Improved Extraction Procedure for Carotenoids from Human Milk

Florian J. Schweigert, Andrea Hurtienne, Katharina Bathe

Institute of Nutritional Science, University of Potsdam, Germany

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Abstract: An improved method for the extraction of the major carotenoids from human milk is described. Carotenoids were extracted from milk first with ethanol and n-hexane. Then, polar xanthophylls were extracted from n-hexane into ethanol/water. The remaining n-hexane was evaporated, the residue combined with the ethanolic milk fraction and the mixture briefly saponified. Carotenoids were extracted from the hydrolysate with n-hexane, combined with the polar xanthophylls from the non-saponified ethanol/water-extract and separated by HPLC. Using this method we were able to significantly improve the recovery of xanthophylls such as lutein and zeaxanthin from human milk. The recovery rate of all carotenoids was > 90%. This method might not only be of value for milk but should be especially useful in the extraction of carotenoids from human tissues such as the adipose tissue.

Key words: Xanthophylls, saponification, milk, human, method

Introduction

Milk is a rich source of carotenoids for the new-born [1] which are precursors of vitamin A and/or have antioxidant properties [2]. Contrary to many mammals in which only or primarily β-carotene is present [3, 4], human milk is a rich source of a verity of carotenoids which differ in its chemical composition [5–8]. To estimate the content of carotenoids, these lipophilic components have do be extracted from the matrix milk with organic solvents. Due to the large quantity of fat present in milk, the samples require saponification and/or lipids have to be removed by enzymatic treatment to release the carotenoids from the lipid matrix [7–9]. The alkaline environment, however, poses a risk especially for the polar carotenoids lutein and zeaxanthin for which losses of up to 40 % have been reported [9].

To overcome this problem, a new step was introduced into the milk extraction method, to remove the polar carotenoids but not the total lipids from the organic hexane extract prior to saponification. This greatly improved the recovery of polar carotenoids from human milk without a major increase in time necessary for the extraction procedure.

Material and Methods

Material: Milk was obtained from healthy lactating women recruited from mothers attending the Clinic for Reproductive Medicine, Charité, Campus Virchow, Berlin. The study protocol was approved by the hospital's Ethics Committee and informed written consent was obtained. Milk was stored at -80°C until the time of pooling.

Methods

Extraction I (Saponification): Based on a modified published method [6, 8], to 1 ml of pooled milk 0.5 ml of 12% pyrogallol in ethanol was added, then samples were saponified with 1.5 ml of a mixture of 3 ml of 50% KOH and 5 ml of ethanol. The mixture was vortexed (1 min.) and incubated at 37°C for 120 min. under nitrogen. After incubation, samples were vortexed with 1 ml of n-hexane for 10 min, centrifuged (1500 g, 10 min.) and the upper organic layer was removed. This step was repeated once. To the combined organic extracts 1.5 ml of 0.1 M NaCl solution and 0.5 ml ethanol was added and vortexed. The mixture was centrifuged (1500 g, 10 min.) and after phase separation the organic layer was removed. The remaining lower layer was extracted once again with 1 ml of n-hexane. The combined organic extracts were evaporated under nitrogen at 30°C to dryness. The residue was re-suspended in 0.2 ml iso-propanol in the extraction vials, vortexed (1 min), sonicated (5 min) and centrifuged (1500 g, 2 min.). Samples were transferred into sealable autoinjection vials (300 µl).

Extraction II (Ethanol extraction prior to saponification): To 1 ml of pooled milk 1 ml ethanol and 1 ml of n-hexane was added, vortexed for 10 min. and centrifuged (1500 g, 10 min), then the upper organic layer was removed. The addition of 1 ml of n-hexane was repeated once. The combined n-hexane layers were extracted twice into 1 ml of ethanol: H_2O (9:1; v:v). Thereafter, the remaining n-hexane layer was dried under nitrogen (30°C) and combined with the aqueous phase remaining from the first extraction step. Then 0.5 ml of 12% pyrogallol in ethanol and 0.6 ml of 50% KOH were added. The mixture was vortexed and saponified at 37°C for 120 min under nitrogen. Thereafter, samples were vortexed with 1 ml of n-hexane for 10 min, centrifuged (1500 g, 10 min) and the organic layer was removed. This step was repeated three times. The ethanol and n-hexane extracts were combined and then evaporated under nitrogen at 30°C to dryness. The residue was treated as in extraction procedure I.

HPLC Analysis: For separation and quantification a modified [6] gradient reversed-phase HPLC-system (Waters, Eschborn, Germany) on a C30 column, (5 µm, 250× 4.6 mm; YMC, Wilmington, USA) was used. The solvent system consists of solvent A with methanol (Roth Chemicals Germany):water (90:10; v:v, with 0.4 g/l ammonium acetate in H_2O) and solvent B with methanol:methyl-tert-butyl-ether (Sigma Deisenhofen, Germany): water (8:90:2; v:v:v, with 0.1 g/l ammonium acetate in H_2O). The column flow rate was 1 ml/min. The gradient elution began at 100% solvent A and was linearly decreased to the indicated percentages over the indicated period of time (93% – 1 min; 85% – 1 min; 80% – 1 min; 75% – 8 min.; 45% – 10 min; 13% – 8 min; 7% – 3 min; 1% – 1 min) at 1% solvent A was kept for 12 min and then increased to 100% solvent A within 1 min. The column was re-equilibrated for 9 min under 100% solvent A. Total run time was 55 min. For detection and characterisation a photodiode array detector was used (Model 996, Waters). Carotenoids and α-tocopherol were quantified by measuring the absorption at 450 and 290 nm, respectively. Retinol and all retinyl esters were quantified by measuring the absorption at 325 nm and calculated as retinol equivalents [10] using retinol as external standard. Standards were dissolved in ethanol, checked for purity by HPLC and measured in a spectral photometer using the wave length of the respective maximum. Results were compared when possible to standard reference material 968a (National Institute of Standards Technology (NIST), Gaithersburg, MD, USA).

Carotenoid standards of lutein, zeaxanthin, canthaxanthin, β-cryptoxanthin, lycopene were a kind gift of Hoffmann-La Roche, Switzerland, α-carotene, β-carotene, α-tocopherol, retinol and retinyl palmitate were from Sigma (Deisenhofen, Germany).

Recovery of retinol α-tocopherol and carotenoids was determined by internal standardisation. Authentic standards of carotenoids (lutein, zeaxanthin, canthaxanthin, β-cryptoxanthin, α-carotene, all-*trans*-ß-carotene, lycopene), α-tocopherol and retinol were added to the pooled milk in ethanol at concentrations between 11% and 60% of the concentration present in the analyzed milk pool (Table I). Recovery was calculated by consideration of the amount of carotenoids, α-tocopherol and retinol in the non-spiked milk. Limits of detection were determined using a signal to noise ratio of 6:1.

Results and Discussion

In human milk samples lutein, zeaxanthin, β-cryptoxanthin, α-carotene, all-*trans*-β-carotene and lycopene as well as α-tocopherol and vitamin A were observed. Results for the different methods (Table I) showed, that if only alkaline hydrolysis is used to liberate carotenoids from the lipid matrix, substantial losses of lutein and zeaxanthin can be observed. The amount of degradation was in the same order of magnitude as reported in a previous study that uses both alkaline saponification and enzymatic treatment [9]. As shown previously, the recovery rate of the polar carotenoids was more effected than that of the lipophilic ones. Table II shows in accordance to previous studies [8], that the losses of polar carotenoids were proportional to the duration of saponification. This was fur-

Table I: Comparison of recoveries between two methods for the determination of carotenoids in human milk (% of standards added)*

	Lutein	Zeaxanthin	β-Cryptoxanthin	α-Carotene	β-Carotene	Lycopene	α-Tocopherol	Vitamin A
A	81.6 ± 0.8	74.7 ± 1.1	91.5 ± 0.3	89.4 ± 1.8	91.3 ± 0.4	91.1 ± 1.5	96.8 ± 0.8	99.9 ± 1.8
B	91.6 ± 1.1	93.7 ± 1.9	97.7 ± 2.0	94.2 ± 0.8	93.0 ± 3.0	96.7 ± 3.5	98.6 ± 3.5	98.6 ± 3.5
C	96.4 ± 1.1	96.9 ± 0.8	98.3 ± 1.3	89.9 ± 1.3	93.9 ± 1.2	91.4 ± 2.0	97.0 ± 1.3	101.3 ± 1.5

A: Samples only saponified at 37°C for 120 min, (extraction method I), standards added before saponification to milk (n = 5)
B: Samples only saponified at 37°C for 120 min, (extraction method I), external standards added after saponification (n = 5)
C: Samples extracted additionally with ethanol:water (9:1, v:v) (extraction method II), external standards added to milk (n = 10)
* Absolute concentration (ng/ml) and detection limit (pg) were as follows: lutein 48.0–1.30; zeaxanthin 12.1–0.82; β-cryptoxantin 62.3–0.99; α-carotene 26.1–1.02; β-carotene 71.6–0.95; lycopene 41.4–2.00; vitamin A 932.1–1.61; α-tocopherol 6.6 mg/ml – 26 ng

Table II: Recovery of carotenoids from human milk depending on the duration of saponification (% from time point 0)

	0	15	30	60	120
Lutein	100 ± 2.6	95 ± 3.1	93 ± 1.8	88 ± 3.3	88 ± 2.4
Zeaxanthin	100 ± 4.2	94 ± 4.1	90 ± 5.0	85 ± 3.7	80 ± 4.4
β-Cryptoxanthin	100 ± 2.1	98 ± 4.3	97 ± 2.9	97 ± 6.0	95 ± 2.6
α-Carotene	100 ± 0.8	102 ± 1.9	102 ± 3.4	100 ± 9.4	94 ± 8.7
ß-Carotene	100 ± 3.4	97 ± 4.4	98 ± 4.7	97 ± 6.7	91 ± 4.2
Lycopene	100 ± 2.3	97 ± 4.3	96 ± 2.6	94 ± 5.5	91 ± 2.0
α-Tocopherol	100 ± 2.6	100 ± 3.1	100 ± 1.4	103 ± 5.6	104 ± 1.5
Vitamin A	100 ± 4.0	103 ± 4.9	107 ± 2.5	116 ± 7.0	124 ± 4.7

Each data point is the mean ± SD of five experiments

ther supported by our observation that recovery rates of polar carotenoids were higher if standards were added after saponification.

To overcome the problem of high losses of polar carotenoids during saponification, an additional step was introduced into the extraction protocol with the aim to remove the polar carotenoids from the hexane extract prior to saponification. As shown in Figure 1, the extraction of hexane with ethanol:water (9:1, v:v) results in the removal of most of the lutein (% of total; mean ± SD, n = 6, 93.3 ± 4.6) and zeaxanthin (94.6 ± 1.6). β-Cryptoxanthin was equally distributed between the ethanol and hexane fraction (ethanol: 49.1 ± 0.9). Lowest extraction rate were observed for α-carotene, β-carotene and lycopene (11.7 ± 3.3; 9.9 ± 1.0; 9.7 ± 4.9, respectively). These differences are due to the different structures and polarities of the individual carotenoids.

If the results of the modified method were compared to the mild alkaline hydrolysis method used in this study and to previous methods published concerning this subject, the recovery rate of the lipophilic carotenoids was comparable. But in addition to that, the recovery rate of polar carotenoids was superior. With recoveries of more than 96%, values comparable to or even better than those of less polar carotenoids were reached. The extraction recovery of α-tocopherol and total vitamin A was not effected.

Retinyl esters were not completely hydrolysed to retinol. The major retinyl esters, palmitate and stearate were still present, indicating that length and temperature of saponification were not sufficient for complete hydrolysis [8]. Since both retinol and retinyl esters, however, exhibit the same absorption intensity at 325 nm which can be attributed to the retinol moiety, both can be quantified using retinol as standard and expressing the results for retinyl esters as retinyl equivalents [10].

In conclusion, the introduction of a new extraction step in the assay protocol for carotenoids in human milk greatly improved the recovery. Without a substantial increase in time the extraction of polar xanthophylls was even more efficient as that of the less polar carotenes. With this modification similar recovery rates as in plasma could be observed. This is of specific importance if results between plasma and milk have to be compared. In addition the modification might be applied in the extraction of carotenoids from tissues such as the adipose tissue for which saponification is necessary.

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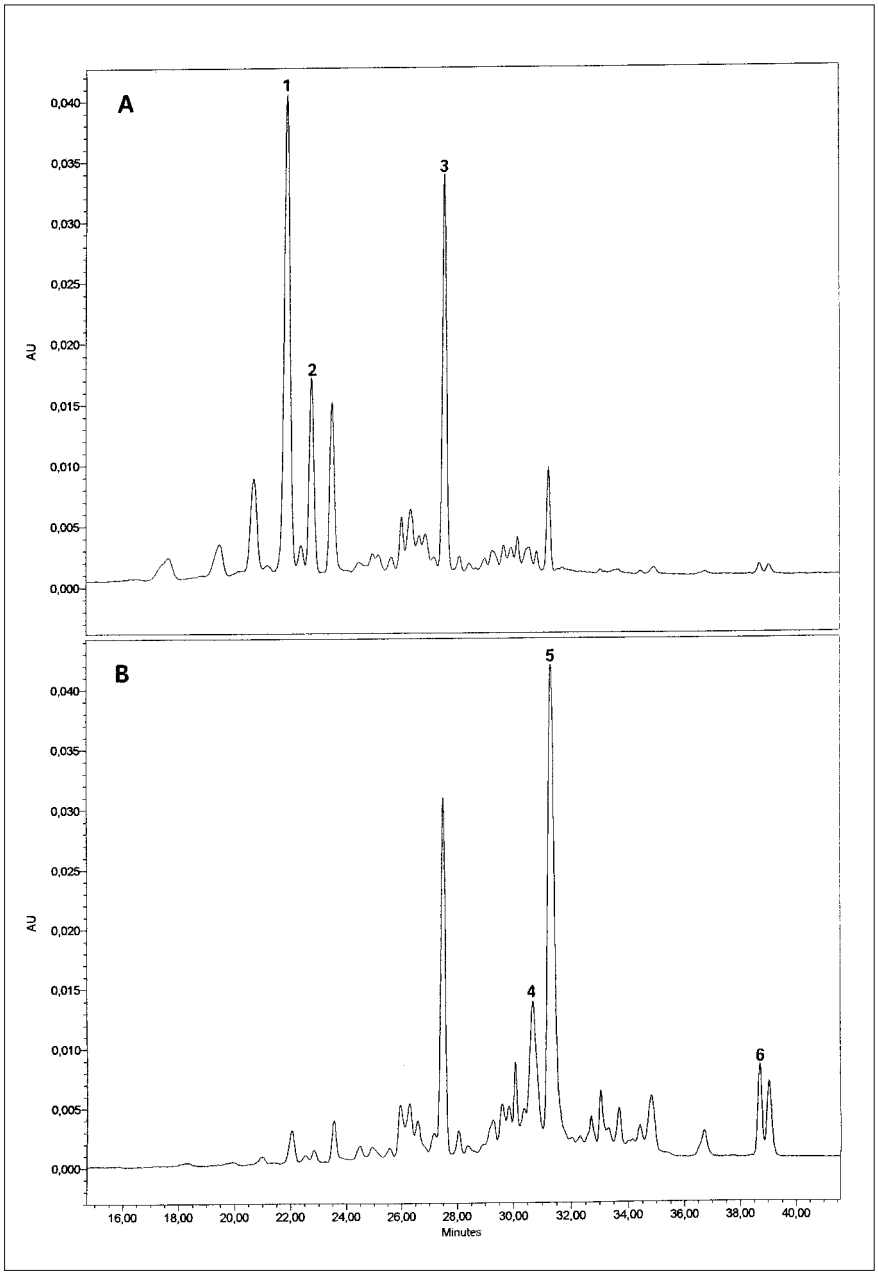

1 – all-trans-lutein;
2 – all-trans-zeaxanthin;
3 – all-trans-cryptoxanthin;
4 – all-trans-α-carotene;
5 – all-trans-β-carotene;
6 – all-trans-lycopene

Figure 1: HPLC chromatogram at 450 nm of milk carotenoids in the ethanol extract (A) and in the saponified n-hexane extract (B).

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Florian J. Schweigert

Institute of Nutritional Science
University Potsdam
Arthur-Scheunert-Allee 114–116
D-14558 Potsdam-Rehbrücke