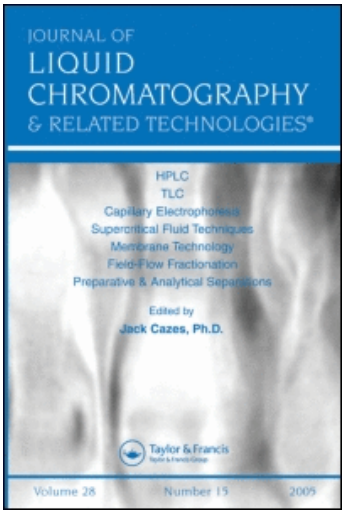

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Quantitative Analysis of Photochemical Reactions Utilizing High Pressure Liquid Chromatography: Linear Polyenes Related to Vitamin A

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QUANTITATIVE ANALYSIS OF PHOTOCHEMICAL REACTIONS UTILIZING HIGH PRESSURE LIQUID CHROMATOGRAPHY: LINEAR POLYENES RELATED TO VITAMIN A

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ABSTRACT

A procedure is described whereby qualitative and quantitative information of a photochemical reaction can be obtained by utilization of high pressure liquid chromatographic methods to separate, identify, and quantify the reaction photoproducts. When coupled with knowledge of the irradiation sources photon flux, which can be obtained using a chemical actinomer, quantitative results are readily calculated. This method has been employed to determine absolute quantum yields for the cis-trans photoisomerizations (ΦPI) of various configurational isomers of linear polyenes related to Vitamin A, and is illustrated using all-trans- and 11-cis-13-demethylretinal. HPLC analyses of photoequilibrium reaction mixtures are presented. Application of other analytical techniques to characterize photochemical reactions is discussed.

INTRODUCTION

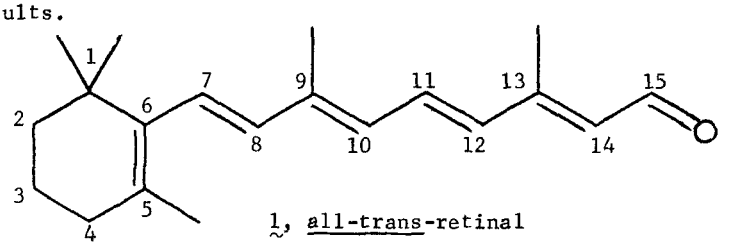
The class of compounds related to the naturally occurring polyene Vitamin A has important biomedical and biochemical relevance. It has been demonstrated that certain retinoids can be utilized to prevent the development of cancer in the skin, respiratory tract, mammary gland, and urinary bladder of laboratory animals, and that they have promising applications for the

prevention of cancer in high-risk human populations (1-3). In addition, select stereoisomers of retinal, the aldehyde of Vitamin A, exist as the light-absorbing component of several proteins. 11-cis-Retinal is the chromophore of the visual protein rhodopsin (4,5), and is present in numerous species of mammalia and amphibia. 13-cis-Retinal and all-trans-retinal exist in various forms of the protein Halo-bacterium halobium, bacteriorhodopsin (6,7), with the latter isomer also present as the chromophore of retinochrome (8,9).

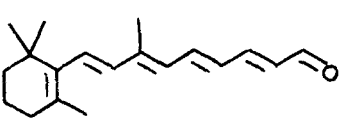
Upon absorption of a photon of light the retinyl chromophore in these proteins undergoes a cis-trans photoisomerization. This process is of particular significance in rhodopsin since it initiates the phenomenon of vision (10). In order to have a better understanding of the nature of the photoisomerization of the 11-cis-retinyl chromophore in the visual protein and the environmental factors that may influence this reaction, we have made a detailed, quantitative examination of the photochemical properties of 11-cis-retinal, all-trans-retinal, and a variety of isomers of synthetic retinals (11-14).

High pressure liquid chromatographic (HPLC) methods have been utilized for both analytical and preparative separations and purifications of a number of retinoids including retinoic acids, retinoates, retinols, retinals, and related compounds (15-27). HPLC methods have also been employed to analyze the photochemical reaction products of the isomeric retinals and its analogs (11-15,28-30). HPLC techniques have been essential to our research by facilitating the isolation and purification of a variety of stereoisomers of the retinals, related linear polyenals, and their synthetic intermediates (25), and by enabling the quantitative determination of photochemical reaction products and the quantum efficiency of product formation. In particular, we have determined absolute quantum yields for the cis-trans photoisomerization of configurational isomers of retinal (structure 1), 13-demethylretinal (2), 14-methylretinal

(3), 10-methylretinal (4), and 10,14-dimethylretinal (5) and wish to present the method that we employed to obtain these results.



1, all-trans-retinal



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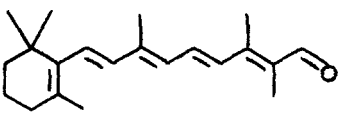

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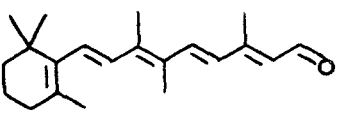

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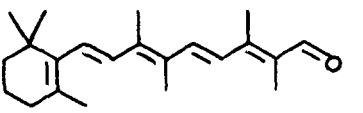

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EXPERIMENTAL

all-trans-Retinal (1) was purchased from Sigma Chemical Company. all-trans-13-Demethylretinal (2) was prepared by manganese dioxide oxidation of the corresponding Vitamin A analog (25), which was synthesized according to the procedure of van den Tempel and Huisman (31). The all-trans isomers of retinal analogs 3-5 were prepared according to the procedure of Tanis, Brown and Nakanishi (23).

Preparative separations were performed on a Waters Prep LC/system 500 liquid chromatograph that was modified for ultraviolet detection by attaching a Waters 440 Absorbance Detector.

Two 12 x 2 inch Waters silica gel columns, 5-8% anhydrous ether in hexane, 250 ml/min, and 365 nm detection were employed as the isolation conditions. Analytical separations were accomplished on a Waters ALC/GPC 204 liquid chromatograph or a Perkin-Elmer Series 1 liquid chromatograph. Separation conditions were a 12 x 1/4 inch Waters μ-porasil column, 2-4% anhydrous ether in hexane, 2-4 ml/min, 1000-2000 psi, and 365 nm ultraviolet detection.

Electronic absorption spectra were recorded on a Cary 14 or Perkin-Elmer 575 Spectrophotometer using quartz cuvettes. Nuclear magnetic resonance spectra were recorded on a HF 250 MHz NMR spectrometer. Irradiations were performed using the 150-W xenon lamp and 1/4 m monochromator of an Aminco-Bowman Spectrofluorimeter or a 450-W medium pressure mercury source and Bausch & Lomb monochromator. All experiments were performed under red lighting.

METHODOLOGY

The advantage of applying high pressure liquid chromatographic (HPLC) techniques to qualitative and quantitative investigations of photochemical reactions can readily be demonstrated. Since 11-*cis*-retinal, the light absorbing chromophore of the visual protein rhodopsin, undergoes a photoisomerization to its *all-trans* form to initiate the phenomenon of vision (10), a study of the photochemistry of 11-*cis*-retinal and isomers of related synthetic polyenes may yield insight into the factors that influence the photoisomerization reaction in rhodopsin. Thus, 11-*cis*-13-demethylretinal is selected as a target molecule for which quantitative photochemical information is sought.

all-trans-Retinal yields 11-*cis*-retinal as a primary photoproduct upon irradiation in a polar solvent (4,12,28,32), hence our strategy is to obtain 11-*cis*-2 photochemically from its corresponding *trans* isomer. Thus, *all-trans*-13-demethylretinal was synthesized, isolated and purified to greater than 99.5% isomer pure using HPLC methods, and characterized by electronic absorption

and nuclear magnetic resonance methods. Figure 1 shows the electronic absorption spectrum of all-trans-2 recorded in 3-methylpentane. After irradiation of an ethanol solution of all-trans-2 for several minutes, HPLC analysis (the ethanol is evaporated prior to analysis), reveals the formation of two new peaks. The intensity of these peaks continue to increase with irradiation time, and additional peaks are formed. These peaks increase in intensity at the expense of the all-trans-2 peak until a constant number and peak-area ratio is obtained. At this point

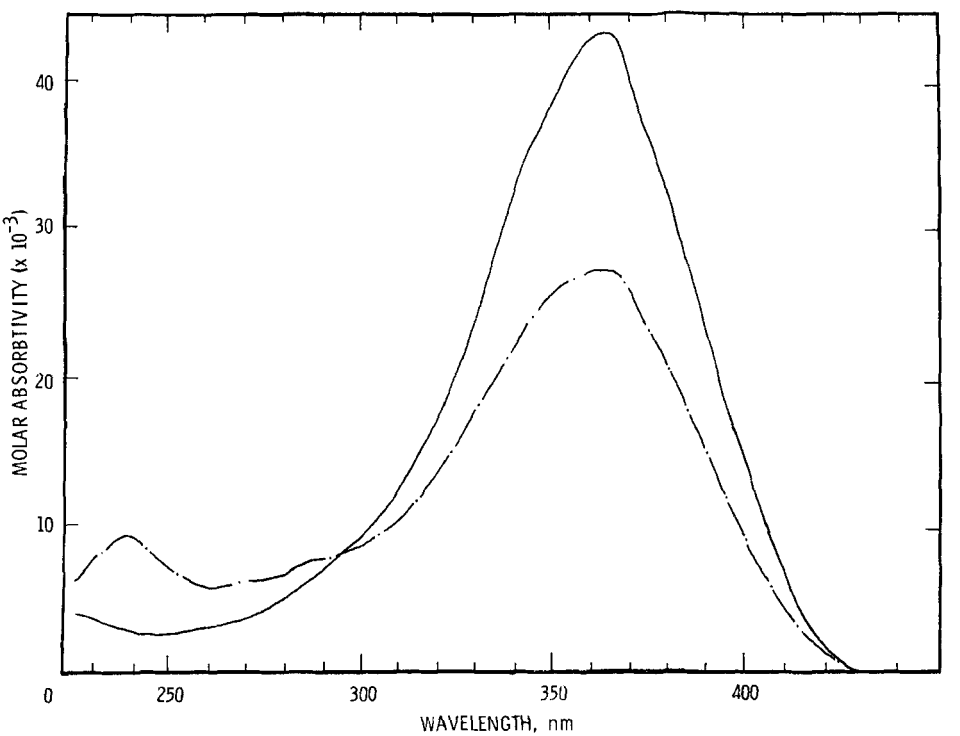


FIGURE 1

Electronic absorption spectrum of all-trans-13-demethylretinal (———) and 11-cis-13-demethylretinal (— • — • —) recorded in 3-methylpentane at room temperature.

a photoequilibrium mixture has been established. Tentative structural assignments of the photoproducts of all-trans-2 are made based upon their relative retention times and analogy to the corresponding retention times of the stereoisomers of the retinals (15,16). Figure 2 reproduces an irradiation-time dependent HPLC analysis of the photochemistry of all-trans-2. Each major photoproduct, greater than ca. 2% relative abundance (Figure 2D), is isolated and purified by HPLC methods and identified using chemical shift and coupling constant data of high resolution nuclear magnetic resonance spectra. Thus, it was determined that 7-cis-, 9-cis- and 11-cis-13-demethylretinal are the major photoproducts present in the photoequilibrium mixture of isomers of 2 (25). Table 1 lists the relative retentions of isomers of polyenes 1-3. Subsequent investigations of the effect of solvent properties and excitation wavelengths on the photoequilibrium composition allows optimization of the yield of 11-cis-2.

Figure 1 displays the electronic absorption spectrum of 11-cis-13-demethylretinal, which was purified to > 99.7% isomer pure using HPLC methods. Upon irradiation of a 3-methylpentane solution of 11-cis-2, HPLC analysis indicates the formation of all-trans-2 as the exclusive photoproduct, a reaction analogous to that of 11-cis-retinal. In order to obtain quantitative information of the efficiency of this photoisomerization HPLC methods are used to analyze the photoproducts.

The quantum yield of a photochemical reaction (ϕ) can be defined as

$$\phi = \frac{\text{number of molecules undergoing a chemical reaction}}{\text{number of photons absorbed}} \quad (1)$$

The number of molecules undergoing a chemical reaction can be expressed in terms of measured variables--C, molar concentration of the solution (obtained from the absorption spectrum and molar absorbtivity); V, volume irradiated in liters; N_A, Avogadro's number; and Δ, the percentage conversion. The percentage

conversion is obtained by integrating the individual HPLC peaks to obtain their relative areas. Multiple planimeter tracings and a disc integrating recorder have been used to determine relative areas. Recently, an electronic integrator has been attached to the absorbance detector. When employing an absorbance detector for HPLC analysis, it is essential to correct each peak for individual detector response in order to obtain accurate quantitative results. For example, from Figure 1 it is evident that ϵ_{365} (the HPLC detection wavelength) differs substantially for all-trans-2 and 11-cis-2. Table 2 lists the normalization factors necessary to correct for the detector response of isomers of polyenes 1-3.

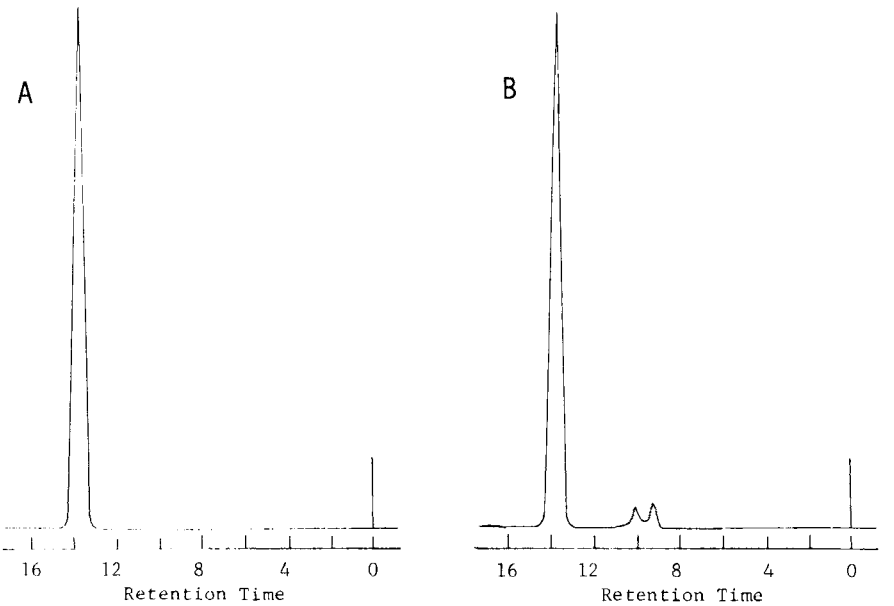
The number of photons absorbed by the solution can be obtained using the chemical actinomer, potassium ferrioxalate (33), to determine the flux of the irradiation source, F, photons per second. The percentage of the light absorbed at the excitation wavelength, A, can be obtained from the absorption spectrum using Beers law. Thus, equation (1) can be expressed in terms of these measured variables,

$$\phi = \frac{N_A CV\Delta}{tFA} \qquad (2)$$

where t is the irradiation time in seconds. For equation (2) to be valid the percentage conversions (Δ) should be kept low (short irradiation times) in order to avoid any secondary photochemistry, and the percentage light absorbed should be high. Our photochemical experiments are generally performed with Δ = 1-3% and A > 90%. Since Δ must be kept low a purity of the starting material of > 99.5% allows HPLC detection and quantification of photoproducts present in as low as 0.1% relative abundance. Thus, accurate, quantitative photochemical measurements can be made by utilizing HPLC techniques and chemical actinometry.

RESULTS AND DISCUSSION

Upon extended irradiation of an ethanol solution of all-trans-13-demethylretinal an equilibrium distribution is established



between all-trans-2 and several configurational isomers. The exact composition is dependent upon the excitation wavelength since the absorption band shapes and molar absorptivities of each isomer differs. Figure 2 illustrates this point for all-trans and 11-cis-2. Table 3 is a summary of the product distribution obtained upon excitation into the first absorption band of all-trans-2. From Table 3, it is evident that the optimal condition for the formation of the target molecule, 11-cis-2, is 390 nm irradiation.

Quantum yields of cis-trans photoisomerization (ΦPI) were determined for all-trans and 11-cis-13-demethylretinal. In a 3-methylpentane solution at room temperature, ΦPI = 0.022 $\pm$ 0.003 (6 trials) for all-trans-2 and ΦPI = 0.66 $\pm$ 0.12 (8 trials) for 11-cis-2 upon 350 nm irradiation. Thus reproducible quantum yields ($\pm$ 20%) may be obtained. These values can be contrasted

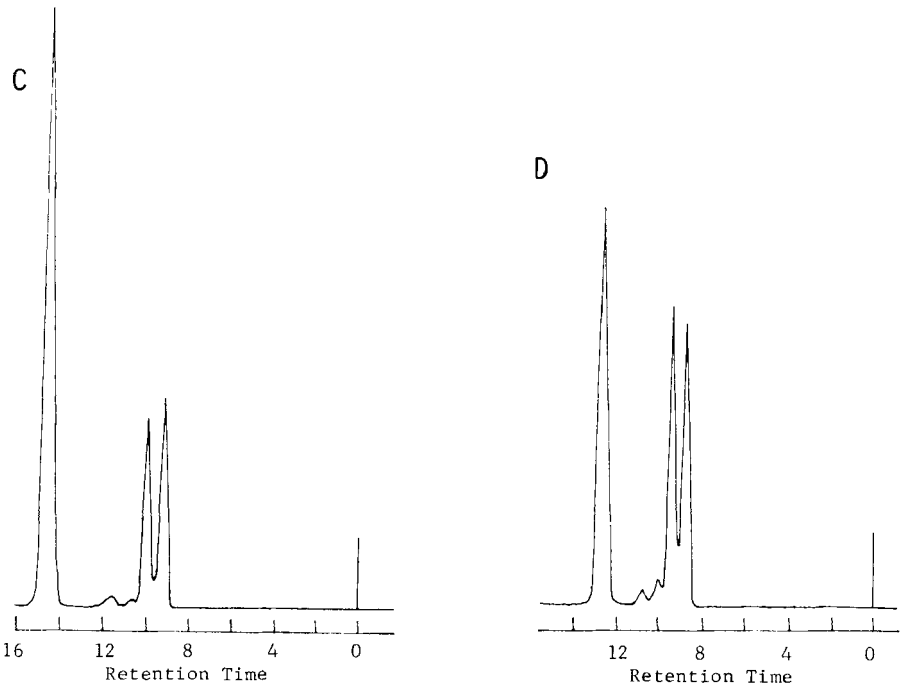


FIGURE 2

High pressure liquid chromatograms of all-trans-13-demethyl-retinal upon 430 nm irradiation in an ethanol solution: A) 0 seconds irradiation; B) 250 seconds irradiation; C) 2000 seconds irradiation; and D) 8000 seconds irradiation (photoquilibrium).

with the corresponding isomers of retinal which have ϕPI values (measured under identical conditions) of 0.08 $\pm$ 0.02 (12) and 0.25 $\pm$ 0.05 (14) for the all-trans and 11-cis isomers, respectively. The primary photoproducts of the trans isomers also differ. Whereas all-trans-1 yields 9-cis and 13-cis-1 in a one to four ratio, all-trans-2 yields 9-cis- and 11-cis-2 in approximately equal amounts.

TABLE 1

Relative Retentions of Isomers of Polyenes 1-3[a]

Isomer	Compound		
	Retinal (1)	13-Demethylretinal (2)	14-Methylretinal (3)
13-cis	0.54	--	0.45
11-cis	0.57	0.52	0.53
9-cis, 13-cis	0.56	--	0.59
9-cis	0.76	0.65	0.66
7-cis	0.84	0.80	0.83
trans	1.00	1.00	1.00

[a]Waters μ-porasil column, 2-4% anhydrous ether in hexane.

TABLE 2

Normalization Factors of Isomers of Polyenes 1-3[a]

Isomer	Compound		
	Retinal (1)	13-Demethylretinal (2)	14-Methylretinal (3)
7-cis	--	2.06	--
9-cis	1.22	1.59	1.25
11-cis	1.80	1.59	2.89
13-cis	1.26	--	1.29
9-cis, 13-cis	1.32	--	1.63
trans	1.00	1.00	1.00

[a]365 nm detection only.

The quantitative photochemical data obtained using HPLC analysis facilitates elucidation of the mechanism of the photoisomerization reaction by offering distinct advantages over other analytical methods such as electronic absorption or nuclear magnetic resonance spectroscopy. Owing to the similarities in the

TABLE 3

Photoequilibrium Composition of Isomers of 13-Demethylretinal[a]

Excitation Wavelength[b] $\lambda_{e,nm}$	Percentage Isomer Distribution[c]			
	all-trans	7-cis	9-cis	11-cis
350	51	3	21	23
390	38	3	22	35
430	37	3	30	27

(a) Obtained upon independent irradiation of ethanolic solutions of all-trans- and 9-cis-13-demethylretinal to this composition; corrected for individual isomer response of the detector; (b) 7 nm bandpass; (c) $\pm$ 0.5; the remainder of the mixture was not analyzed by NMR.

band maxima of the electronic absorption spectra of the isomeric retinals and related linear polyenes, it is difficult to determine the configuration of the photoproducts. This is especially true if more than one photoproduct were to be present, or any product formed in low yields. The absorption technique is inherently sensitive to any uncertainty in the band shape or molar absorptivity of any isomer of the series since the spectrum of the photochemical reaction mixture must be deconvoluted into its component spectra. In addition, since molar absorptivities generally are accurate only to within 5-10%, large percentage conversions are necessary for these reactions and the possibility of secondary photoreactions is enhanced.

There are several limitations to applying proton nuclear magnetic resonance methods to obtain quantitative information about a photochemical reaction. Large quantities of sample are required and the percentage conversions should be high enough to allow facile detection, but prohibit any secondary photoreaction. Its application to linear polyene photochemistry is particularly limited since proton chemical shifts for the various stereo-

isomers of retinal and related analogs (15,25,34) are similar. This is especially true for the alkenic hydrogens of cis-trans configurations of linear polyenes whose spectra of photochemical mixtures would be expected to be particularly complicated. The ability to detect minor photoproducts is also suspect.

Since a range of column packing materials is available and several methods of HPLC detection exist, we believe that the utilization of high pressure liquid chromatographic techniques to obtain qualitative and quantitative data of photochemical reactions can be readily applied to examine a variety of classes of compounds.

ACKNOWLEDGEMENTS

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