ELSEVIER

Journal of Chromatography B, 695 (1997) 209–215

JOURNAL OF CHROMATOGRAPHY B

Simultaneous detection of carotenoids and vitamin E in human plasma

Olaf Sommerburg*, Lun-Yi Zang, Frederik J.G.M. van Kuijk

Department of Ophthalmology and Visual Sciences, University of Texas Medical Branch, Galveston, TX 77555-1067, USA

Received 26 June 1996; revised 29 November 1996; accepted 9 December 1996

Abstract

A simplified method for analysis of the antioxidants carotenoids and vitamin E in human plasma is presented. The method is based on high-performance liquid chromatography with a single column, a flow-rate gradient, and detection at 450 and 290 nm with a diode array detector. It gives good separation of the vitamin E isomers and the major carotenoids in plasma, with a 25 min analysis time. It was found that hydrolysis of triglycerides and cholesterol esters is required to obtain good recovery of non-polar carotenoids such as lycopene, α-carotene and β-carotene. Two methods were used for hydrolysis of the non-polar lipids, saponification with ethanolic KOH and digestion with an enzyme mixture of lipase and cholesterol esterase. It was found that the enzymatic digestion gave the best recoveries, better than 94% for all of the antioxidants, and preserved several carotenoids. A plasma pool is used for day to day calibration of the method, which eliminates the need for stock solutions of carotenoids that are stable for only a month due to oxidative breakdown and their tendency to crystallize when stored at −20°C in organic solvents. © 1997 Elsevier Science B.V.

Keywords: Carotenoids; Vitamins; Vitamin E; Antioxidants

1. Introduction

Carotenoids can act as singlet oxygen quenchers or free radical scavengers [1], as stimulants of immune response [2] and as anticarcinogenic agents [3]. Due to the variety of functions and biological roles, carotenoid research has increased in recent years. Several techniques have been developed to detect carotenoids, and in 1970 Sweeney and Marsh were the first to use a high-performance liquid chromatography (HPLC) method for the separation of carotenoids [4]. Since this time numerous HPLC procedures were developed, using improved technical equipment and column material. The majority of carotenoid separations are currently performed using reversed-phased 5 μm spherical particles packed in 25 cm columns with octyldecylsilica (ODS or C_{18}) packings. However, due to complex mixtures of carotenoids in tissues, it is a challenging analytical problem to separate all polar and nonpolar carotenoids.

The goal of our work was to simplify a basic HPLC method for detection of carotenoids in human tissues and to combine this method with simultaneous detection of tocopherols. The method was designed for human plasma, containing polar and nonpolar carotenoids, such as lutein, zeaxanthin, cryptoxanthins, lycopenes and α- and β-carotene.

*Corresponding author. Present address: Dept. of Pediatric Nephrology, University Hospital Charité, Schumannstrasse 20/21, Humboldt University, D-10098 Berlin, Germany.

PII S0378-4347(97)00036-4

There are a number of good HPLC methods for simultaneous detection of the whole spectrum of carotenoids, retinols and tocopherols [5–14]. Some of these methods require two detectors, one for UV detection of carotenoids and retinols and a fluorescence detector for determination of tocopherols [10,14]. The existing methods are based on chromatographic conditions requiring complicated solvent regimes, with up to three solvent mixtures and as many solvent gradients [10,11], or up to four flow gradients [14], which require longer re-equilibration times between the injections [10,11,14]. In addition, coupled columns, leading to an increased total run time of up to 40–60 min, have been used [8,14]. Separation in different parts of the chromatogram is not always equally good [11], especially for the polar carotenoids, lutein and zeaxanthin [6,9,12–14]. It was the purpose of this study to establish a simpler reliable method using a single column with a diode array detector, a single solvent mix with a flow gradient and a reasonable analysis time, while reaching good separation in all parts of the chromatogram.

2. Experimental

2.1. Reagents

Methanol, hexane and acetonitrile were purchased from EM Science (Gibbstown, NJ, USA). Tocol, β-apo-10′-carotenal and zeaxanthin were a generous gift by Hoffmann–La Roche (Nutley, NJ, USA). Disodium hydrogenphosphate hydrate ($Na_2HPO_4 \cdot H_2O$), β-carotene and lutein standards, O-methylhydroxylamine and O-ethylhydroxylamine were purchased from Fluka (Buchs, Switzerland). Sodium sulfate and sodium chloride (NaCl) were from Fischer Scientific (Fair Lawn, NJ, USA). Cholesterol esterase (from *pseudomonas species*, catalog number C-1403), lipase (from *Chromobacterium Viscosum*, catalog number L-0763), butylated hydroxytoluene (BHT), diethylenetriaminepentaacetic acid (DTPA), Triton X 100, SDS and PIPES disodium salt were purchased from Sigma (St. Louis, MO, USA). Potassium hydroxide (KOH) was from Mallinckrodt (Paris, KY, USA) and ethanol was from Midwest Grain Products (Weston, MI, USA). Ammonium acetate was purchased from Baker (Phillipsburg, NJ, USA). Phosphate buffered saline (PBS) was prepared with 10 m*M* $Na_2HPO_4 \cdot H_2O$ and 0.15 *M* NaCl titrated to pH 7.4. The PBS buffer typically contained 2 m*M* DTPA.

2.2. Sample preparation: extraction of antioxidants from plasma

Human plasma samples were used from a large plasma pool, which was prepared as described before from ethylenediaminetetraacetic acid (EDTA) treated blood [15,16]. Plasma was either extracted directly, or it was saponified with KOH, or it was digested with an enzyme mixture containing triglyceride hydrolase and cholesterol esterase. For direct extraction, 200 μl of the plasma was added to 800 μl PBS buffer with 2 m*M* DTPA (pH 7.4) and vortexed-mixed. Then 1 ml of ethanol containing 0.1 mg BHT/ml was added, and the sample was vortex-mixed for 3 min. For extraction, 2 ml of hexane and 1 ml of hexane containing the internal standards (Tocol and β-apo-10′-carotenal methyloxime) were added to the samples and they were vortex-mixed for 3 min and centrifuged at 1800 *g* for 3 min. The hexane (upper layer) of the sample containing the carotenoids and tocopherols was collected and passed through a Pasteur pipette, prepared with ~150 mg of anhydrous sodium sulfate to remove any traces of moisture. This procedure was repeated with 2 ml of hexane. The extracted antioxidants in hexane are stable for more than 24 h at −20°C and temporarily stored, until the hexane phase was evaporated with argon to dryness. The residue was redissolved in 60 μl mobile phase and 20 μl was injected onto the HPLC column.

To saponify the triglycerides in the plasma, 200 μl was mixed with 800 μl PBS buffer and 1 ml ethanol–BHT as described above, after which 1 ml of saturated ethanolic KOH solution was added and the samples were incubated in a water bath at 60°C for 30 min. After the incubation, the vial was immediately placed in ice and 1 ml of ice-cold distilled water was added to the sample, to cool it down to room temperature. Hexane was used to extract the samples as described above.

The enzyme reagent used for digestion of plasma contains 160 IU/ml of triglyceride hydrolase (lipase) for hydrolysis of triglycerides and 1 IU/ml of

cholesterol esterase for hydrolysis of the plasma cholesterol esters. The enzyme reagent was prepared in PBS buffer pH 7.4 with 2 m*M* DTPA and 0.25% (w/v) Triton X-100. For digestion, 200 μl of plasma was mixed with 1 ml of the enzyme reagent and kept at ambient temperature in the dark for 1 h. After incubation, 100 μl of 5% sodium dodecyl sulfate (SDS) was added and the samples were mixed for 30 s with a vortex mixer. Then 1 ml of ethanol–BHT was added and the samples were vortexed and extracted with hexane as described above.

Recoveries were calculated by the method of internal addition for directly extracted, KOH saponified and enzyme digested plasma samples, as described previously for retinoids [17] and fatty acids [18]. Three separate samples sets were prepared in each experiment consisting of plasma, stock solutions of carotenoids and tocopherols, and plasma spiked with these stock solutions. Plasma was extracted as described above. Stock solutions of carotenoids (containing lutein, zeaxanthin, lycopene and β-carotene) and tocopherols (containing α- and γ-tocopherol), each in 1 ml of hexane with amounts matching the amount in 200 μl plasma, were added to 1 ml of PBS buffer and 1 ml of ethanol–BHT containing 50 μg/ml cholesterol. BHT was added for protection [7,15,17,19]. Cholesterol was added to function as a lipid carrier as described previously for retinoids [17]. Prior to extraction, 1 ml of hexane containing the internal standards was added. Plasma spiked with stock solutions was extracted with 1 ml of hexane containing tocopherols, 1 ml of hexane containing carotenoids and 1 ml of hexane containing internal standards. Peak area ratios were used for calculating the recoveries. Freshly prepared stock solutions were used to calibrate the plasma pool.

2.3. Synthesis of internal standard, β-apo-10′-carotenal methyloxime

The internal standard for carotenoids was prepared by a procedure adapted from the method of Handelman et al. [20]. Typically, 200 μl of a 1 ml/mg solution of β-apo-10′-carotenal in methanol was mixed with 200 μl of 0.1 *M* methylhydroxylamine (in 0.1 *M* PIPES buffer, adjusted to pH 6.7) and incubated for 24 h in a water bath at 37°C. The standard is the O-methylhydroxylamine derivative of β-apo-10′-carotenal and is called apo-10′-carotenal methyloxime. A mixture of *anti* and *syn* isomers is produced, which can be separated using the same chromatographic conditions as described below. The most predominant isomer (*anti*) is collected from different injections with a Gilson FC 205 fraction collector (Gilson Medical Electronics, Middleton, WI, USA), and was used as internal standard. To store the internal standard, the apo-10′-carotenal methyloxime was reextracted in hexane. The absorbance of the stock solution was determined at 450 nm and a dilution was prepared in hexane with an absorbance of 0.01. The internal standard is stable for several months at −20°C. A 1-ml volume of this dilution was used in each analysis.

2.4. High-performance liquid chromatography conditions

The HPLC system consisted of a Beckman System Gold Module 125 programmable solvent pump, a Beckman System Gold Module 168 diode array detector (Beckman Instruments, Palo Alto, CA, USA), and a Rheodyne 7725i injector valve with a 20-μl injection loop (Rheodyne, Cotati, CA, USA). The diode array detector was set in channel A at wavelength 450 nm to detect the carotenoids. The second channel B was set at 290 nm to detect the tocopherols. A protein and peptide 5 μm C_{18} column, 250×4.6 mm I.D. (VYDAC, 218TP54, Hesperia, CA), with a precolumn (40×4.6 mm I.D.), was used. For the mobile phase two solvents were prepared. Solvent A consisted of acetonitrile–methanol (85:15, v/v), with 0.01% (w/v) ammonium acetate, and solvent B was pure hexane. During the run a mix of 97% solvent A and 3% of solvent B was used. The initial flow-rate was 0.55 ml/min. At 10 min, a 5-min flow gradient ramp was executed to a final flow-rate of 1.2 ml/min. At 23 min, the flow-rate was decreased within a 1-min ramp to the initial setting, and the system was equilibrated for 1 min, giving a total analysis time of 25 min.

3. Results

To check the linearity of the HPLC method for lutein, zeaxanthin and β-carotene, a series of dilu-

tions of the calibration stock solutions were prepared and determined by HPLC. The results of the linearity measurements were calculated and correspond to their regression lines: lutein 20–156 ng/ml (r= 0.97), zeaxanthin 17–139 ng/ml (r=0.97) and β-carotene 15–126 ng/ml (r=0.99). The detection limit was defined as the lowest measurable concentration of a standard in a sample which generates a peak with a signal-to-noise ratio of 3 or better. The following amounts injected on the column were determined as detection limits: lutein 1–10 ng, zeaxanthin 1–10 ng, β-carotene 5–10 ng, γ-tocopherol 10–100 ng and α-tocopherol 10–100 ng. The precision of the method was checked by multiple determinations of plasma samples from the plasma pool on different days. The within-day and between-day variations are less than 4% for each antioxidant. The recovery was calculated from the results of internal addition experiments for each of three different extraction methods, as shown in Table 1. The results indicate that the α-tocopherol, lutein and zeaxanthin are also well recovered from plasma without saponification or digestion. However, in order to obtain good recovery of γ-tocopherol, lycopene and β-carotene it is necessary to saponify the samples with KOH or to digest the samples with enzymes, to hydrolyze triglycerides and cholesterol esters.

Typical chromatograms of a human plasma sample extracted after KOH saponification and enzyme digestion, and recorded as two different wavelengths are shown in Fig. 1. Several small peaks around lutein are not present in the KOH digested sample in channel A at 450 nm (Fig. 1A), but could be seen in the enzyme digested sample (Fig. 1C). The identification of these peaks was reported elsewhere [7]. Furthermore, some or all of the tocol would disappear after KOH saponification (Fig. 1B). Retinol can be seen in samples treated with KOH, but could not be seen after enzyme digestion (Fig. 1D), since several large peaks from components in the enzyme solution would now elute in this area. All chromatograms obtained show a clean baseline separation of the analytes of interest. The VYDAC-218TP54 C_{18} protein and peptide column showed a good ability to separate all major carotenoids, including lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene. Baseline separation was also obtained between α- and γ-tocopherol. The amounts of tocopherols and carotenoids found in human plasma are shown in Table 2 and are in agreement with previous reports [7,15,21].

Table 1
Recoveries of antioxidants from plasma after extraction with three different methods

	Direct n=5	KOH n=6	Enzymes n=6
α-Tocopherol	99.9±0.9	96.4±7.1	104.7±4.2
γ-Tocopherol	88.6±0.5	100.8±11.6	106.8±2.6
Lutein	92.4±1.2	93.4±1.7	93.9±2.4
Zeaxanthin	92.1±1.1	91.5±4.7	95.1±2.0
Lycopene	80.7±0.3[a]	91.7±10.8[a]	96.7±4.2
β-Carotene	66.1±1.8	92.7±3.9	99.1±4.5

[a] These recoveries were obtained without adding lycopene since the standard was not available at the time the experiments were carried out. They were estimated by comparing peak height ratios of lycopene/I.S. of the direct and KOH methods with the enzyme method.

4. Discussion

The objective of this study was to develop a HPLC method for the separation and quantification of lipid soluble antioxidants, carotenoids and tocopherols, in human plasma and other tissues. The methods currently available require either multiple solvent gradients, several columns in series or long analysis times [5–14]. We also tried a previously used mobile-phase mixture with isopropanol as solvent B, but it was more difficult to keep stable retention times due to the hygroscopic nature of 2-propanol [17]. This problem is likely to be more severe in humid areas. Using hexane under the conditions described here we obtained better consistency of retention times. Another advantage of hexane over isopropanol is a better separation between lutein and zeaxanthin over the 25-min period. Furthermore, the use of hexane allows a lower flow-rate during the HPLC run with the result of less consumption of mobile phase. Ammonium acetate has been reported to have a protective effect when small amounts of carotenoids are analyzed (less then 20 ng) [7]. We found that adding it to solvent A, only, gave sharper peaks than adding it to both

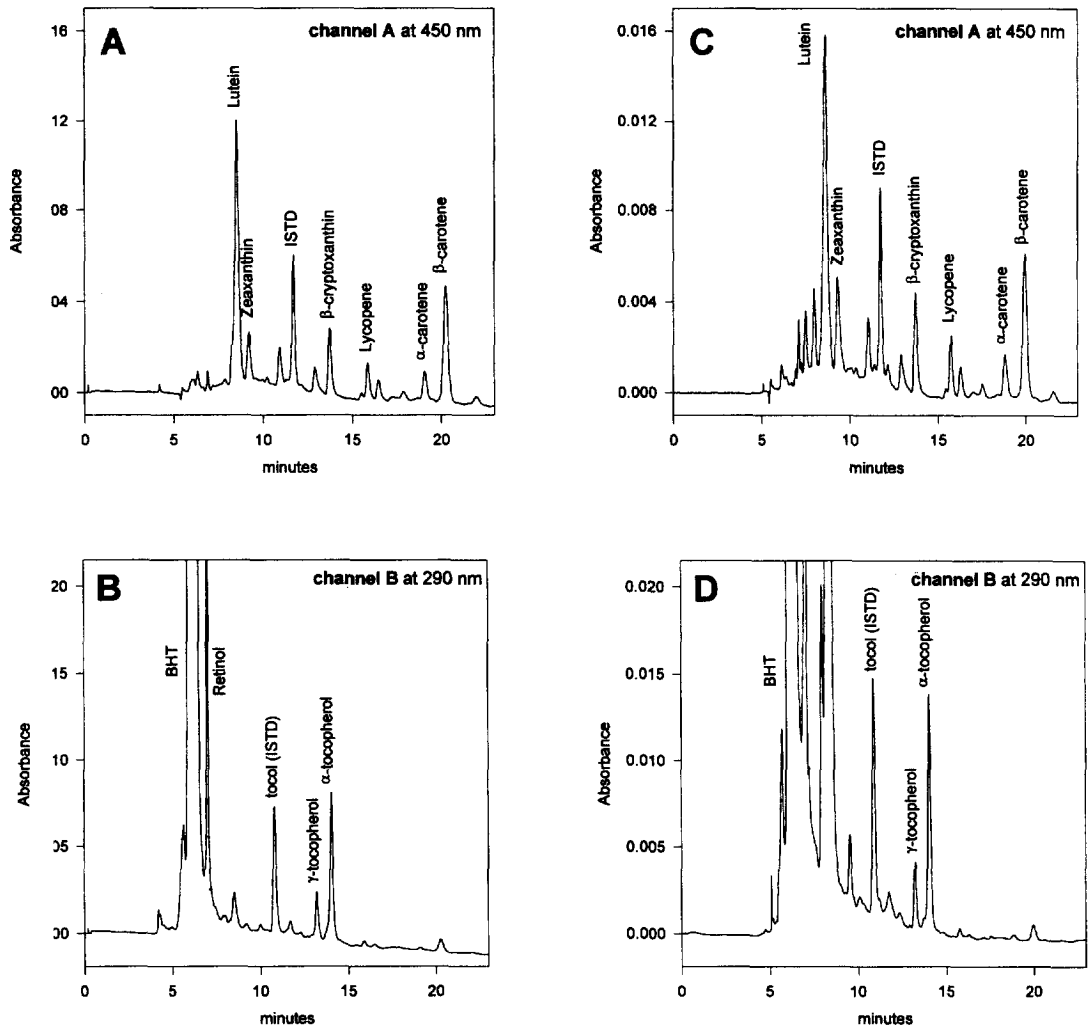

Fig. 1. Chromatograms of a human plasma sample. Chromatogram of extracted carotenoids at 450 nm after KOH saponification (A) and after enzyme digestion (C). Chromatogram of retinol and tocopherols at 290 nm after KOH saponification (B) and after enzyme digestion (D).

Table 2
Absolute concentration of carotenoids and tocopherols in the used plasma pool

	Concentration (μ*M*)	Concentration (μg/l)
α-Tocopherol	29.00±0.52	12 500±231
γ-Tocopherol	5.78±0.14	2410±26
Lutein	0.462±0.020	262.9±11.6
Zeaxanthin	0.065±0.001	37.0±0.2
Lycopene	0.104±0.001	55.8±0.2
β-carotene	0.402±0.004	215.6±2.4

solvents A and B, which is probably due to the poor solubility of ammonium acetate in hexane.

Using a flow gradient instead of a solvent gradient gave a more constant baseline. The detection of retinols and tocopherols in channel B can be improved probably with a programmed wavelength change from 325 to 292 nm in channel B, concurrent with the retention times when the different compounds are eluted.

The sample preparation can be different for the other kinds of human tissues. Nevertheless, the

hexane extraction described above also worked well for simultaneous detection of these antioxidants in human eye tissues, as reported elsewhere [20]. The extraction of carotenoids from blood plasma samples seems to be more complicated. In our experiments we were unable to obtain a reasonable recovery of all carotenoids using a hexane extraction without saponification or digestion of the triglycerides (Table 1), which is contrary to some reports [9–11], but in agreement with others [7,22]. Unsuccessful also were the experiments using a method with hexane and ethyl acetate as extraction solvents, which was published elsewhere [23]. In these experiments we had poor recovery of β-carotene. To reach a satisfactory recovery of the carotenoids we either used alkaline saponification of the plasma triglycerides with KOH followed by a 30-min incubation at 60°C [22], or a 1-h enzymatic digestion of triglycerides at ambient temperature [7]. Detergents without enzyme did not yield satisfactory recovery.

In our first saponification experiments with KOH, the color of the samples turned red. During the hexane extraction this red color extracted into the hexane layer. After separation of the samples by HPLC we observed a new peak at the wavelength of 450 nm, almost co-eluting with the internal standard. In further investigations using solvent blanks without carotenoids, we found that there is a degradation of BHT by KOH, leading to formation of the red colored compounds, and giving the peak co-eluting with the internal standard. Initially, we used 0.5 mg BHT to protect the antioxidants of interest in the plasma sample. By decreasing the concentration of BHT down to 0.05 mg/sample, we could eliminate the formation of red color, and no additional peaks were observed in the chromatograms. There was still a sufficient protective effect [24], and the use of less BHT also improved the detection of retinol. One drawback of the KOH method is that the tocol internal standard was not always preserved in these samples. This could be overcome by using peak area ratios of α- and γ-tocopherol and apo-10′-carotenal methyloxime, thus using this as internal standard for the tocopherols instead of tocol. The ethanolic KOH also destroyed certain carotenoids in the sample (Fig. 1B), which was also found by Handelman et al. [7].

A different method to obtain a good recovery of all carotenoids is the digestion of triglycerides with enzymes [7]. This offers several advantages over the KOH method; no interaction with BHT, preservation of several carotenoids and tocol, sample preparation at room temperature and slightly better recoveries for all carotenoids. The advantages of the KOH method is that it is cheaper, and it is easier to detect retinol. After comparing both methods extensively, we decided to use the enzyme method for routine analysis of fat soluble antioxidants from plasma. Another reason for improved recovery of carotenoids after hydrolysis of the non-polar lipids is that the digested plasma extracts are more soluble in polar organic solvents. The extract must be completely dissolved, preferably in mobile phase, or in a solvent miscible with the mobile phase which does not create chromatographic artifacts [9]. In this study we were able to dissolve the saponified and digested samples directly into the mobile phase used.

Carotenoids are unstable and the stock solutions used for quantitation change in concentration over time because there is oxidative breakdown of the carotenoids, or the carotenoids come out of solution because of the tendency to crystallize when stored in the freezer at −20°C [10]. This can easily lead to erroneous quantitation. To overcome this problem, we suggest to make a human plasma pool with 1000 samples of 0.5 ml each [15,16]. The carotenoid and tocopherol content of the plasma from the pool can then be determined using standards, after which the plasma pool can be used for day to day calibration of the method. This offers an advantage over stock solutions in solvents, since tocopherols and carotenoids have been reported to be stable at −70°C for up to 5 years [25]. We routinely use a plasma pool for quantitative determination of fatty acids [18], protein content of samples, vitamin E [15] and carotenoids.

In conclusion the HPLC method described gives excellent separation and recoveries of 6 major antioxidants, lutein, zeaxanthin, lycopene, β-carotene, γ- and α-tocopherol, within 25 min. The extraction using enzymatic digestion of triglycerides was found to be suitable for the analysis of carotenoids and tocopherols in human plasma. However, the method can also be used to measure carotenoids in human eye tissues.

5. Abbreviations

BHT	Butylated hydroxytoluene
HPLC	High-performance liquid chromatography
DTPA	Diethylenetriamine-pentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulfate
PIPES	Piperazine N,N-bis(2-ethanesulfonic acid)

Acknowledgments

This work was supported by a Grant from the National Eye Institute (No. EY-08818-03A2) to FJGMvK and by a Grant form the German Academic Exchange Service (DAAD, No. D-94-20537) to OS. Dr. van Kuijk is a Research to Prevent Blindness William and Mary Greve Scholar.

References

[1] G.W. Burton, K.U. Ingold, Science 224 (1984) 569.
[2] A. Bendich, J.A. Olson, FASEB J. 3 (1989) 1927.
[3] N.I. Krinsky, J. Nutr. 119 (1989) 123.
[4] J.P. Sweeney, A.C. Marsh, J. Assoc. Off. Anal. Chem. 53 (1970) 937.
[5] J. Arnaud, I. Fortis, S. Blachier, D. Kia, A. Favier, J. Chromatogr. 572 (1991) 103.
[6] A.L. Sowell, D.L. Huff, P.R. Yeager, S.P. Caudill, E.W. Gunter, Clin. Chem. 40 (1994) 411.
[7] G.J. Handelman, B. Shen, N.I. Krinsky, Methods Enzymol. 213 (1992) 336.
[8] A.B. Barua, H.C. Furr, Methods Enzymol. 213 (1992) 273.
[9] K.S. Epler, R.G. Ziegler, N.E. Craft, J. Chromatogr. 619 (1993) 37.
[10] D. Hess, H.E. Keller, B. Oberlin, R. Bonfanti, W. Schuep, Int. J. Vitam. Nutr. Res. 61 (1991) 232.
[11] D.W. Nierenberg, S.L. Nann, Am. J. Clin. Nutr. 56 (1992) 417.
[12] Z. Zaman, P. Fielden, P.G. Frost, Clin. Chem. 39 (1993) 2229.
[13] T. Van Vliet, F. Van Schaik, J. Van Schoonhoven, J. Schrijver, J. Chromatogr. 553 (1991) 179.
[14] A.A. Franke, L.J. Custer, R.V. Cooney, J. Chromatogr. 614 (1993) 43.
[15] G.J. Handelman, L.J. Machlin, K. Fitch, J.J. Weiter, E.A. Dratz, J. Nutr. 115 (1985) 807.
[16] T. Friedrichson, H.L. Kalbach, P. Buck, F.J.G.M. van Kuijk, Curr. Eye Res. 14 (1995) 693.
[17] F.J.G.M. van Kuijk, G.J. Handelman, E.A. Dratz, J. Chromatogr. 348 (1985) 241.
[18] L. Zang, H.L. Kalbach, B. Brown, L.L. Jackson, F.J.G.M. van Kuijk, J. Chromatogr. B 677 (1996) 209.
[19] F.I. Chow, S.T. Omaye, Lipids 18 (1983) 837.
[20] G.J. Handelman, E.A. Dratz, C.C. Reay, F.J.G.M. van Kuijk, Invest. Ophthalmol. Vis. Sci. 29 (1988) 850.
[21] B. Olmedilla, F. Granado, I. Blanco, E. Rojas-Hidalgo, Am. J. Clin. Nutr. 60 (1994) 106.
[22] H.H. Schmitz, C.L. Poor, E.T. Gugger, J.W. Erdman Jr., Methods Enzymol. 214 (1993) 102.
[23] F. Khachik, G.R. Beecher, M.B. Goli, W.R. Lusby, C.E. Daitch, Methods Enzymol. 213 (1992) 205.
[24] R. Singkamani, S. Koottathep, A. Supapang, D.I. Thurnham, Clin. Chem. 35 (1989) 513.
[25] B.K. Edmonds, D.W. Nierenberg, J. Chromatogr. 614 (1993) 169.