Journal of Chromatography, 378 (1986) 45—53
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3070

SEPARATION OF RETINYL ESTERS BY NON-AQUEOUS REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

HAROLD C. FURR*, DALE A. COOPER and JAMES A. OLSON

Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011 (U.S.A.)

(Received October 9th, 1985)

SUMMARY

Rapid separation and sensitive quantitation of vitamin A esters can be achieved by use of an acetonitrile—dichloromethane (80:20) mobile phase with a 5-μm C_{18} column (15 cm × 4.6 mm) and absorbance detection at 325 nm. Either a Waters Resolve or a Rainin Microsorb column was used satisfactorily. Retinyl palmitate is eluted at about 7 min (capacity factor, $k' = 5.5$) at a flow-rate of 1.5 ml/min; retinyl palmitate and retinyl oleate, which are usually difficult to separate, are well resolved (resolution 1.2). Sensitivity (at a signal-to-noise ratio of 10:1) is 8 pmol retinyl palmitate (equivalent to 2.5 ng retinol). Quantitation of total retinyl esters is identical to that determined by a gradient high-performance liquid chromatographic technique over the range 30—1000 ng retinyl esters. Retinyl ester peaks in rat liver extracts were identified by their characteristic light absorption spectra, susceptibility to saponification, and by co-chromatography with authentic standards. Nine vitamin A ester peaks were identified and quantitated in rat liver extracts. A 10-μm Whatman Partisil 10/25 ODS-2 column was used with the same mobile phase to obtain partial resolution of retinyl esters (resolution 1.05 between retinyl oleate and retinyl palmitate; $k' = 11.0$ for retinyl palmitate) and improved retention for retinol ($k' = 2.5$, compared with $k' = 0.6$ for retinol on the 5-μm column).

INTRODUCTION

Dietary vitamin A in excess of immediate tissue requirements is stored as the ester of retinol with long-chain fatty acids; the primary site of such storage is the liver. Futterman and Andrews [1] analyzed the retinyl ester composition of human, rat, calf, sheep, rabbit, cat, frog and trout livers by gas chromatography of the saponified fatty acids (as their methyl esters). They found that the predominant ester in every case was retinyl palmitate, with retinyl stearate and oleate the next most prevalent; small amounts of retinyl linoleate, palmitoleate, myristate, heptadecanoate and pentadecanoate were also found

0378-4347/86/$03.50 © 1986 Elsevier Science Publishers B.V.

(in approximately that order of abundance). Goodman et al. [2] confirmed these results in rat liver by use of argentation thin-layer chromatography and reversed-phase paper chromatography.

More recently, reversed-phase high-performance liquid chromatography (HPLC) has been used to analyze retinyl ester composition: De Ruyter and De Leenheer [3] used methanol with an octadecylsilane column to separate several long-chain retinyl esters, and later used argentation chromatography with methanol—water eluents in an ingenious approach to separation of vitamin A esters [4]. Ross [5] and Bhat and LaCroix [6] have both used acetonitrile—water solvents on reversed-phase columns, but their procedures are time-consuming, requiring 1 h or more to elute retinyl stearate. Non-aqueous reversed-phase HPLC has been used previously for the separation of carotenoids [7—10] and for the chromatography of retinyl palmitate in cod liver oil [11] and in breakfast cereal [12], but to our knowledge it has not been used for the separation of individual retinyl esters. We report here that the use of acetonitrile—dichloromethane solvent mixtures provides a rapid (10 min), satisfactory separation of almost all retinyl esters when used with a 5-μm C_{18} column and can be used for resolution of the most prevalent vitamin A esters on a 10-μm octadecylsilane column.

EXPERIMENTAL

Animals

Weanling female Sprague—Dawley rats (Holtzman, Madison, WI, U.S.A.) were placed in individual cages and given pelleted vitamin A-deficient diet (ICN Nutritional Biochemicals, Cleveland, OH, U.S.A.) and tap water ad libitum. After one week on this diet without supplementation, animals were given a daily oral supplement of retinyl acetate (Sigma, St. Louis, MO, U.S.A.) in 0.1 ml corn oil, providing 5 or 240 μg vitamin A (as retinol) per day (five animals per group). Weight-matched controls received the daily 0.1 ml corn oil only. After fourteen days of vitamin A supplementation, all animals were sacrificed and livers were taken and frozen for later analysis.

Female cross-bred pigs (from the Swine Nutrition Herd, Iowa State University, Ames, IA, U.S.A.), approximately five weeks old, were fed nutritionally complete 14% protein sow feed (Cooper's Mill, Ames, IA, U.S.A.) for eight days ad libitum. Concurrently, oral doses of 436 μg vitamin A (expressed as retinol) were administered on alternate days to enhance storage of vitamin A in the liver. The central portions of the main lobes of each pig liver were removed and frozen at sacrifice.

Tissue retinoid assays

Thawed liver portions (1.0 g) were ground with anhydrous sodium sulfate and extracted with dichloromethane [13]; each liver extract was diluted to 50 ml. Aliquots of liver extracts were concentrated under argon, re-dissolved in 250 μl 2-propanol—dichloromethane (4:1) and analyzed by injection of 100 μl of solution. Gradient reversed-phase HPLC of liver extracts was performed as previously described [14] with a 10-min linear solvent gradient from methanol—water (85:15) to methanol—tetrahydrofuran (50:50) (flow-

rate 2.0 ml/min). An LDC gradient pumping system (Riviera Beach, FL, U.S.A.) and LDC Spectromonitor III variable-wavelength absorbance detector (set at 325 nm) were used, with a μBondapak C_{18} analytical column (Waters Assoc., Milford, MA, U.S.A.) preceded by a guard column. A Shimadzu C-R2AX electronic integrator (Shimadzu, Columbia, MD, U.S.A.) was used to determine peak areas, and retinol and retinyl esters were quantitated by use of appropriate standard curves. All extractions and chromatographic procedures were carried out under F40 Gold fluorescent lights.

Retinyl ester separation by isocratic non-aqueous reversed-phase HPLC

Retinyl esters were separated by reversed-phase HPLC on a Waters Resolve 5-μm C_{18} column (15 cm × 3.9 mm I.D.) using acetonitrile—dichloromethane (80:20) at a flow-rate of 1.5 ml/min. A Rainin Microsorb 5-μm C_{18} column, 15 cm × 4.6 mm I.D. (Rainin, Woburn, MA, U.S.A.) gave similar results, and chloroform could be substituted for dichloromethane in the mobile phase. For some studies, a 10-μm Partisil 10/25 ODS-2 column (25 cm × 4.6 mm I.D.; Whatman Chemical Separations, Clifton, NJ, U.S.A.) was used, with the same mobile phase. An Uptight guard column (Upchurch Scientific, Oak Harbor, WA, U.S.A.) with pellicular octadecylsilane packing (Vydac, The Separations Group, Hesperia, CA, U.S.A.) preceded the analytical column. A single LDC Constametric III pump and a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) were used, with an LDC Spectromonitor III variable-wavelength detector (set at 325 nm); a Shimadzu C-R2AX integrator was used to plot the chromatogram at two attenuations simultaneously and to determine peak areas. Portions of the dichloromethane liver extracts were concentrated and dissolved in 2-propanol—dichloromethane for HPLC analysis. Aliquots of approx. 30—300 ng retinyl esters (corresponding to 2—10 mg liver, depending on liver vitamin A content) were injected. Injection volumes much greater than 10 μl led to impaired peak resolution. Chromatographic conditions are summarized in Table I.

TABLE I

CHROMATOGRAPHIC CONDITIONS

5-μm Column	Waters Resolve 5-μm C_{18} (Waters Assoc.), 15 cm × 3.9 mm
Mobile phase	Acetonitrile—dichloromethane (80:20) at a flow-rate of 1.5 ml/min
Detection wavelength	325 nm
Sensitivity	2.5 ng vitamin A palmitate (expressed as ng retinol), equivalent to 8 pmol
Resolution	1.2 between retinyl oleate and retinyl palmitate
Reproducibility	Within-day coefficient of variation (n=5) ± 0.25% for retinyl palmitate and ± 2.21% for retinyl oleate Between-day coefficient of variation (n=5) ± 0.20% for retinyl palmitate and ± 1.12% for retinyl oleate
10-μm Column	Whatman Partisil 10/25 ODS-2 10-μm C_{18} (Whatman Chemical Separations), 25 cm × 4.6 mm
Mobile phase	Acetonitrile—dichloromethane (80:20) at 1.5 ml/min
Sensitivity	4.3 ng vitamin A palmitate (expressed as ng retinol), equivalent to 15 pmol (at 10:1 signal-to-noise ratio)
Resolution	1.1 between retinyl oleate and retinyl palmitate

Retinyl ester standards for peak identification were synthesized by Dr. Arun Barua from retinol and the appropriate acyl chlorides [15], or by transesterification of retinyl acetate with the appropriate fatty acid methyl ester [16]. 13-*cis* Retinyl palmitate was prepared, purified and characterized by Mr. Gary Landers of this laboratory. Individual retinyl esters in liver extracts were identified by co-chromatography with each ester standard. To further identify chromatographic peaks, liver extracts were saponified, and the hexane extracts of saponification were evaporated and dissolved in 2-propanol—dichloromethane at the same final concentration as the unsaponified extracts and analyzed under the same chromatographic conditions. In addition to chromatographic analysis monitored at 325 nm, some extracts were monitored at 280 nm to facilitate recognition of non-retinoid peaks. A Shimadzu SPD-M1A photodiode-array spectrophotometric detector also was used to confirm the characteristic absorption spectrum of retinyl ester peaks.

Retinyl ester quantitation

The retinol component of each vitamin A ester was quantitated by peak area by using retinyl palmitate (Sigma) as standard. Values are expressed as ng retinol, using $E^{1\%}_{1\,\mathrm{cm}}$ = 1850. To determine agreement for total retinyl ester assay between the gradient and isocratic reversed-phase HPLC techniques, aliquots of various volumes of a single dichloromethane liver extract were concentrated and re-dissolved in 2-propanol—dichloromethane (to give 0.5—100 ng/μl total vitamin A. Aliquots (10 μl) of each concentrate were analyzed by both gradient and isocratic HPLC techniques.

RESULTS AND DISCUSSION

As can be seen in Fig. 1, the non-aqueous reversed-phase HPLC system described here gives rapid separation of all the major retinyl esters. Twelve peaks are routinely detected in liver extracts when the chromatography is monitored at 325 nm. To obviate slight changes in retention time (retention times with this solvent are sensitive to ambient temperature), we have used retention times relative to that of retinyl palmitate (capacity factor, k' = 5.5, uncorrected retention time 7.2 min). Based on co-chromatography with authentic standards, the vitamin A peaks are identified as indicated in Table II. Semi-logarithmic plots of log retention time versus fatty acyl carbon number were linear (but not parallel) for saturated and mono-unsaturated acyl retinyl esters (Fig. 2); on the basis of these plots, peak 8 is tentatively identified as retinyl pentadecanoate and peak 11 as retinyl heptadecanoate. All the peaks identified as retinyl esters were susceptible to saponification, were diminished in vitamin A-depleted animals (Fig. 1) and appeared to have the characteristic light absorption spectrum of a retinyl ester (data not shown). Retinyl laurate and retinyl arachidonate standards were not resolved under these chromatographic conditions. Because retinyl arachidonate is not synthesized in vitro by liver microsomes under conditions in which other retinyl esters are synthesized readily [17], we have provisionally identified peak 4 as retinyl laurate. Futterman and Andrews [1, 16] were unable to find retinyl arachidonate in tissues that they examined.

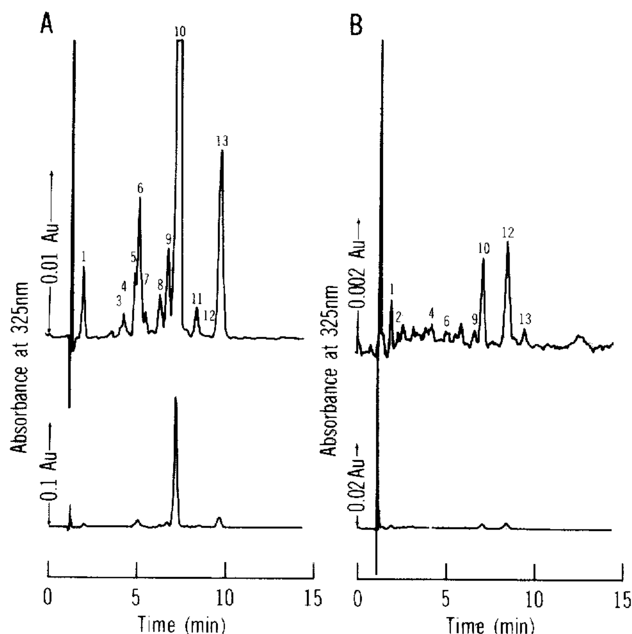

Fig. 1. Isocratic non-aqueous reversed-phase high-performance liquid chromatograms of rat liver extracts, using 5-μm Resolve column. Chromatographic conditions as in Table I; peak identification as in Table II. (A) Liver extract from rat fed 240 μg retinol (as retinyl acetate) per day for fourteen days; aliquot of extract analyzed equivalent to 1.6 mg liver, 281 ng total retinyl esters. Upper trace is chromatogram recorded at attenuation 5, lower trace shows the same chromatogram recorded simultaneously at attenuation 9. (B) Liver extract from rat fed 5 μg retinol (as retinyl acetate) per day for fourteen days; aliquot of extract analyzed equivalent to 11.1 mg liver, 4.0 ng total retinyl esters. Upper trace is chromatogram recorded at attenuation 3, lower trace shows the same chromatogram recorded simultaneously at attenuation 7.

TABLE II

PEAK IDENTIFICATION

Peak No.*	Identification	Relative retention time**
1	Retinol	0.26
2	Non-retinoid	0.36
3	Retinyl linolenate	0.56
4	Retinyl laurate	0.59
5	Non-retinoid	0.69
6	Retinyl linoleate	0.70
7	Retinyl myristate + retinyl palmitoleate	0.75
8	Retinyl pentadecanoate	0.87
9	Retinyl oleate	0.93
10	Retinyl palmitate	1.00
11	Retinyl heptadecanoate	1.16
12	Non-retinoid	1.20
13	Retinyl stearate	1.35

*Peak numbers as indicated in Figs. 1 and 4.

**Relative retention times on Resolve 5-μm C_{18} column.

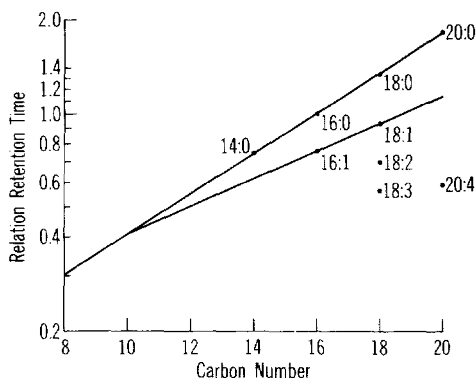

Fig. 2. Relative retention times of retinyl esters. Semi-logarithmic plot of retention times of retinyl ester standards (relative to retinyl palmitate) versus carbon number of fatty acyl component, as determined on Resolve 5-μm C_{18} column. Chromatographic conditions as in Table I.

The selectivity of this method, as of most HPLC methods for vitamin A, depends on the fact that very few other biological compounds absorb light significantly at 325 nm. However, especially in concentrated tissue extracts, some other compounds are also detected. In Fig. 1, the peak eluting at 4.9 min (peak 5, just before retinyl linoleate) appears to have greater light absorbance at 280 nm than at 325 nm and thus does not seem to be a retinyl ester. A peak eluting at 7.2 min (peak 12, between retinyl heptadecanoate and stearate) has maximum optical density at 275 nm. The peak eluting at about 2.6 min (peak 2) also shows an absorbance maximum at about 280 nm and is tentatively identified as α-tocopherol because it co-elutes with an authentic sample.

13-*cis* Retinyl palmitate standard elutes between all-*trans* retinyl oleate and all-*trans* retinyl palmitate; it does not seem to be present in appreciable amounts in these liver extracts. Because peaks are infrequently observed eluting after retinyl stearate, it seems that retinyl arachidate and longer-chain saturated acyl retinyl esters were not present in detectable amounts in these extracts. An α-carotene standard elutes at 0.83 relative to retinyl palmitate, and β-carotene at 0.89 (between retinyl myristate and retinyl pentadecanoate). Carotenoids were shown to be absent from these rat samples by the lack of light absorbance at 450—490 nm (data not shown); however, the presence of these two carotenoids should not interfere with the determination of retinyl esters other than retinyl pentadecanoate. In several human serum samples analyzed by this technique, lycopene was found at 0.58 relative to retinyl palmitate, potentially interfering with the identification and quantitation of retinyl linoleate and laurate. Light absorption at 325 nm by such carotenoids is typically 10% of their maximum light absorption.

Sensitivity of this technique, using our instrumentation, is about 8 pmol retinyl palmitate (equivalent to 2.5 ng retinol) at a signal-to-noise ratio of 10:1, and reproducibility is good (Table I). This method, using a 5-μm end-capped column (i.e., without free silanol groups), may not always be suitable for quantitation of retinol, however; because the retinol peak elutes so near the solvent front (uncorrected retention time 1.4 min; $k' = 0.6$), other com-

ponents in liver extract may contribute to its absorbance. Thus, the previously described gradient HPLC system [14] is currently recommended for quantitation of retinol and total retinyl esters in a single run, unless it can be shown that there are no other contributions to the retinol absorbance peak.

As confirmed by Ross [5], the light absorbance at 325 nm of vitamin A esters is entirely due to their retinyl component. Thus, it is convenient to express mass of retinyl esters as mass of the retinol component, ignoring the mass of the fatty acyl unit. This has further advantages in studies of vitamin A nutriture, making quantities of vitamin A (retinol, retinyl acetate, retinyl palmitate or other retinyl esters) directly comparable. We have adhered to this convention in this study. We compared total retinyl esters (without regard to their fatty acyl composition) as determined by the gradient reversed-phase HPLC technique (Fig. 3). At levels above 30 ng total vitamin A esters, there is excellent agreement between the two methods. The decrease in total retinyl esters at lower levels found by the isocratic method is explained by the failure to detect minute quantities of the minor retinyl esters.

Operating pressures with this solvent system are usually low (47—53 bar). In analyses of some few tissue extracts, we have observed precipitation of lipids within the analytical column, with consequent pressure increases. This problem usually could be alleviated by a brief rinse with methanol.

The separations of the retinyl oleate/palmitate pair and the retinyl myristate/palmitoleate pair have been the most difficult in previous HPLC separations of retinyl esters [3—6]. We had found previously that methanol—tetrahydrofuran—water solvent mixtures could separate the saturated retinyl esters by chain length when used on a 10-μm C_{18} column [14]. De Ruyter and De Leenheer [4] had overcome this problem by incorporating silver salts in the mobile phase. Ross [5] and Bhat and LaCroix [6] found that acetonitrile—water mixtures could provide sufficient separation of retinyl oleate from palmitate, but at the expense of very long elution times (on the order of 1 h). The method presented here, using non-aqueous acetonitrile—dichloromethane,

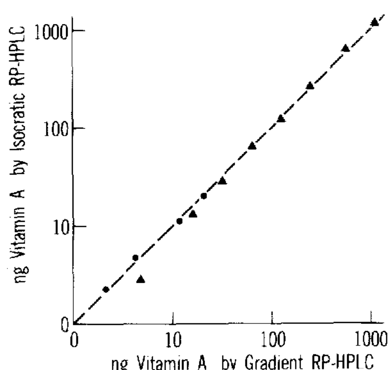

Fig. 3. Correlation of quantitation by gradient reversed-phase HPLC and by isocratic non-aqueous reversed-phase HPLC on 5-μm Waters Resolve column. Aliquots of various volumes of a single liver extract were concentrated to 250 μl, and 10-μl aliquots of each were analyzed by both HPLC methods. Retinol and total retinyl esters were quantitated by the appropriate standard curves for each technique. (●) Retinol; (▲) total retinyl esters; (- -) theoretical 1:1 correspondence between values obtained by each HPLC technique.

provides adequate resolution of all the retinyl esters tested except retinyl myristate/palmitoleate and retinyl laurate/arachidonate. Analysis times are short (capacity factors for retinyl esters from 2.8 to 8.7, all esters eluted within 10 min), providing good sensitivity compared with slower HPLC separations (due to less peak broadening) and permitting the rapid analysis of many samples. The expense and nuisance of argentation chromatography are avoided. Resolution of retinyl oleate and palmitate (calculated at baseline) is 1.2. We have not been able to achieve separation of retinyl oleate from palmitate with methanol—tetrahydrofuran, acetonitrile—tetrahydrofuran or methanol—dichloromethane mobile phases, but acetonitrile—chloroform (80:20) gives results very similar to those of acetonitrile—dichloromethane. (We prefer the use of dichloromethane because of the known health hazards of chloroform; however, dichloromethane-containing solutions should also be used with caution.) The two 5-μm C_{18} columns that we have tested (Rainin Microsorb and Waters Resolve) have given similar results. A 10-μm μBondapak C_{18} column (Waters Assoc.) did not give satisfactory resolution of the esters in preliminary testing with this mobile phase.

A 10-μm column that was useful in the separation of retinyl esters was a Whatman Partisil 10/25 ODS-2 column that was used to analyze pig liver extracts (Fig. 4). As expected, there was a reduction in resolution, but quantitation of total retinyl esters above 60 ng gave results comparable to those obtained by gradient HPLC analysis (data not shown). The lower sensitivity obtained when using the 10-μm column (15 pmol or 4.3 ng retinyl palmitate,

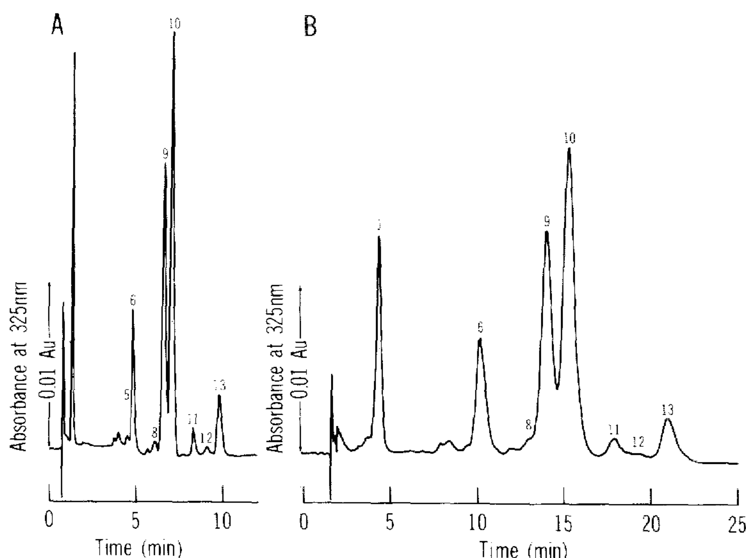

Fig. 4. Comparison of non-aqueous reversed-phase separation of retinyl esters by 5-μm Resolve column and 10-μm Partisil ODS-2 column. Liver extract from pig fed 436 μg retinol on alternate days for eight days. Chromatographic conditions as in Table I; peak identification as in Table II. (A) Separation on Waters Resolve 5-μm C_{18} column. Aliquot of extract analyzed equivalent to 11.6 mg liver, 230 ng total retinyl esters. (B) Separation on Whatman Partisil ODS-2 10-μm C_{18} column. Aliquot of extract analyzed equivalent to 29 mg liver, containing 640 ng total retinyl esters.

expressed as ng retinol component) was compensated for by the ability to inject larger sample volumes without peak distortion. This system resolved five major vitamin A ester peaks, which were determined using criteria similar to those used for 5-μm columns to be the linoleyl, oleyl, palmitoyl, heptadecanoyl and stearoyl esters of retinol. These five retinyl esters compose approx. 96% of the total retinyl esters in pig liver, in agreement with analysis on the 5-μm column. In the samples studied, the inability to resolve minor retinyl ester peaks has little effect on the values obtained for the relative abundance of the major retinyl esters.

Additionally, the Partisil ODS-2 column resolved retinol from the solvent front ($k' = 2.5$) sufficiently for it to be quantitated with more confidence. The greater retention of retinol on the ODS-2 column compared with that on the 5-μm columns tested may be explained by a lack of end-capping on the Partisil ODS-2 column so that an appreciable number of silanol groups remain free and available for hydrogen bonding with retinol.

ACKNOWLEDGEMENTS

We acknowledge the kind help of Dr. Arun Barua and Mr. Gary Landers in providing retinyl ester standards. This work was supported by USDA Competitive Research Grant No. 84-CRCR-1-1418 and by NIH-AM 32793 of the US Public Health Service. This is Journal Paper J-12000 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA, U.S.A.; Project No. 2534.

REFERENCES

1 S. Futterman and J.S. Andrews, J. Biol. Chem., 239 (1964) 4077.
2 D.S. Goodman, H.S. Huang and T. Shiratori, J. Lipid Res., 6 (1965) 390.
3 M.G.M. De Ruyter and A.P. De Leenheer, Clin. Chem., 24 (1978) 1920.
4 M.G.M. De Ruyter and A.P. De Leenheer, Anal. Chem., 51 (1979) 43.
5 A.C. Ross, Anal. Biochem., 115 (1981) 324.
6 P.V. Bhat and A. LaCroix, J. Chromatogr., 272 (1983) 269.
7 H.J.C.F. Nells and A.P. De Leenheer, Anal. Chem., 55 (1983) 270.
8 C.R. Broich, L.E. Gerber and J.W. Erdman, Lipids, 18 (1983) 253.
9 N. Ketrangi, L.A. Kaplan and E.A. Stein, J. Lipid Res., 25 (1984) 400.
10 C.C. Tangney, J. Liq. Chromatogr., 7 (1984) 2611.
11 N.A. Parris, J. Chromatogr., 157 (1978) 161.
12 W.O. Landen, J. Assoc. Off. Anal. Chem., 63 (1980) 131.
13 J.A. Olson, Nutr. Rep. Int., 19 (1979) 807.
14 H.C. Furr, O. Amédée-Manesme and J.A. Olson, J. Chromatogr., 309 (1984) 299.
15 H.S. Huang and D.S. Goodman, J. Biol. Chem., 240 (1965) 2839.
16 S. Futterman and J.S. Andrews, J. Biol. Chem., 239 (1964) 81.
17 H.C. Furr, M.D. Ball and J.A. Olson, Fed. Proc., Fed. Am. Soc. Exp. Biol., 44 (1985) 772.