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Enzymatic hydrolysis, extraction, and quantitation of retinol and major carotenoids in mature human milk

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An improved method for enzymatic hydrolysis, extraction, and high performance liquid chromatography (HPLC) separation of major carotenoids and retinol of mature human milk is described. After pretreatment with bile salts and protease, carotenoids and retinol are released by lipase treatment followed with brief chemical saponification. The HPLC method provides enhanced peak resolution and improved elution profiles. The procedure is nondestructive to provitamin-A carotenoids while effectively hydrolyzing retinyl esters. Using this method, we were able to significantly increase the recovery of both β-carotene and retinol from human milk over previously published procedures. The method is sensitive to picomolar quantities of carotenoids and retinol. Retinol, lutein/zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, and β-carotene are effectively recovered and quantitated from a single 1-mL sample of milk. (J. Nutr. Biochem. 9:178–183, 1998) © *Elsevier Science Inc. 1998*

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Introduction

Healthy mothers provide sufficient retinol in their breastmilk to support normal infant needs. However, the average daily dietary intake of vitamin A (retinol plus β-carotene) by lactating women in developing countries (660 RE/day) is substantially less than the recommended safe level for U.S. lactating women[1] and is often insufficient to support the needs of the infant. Unfortunately, in these countries, breastmilk is the major source of vitamin A for the infant.[2] In addition, breastmilk vitamin A may be useful as a non-invasive and reliable predictor of vitamin A status. Consequently, a sensitive method for simultaneous quantitation of vitamin A and carotenoids in breastmilk is needed.

We[3–5] and others[6] have previously reported methods for quantitation of carotenoids and retinol[1,7] in human milk and colostrum.[8] However, these methods were lengthy and for the assay of retinol, required prolonged exposure to alkali to hydrolyze retinyl esters. Because carotenoids were degraded by lengthy alkaline hydrolysis, separate procedures were necessary for analysis of milk retinol and carotenoids, and therefore both assay time and sample volume needed to measure vitamin A equivalents (vitamin A + provitamin A carotenoids) were doubled. We report here a method that combines enzymatic and chemical hydrolysis, thereby minimizing exposure to alkaline hydrolysis. Using this method, major milk carotenoids and retinol can be quantitated simultaneously in 1 mL of milk. The method can be completed in about half the time required for our previous procedure, is sensitive to picomolar quantities, and provides increased recovery of both β-carotene and retinol from milk.

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Methods and materials

Chemicals

Retinol and carotenoid standards, protease [Type XXV, Streptomyces griseus], lipase [Type VII, Candida rugosa], triethylamine (TEA), retinol, lutein, and lycopene were purchased from Sigma Chemical Co. (St. Louis, MO USA). α-Carotene and β-carotene (>97% purity) were from Fluka Biochemical Co. (Ronkonkoma, NY USA). β-Cryptoxanthin was a generous gift from Dr. Gary Beecher. Standard Reference (SRM) 968b (Fat-Soluble Vitamins and Cholesterol in Human Serum) was purchased from the National Institute of Standards and Technology (NIST) Analytical Chemical Division (Gaithersburg, MD USA). Methanol, hexane, tetrahydrofuran (THF), and acetonitrile (ACN) were purchased from Baxter (Muskegon, MI USA) and ethanol was from Quantum

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Chemical (Tuscola, IL USA). Butylated hydroxytoluene (BHT) was from Aldrich Chemical Co. (Milwaukee, WI) and ammonium acetate was from J.T. Baker Co. (Phillipsburg, NJ). All other reagents were technical grade or better and were purchased from Sigma Chemical Co. or Fisher Scientific Co. (Tustin, CA). All solvents used for HPLC chromatography were HPLC or spectrophotometric grade and were degassed and filtered through a 0.45-μm Fluropore filter (Millipore, MA USA) before use.

Sample collection

Mothers obtained full breast expressions in their homes using an electric breast pump (Ameda Egnell, Cary, IL USA) as previously described.[3,5] Participants were healthy, nonsmoking mothers in the Tucson metropolitan area, whose infants were between 1 and 4 months of age. Samples were held at 4°C in household refrigerators until they could be delivered to the laboratory (2 to 24 hr after expression). All subjects signed informed consent forms in accordance with the University of Arizona Human Subjects Committee.

Construction of the pool

Single full-breast expressions were obtained from seven healthy mothers in the Tucson metropolitan area. Samples from each mother were combined and a 250-mL portion was removed and stored at −80°C until construction of the pool. To construct the pool, the 250-mL portion was thawed at 37°C in an orbital shaker (Model 35127, Lab-line, Inc, Melrose Park, IL USA, 130 oscillation/min, 10 min) and multiple 1-mL portions were removed and stored in darkened vials at −80°C until analysis. A single pool sample was analyzed with each batch of experimental samples and the same pool was used throughout the study.

Hydrolysis

To thawed 1-mL samples, 10 mg $MgCO_3$ (basic salt), and 6 mg bile salt [sodium cholate:sodium deoxycholate, 1:1 (wt/wt)] were added and samples were incubated in an orbital shaking bath for 1 hr. Protease (Streptomyces griseus, 1 mg) and lipase (Candida cylindracea, 10 mg) were then added and incubation continued for an additional hour. Finally, samples were made 2.5 or 4.0 M in KOH by the addition of 0.5 mL or 1.0 mL of KOH:H_2O (1:1 wt/vol) and the mixture incubated for 0.5 hr. All steps were performed at 37°C in subdued lighting.

Extraction

After hydrolysis and saponification, absolute ethanol (0.5 mL) was added to each sample. The vial was then mixed vigorously by vortexing for 30 sec and held at room temperature for 10 min. Next, hexane (2 mL) was added followed by vortexing (1 min) and centrifugation (600 × g, 10 min). The resulting hexane layer was removed and saved in 2.0-mL polypropylene microcentrifuge tubes. The aqueous layer was re-extracted twice with hexane (1 mL). The combined hexane layers were evaporated just to dryness with nitrogen and re-suspended in 250 μL of THF/ACN (15:85, vol/vol). After agitation for 1 min in a water bath sonicator (Sonicor Instrument Corp, Copiague, NY USA), the sample was centrifuged (12,700 × g, 15 sec). Samples (50 μL) were then analyzed by HPLC as described below. All procedures were performed at 25°C.

Analysis of milk lipids

Milk lipid concentrations were determined by "creamatocrit" as described by Lucas et al.[9] and shown by us to give results comparable to that of standard gravimetric methods.[10]

HPLC Analysis

Analyses were performed with two Waters model 510 pumps (Waters Associates, Milford, MA USA), a Beckman 520 autosampler with a 50 μL-loop (Beckman Altex, Palo Alto, CA USA), a Milton Roy model SM4000 programmable UV/VIS detector (Milton Roy, Riviera Beach, FL USA), and a Waters Maxima 820 chromatography workstation equipped with a 5-μm YMC C_{18} reversed-phase column (4.6 × 250 mm). Components were eluted with 95% solvent A [ACN:THF (85:15, vol/vol) with 250 ppm BHT and 0.05% TEA] and 5% solvent B (50 mM ammonium acetate in methanol with 0.05% TEA) at a flow rate of 2.5 mL/min. Total time for a single HPLC analysis was 13 min. As a precautionary measure to prevent lipid buildup, the column was flushed overnight with 100% methanol. The same HPLC column was used throughout the study.

Carotenoids and retinol were quantitated using extinction coefficients $\epsilon_{dl/g\ cm,\ ethanol,\ 325\ nm} = 1850$ and $\epsilon_{dl/g.cm,\ hexane,\ 452\ nm} = 2592$ for retinol and trans β-carotene, respectively. The method was verified using authentic standards obtained from NIST in accordance with their "Round Robin" analytical quality control program (Standard reference material 968b: Fat-soluble vitamins and cholesterol in human serum). Retinol and lutein/zeaxanthin were quantitated using peak heights, lycopene; β-carotene and α-carotene were quantitated by integration of the area under the curve (AUC). Because the HPLC method did not provide baseline resolution of lutein and zeaxanthin, the sum of their concentrations is reported as a single value.

Quantitation

The HPLC system was calibrated at the beginning of the study using standard curves constructed from authentic β-carotene, α-carotene, β-cryptoxanthin, lycopene, lutein, and retinol, and the same curves were used throughout the study. Recovery of retinol and carotenoids was determined by internal standardization as follows. Before hydrolysis, authentic standards of retinol, lutein, lycopene, β-cryptoxanthin, α-carotene, and β-carotene were added to pooled milk for which peak heights and AUC had been determined previously. Recovery was calculated by the formula shown below.

$$[\text{nmol}] = \frac{[\text{nmol in pool} + \text{nmol authentic standard}] - [\text{nmol in pool}]}{[\text{nmol authentic standard}]} \times 100$$

For reliability determinations, pools were analyzed weekly for 6 weeks. Limits of detection were determined using a signal to noise ratio of 3:1. Descriptive statistics were performed using Microsoft Excel 5.0 (Microsoft Corp.)

Results and discussion

Our procedure for extraction, hydrolysis and quantitation of retinol and major carotenoids from human milk is outlined in *Figure 1* and the results of the assay are presented in *Table 1*. The method can detect picomolar quantities of retinol and less than picomolar quantities of carotenoids. Technical variability in measurement of the pool was ≤12% over a period of 6 weeks. Using this procedure, we recovered about 1.5 times more retinol and β-carotene than with our previously published method.[5] A number of methods for extraction and quantitation of retinol from milk have been reported[7,11,12] and these have recently been reviewed.[1,13] Most methods require large sample volumes and all use prolonged alkaline hydrolysis, thus none of these

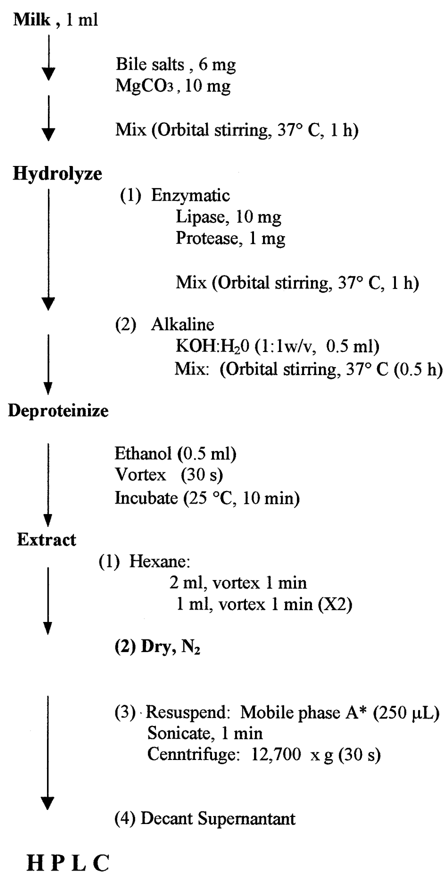

Figure 1 Procedure for hydrolysis and extraction of milk samples.

methods are suitable for the simultaneous quantitation of milk retinol and carotenoids. Retinol concentrations reported here compare favorably with those previously reported and are similar to serum retinol concentrations.

Available methodology for analysis of carotenoids in human milk has also recently been reviewed.[13] More recently, using alkaline hydrolysis, Johnson and coworkers[6] reported extraction and quantitation of β-carotene (but no other carotenoids) using alkaline hydrolysis. These workers reported significantly higher concentrations of β-carotene in milk than those we have observed. Methodology was not the focus of that report and technical details of the method were not discussed, thus we cannot directly compare the results of our two laboratories. Differences in maternal diets and sampling procedures may explain, at least to some extent, the differences in the results. In addition, Granelli and Helmersson[14] recently reported methodology for extraction and quantitation of β-carotene in bovine milk fat. This procedure provides a chromatographic profile similar to that we have previously reported, however, since it is based on 50 to 100 mg milk fat, (equivalent to 1 to 2 liters human milk), it is inappropriate for analysis of breastmilk. Thus, to our knowledge, ours is the only laboratory that has published methodology for simultaneous extraction and quantitation of major carotenoids and retinol in human milk.[3,5,8,15] The present procedure improves our previous method in that it is more rapid, uses milder hydrolysis conditions, requires less sample, and results in improved recovery of carotenoids.

Individual variation in milk carotenoid concentrations is substantial, and lipid concentrations change significantly over a breast emptying.[3,5,16] Thus, foremilk is low in lipid and lipid-soluble components, including carotenoids. Conversely, residual lipid retained in the breast from a previous feeding where full emptying was not achieved can result in elevated carotenoid concentrations. Therefore, for quantitation of milk carotenoids, full breast expressions should be collected if possible. Where this is not possible, to facilitate comparison of data between different laboratories, carotenoids should be reported relative to milk lipid concentrations (*Table 1*).

Freezing and thawing of milk lyses the milk fat globule, and for this reason is the major cause of technical variability in the quantitation of milk carotenoids. This process breaks the emulsion between the fat globule and the aqueous fraction[17] and results in non-uniform adherence of lipids to membrane fragments.[5] Ideally, samples should be analyzed before freezing. However, in most cases, and particularly for large clinical trials, it is necessary to work with frozen samples. Thus, we first investigated ways to solubilize milk lipids prior to extraction.

Bile salts greatly improved dispersion of the thawed samples, resulting in a visually uniform mixture, and as described below, enhanced lipase activity. In addition, as noted by others, magnesium salts[18,19] aided in dispersion of

Table 1 Determination of retinol and major carotenoids in mature human milk

	Concentrations		Assay reliability		
Analyte	μmol/L[1]	nmol/g lipid[1,2]	Recovery % ±SD[1,3]	CV[1,4]	Detection limit (pmol)[5]
Retinol	2.47 ± 0.29	41.1 ± 4.8	97.0 ± 0.03	11.7	5.4
Lutein/zeaxanthin	0.021 ± 0.002	0.36 ± 0.03	61.0 ± 2.40	9.5	2.0
β-Cryptoxanthin	0.014 ± 0.001	0.23 ± 0.01	82.1 ± 6.70	7.1	1.9
Lycopene	0.031 ± 0.002	0.52 ± 0.03	85.5 ± 0.80	6.5	1.5
α-Carotene	0.018 ± 0.002	0.31 ± 0.03	91.0 ± 0.30	11.1	2.1
β-Carotene	0.077 ± 0.007	1.28 ± 0.11	92.0 ± 0.30	9.1	3.0

[1]$n = 6$
[2]60 mg/L
[3]Percent recovery was calculated by internal standardization as described in Methods.
[4]Coefficients of variation (CV) in mean of pooled samples for 7 mothers over 45 days. Pools were analyzed weekly.
[5]Determined using a signal:noise ratio of 3:1.

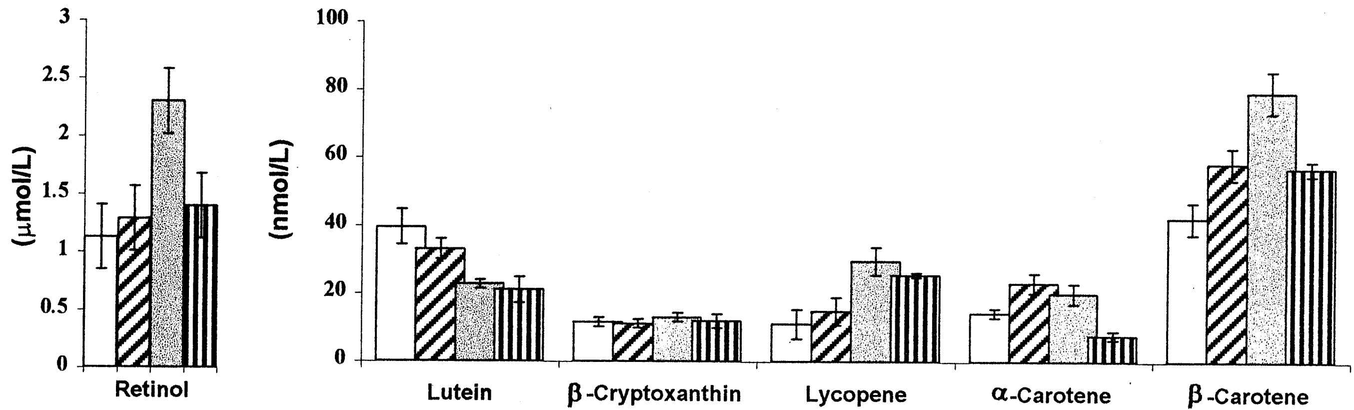

Figure 2 Effect of pretreatments on extraction of retinol and major carotenoids from mature human milk. Bile salt + yeast lipase + (□) no addition (▨) protease (▩) protease + 2.5 M KOH, (▥) protease + 4.0 M KOH. Data points are the mean ± SD of three experiments.

milk samples after freezing and thawing. BHT is frequently used to protect carotenoids against oxidation and is added to our HPLC mobile phase (see below). However, because BHT is oxidized by KOH to unidentified compounds that absorb at 325 nm and co-elute with retinoids, it should be excluded from samples before hydrolysis.

At least 14 retinyl esters are present in human milk.[5,20] Because their fatty acid chains vary from 8 to 18 carbons, the efficiency with which the various esters are extracted varies with the solvent strength of the extraction mixture. Therefore, for quantitation of total retinol in milk, retinyl esters should be hydrolyzed to retinol. Our previous procedure[5] required prolonged alkaline hydrolysis for complete hydrolysis of milk retinyl esters. However, because carotenoids, and particularly α-carotene and lycopene, are significantly degraded by this procedure,[15] it was necessary to analyze carotenoids separately, effectively doubling the assay time and sample volume required. Therefore, we investigated the use of enzymatic hydrolysis to avoid prolonged exposure to alkali so that retinol and carotenoids could be analyzed from a single sample. As is the case for retinyl esters, because of structural differences, individual carotenoids are extracted with different efficiencies by any given solvent. Because our work is primarily concerned with the quantitation of the pro-vitamin A carotenoids, β-cryptoxanthin, α-carotene, and β-carotene, recovery of the more hydrophobic carotenoids was optimized.

Various lipase treatments produced comparable results, including porcine (Sigma L-3126), yeast (Sigma L-8525, L1754), and bacterial lipases (Fluka 62308). For convenience and economy, yeast lipase (Sigma L1754) was chosen for routine experiments. In agreement with earlier reports on adipose tissue,[21] we found that protease treatment (2 units/mL) enhanced the recovery of carotenoids and retinol from milk (*Figure 2*) and that addition of bile salts (7.5 mM) enhanced lipase activity. Addition of higher concentrations of bile salts did not result in further enhancement of lipase activity.

Further treatment with lipase effectively released lutein/zeaxanthin and β-cryptoxanthin from milk. However, this treatment did not completely release lycopene, α-carotene, or β-carotene, and was ineffective in hydrolysis of retinyl esters (*Figures 2* and *4*). Treatment with lipase followed by brief alkaline hydrolysis (2.5 M, 20 min) however, resulted

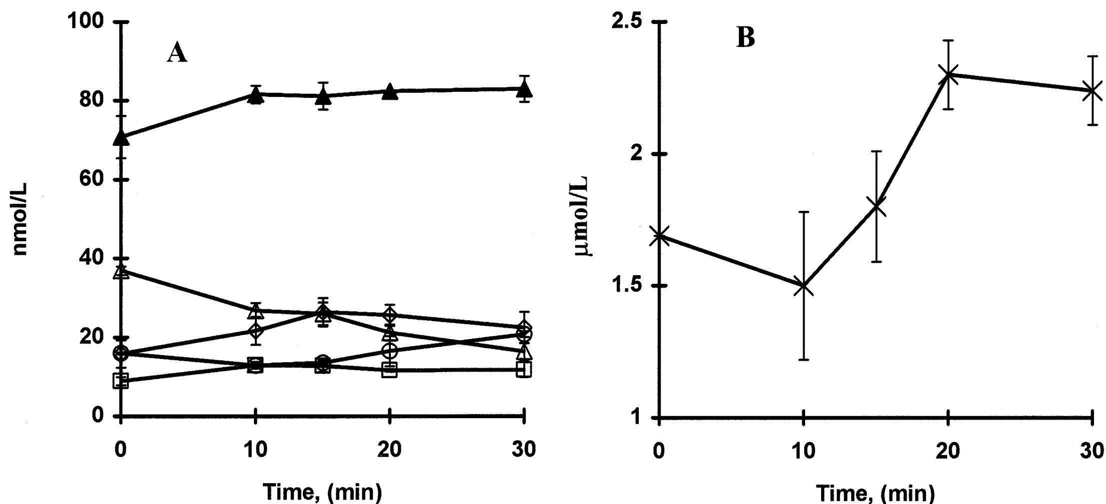

Figure 3 Time dependence of KOH treatment (2.5 M) on recovery of carotenoids and retinol from mature human milk. Each data point is the mean ± SD of six experiments. Panel A: (△) Lutein, (□) β-Cryptoxanthin, (◇) Lycopene, (○) α-Carotene; (▲) β-Carotene. Panel B: (×) Retinol.

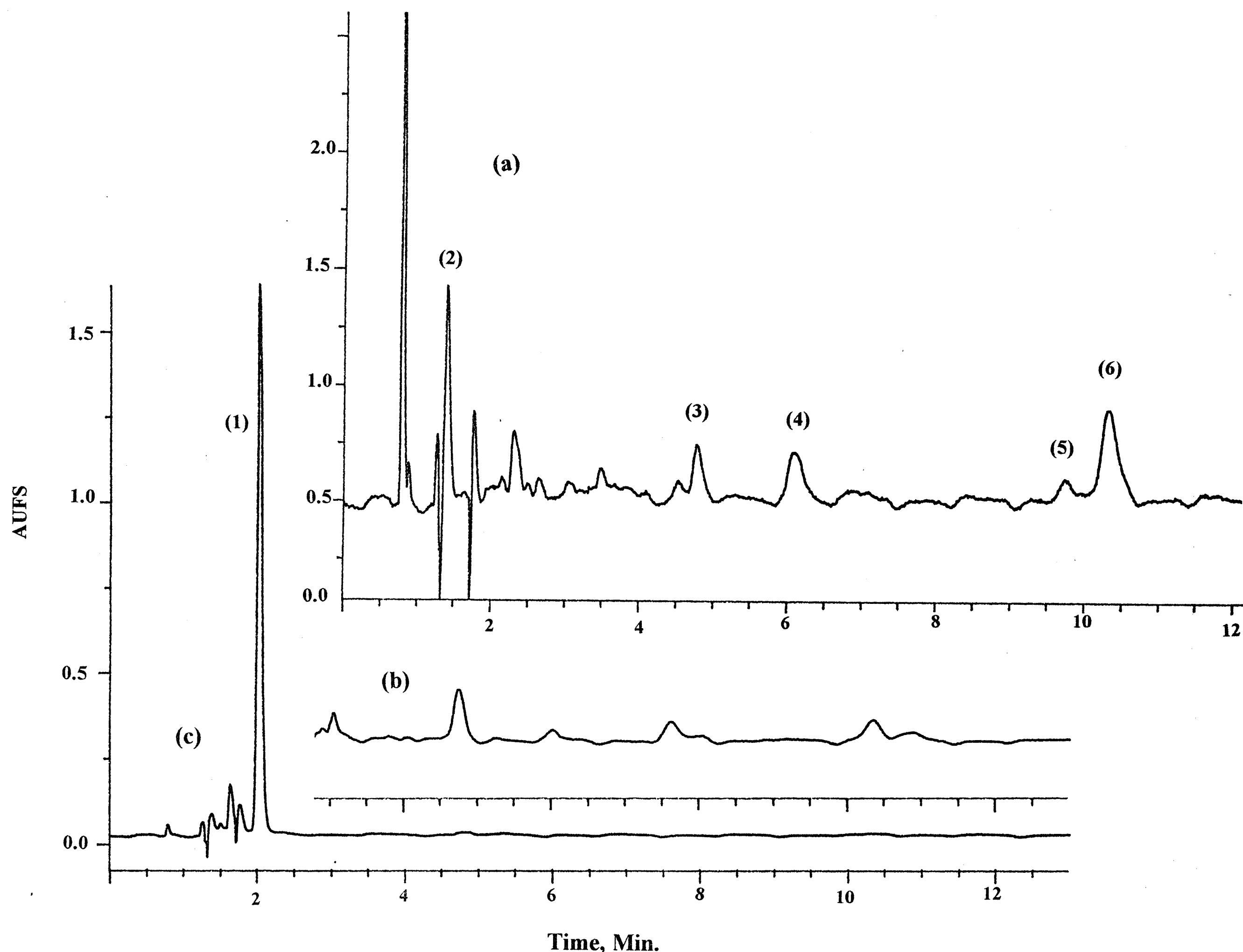

Figure 4 HPLC chromatogram of (a) major milk carotenoids (b) retinyl esters in human milk following enzymatic hydrolysis alone and (c) milk retinol after enzymatic and alkaline hydrolysis. Extracted components were eluted with 95% solvent A (THF/ACN (15/85% vol/vol) with 0.025% BHT (wt/vol) and 0.05% TEA (vol/vol) and 5% solvent B (50 mM ammonium acetate in methanol with 0.05% TEA) at a flow rate of 2.5 mL/min. Carotenoids and retinoids were detected at 452 and 325 nm, respectively. (1) Retinol, k′ = 1.0; (2) lutein/zeaxanthin, k′ = 1.25; (3) β-cryptoxanthin, k′ = 5.9; (4) lycopene, k′ = 8.8; (5) α-carotene, k′ = 15.0; (6) β-carotene, k′ = 16.1.

in release of the major carotenoids and essentially complete hydrolysis of retinyl esters (*Figures 3* and *4*). Xanthophylls (lutein/zeaxanthin) were progressively degraded by alkaline hydrolysis (*Figure 3*). In contrast, recovery of α-carotene and β-carotene was increased after 10 min of hydrolysis and these carotenoids were not degraded by additional hydrolysis up to 30 min. The amount of lycopene extracted was increased after 15 min of hydrolysis, but further hydrolysis led to its degradation. Alkaline hydrolysis for 30 min did not affect release of β-cryptoxanthin from milk. After alkaline hydrolysis for 20 min, retinol concentrations were increased approximately 1.5-fold and no retinyl esters were detected (*Figure 4b*). Retinol concentrations were unchanged after additional hydrolysis up to 30 min. Carotenoids are degraded by prolonged alkaline hydrolysis and their oxidation products may co-elute with retinol,[4,22] spuriously inflating concentrations. Therefore, prolonged alkaline hydrolysis of milk samples containing carotenoids should be avoided.

Our procedure effectively hydrolyses lipid esters in milk at usual concentrations (30 to 75 g/L) in normal human milk.[23] However, where lipid concentrations are higher than 60 g/L, partial hydrolysis products of triglycerides may be extracted and retained on the HPLC column, resulting in increased pressure, distorted baselines and shortened HPLC column life. For best results, we recommend that samples having lipid concentrations >60 g/L be diluted accordingly before assay.

After extraction of carotenoids and retinol from hydrolyzed samples as previously described,[5] the samples were dispersed in the HPLC mobile phase by sonication. Extracted samples were then evaporated just to dryness, suspended in ethanol, mixed with vortexing, incubated at room temperature for 10 min, and particulates removed by centrifugation.

HPLC elution profiles of extracted retinol and carotenoids are shown in *Figure 4*. Our previous mobile phase[5] was modified by the addition of ammonium acetate and triethylamine[24] as recommended by NIST for HPLC analysis of carotenoids in serum. In addition, methanol was replaced with acetonitrile to enhance recovery of lipids from

the column and to provide a more uniform baseline. We have recently reported a method for baseline resolution of milk carotenoids using gradient HPLC analysis.[4] However, because isocratic conditions are preferable for routine assays, this procedure was optimized for isocratic analysis of retinol and the pro-vitamin A carotenoids, which are typically the analytes of primary interest in clinical studies.

For determination of recovery of carotenoids and retinol, authentic standards were added to samples before hydrolysis as described in Methods. As shown in *Table 1*, recovery of retinol, α-carotene, and β-carotene were essentially complete. In addition, lycopene and β-cryptoxanthin were well recovered (>80%). However, the xanthophylls (lutein/zeaxanthin) were poorly recovered. These data agree with those in *Figure 3*, which show effective release of α-carotene, β-carotene, and retinol but progressive degradation of lycopene and lutein/zeaxanthin over time with alkaline hydrolysis. Concentrations of β-cryptoxanthin recovered were unchanged by alkaline hydrolysis. The data do not allow us to determine whether β-cryptoxanthin was incompletely released from the milk matrix or whether it was degraded by our treatment. As discussed above, carotenoids are transported in the milk fat globule, which is lysed to varying degrees by freezing and thawing of milk. Consequently, authentic standards added to milk, particularly after freezing and thawing, cannot completely equilibrate with endogenous carotenoids. Therefore, strictly considered, our procedure measures only recovery of carotenoids in solution, and not that of carotenoids that may remain attached to the milk fat globule after hydrolysis.

In summary, we have developed and validated a method combining enzymatic and chemical hydrolysis for the simultaneous determination of the major carotenoids and retinol in mature human milk. The method is efficient, sensitive, provides efficient recovery of retinol and milk carotenoids and is suitable for their simultaneous analysis. Alkaline hydrolysis can be avoided for analysis of lutein/zeaxanthin, and lycopene, whereas a combination of enzymatic and alkaline hydrolysis is required for efficient release of α-carotene and β-carotene and for complete hydrolysis of retinyl esters.

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References

1. Newman, V. (1994). Vitamin A and breast-feeding: A comparison of data from developed and developing countries. *Food and Nutrition Bulletin* **15,** 161–176
2. Stolzfus, R.J. and Underwood, B.A. (1995). Breast-milk vitamin A as an indicator of the vitamin A status of women and infants. *WHO Bulletin OMS* **73,** 703–711
3. Canfield, L.M., Giuliano, A.R., Neilson, E.M., Yap, H.H., Cui, H.A., and Blashil, B.M. (1997). β-Carotene in breastmilk and serum is increased following a single β-carotene dose. *Am. J. Clin. Nutr.* **66,** 52–61
4. Khachik, F., Spangler, C.J., Smith, J.C., Canfield, L.M., Steck, A., and Pfander, H. (1997). Identification, quantitation, and relative concentrations of carotenoids and their metabolites in human milk and serum. *Anal. Chem.* **69,** 1873–1881
5. Giuliano, A.R., Neilson, E.M., Yap, H., Baier, M., and Canfield, L.M. (1994). Carotenoids of mature human milk: Inter/intraindividual variability. *J. Nutr. Biochem.* **5,** 551–556
6. Johnson, E.J., Qin, J., Krinsky, N.I., and Russell, R.M. (1997). β-Carotene isomers in human serum, breast milk and buccal mucosa cells after continuous oral doses of all-trans and 9-cis β-carotene. *J. Nutr.* **127,** 1993–1999
7. Stoltzfus, R.J., Hakimi, M., Miller, K.W., Rasmussen, K.M., Dawiesah, S., Habicht, J., and Dibley, M.J. (1993). High dose vitamin A supplementation of breast-feeding Indonesian mothers: Effects on the Vitamin A status of mother and infant. *J. Nutr.* **123,** 666–673
8. Patton, S., Canfield, L.M., Huston, G.E., Ferris, A.M., and Jensen, R.G. (1990). Carotenoids of human colostrum. *Lipids* **25,** 159–165
9. Lucas, A., Gibbs, J.A.H., Lyster, R.L.J., and Baum, J.D. (1978). Creamatocrit: Simple clinical technique for estimating fat concentration and energy value of human milk. *Br. Med. J.* **1,** 1018–1020
10. Canfield, L.M., Hopkinson, J.M., Lima, A.F., Martin, G.S., Sugimoto, K., Burr, J., Clark, L., and McGee, D.L. (1990). Quantitation of vitamin K in human milk. *Lipids* **25:** 406–411
11. Chappell, J.E., Francis, T., and Clandinin, M.T. (1986). Simultaneous high performance liquid chromatography analysis of retinol ester and tocopherol isomers in human milk. *Nutr. Research* **6,** 849–852
12. Newman, V. (1995). Vitamin A in breast milk. *Pacific Health Dialog* **2,** 61–64
13. Canfield, L.M., Giuliano, A.R., and Graver, E.J. (1995). Fat soluble vitamins in human milk 1: Vitamin K, vitamin A and the carotenoids. In *Handbook of Milk Composition* (R.G. Jensen, ed.), p. 693–705, Academic Press, New York, NY USA
14. Granelli, K. and Helmersson, S. (1996). Rapid high-performance liquid chromatographic method for determination of β-carotene in milk. *J. Chromatography A* **721,** 355–358
15. Giuliano, A.R., Neilson, E.M., Kelly, B.E., and Canfield, L.M. (1992). Simultaneous quantitation and separation of carotenoids and retinol in human milk by high-performance liquid chromatography. In *Methods in Enzymology* (L. Packer, ed.), p. 391–399, Academic Press, San Diego, CA USA
16. Canfield, L., Giuliano, A., Neilson, E., and Blashill, B. (1997). β-carotene supplementation of normal lactating mothers. β-Carotene in breast milk and serum is increased after a single β-carotene dose. *Am. J. Clin. Nutr.* **66,** 52–61
17. Neville, M.C. (1995). Sampling and storage of human milk. In *Handbook of Milk Composition* (R.G. Jensen, ed.), p. 63–77, Academic Press, New York, NY USA
18. Brockman, H.L., Momsen, W.E., and Tsujita, T. (1998). Lipid-lipid complexes: properties and effects on lipase binding to surfaces. *JAOCS* **65,** 891–896
19. Jensen, R.G. (1994). Nutritional aspects. In *The Lipids of Human Milk* (R.G. Jensen, ed.), p. 187–192, CRC Press, Boca Raton, FL USA
20. Ross, A.C. (1986). Separation and quantitation of retinyl esters and retinol by high-performance liquid chromatography. *Meth. Enzymol.* **123,** 68–74
21. Peng, Y.M., Peng, Y.S., and Lin, Y. (1993). A nonsaponification method for the determination of carotenoids, retinoids and tocopherols in solid human tissues. *Can. Epidemiol. Biomark. Preven.* **2,** 139–144
22. Hu, X., White, K., and Canfield, L. (1996). Inhibition of the growth of breast cancer cells *in vitro* by oxidative metabolites of β-carotene. *FASEB J.* **10,** A490
23. Canfield, L.M., Giuliano, A.R., and Graver, E.J. (1995). Carotenoids, retinoids, and vitamin K in human milk. In *Handbook of Milk Composition* (R.G. Jensen, ed.), p. 693–705, Academic Press, San Diego, CA USA
24. Epler, K.S., Ziegler, R.G., and Craft, N.E. (1993). Liquid chromatographic method for the determination of carotenoids, retinoids and tocopherols in human serum and in food. *J. Chromatography* **619,** 37–48