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Characteristic product ions of acetylene carotenoids by electrospray and nanospray ionization tandem mass spectrometry

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Carotenoids constitute a large group of natural pigments produced by photosynthetic organisms (plants and algae) and non-photosynthetic bacteria and fungi.[1] They are responsible for several biochemical events that regulate the adaptation and survival of different organisms, including light-harvesting, protection against UV radiation, and quenching of reactive oxygen species.[2–5]

In animals, they play important roles in visual communication and sex behavior[6], modulation of the immune system[7], growth factors and intracellular signaling pathways, regulation of cell differentiation[8], and as precursors for the pigment vitamin A.[9]

Carotenoids normally contain a C40 linear and symmetrical skeleton with an extensive polyene chain, formed by head-to-tail condensation of eight isoprene units.[10] These pigments possess unique chemical reactivity and light-absorbing properties due to the long central conjugated polyene chain, a chromophore system with λmax. 400–500 nm. Modifications in the basic structure, such as cyclization, hydrogenation, dehydrogenation, hydroxylation, epoxidation, rearrangement, and addition of lateral groups, give rise to a complex variety of similar compounds with widespread isomerism.[11]

About 750 carotenoids have been isolated from natural sources. More than 250 are of marine origin and show an interesting structural diversity, such as allenic (e.g., fucoxanthin) and all the acetylenic carotenoids (alloxanthin).[12] Albeit most carotenogenesis have been proposed, still little is known about the final biosynthetic steps leading to the formation of both allenic and acetylenic bonds.[13,14]

The economic relevance of carotenoids has driven the development of reliable analytical methodologies to analyze these pigments in food and other biological matrices.[15] Mass spectrometry (MS) is currently the preferred technique for determining the identity of carotenoids in complex samples. It allows one to distinguish between co-eluting compounds, when coupled with chromatographic systems, and it provides structural information on the basis of the molecular masses, using high-resolution instruments (HRMS), and key fragment ions through application of tandem mass spectrometry (MS/MS or MS2).[16–19]

Various approaches have explored carotenoid behavior through different ionization techniques, including electron impact (EI), fast atom bombardment (FAB), chemical ionization (CI), matrix-assisted laser desorption/ionization (MALDI), electrospray (ESI) and atmospheric pressure chemical ionization (APCI).[20–23] EI has been applied to evaluate the fragmentation pathways of carotenoids through radical ion formation and redox reactions.[24–27] Most recently, APCI combined with collision-induced dissociation (CID) experiments have attempted to identify characteristic fragments that can be associated with specific functional groups in the structures of these compounds.[20,22]

Previously, we reported the fragmentation of protonated carotenoids using ESI- and nanospray-CID-MS/MS.[28] The results were compared with the characteristic carotenoid ions proposed by APCI-MS/MS.[20] It was demonstrated that product ions of protonated carotenoids derived from charge-remote electrocyclic and retro-ene eliminations. These mechanisms rely on the correct conformation and distribution of hydrogens, achieved during protonation of the polyene chain, which form a carbocation intermediate and induce cis-trans isomerization.[28] In addition to the characteristic elimination of the aromatic ring from the polyene chain by $8\pi/6\pi$ electron electrocyclization,[29] we also provided diagnostic product-ions for keto-carotenoids, generated by vinyl-allyl cleavage,[30] and epoxy-carotenes, with formation of oxonium ions.[24,28]

Herein we investigate the gas-phase reactions of acetylenic carotenoids by ESI- and nanospray-CID-MS/MS, and provided key product ions for their rapid detection.

The MS/MS analysis of the carotenoid pigments alloxanthin, diadinoxanthin and diatoxanthin (Figure S-1) by two tandem mass spectral techniques* exhibited the product ions *m/z* 217 and 199. Accurate mass measurements by ultra-high-resolution ESI-FTICR MS/MS analysis (see Figures 1A-C) allow unequivocal determination of the molecular formulae of the two product ions, as shown in Table 1. These product ions are only observed in carotenoids that contain acetylenic bonds in the polyene chain. Similar fragments could also be detected on MS/MS spectra deposited in the public MASSBANK database (see Supporting Information).[31]

The fragmentation of acetylenic carotenoids initiated with selection of the protonated molecule $[M+H]^+$ as the precursor ion. Protonation is favored when using protic solvents mixed with 0.5% formic acid prior the ionization. According to previous studies, protonation of carotenoids preferably occurs between C7-C11, even in the presence of oxygen atoms in the xanthophyll.[28,32,33] During the ionization and subsequent CID fragmentation, the internal energy of the analytes increases sufficiently to induce isomerization of the polyene chain.

We observed dehydration of hydroxyl substituents, similar to those previously detected for xanthophylls,[20] and sequential cleavages of the polyene chain, which includes the diagnostic loss of toluene and/or xylene.[29] The formation of the key product ion *m/z* 217 arises by the transfer of the γ-hydrogen atom to an unsaturated center via a six-electron cyclic transition state, resulting in the formation of a conjugated diene and an allyl ion (Figure 1D). Several cleavages of the polyene chain by sequential pericyclic charge-remote reactions are characteristic of carotenoids. Due to the sp orbitals at C7-C8, this retro-ene reaction is chosen to attain the proper structural conformation and the hydrogen requirements. The product ion *m/z* 199 occurs through a further dehydration of *m/z* 217, possibly via a 1,2 elimination.

As a carotenoid epoxide, diadinoxanthin also showed the fragment *m/z* 221, formed by epoxide-furanoxide rearrangement with formation of cyclic oxonium ion, as summarized in Figure 2.[28]

The ultra-high-resolution accurate-mass analysis supports the evidence of $C_{15}H_{21}O^+$ as *m/z* 217 and $C_{15}H_{19}^+$ as *m/z* 199, and corresponds to key fragments for the rapid detection of acetylenic carotenoids. These results expand the set of diagnostic product ions for carotenoids analyzed by ESI-MS/MS, and provide structural information to assist their characterization via collision induced dissociation mass spectrometry.

* Ultra-high resolution accurate-mass ESI-MS/MS analyses were performed on a 7 Tesla Apex IV Fourier-transform ion cyclotron resonance instrument (Bruker Daltonics, Billerica, MA, USA) using an Apollo off-axis ESI source. Samples were directly infused from a syringe pump at 100 μL min^{-1}. The acquisition parameters were: capillary voltage, 4600 V; end plate voltage, 3500 V; capillary exit potential, 200 V and drying gas temperature, 200°C. Spectra were obtained by summing 80 0.5 s scans. CID-MS/MS fragmentation analyses were performed on the isolated parent ions using N_2 as the collision gas. Nanospray ionization analyses were performed on a QStar-XL quadrupole time-of-flight hybrid instrument (Applied Biosystems, Warrington, UK) using a Nanomate 100 automatic chip based nanospray system (Advion Biosciences, Norwich, UK). The QStar acquisition parameters were: ion source gas flow rate, 50 arbitrary units; curtain gas flow rate, 20 arbitrary units; ionspray voltage, 2700 V; declustering potential, 75 V; focusing potential, 280 V (see ESI-MS/MS spectra on Supporting Information).

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Table 1. Accurate masses and calculated molecular formulae of the main product ions observed in the FTICR MS/MS analysis of the $[M+H]^+$ acetylenic carotenoids **1-3** (see Fig. 1A-C).

compound	precursor ion	precursor m/z	product ion m/z	molecular formula
Alloxanthin (**1**)	$[M+H]^+$	565.4036 (-0.9)[a]	217.1582 (-2.3)	$C_{15}H_{21}O^+$
			199.1482 (0.0)	$C_{15}H_{19}^+$
Diatoxanthin (**2**)	$[M+H]^+$	567.4187 (-1.8)	217.1588 (0.5)	$C_{15}H_{21}O^+$
			199.1482 (0.0)	$C_{15}H_{19}^+$
Diadinoxanthin (**3**)	$[M+H]^+$	583.4145 (-0.2)	217.1592 (2.3)	$C_{15}H_{21}O^+$
			199.1488 (3.0)	$C_{15}H_{19}^+$

[a] The mass measurement error (ppm) is given in parentheses.

Figure caption

Figure 1. Selected ESI-FTICR MS/MS spectrum of **(A)** alloxanthin (**1**), precursor ion $[M+H]^+$ *m/z* 565.4036, **(B)** diatoxanthin (**2**), precursor ion $[M+H]^+$ *m/z* 567.4187 and **(C)** diadinoxanthin (**3**), precursor ion $[M+H]^+$ *m/z* 583.4145. **(D)** Proposed fragmentation mechanism for the formation of product ions $C_{15}H_{21}O^+$ (*m/z* 217.1587) and $C_{15}H_{19}^+$ (*m/z* 199.1482).

Figure 2. Main product ions of acetylenic carotenoids by ESI-MS/MS. The bond colored in red represents the site of C-C cleavage.