Effects of Ozone and Oxygen on the Degradation of Carotenoids in an Aqueous Model System

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The effects of ozone and oxygen on the degradation of carotenoids in an aqueous model system were studied. All-*trans* β-carotene, 9-*cis* β-carotene, β-cryptoxanthin, and lycopene were adsorbed onto a C_{18} solid phase and exposed to a continuous flow of water saturated with oxygen or ozone at 30 °C. Carotenoids were analyzed using HPLC with a C_{30} column and a photodiode array detector. Approximately 90% of all-*trans* β-carotene, 9-*cis* β-carotene, and β-cryptoxanthin were lost after exposure to ozone for 7 h. A similar loss of lycopene occurred in only 1 h. When exposed to oxygen, all carotenoids, except β-cryptoxanthin, degraded at lower rates. The degradation of all the carotenoids followed zero-order reaction kinetics with the following relative rates: lycopene > β-cryptoxanthin > all-*trans* β-carotene > 9-*cis* β-carotene. The major degradation products of β-carotene were tentatively identified on the basis of their elution on the HPLC column, UV−Vis spectra, and electrospray LC−MS. Predominant isomers of β-carotene were 13-*cis*, 9-*cis*, and a di-*cis* isomer. Products resulting from cleavage of the molecule were β-apo-13-carotenone and β-apo-14′-carotenal, whereas epoxidation yielded β-carotene 5,8-epoxide and β-carotene 5,8-endoperoxide.

Keywords: *Ozone; oxidation; carotenoids; β-carotene; β-cryptoxanthin; lycopene; degradation*

INTRODUCTION

Carotenoids are naturally occurring red, yellow, and orange pigments which are widely distributed in nature. Carotenoids have drawn considerable attention because of their possible association with a reduced risk of certain types of cancers and cardiovascular disease (Ziegler, 1989; Giovanucci, 1999). The antioxidant action of carotenoids was related to their ability to trap free radicals (Burton and Ingold, 1984; Mortensen and Skibsted, 1997) and quench singlet oxygen (Foote and Denny, 1968; Stahl and Sies, 1993). Depending on the redox potential of the molecule and the surrounding environment, the antioxidant activity of carotenoids may shift to prooxidant activity as reviewed by Palozza (1998).

Ozone is a strong antimicrobial agent and commonly used in place of chlorine to increase the microbiological safety of foods (Kim et al., 1999). The benefits of ozone are based on its ability to reduce microbial loads, oxidize toxic organic compounds, and decrease the biological oxygen demand in the environment. The use of ozone minimizes inorganic waste because the molecule decomposes spontaneously to oxygen (Adler and Hill, 1950). The most common use of ozonation is to disinfect drinking water (Munter et al., 1998). However, ozone has been successfully tested for the preservation of meat (Dondo et al., 1992), poultry (Sheldon and Brown, 1986), shrimp (Chen et al., 1992), and fruits and vegetables (Badiani et al., 1996, Kim and Yousef, 1998, Achen and Yousef, 1999).

Surface discoloration is often reported to result from ozone treatment of broccoli florets (Lewis et al., 1996) and peaches (Badiani et al., 1996). Sakaki and Kondo (1981) reported the destruction of chlorophyll and carotenoids in spinach leaves after ozone treatment. Carrots treated with ozone were lighter in color than nontreated samples (Liew and Prange, 1994). The direct effects of ozone on the degradation of specific carotenoids, however, have not been investigated. The objectives of this research were twofold: (1) to determine the degradation rate of (all-E)-β,β-carotene (all-*trans* β-carotene), (9Z)-β,β-carotene (9-*cis* β-carotene), (all-E, 3R)-β,β-caroten-3-ol (β-cryptoxanthin), and (all-E)-ψ,ψ-carotene (lycopene) exposed to ozone and oxygen in an aqueous model system, and (2) to identify the predominant degradation products of all-*trans* β-carotene formed during exposure to ozone. Structures of the carotenoids studied are shown in Figure 1A.

MATERIALS AND METHODS

Materials. All-*trans* β-carotene and β-cryptoxanthin were obtained from Hoffmann-La Roche, Inc. (Nutley, NJ). Lycopene was provided by Sigma Chemical Co. (St. Louis, MO). The C_{18} Sep-Pak cartridges were obtained from Waters Associates (Milford, MA). A *Dunaliella* algae-derived β-carotene prepara-

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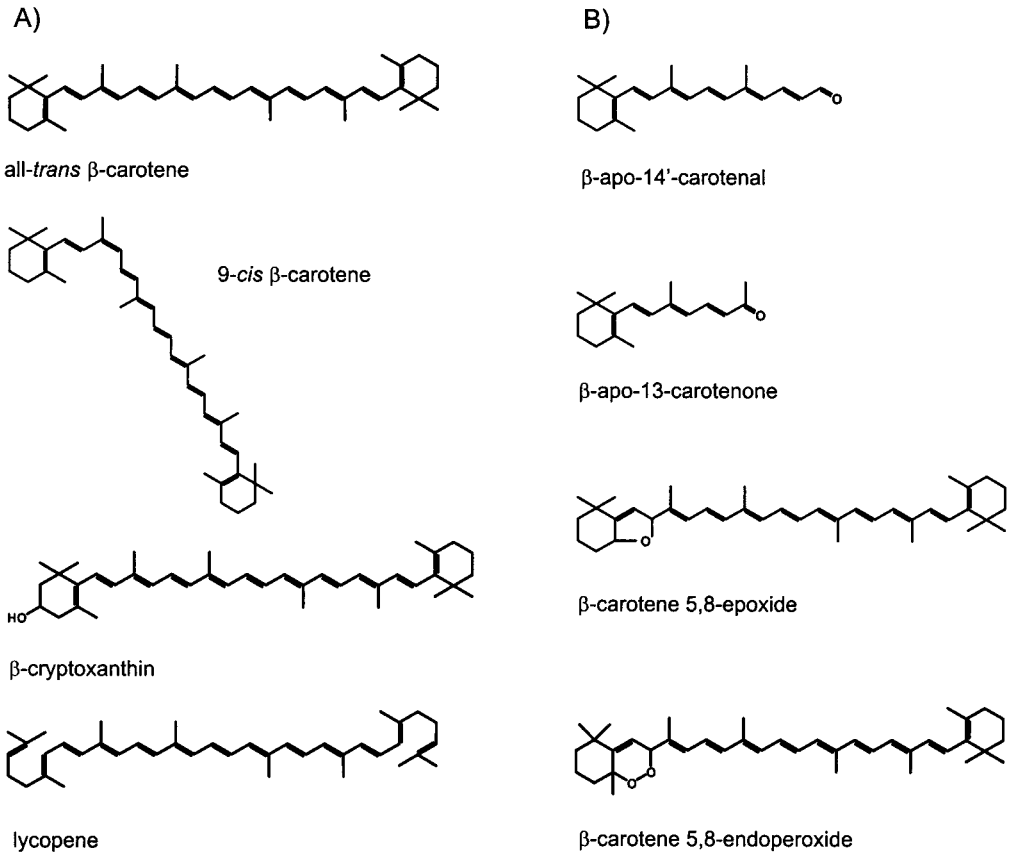

Figure 1. Chemical structures of (A) carotenoids studied and (B) tentatively identified β-carotene degradation products.

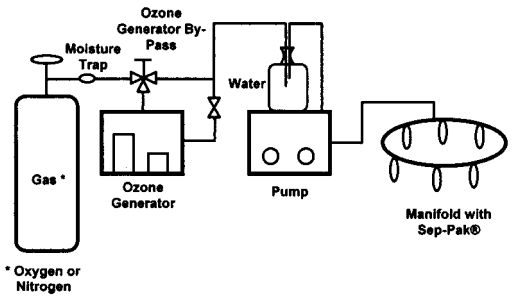

Figure 2. Schematic diagram of the oxidation apparatus.

tion, Betatene (4% β-carotene in soybean oil), was a gift from Betatene, Ltd., Melbourne, Australia.

The 9-*cis* β-carotene isomer was obtained by dissolving a small amount of the *Dunaliella* algae-derived β-carotene preparation in hexane. The mixture was filtered through a 0.45-μm (3 mm) nylon filter (MSI, Westboro, MA) and the hexane was evaporated under nitrogen. The extract was redissolved in the injection solvent of 50:50 methyl-*tertiary*-butyl ether (MTBE)/methanol. The 9-*cis* peak was isolated using a semipreparatory C_{30} reversed-phase column as described later in the Chromatographic Instrumentation section.

Methanol, hexane, and acetone were certified A. C. S. grade, and MTBE was HPLC grade (Fisher Scientific Co., Fairlawn, NJ). Ethanol (USP) was purchased from Aaper Alcohol and Chemical Co. (Shelbyville, Kentucky).

Ozone Generation. Ozone was generated by exposing a continuous flow (200 mL/min) of 100% oxygen to ultraviolet radiation (185 nm). A water trap was positioned at the gas outlet to remove excess moisture that could decrease the efficiency of the ozone generation.

Oxidation Apparatus. Figure 2 is a schematic diagram of the oxidation apparatus used in the study. The system consisted of a gas source, an ozone generator, a Waters model 510 pump (Waters Associates, Milford, MA), and a manifold comprising ports for six Sep-Pak cartridges. The ozone generator was bypassed in experiments where carotenoids were exposed to nitrogen or oxygen. A water flow of 1 mL/min was maintained through each Sep-Pak cartridge.

Ultrafiltered water was saturated with ozone, oxygen, or nitrogen at 30 °C for 12 h prior to each experiment. Nitrogen was used in the experiment as a control and represents a nonoxidative condition. In experiments with nitrogen, the water was degassed under vacuum prior to adding the nitrogen gas to ensure complete removal of gases. A gas dispersion tube with a fritted cylinder was attached to the tubing end to promote maximum dissolution of gas into the water. On the basis of the solubility of each gas in water at saturation (Dean, 1985), the concentrations of ozone, oxygen, and nitrogen in the water at 30 °C were calculated to be 2.3×10^{-4} mol/L, 1.6×10^{-4} mol/L, and 7.3×10^{-4} mol/L, respectively.

Sample Preparation. The C_{18} Sep-Pak cartridges were preconditioned by passing 1 mL each of hexane, acetone, and finally ethanol. Carotenoids were first dissolved in a small amount of tetrahydrofuran (THF) to ensure complete dissolution before addition of ethanol. Ethanol, a polar solvent, was chosen to facilitate transfer of carotenoids onto the nonpolar C_{18} groups of the solid phase. A 50-μL portion of 0.1 mM ethanolic carotenoid was loaded on the preconditioned cartridge and rinsed with 0.1 mL of distilled water prior to each experiment. Under these conditions all carotenoids were retained within the cartridge. Six cartridges loaded with pigments were placed simultaneously on the manifold. Each cartridge was removed from the manifold at specific time intervals. As cartridges were removed, the flow through the remaining cartridges was adjusted to remain constant at 1.0 mL/min. Sampling was done every hour for β-carotene and β-cryptoxanthin, and every 15 min for lycopene.

Upon removal from the manifold, each carotenoid was immediately eluted from the cartridge with acetone into a test tube containing water and hexane. The cartridge was then rinsed with hexane three times, the organic layer was combined, and the solvent was evaporated to dryness under a

Table 1. Chromatographic Conditions for the Separation of β-carotene, Lycopene, and β-cryptoxanthin

carotenoid	time (min)	flow (mL/min)	methyl-*tertiary*-butyl-ether (%)	methanol (%)	detection (nm)
β-carotene (all-*trans* and 9-*cis*)					452
	initial	1.0	0	100	
	18.0	1.0	35	65	
	28.0	1.0	50	50	
	30.0	1.0	70	30	
	35.0	1.0	0	100	
lycopene					472
	initial	1.3	38	62	
	12.0	1.3	38	62	
	25.0	1.3	60	40	
	27.0	1.3	60	40	
	30.0	1.3	38	62	
β-crypto -xanthin		1.0	13	87	449

stream of nitrogen. Samples were stored at −20 °C and analyzed within 24 h. Subdued yellow light was used throughout the experiments.

Chromatographic Instrumentation. Sample analyses were performed using reversed-phase HPLC employing a polymeric analytical scale (4.6 mm i.d. × 250 mm) 5-μm C_{30} reversed-phase column prepared at the National Institute of Standards and Technology (NIST; Gaithersburg, MD) as described by Sander et al. (1994). Purification of standards and isolation of the 9-*cis* β-carotene isomer from the algae preparation were obtained by using a semipreparatory (10 mm i.d. × 250 mm) 5-μm C_{30} reversed-phase column prepared by NIST. The HPLC system consisted of a Waters model U6K injector, two model 510 pumps, a gradient controller model 680, and a photodiode array model 990 detector (Waters Associates, Milford, MA), coupled to a NEC Powermate SX/20 computer (Boxborough, MA). Waters 990 PDA chromatography software (Waters Associates, Milford, MA) was used for data acquisition.

Chromatographic Procedures. A binary mobile phase of MTBE/methanol was used to separate the carotenoids and monitor degradation. Solvents were filtered through a 1.0-μm PTFE laminated filter prior to use. All-*trans* β-carotene, 9-*cis* β-carotene, and lycopene were separated using gradient solvent conditions, whereas β-cryptoxanthin was separated isocratically. Chromatographic conditions for each carotenoid were developed to optimize separations within a reasonable time (Table 1).

Liquid Chromatography−Mass Spectrometry Conditions. The liquid chromatographic procedure was identical to that outlined above, except that a YMC (Wilmington, NC) analytical C_{30} column was used. The mass spectrometry conditions included a Hewlett-Packard (HP) 5989B electrospray spectrometer equipped with an Ion Guide source (Analytica, Branford, CT) and an HP1050 gradient HPLC unit. Column eluent was split postcolumn at a ratio of 1:3 to permit 300 μL/min to enter the mass spectrometer. To enhance ionization, 5% heptafluorobutanol in methanol/MTBE was added postcolumn as previously described by van Breemen (1995). Nitrogen bath gas at 250 °C and 10 L/min was used to enhance solvent evaporation in the atmospheric pressure ion source. Nebulization of the mobile phase was obtained using nitrogen gas at 80 psi.

Kinetic Data Analysis. A plot of the concentration and natural log concentration of carotenoid versus time was used to fit a kinetic model and to obtain reaction rate constants. The plot that gave the best correlation was selected and the slope of the regression lines was the rate constant (k).

Statistical Analysis. The experiment was a complete randomized design. Treatments were arranged as a 3 × 4 factorial with factors given by compound (4 types) and atmospheric conditions (3 types). There were two replicates for each treatment combination, which yielded a total of 24 experimental units used for statistical analysis. Rate constants for the degradation of pigments were analyzed by the general linear models (GLM) procedure in the *Statistical Analysis Systems User's Guide* (1990).

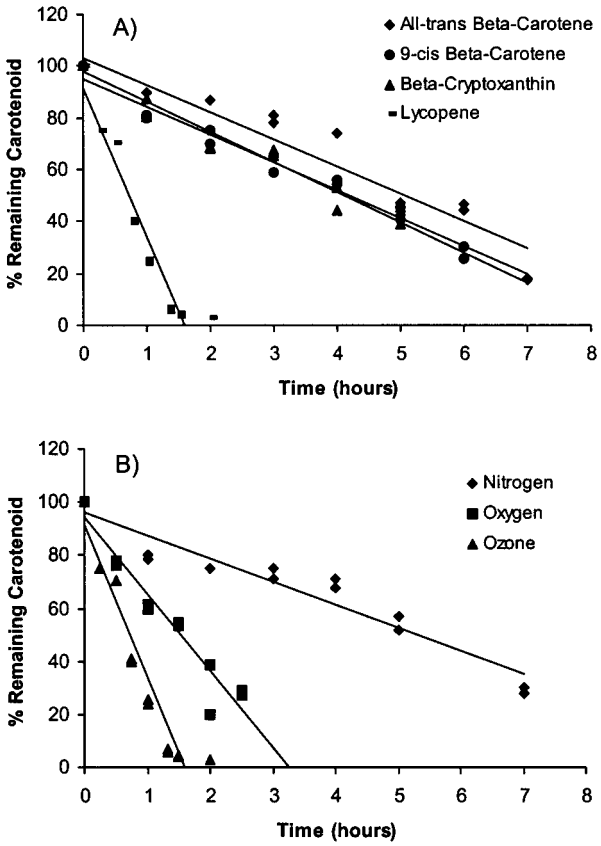

Figure 3. Stability plots of (A) carotenoids after ozone exposure and of (B) lycopene after nitrogen, oxygen, and ozone exposures.

RESULTS AND DISCUSSION

Kinetic and Reaction Rates. Figure 3A illustrates the loss of each carotenoid after ozone exposure and Figure 3B shows the loss of lycopene after nitrogen, oxygen, and ozone exposures. Nitrogen was used as a control and represented a non-oxidative condition. The results, however, showed that some degradation of all carotenoids occurred. The kinetic parameters for carotenoid degradation upon ozone and oxygen exposures (Table 2) were, therefore, corrected with the values found for nitrogen. Under our experimental conditions, all carotenoids degraded following a zero-order kinetic model: $C = C_0 - (k)(t)$]. A zero-order model was also reported for β-carotene exposed to a continuous stream of oxygen in toluene (El-Tinay and Chichester, 1970) and for carotenoids in cyclohexane and ethanol at different temperatures under nitrogen (Mínguez-Mosquera and Jarén-Galán, 1995).

Except for β-cryptoxanthin, ozone (as anticipated) was a stronger oxidant than oxygen. The degradation rates of all-*trans* β-carotene and lycopene were 3 times faster in the presence of ozone than in oxygen. The degradation rate of β-cryptoxanthin in the presence of ozone or oxygen was not different.

There was a marked difference between lycopene and the other carotenoids with regard to stability. In the

Table 2. Kinetic Parameters for β-carotene, 9-*cis* β-carotene, β-cryptoxanthin, and Lycopene[a,b]

	reaction order	rate constant (conc h^{-1})	R^2
all-*trans* β-carotene			
oxygen	0	0.0013 ± 0.0003	0.923
ozone	0	0.0039 ± 0.0005	0.913
9-*cis* β-carotene			
oxygen	0	0.0002 ± 0.0003	0.900
ozone	0	0.0025 ± 0.0003	0.972
β-cryptoxanthin			
oxygen	0	0.0040 ± 0.0005	0.938
ozone	0	0.0044 ± 0.0008	0.904
lycopene			
oxygen	0	0.0107 ± 0.0013	0.934
ozone	0	0.0330 ± 0.0038	0.929

[a] Means ± standard error of means. [b] Values were nitrogen-corrected.

presence of ozone and oxygen, approximately 90% of lycopene was lost after 1 and 2 h, respectively. Under identical conditions, the other carotenoids did not achieve equivalent loss until 7 h of exposure. During ozonation, lycopene degraded 13 times faster than 9-*cis* β-carotene, which was the carotenoid with the lowest rate constant.

In solution, ozone decomposes stepwise to hydroperoxyl (·OOH), hydroxyl (·OH), and superoxide ($\cdot O_2^-$) radicals (Adler and Hill, 1950; Hoigne and Bader, 1975). The reactivity of ozone has been attributed to these radicals. Using UV−Vis−NIR spectroscopy, Cataldo (1996) observed the *trans-cis* isomerization of β-carotene at its 15−15′ position, followed by the formation of β-carotene radical cation during ozonation. The radical cation degraded further to unsaturated and conjugated aldehydes and dialdehydes. The striking similarity between oxygen- and ozone-derived carotenoid degradation products observed by HPLC in our study may indicate that the reactive species in both reactions (i.e., β-carotene radical cations) were similar, but more were present during ozonation. The formation of carotenoid adducts (such as endoperoxides or ozonides) which then further degrade to similar products could also explain the results. Clark et al. (1997) reported the formation of endoperoxide of retinoic acid during the initial stages of all-*trans* retinoic acid oxidation at high oxygen pressure. They suggested that two types of reactions occurred concurrently: one involving autoxidation (radical chain process) to give epoxides, and the other involving a direct reaction with triplet molecular oxygen to give endoperoxides. Recently, Zürcher and Pfander (1999) isolated and characterized two ozonides produced during the oxidation of canthaxanthin (β,β-carotene-4,4′-dione) with *m*-chloroperbenzoic acid and molecular oxygen. These ozonides were suspected to be intermediates in the formation of apocarotenoids, usually found as oxidation products of carotenoids. Using electrospray LC−MS analysis, we detected β-carotene epoxide, endoperoxide, and ozonide in our system.

Carotenoid Structure and Stability. The difference in stability of the carotenoids was partly influenced by the structure of individual carotenoids (Miller et al., 1996; Mortensen and Skibsted, 1997; Woodall et al., 1997). In general, the decreasing number of coplanar conjugated double bonds and the presence of hydroxy and keto groups in carotenoids decrease their reactivity in radical-scavenging reactions. The sequence for radical scavenging activity was carotenes > hydroxycarotenoids > ketocarotenoids. In the present study, the rates of degradation were lycopene > β-cryptoxanthin > all-*trans* β-carotene > 9-*cis* β-carotene. Lycopene was the most effective free radical scavenger, owing to its 11 conjugated double bonds. In β-carotene, 2 of its 11 conjugated double bonds are not coplanar with the polyene chain, thus, it was less reactive than lycopene (Mortensen and Skibsted, 1997; Woodall et al., 1997). Similar results were obtained when carotenoids were heated in oil (Henry et al., 1998). β-Cryptoxanthin differs from β-carotene only in the presence of a hydroxyl group at the C-3 position. This structural modification did not result in a notable change in degradation rate following exposure to ozone. Miller et al. (1996) made a similar observation for β-cryptoxanthin in the presence of a radical cation. The same co-workers found that when each β-ring contains one hydroxyl group (as in zeaxanthin), the stability toward radical cation was reduced significantly.

Geometric Configuration and Stability. The all-*trans* isomer was slightly (1.6x) more sensitive to ozone than the 9-*cis* β-carotene. Interestingly, in the presence of oxygen the 9-*cis* isomer was far less sensitive to oxidation. In a study in which β-carotene was heated in oil (Henry et al., 1998) the stability of the 9-*cis* isomer did not differ significantly from that of all-*trans* β-carotene. In contrast, Jimenez and Pick (1993) found that 9-*cis* β-carotene degraded faster than all-*trans* when exposed to oxidants. Levin and Mokady (1994) showed that systems with 9-*cis* β-carotene had low hydroperoxide accumulation and the 9-*cis* isomer had a higher antioxidant potency than all-*trans* β-carotene. They suggested that the higher reactivity of the cis molecule toward free radicals was due to a higher steric interference between the two parts on the other side of the cis double bond. Currently, there is no apparent explanation for these discrepancies.

Degradation Products of β-Carotene. The degradation products of all carotenoids formed during oxygen exposure were similar to those formed during ozonation and therefore the discussion will focus only on the degradation products of ozonation. Figure 4A and 4B illustrates the chromatographic separation of all-*trans* β-carotene after 5 h of exposure to oxygen and ozone, respectively. The products were identified based on their elution on the HPLC column and UV−vis spectra as well as electrospray LC−MS analysis (Table 3).

The predominant β-carotene isomers formed during ozonation were 13-*cis*, 9-*cis*, and an unidentified *cis* β-carotene. Base on its large shift in the blue region (Table 3), the unidentified *cis* β-carotene isomer was suspected to be a di-*cis* isomer. The structures of the major oxidation products formed during ozonation of β-carotene are shown in Figure 1B. Products resulting from cleavage of the molecule were tentatively identified as 13-apo-β-caroten-13-one (β-apo-13-carotenone, *m/z* 258 $M^{\cdot+}$) and 14′-apo-β-caroten-14′-al (β-apo-14′-carotenal, *m/z* 311, $[M+H]^+$). These findings indicate that β-carotene can be cleaved centrally at double bonds 13,-14 and 13′,14′ to produce β-apo-13-carotenone and β-apo-14′-carotenal, respectively. Handelman et al. (1991) found similar results during autoxidation and azo-bis-isobutyronitrile (AIBN) initiated oxidation of β-carotene.

Epoxides of β-carotene were also formed during ozone exposure. 5,8-Epoxy-5,8-dihydro-β,β-carotene (β-carotene 5,8-epoxide, $C_{40}H_{56}O$; *m/z* 552 $M^{\cdot+}$) and 5,8-epidioxy-5,8-dihydro-β,β-carotene (β-carotene 5,8-endoperoxide, $C_{40}H_{56}O_2$; *m/z* 568 $M^{\cdot+}$) have been tentatively

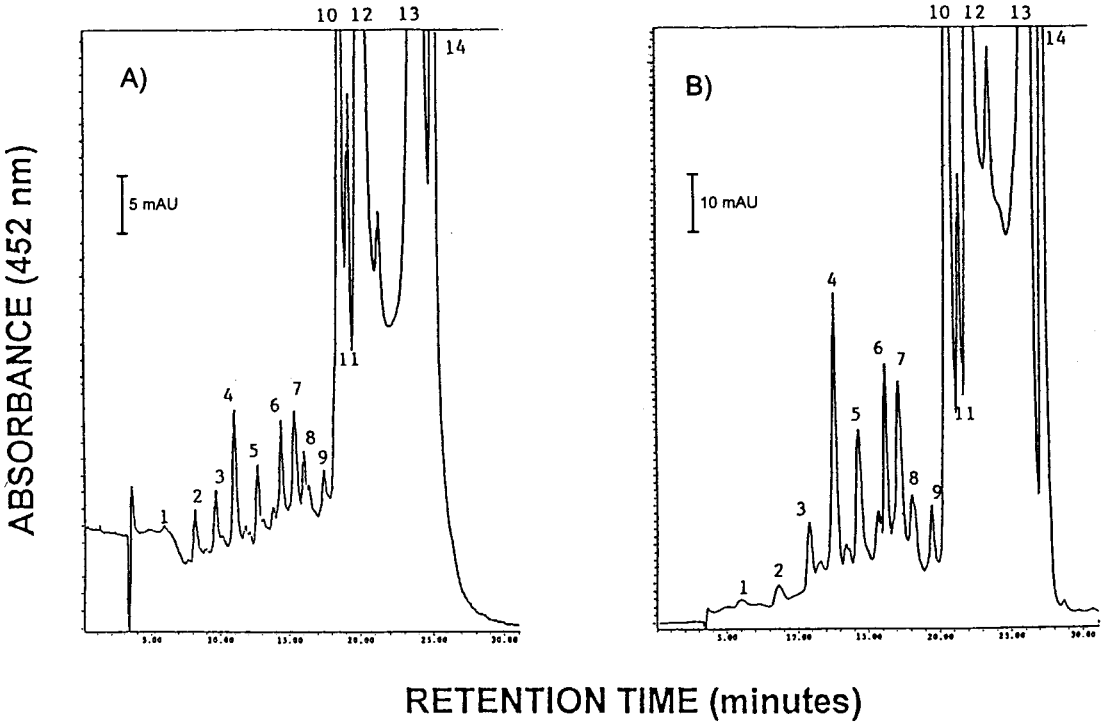

Figure 4. Chromatographic separation of all-*trans* β-carotene after 5 h exposure of (A) oxygen and (B) ozone. **1**, unknown; **2**, β-apo-14′-carotenal; **3**, β-apo-13-carotenone; **4**−**7**, unknown; **8**, β-carotene 5,8-endoperoxide; **9**, β-carotene 5,8-epoxide; **10**, a mono-epoxide (tentative); **11**, a di-*cis* isomer (tentative); **12**, 13-*cis* β-carotene; **13**, all-*trans* β-carotene; and **14**, 9-*cis* β-carotene.

Table 3. UV−Vis Absorbance Maxima and Molecular Weights for β-carotene and Degradation Products[a]

peak	compound	UV−Vis absorbance maxima (nm)[b]	*m/z*
2	β-apo-14′-carotenal	400	311
3	β-apo-13-carotenone	377	258
8	β-carotene 5,8-endoperoxide	315, 400, **423**, 447	568
9	β-carotene 5,8-epoxide	453, **426**, 405	552
11	di-*cis* β-carotene (tentative)	315, 400, **427**, 454	536
12	13-*cis* β-carotene	339, 421, **446**, 473	536
13	all-*trans* β-carotene	425, **450**, 479	536
14	9-*cis* β-carotene	342, 423, **447**, 474	536

[a] Peak numbers correspond to Figure 4. See Experimental Procedures for HPLC and LC−MS methods. [b] Absolute maxima are listed in bold.

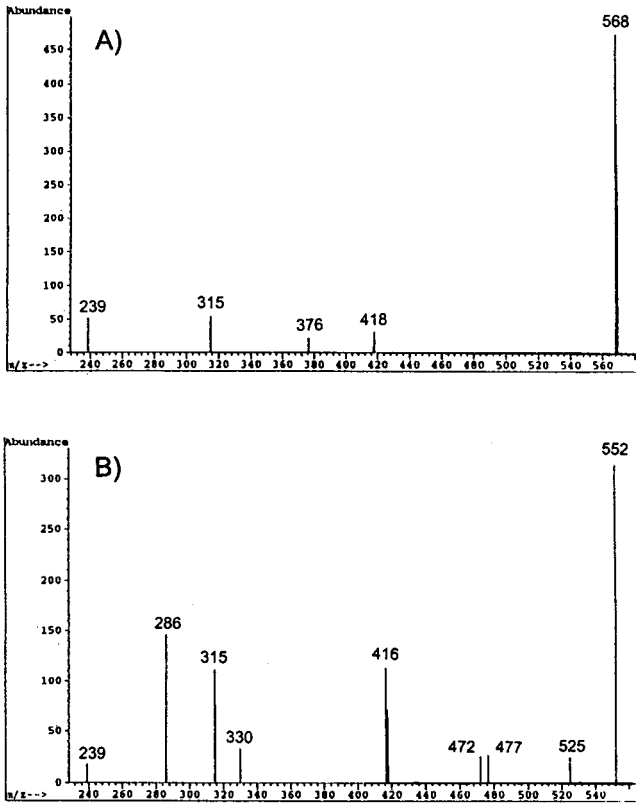

Figure 5. Positive ion electrospray mass spectrum of (A) β-carotene 5,8-endoperoxide and (B) β-carotene 5,8-epoxide

identified as oxidation products (Figure 5). β-Carotene 5,8-endoperoxide has been previously reported as a singlet oxygen (Stratton et al., 1993) and thermal (Marty and Berset, 1988; 1990) oxidation product of β-carotene. The electrospray mass spectra also indicated the presence of a compound with a molecular ion of *m/z* 584. This is an indication that β-carotene combined with an ozone molecule to produce a compound with the empirical formula of $C_{40}H_{56}O_3$. The exact positioning of the oxygen atoms is unclear because more sophisticated techniques such as tandem mass spectrometry and nuclear magnetic resonance (NMR) will be needed for more definitive structure elucidation. Marty and Berset (1988) found a similar compound in an oxidized β-carotene mix and suggested β-carotene-3-ol-5,8,5′,8′-diepoxide and β-carotene-4-ol-5,8,5′8′-diepoxide as possible products.

In summary, there is a marked difference in the degradation kinetics of carotenoids during ozone and oxygen exposures employing the model system used in this study. As anticipated, ozone caused greater degradation than oxygen, because of its reactivity toward highly unsaturated carotenoids. Lycopene is the most susceptible to degradation, followed by β-cryptoxanthin, all-*trans* β-carotene, and 9-*cis* β-carotene. These results confirm that carotenoid stability toward oxidation is a function of both the number of conjugated double bonds and the presence of functional groups. The similarity

between oxygen- and ozone-derived degradation products of β-carotene may indicate that their reaction mechanisms resemble each other. The data generated in this study are representative for the novel model system used: degradation of carotenoids in an aqueous environment while absorbed to a stationary phase. Further research is necessary to determine the application of this model system to carotenoid-containing foods such as fruits and vegetables.

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