Assays for Carotenoids: Linking Breastmilk and Maternal Intakes

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Abstract

Humans are not able to synthesize carotenoids and rely on their dietary intake mainly with vegetables and fruit, algae, and some animal products. The major dietary and plasma carotenoids are β-carotene, α-carotene, lutein, zeaxanthin, lycopene, and β-cryptoxanthin. Carotenoids are transferred to the mammary gland and breastmilk. Maternal dietary intake, geographical locations, age, nutritional status, premature birth, and circadian and within-feeding variations may affect breastmilk carotenoid levels. Those factors, especially circadian and within-feeding variations, should be included in studies assessing breastmilk carotenoids. During handling samples should be protected against oxygen, light, and temperature and stored in a −80 °C. The most recommended for the determination of carotenoids in breastmilk is a normal- or reversed-phase HPLC/UHPLC after specific sample preparation (saponification, hydrolyzation, and extraction). The most recommended method of nutritional data collection is at least a 3-day food record along with FFQ. Additionally, national databases of food carotenoids need to be developed to accurately estimate their intake.

Keywords

α-Carotene · β-Carotene · Breastmilk · β-Cryptoxanthin · Carotenoids · High-performance liquid chromatography (HPLC) · Lutein · Lycopene · Maternal diet · Nutritional assessment · Zeaxanthin

Abbreviations

α-CA	α-carotene
α-CR	α-cryptoxanthin
β-CA	β-carotene
β-CR	β-cryptoxanthin
BMI	body mass index
CD36	cluster of differentiation 36
FFQ	food frequency questionnaire
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
L	lutein
L + Z	lutein+zeaxanthin
LDL	low-density lipoprotein
LY	lycopene
NPC1L1	Niemann-Pick C1-Like 1
NS	nonsignificant
RAE	retinol activity equivalents
SR-BI	scavenger receptor class B type 1
UHPLC	ultra-high-performance liquid chromatography
UPLC	ultra-performance liquid chromatography
USDA	United States Department of Agriculture
Z	zeaxanthin

Introduction

Breastfeeding is a gold standard in infant nutrition: exclusive breastfeeding is recommended up to 6 months of infant life, followed by further breastfeeding up to 2 years or more (WHO 2003; Fewtrell et al. 2017). For child, breastmilk is not only a source of energy and all essential nutrients but also thousands of different bioactive compounds, including immune and growth factors, hormones, and phytochemicals (Miller et al. 2013; Samuel et al. 2020; Ríos et al. 2021). Breastmilk compositions vary and change dynamically during the course of lactation to fit to changing nutritional requirements and developmental needs of the child (Miller et al. 2013; Samuel et al. 2020). Those changes are related to the maternal, infant, and physiological factors, including maternal diet and nutritional status or lactation stage and the phase of single nursing (Miller et al. 2013; Samuel et al. 2020). Breastmilk always is the best and adequate source of nourishment for the infant, also in case when maternal diet and nutritional status are suboptimal (Butte et al. 2001). Breastmilk is a dynamic fluid that can vary its composition depending on individually multiple factors, including maternal diet and food intake (Miller et al. 2013; Ríos et al. 2021). There are several nutrients and bioactive substances in breastmilk that are diet dependent, including vitamins C, B_6, and B_{12}, choline, iodine, selenium, and phytochemicals such as carotenoids (Tsopmo 2018; Ríos et al. 2021). Carotenoids are lipophilic pigment synthesized by plants and some microorganisms (photosynthetic bacteria, some species of archaea and fungi, algae). As mammals, humans are not able to produce carotenoids and rely on their dietary intake, mainly with vegetables and fruits, and some seafood (Böhm et al. 2021). We can distinguish two classes of carotenoids: carotenes (e.g., β-carotene, α-carotene, lycopene) and more polar xanthophylls (e.g., lutein and zeaxanthin (L + Z), β-cryptoxanthin, astaxanthin) (Krinsky and Johnson 2005; Jomova and Valko 2013; Reboul 2019). More than 650 different carotenoids are present in nature, and in the human diet around 50–100 of them are found, whereas in human serum only 6 compounds (β-carotene, α-carotene, lycopene, lutein, zeaxanthin, and β-cryptoxanthin) make up more than 90% of total serum carotenoids (Fig. 1) (Krinsky and Johnson 2005; Rao and Rao 2007; Eggersdorfer and Wyss 2018). Those six carotenoids are also the most common in breastmilk samples due to plasma-breastmilk transport (Schweigert et al. 2004; Meneses and Trugo 2005; de Azeredo and Trugo 2008; Lipkie et al. 2015; Zielinska et al. 2017a; Ríos et al. 2021).

Carotenoid's Absorption and Transport

After ingestion carotenoids are released from the food matrix and diluted in the lipid phase prior to the absorption in the small intestine. Like other lipids, carotenoids are absorbed via passive diffusion or active transport via, e.g., SR-BI, CD36, and NPC1L1 transporters (Krinsky and Johnson 2005; Reboul 2019; Böhm et al. 2021). In the enterocytes carotenoids are incorporated into chylomicrons and secreted into the lymph and further transported to the liver (Krinsky and Johnson 2005; Böhm et al. 2021). From the liver, carotenoids are released to the serum into

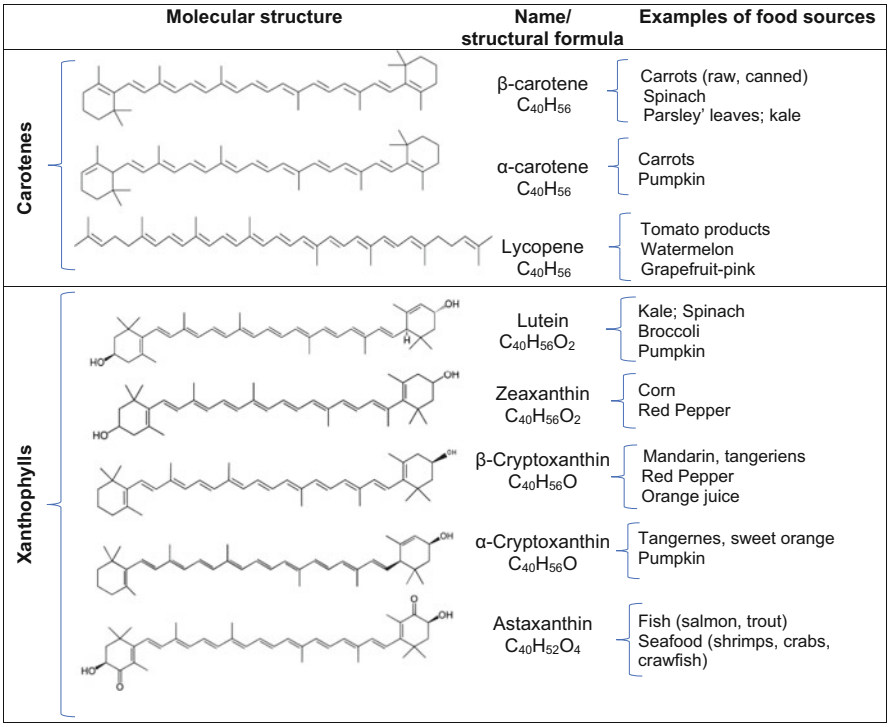

Fig. 1 The chemical structures of the selected carotenoids and their major dietary sources

lipoproteins. Interestingly, carotenes are mainly incorporated into low-density lipoprotein (LDL), whereas xanthophylls are incorporated equally between LDL and high-density lipoproteins (HDL). Moreover, due to different polar characteristics, carotenes are present in the lipoprotein core, whereas xanthophylls are located on its surface, which may explain its equal distribution between LDL and HDL fractions (Krinsky and Johnson 2005; Machado et al. 2019). Carotenoids are accumulated in several organs and tissues, especially the liver, adrenal gland, skin, lung, adipose tissue, brain, retina, prostate, breast, and breastmilk (Böhm et al. 2021). However, the distribution of carotenoids in human organs shows a high specificity depending on the carotenoid type.

Carotenoid Transport into the Breastmilk

In breastmilk, carotenoids are present in 10–120 times lower concentrations than plasma. However, the different serum to breastmilk carotenoid ratio may be observed depending on their polarity (polar xanthophylls have a higher ratio than less polar carotenes) (Meneses and Trugo 2005; Lipkie et al. 2015; Zielinska et al. 2017a; Böhm et al. 2021). Moreover, when the changes in carotenoid levels in

breastmilk were observed, their serum levels were intact (Schweigert et al. 2004; Machado et al. 2019). This indicates that despite the similar fate of carotenoids and fat in the intestine, carotenoids may be transported into breastmilk differentially, independent from fat (Macias and Schweighert 2001; Zielinska et al. 2017a). Possible mechanisms of carotenoid transport from plasma into breastmilk cover intracellular transport and preferential uptake by lipoproteins, especially the HDL fraction (Zielinska et al. 2017a; Machado et al. 2019).

Breastmilk Composition

Breastmilk carotenoids are part of complex and variable lipid fractions (Duan et al. 2019; Samuel et al. 2020), and their profile changes differently compared to plasma (Macias and Schweighert 2001). It is well known that breastmilk fat concentration changes throughout lactation (Samuel et al. 2020). The lowest amount of fat is present in colostrum, but then increases rapidly, remains relatively stable in the mature milk in the first year of lactation, but then increases again (Mitoulas et al. 2002; Miller et al. 2013; Czosnykoska-Łukacka et al. 2018; Samuel et al. 2020). Breastmilk fat levels are also regulated by circadian variations – the highest concentration is observed in the morning and evening (Italianer et al. 2020; Samuel et al. 2020). Another factor determining its concentrations is within-feeding variation (Samuel et al. 2020). Hindmilk (milk at the beginning of single feeding or prefeed milk) contains much lower levels of fat than foremilk (milk at the end of single feeding or postfeed milk) (Khan et al. 2013; Samuel et al. 2020). On the contrary, breastmilk fat levels did not vary between breasts (Mitoulas et al. 2002; Pines et al. 2016) or regarding the volume of produced breastmilk and emptying the breast at the previous feeding (Samuel et al. 2020). Method of breastmilk expression (manual vs. electrically by breast pump) may also influence fat level in milk (Samuel et al. 2020). Results about the relation to the number of breastfeeding or intervals between feeding are inconclusive (Khan et al. 2013; Samuel et al. 2020).

Breastmilk Carotenoids

In the last two decades, breastmilk carotenoids were widely investigated (Zielinska et al. 2017a; Ríos et al. 2021). In many studies, predominant breastmilk carotenoid is lutein (or lutein+zeaxanthin) (Macias et al. 2001; Ríos et al. 2017; Wu et al. 2020) or β-carotene (Machado et al. 2019). Interestingly, a recent study reported for the first time in human colostrum free apocarotenoids and its esters, carotenoid metabolites derived from oxidative or enzymatic cleavage (Zoccali et al., 2020). As shown in Table 1 carotenoid concentrations change within the course of lactation, with the highest level in the colostrum, and decrease especially between the 2nd and 4th weeks of lactation (Jackson et al. 1998; Sommerburg et al. 2000; Macias et al. 2001; Schweigert et al. 2004; Cena et al. 2009; Song et al. 2013; Ríos et al. 2017; Xue et al. 2017; Xavier et al. 2018; Machado et al. 2019; Xavier et al. 2019; Wu et al. 2020;

Table 1 Breastmilk carotenoids according to lactation stage

Study group	Milk sample	Analysis method, unit	Carotenoid level by lactation stage		
			Colostrum	Transitional milk	Mature milk
Cuba $N = 21$ Macias and Schweigert (2001)	10–12 mL of foremilk (morning); manual expression	HPLC nmol/L	β-CA: 125.7 ± 6.37 L: 67.9 ± 44.9 Z: 9.7 ± 6.7 LY: 137.3 ± 86.1 β-CR: 61.1 ± 66.6	β-CA: 44.2 ± 34.1 L: 44.5 ± 36.1 Z: 8.6 ± 5.5 LY: 44.2 ± 34.1 β-CR: 24.8 ± 22.4	β-CA: 36.2 ± 17.2 L: 27.3 ± 16.4 Z: 7.9 ± 7.7 LY: 18.8 ± 2.7 β-CR: 16.6 ± 12.7
Germany $N = 21$ Schweigert et al. (2004)	Total volume from one breast; no data	HPLC nmol/L	β-CA: 423.4 ± 326.6 L: 164.0 ± 84.9 Z: 33.2 ± 84.9 LY: 508.9 ± 421.7 β-CR: 238.8 ± 156.1	–	β-CA: 78.2 ± 46.2 L: 88.1 ± 37.8 Z: 19.5 ± 10.2 LY: 59.8 ± 38.9 β-CR: 60.6 ± 36.7
Italy $N = 21$ Cena et al. (2009)	5–6 mL of milk; breast pump	HPLC nmol/L	L: 280 ± 220	–	L: 110 ± 50
USA $N = 17$ Song et al. (2013)	Total volume from one breast at 10 a.m.–1 p.m.; electric pump	HPLC nmol/L	–	β-CA: 164.3 ± 25.2 α-CA: 59.0 ± 13.5 L: 121.2 ± 20.9 Z: 46.3 ± 5.4 LY: 119.9 ± 18.9 β-CR: 57.4 ± 10.7 α-CR: 57.4 ± 10.7	β-CA: 88.0 ± 23.3 α-CA: 23.2 ± 4.8 L: 56.4 ± 6.8 Z: 21.4 ± 2.5 LY: 49.5 ± 6.4 β-CR: 24.8 ± 4.4 α-CR: 13.5 ± 2.0
USA $N = 20$ Lipkie et al. (2015)	Total volume from one breast at 9 a.m.–1 p.m.; electric pump	HPLC nmol/L	–	β-CA: 71.6 ± 56.7 α-CA: 20.2 ± 10.7 L: 125.5 ± 80.1 Z: 35.5 ± 19.5 LY: 75.2 ± 37.1 β-CR: 35.5 ± 27.1	β-CA: 67.4 ± 54.8 α-CA: 18.6 ± 10.8 L: 89.0 ± 45.7 Z: 26.8 ± 15.1 LY: 55.9 ± 33.2 β-CR: 31.2 ± 21.4

(continued)

Table 1 (continued)

Study group	Milk sample	Analysis method, unit	Carotenoid level by lactation stage		
			Colostrum	Transitional milk	Mature milk
				α-CR: 25.2 ± 11.0	α-CR: 21.1 ± 9.6
Mexico $N = 20$ Lipkie et al. (2015)	Total volume from one breast at 9 a.m.–1 p.m.; electric pump	HPLC nmol/L	–	β-CA: 62.6 ± 30.4 α-CA: 22.2 ± 12.2 L: 166.0 ± 114.9 Z: 50.4 ± 33.1 LY: 60.3 ± 42.7 β-CR: 72.5 ± 61.1 α-CR: 28.0 ± 14.2	β-CA: 40.5 ± 16.5 α-CA: 14.3 ± 8.1 L: 103.2 ± 63.3 Z: 39.6 ± 33.4 LY: 39.7 ± 22.8 β-CR: 43.7 ± 32.2 α-CR: 20.2 ± 13.2
China $N = 20$ Lipkie et al. (2015)	Total volume from one breast at 9 a.m.–1 p.m.; electric pump	HPLC nmol/L	–	β-CA: 83.0 ± 42.7 α-CA: 18.2 ± 8.5 L: 313.7 ± 161.3 Z: 58.8 ± 34.0 LY: 34.7 ± 19.1 β-CR: 53.6 ± 34.6 α-CR: 39.8 ± 15.8	β-CA: 71.6 ± 30.0 α-CA: 11.3 ± 5.6 L: 206.0 ± 93.9 Z: 37.5 ± 17.6 LY: 13.7 ± 13.2 β-CR: 75.4 ± 68.4 α-CR: 31.3 ± 12.3
China $N = 509$ Xue et al. (2017)	Total volume from one breast at 9–11 a.m.; electric pump	U-HPLC μgl/ 100 mL	β-CA: 8.0 (4.7–15.2) L: 5.7 (2.9–10.2) Z: 1.0 (0.5–1.5) LY: 6.3 (4.0–9.9) β-CR: 6.2 (2.4–12.9)	β-CA: 2.8 (2.0–4.4) L: 7.0 (4.6–10.3) Z: 1.4 (1.0–2.2) LY: 2.5 (1.7–4.3) β-CR: 3.4 (1.7–5.7)	β-CA: 1.7 (1.4–3.1) L: 2.9 (0.9–5.9) Z: 0.8 (0.4–1.5) LY: 1.4 (1.1–2.0) β-CR: 1.7 (1.1–2.6)
Spain $N = 30$ Ríos et al. (2017)	Total volume from one breast; no data	U-HPLC nmol/L	β-CA: 754.8 α-CA: 219.6 L: 486.4 Z: 98.1 LY: 854.3 β-CR: 962.6	–	β-CA: 53.3 α-CA: 14.9 L: 199.8 Z: 64.95 LY: 159.9 β-CR: 145.3

(continued)

Table 1 (continued)

Study group	Milk sample	Analysis method, unit	Carotenoid level by lactation stage		
			Colostrum	Transitional milk	Mature milk
Spain $N = 70$ Xavier et al. (2018)	Total volume from one breast; no data	U-HPLC nM	β + α-CA: 1103 (602.3–2238) L: 486.3 (3229–745.8) Z: 106.4 (73.7–141.4) LY: 1065 (483.0–1846)	–	β + α-CA: 84.8 (59.4–315.0) L: 195.9 (150.3–270.8) Z: 59.9 (38.4–01.0) LY: 192.7 (118.8–221.2)
Brazil $N = 19$ Machado et al. (2019)	10 mL in morning; manual expression	HPLC µmol/L	–	β-CA: 0.17 ± 0.02 α-CA: 0.04 ± 0.01 L + Z: 0.07 ± 0.02 LY: 0.04 ± 0.01	β-CA: 0.14 ± 0.3 α-CA: 0.03 ± 0.01 L + Z: 0.06 ± 0.01 LY: 0.04 ± 0.00
Switzerland $N = 28$ Redeuil et al. (2021)	Total volume from one breast in morning; electric pump	UPLC µgl/ 100 mL	β-CA: 38.9 ± 37.9 L: 7.98 ± 4.93 Z: 1.63 ± 0.94 LY: 25.7 ± 19.1 β-CR: 15.3 ± 8.53	β-CA: 9.18 ± 7.61 L: 7.94 ± 6.33 Z: 2.08 ± 1.38 LY: 5.70 ± 3.40 β-CR: 7.04 ± 5.20	β-CA: 4.53 ± 3.48 L: 4.31 ± 2.52 Z: 1.12 ± 0.68 LY: 2.34 ± 1.2 β-CR: 4.39 ± 4.17
China $N = 42$ Wu et al. (2020)	Total volume from both breasts at 9–11 a.m.; electric pump	HPLC µgl/ 100 mL	β-CA: 11.1 (6.5–23.3) L: 7.1 (5.1–13.0) Z: 2.2 (1.3–3.3) LY: 11.9 (7.1–17.4) β-CR: 3.9 (1.5–7.0)	β-CA: 3.1 (1.8–5.3) L: 9.5 (6.8–13.1) Z: 2.2 (1.3–2.9) LY: 1.3 (0.8–2.5) β-CR: 2.0 (1.3–3.6)	β-CA: 1.8 (1.0–3.1) L: 4.6 (2.9–7.6) Z: 1.1 (0.7–1.9) LY: 0.6 (0.3–1.0) β-CR: 0.8 (0.5–2.1)

α-CA = α-carotene. α-CR = α-cryptoxanthin. β-CA = β-carotene, β-CR = β-cryptoxanthin. HPLC = high-performance liquid chromatography. L = lutein. LY = lycopene. L + Z = lutein +zeaxanthin. UHPLC = ultra-high-performance liquid chromatography. UPLC = ultra-performance liquid chromatography. Z = zeaxanthin

Redeuil et al. 2021). Interestingly, this decrease is not observed in serum, which confirms the specific mechanisms of carotenoid transport into milk (Machado et al. 2019). Moreover, a higher drop is observed in carotenes than in the more polar xanthophylls (88.67% vs. 35.92%) (Macias and Schweigert 2001; Schweigert et al. 2004; Wu et al. 2020; Redeuil et al. 2021; Ríos et al. 2021; Sun et al. 2021), as well

as vitamin A non-precursors (Lipkie et al. 2015). Carotenoid levels in mature milk (Table 2) are relatively stable and did not vary across the course of lactation but may be influenced by other factors (Song et al. 2013; Lipkie et al. 2015; Zielinska et al. 2019a). It is not exactly known how factors affecting fat concentrations determine breastmilk carotenoids. Previous studies have shown that carotenoids are probably transported into milk independently to fat (Macias and Schweighert 2001; Schweigert et al. 2004; Machado et al. 2019; Wu et al. 2020) and its content might be not related to fat in mature milk (Duan et al. 2019). However, in one study xanthophylls, but not carotenes, were correlated with milk fat (Wu et al. 2020), while in the study by Giuliano et al. (1994), α- and β-carotene, lycopene, and β-cryptoxanthin were correlated with milk fat. Also, within-feeding variations in breastmilk carotenoid levels were reported (Jackson et al. 1998; Hampel et al. 2017). Jackson et al. (1998) showed that hindmilk contained 25% more total carotenoids than mid- and foremilk. These authors investigated also the diurnal patterns and showed a nonsignificant trend of higher carotenoids at midday (Jackson et al. 1998). In a recent study circadian variation in vitamin A levels (β-carotene + retinol) was also reported but diminished after adjustment for fat (Hampel et al. 2017). The most important determinants of breastmilk carotenoids are geographical location, which is related to differences in dietary patterns and carotenoid intake (Canfield et al. 2003; Jackson and Zimmer 2007; Lipkie et al. 2015; Nguyen et al. 2020; Ríos et al. 2021). Other factors that may affect breastmilk carotenoids are maternal age (de Azeredo and Trugo 2008; Denić et al. 2019), preterm birth (Xavier et al. 2018; Redeuil et al. 2021), and maternal nutritional status (BMI; overweight or obesity) (Panagos et al. 2016; Zielinska et al. 2019a).

Carotenoid Intake During Lactation

In European countries intakes of β-CA, α-CA, L/Z, β-CR, and LY are about 1.5–8.8, 0.25–7.7, 0.78–3.25, 0.17–1.36, and 1.64–8.05 g/d, respectively (Böhm et al. 2021). Currently, only a few studies investigated carotenoid intake during lactation with similar results to those observed in non-lactating individuals (Table 3). Similarly to breastmilk carotenoids, their intake varies between different populations (Meneses and Trugo 2005; Cena et al. 2009, Panagos et al. 2016; Xue et al. 2017; Kim et al. 2018; Xu et al. 2019; Zielinska et al. 2019a). Currently there are no official nutritional recommendations for carotenoid intake (Böhm et al. 2021). Previously, in non-lactating (Böhm et al. 2021) and lactating (Cena et al. 2009) individuals, carotenoid intake was linked to plasma/serum concentrations. Maternal plasma carotenoids were correlated to their breastmilk levels (Schweigert et al. 2004; Meneses and Trugo 2005; Sherry et al. 2014, Lipkie et al. 2015; Machado et al. 2019; Xu et al. 2019). However, results about associations between maternal dietary intake and breastmilk carotenoids are ambiguous: some confirm a strong association (Cena et al. 2009; Panagos et al. 2016; Kim et al. 2018, Zielinska et al. 2019a; Machado et al. 2019), and some did not (Meneses and Trugo 2005; Xue et al. 2017; Xu et al. 2019). Despite the discrepancies in the observational studies, several

Table 2 Breastmilk carotenoids in mature milk

Study group	Milk sample	Analysis method, unit	Specific carotenoids in mature milk				
			β-CA	α-CA	L + Z	LY	β-CR
Australia $N = 51$ Canfield et al. (2003)	Total volume from one breast between 13:00 and 17:00; electric pump	HPLC µmol/L	0.060 ± 0.007	0.034 ± 0.003	0.027 ± 0.002	0.031 ± 0.002	0.024 ± 0.002
Canada $N = 55$ Canfield et al. (2003)			0.036 ± 0.003	0.036 ± 0.003	0.030 ± 0.001	0.030 ± 0.002	0.027 ± 0.003
Chile $N = 49$ Canfield et al. (2003)			0.044 ± 0.004	0.024 ± 0.002	0.057 ± 0.005	0.021 ± 0.002	0.016 ± 0.002
Japan $N = 50$ Canfield et al. (2003)			0.062 ± 0.005	0.045 ± 0.004	0.077 ± 0.002	0.023 ± 0.002	0.080 ± 0.008
Mexico $N = 50$ Canfield et al. (2003)			0.051 ± 0.005	0.031 ± 0.002	0.044 ± 0.003	0.032 ± 0.002	0.057 ± 0.008
Philippines $N = 60$ Canfield et al. (2003)			0.022 ± 0.002	0.041 ± 0.010	0.035 ± 0.003	0.016 ± 0.002	0.012 ± 0.001
UK $N = 50$ Canfield et al. (2003)			0.048 ± 0.003	0.031 ± 0.003	0.027 ± 0.002	0.034 ± 0.002	0.012 ± 0.001
Brazil $N = 49$ Meneses and Trugo (2005)	Total volume from one breast; handheld breast pump	HPLC µmol/L	0.018 ± 0.002	–	0.006 ± 0.001	–	–
Korea $N = 34$ Duan et al. (2019)	Lack of data	HPLC µg/100 g	1.68 (0.49–9.46)	0.19 (0.03–1.07)	3.85 (1.15–9.68)	0.29 (0.00–1.22)	3.60 (0.53–10.96)
China $N = 56$ Xu et al. (2019)	Total volume from one breast between 13:00 and 16:00; manual pump	HPLC µg/dL	–	–	L: 8.2 ± 0.65 *trans*-L: 7.7 ± 0.61 Z: 2.0 ± 0.17	–	–

Korea $N = 98$ Kim et al. (2018)	150 ml; breast pump	HPLC μg/dL	–	–	3.50 ± 3.71	–	–
Poland $N = 53$ Zielinska et al. (2019)	Pooled sample from 4 samples collected 4 times during the day (6:00–12:00; 12:00–18:00; 18:00–24:00; 24:00–6:00) via collection of the same amount of pre- and postfed milk; manually or breast pump	HPLC nmol/L	33.1 (32.9–33.3)	–	33.0 (24.1–41.8)	111.2 (105.0–117.3)	–
China $N = 111$ Nguen et al. (2020)	50–150 ml (lack of other data); manually or breast pump	HPLC μgl/L	–	–	66.1 ± 51.6	–	–
Korea $N = 155$ Nguen et al. (2020)			–	–	41.3 ± 27.4		
Pakistan $N = 97$ Nguen et al. (2020)			–	–	47.6 ± 58.9	–	–
Vietnam $N = 92$ Nguen et al. (2020)			–	–	49.7 ± 47.9	–	–
Cambodia $N = 23$ Whitefield et al. (2020)	Total volume from one breast; electric pump	HPLC μgl/L	63.4 (41.4–97.1)	11.4 (8.59–15.2)	56.1 (40.7–77.3)	8.87 (7.22–10.9)	10.2 (7.36–13.9)
Indonesia $N = 212$ Gibson et al. (2020)	Total volume from one breast at morning; handheld breast pump	HPLC μgl/L	21 (14–32)	9.6 (6.2–12.6)	–	–	26 (17–43)

α-CA = α-carotene. β-CA = β-carotene, β-CR = β-cryptoxanthin. HPLC = high-performance liquid chromatography. L = lutein. LY = lycopene. L + Z = lutein+zeaxanthin. Z = zeaxanthin

Table 3 Carotenoid intake during lactation

Study group	Nutritional assessment method	Dietary supplements assessed	Nutritional database used	Assessed carotenoids, units	Carotenoid dietary intake	Associations to breastmilk carotenoids
Multinational study Canfield et al. (2003)	24 h food record	No	No	Rank order of intake	No quantitative data; qualitative analysis of dietary sources and rank order of carotenoids according to its intake	Rank order in breastmilk and dietary carotenoids was similar and suggests an association
Brazil $N = 49$ Meneses and Trugo (2005)	FFQ	No	ESHA database	Provitamin A carotenoids, μg RAE/d	301 ± 184	NS
Italy $N = 21$ Cena et al. (2009)	2 × quantitative 30-item FFQ covering past month (3- and 30-day T0; T1)	No	No data	L, μg/d	T0 L: 1209 ± 157 T1 L: 1258 ± 102	T0: $r = 0.94$, $p \leq 0.001$ T1: $r = 0.82$, $p \leq 0.001$
USA $N = 21$ Panagos et al. (2016)	3-day food record 1 week before visit	No	USDA	β-CA, α-CA, LY, L + Z, CR, mg/ 1000 kcal	β-CA: 2.35 ± 3.19 α-CA: 0.31 ± 0.35 LY: 1.63 ± 1.55 L + Z: 2.08 ± 2.92 CR: 0.11 ± 0.15	L: $\beta = 0.41$, $p < 0.01$
China $N = 509$ Xue et al. (2017)	1 × 24 h dietary record	Yes	Chinese and Japanese food composition tables	Total carotenoids	No data	NS

Korea $N = 98$ Kim et al. (2018)	3-day food record	Yes	USDA	L, β-CA mg/day	β-CA: 4.33 ± 2.61 L: 4.71 ± 3.11	$\beta = 0.3653$, $p = 0.0022$
China $N = 56$ Xu et al. (2019)	1 × 3-day food record	No	USDA	L + Z, mg/d	3.3 ± 0.41	NS
Poland $N = 53$ Zielinska et al. (2019)	2 × 3-day food record (3- and 6-month T1;T2)	Yes	USDA	β-CA, LY, L + Z, μg/d	T1: β-CA: 4480.8 (3575.0–5386.7) LY: 7898.3 (5465.2–10329.5) L + Z: 2945.2 (1910.8–3979.6) T2: β-CA: 3441.9 (5037.5–9474.1) LY: 7255.8 (5037.5–9474.1) L + Z: 3739.3 (2834.9–4643.7)	T1: β-CA: $\beta = 0.407$, $p \leq 0.01$ LY: $\beta = 0.415$, $p \leq 0.01$ L + Z: $\beta = 0.730$, $p \leq 0.001$ T2: β-CA: $\beta = 0.428$, $p \leq 0.001$ LY: $\beta = 0.401$, $p \leq 0.01$ L + Z: $\beta = 0.644$, $p \leq 0.001$
Brazil $N = 19$ Machado et al. (2019)	2 × 24 h dietary record	No	USDA	β-CA, α-CA, LY, L + Z, μg/d	β-CA: 3249 (1408–6707) α-CA: 1053 (56–3712) LY: 1854 (302–6472) L + Z: 2446 (872–4873)	β-CA: $\beta = 2.52 \times 10^{-5}$, $p \leq 0.05$
Serbia $N = 19$ Denić et al. (2019)	1 × 7-item FFQ covering pregnancy and lactation	No	–	–	–	β-CA correlated to vegetable and fruit intake

α-CA = α-carotene. α-CR = α-cryptoxanthin. β-CA = β-carotene. β-CR = β-cryptoxanthin. FFQ = food frequency questionnaire. L = lutein. LY = lycopene. L + Z = lutein+zeaxanthin. RAE = retinol activity equivalent. USDA = United States Department of Agriculture. Z = zeaxanthin

intervention studies confirmed a strong association between dietary intake of carotenoids and their breastmilk levels (Sherry et al. 2014; Nagayama et al. 2014; Haftel et al. 2015; Schaefer et al. 2020).

Assays for Carotenoids: Methodological Implications

Breastmilk Sample Collection

Studies assessing breastmilk carotenoids used a variety of breastmilk collection methods, sometimes not even described properly (Tables 1 and 2). Differences in milk sampling protocol may make it difficult to compare the results and explain discrepancies in carotenoid level between studies (Zielinska et al. 2017a; Denić et al. 2019). To minimize the bias regarding sampling, the selecting of optimal and appropriate milk collection method (and its description) is crucial in the case of breastmilk carotenoid assessment (Miller et al. 2013; Zielinska et al. 2017a). There is no recommended, universal method of breastmilk sampling for milk composition studies (Miller et al. 2013). Milk collection protocol adapted for studies regarding carotenoids (and other fat-soluble compounds) should be standardized among all study subjects and several conditions considered, including longitudinal, circadian, and within-feeding variations in milk composition, the volume of milk consumed at the previous feeding, intervals between feedings, expression method (e.g., availability of breast pumps), physiological milk let-down, and infant's nutritional needs (Miller et al. 2013; Samuel et al. 2020; Casavale et al. 2019). Miller et al. (2013) characterized the most often used milk sampling protocols: full breast expression, expression of the alternate breast, mid-feed sampling, fore- and hindmilk sampling, and foremilk (or hindmilk) sampling. In studies assessing carotenoids, full breast expression was used most often (Schweigert et al. 2004; Song et al. 2013; Lipkie et al. 2015; Xue et al. 2017; Ríos et al. 2017; Xavier et al. 2018; Redeuil et al. 2021; Wu et al. 2021; Canfield et al. 2003; Meneses and Trugo 2005; Whitefield et al. 2020; Gibson et al. 2020). Several studies used fore- or hindmilk sampling (Macias and Schweigert 2001; Cena et al. 2009; Machado et al. 2019; Nguen et al. 2020) and one study fore- and hindmilk sampling four times per day (Zielinska et al. 2019a). Full breast expression is a relative optimal method that eliminates the within-feeding variation in milk composition but does not consider circadian variations, so the time of sampling should be specified in one study group (Miller et al. 2013). Fore- or hindmilk sampling does consider neither circadian variation nor within-feeding variation, so it is less applicable (Miller et al. 2013). Fore- and hindmilk sampling eliminates bias related to within-feeding variations in milk composition and requires a relatively small amount of milk, so it is not related with the nutritional risk for infant (Miller et al. 2013). Repeatable collections within the 24-h period will minimize bias related to circadian variation (Zielinska et al. 2019a), so this method is more applicable than the previous one. Regardless of the chosen sampling protocol method of milk expression, data about breast from the sample

was expressed, the volume of collected sample and infant feeding data should be also specified in further studies (Samuel et al. 2020).

Breastmilk Sample Handling and Storage

Handling of collected breastmilk samples is another critical step in milk analysis (Miller et al. 2013; Samuel et al. 2020). Carotenoids are susceptible to oxygen, light, temperature, and prooxidant metal, so invalid sample handling and storage may lead to carotenoid loss due to its autooxidation or *cis-trans* isomerization (Jackson et al. 1998; Canfield et al. 2003; Rodríguez-Bernaldo de Quirós and Costa, 2006; Amorim-Carrilho et al. 2014). Breastmilk samples should be expressed immediately into the collection container, protected against oxygen and light, and kept in the cooling condition during transport to the laboratory (Samuel et al. 2020). Before preparing aliquots for further analysis breastmilk should be homogenized with gentle swirls due to the even distribution of fat into samples (Miller et al. 2013). The milk sample should be divided into smaller aliquots and then stored as soon as possible at −80 °C until further analysis. A previous study showed that 1 year of storage in −70 °C resulted in the decrease of β-cryptoxanthin, but other carotenoids were stable (Jackson et al. 1998). It is important to avoid freeze-thaw cycles that lead to lipolysis activation. Before analysis stored samples should be thawed at room temperature or in a water bath (37 °C) (Samuel et al. 2020).

Breastmilk Analysis

Analysis of breastmilk carotenoids covers several stages (Fig. 2). The most popular method of breastmilk carotenoids analysis is a normal/reversed-phase HPLC/UHPLC analysis which requires a specific sample preparation (Table 4). The applied sample preparation methods vary depending on the analytical material and chemical form of carotenoids. In plants tissues, except bell pepper fruits, carotenoids abundant mostly in free form. Animals tissue, including breastmilk, contain mostly fat-esterified carotenoids form. From chemical point of view they are more stabile, but for analysis purposes saponification process should be used. For the extraction of free carotenoids solvents such as hexane or acetone are sufficient, but for breastmilk samples prior to extraction saponification and hydrolysis of the ester bonds have to be conducted (Amorim-Carrilho et al. 2014) with a strong base solution. For the saponification of breastmilk samples, the most commonly used reagent is potassium hydroxide (30–60%) in methanol and for hydrolysis – ethanol. However, saponification may result in the loss of transformation of carotenoids, so the optimal parameters of temperature and time are crucial (Jackson et al. 1998; Amorim-Carrilho et al. 2014). For the prevention of carotenoid loss during sample preparations, a variety of reagents were added, whereas the most common were butylated-hydroxytoluene (BHT) and pyrogallol (Table 4; Amorim-Carrilho et al. 2014). After saponification and hydrolysis, breastmilk carotenoids have to be extracted from

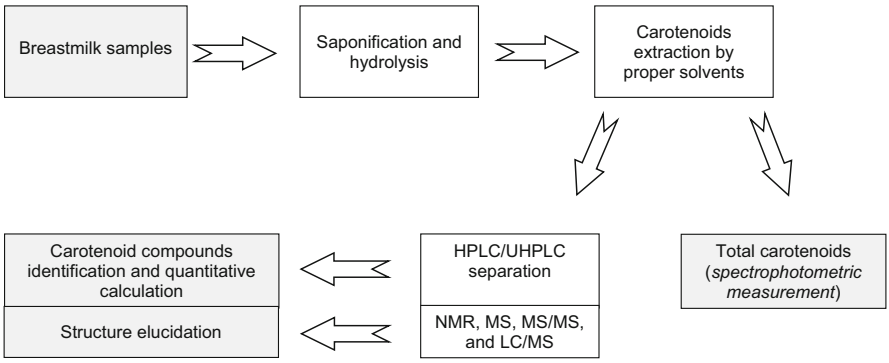

Fig. 2 Scheme of sample preparation and carotenoid analysis. HPLC = high-performance liquid chromatography, LC/MS = liquid chromatography-mass spectrometry, MS = mass spectrometry, MS/MS = tandem mass spectrometry, NMR = nuclear magnetic resonance spectroscopy, UHPLC = ultra-high-performance liquid chromatography

breastmilk sample, usually by n-hexane in 1–3 repeatable cycles (Table 4; Amorim-Carrilho et al. 2014). After those procedures the collected extract of breastmilk sample is purified from the extraction solvent by evaporating under pressure and re-dissolving the sample in the final solvent (usually hexane or mobile phase). The sample prepared in this way can be used for the measurement of total carotenoids (in the spectrophotometric method) or for the separation and qualitative and quantitative identification in the HPLC/UHPLC system. Based on retention time and pure carotenoid standards prepared, the sample will be identified quantitatively and/or qualitatively.

Carotenoids absorb light very strongly and have intense absorption ranges in the visible light regime. The features of the UV/Vis spectrum present the characteristic information and these can be use for qualitative and quantitative analysis of carotenoids. The first and most important feature is the wavelength. For carotenoids, it is 445–475 nm (Miller et al. 2013). The second feature is the shape and arrangement of the spectrum, and the third is the absorbance value. All this information can be obtained graphically (spectrum illustration) and presented numerically (measurable value). The type of solvent used for the mobile phase should be taken into account when determining carotenoids in breastmilk samples. For practical reasons, light absorption is best for most carotenoids; they are similar in hexane, diethyl ether, and acetonitrile. Other solvents absorb light, so there are bathochromic shifts. The presence of water is undesirable. This can also affect light absorption. The expected concentration of carotenoids in the examined sample is also of great importance (Amorim-Carrilho et al. 2014).

In general, carotenoids are particularly difficult to analyze accurately due to their lipophilicity, instability, structural similarity, and/or scarcity of certified reference materials, which poses problems of accuracy and comparability of obtained results.

Table 4 Analytical methods for carotenoid assessment in breastmilk samples

Study	Analyte	Analytical method		
		Milk fat analysis	Extraction protocol	Detection system
USA $N = 23$ Jackson et al. (1998)	β-CA, α-CA, L + Z, LY, β-CR	–	Saponification: 2 mL of mixed milk +1.5 ml of 50% (wt./v) KOH + 2.5 ml ethanol. Water bath (2 h, 45 °C). 3x extraction: 3 ml hexane +0.025% (wt./v) BHT. Evaporation of hexane residue under nitrogen, then dissolved in mobile phase	HPLC RP (reversed phase) UV-Vis detectors Column: C18 (3.9 × 300 mm, 4 μm) No guard column Mobile phase: isocratic acetonitrile/methanol/THF (50:45:5; (v/v/v) Injection: 200 μl Flow rate: 2.5 ml/min Time: 20 min
Cuba $N = 21$ Macias and Schweigert (2001)	β-CA, α-CA, L, Z, LY, β-CR	–	Saponification: 1 ml milk +500 μl 12% pyrogallol in ethanol+1.5 ml 50% KOH and ethanol (3:5; v/v), vortex, incubation (2 h, 37 °C, under nitrogen). 2x extraction: +1 ml n-hexane (10 min, centrifugation); +1.5 ml 0.1 M NaCl +500 μl ethanol, vortex (centrifugation). Evaporation of hexane residue under nitrogen, then dissolved in 200 μl isopropanol, vortexed and sonicated	HPLC RP (reversed phase) Column: C30 (250 × 4.6 mm, 5 μm) Mobile phase: A (methanol/methyl-tert-butyl-ether/water, 83:15:2; v/v/v); B (methanol/methyl-tert-butyl-ether/water, 8:90:2; v/v/v) both with 1.5% ammonium acetate in H_2O Gradient elution Flow rate: 1 ml/min
Germany $N = 21$ Schweigert et al. (2004), Schweigert et al. (2000)	β-CA, α-CA, L, Z, LY, β-CR, 9-cis-β-CA, canthaxanthin	–		HPLC RP (reversed phase) Column: C30 (250 × 4.6 mm, 5 μm) and C18 pre-column Mobile phase: A (methanol/water, (90:10; v/v), with 0.4 g ammonium acetate in 1 l H_2O); B (methanol/methyl-tert-butyl-ether/water (8:90:2; v/v/v), with 0.1 g ammonium acetate in 1 l H_2O)

(continued)

Table 4 (continued)

Study	Analyte	Analytical method		
		Milk fat analysis	Extraction protocol	Detection system
Multinational study Canfield et al. (2003), Liu et al. (1998)	β-CA, α-CA, L + Z, LY, β-CR	Creamatocrit	Hydrolysis and saponification: 1 ml milk +10 mg $MgCO_3$ + 6 mg bile salt. Incubation in an orbital shaker (1 h); + 1 mg or protease +10 mg of lipase; incubation (1 h); + 1 ml KOH:H_2O (1:1; wt./v); incubation (0.5 h, 37 °C). Extraction: +0.5 ml of ethanol (vortexing, 30 s, room temperature/10 min); + 2 ml hexane; vortexing (1 min); evaporation under nitrogen, then dissolved in 250 µl o THF/CAN (15:85; v/v); centrifuging (12,700x*g*, 15 s.)	HPLC UV-Vis detectors Column: C18 (4.6 × 250 mm, 5 µm) Mobile phase: 95%A (ACN:THF (85:15; v/v); 5% B (50 mM ammonium acetate in methanol with 0.05% TEA) Flow rate: 2.5 ml/min Time: 13 min
Brazil $N = 49$ Meneses and Trugo (2005)	β-CA, L + Z	Creamatocrit		HPLC UV-VIS Column: C18 (4.6 × 250 mm, 5 µm) Mobile phase: 95% A (ACN:THF, 85:15; v/v), 5% B (50 mmol/L ammonium acetate in methanol) Injection: 20 µl Flow rate: 2 ml/min
Brazil $N = 19$ Machado et al. (2019)	β-CA, α-CA, L + Z, LY	Creamatocrit		HPLC UV-Vis detectors Column: C18 (4.6 × 150 mm) Mobile phase: ACN/THF+ 15 mM methanolic ammonium acetate, 65:25:10, v/v/v) Injection: 50 µl Flow rate: 0.9 ml/min
Italy $N = 21$ Cena et al. (2009)	L	–	Saponification: 2 ml milk +0.5 ml of KOH (40% in methanol) + 0.1 ml β-apo-8′-carotenal in methanol, incubation in water bath (30 min, 45 °C). 3 x extraction: + 1.5 ml hexane +0.01% BHT wt./v; evaporation under nitrogen, then dissolved in 0.5 ml of isopropanol-hexane (10: 90; v/v)	Column: C18 (4 × 250 mm, 4 µm) Mobile phase: mixture of A (2-propanol), B (hexane) Gradient elution Flow rate: 1 ml/min

(continued)

Table 4 (continued)

Study	Analyte	Analytical method		
		Milk fat analysis	Extraction protocol	Detection system
USA $N = 17$ Song et al. (2013)	β-CA, α-CA, L, Z, LY, β-CR; α-CR	Creamatocrit	Saponification: 0.75 ml + 0.3 ml 30% methanolic KOH (15 min, ambient temperature); 3 x extraction: 3:1 petroleum ether +0.1% 2,6-di-tert-butyl-4-methylphenol: acetone; evaporation under vacuum, then dissolved in 150 µl of 1:1 ethyl acetate and methanol before analysis	HPLC DAD detectors Column: C30 (2 × 150 mm) + guard column (2 x 50 mm)
USA $N = 20$ Lipkie et al. (2015)	β-CA, α-CA, L, Z, LY, β-CR, α-CR	Creamatocrit	Saponification: 0.7 ml milk +1.3 ml 0.9% NaCl +2 ml ethanol + internal standard; shaken 10 min. 2 x extraction: + 2 ml (9:1; v/v) hexane/ethyl acetate +0.1% BHT. Evaporation under nitrogen at 35 °C, then placed on ice. Saponification: + 2 ml 10% wt./v KOH, incubation (1 h, 37 °C); quenched with 2 ml chilled water; re-extraction 9:1 ethanolic extract, dried, resolubilized in 50 µl ethyl acetate +50 µl methanol; centrifugation (14,000 rpm/5 min)	HPLC DAD detectors Column: C30 (2 × 150 mm) Mobile phase: mixture of 2 mM ammonium acetate and ethyl acetate
USA $N = 21$ Panagos et al. (2016)	β-CA, LY, L, Z, β-CR	Ultrasound technique	Saponification: 4 ml milk +5 ml ethanol +3 ml 50% wt./v) KOH; sonication in a water bath, saponification in an orbital shaker (25 °C, 130 oscillations/min: β-CA, L, Z, CR: 16 h, α-CA, LY: 0.5 h); 2 x extraction +4 ml hexane, vortexing and sonicating (5 min); evaporation under nitrogen, then dissolved in 2.5 ml ethanol and 3.5 ml of	HPLC RP (reversed phase) Column: C30 (150 × 4.6 mm, 3 µm) Mobile phase: 10% THF, 90% methanol (9:1; v/v), and 0.5 g/l BHT Flow rate: 1.6 ml/min

(continued)

Table 4 (continued)

Study	Analyte	Analytical method		
		Milk fat analysis	Extraction protocol	Detection system
			H_2O, centrifugation 600x*g*/10 min; hexane layers evaporated in nitrogen and dissolved in 200 µl of THF methanol (20:80, v/v)	
China *N* = 509 Xue et al. (2017)	β-CA, α-CA, LY, L, Z, CR	–	Saponification: 1 ml milk +5 µl ethanol + BHT (79 g/l) + 10 µl aqueous solution of deferoxamine mesylate (10 mg/ml) + 4 ml methanol +1 ml KOH (30%, v/v), mixing, water bath (30 min, 37 °C); cooling on ice; 2x extracting: + 5 ml hexane +350 mg BHT/l, mixing, 30 s, centrifugation (2500 rpm/10 min, 4 °C); hexane layers evaporated in nitrogen and dissolved in 70 µl dioxane/ethanol (1:1; v/v) + 70 µl acetonitrile; centrifuging 2500 rpm / 10 min	UHPLC UV-Vis detectors Column: C18 (2.1 mm × 150 mm, 1.8 µm) Mobile phase: A (0.5 M ammonium acetate in water) Flow rate: 0.4 ml/min
Spain *N* = 30 Ríos et al. (2017)	β-CA, α-CA, L, Z, LY, β-CR; xanthophyll esters	Solvent extraction followed by gravimetry	2 x extraction: 3 ml milk +6 ml methanol, vortex 20 min, cooled −20 °C, centrifugation (10,000 x *g*, 5 min, 4 °C); upper layer was discarded +5 ml diethyl ether +2 ml hexane, vortexing, centrifuging (10,000 x *g*, 5 min, 4 °C); evaporation on rotatory evaporator (25 °C), dissolved in 1 ml hexane; SPE column, elution with 30 ml hexane; evaporation on rotary evaporator (25 °C), dilution in 1 ml methanol/methyl-tert-butyl-ether (8:2; v/v)	HPLC-MS Column: C30 (250 × 4.6 mm, 3 µm) Mobile phase: methyl-tert-butyl-ether/water (A: 81:15:14; B: 7:90:3) Gradient elution Injection: 30 µl Flow rate: 1 ml/min
Spain *N* = 70 Xavier et al. (2018)	α + β-CA, L, Z, LY, β-CR; xanthophyll esters			UHPLC

(continued)

Table 4 (continued)

Study	Analyte	Analytical method		
		Milk fat analysis	Extraction protocol	Detection system
China $N = 56$ Xu et al. (2019)	L, Z, trans-L	–	Milk sample + tetrahydrofuran; Saponification: + 5% methanolic KOH; extraction: 50 mg BHT +200 ml dichloromethane +400 ml petro ether +400 ml hexane	HPLC PAD detectors Column: C30 (150 × 4.6 mm, 3 μm) Mobile phase: methyl-tert-butyl-ether Gradient elution Injection: 50 μl Flow rate: 1 ml/min
Korea $N = 98$ Kim et al. (2018)	L	–	1 ml milk +8 ml acetone +0.1% BHT; incubation (20 min, 37 °C); centrifugation (3000 rpm, 10 min); filtrating through a syringe filter	HPLC RP (reversed phase) Column: C30 (250 × 4.6 mm, 5 μm) Mobile phase: methanol/methyl-tert-butyl-ether Gradient elution Flow rate: 1 ml/min
Korea $N = 34$ Duan et al. (2019)	β-CA, α-CA, LY, L + Z, β-CR	MilkoScan FT2	5 g of milk +10 ml pyrogallol solution (6% in ethanol); vortex (2 min), evaporation with nitrogen (1 min); sonication (10 min) + 8 ml KOH (60%); vortex (2 min), water bath 60 min, 75 °C, 100 rpm) + 30 ml 3% NaCl +15 ml hexane/ethyl acetate (85:15; v/v, 0,01 BHT), vortex	HPLC UV-Vis detectors Column: C18 (250 × 4.6 mm, 5 μm) Mobile phase: acetonitrile/methanol/methylene, (75:20:5, v/v/v) Gradient elution Injection: 20 μl Flow rate: 1 ml/min Time: 40 min
Poland $N = 53$ Zielinska et al. (2019)	β-CA, LY, L + Z	MIRIS HMA	Saponification: 2 ml milk +500 μl 12% pyrogallol +50 μl 1% ascorbic acid in 0.1 M HCL, + 1.5 ml 30% KOH + 2.5 ml ethanol, vortexing (30 sec), incubation in water bath (40 min, 50 °C); ice cooling; 3 x extraction: + 1 ml NaCl +1 ml n-hexane; shaking 3 min; centrifugation (8000 rpm, 10 min, 4 °C); evaporation in vacuum (30 min, 30 °C); dissolved in 0.5 ml hexane	HPLC UV-Vis detectors Column: C18 (250 × 4.6 mm) Mobile phase: A (acetonitrile/methanol, 90:10, v/v) and B (methanol/ethyl acetate (34:16, v/v) Injection: 100 μl Flow rate: 1 ml/min

(continued)

Table 4 (continued)

Study	Analyte	Analytical method		
		Milk fat analysis	Extraction protocol	Detection system
Multinational study Nguen et al. (2020)	L	–	Saponification: 2 ml milk +4 ml ethanol (0.1% wt./v) + 1 ml NaCl (2% wt./v) + 4% ascorbic acid +1 ml KOH (60%, wt./v), incubation (70 °C, 60 min), cooling in ice water. 2 x extraction: 5 ml hexane, + 0.1% NaCl; evaporation under vacuum (40 °C, 30 min), dissolved in 100 µl isopropanol/hexane (75: 25 v/v; 0.025% BHT)	HPLC UV-Vis detectors Column: C30 (150 × 4.6 mm, 5 µm) Mobile phase: A (methanol/ acetonitrile/water, 4: 5:1; v/v/v) B (methyl-tert-butyl) Gradient elution Flow rate: 1 ml/min
Cambodia $N = 23$ Whitefield et al. (2020), Turner and Burri (2012)	β-CA, α-CA, LY, L + Z, β-CR	SpectraStar 2600 XT Neonatal Analyzer	Saponification: 100 µl of milk +0.1% BHT in ethanol +10% pyrogallol in ethanol +200 µl 15% KOH. Extraction: + 4 ml hexane, evaporation, dissolved in 50 µl acetonitrile	HPLC UV-Vis detectors Column: C18 (100 × 2.1 mm, 3.5 µm) Injection: 20 µl
Bangladesh $N = 18$ Hampel et al. (2017)	β-CA	Creamatocrit		HPLC UV-Vis detectors Column: C18 (100 × 2.1 mm, 3.5 µm) Injection: 25 µl Flow rate: 0.6 ml/min
Indonesia $N = 212$ Gibson et al. (2020)	β-CA, α-CA, β-CR	Creamatocrit		
Switzerland $N = 28$ Redeuil et al. (2021), Levêques et al. (2019)	β-CA, L, Z, LY, β-CR	–	750 µl milk +5 µl BHT ethanolic solution (79 g/ l) + 10 µl deferoxamine mesylate aqueous solution (10 mg/ml) + 1 ml ethanol +25 µl internal standard, mixing; 2 x extraction: + 2.5 ml n-hexane-ethyl acetate (90:10; v/v) with 350 mg/l BHT, mixing (4 min), centrifugation (1200 x g, 10 min, 4 °C); + n-hexane-ethyl acetate (90: 10; v/v), evaporation and dissolved in 125 µl isooctane-ethyl acetate (90:10; v/v), centrifugation (11,000 x *g*, 10 min, room temperature)	HPLC UV-Vis detectors Mobile phase: A (n-hexane) B (n-hexane-dioxane, 50:50, v/v + 0.001% acetic acid) Gradient elution Injection: 5 µl Flow rate: 0.3–0.4 ml/min Time: 22 min

(continued)

Table 4 (continued)

Study	Analyte	Analytical method		
		Milk fat analysis	Extraction protocol	Detection system
China $N = 42$ Wu et al. (2020), Schimpf et al. (2017)	β-CA, L, Z, LY, β-CR	–	0.5 ml milk +4 ml water +0.5 g sodium ascorbate, 10 ml methanol + 1 ml tetrahydrofuran. Saponification: 1 ml aqueous KOH (45%, wt./v), water bath (65 °C, 15 min); extraction: +10 ml hexane-methyl-tert-butyl-ether (3:1; v/v); evaporation and dissolved in 400 µl hexane-methyl-tert-butyl-ether	HPLC UV-Vis detectors Column: C30 (250 x 4.6 mm, 3 µm) Mobile phase: A (methanol) B (hexane-methyl-tert-butyl-ether) Gradient elution Injection: 5 µl Flow rate: 0.3–0.4 ml/min Time: 22 min

ACN = acetonitrile. BHT = butylatedhydroxytoluene. α-CA = α-carotene. α-CR = α-cryptoxanthin. β-CA = β-carotene. β-CR = β-cryptoxanthin. HCl = hydrochloric acid. HPLC = high-performance liquid chromatography. KOH = potassium hydroxide. L = lutein. LY = lycopene. L + Z = lutein+zeaxanthin. UHPLC = ultra-high-performance liquid chromatography. HPLC-MS = high-performance liquid chromatography-mass spectrometry. THF = tetrahydrofuran. UPLC = ultra-performance liquid chromatography. Z = zeaxanthin

Nutritional Assessment

Data about maternal nutrition was collected using a variety of methods (Table 3): FFQ covering different periods (Meneses and Trugo 2005; Cena et al. 2009; Denić et al. 2019), 24-food record (Canfield et al. 2003; Xue et al. 2017; Machado et al. 2019), and 3-day dietary record (Panagos et al. 2016; Kim et al. 2018; Xu et al. 2019; Zielinska et al. 2019a) repeated one (Canfield et al. 2003; Meneses and Trugo 2005; Panagos et al. 2016; Xue et al. 2017, Kim et al. 2018; Xu et al. 2019; Denić et al. 2019) or more times. Studies evaluated the association between breastmilk carotenoids and the intake of specific food groups (Canfield et al. 2003; Denić et al. 2019) or the intake of total carotenoids (Meneses and Trugo 2005) or specific carotenoid (Cena et al. 2009; Xue et al. 2017; Panagos et al. 2016; Kim et al. 2018; Xu et al. 2019; Zielinska et al. 2019a; Machado et al. 2019). Studies assessing carotenoid intake mainly calculated it based on USDA food database because specific national food databases are often lacking in data about carotenoids in different foods (Amorim-Carrilho et al. 2014; Panagos et al. 2016; Xue et al. 2017; Kim et al. 2018; Xu et al. 2019; Zielinska et al. 2019a; Machado et al. 2019). Unfortunately, data collection in a short period of time, lack of national database of carotenoid contents in foods, or collection of data via FFQ may lead to inaccurate evaluation of carotenoid intake (Amorim-Carrilho et al. 2014). In consequence, this may lead to a lack of bias in the analysis of association between maternal intake of carotenoids and its breastmilk concentrations.

Data Collection

The most recommended method of the collection of dietary data is at least a 3-day dietary record, which allows to collect all abundant information about consumed foods, its portion size, and its preparation method, as well as minimize the bias related to day-to-day variation, allowing the calculation of energy and nutrient intake (Shim et al. 2014; Ortega et al. 2015; Burrows et al. 2017). Carotenoid's bioavailability increases in the presence of dietary fat and after selected food processing (e.g., chopping and cooking), but reduces in the presence of dietary fiber (Reboul 2019; Böhm et al. 2021), especially soluble fractions (pectin) (Hamułka 2009). Also, individual genetic characteristics related to intestinal transporters and digestion enzymes may influence its bioavailability (Krinsky and Johnson 2005; Böhm et al. 2021). The 3–7-day records allow obtaining all the nutritional data that influence carotenoid bioavailability. However, this method is related to higher costs and time and it is difficult for participants; when the use of the 3–7-day dietary record is impossible, then the use of semiquantitative FFQ may be useful (Shim et al. 2014). Another possibility is a qualitative analysis of maternal nutrition. Previous study confirmed correlations between FFQ and plasma carotenoids in plasma. Moreover, FFQ covers a longer period of time, so it applies to many types of vegetables and fruits consumed seasonally (Burrows et al. 2017; Yuan et al. 2018). Previously it was shown that fruit and vegetable intake assessed by FFQ was correlated with plasma carotenoids (Burrows et al. 2015), which are a good biomarker of their intake (Baldrick et al. 2011). However, the use of FFQ should be validated and it should include not only different types of vegetables and fruits but also different methods of their preparation as these can affect carotenoid bioavailability (Burrows et al. 2015; Dias et al. 2018). The utilization of semiquantitative or qualitative FFQ is especially useful when the study has a cross-sectional design and assessed the maternal nutrition and biomarkers (Burrows et al. 2017). In the studies conducted, biomarkers may not reflect the same period as in nutritional assessment, e.g., 24-food record, so using both methods of collection of nutritional data may be beneficial. Another important issue, which should be considered during nutritional assessment, is the usage of dietary supplements, which is very common among pregnant and lactating individuals. As many supplements contain carotenoids (Böhm et al. 2021), assessment should include also quantitative evaluation of carotenoid intake via dietary supplements.

Carotenoid Database

A proper assessment of semiquantitative and quantitative data requires a valid database of carotenoid level and isoforms in nationally available food products, including different crop varieties (Amorim-Carrilho et al. 2014; Dias et al. 2021). So, the development those databases covering different food products and methods of preparation is a crucial step in the studies assessing carotenoid intake, because carotenoid levels are different in raw and processed products (Dias et al. 2018).

Moreover, it is important to include less common carotenoids, such as astaxanthin, phytoene, phytofluene, capsanthin, canthaxanthin, and fucoxanthin (Böhm et al. 2021; Liu et al. 2021).

Considering that foods contain different carotenoids in different levels, it is recommended to consume a diversified diet to obtain appropriate levels of the major health-promoting dietary carotenoids.

Applications to Prognosis, Other Diseases, or Conditions

In this chapter, we reviewed studies and methods assessing breastmilk carotenoids and their maternal intake. Previous studies showed that breastmilk carotenoids are highly correlated with infant carotenoid status (Henriksen et al. 2013; Sherry et al. 2014; Lipkie et al. 2015; Sun et al. 2021). Moreover, before complementary feeding starts breastmilk is the only source of carotenoids for infants, as the infant formula is not supplemented with carotenoids (Jewell et al. 2004), and breastfed infants have higher plasma carotenoid levels than those formula fed (Sommerburg et al. 2000; Bettler et al. 2010). Interestingly, breastfeeding may have a longitudinal impact on children's carotenoid status in childhood (Liu et al. 2021). Currently, the potential health benefits of carotenoids in early postnatal development were mostly investigated for lutein and zeaxanthin (Giordano and Quadro 2018). Carotenoids are very effective antioxidants and immunomodulators (Krinsky and Johnson 2005; Jomova and Valko 2013; Zielinska et al. 2017a, b; Giordano and Quadro 2018). Xanthophylls, lutein, zeaxanthin, and β-cryptoxanthin are accumulated in retina and brain regions related to cognitive function and probably are crucial for optimal neurodevelopment and visual performance (Henriksen et al. 2013; Jeon et al. 2018). Breastmilk and maternal lutein were associated with a better recognition memory in infancy (Cheatham and Sheppard 2015) and visual acuity (Lai et al. 2020). In other studies, breastmilk β-carotene was related to infant motor development at the 6 months of life (Zielinska et al. 2019b). It was hypothesized that lutein and zeaxanthin may protect premature infants against elevated oxidative stress and diseases related to prematurity, such as retinopathy of prematurity but results are inconclusive and further research is needed (Cota et al. 2020). There is no doubt that higher intake of carotenoids, especially with vegetables and fruits, will be also associated to health benefits in mothers, including reduced risk of noncommunicable diseases and maintenance of cognitive performance (Fig. 3) (Krinsky and Johnson 2005; Johnson 2014; Zielińska et al. 2017b; Giordano and Quadro 2018).

Mini-Dictionary of Terms

- ***Breastmilk***. *Complex and dynamic biological fluid rich in nutrients and bioactive compounds produced in mammary glands during lactogenesis.*
- ***Carotenes.*** *Non-oxygenated, less polar, the fraction of carotenoids,* e.g. *β-carotene, α-carotene, lycopene.*

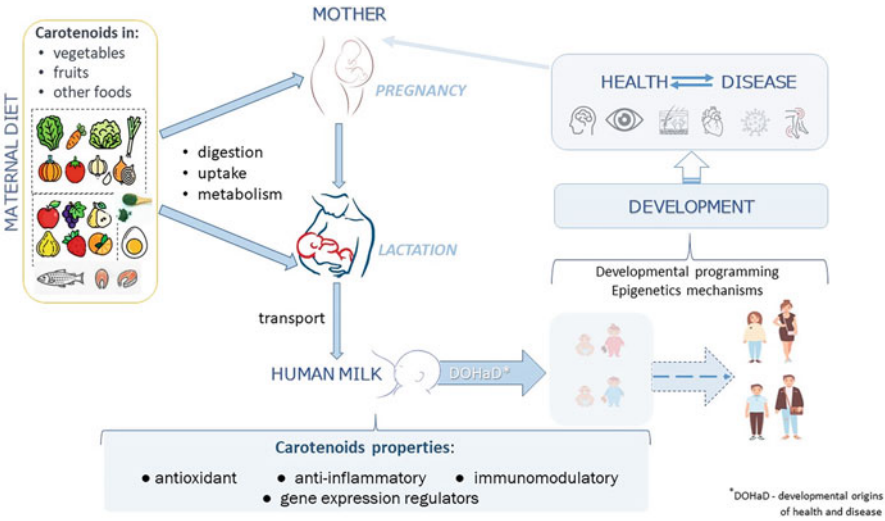

Fig. 3 Association between maternal carotenoid intake, breastmilk, and children development and health. DOHaD = Developmental Origin of Health and Disease. Skin by Mahmure Alp from the Noun Project

- ***Carotenoids.*** *Fat-soluble, natural pigments produced by plants and microorganisms and are responsible for the color of a variety of foods. Some carotenoids have provitamin A properties (β-carotene, α-carotene,* β-cryptoxanthin).
- ***Colostrum.*** *Breastmilk is produced in low volume after birth: low in fat, high in protein, immune factors. Thanks to high levels of carotenoids, colostrum is often yellow or orange in color.*
- ***Foremilk.*** *The first milk at the beginning of every single feeding. Compared to hindmilk it contains a little amount of fat (1–2%).*
- ***Hindmilk.*** *Breastmilk at the end of every single feeding. Compared to foremilk it contains more fat and carotenoids.*
- ***Mature milk.*** *Breastmilk produced since 3–4 weeks postpartum*
- ***Transitional milk.*** *Breastmilk produced after colostrum and mature milk. The fat concentrations, energy value, and volume gradually increase in transitional milk.*
- **Xanthophylls.** Oxygenated hydrocarbon molecules, more polar than carotenes, e.g., lutein and zeaxanthin (L + Z), β-cryptoxanthin, and astaxanthin.

Key Facts of Carotenoids in the Breastmilk

- Humans cannot synthesize carotenoids and rely on their carotenoid intake with diet, mainly vegetables and fruits, algae, or some animal foods, e.g., egg yolk, salmon, and rainbow trout.
- Major dietary, plasma, and breastmilk carotenoids are β-carotene, α-carotene, lutein, zeaxanthin, lycopene, and β-cryptoxanthin.

- Breastmilk carotenoids are related to maternal dietary intake and influence infant carotenoid status.
- Dietary carotenoids may influence infant growth and development and health in later life through metabolic programming and epigenetic mechanisms.

Summary Points

- Breastmilk carotenoids vary between different populations and geographical locations and according to circadian and within-feeding variations in breastmilk composition, maternal nutritional status, age, and premature birth.
- Breastmilk sample collection protocol for carotenoid analysis should take into account circadian and within-feeding variation in milk composition, data about infant feeding, and method of milk expression.
- During storage and handling breastmilk samples should be protected against oxygen, light, and temperature. Between collection and analysis samples should be stored in a very low temperature (−80 °C) for as short period of time as possible.
- The most popular method of breastmilk carotenoid analysis is HPLC or U-HPLC. Prior to analysis breastmilk samples should be saponificated, hydrolyzed, and extracted.
- The best protocol for collection of nutritional data is at least a 3-day food record and additional FFQ (if repeated records are not possible). Development of national databases of food carotenoids is necessary.

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