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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.
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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

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