ELSEVIER

Journal of Chromatography B, 707 (1998) 69–79

JOURNAL OF CHROMATOGRAPHY B

# Reversed-phase gradient high-performance liquid chromatographic procedure for simultaneous analysis of very polar to nonpolar retinoids, carotenoids and tocopherols in animal and plant samples

Arun B. Barua*, James A. Olson

*Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011, USA*

Received 20 August 1997; received in revised form 26 November 1997; accepted 28 November 1997

**Abstract**

A reversed-phase gradient high-performance liquid chromatographic (HPLC) procedure, which utilizes gradient elution and detection by a photodiode-array detector, has been developed to analyze simultaneously very polar retinoids, such as 4-oxo-retinoyl-β-glucuronide, retinoyl β-glucuronide and 4-oxo-retinoic acid; polar retinoids, such as retinoic acid and retinol; nonpolar retinoids, such as retinyl esters; along with xanthophylls, monohydroxy carotenoids, hydrocarbon carotenoids, and tocopherols. The procedure has been applied to the simultaneous analysis of retinoids, carotenoids, and tocopherols present in human serum and liver, rat serum and tissues, and for carotenoids in a number of fruits and vegetables. Bilirubin present in human serum can also be simultaneously analyzed. By this gradient HPLC procedure, 3,4-didehydroretinyl ester (vitamin $A_2$ ester) has been identified as a minor constituent in a human liver sample. Lycopene was identified as a major carotenoid in one specimen of papaya fruit, and 5,6,5′,6′-diepoxy-β-carotene was characterized as a major carotenoid in one specimen of mango fruit. © 1998 Elsevier Science B.V.

*Keywords:* Retinoids; Carotenoids; Tocopherols

## 1. Introduction

High-performance liquid chromatography (HPLC) has become the method of choice for the analysis of retinoids, carotenoids and tocopherols in animal and plant tissues. Because the structure and polarity of these analytes are very different, however, the separation and quantification of all the analytes in a single run in a reasonable amount of time are very difficult. Several reversed-phase HPLC procedures that simultaneously analyze retinol, α- and γ-tocopherols, retinyl esters and the carotenoids that occur in human and animal serum and tissues [1–11] do not separate more polar metabolites satisfactorily.

Retinol (ROL) is metabolized to a number of polar metabolites, namely, retinoic acid (RA), 4-oxo-retinoic acid, retinoyl β-glucuronide (RAG) and 5,6-epoxy-retinoic acid [for a review, see [12]], some of which play important physiological roles in the overall functions of vitamin A. Methods that are available for the analysis of polar metabolites of RA cannot analyze simultaneously the less polar retinyl esters, whereas methods that can separate ROL and its esters cannot be used to separate the more polar retinoids [for a review, see [13]]. Recently, polar retinoids and nonpolar retinyl esters were resolved

*Corresponding author.

0378-4347/98/$19.00 © 1998 Elsevier Science B.V. All rights reserved.
*PII* S0378-4347(97)00614-2

by HPLC, but only in a total run time of 115 min [14]. Methods that have been described for the analysis of carotene cleavage products, including ROL and RA [15–17], also do not seem to be suitable for the analysis of very polar retinoids, such as 4-oxo-retinoic acid and RAG [18,19]. In our laboratory, attempts to analyze RAG on $C_{18}$ columns using a solvent mixture containing varying proportions of acetonitrile, dichloromethane and methanol containing 0–10% water and ammonium acetate, were unsuccessful. RAG was either retained on the column or was completely degraded.

In recent years, the use of a photodiode-array (PDA) detector during HPLC analysis [9,11,20] has allowed the identification of analytes that escape detection during analysis by a single-wavelength detector or by detectors set at fixed wavelengths. Thus, by use of a PDA detector, it has been possible to demonstrate the occurrence of colorless carotenoids like ς-carotene, phytoene and phytofluene [9,11], which absorb at wavelengths of ≤400 nm, in biological samples.

In the present study, we developed a reversed-phase gradient HPLC procedure for the analysis of very polar, polar and nonpolar retinoids and carotenoids along with tocopherols and bilirubin in a single run in a reasonable amount of time. The usefulness of the procedure in studying the metabolism of RA in humans and of RAG and β-carotene in the rats will be described. Also, we describe a simplified procedure for the extraction of these analytes from animal and plant tissues.

## 2. Experimental

### 2.1. Solvents and reagents

HPLC-grade methanol and reagent-grade dichloromethane and 2-propanol were purchased from Fisher Scientific (Fairlawn, NJ, USA). All-*trans* 4-oxo-retinoyl β-glucuronide (ORAG) and all-*trans* retinoyl-β-glucuronide (RAG) were synthesized as described previously from this laboratory [21]. All-*trans* 4-oxo-retinoic acid (ORA) and all-*trans* retinoic acid (RA) were gifts from Hoffman La-Roche, NJ, USA. Methyl retinoate (MR) was prepared by treating RA with a solution of diazomethane in diethyl ether. 5,6-Epoxyretinoic acid (ERA) and 5,6,5′,6′-diepoxy-β-carotene were prepared by reaction of RA and β-carotene, respectively, with 3-chloroperoxybenzoic acid. 3,4-Didehydroretinol and its acetate were graciously provided by Dr. S. Tanumihardjo. β-Carotene, α- and γ-tocopherols, retinol (ROL), retinyl acetate (RAC) and bilirubin were purchased from Sigma (St. Louis, MO, USA). Lutein was a gracious gift from Kemin Industries (Des Moines, IA, USA). Prior to their use as standards, all compounds were analyzed and purified by HPLC, whenever necessary.

### 2.2. HPLC system

The HPLC system consisted of the following Waters (Milford, MA, USA) equipment: an automated sample injector (WISP 710B), two Model 510 pumps, an automated gradient controller, a model 991 photodiode-array detector, and a model 5200 printer–plotter. The data generated in the 3D (DT3) mode, were stored and processed by a NEC Powermate SX Plus computer (NEC Technology, Boxborough, MA, USA) (PDA 991 System). Some of the samples were later analyzed by a Model 996 PDA detector equipped with a 717 plus autosampler, a MILLENIUM 2010 chromatography manager, a Model 486/33I NEC Powermate and a Hewlett-Packard (Houston, TX, USA) Laser Jet series II printer (PDA 996 System).

A 3-μm Microsorb-MV column (100×4.6 mm) (Rainin, Woburn, MA, USA) was used, which was preceded by a guard column of $C_{18}$ material (Upchurch Scientific, Omaha, NE, USA). The solvent system consisted of methanol–water (3:1, v/v) containing 10 m*M* ammonium acetate (solvent A) and methanol–dichloromethane (4:1, v/v) (solvent B). A linear gradient from solvent A (100%) to solvent B (100%) was applied over a period of 15–20 min, followed by isocratic elution with solvent B (100%) for an additional 15–20 min. The flow-rate was 0.8 ml/min. At the end of the run, the gradient was reversed to initial conditions by applying a linear gradient of 5 min. The column was then allowed to equilibrate for 10 min with solvent A before the next injection.

*2.2.1. Metabolism of orally administered RA in humans*

Human volunteers were dosed with RA suspended in corn oil (50 mg/person). Plasma samples (500 μl) were analyzed at various times after the dose. The details of the experimental procedures have been described elsewhere [22].

*2.2.2. Metabolism of RAG in rats*

In brief, rats of different vitamin A status were dosed with RAG (3 mg/rat) and blood and tissues were analyzed for retinoids at various times after the dose. The details of these experiments have been described elsewhere [23].

*2.2.3. Metabolism of β-carotene in rats*

In these studies, details of which will be published elsewhere, the metabolism of β-carotene in vitamin A-sufficient and vitamin A-deficient rats was examined. Rats were dosed orally with β-carotene (3 mg/rat) suspended in peanut oil, and their blood and other tissues were collected and analyzed for carotenoids and retinoids at various times after the dose.

*2.3. Extraction from serum*

Human or rat serum was extracted by a modification [24] of a published procedure [11] as follows: the extraction solvent mixture was prepared by mixing 2-propanol and dichloromethane (2:1, v/v) containing retinyl acetate (about 6 n*M*), which was used as an internal standard, and the antioxidant, butylated hydroxytoluene (BHT) (1 m*M*). A measured volume of serum (20–100 μl) (1 vol) was placed in a conical centrifuge tube (1.5-ml capacity). The extraction solvent mixture (3 vol), was added, followed by the addition of glacial acetic acid (1 μl/20 μl serum). The mixture was vortexed for 30 s, then centrifuged for 1 min in an Eppendorf microcentrifuge (Model 5415). The supernatant solution was pipetted out and transferred into a glass autosampler insert (300-μl capacity, Waters). An aliquot of 20–70 μl was injected onto the HPLC system. In the absence of acetic acid, the recovery of RA was only 67±1.5% [11], but by adding acetic acid to the extraction solvent, the recovery of RA was increased to 98±5% [24].

To examine the UV–visible spectrum of very weak peaks in the chromatogram, the extract from 100 μl or more of serum was evaporated to dryness under a stream of argon. Occasionally, when a trace of solvent was difficult to remove, addition of methanol followed by a stream of argon expedited evaporation. The residue was dissolved in 50–100 μl of 2-propanol–dichloromethane (2:1, v/v), and an aliquot was injected. When larger volumes (>0.5 ml) of serum were used, extraction was carried out with ethanol, ethyl acetate and hexane as described previously [11], but in the presence of acetic acid.

*2.4. Extraction from animal and plant tissues and a multivitamin tablet*

Human and rat liver (100–200 mg), and other tissues (200 mg– 2 g), or the pulp of fruits and fresh vegetables (2–5 g) were first finely minced and then ground or homogenized in 3–5 ml of 2-propanol–dichloromethane (2:1, v/v). The mixture was transferred into a 20-ml vial, and the volume was increased to about 10 ml with 2-propanol–dichloromethane (2:1, v/v). The vial was stoppered, vortexed for 1 min, and then kept under argon at −20°C overnight. The next day, the mixture was vortexed for 1 min and returned to the freezer. On the third day, the mixture was vortexed, then centrifuged or filtered, and the supernatant solution or the filtrate was evaporated to dryness in a rotary evaporator. The residue was dissolved in 200 μl 2-propanol–dichloromethane (2:1, v/v). An aliquot of 20–40 μl was injected onto the HPLC system.

A multivitamin tablet (Centrum) was ground to a powder, and the powder was transferred to a 20-ml vial. A 10-ml volume of 2-propanol–dichloromethane solution was added, and the mixture was treated as described above. On the third day, the mixture was vortexed; then about 500 μl of the solution was centrifuged, and a 50-μl aliquot was injected onto the HPLC system.

## 3. Results

*3.1. Reference compounds*

The spectrum index plot, obtained by use of the PDA 991 System and a gradient time of 15 min, of a

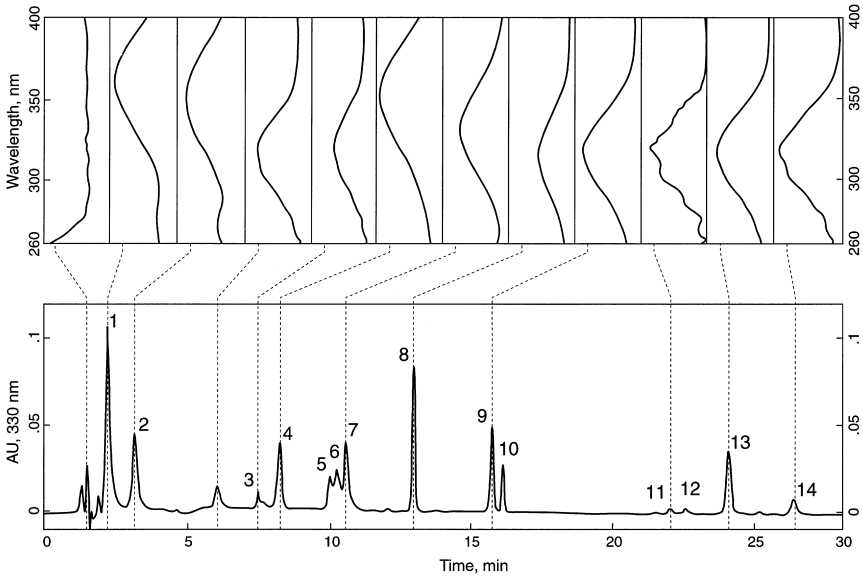


Fig. 1. Spectrum index plot of a standard mixture of retinoids obtained by reversed-phase gradient HPLC equipped with the PDA 991 System, as described in Section 2. Peak identification: 1, all-*trans* 4-oxo-retinoyl β-glucuronide; 2, all-*trans* 4-oxo-retinoic acid; 3, all-*trans* 5,6-epoxyretinoic acid; 4, all-*trans* retinoyl β-glucuronide; 5, 13-*cis* retinoic acid; 6, 9-*cis* retinoic acid; 7, all-*trans* retinoic acid; 8, all-*trans* retinol; 9, all-*trans* retinyl acetate; 10, methyl retinoate; 11, retinyl ester; 12, retinyl linolenate; 13, retinyl palmitate; 14, retinyl stearate.

mixture of various reference retinoids that generally occur in serum and other animal tissues, is shown in Fig. 1. Very polar retinoids, such as ORAG (peak 1) and ORA (peak 2) clearly separated well from the polar retinoids, RA (peaks 5–7) and ROL (peak 8), as well as from nonpolar retinyl esters (peaks 11–14). ERA (peak 3) and RAG (peak 4) also separated well from each other. The carotenoids and tocopherols, whose chromatograms are not shown in Fig. 1, also resolved well from each other, thereby making the gradient procedure suitable for simultaneous analysis of all of these analytes in a single run.

In Table 1, the retention times of the retinoids, carotenoids, and α- and γ-tocopherol standards are shown under one experimental condition. When tissue extracts were analyzed, slight variations of retention times were occasionally observed. Therefore, in each analysis the identification of the compound in each peak was confirmed by study of its absorption spectra by use of the PDA. Only partial separation of 9-*cis*-, 13-*cis*- and all-*trans*-RA was achieved. In the absence of 9-*cis*-RA (Fig. 1, peak 6), however, 13-*cis*-RA (Fig. 1, peak 5) and all-*trans*-RA (Fig. 1, peak 7) separated well from each

Table 1
Retention times ($t_R$) of retinoids, carotenoids and tocopherols

| Analyte | $t_R$ (min) |
|---|---|
| All-*trans* 4-oxoretinoyl β-glucuronide | 2.1 |
| All-*trans* 4-oxoretinoic acid | 3.1 |
| All-*trans* 5,6-epoxyretinoic acid | 7.4 |
| All-*trans* retinoyl β-glucuronide | 8.7 |
| 13-*cis* Retinoic acid | 9.9 |
| 9-*cis* Retinoic acid | 10.2 |
| All-*trans* retinoic acid | 10.5 |
| All-*trans* retinol | 12.9 |
| All-*trans* retinal | 13.8 |
| All-*trans* retinyl acetate | 15.7 |
| All-*trans* lutein | 16.1 |
| γ-Tocopherol | 18.2 |
| α-Tocopherol | 18.7 |
| All-*trans* methyl retinoate | 16.3 |
| All-*trans* retinyl palmitate | 24.1 |
| All-*trans* retinyl stearate | 26.4 |
| All-*trans* lycopene | 26.5 |
| All-*trans* β-carotene | 27.1 |

HPLC was carried out on a Rainin 3-μm Microsorb-MV column (100 ×4.6 mm) by use of PDA 991 system and a linear gradient of methanol–water (75:25, v/v) containing 10 m*M* ammonium acetate) to methanol—dichloromethane (4:1, v/v) in 15 min, followed by isocratic elution with the later solvent mixture for an additional 15 min at a flow-rate of 0.8 ml/min.

other. The procedure was then applied to the analysis of retinoids, carotenoids, tocopherols and bilirubin in human serum and liver, rat serum and other tissues, in several fruits and vegetables and in a multivitamin tablet.

### 3.2. Human and rat serum and tissues

Extraction of retinoids from the serum collected 3 h after an oral dose of RA to a human volunteer, followed by analysis with the PDA 996 System and a gradient time of 20 min, gave the chromatograms shown in Fig. 2. Chromatogram 2A was monitored at 290 nm for tocopherols, chromatogram 2B at 340 nm for retinoids, and chromatogram 2C was monitored at 445 nm for the carotenoids. The compound in each peak was identified by comparing the absorption spectrum and retention time of each compound with authentic standards. The separation of γ-tocopherol (peak 16) and α-tocopherol (peak 17) is shown in Fig. 2A. Peak 15 was due to BHT. In Fig. 2B, endogenous ROL (peak 8) separated well from the administered RA (peak 7) and the internal standard retinyl acetate (peak 9). In this serum sample, it was possible to detect some 4-oxo-RA (peak 2) as well as RAG (peak 4). Bilirubin appeared as a broad peak (Fig. 2C, peak 18) just prior to RAG and RA, but did not interfere with their detection at 300–350 nm. The separation of lutein (peak 19), zeaxanthin (peak 20), β-cryptoxanthin (peak 22), lycopene (peak 23), α-carotene (peak 24) and β-carotene (peak 25) is shown in Fig. 2C. Peak 21 was identified in earlier studies as 2′,3′-anhydrolutein [9,11]. When the chromatograms were plotted at 345 and 400 nm, it was also possible to detect some ς-carotene ($\lambda_{max}$ 425, 400, 380 nm in solvent B) and phytofluene ($\lambda_{max}$ 365, 345, 330 nm in solvent B), respectively, in human serum.

The same procedure was used in studying the metabolism of orally administered RAG in rats of different vitamin A status [23]. Serum, liver, intestines, spleen, brain, heart, skeletal muscle, kidney and lung tissues were analyzed for all the polar and nonpolar retinoids. The details of these analyses have been described elsewhere [23]. It has been demonstrated that the present procedure could separate the polar retinoids, such as RAG and RA, along with the nonpolar retinoids, namely the retinyl esters very well. In this study, the usefulness of retinyl acetate as an internal standard in quantifying the analytes was demonstrated.

A sample of liver was obtained during an autopsy from a human patient who died from a genetic disease, Down's syndrome. The normal diet of the patient is not known. However, the patient had received large supplements of vitamin A for several months prior to death. Although the range of liver vitamin A concentration in a healthy population varies form 20–400 μg/g liver, we found a very high level of retinyl esters (2297 μg/g liver) in this liver sample (Fig. 3). At least six retinyl esters (peaks 11–14 and peaks marked RE) were identified from their characteristic absorption spectra. The concentration of retinyl palmitate (peak 13) was the highest (1753 μg/g liver), followed by retinyl linolenate (274 μg/g, peak 12) and retinyl stearate (161 μg/g, peak 14). It was also possible to identify 3,4-didehydroretinyl ester (peak marked DR, Fig. 3), presumably as its palmitate (vitamin $A_2$ palmitate) as a minor retinoid (35 μg/g liver, peak marked DR), on the basis of its characteristic absorption spectrum ($\lambda_{max}$ 330, 275, ~265 nm in the HPLC solvent mixture) as shown as an insert in Fig. 3. Although the absorption spectrum of the ester was identical to that of 3,4-didehydroretinyl acetate, its elution time as expected was much later than the acetate which eluted just prior to retinyl acetate (not shown). Saponification of the ester fraction gave 3,4-didehydroretinol, which eluted just prior to ROL during HPLC, and gave the characteristic absorption spectrum of the compound.

Extraction of the carotenoids and retinoids from serum and tissues of rats dosed with β-carotene followed by HPLC analysis, showed that β-carotene was converted predominantly to ROL and to some RA by vitamin A deficient rats. The chromatogram of an extract of the small intestine and its contents from a vitamin A deficient rat 3 h after the dose, obtained with the PDA 996 system and a gradient time of 20 min, is shown in Fig. 4A. It can be seen that RA (peak 7), ROL (peak 8) and retinyl esters (peaks 11–13) were formed from the administered β-carotene (peak 25). Because the dose of β-carotene was huge, a substantial amount of β-carotene was present in the intestinal contents and could be seen in the chromatogram (peak 25), even

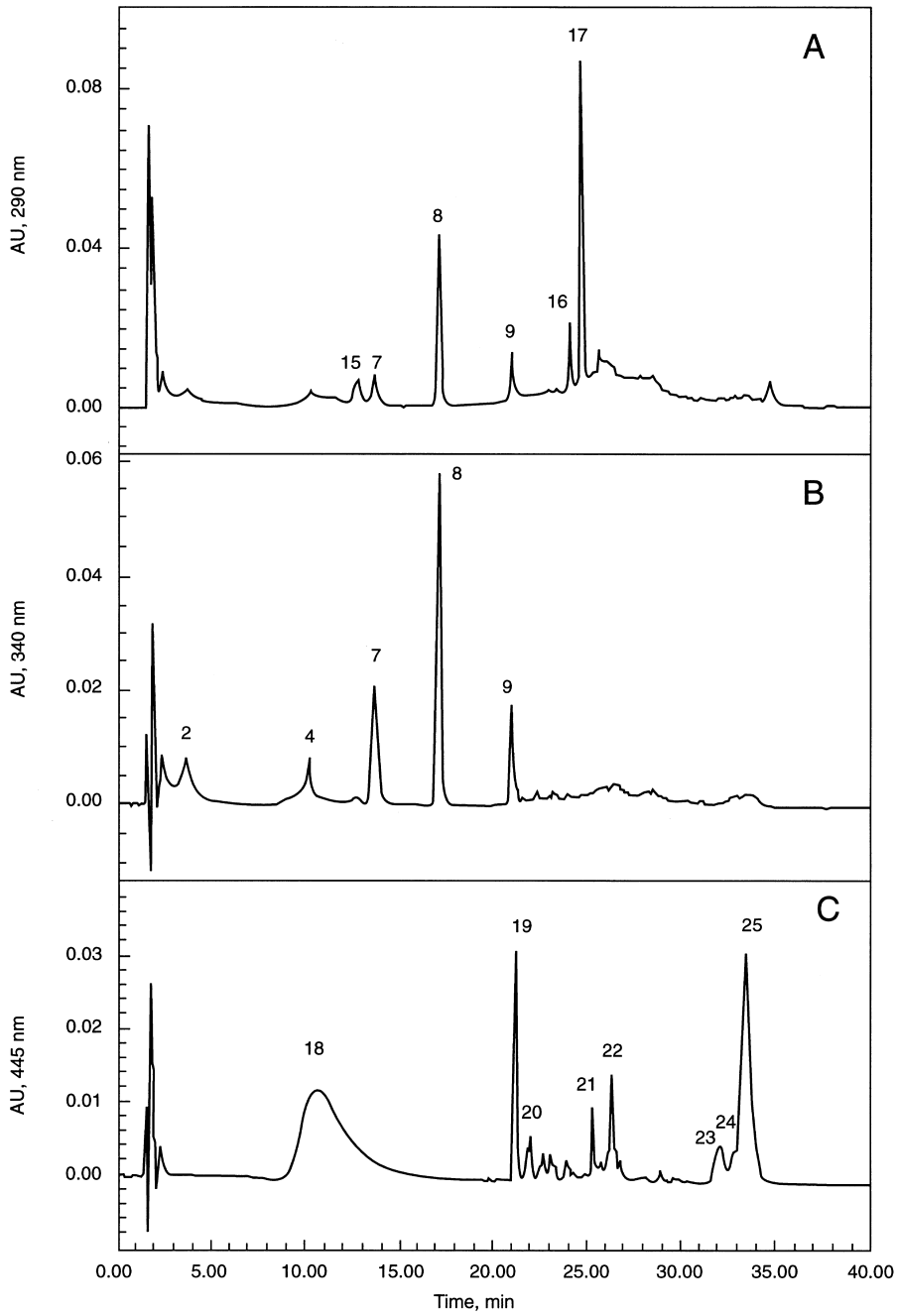


Fig. 2. Reversed-phase HPLC elution profiles of tocopherols (A), retinoids (B) and carotenoids (C) present in human plasma (200 μl). Blood was collected 3 h after an oral dose of retinoic acid. The chromatogram was obtained by use of the PDA 996 System and a gradient time of 20 min, as described in Section 2. Peak identification: 2, 4-oxo-retinoic acid; 4, retinoyl β-glucuronide; 7, retinoic acid; 8, retinol; 9, retinyl acetate; 15, BHT; 16, γ-tocopherol; 17, α-tocopherol; 18, free bilirubin; 19, lutein; 20, zeaxanthin; 21, 2′3′-anhydrolutein; 22, β-cryptoxanthin; 23, lycopene; 24, α-carotene; 25, β-carotene.

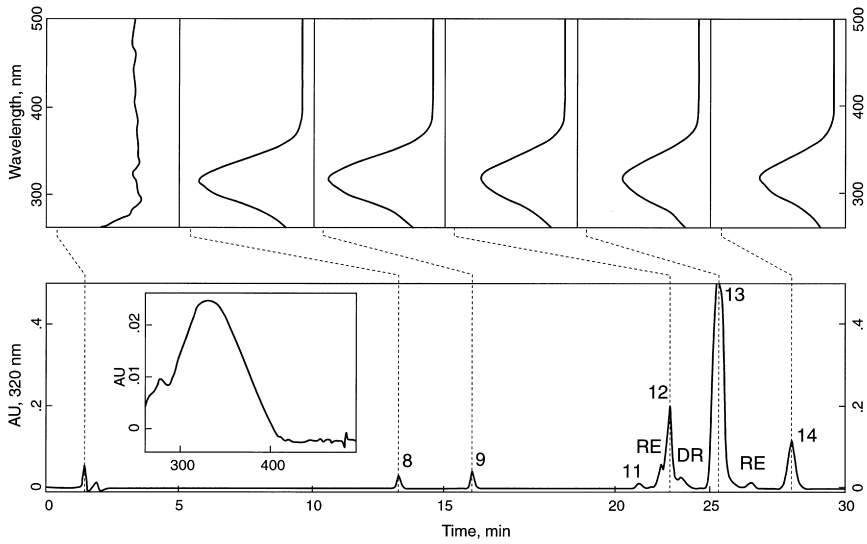


Fig. 3. Spectrum index plot of retinoids present in human liver obtained by reversed-phase gradient HPLC equipped with the PDA 991 System, as described in Section 2. Peak identification: 8, retinol; 9, retinyl acetate; 11 and RE, retinyl esters; 12, retinyl linolenate; 13, retinyl palmitate; 14, retinyl stearate; DR, 3,4-didehydroretinyl ester. The inset spectrum is of dehydroretinyl ester present in peak DR.

though it was monitored at 330 nm. The absorption spectra of the four major peaks, namely, RA, ROL, retinyl ester and β-carotene are shown in Fig. 4B.

### *3.3. Vegetables and fruits*

The gradient HPLC procedure was also applied to the analysis of carotenoids in fresh tomato and ketchup, in spinach, and in papaya and mango fruits. The primary purpose was to demonstrate the applicability of the presently described extraction and analysis procedures for the analysis of the carotenoids present in these samples. Thus, no attempt was made to characterize all the carotenoid peaks seen in each chromatogram.

An extract of fresh ripe garden tomato contained at least 23 carotenoids, judging from their characteristic absorption spectra. The total carotenoid content estimated from the absorption at 445 nm was found to be 140 μg/g fresh pulp. The major carotenoids were lutein (16%), lycopene (22%), β-carotene (31%) and phytofluene ($\lambda_{max}$ 365, 345, 330 nm in solvent B; 18%). The carotenoids found in tomato ketchup (Heinz) included lycopene (56%), lutein (6%) and β-carotene (15%), but not phytofluene.

Fresh spinach leaves contained, besides several chlorophyll peaks, 15 carotenoids — four of them major, two medium and nine minor. The total carotenoid content was found to be 222 μg/g pulp. The four major carotenoids were neoxanthin (11%), violaxanthin (20%), lutein (60%) and β-carotene (23%).

A specimen of papaya fruit (red mutant grown in Mexico) purchased from a local grocery store contained 251 μg/g pulp of total carotenoids. Among the >32 carotenoids in the extract were five major and twenty minor carotenoids, along with seven carotenoids of medium concentration. The concentration of lycopene was highest (25%), followed by ς-carotene (20%), β-carotene (16%), phytofluene (15%) and β-cryptoxanthin (10%).

The total carotenoids present in a sample of mango grown in Mexico was 175 μg/g pulp. The chromatogram obtained by use of the PDA 991 System and a gradient time of 15 min is shown in Fig. 5. Among the thirty-six carotenoids present were three major, six medium and twenty-seven minor carotenoids, as judged from their characteristic absorption spectra. Treatment of the crude extract with traces of dilute HCl resulted in a blue shift of the absorption peaks of several carotenoids by 15–50

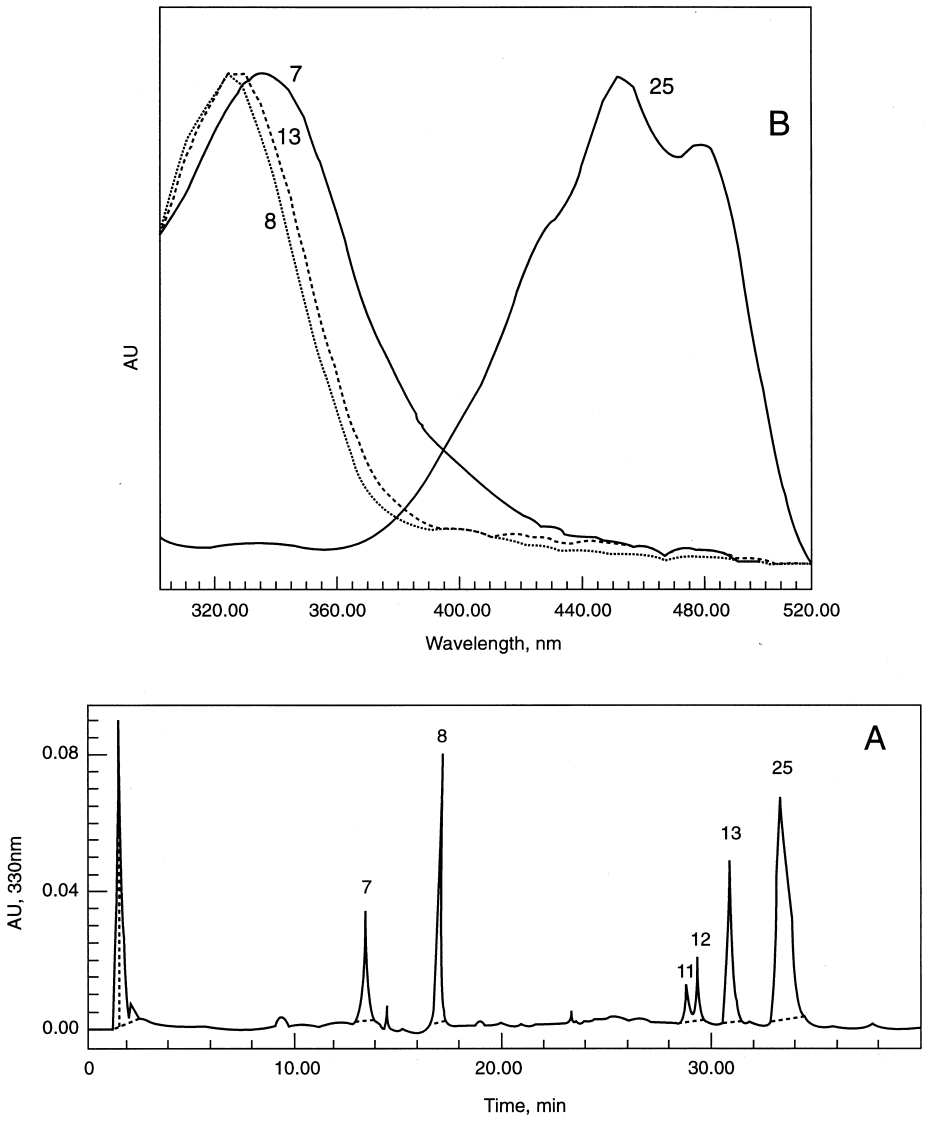


Fig. 4. Reversed-phase HPLC profile (A) of metabolites of β-carotene isolated from the small intestine and its contents of a vitamin A-deficient rat 3 h after an oral dose of β-carotene. The chromatogram was obtained by use of PDA 996 system and a 20-min linear gradient. Peak identification: 7, retinoic acid; 8, retinol; 11–13, retinyl esters, 25, β-carotene. The absorption spectra of the four major metabolites obtained with the PDA are shown in the upper panel (B).

nm, thereby indicating the presence of 5,6-monoepoxy- and 5,6,5′6′-diepoxy-structures. A major carotenoid in mango was characterized as 5,6,5′,6′-diepoxy-β-carotene (24%, peak 26) from its absorption spectrum ($\lambda_{max}$ 470, 438, 410 nm in the HPLC solvent mixture), which shifted to 422, 397 and 370 nm on treatment with a trace of dilute HCl, and by comparison of its chromatographic behavior with the authentic compound. Another major peak (11%, peak 27) showed $\lambda_{max}$ 465, 434 and 406 nm, which shifted to 445, 418 and 392 nm on treatment with a trace of dilute HCl. This carotenoid most likely is a

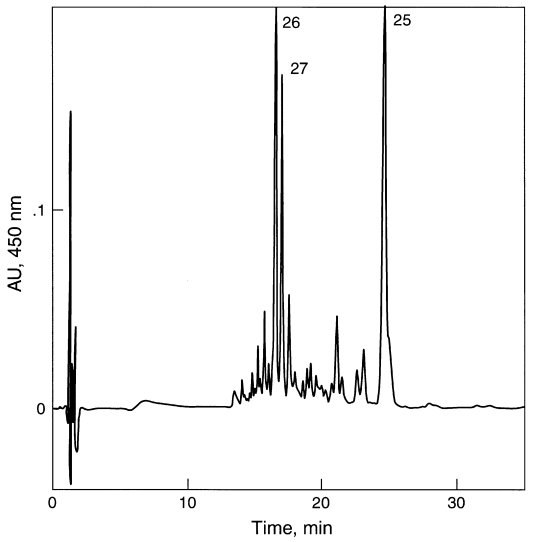


Fig. 5. Chromatogram of mango extract obtained by reversed-phase gradient HPLC using PDA 991 and a gradient time of 15 min as described in Section 2. Peak identification: 25, β-carotene; 26, 5,6,5′6′-diepoxy-β-carotene; 27, unidentified epoxycarotenoid.

5,6-epoxide, but its identity is not yet known. The predominant carotenoid in mango was β-carotene (32%, peak 25).

### *3.4. Multivitamin tablet*

The bottle containing the multivitamin tablets (Centrum) was labeled as containing 5000 I.U. of vitamin A, 40% of which was present as β-carotene. Our analysis revealed that retinyl acetate was the predominant form of vitamin A present. Traces of oxidation products of vitamin A, as judged from their spectra, were also present. Quantitation of retinyl acetate showed that there was 1025 μg/tablet or 3106 I.U. of retinyl acetate, very close to the estimated amount of 3000 I.U. Similarly, each tablet contained 625 μg β-carotene which is equivalent to 1040 $I.U._c$ of β-carotene, or about half of that claimed to be present.

## 4. Discussion

In this study, by use of a PDA detector and a reversed-phase gradient HPLC procedure, very polar, polar and nonpolar retinoids, carotenoids and tocopherols that generally occur in human serum and tissues, could be separated and analyzed in a single run within 30–40 min. An additional 15 min was necessary for re-equilibration of the column back to its initial condition. If the separation of carotenoids along with less polar retinoids was the primary goal, the gradient time from solvent A to solvent B could be reduced from 15–20 min to 5–10 min, thereby reducing the total analysis time. This modification did not affect the resolution of ROL, retinyl esters, tocopherols and the carotenoids, but did affect the separation of polar retinoids.

On the other hand, if resolution of polar retinoids was most important, the gradient from solvent A to solvent B can be modified. Thus, at a flow-rate of 0.8 ml/min, a linear gradient of solvent A (100%) to solvent B (50%) in 8 min and (100%) in the next 7 min was used. Thereafter, solvent B alone was used for an additional 20 min. This regimen resulted in the elution of ORA at 5.2 min, ROL at 15.7 min, RAC at 18.5 min and β-carotene at 29 min. Such a modification resulted not only in a more satisfactory resolution of polar compounds, but of nonpolar compounds as well. Solvent compositions can also be varied slightly in order to further resolve analytes of interest.

The gradient procedure described here is superior for several reasons to purely isocratic procedures [10,11,24] and to gradient procedure [25] reported from this laboratory in the past. The polar retinoids ORAG and ORA, which were difficult to separate from the solvent front in past isocratic as well as gradient procedures, could be separated by our new procedure. Separation of RA from RAG was better, and even their isomers were partially separated. In the isocratic procedures [11], bilirubin in serum often did not separate well from lutein, making quantitation of lutein difficult. On the other hand, the separation of carotenoids was not satisfactory in the described gradient procedure [25]. In the gradient procedure described in this paper, bilirubin was well separated from lutein and the quantitation of lutein and other hydroxycarotenoids was better than in the previously described method [11]. However, the resolution of the hydrocarbon carotenoids, lycopene, α-carotene and β-carotene was not superior, but reasonably acceptable values could be obtained.

The extraction method was also applied to other animal and plant tissues. Small sample sizes of ground or homogenized tissues remained in contact with the extraction solvent mixture at −20°C for up to 3 days. Although in most cases, a satisfactory recovery of analytes was obtained by allowing the tissues to stand with the solvent mixture overnight, occasionally it was necessary to wait longer to obtain a satisfactory recovery. Therefore, for routine analysis, the samples were kept with the solvent mixture for at least 2 nights at −20°C. A recovery of 93±8% was achieved. When kept under argon at −20°C in the dark, no isomerization or degradation of analytes was noticed up to 4 days.

The reversed-phase gradient procedure described here offers several advantages over other methods described by us and others. The present method can separate a full range of very polar to nonpolar retinoids, tocopherols and carotenoids. It is easier to regenerate the column, because compounds of all types of polarity are eluted during each run. The use of a PDA detector enabled analysis of a variety of analytes of interest, including several that have not yet been identified. Ammonium acetate, which is used to reduce the tailing of compounds with carboxyl functions such as RA and RAG, had several advantages over other buffering systems, such as phosphates and acetic acid, in being chemically stable, readily soluble in methanol and water, and suitable for masking the residual silanol functions [26]. Thus, symmetrical peaks and better separation of analytes were possible.

Although the HPLC procedure described here for the simultaneous analysis of very polar to nonpolar retinoids, along with carotenoids and tocopherols present in human and other animal serum and tissues is quite usable, it represents a compromise. Simpler, less time consuming, more efficient methods have been published (for reviews, see [13,26–29]) for the analysis of any single class of compounds in situations where the other classes are either not present or do not require analysis.

To our knowledge, this is the first report of the occurrence of 3,4-didehydroretinyl ester in human liver. Because 5,6-epoxy-β-carotene is more potent than β-carotene in inducing the differentiation of HL-60 cells [30], the presence of sizable amounts of epoxy carotenoids in mango is of interest.

## Acknowledgements

This study was supported by grants from the US Department of Agriculture (USDA-ISU-CDFIN 96-34115-2835 and USDA-NRI-CGP 94-37200-0490), the National Institutes of Health, USA (NIH DK 39733). Journal paper No. J-17551 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 3335, and supported by Hatch Act and State of Iowa funds.

## References

[1] K.W. Miller, C.S. Yang, Anal. Biochem. 145 (1985) 21.
[2] D.B. Milne, J. Botnen, Clin. Chem. 32 (1986) 874.
[3] L.A. Kaplan, J.A. Miller, E.A. Stein, J. Clin. Lab. Anal. 1 (1987) 147.
[4] W.A. MacCrehan, E. Schonberger, Clin. Chem. 33 (1987) 1585.
[5] P.M. Van Haard, R. Engel, T. Postma, Biomed. Chromatogr. 2 (1987) 79.
[6] D.I. Thurnham, E. Smith, P.S. Flora, Clin. Chem. 34 (1988) 377.
[7] E.D. Brown, A. Rose, N. Craft, K.E. Seidel, J.C. Smith, Clin. Chem. 35 (1989) 310.
[8] T. van Vliet, F. Van Schaik, J. Van Schoonhoven, J. Schrijver, J. Chromatogr. 553 (1991) 179.
[9] F. Khachik, G.R. Beecher, M.B. Goli, W.R. Lusby, C.A. Daitch, Methods Enzymol. 213 (1992) 205.
[10] A.B. Barua, H.C. Furr, D. Janick-Buckner, J.A. Olson, Food Chem. 46 (1993) 419.
[11] A.B. Barua, D. Kostic, J.A. Olson, J. Chromatogr. 617 (1993) 257.
[12] W.S. Blaner, J.A. Olson, in: M.B. Sporn, A.B. Roberts, D.S. Goodman (Editors), The Retinoids, Raven Press, New York, 1994, p. 229.
[13] H.C. Furr, A.B. Barua, J.A. Olson, in: M.B. Sporn, A.B. Roberts, D.S. Goodman (Editors), The Retinoids, Raven Press, New York, 1994, p. 179.
[14] S. Kang, E.A. Duell, G.J. Fisher, S.C. datta, Z. Wang, A.P. Reddy, A. Tavakkol, J.Y. Yi, C.E.M. Griffits, J.T. Elder, J.J. Voorhees, J. Invest. Dermatol. 105 (1995) 549.
[15] G. Tang, X. Wang, R.M. Russell, N.I. Krinsky, Biochemistry 30 (1991) 9829.
[16] X. Wang, G. Tang, J.G. Fox, N.I. Krinsky, R.M. Russell, Arch. Biochem. Biophys. 285 (1991) 8.
[17] T. van Vliet, W.H.P. Schreurs, H. van den Berg, Am. J. Clin. Nutr. 62 (1995) 110.
[18] C. Eckhoff, W. Wittfoht, H. Nau, W. Slikker, Biomed. Environ. Mass Spectrom. 19 (1990) 428.
[19] H. Nau, in: M.A. Livrea, L. Packer (Editors), Retinoids, Marcel Dekker, New York, 1993, p. 329.

[20] A.P. De Leenheer, H.J. Nelis, Methods Enzymol. 213 (1992) 251.
[21] A.B. Barua, C.A. Huselton, J.A. Olson, Syn. Comm. 26 (1996) 1355.
[22] A.B. Barua, P.K. Duitsman, D. Kostic, M. Barua, J.A. Olson, Int. J. Vit. Nutr. Res. 67 (1997) 423.
[23] A.B. Barua, P. Duitsman, J.A. Olson, J. Nutr. Biochem. in press.
[24] A.B. Barua, D. Kostic, M. Barua, J.A. Olson, J. Liq. Chromatogr. 18 (1995) 1459.
[25] A.B. Barua, J.A. Olson, Biochem. J. 263 (1989) 403.
[26] A.P. De Leenheer, W.E. Lambert, E. Meyer, in: M.A. Livrea, L. Packer (Editors), Retinoids, Marcel Dekker, New York, 1993, p. 551.
[27] H.C. Furr, A.B. Barua, and J.A. Olson, in: A.P. De Leenheer, W.E. Lambert, H.J. Nelis (Editors), Modern Chromatographic Analysis of Vitamins, Marcel Dekker, New York, 1992, p. 1.
[28] J.K. Lang, M. Schillaci, B. Irvin, in: A.P. De Leenheer, W.E. Lambert, H.J. Nelis (Editors), Modern Chromatographic Analysis of Vitamins, Marcel Dekker, New York, 1992, p. 153.
[29] N.E. Craft, Methods Enzymol. 213 (1992) 185.
[30] P. Duitsman, B. Becker, A.B. Barua, J.A. Olson, FASEB J. 10 (1996) A732.