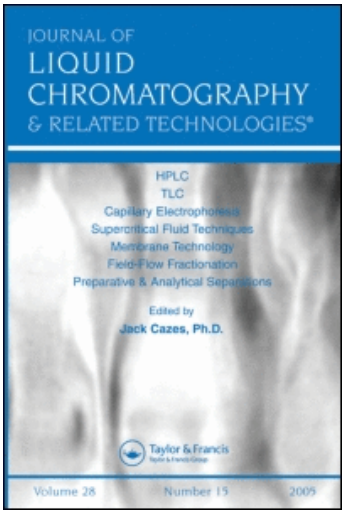

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Simultaneous Profiling and Identification of Carotenoids, Retinols, and Tocopherols by High Performance Liquid Chromatography Equipped with Three-Dimensional Photodiode Array Detection

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SIMULTANEOUS PROFILING AND IDENTIFICATION OF CAROTENOIDS, RETINOLS, AND TOCOPHEROLS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY EQUIPPED WITH THREE-DIMENSIONAL PHOTODIODE ARRAY DETECTION

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ABSTRACT

A high performance liquid chromatography (HPLC) system was developed for the simultaneous separation and identification of carotenoids, retinol, its derivatives, and tocopherols. These lipophilic and semilipophilic products were chromatographed on a C18 Vydac column and were monitored and identified using an on-line, three-dimensional photodiode array detector, which permitted profiling on the 200 to 800 nm absorption spectrum of any chromatographic peak in less than 1 sec at a high resolution of 1.2 nm. Identification of peaks and overlapping peaks is conducted by subsequent spectra analysis by the use of a powerful computer station combining Microsoft Windows™ and a chromatography manager. This methodology was successfully applied to extracts of different origins such as plants, sera, tissues and others, memorizing each spectral analysis for processing and comparing within itself or with previously stored data. Quantitative and qualitative processing of the spectral analyses allowed the build-up of a new database of the full absorption profile for each indicated product in comparison to previously published data.

INTRODUCTION

High pressure liquid chromatography (HPLC) is being employed as the currently most accepted technique for the separation and identification of carotenoids [1]. The HPLC method is most accepted for the elucidation and quantification of carotenoids in prokaryotes, eukaryotes, as well as in any specimen whose contents are under question. Advances in the HPLC method for the separation of carotenoid and retinol mixtures have been summarized and reviewed intensively recently [1, 2]. However, the question of proper peak identification is still open for further analysis. Classical HPLC equipment runs on one to four channels, and the carotenoids are compared to authentic standards by the retention time at the indicated channels. Improved analysis is supported by the use of off-line spectrophotometric techniques such as fluorescence, NMR, mass spectrometry and others.

The recent introduction of the HPLC on-line photodiode array detector permits complete absorption spectrum detection of each chromatographic peak in a very short time of less than 1 sec. The photodiode array detectors owe their spectral superiority to the reverse-optic configuration and the array of photodiodes, each dedicated to a particular band of wavelengths, as well as to the attached computer for data processing [3]. The technology of the photodiode array detectors in the last two years was developed to improve the integrity of the chromatographic results to reach better resolution and sensitivity on the full spectral map through continuous monitoring and acquisition of all UV/Vis wavelengths. The modern, advanced photodiode arrays need a high level of chromatography manager and developed software to give full automation and quantification of the processed, three-dimensional, spectral data.

Since carotenoids possess special characteristic absorption, and because small chemical or structural modifications may slightly alter their spectra, carotenoids are most appropriate candidates for identification by the combined technique of HPLC - photodiode array detection.

In the present study we analyzed and identified a variety of carotenoids, retinols and tocopherols by using a reversed phase HPLC

separation system equipped with a three-dimensional, sensitive photodiode array detector and an upbased chromatogram manager, a system of improved potential for future analysis of lipophilic carotenoids and related products.

MATERIALS AND METHODS

HPLC System

The HPLC System was equipped with a 7725i Rheodyne Injector (Rheodyne Inc., Cotati, CA, USA) on a Waters HPLC system (Millipore, Marlborough, MA, USA), including pumps 501 and 510, a Waters 996 photodiode array detector attached to a Waters Millennium 2010, Version 2.0, Chromatography Manager, on a compatible IBM computer 486 DX2 66 strengthened with Microsoft Windows™ supported by hard disk of IJ, 33 mega RAM and connected to an HP Deskjet 1200C plotter.

Column and Solvents

The column was a Vydac 201 TP 54 stainless steel column of 25 cm×4.6 mm (i.d.) packed with C18 reversed phase material of 5 μm particle size, 300 Å pore size (The Separation Group, Hysperia, CA, USA). The column was protected by a 5 cm C18 ODS (4) guard column (Shimadzu, Kyoto, Japan) and with a small preguard column, Waters Guard-Pak, inserted with a C18 μBondapak cartridge.

Elution was performed at ambient temperature (25±3°C) with an isocratic solvent, methanol: acetonitrile, HPLC grade (9:1, v/v) at a constant flow of 1.0 ml/min. Mobile phase was flushed with nitrogen to avoid air gassing in the solvents. With proper use, the column retained its elution profile for a long period of continuous use. A gradual slight increase of retention time after about 100 injections of sample was reversed by washing the column with methanol: acetonitrile: methylene chloride (8:1:1, v/v/v).

Standards

Synthetic lutein, zeaxanthin, canthaxanthin, β-apo-8-carotenal, β-crypoxanthin, echineone, all-trans α-carotene, all-trans β-carotene, 9,15-dicis β-carotene, all-trans γ-carotene, 15-cis β-carotene and all-trans zeta-carotene were provided by Hoffmann La Roche, Basel, Switzerland. Lutein from alfalfa, lycopene from tomato, α-carotene from carrots, retinol, retinal, 9-cis retinal, 13-cis retinoic acid and α-tocopherol were from Sigma, St. Louis, MO, USA. 9-cis β-carotene, all-trans phytoene, 9-cis phytoene, all-trans and 9-cis phytofluene, violaxanthin and zeaxanthin from *Dunaliella* were self extracted and separated. All standards were kept at -70°C under N_2, dried by a stream of N_2 prior to analysis and injected into the HPLC in methylene chloride.

Carotenoids from *Dunaliella*

Pigments of *Dunaliella* grown for selective induction and accumulation of the necessary carotenoids were extracted from the algal pellet with ethanol/hexane [4, 5]. Carotenoids were pre-separated and pre-purified by using a preparative HPLC column, Vydac 218 TP 1022, a stainless steel C18 column of 10 μm particle size (The Separation Group, Hysperia, CA, USA), followed by dehydration under N_2 and redissolution in methylene chloride for analysis by the analytical HPLC column. *Dunaliella* was used to produce and accumulate the indicated carotenoids and their equivalent stereoisomers [4, 5]. All carotenoids were kept at -70°C under N_2, dried by a stream of N_2 prior to analysis and injected into the HPLC in methylene chloride.

Carotenoids and Vitamins in Human Serum

Five healthy young male humans between the ages of 20 to 25 donated blood after overnight fasting. These men were non-smokers and

were not vegetarians. Blood samples of up to 5 ml were collected and the blood was separated. The serum was stored at -70°C under N_2 until analysis, usually within 7 days. To one ml of serum, 2.5 ml of ethanol were added, and after 5 min of mixing, the vitamins were extracted with 5 ml n-hexane, mixed vigorously and phase separated centrifugally at 2,000×g for 5 min. The upper phase was removed and the water/ethanol phase was extracted a second time with 3 ml n-hexane. The two hexane extracts were combined and evaporated by a stream of N_2. The dried residues were dissolved in a minimal volume of methylene chloride prior to injection.

RESULTS AND DISCUSSION

Column and Solvents

The major aim of this study was to identify and verify the HPLC peaks of various carotenoids and retinols. To reach this goal, one reversed phase column was selected, Vydac 201 TP 54, and one set of solvents (methanol: acetonitrile, 9:1, v/v) was used to elute the samples on this column. The system ran isocratically under constant conditions to allow repetition of many HPLC runs representing only slight modifications in the profile of the chromatograms while retaining the elution time at its initial setting. All HPLC runs were set at a flow rate of 1.0 ml/min at a column pressure of 800±50 psi. The constant pressure was necessary to reach similar elution profiles, while a gradual increase in the column pressure was usually reflected by a plus shift in the retention time, an indication of the necessity of column wash. The two inert solvents, methanol and acetonitrile, with no reactive solvents such as ethyl acetate or ammonium acetate [6], ensured highly satisfactory recovery of all carotenoids and retinoids. The decision to omit more volatile solvents such as dichbromethane or hexane was related to the gradual evaporation of these solvents when stored in the HPLC containers with a flush of N_2. Moreover, the Millennium Maxplot full spectra analysis, as described, requires solvents of very low UV wavelength maxima of which methanol,

λmax = 205 nm, and acetonitrile, λmax = 190 nm, are good choices, while methylene chloride, λmax =233 nm, ethyl acetate, λmax =256 nm, and many other organic solvents may interfere in monitoring and acquisition of the low UV wavelengths between 190 to 250 nm. Under the above preset conditions, the Vydac column showed good results on runs of up to about 30 min. The peak orientation, to achieve ideal integration of valley to valley with no peak profile distortion was best between 0 to 15 min, good between 15 to 20 min, and satisfactory between 20 to 30 min of retention time. Above that time range the orientation of most peaks was gradually distorted, being unsatisfactory for direct peak resolution.

High Resolution Photodiode Array Chromatography

Improved technique of the modern photodiode array detector provides higher resolution and sensitivity of the detection system. This technique gives the full spectral map of each peak through continuous monitoring and acquisition of all UV/VIS wavelengths, which with the Chromatograph Manager provides detailed 3-D graphic covering of the spectrum from 200 to 800 nm. The advanced diodes spectrum techniques improves the optical resolution down to 1.2 nm, a sensitive detection level that permitted minute differences in the spectral shape to achieve compound identification and confirmation. The post-run analysis by the Chromatogram Manager, which combines the power of Microsoft Windows™ and DOS, allows detailed analysis of each peak in the chromatogram for comparison with reference spectra already stored and memorized. Out of many different 3-D chromatographic runs using this advanced detection capability in our laboratory, over the last 12 months, two groups of samples are illustrated: an authentic carotenoid standard, synthetic 15-cis β-carotene along with an extract of human serum. The 3-D graphic profile of the HPLC run of 15-cis β-carotene is illustrated in Fig. 1, covering spectra from 250 to 600 nm at 1.2 nm resolution. The colored 3-D cube can be rotated in any direction and angle to allow a direct view of the spectrum from any side, with a clear side or topographic view of the

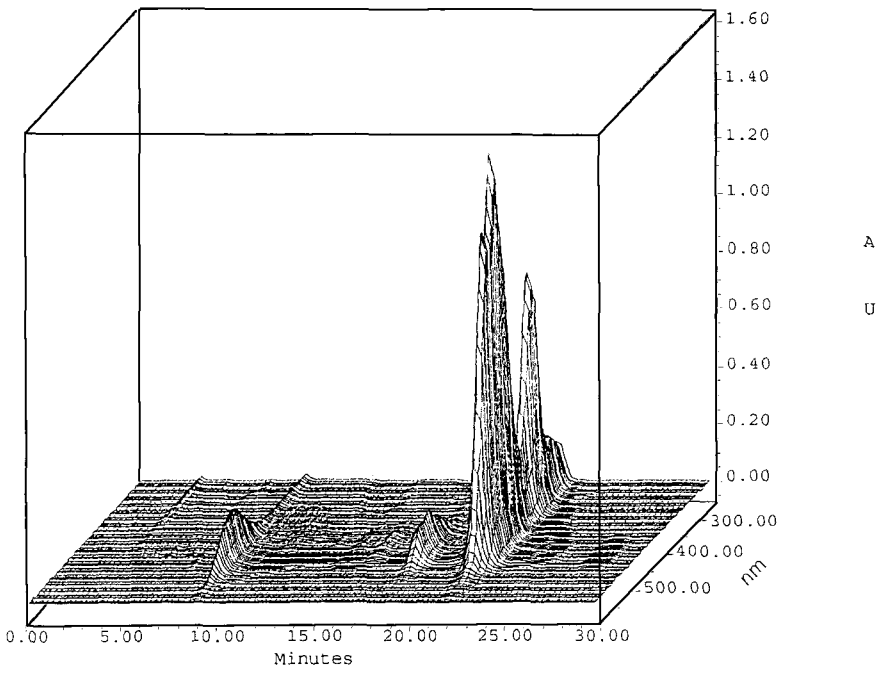

FIGURE 1. 3-D graphic profile of 15-cis β-carotene on C18 reversed phase column. Eluent was methanol: acetonitrile (9:1, v/v) at a flow rate of 1 ml/min and the absorbance was monitored from 250 to 600 nm at a resolution of 1.2 nm.

different components and their absorption intensity. Post-run analysis can be used to identify the different components on the time axis at any selected derivation wavelength channel. The β-carotene peak, at 21.8 min, presenting absorption maxima at 336.6, 450.9 and 470.3 nm, is the 15-cis β-carotene, while the other minor two peaks at 8.6 min, λmax = 298.4, 475.2 nm, and at 18.5 min, λmax = 274.6, 417.0, 450.8, 475.1 were identified as canthaxanthin and all-trans β-carotene, respectively. The content of these contaminant products in the 15-cis β-carotene standard was low, as calculated by the peak areas integration, and did not exceed 5% (w/w).

The wavelength maxima obtained by our HPLC 3-D photodiode array system to many standards was compared to the literature data (Table 1), providing more accurate elution time and absorption maxima for each standard assayed. The absorption maxima illustrated in Table 1 were repeatable for each standard or component, both in chromatography and in absorption spectra. Thus, identification of unknown matching peaks of biological extracts was a relatively simple task by using the acquired memorized database of various standards. This was better illustrated on runs when the retention time shifted aside from its original setting and could not be used solely for peak identification.

Dunaliella bardawil, a unicellular green alga, was already thoroughly studied for its content of carotenoids and other photosynthetic pigments [4, 7]. This species is known for the accumulation of large amounts of all-trans β-carotene and 9-cis β-carotene in a content and ratio related to the light absorbed during one cell cycle. The HPLC 3-D plot of the algal extract showed clear profiles of a few prominent carotenoids (Table 1), with major peaks at 16.5 min, λmax = 436.4, 446.0, 475.2 nm, α-carotene; at 18.5 min, λmax = 417.0, 450.8, 475.1 nm, all-trans β-carotene; and at 20.7 min, λmax = 268.4, 341.5, 436.4, 446.0, 472.7, 9-cis β-carotene. The identical isomeric precursors of β-carotene were also assayed in phytoene-rich *Dunaliella* and are listed in Table 1. The ease of analysis and accuracy in identification of the plant and algal pigments allows the establishment of an HPLC library of "fingerprints" of many pigments known or in question.

The photodiode array - Millennium analysis of human serum is illustrated in Fig. 2. The 3-D plot clearly resolved the different components in the serum extract exhibiting above all two major peaks, both at the hydrophilic side of the elution. Maxplot derivation revealed that these two peaks are retinol, λmax = 323.5 nm at 3.8 min, and α-tocopherol, λmax = 291.3 nm at 6.9 min, closest to γ-tocopherol, λmax = 294.9 at 6.2 min (Table 1). Using the computer software to run the λmax of each observed peak indicated that the major carotenoids in this human serum are the plant origin, hydroxy carotenoid, violaxanthin, λmax = 443.6 nm at 5.3 min, and the fruit origin β-cryptoxanthin, λmax = 450.8 nm at 10.5 min. All other peaks between 4.5 min to the end of the chromatogram,

TABLE 1.
Carotenoids, Retinoids and Tocopherols Assayed by HPLC Photodiode Array in Comparison to Literature Data

Carotenoid and origin	Ret. time (min)	Absorption maxima (nm) Waters 3-D system	Absorption max. (nm) Literature	Ref
β-apo-8-carotenol (synthetic)	9.3	269.9, 465	463	13
canthaxanthin (synthetic)	9.1	475.2	474-478	13
all-trans α-carotene (synthetic)	18.0	269.9, 331.9, 431.5, 441.2, 467.0	422, 442, 471	19
all-trans α-carotene (carrots)	16.5	269.9, 436.4, 446.0, 475.2	--	
all-trans β-carotene (synthetic)	18.5	275.4, 417.0, 450.8, 475.1	429, 449, 475	19
9-cis β-carotene (*Dunaliella*)	20.7	268.4, 341.5, 436.4, 446.0, 472.7	445	20
15-cis β-carotene (synthetic)	21.8	336.6, 450.9, 470.3	448	20
9,15-dicis β-carotene (synthetic)	19.7	284.2, 336.6, 446.0, 465.2	--	
all-trans γ-carotene (synthetic)	21.4	280.3, 441.0, 463.0, 490.2	437, 460, 490	13
all-trans ζ-carotene (*Dunaliella*)	18.3	236.2, 378.0, 399.6, 424.1	295, 377, 398, 422	19
9-cis ζ-carotene (*Dunaliella*)	19.5	236.1, 374.1, 395.2, 420.1	295, 374, 395, 419	19
β-cryptoxanthin (synthetic)	10.5	274.7, 426.8, 450.8, 480.1	425, 449-452, 473-8	13
echinenone (synthetic)	11.6	299.4, 465.5	457-461	13
lutein (alfalfa)	5.0	269.9, 331.9, 446.1, 475.2	422, 443, 470	19
lycopene (tomato)	25.3	293.7, 263.9, 446.1, 470.3, 504.4	444, 470, 502	13
all-trans phytoene (*Dunaliella*)	18.9	277.8, 286.1, 296.0	276, 286, 297	13
9-cis phytoene (*Dunaliella*)	18.0	277.8, 286.1, 296.0	276, 284, 294	19
phytofluene, all-trans & 9-cis (*Dun.*)	18.8, 19.2	331.4, 348.2, 368.2	329, 346, 365	19
Violaxanthin (*Dunaliella*)	4.8	267.5, 329.4, 443.6, 471.0	414, 441, 471	13
Zeaxanthin (*Dunaliella*)	5.3	276.1, 425.9, 450.3, 477.3	341, 427, 448, 475	19
Retinoids:				
Retinol (synthetic)	3.8	327.1	--	
Retinol (serum)	3.8	323.5	325	21
9-cis retinol (synthetic)	3.85	322.3	--	
all-trans retinoic acid (synthetic)	3.0	336.6	--	
13-cis retinoic acid (synthetic)	3.2	341.4	--	
Vitamin E:				
α-tocopherol (synthetic)	6.9	291.3	292	22
γ-tocopherol (synthetic)	6.2	294.8	294	22

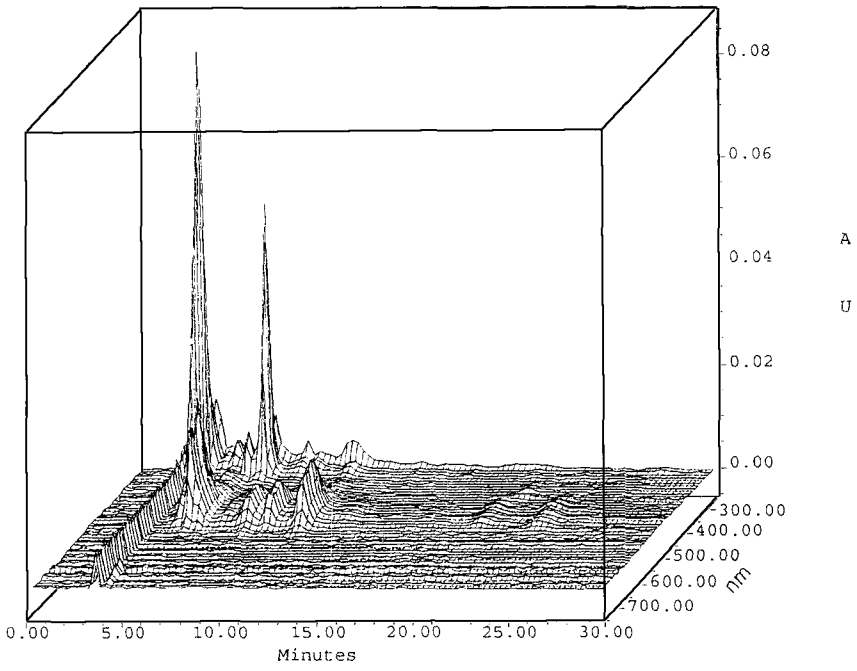

FIGURE 2. 3-D graphic profile of human extract. Separation conditions were as described under Figure 1.

aside from the two tocopherols, were identified as carotenoids, presenting typical absorption maxima around 450 nm. The last observed two peaks at 21.5 min and at 23.5 min were the hydrocarbons all-trans α-carotene and all-trans β-carotene, respectively. β-carotene usually overlapped a small peak of phytofluene clearly detected by the UV absorbing peak of λmax = 286.1 nm. Lycopene isomers, which elute on the Vydac column after the cis stereoisomers of β-carotene [8, 9] were not detected at the indicated chromatogram at any sensitivity. Attempts to search for lycopene in serum prior to α-carotene [6] were negative. The standard lycopene of tomato extract which was used in our study exhibited a clear peak at 25.3 min with λmax = 470.3 nm. Lycopene standard and lycopene in the serum extracts were always eluted late after α-carotene and β-

carotene. The HPLC profile of the presented human serum was similar to many other human sera extracts which were analyzed by our photodiode array and the variations were mainly in the composition and content of the hydroxy and keto carotenoids and less in the profile of the hydrocarbon carotenoids. The list of the different polar carotenoids is similar to that described in a few previous manuscripts [6, 9-20] and include α-cryptoxanthin stereoisomers of lutein and zeaxanthin. α-carotene in the human serum, like the α-carotene in the alga *Dunaliella*, resembles the authentic standard α-carotene from carrots, λmax = 446.0 nm, and was significantly different from the synthetic α-carotene, λmax = 441.2 nm. The variation between different batches of standards of α and β-carotene was noted earlier [12]. The photodiode array and the memorized data were very useful when interpreting such minute differences between stereoisomers of carotenoids and between the α and β configuration.

The introduction of this improved 3-D liquid chromatography detector and the chromatograph manager revealed many peaks of unknown or partially known carotenoids for identification and evaluation. Samples either from plants and human plasma of low content of carotenoids can be detected and analyzed at a fine separation level of cis stereoisomers and of other lipophilic extracted products even when overlapping occurs. The simultaneous analysis of retinol, tocopherols and carotenoids [11, 17] is simple, rapid and accurate. It was of interest to note that the early eluted peak at 3.1 min with absorption maxima varying from 240 nm to 350 nm was always related to the oxidation of the carotenoid studied. We assume that the oxidized carotenoids are relatively polar with early elution properties and shorter absorption maxima. The developed new diode array detection will allow future analysis of different oxidized carotenoids on the polar side of the reversed phase elution column along with better analysis of all other detected peaks of non-oxidized components. Altogether, the developed fine art of HPLC separation by using the modern technique of diodes and computer hardware and software allows the analyzers a wide range of methodologies to identify samples with certainty and reliability.

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