Research Article

Xanthophyll esters accumulate in the human mammary gland

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Abbreviations: HPLC, high-performance liquid chromatography; **SPE**, solid phase extraction; **APCI**, atmospheric pressure chemical ionization; **TOF**, time of flight; **MFGs**, milk fat globules

Abstract

Scope: Carotenoids in human milk are associated with other lipid counterparts in several metabolic processes. One interesting association that has not been demonstrated to date is the presence of xanthophyll esters. Colostrum and mature milk samples were analysed to determine the occurrence of xanthophyll esters and identify the compounds. Thus, the association of the amounts of these compounds with lactation and whether they are significant contributors to the carotenoid profile of human milk was assessed.

Methods and results: Pre-term and term delivering mothers were included in the study to donate colostrum at 3-5-days postpartum and mature milk at 15-days postpartum. Carotenoids extracts were subjected to a clean-up procedure to remove the triacylglycerol fraction and then analysed by HPLC-MS^n. Identification of xanthophyll esters was achieved by considering their chromatographic behaviour, UV-visible characteristics and MS^n features.

Conclusion: Xanthophyll esters are significant contributors to the carotenoid profile in the colostrum, while mature milk does not contain these compounds. Therefore, fatty acid acylation to xanthophylls is activated during the accumulation of carotenoids in the human mammary gland. The sharp decline in the amount of xanthophyll esters in mature milk indicates that the lipophilic components are those recently incorporated in the mammary epithelium.

1 Introduction

Efforts have been made worldwide to reinforce breastfeeding of infants from birth to 4-6th months of age, and thereafter, breastfeeding should be completed with complementary foods to supplement the limited nutrients in human milk[1,2]. This determination by international health agencies is based on knowledge that human milk is the ideal nutrition for newborns, supporting healthy growth and development through a wide array of nutrients and non-nutrient factors; breast milk also reduces infant morbidity and mortality[3]. Most references made to the composition of human milk include carotenoids as the representative lipid constituents present in colostrum and in mature milk[4,5]. Diet is the only origin of these compounds, and after delivery, maternal milk provides them to the infant, where they play different roles. Carotenoids with a β-ionone ring structure are vitamin A precursors that potentially complement the source of vitamin A for the newborn[6]. These carotenoids and the non-provitamin A lycopene, lutein and zeaxanthin, which are supplied to the nursing infant in human milk, have antioxidant activities and may enhance the immune system[7]. Lutein and zeaxanthin have received special attention, as they prevent retinal damage through blue-light filtration and their antioxidant properties, which is of significant benefit during the first months of life until complete development of the eye is achieved[8,9]. Several studies have reported the direct correlation between the dietary carotenoid supply and qualitative pattern of human milk, resulting in significant differences among the populations of different countries, and even extreme variations in concentration are observed within the same region[10-14]. Within individuals, there is large variability in carotenoid content, depending on maternal dietary patterns, feeding patterns and the stage of lactation.

Carotenoids in human milk are particularly associated with other lipid counterparts in several ways. Although the transport and processing of carotenoids in the mammary gland and following secretory mechanisms to milk are not fully understood, it has been observed that the amount and class of lipids and specific distribution of carotenoids into lipoproteins, in which

they are transported[15], are determining factors that should contribute to different carotenoid patterns during their accumulation in the mammary gland and in the subsequent stages of lactation (colostrogenesis and lactogenesis). Indeed, the same receptors that mediate the selective uptake of lipoproteins and cholesteryl ester, scavenger receptor class B type I (SR-BI) and cluster determinant 36 (CD36), are shown to actively facilitate the cellular uptake of carotenoids in human epithelial tissues, such as those of the intestine and kidney[16,17]. Consequently, the same receptors could be involved in the selective transport of carotenes and xanthophylls in the epithelium of the mammary gland as well. Furthermore, some differences in the carotenoid concentration within and between individuals or among different laboratories are corrected when the figures are reported relative to the milk lipid content[18]. However, another association among carotenoids and lipids that has been speculated, although not demonstrated to date, is the presence of carotenol fatty acid esters in human milk. Fatty acids esterify hydroxyl xanthophylls very frequently in most plant tissues, including fruits and vegetables that are common in our diet[19]. However, xanthophyll esters are hydrolysed in the small intestine before absorption, so that any amount or a very low amount of xanthophyll esters is found in the serum or the peripheral tissues, such as human skin[20,21]. Therefore, re-esterification seems to be unfeasible, at least in serum, but not in the intestinal epithelium, where the esterification of xanthophylls from dietary sources has been demonstrated *in vitro* and follows a similar pathway to that of cholesterol trafficking, including de-esterification in the intestinal lumen, cellular absorption and re-esterification by some acyltransferases and, finally, transport in chylomicra to the liver.

Information regarding carotenol fatty acid esters has only been obtained from the analysis of extracts prepared without alkaline saponification. This step is commonly applied to obtain free carotenoid forms, simplifying the identification and quantification of the possible wide range of bound combinations among fatty acids and xanthophylls initially present in the sample. Additionally, chemical saponification removes triacylglycerides and other lipid constituents that

may interfere with analysis, particularly when mass-spectrometry is applied, and that may accumulate in the column, reducing its analytical performance and lifetime.

The aim of this work was to investigate the presence of carotenol fatty acid esters in human milk at two stages of lactation, colostrum and mature milk. Hence, it will be possible to evaluate the association of the levels of these compounds with lactogenesis and whether they are significant contributors to the carotenoid profile of human milk.

2 Materials and Methods

2.1 Chemicals and reagents. Solvents for HPLC-MS (methanol, methyl *tert*-butyl ether and water) and solvents of analytical grade (diethyl ether, hexane, S_2C) were obtained from Panreac (Barcelona, Spain). KOH and NaCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solid-phase extraction columns (SPE) were obtained from Restek (Bellefonte, PA, USA). Lutein, zeaxanthin, lycopene, β-carotene and β-cryptoxanthin standards were obtained from Extrasynthese (Genay Cedex, France). Oleoresins from paprika (*Capsicum annuum* L.) and marigold (*Tagetes erecta* L.) were a gift from Evesa (La Línea de la Concepción, Cadiz, Spain) and Kemin (Lisbon, Portugal), respectively.

2.2 Samples. Breast milk samples from 30 mothers were collected at the Unidad de Neonatología of the Hospital Universitario Virgen del Rocío (Sevilla, Spain). Women were eligible to join the study if they had no chronic diseases and were nonsmokers on a regular diet without supplements. Pre-term (30-36th week of pregnancy) and term (37-42^{on} week of pregnancy) delivering mothers were included to donate colostrum at 3-5-days postpartum. The same mothers provided mature milk samples at 15-days postpartum, so that dependent samples were obtained for data comparison. The total milk volume of one breast was collected into a polypropylene bottle. After collection, the samples were transported directly to the laboratory and stored at -80ºC until analysis. The subjects provided informed consent after the study protocol was approved by the Ethics Committee of the Hospital Universitario Virgen del Rocío

and the Bioethics Subcommittee of the Spanish National Research Council (AGL2013-42757R).

2.3 Extraction of carotenoid fraction. The experimental conditions described previously were used with slight modifications[22]. Human milk (3 mL) was vortex-mixed with methanol (6 mL) for 2 min and cooled at -20 ºC for 20 min. Subsequently, the cooled mixture was centrifuged at 10,000×g and 4 ºC for 5 min, and the upper layer was discarded. Diethyl ether (5 mL) and hexane (2 mL) were added to the pellet, and the sample was vortex-mixed for 2 min. Then, 5 mL of NaCl 10% (w/v) were added, and the sample was vortex-mixed again for 2 min. After centrifugation (10,000×g and 4 ºC for 5 min), the organic layer was isolated in a rotatory flask, the water layer as discarded, and the pellet as extracted again as described above. The combined organic extracts were evaporated to dryness in a rotatory evaporator at 25 ºC, and the residue was dissolved in 1 mL of hexane. For analysis of the xanthophyll esters by HPLC-MS, the sample was applied to a graphitized carbon SPE column (500 mg, 6 mL) previously activated with 30 mL of methanol and equilibrated with 30 mL of hexane. Once the sample was placed on the SPE column, the triacylglyceride fraction was eluted with 30 mL of hexane. The carotenoid fraction retained in the column as eluted with 30 mL of S_2C and recovered in a flask. The solvent was removed in a rotatory evaporator at 25 ºC, and the residue was dissolved in 1 mL of methanol:methyl *tert*-butyl ether (8:2). This solution was filtered through a 0.22-μm filter and stored at -20 ºC until analysis[23].

2.4 Chemical hydrolysis of human milk. The experimental conditions for chemical hydrolysis of xanthophyll esters have been described previously[24] and were used with slight modifications. Human milk (3 mL) was mixed with 3 mL of KOH:methanol (20% w/v), and the mixture was incubated for 1 h. After hydrolysis, 6 mL of methanol were added, and the mixture was vortex-mixed for 2 min and cooled at -20 ºC for 20 min. Subsequently, the cooled mixture as centrifuged at 10,000×g and 4 ºC for 5 min, and the upper layer was discarded. Diethyl ether (5 mL) and hexane (2 mL) were added to the pellet and vortex-mixed for 2 min. Then, 5 mL of

NaCl 10% (w/v) were added, and the sample was vortex-mixed again for 2 min. After centrifugation (10,000×g and 4 ºC for 5 min), the organic layer was washed with water until a neutral pH was reached. Finally, the organic extract was evaporated to dryness in a rotatory evaporator at 25 ºC, and the residue was dissolved in 1 mL of methanol:methyl *tert*-butyl ether (8:2). This solution was filtered through a 0.22-μm filter and stored at -20 ºC until analysis.

2.5 Determination of the lipid content. The lipid content of human milk samples was determined according to the solvent extraction procedure followed by gravimetry[25].

2.6 Liquid chromatography-Mass Spectrometry. The carotenoid profile in human milk samples was separated using a Dionex Ultimate 3000RS U-HPLC (Thermo Fisher Scientific, Waltham, MA, USA) as previously described[26]. Briefly, a C30 reversed-phase YMC (analytical column (250 mm×4.6 mm (i.d.), YMC, Europe, Schermbeck, Germany) with a 3-μm particle size was used for separation. The mobile phases were mixtures of methanol:*tert*-butyl methyl ether:water (A, 81:15:4 and B, 7:90:3). The program started with 10 min of isocratic A (100%), followed by a gradient of B at 50% for 40 min, B at 100% for 50 min, A at 100% for 55 min and isocratic A at 100% from 55 to 60 min. The flow rate was 1 mL/min, and the injection volume was 30 μL. The UV-visible spectra were recorded from 350 to 600 nm with a photodiode array spectrometer. A split post-column of 0.4 mL/min was introduced directly on the mass spectrometer ion source. Mass spectrometry was performed using a micrOTOF-QII high resolution time-of-flight mass spectrometer (UHR-TOF) with qQ-TOF geometry (Bruker Daltonics, Bremen, Germany) equipped with an APCI source. The instrument was operated in positive ion mode using a scan range of *m/z* 50-1200. Mass spectra were acquired using broad band Collision Induced Dissociation (bbCID) mode, providing MS and MS/MS spectra simultaneously. The instrument control was performed using Bruker Daltonics Hystar 3.2 software. The in-house mass database created *ex professo* contains the monoisotopic masses, elemental composition and, optionally, the retention time and characteristic product ions for 360 carotenes, xanthophylls and xanthophyll esters. Data evaluation was performed with the Bruker

Daltonics DataAnalysis 4.0 software. From the HPLC/qTOF-MS data, automated peak detection of the extracted ion chromatograms expected for the $[M+H]^+$ ion of each compound in the database was accomplished with a script command editor. High-resolution mass spectrometry measurements were completed on the basis of mass accuracy and in combination with the isotopic pattern in the SigmaFit algorithm. This algorithm provides a numerical comparison of the theoretical and measured isotopic patterns (mSigma value). Only positive records with mass accuracy and mSigma values below the threshold limits (5 ppm and 50 for the mass error and mSigma value, respectively) were included in the final identification table. The experimental mass spectrum characteristics and the MS^2 data were compared with the data available in the literature for carotenoid identification in the human milk samples[26-32]. The behaviour of the colostrum xanthophyll esters in the MS and MS^2-based reactions was compared with that of vegetable xanthophyll esters in the reference materials (paprika and marigold oleoresins), as previously described[26, 33-35]. Stock solutions were prepared for β-carotene, β-cryptoxanthin, lutein, and lycopene at a concentration of approximately 25 mg/L. Once the exact concentration was determined, working stock solutions for external calibration curves were prepared at 5 concentration levels ranging 0.150 to 10 mg/L. The content of xanthophylls and carotenes were determined in the unsaponified extract. The xanthophyll esters content was expressed as the corresponding free xanthophyll equivalents [32, 33].

2.7 Statistical analysis. Values are reported as the median and range or as percentages. The data were analysed using a non-parametric statistical procedure in the SPSS software (IBM® SPSS® Statistics, version 22). The differences between the carotenoid content in human milk samples at the two stages of lactation were evaluated using the Wilcoxon test for paired data, with the significance set at $P<0.05$.

3 Results

Figure 1a depicts the HPLC trace of a typical direct extract of a human colostrum sample. The carotenoid profile contains the xanthophylls zeaxanthin, lutein and β-cryptoxanthin as well as the carotenes α-carotene, β-carotene and lycopene. Other representative chromatographic peaks show the UV-visible pattern of the xanthophylls β-cryptoxanthin, zeaxanthin or lutein, and they disappear when a portion of the direct extract is saponified, as shown in Figure 1b. With these hints, those chromatographic peaks should correspond to xanthophyll esters, that is, combinations of different fatty acids bound to the previously mentioned xanthophylls. Identification of the xanthophyll esters was performed by assessing the chromatographic behaviour of the compounds on the C30 column, their UV-visible spectrum and their MS and MS^2 spectra and comparing it with the data reported in the literature when possible and profiles in reference materials when available. Additionally, high-resolution mass spectrometry data for each xanthophyll ester, the mass error and mSigma values helped support the identification of the compound in the sample, as those data provide a numerical comparison of the theoretical and measured mass value and isotopic pattern for the assumed protonated molecule. The results shown in Table 1 indicate the xanthophylls esters identified in colostrum samples, including their chromatographic, UV-visible and mass spectroscopic characteristics. The nature of the fatty acid bound to a xanthophyll was ascertained by those product ion(s) arising from the protonated molecule, which significantly contribute to the MS^2 profile of xanthophyll esters. Figure 2 shows the MS spectra of the representative esters of lutein/zeaxanthin identified in human colostrum samples. Thus, the fragmentation reaction from the lutein/zeaxanthin monoester protonated molecule $[M+H]^+$ yields a product ion at *m/z* 551.4253, the calculated mass corresponding to the $[M+H\text{-fatty acid}]^+$, from which the mass of the fatty acid originally bound to lutein is obtained. Occasionally, in some of the MS^2 spectra of the lutein/zeaxanthin monoesters, the main product ion arising from the protonated molecule was observed at *m/z* 549.4096 (calculated mass, Figure 2b). This product ion corresponds to the loss of the fatty acid

and a subsequent γ-charge site rearrangement instead of the most frequent α-charge site. In the case of β-cryptoxanthin monoesters, the fragmentation reaction yields a product ion at *m/z* 535.4304 corresponding to $[M+H\text{-fatty acid}]^+$, which allows the identification of the fatty acid bound to β-cryptoxanthin. The identification of various signals corresponding to xanthophyll ester, which disappears from the chromatogram once the sample is saponified, was not possible in some samples because of their low intensity or low-quality MS or MS^2 spectra. Additionally, not all of the xanthophylls esters identified in Table 1 were present in all samples at once, and their quantitative contribution to the carotenoid profile was also sample-dependent. Therefore, to estimate the significance of the xanthophyll esters to the quantitative profile of carotenoids in colostrum and mature milk, the amounts of xanthophyll esters in each sample were added and considered a single group. Then, a statistical study was performed with those figures. Table 2 shows the data regarding the carotenoid content, including the free xanthophylls, carotenes, xanthophylls ester group, and total carotenoid content, distinguishing colostrum (3-5 days postpartum) and mature milk (15 days postpartum). Thus, the total carotenoid content in colostrum (4328.7 nmol/L) was much higher than that in mature milk (657.9 nmol/L), as mature milk represents 14.5% of the colostrum group. The content of the di-hydroxylic xanthophylls zeaxanthin and lutein showed the lowest reduction in mature milk, although with significant decreases ($P<0.05$) of 34% and 65%, respectively, while the carotene content in mature milk represents 10-15% of the colostrum, which is also a significant reduction (85-90%, $P<0.05$). However, the highest reduction in carotenoid content during lactogenesis corresponded to xanthophyll esters, which showed a decrease of 100% from colostrum (504.0 nmol/L) to mature milk (0). Indeed, xanthophyll esters were only observed in two mature milk samples, while traces or no amounts were found in the rest of mothers. These trends are reproduced independently, no matter the unit used to represent the data (either per L of milk or per g of fat in milk). The milk fat concentration was 3-5%. The lutein and zeaxanthin contents were significantly correlated either in colostrum ($R^2_{adj}=0.669$, $P<0.01$) or mature milk ($R^2_{adj}=0.579$,

243 $P<0.01$). Some relationships among different carotenoids were analysed to probe the significant
244 biochemical changes in the carotenoid composition during lactation. Thus, the ratio of the free
245 xanthophyll content to the sum of the carotene and xanthophyll ester contents increased
246 significantly from 0.55 in colostrum to 1.57 in mature milk. However, the ratio of the total
247 xanthophyll content (free and esterified) to the total carotene content showed no significant
248 differences during the progression of lactation, as it was 1.57 in colostrum and 1.57 in mature
249 milk.

250 **4 Discussion**

251 The qualitative profile of the carotenoids in colostrum and mature human milk described here
252 for Spanish mothers is comparable to those reported in previous works, with wide variations in
253 contents among donors and the characteristic significant decrease in concentration with the
254 progress of lactation, already reported in literature[10, 18, 37-39]. Thus, quantitatively, the data
255 are higher than those described in several multinational studies, for colostrum and mature milk,
256 but they are similar to the carotenoid content described for Northern Ireland mothers[40]. These
257 data should be correlated with differences and similarities in dietary carotenoid intakes among
258 geographical regions, depending on food availability and culture. Spain and Northern Ireland
259 populations have the same intake levels of fruit and vegetables (>400 g/d), while lower intake
260 values[41] are observed in countries with lower breast milk carotenoid contents[37-39].
261 This is the first study to report that the significant presence of xanthophyll esters in the initial
262 stage of lactation (colostrum) almost disappears in mature milk. Xanthophyll esters have a
263 substantial content in our diet[19, 42], but they are hydrolysed before absorption in the intestinal
264 lumen[42, 43]. Thus, no amounts or very low amounts of xanthophylls esters have been
265 described to circulate in human serum [20, 44, 45] and these references did not include a
266 saponification step that would have masked their presence. The analysis of some other human
267 tissues revealed the absence of xanthophyll esters, although several studies do include the
268 saponification step in sample preparation[15, 27], with the exception of the analysis of

carotenoids in human skin, where xanthophyll esters accumulate, but in low amounts[21]. Therefore, the possibility of a noteworthy re-esterification process was not anticipated.

Here, we show that one-third of the total xanthophyll content in the colostrum is esterified meaning that fatty acid acylation to xanthophylls is activated during the accumulation of carotenoids in the human mammary gland. The presence of xanthophyll esters in the colostrum and their subsequent decline during lactation are compatible with the accepted lipid secretion pathway in mammary epithelial cells. Thus, the lipid bodies formed by newly synthesized lipid molecules are transported from the cytoplasm to the apical region of the cell, where they are secreted as milk fat globules (MFGs) enveloped by the cellular membrane[46, 47]. Hence, several lipid constituents of MFGs are characteristic membrane lipids that are associated with the lipid droplets in milk during secretion. Indeed, it has been demonstrated that carotenoids are distributed on the surface of the MFGs[10, 48]; thus, they are originally constituents of the cellular membrane of the mammary epithelium. At the initial stage of lactation, the secretion of colostrum contents the MFG membrane carotenoids accumulated in the mammary epithelium, and our data show that most of them are carotenes and xanthophyll esters. With the progress of lactation, the profile of the MFG membrane carotenoids is dominated by free xanthophylls and carotenes and lacks xanthophyll esters. Once lactation starts, milk secretion is a continuous process and the MFG membrane lipids are those recently incorporated into the mammary epithelium[46]. In such an intensive refurbishment process, there is no time (or need) to perform some biochemical processes, such as fatty acid acylation to xanthophylls.

These results lead to several new questions regarding the biochemical scenario of xanthophyll esterification in human tissues, including the candidate enzyme(s) involved in the synthesis of xanthophyll esters, factors that modulate the kinetics of the esterification reaction, and whether there are any biological implications for the esterification of structurally-related compounds, such as retinol, which esterification has been demonstrated to take place by mammary gland microsomes from the lactating rat[49] or other lipophilic substances (cholesterol), which are also released as fatty acid esters during lactation[50].

4 Concluding remarks

Xanthophyll esters are great contributors to the carotenoid profile at the initial stage of lactation, while mature milk does not possess these compounds. Therefore, fatty acid acylation to xanthophylls is activated during the accumulation of carotenoids in the human mammary gland, and the sharp decline in the xanthophyll esters content in mature milk indicates that the lipophilic components, including carotenoids, are those that have recently been incorporated into the mammary epithelium.

Author contributions: E. D. S., I. A. V., and J. A. M. carried out all human studies and sample collection. J. J. R., J. G. F., A.A.O.X, and M. J. G. performed the analytical studies. J. A. M., J. G. F., and A. P. G. supervised the study. A.A.O.X, J. G. F. and A. P. G. wrote the manuscript.

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Table 1.- The xanthophyll esters identified in human colostrum samples according to their chromatographic behaviour, UV-vis, and mass spectrometry characteristics as determined by HPLC-APCI$^+$-qTOF.

Carotenoid	R_t (min)	Elemental composition	$[M+H]^+$ calc. (Da)	$[M+H]^+$ meas. (Da)	λ_{max} (nm)	*m/z* found (Da)
Lutein-linolenic ester	34.6	$C_{58}H_{85}O_3$	829.6493	829.6415 [a]	421, 442, 472	829.6415 $[M+H]^+$, 577.4410 $[M+H\text{-}18\text{-}C_{16}H_{27}O]^+$, 549.4089 $[M+H\text{-}FA]^+$, 523.4295 $[M+H\text{-}18\text{-}C_{19}H_{29}O_2]^+$, 495.3633 [M+H-FA-56]
Zeaxanthin-linolenic ester	35.4	$C_{58}H_{85}O_3$	829.6493	829.6422 [a]	427, 450, 476	829.6422 $[M+H]^+$, 551.4259 $[M+H\text{-}FA]^+$
Lutein-arachidonic ester	36.2	$C_{60}H_{86}O_3$	855.6650	855.6595 [a]	421, 442, 472	855.6595 $[M+H]^+$, 579.4201 $[M+H\text{-}18\text{-}C_{19}H_{30}]^+$, 551.4260 $[M+H\text{-}FA]^+$, 523.4290 $[M+H\text{-}18\text{-}C_{21}H_{30}O_2]^+$, 495.3693 [M+H-FA-56]
Zeaxanthin-arachidonic ester	37.1	$C_{60}H_{86}O_3$	855.6650	855.6573 [a]	427, 450, 476	855.6573 $[M+H]^+$, 551.4254 $[M+H\text{-}FA]^+$, 495.3648 [M+H-FA-56]
Lutein-linoleic ester	37.8	$C_{58}H_{86}O_3$	831.6656	831.6680 [a]	425, 446, 476	831.6680 $[M+H]^+$, 551.4255 $[M+H\text{-}FA]^+$, 495.3661 [M+H-FA-56]
Zeaxanthin-linoleic ester	38.5	$C_{58}H_{86}O_3$	831.6656	831.6659 [a]	427, 450, 476	831.6659 $[M+H]^+$, 551.4259 $[M+H\text{-}FA]^+$, 495.369 [M+H-FA-56]
β-Cryptoxanthin-linolenic ester	40.7	$C_{58}H_{84}O_2$	813.6544	813.6581 [a]	428, 450, 477	813.6581 $[M+H]^+$, 535.4256 $[M+H\text{-}FA]^+$
β-Cryptoxanthin-linoleic ester	41.8	$C_{58}H_{86}O_2$	815.6701	815.6728 [a]	428, 450, 477	815.6728 $[M+H]^+$, 535.4258 $[M+H\text{-}FA]^+$
Lutein-oleic ester	43.7	$C_{58}H_{88}O_3$	833.6806	833.6837 [a]	420, 448, 474	833.6837 $[M+H]^+$, 577.4409 $[M+H\text{-}18\text{-}C_{16}H_{30}O]^+$, 551.4255 $[M+H\text{-}FA]^+$
β-Cryptoxanthin-oleic ester	44.5	$C_{58}H_{88}O_2$	817.6857	817.6852 [a]	428, 450, 477	817.6852 $[M+H]^+$, 535.4253 $[M+H\text{-}FA]^+$
Lutein-eicosenoic ester	45.8	$C_{60}H_{92}O_3$	861.7119	861.7094 [a]	420, 448, 474	861.7094 $[M+H]^+$, 579.4205 $[M+H\text{-}18\text{-}C_{19}H_{36}]^+$, 551.4253 $[M+H\text{-}FA]^+$
Zeaxanthin-eicosenoic ester	46.7	$C_{60}H_{92}O_3$	861.7119	861.7104 [a]	428, 451, 476	861.7104 $[M+H]^+$, 551.4259 $[M+H\text{-}FA]^+$

[a]Experimental *m/z* with a mass error and mSigma values below the threshold limits (5 ppm and 50, respectively). [b]FA= fatty acid.

Table 2.- The carotenoid contents of human colostrum and mature milk samples in nmol per volume or fat content. The values presented are the median and range in parenthesis.

	nmol/L milk		nmol/g fat	
	Colostrum	Mature milk	Colostrum	Mature milk
Lutein	486.4 (1588.1)[a]	199.8 (366.5)	22.43 (59.94)[a]	4.685 (12.53)
Zeaxanthin	98.11 (176.06)[a]	64.95 (78.26)	3.575 (12.20)[a]	1.785 (9.49)
β-Cryptoxanthin	962.6 (3075.6)[a]	145.3 (380.7)	34.64 (149.9)[a]	3.227 (14.27)
Lycopene	854.3 (3931.4)[a]	159.9 (499.5)	43.41 (145.0)[a]	3.835 (16.95)
α-Carotene	219.6 (727.0)[a]	14.86 (82.04)	9.227 (90.41)[a]	0.429 (1.76)
β-Carotene	754.8 (2153.8)[a]	53.28 (260.1)	30.08 (320.6)[a]	1.448 (5.58)
Xanthophylls esters	504.0 (3364.6)[a]	0 (152.7)	26.04 (154.4)[a]	0 (5.28)
Total	4328.7 (13643)[a]	657.9 (1151.4)	179.6 (800.46)[a]	15.99 (41.69)

[a]The data in the colostrum are significantly higher ($P<0.05$) than the corresponding values in mature milk.

Figure legends

Figure 1.- Chromatograms observed at λ=450 nm of a typical of human colostrum sample corresponding to the crude (a) or saponified (b) extract. Peak identification: 1: lutein; 1': *cis*-lutein; 2: zeaxanthin; 2': *cis*-zeaxanthin; 3: β-cryptoxanthin; 4: lutein-arachidonic ester; 5: lutein-linoleic ester; 6: zeaxanthin-linoleic ester; 7: α-carotene; 8: β-carotene; 8', *cis*-β-carotene; 9: lutein-eicosenoic ester; 10: zeaxanthin-eicosenoic ester; 11: lycopene; and 11': *cis*-lycopene.

Figure 2.- The mass spectra of lutein-oleic ester (a), lutein-linolenic acid (b); zeaxanthin eicosenoic ester (c) and lutein-arachidonic ester (d). The black diamond indicates the protonated molecule $[M+H]^+$ (a: 833.6837 Da; b: 829.6415 Da; c: 861.7094 Da; and d: 855.6595 Da). Some characteristic product ions are observed as described in Table 1.