the concentration of several flavonoids over time, such as epigallocatechin gallate, could not be fully examined because of their detection in only a limited number of samples (Table 3). However, in general, minimal changes in flavonoid content over time were noted in human milk collected at different stages of lactation. Specifically, epicatechin, epicatechin gallate, hesperetin, quercetin, and naringenin contents were not significantly different among weeks 1, 4, and 13 ($P > 0.05$). However, the kaempferol concentration was found to increase significantly with the week of lactation (Table 3). Specifically, the kaempferol concentration significantly increased from weeks 1 to 13 ($P < 0.001$) and from weeks 4 to 13 ($P < 0.005$). An increase was also noted from weeks 1 to 4, but this increase did not quite reach statistical significance ($P = 0.06$).

Table 2
Coefficients of variation and limits of detection for flavonoid and carotenoid extractions

	Intraday CV (%)	Interday CV (%)	LOD (nmol/L human milk)
Epicatechin	4.3 ± 0.2	12.3 ± 1.0	13.5
Epicatechin gallate	13.6 ± 4.5	16.6 ± 4.7	6.2
Epigallocatechin*	4.7 ± 0.6	4.0 ± 1.3	31.4
Epigallocatechin gallate*	1.2 ± 0.7	9.1 ± 2.8	50.3
Naringenin	5.4 ± 4.9	7.1 ± 3.7	10.2
Kaempferol	9.5 ± 1.6	9.3 ± 5.0	0.4
Hesperetin	7.4 ± 1.7	9.3 ± 3.1	22.2
Quercetin	10.9 ± 3.0	13.7 ± 1.8	8.2
Lutein	6.5 ± 3.7	15.9 ± 3.3	0.3
Zeaxanthin	0.3 ± 0.2	15.1 ± 2.8	0.3
α-Cryptoxanthin	5.4 ± 2.8	12.1 ± 2.4	0.4
β-Cryptoxanthin	10.0 ± 8.3	13.1 ± 6.9	0.4
α-Carotene	18.8 ± 4.5	16.2 ± 3.8	0.3
β-Carotene	1.6 ± 0.8	2.3 ± 0.4	0.4
Lycopene	1.1 ± 0.9	3.4 ± 2.5	0.3

CV, coefficient of variation; LOD, limit of detection

* Epigallocatechin and epigallocatechin gallate were not detected in the pooled milk sample used for the calculation of the coefficients of variation. Thus, the intraday and interday coefficients of variation were estimated based on the extraction of the pooled human milk sample spiked with the green tea extract.

Carotenoid content of human milk

A representative chromatogram depicting the major carotenoids present in human milk is shown in Figure 3. The β-carotene and lutein were the most abundant carotenoids in these human milk samples (Table 4). A sharp decrease in carotenoid content was noted as a function of lactation stage (Table 4). The β-carotene levels in human milk decreased significantly ($P < 0.05$) from 164.3 ± 25.2 to 88.0 ± 23.3 nmol/L from weeks 1 to 13, respectively. Similar decreases in carotenoid content were noted from weeks 1 to 4 for α-carotene (59.0 ± 13.5 to 19.2 ± 3.0 nmol/L, $P < 0.005$), α-cryptoxanthin (30.6 ± 4.8 to 16.8 ± 3.8 nmol/L, $P < 0.005$), β-cryptoxanthin (57.4 ± 10.7 to 27.5 ± 4.8 nmol/L, $P < 0.005$), lycopene (119.9 ± 18.9 to 68.0 ± 16.3 nmol/L, $P < 0.05$), lutein (121.2 ± 20.9 to 61.9 ± 10.9 nmol/L, $P < 0.05$), and zeaxanthin (46.3 ± 5.4 to 22.8 ± 2.7 nmol/L, $P < 0.0001$; Table 4). Additional decreases in carotenoid content were observed from weeks 4 to 13, but these were not found to be statistically significant.

The carotenoid content of human milk also was expressed by its lipid content (Table 5). The lipid content in milk ranged from 22.20 to 89.93 g/L (Fig. 4). As before, β-carotene and lutein were highest in human milk and ranged from 0.5 to 9.7 and from 0.9 to 6.8 nmol/g of lipid in week 1 of lactation, respectively. The ranges of zeaxanthin, α-cryptoxanthin, β-cryptoxanthin, α-carotene and lycopene were found to be 0.5 to 2.1, 0.2 to 2.1, 0.4 to 5.8, 0.3 to 7.2, and 0.9 to 10.4 nmol/g of lipid, respectively, in week 1 of lactation. When normalized for lipid content, β-carotene and lutein had the highest concentration throughout all weeks of lactation and showed a significant decrease from weeks 1 to 13 ($P < 0.05$). Similarly, significant decreases were noted in α-carotene (1.6 ± 0.5 to 0.4 ± 0.0 nmol/g of lipid, $P < 0.01$), α-cryptoxanthin (0.7 ± 0.1 to 0.3 ± 0.1 nmol/g of lipid, $P < 0.01$), β-cryptoxanthin (1.4 ± 0.3 to 0.5 ± 0.1 nmol/g of lipid, $P < 0.01$), lycopene (3.0 ± 0.6 to 1.2 ± 0.2 nmol/g of lipid, $P < 0.01$), and zeaxanthin (1.1 ± 0.1 to 0.4 ± 0.0 nmol/g of lipid, $P < 0.001$) from weeks 1 to 4. No significant difference was observed in carotenoid content from weeks 4 to 13 when expressed as milk lipid content (Table 5).

Discussion

Flavonoids in human milk

Although previously reported in bovine milk [16], to our knowledge, this is the first study to identify and quantify flavan-3-ols, flavonols, and flavanones in human milk. The samples included in this study were obtained from 17 free-living mothers at 1, 4, and 13 wk postpartum. The selection of these time points and samples was based on the availability of matched samples for each mother at each time point of early, mid, and later stage

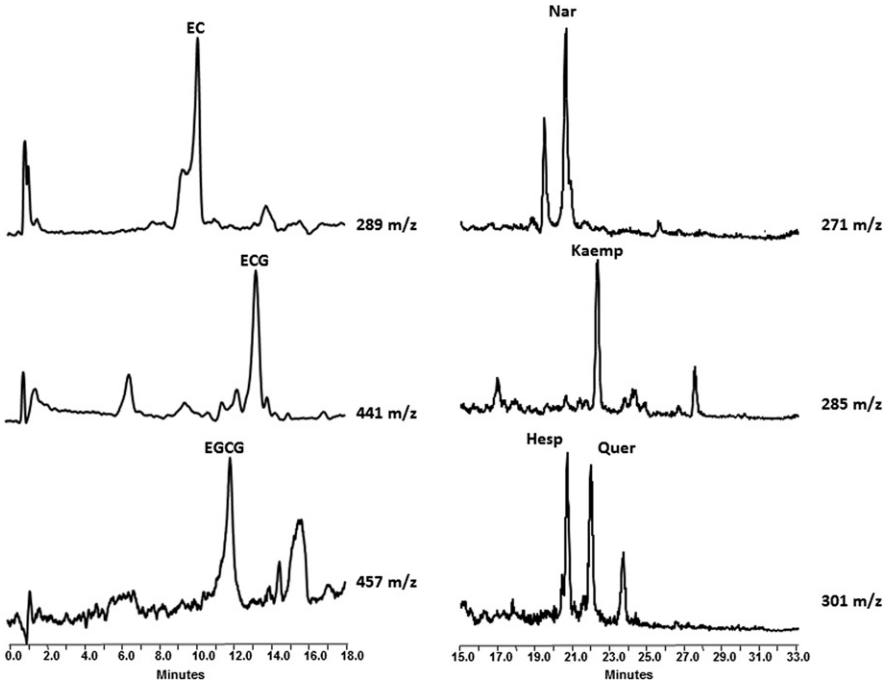


Fig. 2. Representative liquid chromatographic–mass spectrometric selected ion response chromatograms representing the profile of select flavonoids detected in human milk. Selected ion response chromatograms correspond to catechins including epicatechin, epicatechin gallate, and epigallocatechin gallate ($[M-H]^- = m/z$ 289, 441, and 457, respectively) and flavonols and flavanones including naringenin, kaempferol, hesperetin, and quercetin ($[M-H]^- = m/z$ 271, 285, 301, and 301, respectively). EC, epicatechin; ECG, epicatechin gallate; EGCG, epigallocatechin gallate; Hesp, hesperetin; Kaemp, kaempferol; Nar, naringenin; Quer, quercetin.

of lactation. Although dietary records were not collected from these women, these data suggest that dietary flavonoids are transferred from the blood into human milk at significant levels (nanomoles per liter). Previous studies measuring the flavonoid content of human milk have been limited to assessing one class of flavonoids, isoflavones, and specifically following milk levels in response to dietary interventions with soy [15]. In these studies, Franke et al. [13,15] reported levels of daidzein and genistein in human milk at 80 to 110 and 30 to 50 nmol/L, respectively, after the consumption of soy-rich diets. These levels are similar to those found for flavan-3-ols, flavonols, and flavanones in the present study (Table 3), suggesting that these flavonoids are likely absorbed and transferred to human milk rapidly from the mother's diet and present in the diets of nursed infants. Furthermore, the generally consistent levels of flavonoids during the three periods assayed in the present study suggest that the accumulation of these compounds in human milk is not affected by the lactation stage. Considering the transient nature of flavonoids in biological fluids and tissues [22], the human milk profiles may be more reflective of daily dietary exposure and not an accumulation of flavonoids in these fluids.

The qualitative flavonoid profile of human milk was also of particular interest. The eight flavonoids assessed in these samples were selected after a preliminary assessment of pooled milk samples. In addition, catechin, gallocatechin, catechin gallate, gallocatechin gallate, peonidin glucosides, malvidin glucosides, petunidin glucosides, cyanidin glucosides, and delphinidin glucosides were included in the preliminary assessment; however, they were not detected and thus were excluded from the present study. Flavan-3-ols (epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate), flavonols (quercetin and kaempferol), and flavanones (naringenin and hesperetin) were selected based on their predominance in these human milk samples and general prevalence in commonly

Table 3
Flavonoid concentration (nanomoles per liter) in human milk samples collected from free-living women at weeks 1, 4, and 13 postpartum*

Flavonoid	Week 1				Week 4				Week 13			
	n†	Range‡	Mean‡	SEM‡	n	Range	Mean	SEM	n	Range	Mean	SEM
Epicatechin	8	68.3–120.5	90.5[a]	7.1	5	63.7–828.5	249.2[a]	145.3	8	73.0–136.0	95.5[a]	7.9
Epigallocatechin	–	–	–	–	–	–	–	–	–	–	–	–
Epicatechin gallate	17	55.7–609.3	189.5[a]	36.7	11	62.2–645.6	236.6[a]	60.1	14	56.5–492.7	230.6[a]	39.7
Epigallocatechin gallate	3	425.5–2364.7	1118.8[a]	624.3	–	–	–	–	5	215.1–1683.8	667.2[a]	267.3
Naringenin	17	82.9–542.6	252.1[a]	29.6	17	98.1–722.0	210.4[a]	39.3	15	64.1–447.0	196.6[a]	26.8
Kaempferol	17	7.8–34.0	15.7[a]	1.7	17	8.9–53.6	23.1[a]	2.9	17	17.7–71.4	34.8[b]	3.30
Hesperetin	12	107.1–1272.8	459.2[a]	123.6	13	79.9–1603.1	393.6[a]	119.5	11	74.8–704.7	352.0[a]	72.3
Quercetin	17	40.0–77.6	48.1[a]	2.2	17	33.1–108.6	59.8[a]	5.8	17	32.5–95.9	50.9[a]	3.4

Different superscript letters indicate a significant difference in flavonoid content in human milk at different lactation stages ($P < 0.05$).

* See Table 1 for demographic data.

† Number of samples in which the specific flavonoid was positively detected; $n = 17$ would indicate that the specific flavonoid was detected in all milk samples.

‡ Range, mean, and SEM were calculated based on the number of samples in which the compound was detected.

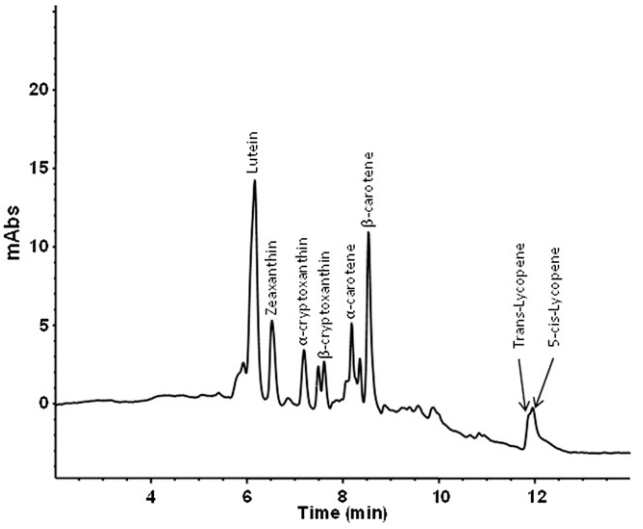


Fig. 3. Representative liquid chromatographic–diode array detector chromatograms representing the profile of select carotenoids detected in human milk. The signal at 450 nm is depicted. mAbs, milli-absorbance.

consumed fruits, vegetables, and beverages [23,24]. Of these polyphenols, naringenin and hesperetin were found at the highest levels compared with flavan-3-ols and flavonols. Flavanones are particularly abundant in citrus fruits and juices, such as oranges and grapefruits [25]. In contrast, epigallocatechin gallate was found at high levels but only in eight individuals. Nursing mothers typically are advised to avoid flavan-3-ol–containing foods/beverages such as tea because of the caffeine content. Considering that tea consumption may be restricted but not completely avoided, the prevalence of decaffeinated tea products, and the high content of epigallocatechin gallate in tea [26], it is not surprising that a select group of individual samples exhibited appreciable levels of epigallocatechin gallate.

It is unclear if the flavonoid profile found in the present study is due to a specific dietary exposure to specific flavonoids such as naringenin and hesperetin or to other factors including the time of collection, the kinetics of deposition, and the metabolism and stability of specific polyphenols in the milk matrix. In fact, the general instability of many flavonoids to near neutral pH conditions is well documented [27]. In addition, the relative difference in antioxidant activity of the flavonoids may influence their presence in human milk. For instance, catechins and flavonols have similar antioxidant activity, whereas flavanones have less antioxidant activity [28]. Thus, catechins and flavonols in human milk more likely are susceptible to oxidative reactions, whereas flavanones may remain intact for longer periods. Therefore, naringenin and hesperetin may be present in high levels in most mothers because of their higher oxidative stability compared with other flavonoids, a possibility that merits further investigation.

In addition, the oxidation of flavonoids in milk-based systems may be further affected by an association with specific macronutrients such as protein. Previous studies have suggested that proteins in milk can interact with catechins by proline-rich peptide segments [29] and that these interactions alter the oxidative stability of specific flavonoids [30]. It is plausible that flavonoids or their metabolites present in human milk associate with proline-rich segments of milk proteins, increasing their stability to oxidative conditions in the milk. The extent to which the differences in these associations and stability alter the flavonoid profile of human milk and, by extension, availability to the infant merits further study.

Furthermore, flavonoids are typically present in biological tissues and fluids as various metabolites, not as their native aglycones or glycosides. Although the chemical nature of the metabolites is specific to each flavonoid, typically flavonoid metabolites are found in plasma and tissues as glucuronides, methyl glucuronides, sulfates, and methyl sulfates [31,32]. Because flavonoid conjugation to these metabolites may occur in the small intestines and the liver, it is likely that the flavonoid metabolites are available to be transferred into the milk before hepatic metabolism. Although further research is needed to determine the presence and relative concentration of specific flavonoid metabolites in human milk, the consistent presence of specific flavonoids in human milk samples collected over a 13-wk period from free-living mothers suggests that these compounds are being effectively transferred into human milk and made available for absorption by a nursing infant.

Carotenoid content of human milk

Although the lipid and carotenoid content in human milk has been reported previously, few studies have focused on their concentration from 1 to 13 wk postpartum. In general, the lipid content and variability were similar to those previously shown for human milk at later stages of lactation [33]. In addition, the fluctuation of milk lipid content 1 to 13 wk postpartum was similar to that observed by Michaelsen et al. [34]. Schweigert et al. [11] and Jewell et al. [9] examined milk carotenoid concentrations 1 to 41 d postpartum. Studies by Canfield et al. [35] and Lietz et al. [36] examined a wider range of time than

Table 4
Carotenoid concentration (nanomoles per liter) in human milk samples collected from free-living women at weeks 1, 4, and 13 postpartum*

Carotenoid	Week 1				Week 4				Week 13			
	n†	Range‡	Mean‡	SEM‡	n	Range	Mean	SEM	n	Range	Mean	SEM
Lutein	17	58.1–412.9	121.2[a]	20.9	15	16.8–193.4	61.9[b]	10.9	15	19.7–116.1	56.4[b]	6.8
Zeaxanthin	17	19.4–115.4	46.3[a]	5.4	15	11.9–52.6	22.8[b]	2.7	15	9.3–44.4	21.4[b]	2.5
α-Cryptoxanthin	16	11.9–72.6	30.6[a]	4.8	15	2.2–50.6	16.8[b]	3.8	16	2.3–33.1	13.5[b]	2.0
β-Cryptoxanthin	16	9.4–175.4	57.4[a]	10.7	17	2.1–70.7	27.5[b]	4.8	17	6.8–77.6	24.8[b]	4.4
α-Carotene	16	12.0–220.6	59.0[a]	13.5	14	7.3–46.5	19.2[b]	3.0	15	3.7–80.2	23.2[b]	4.8
β-Carotene	17	17.3–327.8	164.3[a]	25.2	16	10.7–377.4	104.4[a,b]	27.7	17	8.5–352.6	88.0[b]	23.3
Lycopene	17	30.5–317.5	119.9[a]	18.9	16	9.0–256.6	68.0[b]	16.3	17	19.2–103.0	49.5[b]	6.4

Different superscript letters indicate a significant difference in flavonoid content in human milk of different lactation stages ($P < 0.05$).

* See Table 1 for demographic data.

† Number of samples in which the specific carotenoid was positively detected; $n = 17$ would indicate that the specific flavonoid was detected in all milk samples.

‡ Range, mean, and SEM were calculated based on the number of samples in which the compound was detected.

Table 5
Carotenoid concentration (nanomoles per gram of lipid) in human milk samples collected from free-living women at weeks 1, 4, and 13 postpartum*

Carotenoid	Week 1				Week 4				Week 13			
	n†	Range‡	Mean‡	SEM‡	n	Range	Mean	SEM	n	Range	Mean	SEM
Lutein	17	0.9–6.8	2.9[a]	0.4	15	0.5–2.3	1.1[b]	0.1	15	0.5–3.6	1.4[b]	0.2
Zeaxanthin	17	0.5–2.1	1.1[a]	0.1	15	0.3–0.6	0.4[b]	0.0	15	0.2–1.7	0.6[b]	0.1
α-Cryptoxanthin	16	0.2–2.1	0.7[a]	0.1	15	0.1–0.8	0.3[b]	0.1	16	0.1–0.8	0.3[b]	0.1
β-Cryptoxanthin	16	0.4–5.8	1.4[a]	0.3	17	0.1–1.0	0.5[b]	0.1	17	0.2–1.9	0.6[b]	0.1
α-Carotene	16	0.3–7.2	1.6[a]	0.5	14	0.2–0.7	0.4[b]	0.0	15	0.1–2.0	0.6[b]	0.1
β-Carotene	17	0.5–9.7	3.8[a]	0.7	16	0.2–8.2	2.1[a,b]	0.6	17	0.2–8.6	2.0[b]	0.6
Lycopene	17	0.9–10.4	3.0[a]	0.6	16	0.3–4.0	1.2[b]	0.2	17	0.5–2.5	1.1[b]	0.1

Different superscript letters indicate a significant difference in flavonoid content in human milk of different lactation stages ($P < 0.05$).
* See Table 1 for demographic data.
† Number of samples in which the specific carotenoid was positively detected; $n = 17$ would indicate that the specific flavonoid was detected in all milk samples.
‡ Range, mean, and SEM were calculated based on the number of samples in which the compound was detected.

the previously mentioned studies, but their focus was specifically on β-carotene and red palm oil supplements, respectively. In the present study, human milk carotenoid content was found to decrease over lactation stages but generally had less variability in content compared with flavonoids. This is consistent with previous studies that examined the two classes of phytochemicals in plasma [37].

The α-carotene, α/β-cryptoxanthin, lycopene, lutein, and zeaxanthin significantly decreased with increasing stages of breast-feeding, specifically from 1 to 4 and from 1 to 13 wk but not 4 to 13 wk postpartum. These data suggest that carotenoid content in human milk decreases during the early stages of lactation but then only modestly decreases during the next 9 wk. These data are similar to those obtained by Schweigert et al. [11] during a shorter period. Schweigert et al. reported significant decreases in carotenoid concentration in human milk 4 to 19 d postpartum. The significantly greater carotenoid concentration in human milk during the first week postpartum likely was caused in part by the storage of milk before breast-feeding of the infant. Before the first week postpartum, human milk is not being eliminated from the breasts and carotenoids may have accumulated this period. Once the infant is breast-fed, the milk repositories are continuously emptied; thus, carotenoids do not accumulate for a longer period.

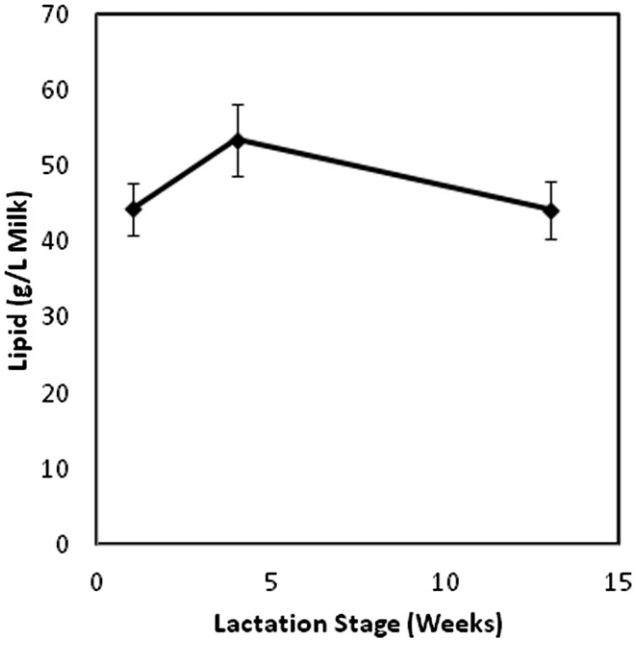


Fig. 4. Change in milk lipid content across 1, 4, and 13 wk of lactation. Data represent mean ± SEM for 17 individual samples at each time point.

Lutein, β-carotene, and total lycopene showed the highest concentration throughout every week in this study. These results are consistent with the carotenoid content of typical Western diets. Canfield et al. [10] examined carotenoid concentrations in breast milk from nine nations, including the USA and the United Kingdom [10]. These two countries had a similar qualitative profile, where lutein, β-carotene, and total lycopene were the most abundant. Similarly, Jewell et al. [9] examined human milk samples from mothers in Ireland and found that lutein and β-carotene had the highest concentrations, although total lycopene was not measured.

Conclusions

Flavonoids and carotenoids were identified and quantified in human milk from 17 mothers during 1, 4, and 13 wk postpartum. To our knowledge, this is the first study to report flavonoids in human milk. Further research is required to better understand the role these phytochemicals may play in infant health.

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