Spectroscopic studies of neutral and chemically oxidized species of β-carotene, lycopene and norbixin in CH_2Cl_2: Fluorescence from intermediate compounds

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ABSTRACT

Radical cations, dications and oxidized intermediate species of three carotenoids, namely, β-carotene, lycopene and norbixin, were generated in CH_2Cl_2 solutions via chemical oxidation using anhydrous $FeCl_3$. UV–vis, fluorescence and fluorescence-excitation spectroscopic studies were performed to understand and compare the nature of intermediate species generated during the chemical oxidation process and subsequent degradation. The intense emission observed at 550 nm can be assigned to the $S_2 \rightarrow S_0$ ($1^1B_u \rightarrow 1^1A_g$) transition of the carotenoid molecules. The 350 nm excitation during the oxidation process for β-carotene, lycopene and norbixin exhibit intense fluorescence peaks at 492 nm, 493 nm and 500 nm, respectively. These peaks are assigned to intermediate peroxy/epoxy compounds of the three molecules that are formed with molecular oxygen prior to the formation of oxidized short-chain stable compounds.

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1. Introduction

Carotenoids (Car) are linear C_{40} tetraterpenoid hydrocarbons composed of eight C_5 isoprene units. The extended conjugated polyene chain with a delocalized π electrons system serves as the light absorbing chromophore [1–4], which imparts brilliant colors to these molecules. Carotenoids are multifunctional naturally occurring pigments found in many plants, animals, algae, among others, [5,6] and they participate in redox reactions as electron donors, generating Car radical cations (Car$^{\bullet +}$) [7,8]. These reactive Car$^{\bullet +}$ species are involved in many reactions, one of which is the reaction with O_2 to produce the epoxide form of Car$^{\bullet +}$, which subsequently undergoes cleavage to form apo-carotenals, apo-carotenones and other various oxidized products [9–13]. The potential health risks associated with these molecules were recently reported in an article [12] that indicated that the oxygenated products of β-carotene that were generated in vitro increased the oxidative stress of isolated rat liver. The ability of Car to quench singlet oxygen ($^1O_2$), especially under photosensitized oxidation conditions to form Car$^{\bullet +}$ oxygen adducts, were also reported by many workers [11–13]. However, the role of oxygenated Car in biological systems has not been fully explained.

Further, the electronic absorption properties of many Car species and their radical species have been extensively studied [14–16]. However, their luminescence properties have not been addressed adequately to our knowledge. In this study, both electronic absorption and fluorescence spectroscopic techniques were used to gain a better understanding of the chemical oxidation process of Car. It is essential to elucidate the role of epoxy Car, especially in the area of biological research because oxidation products may have a beneficial or a harmful effect on human health. Three Car species, namely, β-carotene, lycopene and norbixin (Fig. 1), were investigated in this work, and anhydrous ferric chloride ($FeCl_3$) in CH_2Cl_2 was used as the oxidizing agent.

2. Experimental

2.1. Sample preparation and HPLC characterization

β-carotene (I) of a purity $\geq 93\%$ was purchased from Sigma chemicals (PVT) Ltd. Lycopene (II) was extracted from well-matured fruits of watermelon "Dark Belly" species according to the method described in Rodriguez-Amaya [2,3]. Norbixin (III) was extracted [17] from the outer coating of the Annatto seeds of the Bixa orellana tree by washing with hexane, followed by solvent removal by rotary evaporation. Aqueous alkali was added to the

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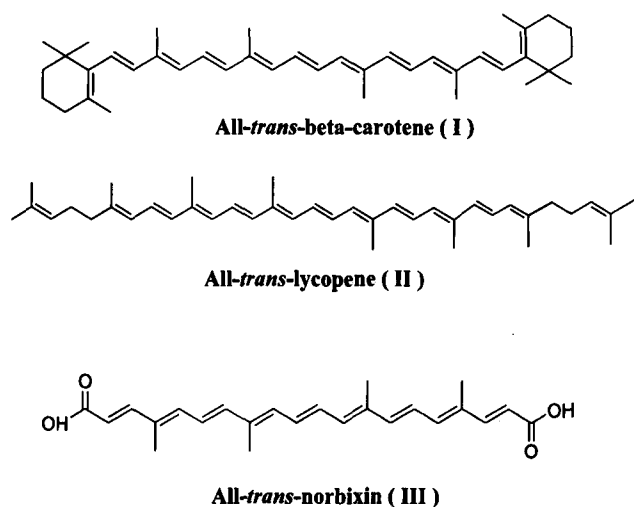 E-mail address: pradeep@sjp.ac.lk (P.M. Jayaweera).

Fig. 1. Molecular structures of the all-*trans* carotenoids (I) β-carotene, (II) lycopene and (III) norbixin.

resultant powder and then heated for hydrolysis, followed by cooling. An aqueous solution was filtered and acidified with 5 M HCl to precipitate norbixin, washed with cold hexane, and then dried to obtain solid norbixin. All of the samples were N_2 purged and stored at −20 °C. Compounds were characterized using UV–vis spectrophotometry and HPLC techniques. A HPLC series 1200 (Agilent, Waldbronn Germany) apparatus equipped with a multi-wavelength/photodiode array detectors was used for chromatographic analysis. The mobile phase consisting acetonitrile, methanol and ethyl acetate with 0.05% (v/v) triethylamine was used at a flow rate 0.5 ml/min. Anhydrous ferric chloride ($FeCl_3$) and HPLC grade dichloromethane (CH_2Cl_2) were purchased from Hemsons International and Sigma Chemicals (PVT) Ltd., respectively.

2.2. UV–vis and fluorescence spectroscopy

The electronic absorption spectra were recorded using a Labomed, Inc., Spectro UV–vis double beam spectrophotometer Model UVD 2960. Fluorescence experiments were performed at 25 °C using a Thermo Scientific Lumina fluorescence spectrometer. The excitation wavelength used throughout this work was 350 nm. The probing wavelengths of the fluorescence excitation scans were recorded at 550 nm. The cuvette holder with the Peltier heater was used to obtain spectra at different temperatures in the range of 10–40 °C. Fluorescence quantum yields of I–III were measured using quinine sulfate as the standard [18]. Anhydrous $FeCl_3$ in CH_2Cl_2 and all of the solvents used throughout this work were scanned to confirm that they are free from fluorescing impurities.

3. Results and discussion

3.1. HPLC analysis of carotenoids

Fig. 2(A)–(C) shows the HPLC chromatograms of (I)–(III), respectively. HPLC chromatograms obtained for extracted lycopene and norbixin samples indicate that they have purities of approximately ≥ 96% and ≥ 90%, respectively. The purity of the samples was confirmed by using photodiode array spectra [2,3,19]. Sharp peaks without any shoulders indicate that molecules (I)–(III) are predominantly in all *trans* configuration.
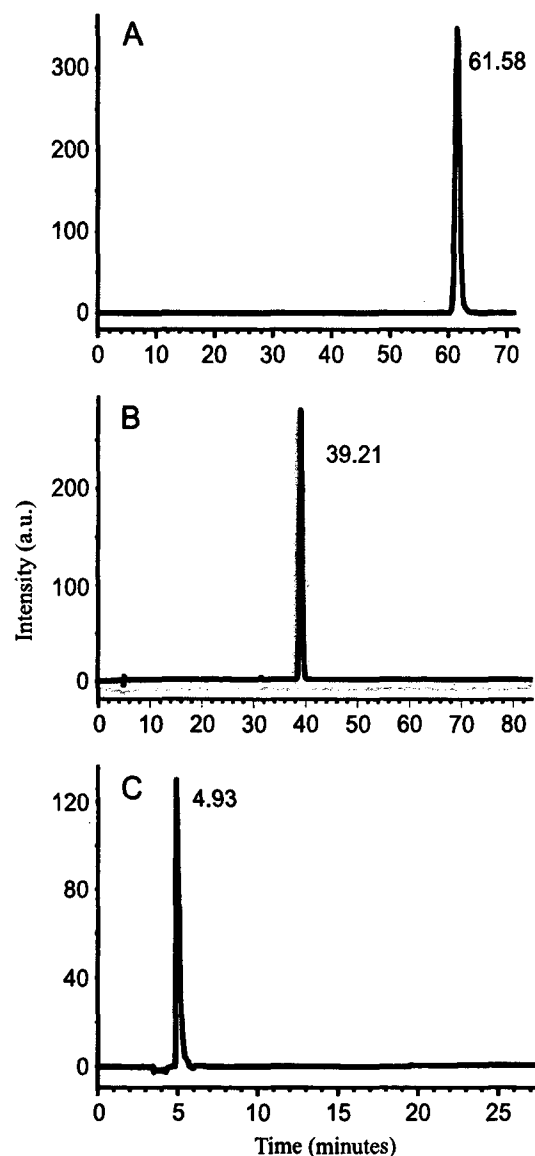
Fig. 2. HPLC chromatogram of (A) standard β-carotene, (B) lycopene extracted from watermelon and (C) norbixin extracted from Annatto seeds.
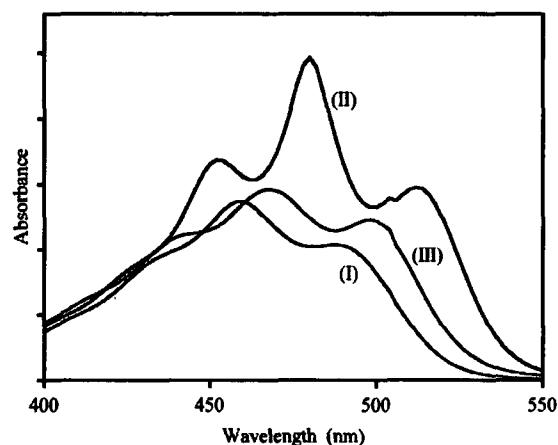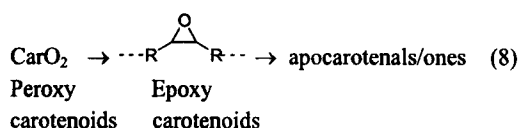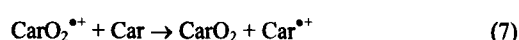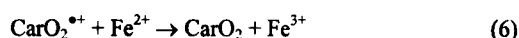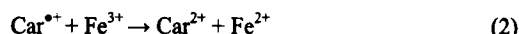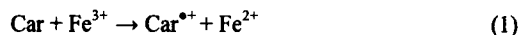
Fig. 3. UV–vis/NIR spectra of the carotenoids (I) β-carotene, (II) lycopene and (III) norbixin in CH_2Cl_2.

3.2. Electronic absorption spectra

Electronic absorption spectra of I–III in CH_2Cl_2 are shown in Fig. 3. The absorption spectra show the characteristic, strongly allowed $S_0 \rightarrow S_2$ electronic transition [20,21] with a vibronic structure corresponding to the 0–2, 0–1 and 0–0 transitions in the 400–500 nm region. A Franck–Condon maximum intensity peak corresponding to the (0–1) band in the strongly allowed $S_0 \rightarrow S_2$ electronic transition was observed in the range of 460–480 nm for three carotenoids. β-carotene and lycopene have eleven conjugated carbon–carbon double bonds, whereas norbixin has nine carbon–carbon double bonds. The absence of terminal rings in lycopene causes the absorption spectrum to be red-shifted by ~20 nm relative to β-carotene [5]. The extension of conjugation in β-carotene to the β ring causes the above described shift.

Radical cations ($Car^{•+}$) of I–III were generated by means of the chemical oxidation method [22] using anhydrous $FeCl_3$ in CH_2Cl_2. The chemical oxidation can be represented by following manner $Car + FeCl_3 \rightarrow Car^{•+} + FeCl_2 + Cl^-$. The orange color of the neutral carotenoid solution turns to a bright bluish color upon addition of $FeCl_3$ which lasts for several minutes, indicating the formation of fairly stable intermediate species that faded into a pale yellow solution. Scheme 1 shows the possible oxidative degradation pathways that carotenoids can go through in the presence of molecular oxygen. Fig. 4 shows the UV–vis/NIR spectral changes that occur when spectrophotometric titrations were performed with $FeCl_3$. Kispert et al. [7,8,15] have extensively studied the spectroscopic and electrochemical properties of canthaxanthin and β-carotene and their radical species generated via $FeCl_3$ and electrochemical oxidation. However, to our knowledge, few published data are available for II and III. In this study, II and III were isolated from natural plant sources and a subsequent spectroscopic characterization was performed. Compound I was used for comparison purposes. The absorption peaks appear in the near IR region (Fig. 4b) with the addition of two equivalents of $FeCl_3$ in all three spectra due to the $D_0 \rightarrow D_3$ transition of the radical cation [23]. The intensity of these radical cation peaks decreases with the further addition of $FeCl_3$ and new blue-shifted absorption peaks appear (see Fig. 4(A)(e)–(C)(e)) for all three compounds. These new peaks appearing at 800 nm, 850 nm and 720 nm can be assigned to the dication (Car^{2+}) for I–III, respectively [14–16]. However, for lycopene, a distinct shoulder appears at approximately 700 nm, which can be assigned to a dimeric or didehydrodimer entity [7] or perhaps an oxidized aggregate of lycopene, and this shoulder is clearly evident when a very high molar excess of anhydrous $FeCl_3$ was added into the solution.

$$Car + Fe^{3+} \rightarrow Car^{•+} + Fe^{2+} \tag{1}$$

$$Car^{•+} + Fe^{3+} \rightarrow Car^{2+} + Fe^{2+} \tag{2}$$

$$Car^{•+} \rightarrow {}^\#Car^{•} + H^+ \tag{3}$$

$$Car^{2+} \rightarrow {}^\#Car^+ + H^+ \tag{4}$$

$$Car^{•+} + O_2 \rightarrow CarO_2^{•+} \tag{5}$$

$$CarO_2^{•+} + Fe^{2+} \rightarrow CarO_2 + Fe^{3+} \tag{6}$$

$$CarO_2^{•+} + Car \rightarrow CarO_2 + Car^{•+} \tag{7}$$

$$CarO_2 \rightarrow \cdots R \overset{O}{\overset{\triangle}{\diagup}} R \cdots \rightarrow apocarotenals/ones \tag{8}$$

Peroxy   Epoxy
carotenoids   carotenoids

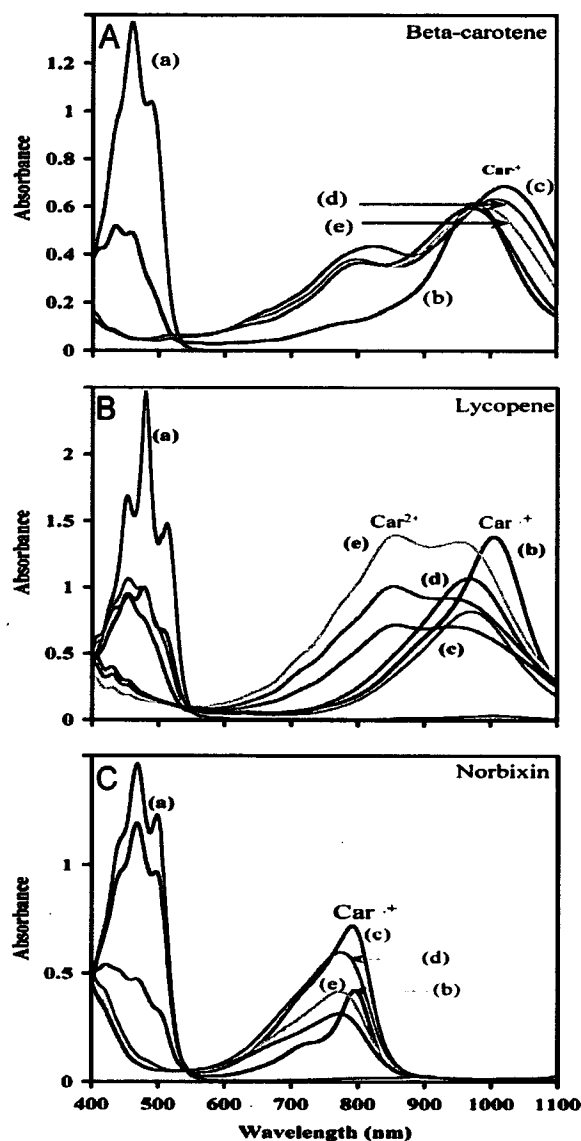**Scheme 1.** $FeCl_3$-induced oxidative degradation reactions of carotenoids in CH_2Cl_2.

Fig. 4. UV–vis/NIR spectra of (A) β-carotene, (B) lycopene and (C) norbixin in CH_2Cl_2 (a) carotenoids, (b) after adding two equivalents of $FeCl_3$, (c)–(e) after 4-, 8-, and 12-min time intervals.

Fluorescence spectra of I–III are shown in Fig. 5(A)(a)–(C)(a), respectively. The corresponding fluorescence excitation spectra of I–III are shown in Fig. 5(A)(b)–(C)(b), respectively, at 25 °C. The fluorescence spectra are broad and do not exhibit the vibronic features as they were recorded at 25 °C. Fujii et al. [24] investigated the fluorescence spectroscopy of the all-*trans*-anhydrorhodovibrin and spirilloxanthin, which have twelve and thirteen conjugated double bonds, respectively. Based on previous work [21,24], we can assign the fluorescence spectra of I–III at approximately 550 nm to the $S_2 \rightarrow S_0$ ($1^1B_u \rightarrow 1^1A_g$) allowed transition. The striking feature of this transition is that, when excited at 350 nm, the blue color solution, i.e., Car with anhydrous $FeCl_3$, exhibits a very strong blue shifted fluorescence (Fig. 5(A)(c)–(C)(c)) for all three molecules investigated in this work. The emission spectra of oxidized species of I and II exhibit an ~70 nm blue-shift compared to the emission of the corresponding Car. However, for III, the observed shift is approximately 50 nm and is presumably due to the two terminal carboxylic groups and the lower number of conjugated π bonds in III. Table 1 summarizes the fluorescence data. For excitations into the NIR, the $D_0 \rightarrow D_3$ transition (~800 nm) of radical cations do not exhibit any emission further to lower wavelength region < 900 nm.
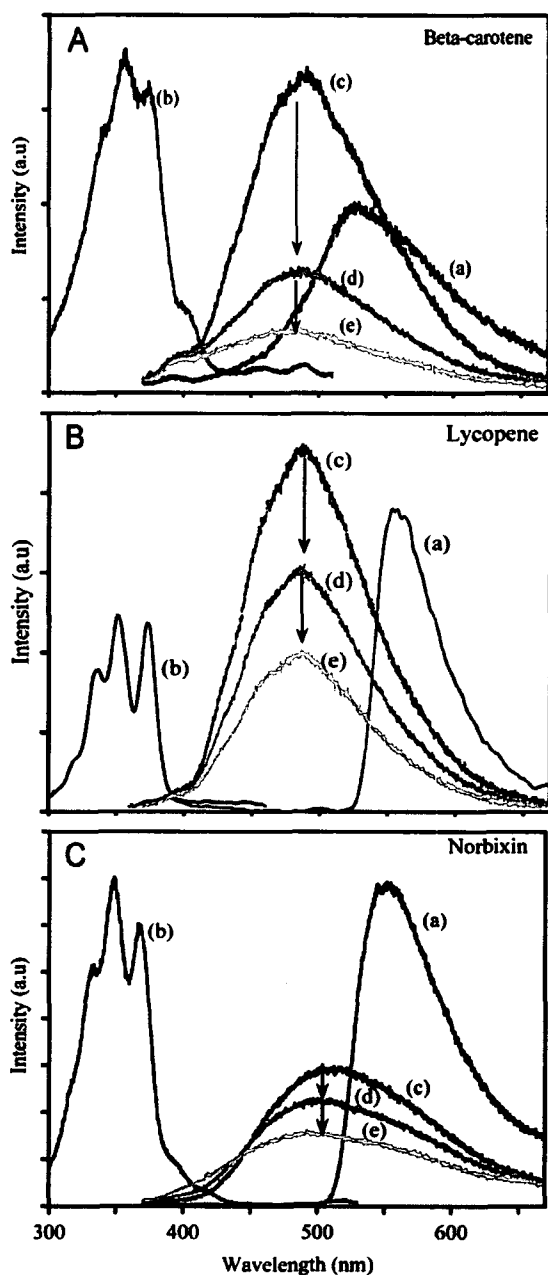
Fig. 5. Fluorescence spectra of (A)(a) β-carotene, (B)(a) lycopene and (C)(a) norbixin in CH₂Cl₂. Fluorescence excitation spectra of (A)(b) β-carotene, (B)(b) lycopene and (C)(b) norbixin in CH₂Cl₂. Probing: 550 nm. Fluorescence spectra with the addition of FeCl₃/CH₂Cl₂ into (A) β-carotene, (B) lycopene and (C) norbixin. Equivalent added (c) 0.3, (d) 1.0 and (e) 1.5. Excitation: 350 nm.

Table 1
Fluorescence wavelength maxima of neutral carotenoids and their chemically oxidized species.

Carotenoid	λ_{max}^{fluo} in CH2Cl2 (nm)	
	Neutral carotenoid	**Peroxy/epoxy carotenoid**
β-Carotene	550	493
Lycopene	555	492
Norbixin	552	500

The fluorescence excitation spectra obtained for Car (probing at 550 nm) and for the oxidizing I–III (probing at 492 nm, 493 nm and 500 nm) exhibit characteristic shapes similar to the electronic absorption spectra of Car molecules. Similar excitation spectra for

both Car and oxidized Car species suggest that the main carbon–carbon backbone remains intact during the fluorescence process. Furthermore, Fig. 5(A)(c–e)–(C)(c–e) shows the decay of the blue shifted emission when fluorescence titrations are performed with saturated anhydrous FeCl₃ in CH₂Cl₂, suggesting that the emitting species are intermediate in nature and are not due to stable decomposed products of molecules I–III. The quantum yield (ϕ_F) and Stokes shift ($\Delta\lambda_S$) values for I–III were estimated by following the method described in Ref. [25–27] and using the following equation:

$$\phi_S = \phi_{ref}\left(Grad_S/Grad_{ref}\right)\left(\eta_S^2/\eta_{ref}^2\right)$$

where ϕ_S and ϕ_{ref} are the fluorescence quantum yields, $Grad_S$ and $Grad_{ref}$ are the gradients of the plots of integrated florescence intensity vs. absorbance and η_S and η_{ref} are the refractive indices of the solvent used for the sample and the standard sample (i.e., reference), respectively. For molecules I–III, the estimated quantum yields are 2.1×10^{-4}, 1.6×10^{-2} and 2.8×10^{-4}, respectively. Stokes shift values, $\Delta\lambda_S = \lambda_{F(max)} - \lambda_{A(max)}$ were calculated using the wavelength maxima of fluorescence ($\lambda_{F(max)}$) and absorbance ($\lambda_{A(max)}$) data. The Stokes shift values for I–III are 90 nm, 75 nm and 84 nm, respectively. All the experimental data were collected in CH₂Cl₂ solutions at 25 °C. The reported literature [28] quantum yield values and our values are comparable. The highest quantum yield is exhibited by II, and I and III
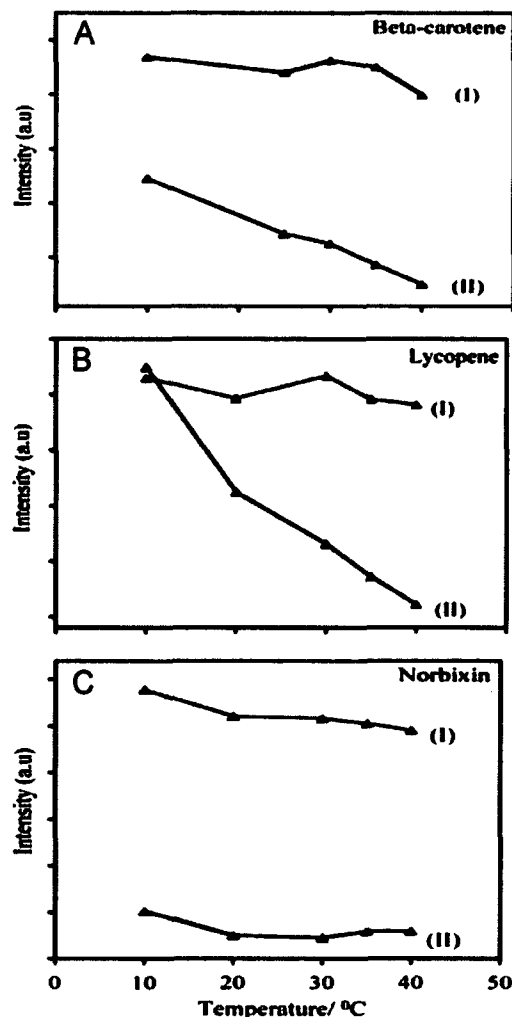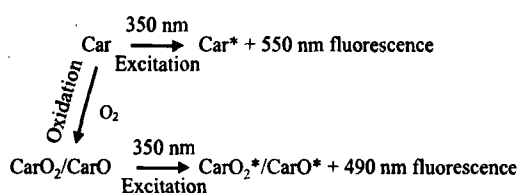

Fig. 6. Fluorescence intensity variation at different temperatures for (A) β-carotene, (B) lycopene and (C) norbixin in CH₂Cl₂ (I) carotenoids and (II) oxidized species generated by chemical oxidation.

$$\text{Car} \xrightarrow[\text{Excitation}]{350 \text{ nm}} \text{Car*} + 550 \text{ nm fluorescence}$$

$$\text{CarO}_2/\text{CarO} \xrightarrow[\text{Excitation}]{350 \text{ nm}} \text{CarO}_2\text{*}/\text{CarO*} + 490 \text{ nm fluorescence}$$

(with "Oxidation" and "O$_2$" along the vertical arrow)

Scheme 2. Fluorescence from Car and peroxy/epoxy Car compounds.

exhibit lower quantum yields. The highest Stokes shift was observed for I, and the lowest was observed for II.

To understand the nature of the blue shifted emission, experiments were performed at different temperatures in the range of 10–40 °C. Fig. 6 shows the fluorescence intensity variations for both Car and oxidized Car species at different temperatures with the addition of anhydrous FeCl$_3$. Note that with increasing temperature, the fluorescence intensity of the blue shifted peak decays faster when compared with the corresponding fluorescence intensity of Car, which remains more or less at a constant level. The results indicate that the emitting species is unstable in nature and decomposes at elevated temperatures. The emission is not due to a stable decomposed product(s) of molecules I–III. Purging of nitrogen gas prior to the chemical oxidation does not produce intense blue shifted fluorescence peaks for all three molecules. This result may well be due to the removal of dissolved oxygen from the medium and the resulting low yield of peroxy/epoxy compound formation. Therefore, it is clear that the blue shifted fluorescence that is observed is due to an oxygenated form of Car and is most likely the peroxy/epoxy form of I–III. To verify that the blue shifted emission is not arising from the isomerized forms of I–III, the samples were heated for various time intervals to induce thermal isomerization [29] and were scanned for fluorescence signals. Fluorescence scans reveal no appearance of new peaks, only the gradual reduction in the fluorescence intensities observed for the neutral molecules.

Detection of two different emitting wavelengths (see Scheme 2) for Car and peroxy/epoxy Car enables numerous possibilities in many areas, especially in understanding the biological functions [30] of these molecules. The oxidative degradation mechanism of Car proceeds *via* the formation of peroxy/epoxy compounds as intermediate compounds by transferring oxygen atoms to Car without changing its molecular structure.

4. Conclusion

Electronic absorption and fluorescence spectroscopic studies on β-carotene, lycopene and norbixin have provided evidence that molecules can be chemically oxidized to form fairly stable intermediate species with FeCl$_3$ in CH$_2$Cl$_2$. Oxidation proceeds with the formation of a number of intermediate species, such as radical cations, dications, didehydrodimers and epoxy/peroxy compounds. Fluorescence peaks observed at 550 nm can be assigned to the $S_2 \rightarrow S_0$ ($1^1B_u \rightarrow 1^1A_g$) transition of the Car molecules, whereas fluorescence detected at 492 nm, 493 nm and 500 nm during chemical oxidation process were assigned to a peroxy/epoxy form of the intermediate species of β-carotene, lycopene and norbixin, respectively.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jlumin.2014.08. 036.

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