International Journal of Mass Spectrometry 312 (2012) 163–172

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Atmospheric pressure chemical ionization tandem mass spectrometry of carotenoids

Richard B. van Breemen*, Linlin Dong, Natasa D. Pajkovic[1]

Department of Medicinal Chemistry and Pharmacognosy, University of Illinois College of Pharmacy, Chicago, IL 60612, United States

ARTICLE INFO

Article history:
Received 29 April 2011
Received in revised form 23 July 2011
Accepted 27 July 2011
Available online 4 August 2011

Keywords:
Carotenoid
Carotene
Xanthophyll
Lycopene
APCI
Tandem mass spectrometry

ABSTRACT

Carotenoids are natural pigments synthesized by plants and photosynthetic microorganisms, some of which, like β-carotene, are precursors of vitamin A, and others such as lutein and lycopene might function in the prevention of age-related macular degeneration and prostate cancer, respectively. Mass spectrometry provides high sensitivity and selectivity for the identification and quantitative analysis of carotenoids in biological samples, and previous studies have described how atmospheric pressure chemical ionization (APCI) offers distinct advantages over electrospray and fast atom bombardment for the analysis of specific carotenoids. Since APCI product ion tandem mass spectra have been reported for only a few carotenoids, a detailed investigation of twelve carotenes and xanthophylls was carried out using both positive ion and negative ion APCI tandem mass spectrometry with collision-induced dissociation. Using protonated molecules as precursor ions in positive ion mode and radical anions in negative ion mode, characteristic fragment ions were identified that may be used to distinguish between carotenoids.

1. Introduction

Carotenoids are polyisoprenoid compounds consisting of two groups, hydrocarbon carotenes and oxygenated derivatives called xanthophylls. The extended system of conjugated double bonds of carotenoids produce characteristic UV and visible absorption spectra (including yellow, orange and red) with absorption maxima in the range of 400–500 nm [1]. Synthesized by photosynthetic plants and microorganisms, more than 600 carotenoids have been identified [2].

In plants, carotenoids protect plants from photo-oxidative damage and transfer energy to chlorophyll in the process of photosynthesis [3]. In addition to these functions, approximately 50 carotenoids are precursors of vitamin A which is essential for vision, cellular differentiation and embryological development. It has been suggested that carotenoids might function as *in vivo* antioxidants, prevent age-related macular degeneration [4] and prevent various forms of cancer including prostate cancer [5].

The identification and quantitative analysis of carotenoids can be challenging due to their instability in the presence of light, heat and oxygen. In biological samples such as human serum and tissue, carotenoids are often present at low concentrations and in the presence of potentially interfering compounds. Since carotenoids are unstable at the high temperatures used for gas chromatography, high-performance liquid chromatography (HPLC) is usually used for their separation, and UV/vis absorbance and/or mass spectrometry is used for the detection and characterization [6]. In particular, the application of liquid chromatography–mass spectrometry (LC–MS) to the analysis of carotenoids offers the advantages of high sensitivity and superior selectivity that are essential for the identification and quantitation of carotenoids.

Among the ionization techniques compatible with LC–MS, the most successful for carotenoid studies have been electrospray [7] and atmospheric pressure chemical ionization (APCI) [8]. Although carotenes and xanthophylls form molecular ions or protonated molecules during positive ion electrospray, the hydrocarbon carotenes do not ionize when using negative ion electrospray. However, APCI forms abundant positively or negatively charged molecular ions or protonated and deprotonated molecules of both carotenes and xanthophylls.

Although mass spectrometry has been used for carotenoid analysis for many years, there have been few tandem mass spectrometric studies of these compounds. Previously, the most comprehensive tandem mass spectrometric analysis of carotenoids utilized fast atom bombardment (FAB) in studies of 17 carotenoids [9]. Using FAB, molecular ions of both xanthophylls and carotenes were detected in positive mode, but no carotenoid ions were detected during negative ion FAB mass spectrometry.

* Corresponding author at: Department of Medicinal Chemistry and Pharmacognosy, University of Illinois College of Pharmacy, 833 S. Wood St., Chicago, IL 60612, United States. Tel.: +1 312 996 9353; fax: +1 312 996 7107.
E-mail address: Breemen@uic.edu (R.B. van Breemen).
[1] Current address: Merck & Co., 770 Sumneytown Pike, West Point, PA 19486, United States.

1387-3806/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijms.2011.07.030

Since atmospheric pressure ionization techniques have replaced FAB for most mass spectrometric applications and since, among these ionization techniques, only APCI can form both positively charged and negatively charged molecular ion species of carotenoids, we carried out tandem mass spectrometric analysis of a series of twelve carotenoids using APCI. Product tandem mass spectra of protonated carotenoids obtained using APCI MS–MS produced fragment ions that were similar to the tandem mass spectra of radical cations observed previously using FAB, but there were some significant differences. Although negative ion tandem mass spectra of carotenoids obtained using APCI MS–MS exhibited some similar fragmentation, unique and complementary fragmentation pathways were observed that may be used for carotenoid characterization, identification and measurement.

2. Materials and methods

2.1. Materials

HPLC-grade methanol, acetonitrile and hexane were purchased from Fisher Scientific (Fair Lawn, NJ). Lycopene (ψ,ψ-carotene), α-carotene ((6′R)-β,ε-carotene) and β-carotene (β,β-carotene) were purchased from Sigma–Aldrich (St. Louis, MO). [$^{13}C_6$]-β-Carotene was a gift from Johan Lugtenburg of Leiden University (Leiden, The Netherlands). Astaxanthin ((3S,3′S)-3,3′-dihydroxy-β,β-carotene-4,4′-dione), β-cryptoxanthin ((3R)-β,β-carotene-3-ol), zeaxanthin ((3R,3′R,6′R)-β,β-carotene-3,3′-diol), capsanthin ((3*R*,3′*S*,5′*R*)-3,3′-dihydroxy-β,κ-caroten-6′-one), lutein ((3R,3′R,6′R)-β,ε-carotene-3,3′-diol), and helenien ((3*R*,3′*R*)-β,β-carotene-3,3′-diol-dipalmitate) were purchased from ChromaDex (Santa Ana, CA). Echinenone was purchased from CaroteNature (Lupsingen, Switzerland), and β-apo-8′-carotenal (8′-apo-β-caroten-8′-al) was purchased from Hoffman-La Roche (Nutley, NJ). γ-Carotene was isolated from a tomato oleoresin (LycoRed, Beer-Sheva, Israel) using reversed phase HPLC as described previously [9]. Chemical structures of these carotenoids are shown in Fig. 1.

2.2. Methods

Positive and negative ion APCI mass spectra and product ion tandem mass spectra were obtained using a Thermo (San Jose, CA) Quantum triple quadrupole mass spectrometer, and accurate mass measurements of product ions were confirmed using a Waters (Manchester, UK) Q-TOF-2 or QTOF Synapt quadrupole time-of-flight hybrid mass spectrometer. Stock solutions of 100 μM were prepared under dim light by dissolving 1 mg of each carotene or xanthophyll in hexane or methanol, respectively. Each stock solution was diluted to a concentration of approximately 2 μM using methanol prior to positive ion APCI or acetonitrile prior to negative ion APCI. Carotenoid solutions were introduced into the mass spectrometer by flow-injection or direct infusion at a solvent flow rate of 200–250 μL/min. The ion source parameters were optimized for each carotenoid using positive ion or negative ion APCI.

For the experiments carried out on the Q-TOF-2 quadrupole time-of-flight hybrid mass spectrometer, the optimum corona current was between 3–5 μA during positive ion APCI and 4–6 μA during negative ion APCI. The cone voltage was optimized to

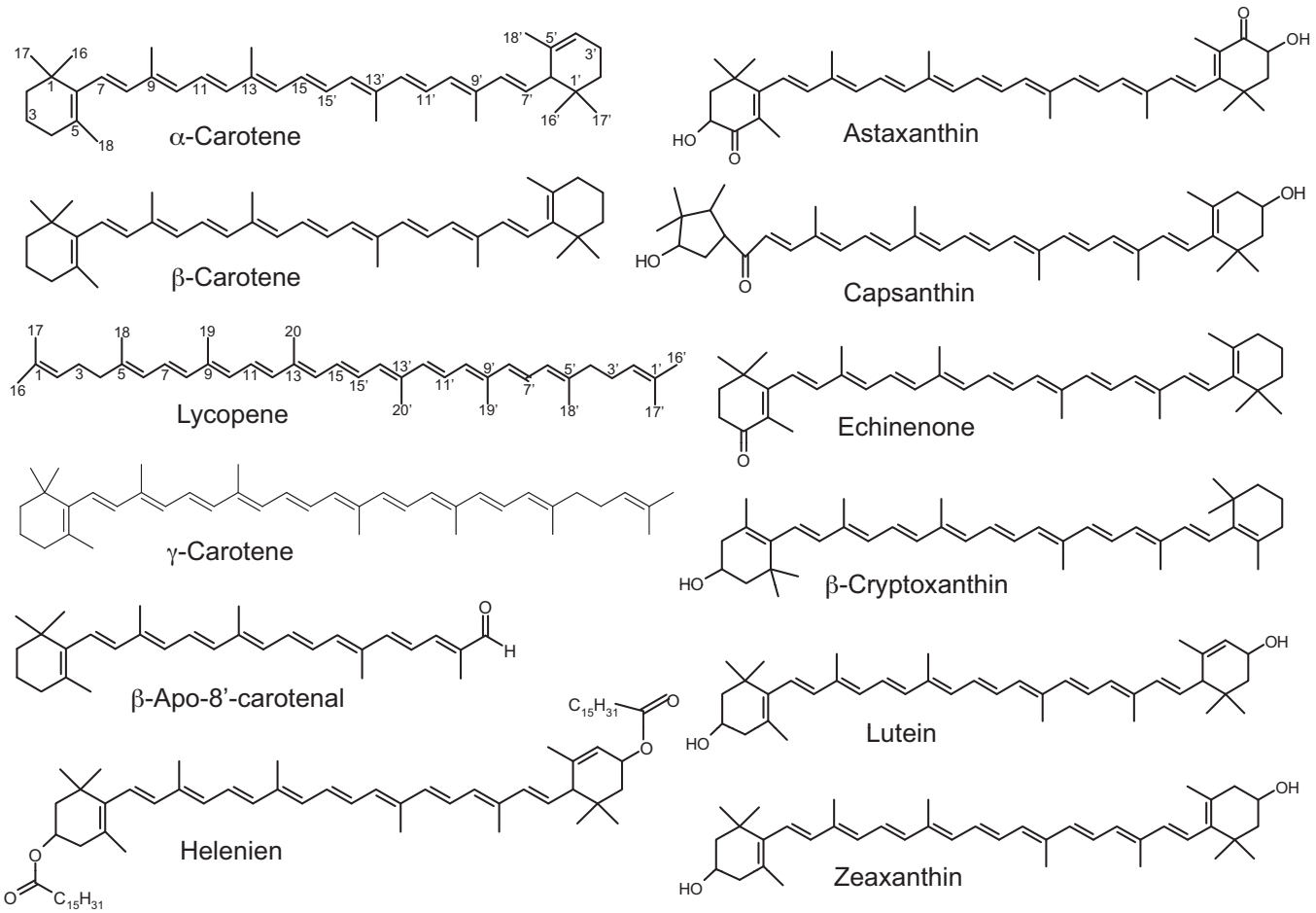

Fig. 1. Carotenoid chemical structures.

30–40 V. Argon was used as the collision gas and the collision-induced dissociation energies were 15–40 eV. The instrumental operating parameters for the TSQ Quantum triple quadrupole were as follows: discharge current 5–8 μA for positive and 8–10 μA for negative ion APCI; APCI vaporizer temperature 350–400 °C; and capillary temperature 300 °C. Positive and negative ion APCI mass spectra were recorded followed by the corresponding product ion tandem mass spectra of the protonated molecules or the molecular anions, respectively.

3. Results

3.1. APCI MS

Protonated molecules were detected as the base peaks in the positive ion APCI mass spectra of all of the carotenes and xanthophylls except for those of lutein and helenien, in which the base peaks corresponded to the loss of water, $[MH-18]^+$, or palmitic acid, $[MH-C_{16}H_{31}COOH]^+$, respectively. When using positive ion APCI, molecular ions and/or protonated molecules may be observed depending on the solvent composition [8], and the use of protic solvents such as methanol as in this investigation favored the formation of protonated carotenoids. In the negative ion APCI mass spectra of all the carotenes and xanthophylls, except that of helenien, molecular ions were the base peaks. The base peak of helenien, which is a palmitoyl ester, corresponded to palmitate. Deprotonated molecules were also detected in all of the negative ion APCI mass spectra, especially those of the carotenes, but were less abundant than the molecular ions.

3.2. APCI MS–MS

Since most carotenoids showed little fragmentation during APCI mass spectrometry, CID with product ion tandem mass spectrometry was used to form fragment ions that might be helpful for structural characterization. Positive ion APCI tandem mass spectra of the carotenes and selected xanthophylls are shown in Figs. 2 and 3, respectively, and negative ion APCI tandem mass spectra of carotenes and selected xanthophylls are shown in Figs. 4 and 5, respectively. The tandem mass spectra of all twelve carotenoids are summarized in Table 1 (positive ion APCI) and Table 2 (negative ion APCI).

3.2.1. β-Carotene and [$^{13}C_6$]-β-carotene

In the positive ion APCI tandem mass spectrum of β-carotene (Fig. 2 and Table 1), an ion of low abundance was observed at *m/z* 445 corresponding to the elimination of toluene, $[MH-92]^+$, from the protonated molecule. A corresponding ion was detected at *m/z* 448 in the tandem mass spectrum of [$^{13}C_6$]-β-carotene. Comparing the tandem mass spectra of β-carotene and [$^{13}C_6$]-

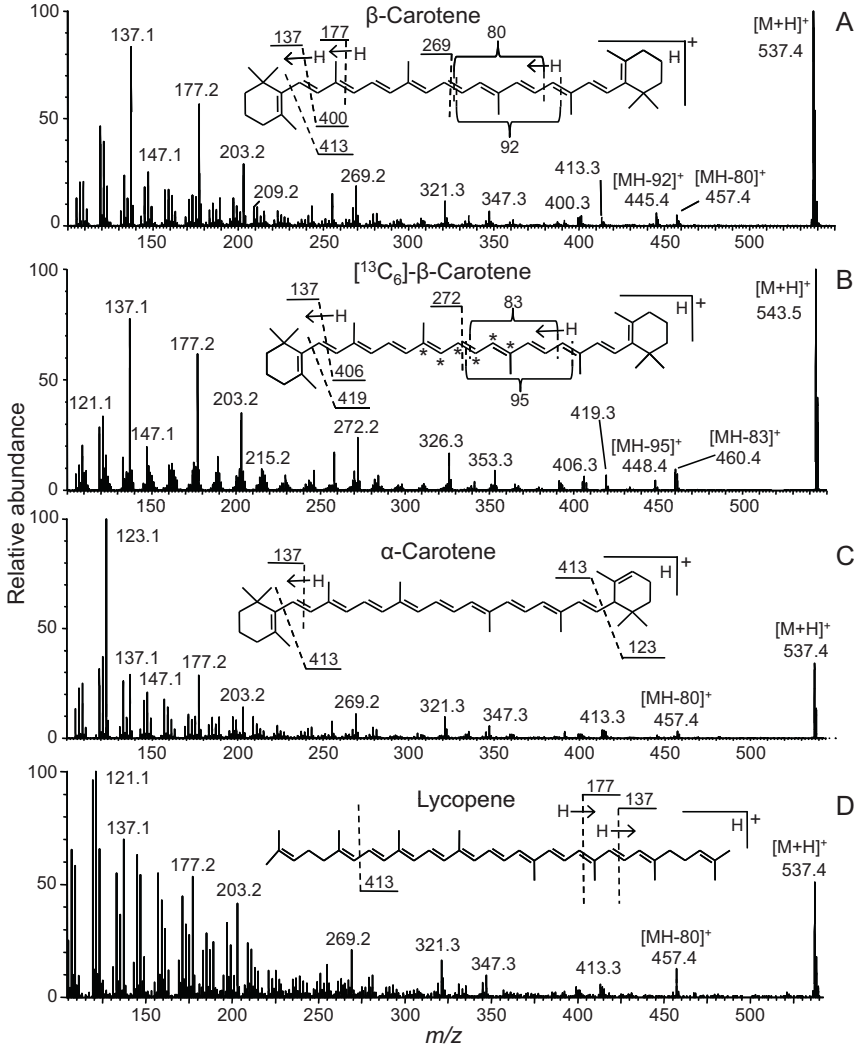

Fig. 2. Positive ion APCI tandem mass spectra of carotenes. (A) β-Carotene; (B) [$^{13}C_6$]-β-carotene; (C) α-carotene; and (D) lycopene.

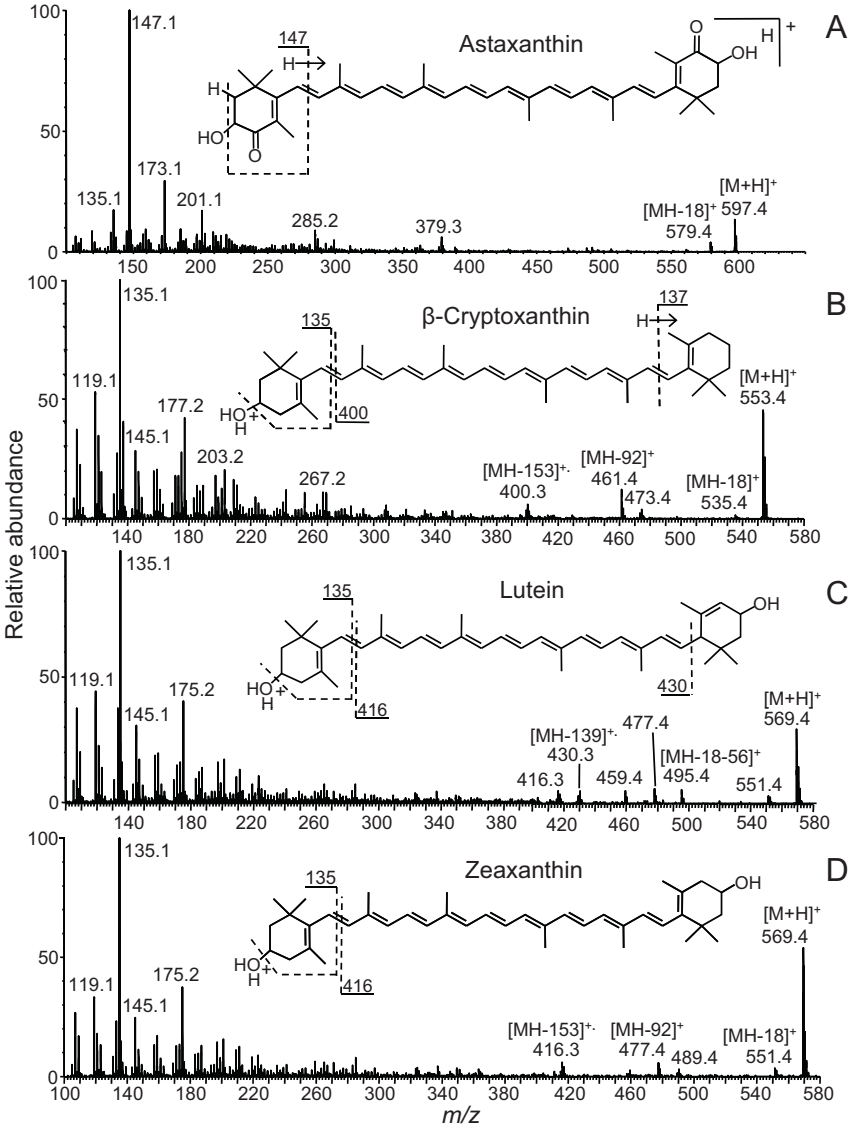

Fig. 3. Positive ion APCI tandem mass specta of selected xanthophylls. (A) Astaxanthin; (B) β-cryptoxanthin; (C) lutein; and (D) zeaxanthin.

Table 1
Positive ion APCI product ion tandem mass spectra with collision-induced dissociation of protonated carotenoids.

Carotenoid	Formula	$[M+H]^+$	$[MH-H_2O]^+$	$[MH-C_6H_8]^+$	$[MH-C_7H_8]^+$	Other significant ions
β-Apo-8′-carotenal	$C_{30}H_{40}O$	417.3[a] (100)	399.3 (4.5)		325.3 (7.9)	95.1 (43.7); 119.1 (58.0); 157.1 (47.8); 293.2 (14.4); 338.3 (10.0); 361.3 (5.6); 389.3 (2.1)
Astaxanthin	$C_{40}H_{54}O_4$	597.4 (13.2)	579.4 (3.8)			147.1 (100); 173.1 (29.2); 201.1 (17.0); 285.2 (8.6); 379.3 (5.9)
Capsanthin	$C_{40}H_{56}O_3$	585.4 (100)	567.4 (22.2)	505.4 (3.6)	493.4 (4.9)	109.1 (77.5); 127.1 (63.1); 135.1 (10.2); 219.2 (12.5); 475.4 (4.7); 487.4 (2.2)
α-Carotene	$C_{40}H_{56}$	537.4 (34.5)		457.4 (3.2)		**123.1 (100)**;[b] 137.1 (29.3); 177.2 (28.9); 413.3 (3.8)
β-Carotene	$C_{40}H_{56}$	537.4 (100)		457.4 (4.6)	445.4 (5.9)	137.1 (83.0); 177.2 (56.8); 269.2 (19.0); 400.3 (4.4); 413.3 (3.5)
$[^{13}C_{10}]$-β-Carotene	$^{12}C_{30}{}^{13}C_{10}H_{56}$	543.5 (100)		460.4 (10.0)	448.4 (4.9)	137.1 (77.4); 177.2 (61.8); 272.2 (24.6); 406.3 (5.8); 419.3 (6.4)
γ-Carotene	$C_{40}H_{56}$	537.4 (100)		457.4 (7.9)		119.1 (73.9); 137.1 (45.2); 145.1 (55.9); 177.2 (50.5); 255.2 (20.0); 269.2 (7.3); 399.3 (7.9)
β-Cryptoxanthin	$C_{40}H_{56}O$	553.4 (45.3)	535.4 (1.4)	473.4 (4.0)	461.4 (12.4)	119.1 (53.1); 135.1 (100); 177.2 (41.9); 400.3 (6.3)
Echinenone	$C_{40}H_{54}O$	551.4 (91.0)		471.4 (8.4)	459.4 (31.3)	133.1 (38.1); 203.1 (100); 255.2 (32.7); 495.4 (1.6); 536.4 (2.7)
Helenien	$C_{72}H_{116}O_4$	1045.9 (91.8)			953.8 (18.4)	135.1 (100); 175.1(63.2); 201.2 (56.8); 267.2 (41.2); 331.2 (24.5); 411.3 (30.8); 533.4 (67.3); 697.6 (27.0); 789.7 (68.4); 817.7 (19.6)
Lutein	$C_{40}H_{56}O_2$	569.4 (29.8)	551.4 (2.8)		477.4 (5.9)	119.1 (44.2); 135.1 (100); 175.2 (40.6); 416.3 (5.1); **430.3 (4.9)**; 459.4 (5.3); **495.4 (5.5)**
Lycopene	$C_{40}H_{56}$	537.4 (50.6)		457.4 (12.1)		121.1 (100); 137.1 (62.9); 177.2 (53.1); 413.3 (4.9)
Zeaxanthin	$C_{40}H_{56}O_2$	569.4 (54.1)	551.4 (3.6)	489.4 (3.3)	477.4 (5.7)	119.1 (33.2); 135.1 (100); 175.2 (37.6); 416.3 (6.0), 459.4 (2.6)

[a] *m/z* (relative abundance).
[b] Bold-face indicates fragment ions of particular utility for distinguishing isomers.

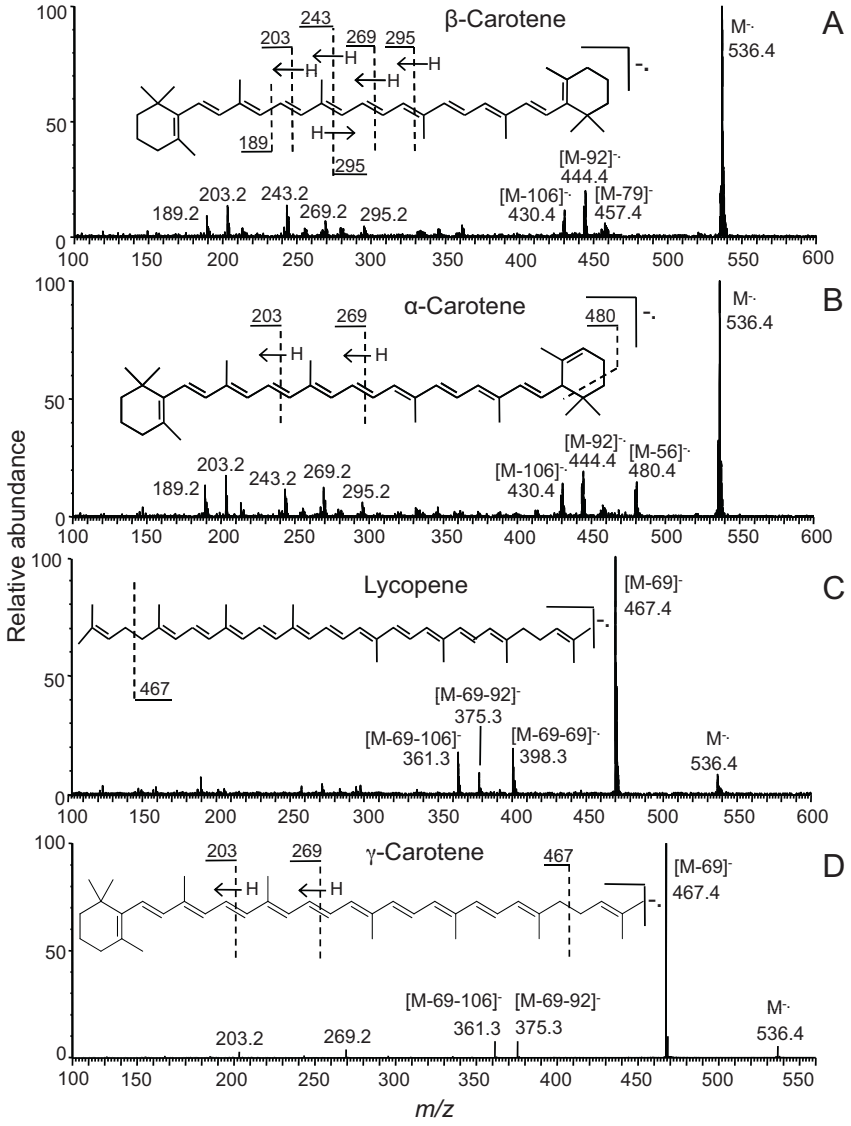

Fig. 4. Negative ion APCI tandem mass specta of carotenes. (A) β-Carotene; (B) α-carotene; (C) lycopene; and (D) γ-carotene.

Table 2
Negative ion APCI product ion tandem mass spectra with collision-induced dissociation of carotenoid molecular anions.

Carotenoid	Formula	M^-	$[M-H_2O]^-$	$[M-C_7H_8]^-$	$[M-C_8H_{10}]^-$	Other significant ions
β-Apo-8′-carotenal	$C_{30}H_{40}O$	416.3[a] (100)				239.2 (10.0); 280.2 (4.3); 359.3 (2.8); 401.3 (4.9)
Astaxanthin	$C_{40}H_{54}O_4$	596.4 (100)		504.4 (49.6)	490.4 (16.0)	167.0 (54.8); 203.0 (25.3); 233.1 (53.9); 297.2 (26.5); 323.3 (10.8); 337.3 (38.4); 363.3 (22.5); 389.3 (47.7); 429.4 (9.6); 581.4 (9.3)
α-Carotene	$C_{40}H_{56}$	536.4 (100)		444.4 (19.0)	430.4 (13.7)	189.2 (13.5); 203.2 (17.7); 243.2 (11.5); 269.2 (12.5); 295.2 (6.2); 413.4 (2.2); 457.4 (4.7); **480.4 (15.0)**[b]
β-Carotene	$C_{40}H_{56}$	536.4 (100)		444.4 (19.6)	430.4 (11.4)	189.2 (9.3); 203.2 (13.4); 243.2 (13.6); 269.2 (7.0); 295.2 (3.9); 457.4 (5.4)
γ-Carotene	$C_{40}H_{56}$	536.4 (5.1)				203.2 (2.3); 269.2 (3.3); 361.3 (8.1); 375.3 (7.4); **467.4 (100)**
Capsanthin	$C_{40}H_{56}O_3$	584.4 (100)	569.4 (1.4)	492.4 (2.5)	478.3 (1.4)	151.1 (11.1); 169.1 (23.8); 209.2 (7.9); 235.2 (30.3); 246.2 (25.2); 273.2 (18.6); 299.2 (14.7); 325.2 (15.9); 365.2 (8.3); 375.3 (46.1); 391.3 (29.1)
β-Cryptoxanthin	$C_{40}H_{56}O$	552.4 (100)	534.4 (39.4)			203.1 (5.6); 243.1 (5.1); 269.1 (5.1); 519.4 (37.0)
Echinenone	$C_{40}H_{54}O_2$	550.4 (100)		458.4 (14.4)	444.3 (5.3)	151.1 (5.7); 191.1 (5.8); 217.2 (4.4); 373.3 (8.3); 535.4 (2.6)
Helenien	$C_{72}H_{116}O_4$	1044.9 (3.1)				255.2 (100)
Lutein	$C_{40}H_{56}O_2$	568.5 (100)	550.5 (36.6)			137.1 (8.2); **429.4 (2.6)**; **512.4 (2.7)**; 535.4 (29.3)
Lycopene	$C_{40}H_{56}$	536.4 (8.4)				361.3 (17.6); 375.3 (9.0); **398.3 (19.3)**; **467.4 (100)**
Zeaxanthin	$C_{40}H_{56}O_2$	568.5 (100)	550.5 (82.6)			137.1 (3.2); 187.0 (5.6); 201.1 (9.4); 267.1 (2.7); 362.1 (4.2); 466.3 (6.0), 535.4 (67.2)

[a] *m/z* (relative abundance).
[b] Bold-face indicates fragment ions of particular utility for distinguishing isomers.

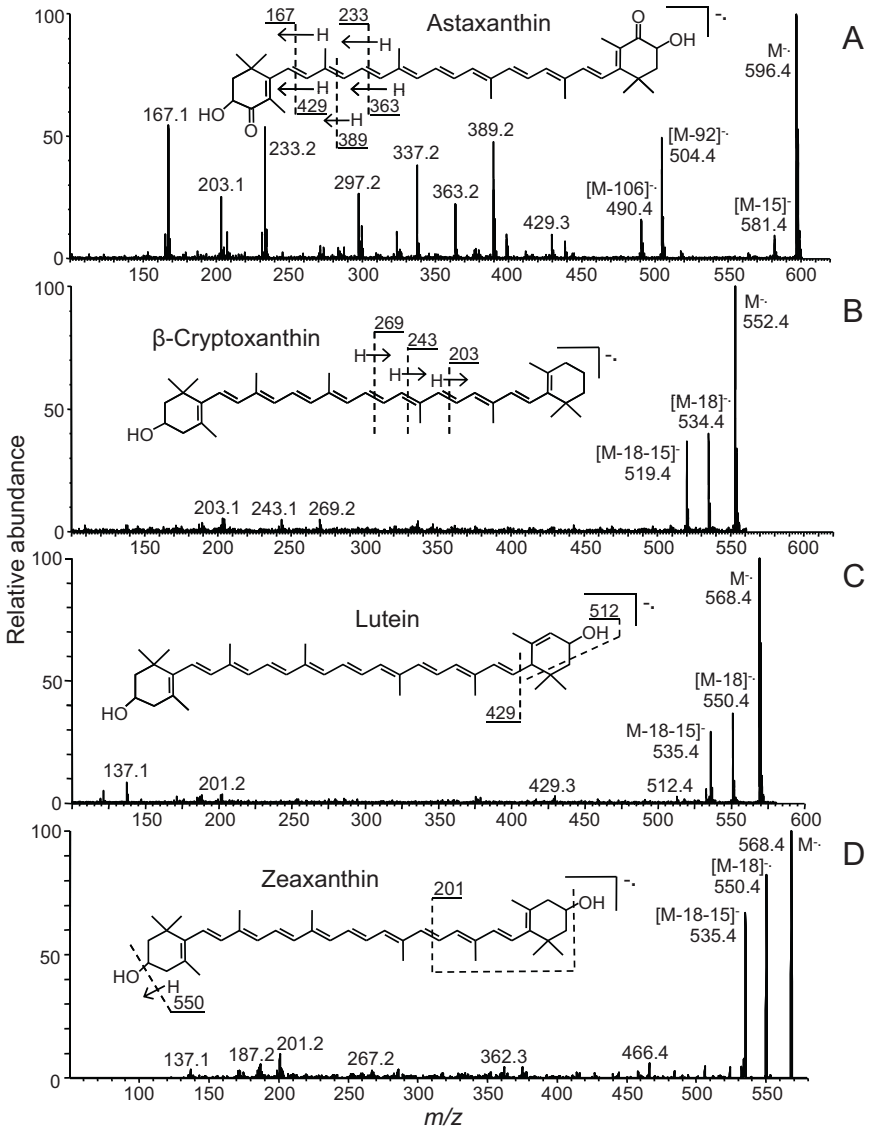

Fig. 5. Negative ion APCI tandem mass spectra of selected xanthophylls. (A) Astaxanthin; (B) β-cryptoxanthin; (C) lutein; and (D) zeaxanthin.

β-carotene, it is evident that toluene is lost from the central part of the polyene chain as originally proposed by Schwieter and coworkers [10]. However, these ions were of low abundance, compared to the positive ion FAB tandem mass spectra of β-carotene [9], presumably because the precursor ions formed by APCI were protonated molecules instead of radical cations. Loss of methyl-cyclopentadiene, $[MH-80]^+$ at *m/z* 457 for β-carotene and $[MH-83]^+$ at *m/z* 460 for $[^{13}C_6]$-β-carotene (Fig. 2 and Table 1), was another fragment ion formed by elimination of the central part of the polyene chain. Since loss of methyl-cyclopentadiene was accompanied by a hydrogen transfer or was facilitated by protonation at this site, this fragmentation pathway appears to be unique to protonated carotenoids and was not observed in the tandem mass spectra of carotenoid molecular ions, $M^{+\bullet}$ [9].

The fragment ion of *m/z* 413 (*m/z* 419 in the positive ion APCI tandem mass spectrum of $[^{13}C_6]$-β-carotene) was formed by loss of a β-ionone moiety from the protonated molecule (Fig. 2). Elimination of the ring with an additional methylene group, $[MH-137]^{+\bullet}$, resulted in the formation of fragment ions observed at *m/z* 400 and *m/z* 406 in the tandem mass spectra of β-carotene and $[^{13}C_6]$-β-carotene, respectively. Cleavage of the 7,8-bond (numbering scheme in Fig. 1) with the charge remaining on the β-ionone moiety instead resulted in the formation of ion of *m/z* 137, $[MH-400]^+$ which was the most abundant fragment ion in the positive ion tandem mass spectra of β-carotene and $[^{13}C_6]$-β-carotene. Fragmentation at the 9,10-bond produced the abundant ion of *m/z* 177 (Fig. 2). The remaining fragment ions in the low mass range of the tandem mass spectra of both labeled and unlabeled β-carotene were identical, which indicated that they originated from the terminal part of these molecules containing no ^{13}C label (Fig. 2 and Table 1).

Fragment ions were detected only in low abundance in the negative ion APCI tandem mass spectrum of β-carotene (Fig. 4). Product ions corresponding to the loss of toluene and xylene molecules and a methyl-cyclopentadienyl radical from the molecular anion were detected at *m/z* 444, 430 and 457, respectively (Table 2). These fragment ions had also been reported in the positive ion EI [10] and, except for $[M-xylene]^{+\bullet}$, in the positive ion FAB mass spectra [9] of β-carotene. Loss of toluene, $[M-92]^+$, was the most abundant fragment ion in the negative ion APCI tandem mass spectrum of β-carotene. In the low mass region of the negative ion APCI tandem mass spectrum, fragment ions resulting from the cleavage of 11,12-, 13,14-, 15,15′, and 14′,13′-carbon–carbon double bonds accom-

panied by hydrogen transfer to the ion were detected at *m/z* 203, 243, 269, and 295, respectively (Table 2 and Fig. 4). The fragment ion of *m/z* 189 was probably formed by cleavage of the 10,11-carbon bond without hydrogen transfer. These ions had not been reported using positive ion FAB MS–MS [9] or positive ion EI mass spectrometry [10].

3.2.2. α-Carotene

A provitamin A carotenoid, α-carotene differs from β-carotene only by the position of a double bond in one of the terminal rings, an α-ionone moiety (Fig. 1). Most of the fragment ions observed in the positive ion APCI product ion tandem mass spectrum of α-carotene (Fig. 2) were the same as those for β-carotene (*e.g.*, *m/z* 137, *m/z* 413 and *m/z* 457). However, the most abundant fragment ion in the positive ion tandem mass spectrum of α-carotene, corresponding to the α-ionone moiety of *m/z* 123, was not observed in the positive ion APCI mass spectrum of β-carotene. Formation of the ion of *m/z* 123 was facilitated by the position of the double bond in the terminal ring, which helped stabilize the resulting carbocation. Since this ion was not observed in the positive ion APCI tandem mass spectrum of β-carotene, γ-carotene, or lycopene (Fig. 2 and Table 1), it may be used to distinguish α-carotene from these isomeric carotenes. The ion of *m/z* 123 was not reported previously as an abundant ion in the FAB tandem mass spectrum of α-carotene [9] and was not observed using negative ion APCI tandem mass spectrometry (Table 2).

The negative ion APCI tandem mass spectrum of α-carotene (Fig. 4) was similar to the corresponding negative ion tandem spectrum of β-carotene and included ions of *m/z* 189, 203, 243, 269, 295, 430, 444, and 457. The main difference between the negative ion APCI tandem mass spectra of α-carotene and β-carotene was the fragment ion of *m/z* 480 which was detected only in the negative ion APCI tandem mass spectrum of α-carotene (Table 2). The ion of *m/z* 480 was formed by retro-Diels-Alder fragmentation of the α-ionone moiety and had been observed in the positive ion FAB mass spectrum of α-carotene [9].

3.2.3. Lycopene

An acyclic carotene without provitamin A activity, lycopene fragmented during positive ion APCI tandem mass spectrometry to form numerous low mass ions (*e.g.*, *m/z* 121, *m/z* 137 and *m/z* 177) that represented cleavages of the polyene chain (Fig. 2). However, no unique fragment ions which could differentiate lycopene from α-carotene or β-carotene were observed during positive ion APCI tandem mass spectrometry. This was different from positive ion FAB tandem mass spectrometry [9] in which a unique lycopene ion was formed, $[M-69]^+$, corresponding to elimination of a terminal isoprene group from the molecular ion. Loss of the terminal isoprene group is free radical-directed and was therefore an unfavorable fragmentation pathway for protonated lycopene. Loss of methyl-cyclopentadiene was observed at *m/z* 457 but not loss of toluene at *m/z* 445 (Table 1).

In the negative ion APCI product ion tandem mass spectrum of lycopene, the base peak was detected at *m/z* 467 (Fig. 4) and was formed by the elimination of a terminal isoprene group from the molecular ion. This fragment ion, $[M-69]^-$, has been reported in previous studies of lycopene using positive ion FAB tandem mass spectrometry [9] and positive ion EI mass spectrometry [10]. The second most abundant product ion corresponded to the loss of two isoprene groups from the molecular anion and was observed at *m/z* 398 (Fig. 4 and Table 2). Other product ions corresponded to elimination of an isoprene group and a molecule of toluene, $[M-69-92]^-$ at *m/z* 375, and elimination of an isoprene group plus xylene, $[M-69-106]^-$.

3.2.4. γ-Carotene

A provitamin A carotenoid, γ-carotene contains a β-ionone moiety like α-carotene and β-carotene, but the other terminus is acyclic like lycopene (Fig. 1). During positive ion APCI tandem mass spectrometry, the fragmentation of γ-carotene was similar to that of lycopene, α-carotene and β-carotene with abundant ions of low mass such as *m/z* 137 and *m/z* 177 and ions of lower abundance at higher mass such as $[M-80]^{+\bullet}$ of *m/z* 457 and *m/z* 269 (Table 1). No unique fragment ions were detected during positive ion APCI that distinguished γ-carotene from lycopene, α-carotene or β-carotene.

Like lycopene, the negative ion APCI tandem mass spectrum of γ-carotene contained a base peak of *m/z* 467 corresponding to the loss of a terminal isoprene group, and ions of *m/z* 375 and *m/z* 361 formed by loss of an isoprene group plus toluene or loss of an isoprene group plus xylene (Fig. 4 and Table 1). Since γ-carotene contains a β-ionone group, ions of *m/z* 203 and *m/z* 269 were detected that include this moiety as observed in the negative ion APCI tandem mass spectra of α-carotene and β-carotene (Fig. 4). Therefore, γ-carotene can be distinguished from lycopene, α-carotene and β-carotene by the detection of ions that characterize both the acyclic terminus of lycopene (*m/z* 467, *m/z* 375 and *m/z* 361) and the β-ionone moiety of provitamin A compounds (*m/z* 203 and *m/z* 269).

3.2.5. β-Cryptoxanthin

β-Cryptoxanthin is a provitamin A carotenoid that is similar in structure to β-carotene except for the presence of a hydroxyl group on one of the two rings Fig. 1). Some fragmentation pathways during positive ion APCI mass spectrometry were similar to those of β-carotene (Table 1) and included loss of toluene at *m/z* 461 and elimination of methyl-cyclopentadiene at *m/z* 473. Elimination of water from the protonated molecule, which is characteristic of hydroxylated xanthophylls [9], was observed at *m/z* 535, and loss of the hydroxylated ring with cleavage at the 7,8 carbon–carbon bond from the protonated molecule was observed at *m/z* 400. Like positive ion APCI tandem mass spectra of lutein and zeaxanthin, the base peak of β-cryptoxanthin was detected at *m/z* 135 and corresponded to the dehydrated terminal ring with cleavage at the 7,8 carbon–carbon bond (Fig. 3).

Two abundant fragment ions were detected in the negative ion APCI tandem mass spectrum of β-cryptoxanthin, $[M-H_2O]^{-\bullet}$ at *m/z* 534 and $[M-H_2O-CH_3]^-$ at *m/z* 519 (Fig. 5). As in the corresponding tandem mass spectra of the provitamin A carotenoids α-carotene, β-carotene and γ-carotene (Fig. 4 and Table 2), several fragment ions of low abundance corresponding to cleavages of the polyene chain were detected at *m/z* 269 and *m/z* 203.

3.2.6. Zeaxanthin

Zeaxanthin is a xanthophyll that is similar in structure to β-carotene except that it contains a hydroxyl group on each of the β-ionone rings (Fig. 1). During positive ion APCI product ion tandem mass spectrometry, the protonated molecule of zeaxanthin showed a characteristic loss of water at *m/z* 551 but at low abundance. Like β-carotene, losses of methyl-cyclopentadiene and toluene were observed in low abundance at *m/z* 489 and *m/z* 477, respectively (Fig. 3 and Table 1). A fragment ion corresponding to the elimination of a hydroxylated terminal ring with cleavage of the 7,8 carbon–carbon bond was observed at *m/z* 416 which is similar to the ion of *m/z* 400 in the tandem mass spectrum of β-cryptoxanthin. Like β-cryptoxanthin, abundant fragmentation of the polyene chain was observed. The base peak of *m/z* 135 corresponded to a dehydrated terminal ring with cleavage at the 7,8 carbon–carbon bond, and the next most abundant fragment ion of *m/z* 175 was the dehydrated terminal ring like that of *m/z* 135 except that cleavage occurred at the 9,10 carbon–carbon bond (Fig. 3).

Like β-cryptoxanthin, the negative ion APCI product ion tandem mass spectrum of zeaxanthin was dominated by the loss of water, $[M-18]^{-\bullet}$ at *m/z* 550 and loss of water plus a methyl radical, $[M-18-15]^-$ at *m/z* 535 (Fig. 5). Fragmentation of the polyene chain of zeaxanthin produced ions of low abundance at *m/z* 187, 201 and 267 (Table 2). The immediate precursor of these ions was probably the dehydrated ion of *m/z* 550. No unique fragment ions were observed in the negative ion APCI product ion tandem mass spectrum of zeaxanthin that could be used to distinguish it from isomeric lutein.

3.2.7. Lutein

Lutein differs from zeaxanthin only by the position of a carbon–carbon double bond in one of the rings and is structurally similar to α-carotene except that the rings are hydroxylated (Fig. 1). During positive ion APCI tandem mass spectrometry, lutein formed abundant fragment ions below *m/z* 300 that were similar to those of zeaxanthin including a base peak of *m/z* 135. Like zeaxanthin, fragment ions were also observed at *m/z* 551, 477, and 416 (Fig. 3 and Table 1). However, a unique lutein fragment ion of *m/z* 495 was formed by the loss of water from the protonated molecule and retro-Diels-Alder fragmentation of the α-ionone ring, $[MH-18-56]^+$. Another unique lutein ion of *m/z* 430 was formed by the elimination of the α-ionone ring. Therefore, the fragment ions of *m/z* 495 and *m/z* 430 may be used to distinguish lutein from zeaxanthin (Fig. 3 and Table 1).

The most abundant fragment ions in the negative ion APCI product ion tandem mass spectrum of lutein were detected at *m/z* 550 and *m/z* 535 and corresponded to $[M-H_2O]^{-\bullet}$ and $[M-H_2O-CH_3]^-$ (Fig. 5). Although of low abundance (Table 2), an ion of *m/z* 429 was formed by elimination of the terminal ring containing the unconjugated carbon–carbon double bond and was similar to the fragment ion of *m/z* 428 reported previously for lutein using positive ion FAB tandem mass spectrometry [9]. A retro-Diels-Alder fragment ion of *m/z* 512 was also observed at low abundance (Fig. 5 and Table 2). These fragmentation pathways were not detected in the negative ion APCI MS–MS spectrum of zeaxanthin.

3.2.8. Capsanthin

The most abundant fragment ions in the positive ion APCI tandem mass spectrum of capsanthin were observed at *m/z* 127 and *m/z* 109 and corresponded to the five-membered ring and a dehydrated five-membered ring, respectively (Table 1). As in the tandem mass spectra of lutein, zeaxanthin and β-cryptoxanthin (Fig. 3), an ion of *m/z* 135 was observed that is characteristic of carotenoids containing a hydroxylated β-ionone ring. Fragment ions corresponding to the loss of water, methyl-cyclopentadiene, toluene, methyl-cyclopentadiene plus water, and toluene plus water were observed at *m/z* 567, 505, 493, 487, and 475, respectively (Table 1).

In the negative ion APCI tandem mass spectrum of capsanthin (Table 2), most fragment ions contained the carbonyl group and the five-membered ring due to stabilization of the negative charge by the carbonyl oxygen. The ion of *m/z* 169 contained the carbonyl group and was probably formed by the cleavage of the 7,8-bond accompanied by a hydrogen transfer. A series of abundant fragment ions containing the carbonyl group and five-membered ring were observed at *m/z* 391, 365, 325, 299, 273, and 246, and were formed by cleavage of the polyene chain with a hydrogen transfer to the neutral leaving group (Table 2). The most abundant product ion was detected at *m/z* 375 and was likely formed as a result of cleavage of the 9,10-carbon bond accompanied by a hydrogen transfer to the leaving group. A complementary ion of *m/z* 209 was observed with the charge on the fragment containing the carbonyl group.

3.2.9. Astaxanthin

The base peak of the positive ion APCI tandem mass spectrum of astaxanthin was detected at *m/z* 147 and corresponded to a dehydrated terminal ring with cleavage of the 7,8 carbon–carbon bond (Fig. 3). A similar ion of *m/z* 201 was formed by fragmentation at the 10,11 carbon–carbon bond with loss of water from the ring. Loss of water from the protonated molecule of *m/z* 597 was observed at *m/z* 579. Loss of toluene was not observed from the protonated molecule, although such fragmentation had been reported previously for astaxanthin molecular ions using FAB tandem mass spectrometry [9].

In the negative ion APCI product ion tandem mass spectrum of astaxanthin, the two most abundant fragment ions were detected at *m/z* 167 and *m/z* 233 and corresponded to cleavage of the 7,8-carbon–carbon bond or cleavage of the 11,12-bond with hydrogen transfer from the leaving group to the ion (Fig. 5). Complementary ions, $[M-167]^-$ and $[M-233]^-$, were detected at *m/z* 429 and *m/z* 363 in the negative ion APCI tandem mass spectrum of astaxanthin, and similar fragment ions have been reported for the positive ion EI [11] and FAB tandem mass spectra of astaxanthin [9]. Additional polyene fragments ions in this sequence were observed at *m/z* 389, 323, and 297 (Fig. 5 and Table 2). A fragment ion of *m/z* 504, formed by the loss of toluene from the molecular ion, was detected in high relative abundance (50%), and losses of a methyl radical, $[M-15]^-$, and xylene, $[M-106]^{-\bullet}$, were detected at *m/z* 581 and *m/z* 490, respectively. Another abundant ion of *m/z* 337 was detected that corresponded to loss of toluene from the ion of *m/z* 429, $[M-167-92]^-$.

3.2.10. Helenien

Helenien is a dipalmitoyl ester of zeaxanthin (Fig. 1), and collision-induced dissociation of the protonated molecule produced ions of *m/z* 789 and *m/z* 533 corresponding to losses one or two molecules of palmitic acid, respectively (Table 1). As in the product ion tandem mass spectrum of zeaxanthin, the base peak was observed at *m/z* 135, and another abundant ion was detected at *m/z* 175. Loss of toluene from the protonated molecule of helenien resulted in the formation of the ion of *m/z* 953, and elimination of toluene from the ion of *m/z* 789 formed the ion of *m/z* 697. During negative ion APCI tandem mass spectrometry of the palmitoyl carotenoid ester helenien, the molecular ion was of low abundance due to extensive in-source fragmentation (Table 2). The base peak of *m/z* 255 in the tandem mass spectrum corresponded to palmitate, and no other abundant fragment ions were observed.

3.2.11. Echinenone

During positive ion APCI with CID, the base peak in the MS–MS spectrum of protonated echinenone (*m/z* 551) was detected at *m/z* 203 (Table 1) due to fragmentation at the 10,11-carbon bond with the charge remaining on the ketone moiety. Note that during EI [11], loss of an uncharged radical containing the ketone, $[M-203]^+$, was observed instead and is characteristic of carotenoids containing a keto group conjugated to the polyene chain. Retro-Diels-Alder fragmentation of the ring containing the ketone, $[MH-56]^+$, formed the product ion of *m/z* 495, and loss of a methyl radical was detected at *m/z* 536. Finally, an abundant fragment ion of *m/z* 459 was formed by elimination of toluene, $[MH-92]^+$, and loss of methyl-cyclopentadiene, $[MH-80]^+$, was detected at *m/z* 471 (Table 1).

During negative ion APCI tandem mass spectrometry, the molecular anion of echinenone (*m/z* 550) eliminated molecules of toluene (*m/z* 458) and xylene (*m/z* 444) (Table 2). Loss of a methyl radical was also observed at *m/z* 535. Fragmentation of the polyene chain with the charge on the ketone occurred with a hydrogen transfer to form ions of *m/z* 151, *m/z* 191, *m/z* 217, and *m/z* 373. The selective formation of ions containing the oxygen atom during negative ion

APCI was probably due to the favorable stabilization of the negative charge by the carbonyl oxygen.

3.2.12. β-Apo-8′-carotenal

The fragmentation pattern of protonated β-apo-8′-carotenal (*m/z* 417) formed during APCI was similar to that of its molecular ion observed during FAB tandem mass spectrometry [9] except for the detection of a fragment ion of *m/z* 361 corresponding to elimination of the terminal aldehyde group, $[MH-C_3H_4O]^+$ (Table 1). Ions of *m/z* 399 and 389 were detected corresponding to losses of water and carbon monoxide, respectively. Elimination of toluene from the protonated molecule was observed at *m/z* 325 (Table 1).

β-Apo-8′-carotenal did not fragment extensively during negative ion APCI tandem mass spectrometry (Table 2). A fragment ion corresponding to the loss of a methyl radical, $[M-15]^-$, from the molecular anion of *m/z* 416 was detected at *m/z* 401, and cleavage of the polyene chain produced fragment ions of *m/z* 280 and *m/z* 239 with the charge remaining on the aldehyde moiety. The fragment ion of *m/z* 359 was formed by cleavage of the polyene chain with elimination of $^{\bullet}C_3H_5O$.

4. Discussion

Solvent composition influences carotenoid ionization during APCI [8], and use of the protic solvent methanol as the primary solvent for positive ion APCI facilitated the formation of protonated molecules instead of molecular ions during this investigation. Although deprotonated carotenoids can be formed in addition to molecular ions when using a solvent system containing methanol, methyl *tert*-butyl ether and ammonium acetate [8], the use of acetonitrile during negative ion APCI in this study produced molecular anions. Previously, it was reported that positive ion FAB ionization of carotenoids formed molecular ions but not protonated molecules, negative ion FAB analysis produced no carotenoid ions [12,13], and the use of positive ion EI produced carotenoid molecular ions [11].

Molecular anions were usually the base peaks of the negative ion APCI tandem mass spectra, but the corresponding protonated molecules were rarely the base peaks of the positive ion APCI tandem mass spectra. A possible explanation for the lack of fragmentation observed during negative ion APCI with collision-induced dissociation might be that molecular anions, formed by capture of low energy electrons [8], were less energetic than the protonated molecules formed during positive ion APCI. Another possibility might be that due to differences in charge localization between the protonated molecules and the molecular anions, the molecular anions were generally more stable. Abundant product ions corresponding to fragmentation of the polyene chain were characteristic of all of the positive ion APCI tandem mass spectra (Table 1) but were observed in few of the corresponding negative ion tandem mass spectra (Table 2). Low mass ions corresponding to fragmentation of the polyene chain were also reported to be characteristic of positive ion EI [11] and FAB tandem mass spectrometry of carotenoids [9].

Elimination of neutral molecules of toluene, $[MH-92]^+$, and methyl-cyclopentadiene, $[MH-80]^+$, from protonated carotenoids was observed in most of the positive ion APCI tandem mass spectra but at low relative abundance. In positive ion FAB tandem mass spectra of carotenoids, elimination of toluene from the molecular ions was more abundant, and loss of xylene, $[M-106]^{+\bullet}$, was often observed instead of $[M-80]^{+\bullet}$. The polyene carotenoid chain is the origin of the neutral molecules of toluene and xylene which are formed through free radical rearrangement as reported by Schwieter et al. [10], and this mechanism is supported by our studies using ^{13}C-labeled β-carotene (Fig. 2). Free radical driven fragmentation pathways should be less favorable for protonated carotenoids than for the corresponding molecular ions. Although losses of toluene or xylene from the molecular anions were observed in less than half of the negative ion APCI mass spectra of carotenoids (Table 2), these product ions, when present, were more abundant than in the corresponding positive ion tandem mass spectra.

Previously, we reported how positive ion FAB mass spectrometry may be used to characterize xanthophylls and carotenes and to distinguish between isomeric carotenoids. The ability to obtain complementary negative ion and positive ion tandem mass spectra during APCI provided some additional structural information that was not observed using positive ion FAB mass spectrometry. For example, xanthophylls containing one or more hydroxyl groups eliminated water during positive ion APCI tandem mass spectrometry, probably facilitated by protonation of the hydroxyl group during ionization, but this fragmentation pathway was not always observed during negative ion APCI MS–MS or during positive ion FAB, probably since loss of water from a molecular ion instead of a protonated molecule would require a hydrogen rearrangement.

Isomeric α-carotene, β-carotene, γ-carotene, and lycopene may be distinguished using negative ion APCI tandem mass spectrometry by observing the characteristic α-carotene ion of *m/z* 480 formed by retro-Diels-Alder fragmentation of the α-ionone moiety, the ion of *m/z* 467 formed by elimination of a terminal isoprene group (lycopene and γ-carotene), and the ion of *m/z* 398 formed by elimination of two terminal isoprene groups (lycopene only) (Fig. 4). During positive ion APCI, the base peak of the tandem mass spectrum of α-carotene was detected at *m/z* 123, which corresponds to the α-ionone moiety and distinguishes this compound from isomeric β-carotene or lycopene (Fig. 2). Note that the ion of *m/z* 123 was not observed using FAB tandem mass spectrometry [9]. Finally, lutein and zeaxanthin may be distinguished by the observation of unique ions in the lutein tandem mass spectra including the ions of *m/z* 429 (negative ion APCI) and *m/z* 430 (positive ion APCI), formed by loss of the α-ionone moiety, and the retro-Diels-Alder fragment ions of *m/z* 512 during negative ion APCI (Fig. 5) and *m/z* 495 (retro-Diels-Alder fragmentation plus loss of water) during positive ion APCI (Fig. 3).

5. Conclusions

Systematic studies of carotenoid fragmentation pathways have been reported previously using positive ion EI [11] and FAB [9] but not APCI. Due to problems with pyrolysis and interfacing to HPLC systems, APCI has replaced EI for carotenoid studies. APCI and electrospray have replaced FAB for carotenoid studies due to enhanced sensitivity and ease of interfacing with HPLC [6,8], and APCI is preferred to electrospray for carotenoid LC–MS analysis due to its linearity of response and wider dynamic range [14]. Unlike FAB and electrospray which can produce molecular ion species but not negatively charged ions of all carotenoids, APCI can form protonated molecules as well as molecular anions, $M^{-\bullet}$ As shown during this investigation, the advantage of generating positively and negatively charged molecular ion species of carotenoids is that each type of precursor ion can produce complementary structural information, and some of the product ions formed during negative ion APCI of carotenoids have not be reported previously. Together these data more completely characterize each carotenoid and may be used to differentiate isomeric compounds. These tandem mass spectra may also guide the development of LC–MS–MS assays for the selective quantitative analysis of carotenoids by enabling the identification of precursor ion/product ion pairs for selected reaction monitoring as illustrated in the LC–MS–MS assay developed in this laboratory for lycopene [15].

Acknowledgements

This research was supported by grants R01 CA101052 from the National Cancer Institute and P50 AT00155 provided to the UIC/NIH Center for Botanical Dietary Supplements Research by the Office of Dietary Supplements and the National Center for Complementary and Alternative Medicine.

References

[1] G. Britton, Structure and properties of carotenoids in relation to function, FASEB J. 9 (1995) 1551–1558.
[2] O. Straub, Lists of natural carotenoids, in: H. Pfander (Ed.), Key to Carotenoids, Birkhauser Verlag, Basel, 1987.
[3] B. Demmig-Adams, A.M. Gilmore, W.W. Adams Jr., Carotenoids 3: in vivo function of carotenoids in higher plants, FASEB J. 10 (1996) 403–412.
[4] S. Carpentier, M. Knaus, M. Suh, Associations between lutein, zeaxanthin, and age-related macular degeneration: an overview, Crit. Rev. Food Sci. Nutr. 49 (2009) 313–326.
[5] R.B. van Breemen, N. Pajkovic, Multitargeted therapy of cancer by lycopene, Cancer Lett. 269 (2008) 339–351.
[6] R.B. van Breemen, Innovations in carotenoid analysis using liquid chromatography/mass spectrometry, Anal. Chem. 68 (1996) 299A–304A.
[7] R.B. van Breemen, Electrospray liquid chromatography–mass spectrometry of carotenoids, Anal. Chem. 67 (1995) 2004–2009.
[8] R.B. van Breemen, C.-H. Huang, Y. Tan, L.C. Sander, A.B. Schilling, Liquid chromatography/mass spectrometry of carotenoids using atmospheric pressure chemical ionization, J. Mass Spectrom. 31 (1996) 975–981.
[9] R.B. van Breemen, H.H. Schmitz, S.J. Schwartz, Fast atom bombardment tandem mass spectrometry of carotenoids, J. Agric. Food Chem. 43 (1995) 384–389.
[10] U. Schwieter, H.R. Bolliger, L.H. Chopart-dit-Jean, G. Englert, M. Kofler, A, v. Konig, G. Planta, R. Ruegg, W. Vetter, O. Isler, Synthesen in der Carotinoid-Reihe, Chimia 19 (1965) 294–302.
[11] C.R. Enzell, G.W. Francis, S. Liaaen-Jensen, Mass spectrometric studies of carotenoids. 2. A survey of fragmentation reactions, Acta Chem. Scand. 23 (1969) 727–750.
[12] H. Schmitz, R.B. van Breemen, S.J. Schwartz, Fast-atom bombardment and continuous-flow fast-atom bombardment mass spectrometry in carotenoid analysis, Methods Enzymol. 213 (1992) 322–336.
[13] R.B. van Breemen, H.H. Schmitz, S.J. Schwartz, Continuous-flow fast atom bombardment liquid chromatography/mass spectrometry of carotenoids, Anal. Chem. 65 (1993) 965–969.
[14] R.B. van Breemen, Liquid chromatography/mass spectrometry of carotenoids, Pure Appl. Chem. 69 (1997) 2061–2066.
[15] L. Fang, N. Pajkovic, Y. Wang, C. Gu, R.B. van Breemen, Quantitative analysis of lycopene isomers in human plasma using high-performance liquid chromatography–tandem mass spectrometry, Anal. Chem. 75 (2003) 812–817.