Fast Atom Bombardment Tandem Mass Spectrometry of Carotenoids

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Positive ion fast atom bombardment (FAB) tandem mass spectrometry (MS–MS) using a double-focusing mass spectrometer with linked scanning at constant B/E and high-energy collisionally activated dissociation (CAD) was used to differentiate 17 different carotenoids, including β-apo-8′-carotenal, astaxanthin, α-carotene, β-carotene, γ-carotene, ζ-carotene, canthaxanthin, β-cryptoxanthin, isozeaxanthin bis(pelargonate), neoxanthin, neurosporene, nonaprene, lutein, lycopene, phytoene, phytofluene, and zeaxanthin. The carotenoids were either synthetic or isolated from plant tissues. The use of FAB ionization minimized degradation or rearrangement of the carotenoid structures due to the inherent thermal instability generally ascribed to these compounds. Instead of protonated molecules, both polar xanthophylls and nonpolar carotenes formed molecular ions, $M^{+\cdot}$, during FAB ionization. Following collisionally activated dissociation, fragment ions of selected molecular ion precursors showed structural features indicative of the presence of hydroxyl groups, ring systems, ester groups, and aldehyde groups and the extent of aliphatic polyene conjugation. The fragmentation patterns observed in the mass spectra herein may be used as a reference for the structural determination of carotenoids isolated from plant and animal tissues.

Keywords: *Carotenoids; mass spectrometry; fast atom bombardment; tandem mass spectrometry; MS–MS; β-apo-8′-carotenal; astaxanthin; α-carotene; β-carotene; γ-carotene; ζ-carotene; canthaxanthin; β-cryptoxanthin; isozeaxanthin bis(pelargonate); lutein; lycopene; neurosporene; nonaprene; neoxanthin; phytoene; phytofluene; zeaxanthin*

INTRODUCTION

Hydrocarbon carotenes and oxygenated xanthophylls comprise the highly conjugated class of pigments known as carotenoids (Isler, 1971). Many key biological roles are ascribed to various carotenoids in both the plant (Goodwin, 1980) and animal kingdoms (Goodwin, 1984). In addition, a growing body of evidence acquired over the past decade suggests that carotenoids may function to prevent and/or attenuate certain deleterious human health conditions (Olson, 1992).

More than 500 individual carotenoid structures occurring in nature have been characterized (Straub, 1987), and complex mixtures are often present in biological tissues. High-performance liquid chromatography (HPLC) coupled with photodiode array detection is currently the preferred method for determining the identity of carotenoids present in biological tissues (Taylor et al., 1989). However, unequivocal determination of carotenoid structures using this method is often difficult due to confounding factors associated with the analysis of certain biological tissues (van Breemen, 1993). Therefore, a powerful complementary analytical technique such as mass spectrometry should be employed to provide confirmation of the carotenoid identity (Davies, 1976).

Several ionization methods have been reported for the mass spectrometric analysis of carotenoids including electron impact (Vetter et al., 1971; Moss and Weedon, 1976), negative ion chemical ionization (Lusby et al., 1992), and fast atom bombardment (FAB) (Caccamese and Garozzo, 1990). Electron impact and chemical ionization require sample vaporization prior to ionization, which is a disadvantage in the analysis of the thermally labile and nonvolatile carotenoids (Vetter et al., 1971). FAB mass spectrometry has become a standard method for the analysis of thermally labile and nonvolatile compounds (Fenselau and Cotter, 1987) and is therefore an appropriate technique for carotenoid analysis. We have previously reported the successful application of MS–MS analysis using FAB ionization for the identification of carotenoids as well as the first application of continuous-flow FAB LC–MS (Schmitz et al., 1992) and continuous-flow FAB LC–MS–MS (van Breemen et al., 1993) to carotenoid analysis. Now we report an extensive investigation using FAB MS–MS to differentiate individual carotenoids based on characteristic features of their chemical structure.

REAGENTS

The trivial names of individual carotenoids are used throughout this paper and are accompanied at first mention by their semisystematic names as recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (Straub, 1987). β-Apo-8′-carotenal (8′-apo-β-caroten-8′-al), astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), canthaxanthin (β,β-carotene-4,4′-dione), β-cryptoxanthin [(3*R*)-β,β-caroten-3-ol], and β-carotene (β,β-carotene) were obtained from Hoffman-La Roche (Nutley, NJ). α-Carotene [(6′*R*)-β,ε-carotene) was purchased from Sigma Chemical Co. (St. Louis, MO). Nonaprene (nonapreno-β-carotene) and isozeaxanthin bis(pelargonate) (β,β-carotene-4,4′-diol dipelargonate) were synthesized and kindly provided by Dr. Fred Khachik of the U.S. Department of Agriculture.

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Lutein [(3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol] and neoxanthin [(3*S*,5*R*,6*R*,3′*S*,5′*R*,6′*S*)-5′,6′-epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol] were isolated from spinach (Khachik et al., 1986) by first isolating the more polar xanthophyll fraction from the acetone extract using open column chromatography on alumina (Davies, 1976; Britton, 1985), followed by purification (twice) using semipreparative reversed-phase HPLC. The HPLC column (10 mm × 25 cm) was packed with polymerically bonded C_{18} on 5 μm silica particles (Vydac, Hesperia, CA), and the mobile phase was composed of methanol/acetonitrile/tetrahydrofuran/water (85:9:1:5 v/v/v/v). The HPLC column flow rate was 1.0 mL/min.

γ-Carotene (β,ψ-carotene) and neurosporene (7,8-dihydro-ψ,ψ-carotene) were isolated from oncum, an Indonesian soybean fermentation product, which is produced by the action of a mold from the genus *Neurospora*. Oncum was extracted using dichloromethane and subsequently chromatographed on alumina. The respective fractions containing either neurosporene or γ-carotene were then chromatographed on the semipreparative HPLC column used above with a mobile phase of methanol/tetrahydrofuran (90:10 v/v) at a flow rate of 1.5 mL/min. Final purification of neurosporene and γ-carotene was carried out twice using HPLC with a calcium hydroxide column (4.6 mm × 25 cm) and an isocratic mobile phase (0.7 mL/min) consisting of 10% acetone in hexane (v/v) for neurosporene or of 5% acetone in hexane (v/v) for γ-carotene.

Phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene), phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene), and ζ-carotene (7,8,7′,8′-tetrahydro-ψ,ψ-carotene) were isolated from a hexane/acetone (50:50 v/v) extract of fresh carrot tissue using open column chromatography on alumina followed by HPLC using a calcium hydroxide column. The HPLC mobile phase employed for isolation of ζ-carotene was 4% acetone in hexane (v/v) and 0.1% acetone in hexane (v/v) for both phytofluene and phytoene. Lycopene was isolated from tomatoes by using open column chromatography on alumina (Simpson et al., 1985).

The purity of each carotenoid used in this study was confirmed either spectrophotometrically using a Shimadzu UV 240 recording spectrophotometer (Kyoto, Japan) or by HPLC analysis using photodiode array detection. The HPLC system used during this study consisted of a Waters (Milford, MA) Model 510 pump, a Model U6K injector, and a Model 990 photodiode array detector equipped with an APC IV series computer (NEC Information Systems Inc., Boxborough, MA).

APPARATUS AND PROCEDURE

The calcium hydroxide column was packed in-house using calcium hydroxide (Aldrich Chemical Co., Milwaukee, WI) sieved through 500 mesh and equilibrated to 44% relative humidity in a K_2CO_3 saturation chamber for 48 h. Calcium hydroxide (6 g) was suspended in 50 mL of 0.5% acetone in hexane and sonicated for 10 min. Slurry packing of the column was then carried out using a column packing reservoir (Scientific Systems, Corp., Baton Rouge, LA) pressurized to 2600 psi using a Haskel air-driven fluid pump (Haskel, Inc., Burbank, CA). Approximately 100 mL of packing solvent was allowed to percolate through the column while under packing pressure.

Positive ion FAB mass spectra were obtained using a JEOL (Tokyo, Japan) JMS-SX102 double-focusing mass spectrometer. The ionization source was maintained at room temperature. Xenon fast atoms at 6 kV were used for FAB ionization. The accelerating voltage was 10 keV, and the instrument resolution was 1000 for all measurements. The range *m/z* 1–1000 was scanned over approximately 10 s. Approximately 1 μg of each carotenoid was mixed with 1–2 μL of the FAB matrix, 3-nitrobenzyl alcohol, on the FAB sample stage per analysis.

MS–MS analyses were carried out using linked scanning at constant B/E and collisionally activated dissociation (CAD). Fragmentation of the precursor ion was enhanced by CAD using helium gas in the first field-free region of the double-focusing mass spectrometer. The helium gas pressure was adjusted so that the abundance of the selected ion precursor was attenuated 70%. Five successive linked scans at constant B/E were signal averaged to produce each MS–MS spectrum.

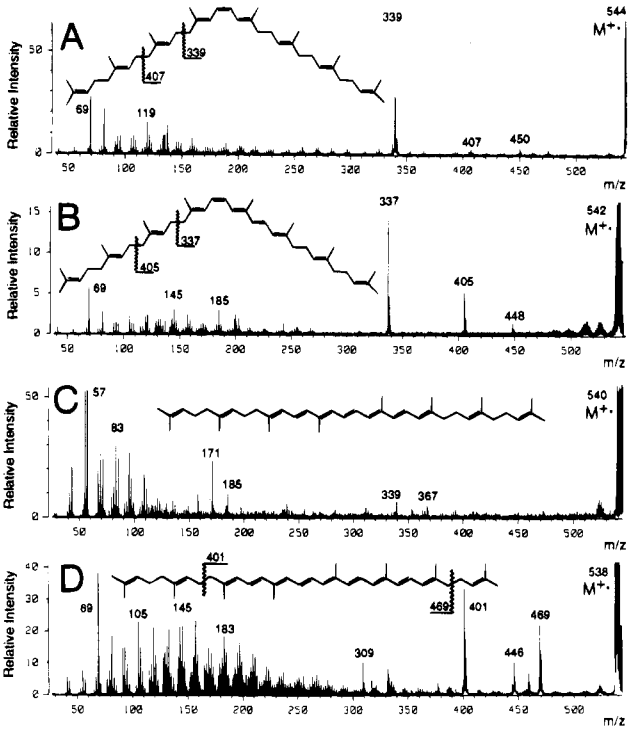

Figure 1. Positive ion MS–MS spectra obtained using FAB ionization, collisionally activated dissociation (CAD) of molecular ions, and linked scanning at constant B/E of the fragment ions for the lycopene metabolic precursors (A) phytoene, (B) phytofluene, (C) ζ-carotene, and (D) neurosporene.

RESULTS AND DISCUSSION

FAB ionization produced abundant molecular ions of both nonpolar carotenes and polar xanthophylls with minimal fragmentation and no detectable thermal decomposition. Although protonated molecules are typically produced during FAB ionization, radical cations, $M^{\cdot+}$, were exclusively formed using the FAB matrix, 3-nitrobenzyl alcohol, probably because of the ease of oxidation of the highly conjugated carotenoids. To obtain structurally significant fragment ions that could be used to distinguish each carotenoid, collisionally activated dissociation was carried out to promote fragmentation and the MS–MS technique of linked scanning at constant B/E was used to separate and record the fragment ions. FAB MS–MS spectra of carotenoids analyzed in this study are shown in Figures 1–4. The interpretation and discussion of these mass spectra appear in the following two sections.

Carotenes. During plant biosynthesis, the terpenoid carotenes are synthesized from isopentenyl pyrophosphate and sequentially unsaturated to form phytoene, phytofluene, ζ-carotene, neurosporene, and then lycopene (Goodwin, 1980). Subsequently, cyclization and isomerization reactions lead to the formation of γ-carotene, α-carotene, or β-carotene. The MS–MS spectra of these compounds and the synthetic β-carotene analog, nonaprene, are discussed in sequence below.

Phytoene. The most abundant fragment ion in the MS–MS spectrum of the phytoene molecular ion at *m/z* 544 was detected at *m/z* 339 and corresponded to cleavage of one of the two sp^3–sp^3 bonds allylic to the conjugated triene (see structure and mass spectrum in Figure 1A). The formation of this fragment ion, [M

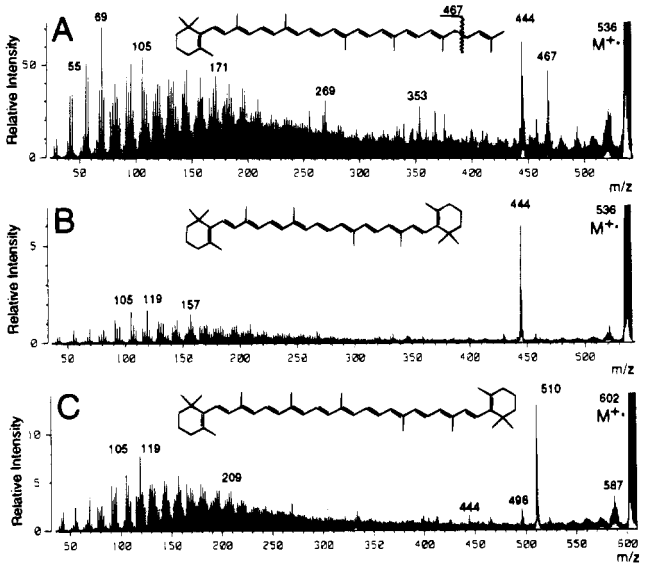

Figure 2. Positive ion FAB MS–MS spectra (linked scanning at constant B/E with CAD) of the carotenes (A) γ-carotene, (B) β-carotene, and (C) nonaprene.

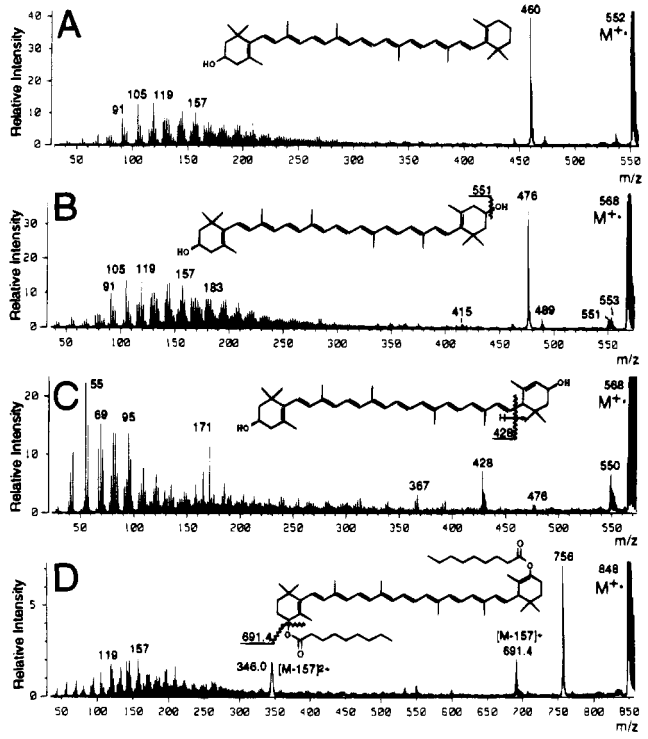

Figure 3. FAB MS–MS mass spectra (linked scanning at constant B/E with CAD) of the alcohol- and ester-containing xanthophylls (A) β-cryptoxanthin, (B) zeaxanthin, (C) lutein, and (D) isozeaxanthin bis(pelargonate).

$-205]^+$, was favored because of the incomplete conjugation of the carbon skeleton of these compounds. Although elimination of a neutral molecule of toluene, $[M - 92]^+$, was commonly observed in lycopene, β-carotene, and other highly conjugated compounds, $[M - 94]^+$ was observed at *m/z* 450 in the tandem mass spectrum of phytoene. This unusual ion, which might have been formed by elimination of a heptadiene neutral or loss of toluene plus a molecule of hydrogen, was also indicative of incomplete conjugation of the carotenoid.

Phytofluene. Similar in structure to phytoene, phytofluene contains one additional carbon–carbon double bond, and therefore the molecular ion was detected two units lower at *m/z* 542 (Figure 1B). As in the tandem

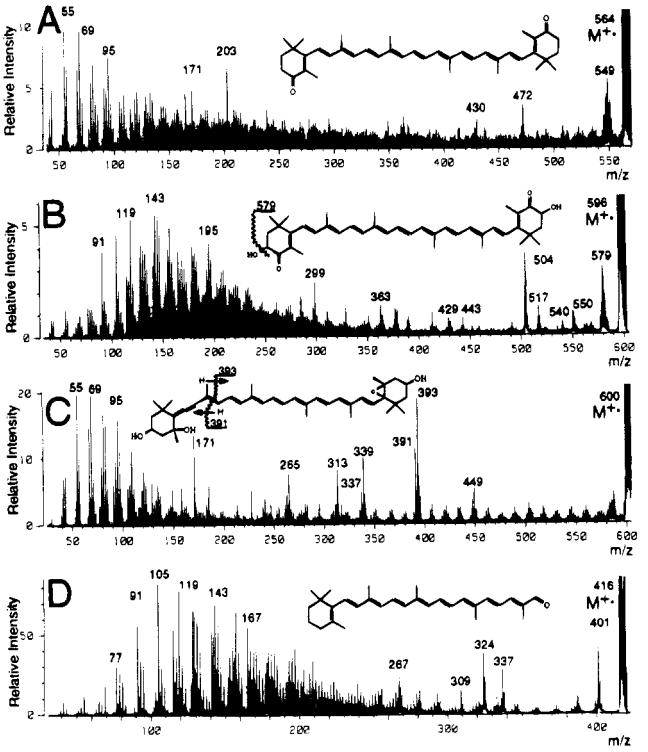

Figure 4. FAB MS–MS mass spectra (linked scanning at constant B/E with CAD) of the xanthophylls (A) cantha-xanthin, (B) astaxanthin, (C) neoxanthin, and (D) β-apo-8′-carotenal.

mass spectrum of phytoene, $[M - 205]^+$ was the most abundant fragment ion and was detected at *m/z* 337. Instead of loss of toluene, $[M - 94]^+$ was observed at *m/z* 448. Both of these fragment ions indicate that the extent of conjugation in phytofluene is much less than in lycopene or β-carotene. Another unusual feature of the tandem mass spectrum of phytofluene (Figure 1B) was the abundant ion at *m/z* 405, formed by loss of a terminal $C_{10}H_{17}$, $[M - 137]^+$. An analogous but much less abundant fragment ion was observed at *m/z* 407 in the tandem mass spectrum of phytoene.

ζ-Carotene. Except for abundant fragment ions below *m/z* 200, almost no significant fragmentation was observed in the MS–MS spectrum of ζ-carotene (Figure 1C). The most abundant fragment ions were detected at *m/z* 55 and 57. It should be noted that due to very small sample size, any fragment ions of low abundance, i.e., <5% relative to the ions at *m/z* 55 and 57, would not have been detected in this analysis due to the background noise level. Ions of approximately 10% abundance (relative to *m/z* 55 and 57) were detected at *m/z* 339 and 367, but their structures were not determined.

Neurosporene. The immediate metabolic precursor to lycopene, neurosporene, differs from lycopene by one saturated double bond (see structure in Figure 1D). Similarly, neurosporene contains one more carbon–carbon double bond than its immediate precursor, ζ-carotene. Like ζ-carotene, the most abundant fragment ions were detected below *m/z* 200 (Figure 1D). However, fragmentation patterns similar to lycopene were also detected, including $[M - 69]^+$ at *m/z* 469 and $[M - 92]^+$. The abundant ion at *m/z* 401, $[M - 137]^+$, corresponded to cleavage of the sp^3–sp^3 bond between carbons 8 and 9 and was not likely to form from lycopene because of unsaturation at this site.

Lycopene. Caccamese and Garozzo (1990) reported

that $[M - 92]^+$ at *m/z* 444 and $[M - 69]^+$ at *m/z* 467 were the only fragment ions observed in the MS–MS spectrum of the molecular ion of lycopene and that the fragment ion at *m/z* 444 was more abundant than the ion at *m/z* 467. This is in contrast to previous reports in which MS–MS spectra of lycopene were obtained during LC–MS–MS (van Breemen et al., 1993) as well as using a direct insertion probe following chromatographic purification (Schmitz et al., 1992). The lycopene mass spectra obtained during these previous studies and the present investigation [data not shown; see van Breemen et al. (1993)], showed that the lycopene ion at *m/z* 467 is the most abundant fragment ion. Because β-carotene fragments during collisionally activated dissociation to form ions at *m/z* 444 but not at *m/z* 467 (Figure 2B), contamination of the lycopene sample by β-carotene would increase the abundance of the signal at *m/z* 444 relative to the *m/z* 467 ion. Another indication of contamination of the lycopene sample by β-carotene would be detection of the β-carotene fragment ion at *m/z* 457. Conversely, detection of *m/z* 467 in the tandem mass spectrum of β-carotene would indicate contamination by lycopene. Corresponding to fragmentation of the polyene chain, a series of abundant fragment ions 14 u apart were detected below *m/z* 210 including *m/z* 91, 105, 119, 143, 157, 171, 185, and 209. Another series of ions was detected at *m/z* 131, 145, 169, and 183. [The mass spectra discussed in this section have been published elsewhere. See Caccamese and Garozzo (1990) and van Breemen et al. (1993).]

γ-Carotene. The molecular ion of γ-carotene at *m/z* 536 is isobaric with lycopene, β-carotene, and α-carotene. Structurally, γ-carotene contains one open-chain terminus similar to lycopene and one β-ionone ring like β-carotene (see structure and MS–MS spectrum in Figure 2A). Because of these structural features, the fragmentation patterns of γ-carotene show a resemblance to the fragment ions of both lycopene [see MS–MS spectrum in van Breemen et al. (1993)] and β-carotene (Figure 2B). However, γ-carotene may be distinguished from β-carotene by noting the presence of the ion at *m/z* 467 in addition to the most abundant fragment ion at *m/z* 444 and from lycopene by observing that the fragment ion at *m/z* 444 is more abundant than the ion at *m/z* 467 (see Figure 2). The ion at *m/z* 444 corresponded to loss of a molecule of toluene from the molecular ion, and the fragment ion, $[M - 69]^+$ at *m/z* 467, was formed by elimination of the terminal isoprenyl radical (Figure 2A).

β-Carotene. As reported by Caccamese and Garozzo (1990) and Taylor et al. (1990), the most abundant fragment ion in the tandem mass spectrum of β-carotene was observed at *m/z* 444 and corresponds to elimination of a neutral molecule of toluene (see Figure 2B). The next most significant fragment ions included *m/z* 55, 69, 91, 105, 119, 143, and 157, which showed abundances of approximately 11–28% relative to the ion at *m/z* 444 (Figure 2B). Formed by fragmentation of conjugated carbon–carbon double bonds, these low molecular weight fragment ions are common to all of the carotenoids investigated but have not been reported in the tandem mass spectra obtained by other investigators. In the tandem mass spectra of partially saturated carotenes, such as phytoene and phytofluene, low molecular weight fragment ions showed an uneven distribution (compared to β-carotene), suggesting that charge-remote fragmentation might be one of the mechanisms responsible for the formation of these ions. Charge-remote fragmentation proceeds by cleavage of an alkyl chain at an uncharged site with elimination of hydrogen and formation of an ion containing a terminal alkene (Jensen et al., 1985). In the studies of Jensen et al. (1985), interruptions in the pattern of charge-remote fragmention were used to identify locations of double bonds. However, in the tandem mass spectra of carotenoids, interruptions in the pattern of low mass fragment ions indicate saturated carbon–carbon bonds located within a polyene chain instead of double bonds within an alkyl chain.

No fragmentation of the terminal rings was observed. However, a fragment ion was detected in low abundance at *m/z* 399, $[M - 137]^+$, which corresponded to elimination of a terminal ring plus a methylene group. Loss of a methyl group was observed at *m/z* 521. Another fragment ion of low abundance was observed at *m/z* 429 and was probably the result of elimination of both toluene and a methyl group. Finally, a fragment ion was observed in low abundance at *m/z* 489, $[M - 79]^+$, which was probably the result of elimination from the extended polyene chain.

α-Carotene. Like β-carotene and γ-carotene, the most abundant fragment ion in the MS–MS spectrum of α-carotene corresponded to the elimination of a molecule of toluene to produce the ion at *m/z* 444. [Data not shown. See MS–MS spectrum in van Breemen et al. (1993) or Schmitz et al. (1992).] Other similarities to the tandem mass spectrum of β-carotene included an abundant series of fragment ions below approximately *m/z* 250 and the loss of a methyl group from the molecular ion, $[M - 15]^+$ at *m/z* 521. However, the fragment ions at *m/z* 480 and 388, corresponding to $[M - 56]^+$ and $[M - 148]^+$, uniquely identify this compound as α-carotene instead of its isomers β-carotene, lycopene, or γ-carotene. The ion $[M - 56]^+$, was formed by retro-Diels–Alder fragmentation (Caccamese and Garozzo, 1990), and $[M\text{-}148]^+$ is probably the result of both retro-Diels–Alder fragmentation and elimination of toluene, $[M - 56 - 92]^+$.

Nonaprene. A synthetic C_{45} analog of β-carotene, nonaprene contains an additional isoprene unit that lengthens the conjugated polyene system by two double bonds (see structure in Figure 2C). The terminal rings are identical to β-carotene (Figure 2B). Like β-carotene, the linked scan at constant B/E of the molecular ion of nonaprene shows predominantly loss of toluene from the polyene chain and no ring fragmentation (Figure 2C). The series of ions below *m/z* 200 is similar to that of β-carotene. The linked scan of nonaprene showed one unexpected fragment ion at *m/z* 444, which corresponded to loss of toluene plus one isoprene unit from the molecular ion, $[M - 158]^+$.

Xanthophylls. Carotenoids containing oxygen belong to the group called xanthophylls. Included in the set of xanthophyll MS–MS spectra discussed below are alcohols, ketones, a diester, an aldehyde, and multifunctional compounds containing combinations of alcohol, ketone, and epoxide moieties. The compounds are discussed in order of increasing complexity beginning with the alcohol β-cryptoxanthin, and the aldehyde β-apo-8′-carotenal is discussed last.

β-Cryptoxanthin. The structure of β-cryptoxanthin is identical to that of β-carotene except that one of the β-ionone rings is hydroxylated (see structure in Figure 3A). The MS–MS fragmentation pattern of β-cryptoxanthin is similar to the tandem mass spectrum of β-carotene (Figure 3A). For example, the most abun-

dant fragment ion at *m/z* 460 was formed by loss of toluene, $[M - 92]^+$, as in the tandem mass spectrum of β-carotene. The only other abundant fragment ions were observed below *m/z* 200. Some fragment ions of very low relative abundance were observed at *m/z* 535, 537, and 445, indicating loss of either a hydroxyl radical, a methyl radical, or toluene and a methyl radical from the molecular ion.

Zeaxanthin. Zeaxanthin contains a symmetrical carbon skeleton similar to that of β-carotene and β-cryptoxanthin but differs from these two carotenoids in that both β-ionone rings are hydroxylated (see structure in Figure 3B). Like β-carotene, loss of toluene, $[M - 92]^+$, gave rise to the most abundant fragment ion in the MS–MS spectrum of zeaxanthin as previously reported by Caccamese and Garozzo (1990) (Figure 3B). However, we report for the first time numerous other fragment ions produced by high-energy collisionally activated dissociation followed by linked scanning at constant B/E of the molecular ion of zeaxanthin. For example, a series of abundant fragment ions were detected below *m/z* 200, which corresponded to cleavage within the conjugated double-bond system. Of much lower abundance, additional fragment ions were detected at *m/z* 553 and 551, corresponding to loss of methyl or hydroxyl radicals from the molecular ion. The observation of an ion at 551, $[M - 17]^+$, suggests that the carotenoid contains a hydroxyl functional group. This type of fragment ion was not observed in the carotene mass spectra. Like $[M - 92]^+$, the ion at *m/z* 489, $[M - 79]^+$, is the result of elimination from an extended polyene chain. The ion at *m/z* 415, $[M - 153]^+$, corresponds to elimination of a hydroxylated ring plus an additional methylene group and is analogous to the $[M - 137]^+$ ion in the linked scan at constant B/E of β-carotene.

Lutein. Isomeric with zeaxanthin, lutein differs from zeaxanthin only by the position of a carbon–carbon double bond within one of its terminal rings, which is not in conjugation with the other double bonds. Therefore, the carbon skeleton of lutein resembles the asymmetrical α-carotene (see structure in Figure 3C). As a result, loss of toluene, $[M - 92]^+$, at *m/z* 476 was less abundant in the tandem mass spectrum of lutein compared to zeaxanthin. Also, a unique lutein fragment ion was observed at *m/z* 428 corresponding to loss of the terminal ring plus a proton. Because the fragment ion at *m/z* 428 was not observed in the zeaxanthin tandem mass spectrum, the lutein ring that was eliminated probably contained the unconjugated carbon–carbon double bond. Observation of the fragment ion at *m/z* 428 can be used to distinguish lutein from zeaxanthin.

Isozeaxanthin Bis(pelargonate). Like zeaxanthin, the molecular ion of isozeaxanthin bis(pelargonate) fragmented during collisionally activated dissociation to eliminate a neutral molecule of toluene, $[M - 92]^+$, as the most abundant fragment ion (Figure 3D). Other similarities include an abundant series of fragment ions below approximately *m/z* 250 and an ion, $[M - 15]^+$, in low abundance. Unlike zeaxanthin, no loss of $[M - 17]^+$ was observed, because the hydroxyl groups were esterified (see structure in Figure 3D). Ester fragmentation produced two ions which distinguished isozeaxanthin bis(pelargonate) from all other carotenoids investigated, $[M - 157]^+$ and $[M - 157]^{2+}$. Cleavage of the bond between the ester oxygen and the isozeaxanthin ring produced an ion at *m/z* 691.4 (or *m/z* 346.0 for the doubly charged ion) and a neutral free radical, $C_9H_{17}OO^{\cdot}$. Additional evidence in support of the observation of the doubly charged ion at *m/z* 346 was provided by the 0.5 u spacing of the isotope cluster. Ester cleavage was the second most abundant fragmentation pathway.

Canthaxanthin. The MS–MS spectrum of canthaxanthin produced abundant fragment ions below *m/z* 200 (see Figure 4A). Perhaps the electron withdrawing carbonyl groups (see structure in Figure 4A) reduced the π electron density of the carbon skeleton and thereby increased the likelihood of fragmentation of the conjugated polyene system. Except for loss of toluene forming the ion detected at *m/z* 472, few other fragment ions were observed above *m/z* 200. Although their structures could not be determined, two ions of low abundance were detected at *m/z* 430 and 363 corresponding to $[M - 134]^+$ and $[M - 101]^+$.

Astaxanthin. In addition to the usual elimination of neutral toluene from the molecular ion, $[M - 92]^+$, and a series of abundant low molecular weight fragment ions, extensive fragmentation of the terminal rings was observed in the tandem mass spectrum of the molecular ion of astaxanthin (see structure and MS–MS spectrum in Figure 4B). Elimination of neutral molecules of C_2H_4O or C_4H_8 from the ring produced ions at *m/z* 550 or 540, respectively. Loss of hydroxyl radical, $[M - 17]^+$, was among the most abundant fragment ions of this type observed in any of the carotenoid MS–MS spectra and is also indicative of the presence of hydroxyl functional groups. Because ring fragmentation was absent in the mass spectra of β-carotene and related compounds, these ring fragment ions are indicative of the oxygenated structure of the astaxanthin rings. In addition, elimination of a terminal ring and elimination of a ring plus a methylene group was observed to form ions at *m/z* 443 and 429, respectively. The molecular weight of these neutral fragments is also indicative of the oxygenated structure of the rings.

Neoxanthin. Containing an allene (see structure in Figure 4C), the tandem mass spectrum of neoxanthin contained several unique fragment ions. For example, cleavage of the double bond allylic to the allene produced abundant fragment ions at *m/z* 393 and 391, accompanied by a hydrogen transfer (Figure 4C). Alternatively, one or both of these ions might be the result of loss of the ring from the other end of the molecule, which contained an epoxide. Another unusually abundant fragment ion was formed by cleavage at one of two sites near the center of the molecule, $[M - 287]^+$, to form an ion at *m/z* 313. No loss of toluene was observed. Although the structures were unknown, other abundant ions were observed at *m/z* 449 (26.3%), 339 (50.4%), and 337 (24.4%) (abundances are expressed relative to the fragment ion at *m/z* 69).

β-Apo-8′-carotenal. Like most of the carotenoids containing either ketones, aldehydes, or an acyclic terminus, the most abundant fragment ions in the MS–MS spectrum of β-apo-8′-carotenal were in the low mass range, *m/z* 55–157 (see structure and mass spectrum in Figure 4D). These low mass ions were formed by cleavage within the conjugated carbon–carbon double bond system with transfer of a hydrogen to the neutral fragment. Although abundant, the fragment ions in the range *m/z* 55–157 were not mentioned by Caccamese and Garozzo (1990) when they reported the linked scan at constant B/E of this carotenoid. Elimination of toluene, loss of a methyl group, and combined elimination of toluene and a methyl group from the molecular ion were also significant fragmentation pathways. Other fragment ions at *m/z* 279, 267, 333, 347, and 360 were

formed by fragmentation of the conjugated polyene chain with a hydrogen transfer to the neutral fragment. The fragment ion at *m/z* 337, $[M - 79]^+$, was formed by loss of $^{\cdot}C_6H_7$ from the polyene chain (Caccamese and Garozzo, 1990).

Conclusions. Using positive ion FAB, molecular ions of nonpolar carotenes and polar xanthophylls were produced, fragmented using high-energy CAD, and the resulting fragment ions were recorded using the MS–MS technique of linked scanning at constant B/E. Although molecular weight data alone are useful in the identification of carotenoids, the fragmentation patterns contain structural information that may be used to further characterize each carotenoid and distinguish isomers. Characteristic fragment ions were identified that may be used to confirm the presence of functional groups such as alcohols, aldehydes, rings, and the extent of conjugation of the polyene chain. Although many carotenoid fragment ions are reported in this investigation for the first time, some of the fragment ions are identical to those reported using electron impact mass spectrometry such as $[M - 92]^{+\cdot}$ for β-carotene (and other carotenoids), $[M - 56]^{+\cdot}$ for α-carotene, $[M - 79]^+$ for zeaxanthin, $[M - 69]^+$ for lycopene, and $[M - 137]^+$ for phytoene (Moss and Weedon, 1976). However, pyrolysis products that form during electron impact ionization such as loss of xylene, $[M - 106]^{+\cdot}$, loss of water, $[M - 18]^{+\cdot}$, and $[M - 154]^{+\cdot}$ were not observed in the FAB tandem mass spectra. Furthermore, MS–MS reduces background noise and eliminates ions due to impurities in the sample, which may interfere with the interpretation of electron impact data. To date, none of these mass spectrometric techniques have been used to distinguish between geometrical isomers, such as *cis/trans* double-bond configurations. This set of carotenoid MS–MS spectra extends our previous studies and will serve as a reference for structural confirmation of carotenoids isolated from biological sources.

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LITERATURE CITED

Britton, G. General Carotenoid Methods. *Methods Enzymol.* **1985**, *111*, 113–149.

Caccamese, S.; Garozzo, D. Odd-electron Molecular Ion and Loss of Toluene in Fast Atom Bombardment Mass Spectra of Some Carotenoids. *Org. Mass Spectrom.* **1990**, *25*, 137–140.

Davies, B. H. Analytical Methods—Carotenoids. In *Chemistry and Biochemistry of Plant Pigments*; Goodwin, T. W., Ed.; Academic Press: London, 1976; Part 4, pp 38–165.

Fenselau, C.; Cotter, R. J. Chemical Aspects of Fast Atom Bombardment. *Chem. Rev.* **1987**, *87*, 501–512.

Goodwin, T. W. Functions of Carotenoids. In *The Biochemistry of the Carotenoids*, 2nd ed.; Chapman and Hall: New York, 1980; Vol. I, pp 77–95.

Goodwin, T. W. Mammals. In *The Biochemistry of the Carotenoids*, 2nd ed.; Chapman and Hall: New York, 1984; Vol. II, pp 173–195.

Isler, O. *Carotenoids*; Birkhauser Verlag: Basel, 1971.

Jensen, N. J.; Tomer, K. B.; Gross, M. L. Gas-Phase Ion Decompositions Occurring Remote to a Charge Site. *J. Am. Chem. Soc.* **1985**, *107*, 1863–1868.

Khachik, F.; Beecher, G. R.; Goli, M. B.; Lusby, W. R.; Smith, J. C., Jr. Separation and Identification of Carotenoids and Their Oxidation Products in the Extracts of Human Plasma. *Anal. Chem.* **1992**, *64*, 2111–2122.

Lusby, W. R.; Khachik; F., Beecher, G. R.; Lau, J. Gas-Phase Reaction Mass Spectrometric Analysis of Carotenoids. *Methods Enzymol.* **1992**, *213*, 111–129.

Moss, G. P.; Weedon, B. C. L. Chemistry of the Carotenoids. In *Chemistry and Biochemistry of Plant Pigments*, 2nd ed.; Goodwin, T. W., Ed.; Academic Press: New York, 1976; pp 149–224.

Olson, J. A. Carotenoids and Vitamin A: an Overview. In *Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications*; Ong, A. S. H., Packer, L., Eds.; Birkhauser Verlag: Basel, 1992; pp 178–192.

Schmitz, H. H.; van Breemen, R. B.; Schwartz, S. J. Applications of Fast Atom Bombardment Mass Spectrometry (FAB-MS) and Continuous-Flow FAB-MS to Carotenoid Analysis. *Methods Enzymol.* **1992**, *213*, 322–336.

Simpson, K. L.; Tsou, S. C. S.; Chichester, C. O. Carotenes. In *Methods of Vitamin Assay*; Augustin, J., Klein, B. P., Becker, D., Venugopal, P. B., Eds.; Wiley: New York, 1985; pp 185–220.

Straub, O. Lists of Natural Carotenoids. In *Key to Carotenoids*; Pfander, H., Ed.; Birkhauser Verlag: Basel, 1987.

Taylor, R. F.; Farrow, P. E.; Yelle, L. M.; Harris, J. C.; Marenchic, I. G. Advances in HPLC and HPLC-MS of Carotenoids and Retinoids. In *Carotenoids: Chemistry and Biology*; Krinsky, N. I., Mathews-Roth, M. M., Taylor, R. F., Eds.; Plenum Press: New York, 1990; pp 105–123.

van Breemen, R. B.; Schmitz, H. H.; Schwartz, S. J. Continuous-Flow Fast Atom Bombardment Liquid Chromatography/Mass Spectrometry of Carotenoids. *Anal. Chem.* **1993**, *65*, 965–969.

Vetter, W.; Englert, G.; Rigassi, N.; Schwieter, U. Spectroscopic Methods. In *Carotenoids*; Isler, O., Gutmann, H., Solms, V., Eds.; Birkhauser Verlag: Basel, 1971; pp 189–266.

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