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EFFECT OF CHARGE GENERATION IN ESI SOURCE ON THE NEUTRAL AROMATIC ELIMINATION MECHANISM IN XANTHOPHYLLS

EFEITO DA GERAÇAO DE CARGAS NA FONTE DE ESI SOBRE O MECANISMO DE ELIMINAÇAO AROMATICA NEUTRA EM XANTOFILAS

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1. ABSTRACT

Carotenes and xanthophylls are natural pigments with high economic relevance in chemical, cosmetics, food and pharmaceutical industries. Systematic studies of carotenoid fragmentation pathways have demonstrated that the neutral elimination of aromatic ring from the polyene chain by electrocyclization reaction produced diagnostic ions to rapidly identify their presence in mixture. However, carotenes and xanthophylls also showed the ability to produce both protonated and radical molecular species, opening up multiple acid-base and/or redox fragmentation, which hamper carotenoids elucidation. Here we investigate the ionization and fragmentation of the radical/protonated and sodiated parent masses $[M]^{+\bullet}$/$[M+H]^+$ and $[M+Na]^+$ of two natural xanthophylls (canthaxanthin and fucoxanthin) and the synthetic apo-⍰-carotene using a Fourier-transform ion-cyclotron resonance mass spectrometer (FTICR MS). The MS/MS analysis showed that sodium adduct yielded better fragmentation of the diagnostic aromatic ring elimination in a simpler MS/MS spectra, whereas molecular ion and protonated molecule resulted in a multitude of fragments involving additional charge-remote fragmentations and direct cleavages of the conjugated π-system by retro-ene and vinyl-allyl reactions. The results suggested that Na^+ promoted electrocyclic aromatic ring elimination by assisting the correct orbital conformation of the polyene chain, giving clearly fragments to the unambiguous determination of carotenoids in biological samples.

Keywords: Carotenoids. Xanthophylls. Pericyclic rearrangement. Electrocyclization. Aromatic ring elimination. FTICR MS.

1. INTRODUCTION

Carotenoids are 40-carbon isoprene derivatives with a conjugated polyene chain - usually up to 15 double bonds. These molecules are widely distributed in nature where they play a key role as antioxidants, singlet oxygen quenchers, membrane stabilisers and light harvesters in photosynthetic organisms.[1-5]

Several analytical methods have been developed to detect and quantify carotenoids based on their chemical reactivity and light-absorbing properties.[6, 7] Liquid chromatography coupled with photodiode array (LC-UV) is by far the most common method, but the spectra similarity demand a complete LC resolution to assure their identity.[6, 8] Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) can improve identification of carotenoids by providing unique structural features in small amounts of very low concentration samples.[6, 9]

Carotenoids have been analyzed by MS using different ionization methods including electron impact (EI), fast atom bombardment (FAB), matrix-assisted laser desorption/ionization (MALDI), electrospray (ESI), atmospheric pressure chemical ionization (APCI).[9-12]

Recently, it was reported the full fragmentation of 16 carotenes and xanthophylls by electrospray ionization tandem mass spectrometry (ESI-CID-MS/MS) and nanospray tandem mass spectrometry (nanoESI-CID-MS/MS) where it was demonstrated that fragmentation of protonated carotenoids produced a multitude of fragments derived from charge-remote fragmentations and pericyclic rearrangements, such as electrocyclic and retro-ene eliminations dependent on cis-trans isomerization and the formation of several conjugated polyene carbocation intermediates.[13]

Additionally, previous studies had indicated that carotenoids and xanthophylls can be ionized by a radical process due to lower oxidation potentials (<1.0 V versus Ag/AgClsat), forming molecular ion besides protonated molecule in ESI (positive mode) source.[14-16] This ability to produce radical molecular species by loss of one or two electrons open up multiple fragmentation routes based on distinguished gas-phase mechanisms - acid-base and/or redox reactions - which hamper structural elucidation.[13]

Despite all information regarding carotenoids ionization and fragmentation, their structural diversity, the presence of *cis/trans*-isomers and the formation of radical molecular ion still make identification of carotenoids by LC-MS, a challenge.

In order to rapidly identify carotenoids in mixture, this work proposes to develop a novel method focused on the specific neutral elimination of aromatic ring in carotenoids.

Generally, the presence of carotenoids can be achieved based on the detection of toluene (92 u) and xylene (106 u) neutral losses.[13, 17, 18] However, ionization of carotenes and xanthophylls into $[M]^{+\bullet}$, $[M+H]^+$ and/or $[M+Na]^+$ results in multiple fragmentation pathways and an unclear generation of diagnostic aromatic ring neutral losses, competing with other charge-remote fragmentations and pericyclic rearrangements, such as retro-ene eliminations.[13]

Here we investigate the ionization and fragmentation of the radical/protonated and sodiated parent masses $[M]^{+\bullet}/[M+H]^+$ and $[M+Na]^+$ of two natural xanthophylls, canthaxanthin (**1**) and fucoxanthin (**2**) and the synthetic apo-⍰-carotene (**3**), figure 1, using an Fourier-transform ion-cyclotron resonance mass spectrometer (FTICR MS) focused on the systematic neutral losses of toluene (92 u) and xylene (106 u).

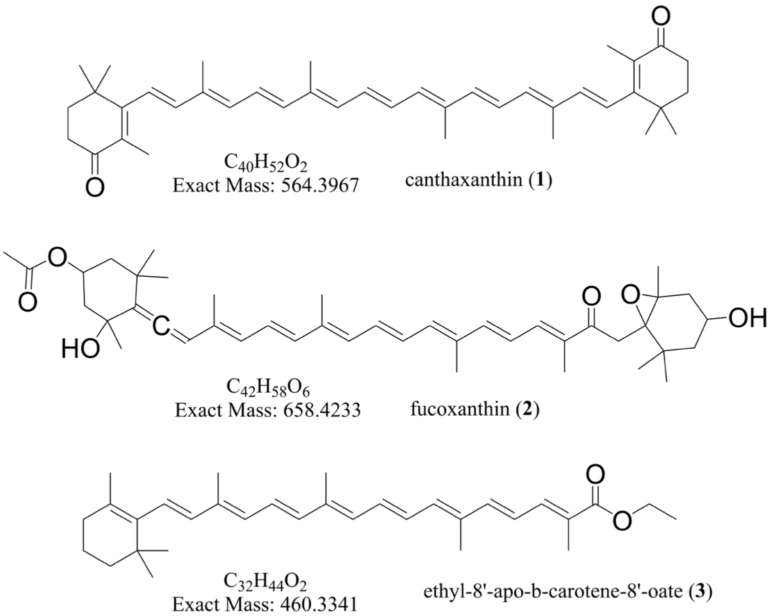

Figure 1. The structures of analyzed polyene (1) canthaxanthin, (2) fucoxanthin and (3) 2, ethyl-8'-apo-β-carotene-8'-oate.

2. EXPERIMENTAL

Canthaxanthin and ethyl-8'-apo-β-carotene-8'-oate were obtained from CaroteNature (Lupsingen, Switzerland). Fucoxanthin was obtained from Sigma-Aldrich (St Louis, MO, USA). Methanol and acetonitrile (HPLC grade) were obtained from Aldrich (Gillingham, Dorset, UK). Deionised water was used throughout the study. Samples were prepared in methanol/water or methanol/acetonitrile/water (50:25:25% v/v) using 0.1 μg mL^{-1} as the final concentration. For the analysis in acidic solutions, diluted standards were prepared in the appropriate solvent with the addition of 0.05% of formic acid.

Electrospray ionisation analyses were performed on an Apex 4 7.0 Tesla Fourier-transform ion-cyclotron resonance mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Samples were directly infused into the Apollo off-axis ESI source using a syringe pump at 100 μL h-1. The acquisition parameters were: capillary voltage, 4600V; end plate voltage, 3500 V; capillary exit potential, 200 V (except were indicated otherwise) and drying gas temperature, 200 °C. Spectra were obtained by summing 8 x 0.5 s scans.

3. RESULTS AND DISCUSSION

The analysis of carotenoids in ESI-FTICR-MS resulted in the formation of radical $M^{+\bullet}$, protonated $[M+H]^+$ and sodiated $[M+Na]^+$ parent ions. Although the detection of both molecular ion and protonated molecule have already been reported in polyenes, and justified by their lower oxidation potential (<1.0 V versus Ag/AgCl$_{sat}$), works describing the formation of sodium adducts in free carotenoids by ESI positive mode were not found.[8, 14-16]

The formation of different ion types on ESI-mass spectra is the result of concomitant acid-base, cation coordination and/or redox reactions occurring in the ionization source. The balance between these processes is directly dependent on experimental parameters such as solvent composition, as well as on intrinsic carotenoids properties, e.g., the presence of oxygen atoms (in case of xanthophylls) and the charge delocalization through the conjugated polyene.[8]

Roughly, MS/MS carotenoid identification comprises the diagnostic neutral losses of toluene (92 u) and xylene (106 u).[17, 19] However, other several fragments can also be produced, mainly involving charge-remote fragmentations, as dehydration, and polyene cleavage based on pericyclic rearrangements as retro-ene elimination (assisted or not by sigmatropic hydrogen shift).[13] This simultaneous generation of multiple ions can hamper structural elucidation through increased complexity and also suppressing the diagnostic aromatic ring fragments.[13, 19]

Considering that carotenoids have the capacity to form radical, protonated and/or sodiated molecules, the understanding of the multiple fragmentation pathways with distinguished gas-phase mechanisms from the selective collision induced dissociation (CID) analysis of $M^{+\bullet}$, $[M+H]^+$ and $[M+Na]^+$ can give rise to characteristic MS/MS profiles and enhanced the detection of target ions, in our case, the neutral losses of 92 and 106 u.[9, 13, 20]

According to the major product ions from tandem mass spectra (available in the Supporting Information and are summarized in Table 1), all molecules produced distinct fragment structures for $[M+H]^+$ and $[M+Na]^+$, with sodium adduct yielding better fragmentation of the diagnostic aromatic elimination in a simpler MS/MS spectra, whereas the protonated molecules resulted in a multitude of fragments involving additional charge-remote fragmentations and direct cleavages of the conjugated π-system by retro-ene and vinyl-allyl reactions.

Table 1. Tabulated data for the positive ion CID-MS/MS product ions from both the ESI-FTICR analysis of the protonated and sodiated carotenoids.

Carotenoid		observed m/z	Electrocyclic reaction**	other abundant ions
1	[M+H]+	565.4039	473 , 459, 441	203, 215, 255, 269, 293
	[M+Na]+	587.3871	495, 481	277
2	[M+H]+	659.4306	549, 531	391, 355, 275, 213, 193, 149
	[M+Na]+	681.4164	589	449, 357, 327, 221, 215, 175
3	[M+H]+	461.3414	-	337, 291, 235, 221, 171, 157, 119
	[M+Na]+	483.3206	391	327. 295, 202, 189, 165, 152, 115

*** Ions represent elimination of toluene (92 u), xylene (106 u) and possible additional losses of water.*

In the case of canthaxanthin, a xanthophyll with one keto group in each ionone ring, the $[M+H]^+$ produced the neutral elimination of toluene and xylene at low intensities (*m/z* 473 and 459, respectively), whereas these diagnostic fragments were the main ions of $[M+Na]^+$ (*m/z* 495 as base peak and 481), as depicted in figure 2. Additionally, the protonated form showed ions related to dehydration and cleavage of polyene chain.

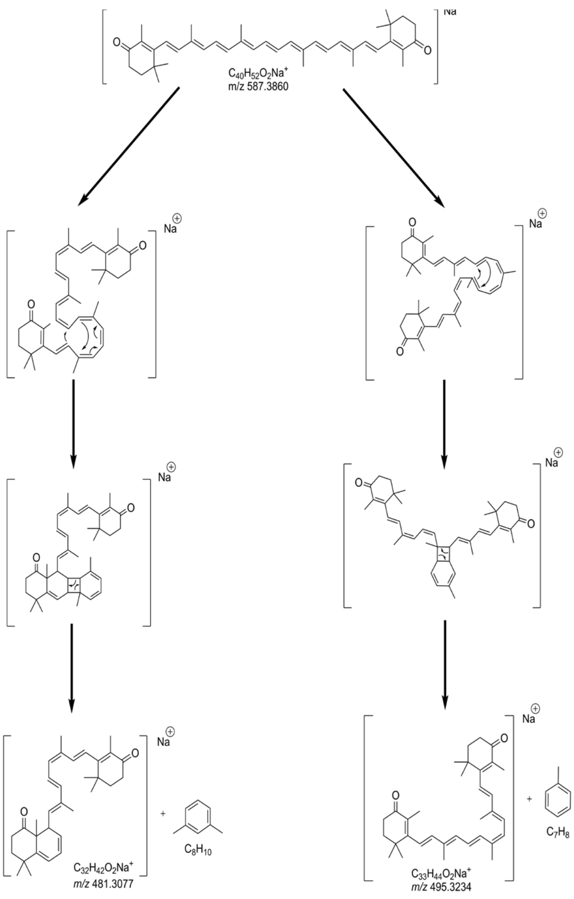

Figure 2. Proposed neutral losses of toluene and xylene from sodiated canthaxanthin adduct.

Similar results were obtained for fucoxanthin, a xanthophyll with an unusual allenic bond besides epoxyl, hydroxyl and carbonyl moieties. The major fragments of $[M+H]^+$ were related to dehydration and the neutral loss of acetic acid (60 u) by 1,4 elimination, while the aromatic ring were not observed. On the other hand, $[M+Na]^+$ showed the neutral loss of 92 u (*m/z* 589) although at low intensity, as indicated in figure 3.

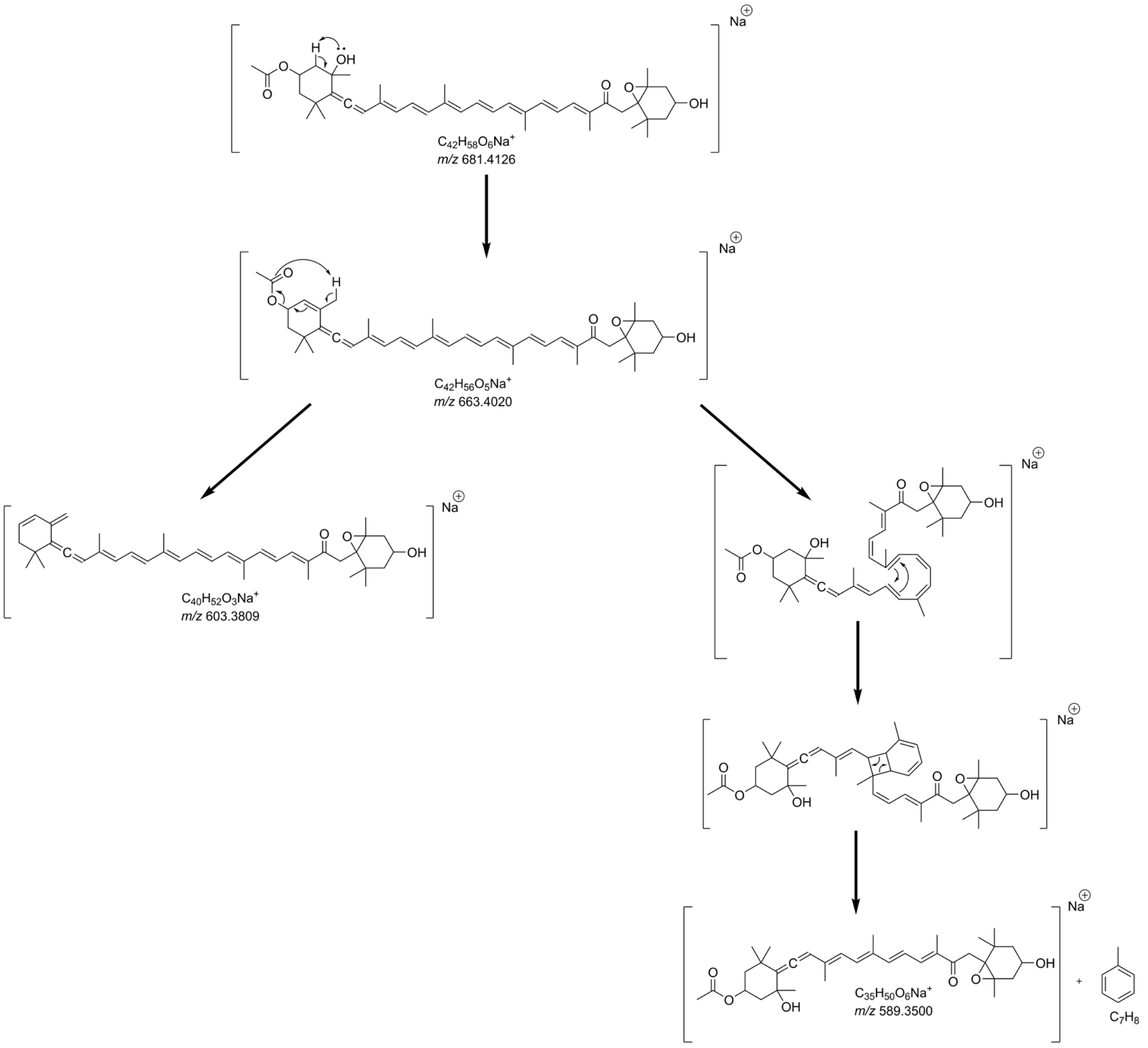

Figure 3. Proposed neutral losses of toluene from sodiated fucoxanthin adduct.

Ethyl-8′-apo-β-carotene-8′-oate also generated different MS/MS spectra. For $[M+H]^+$ several fragments were observed; however none represented characteristic aromatic ring eliminations. In case of $[M+Na]^+$, represented in figure 4, the elimination of toluene (*m/z* 391) is clearly detected in a simpler MS/MS spectrum.

Coordination reactions have already been described to enhance the detection of target substances and to understand fragmentation pathways using different ions such as silver, sodium, cobalt, nickel, copper, manganese, zinc, calcium, barium, potassium, lithium and others.[21, 22] The chemical interaction of the metal atom through formation of metal complexes with ions generated in ESI source induces variations in the geometry and symmetry of coordinated molecules which can affected the intensity and number of fragment ions. In carotenoids, the neutral losses of 92 and 106 u, explained

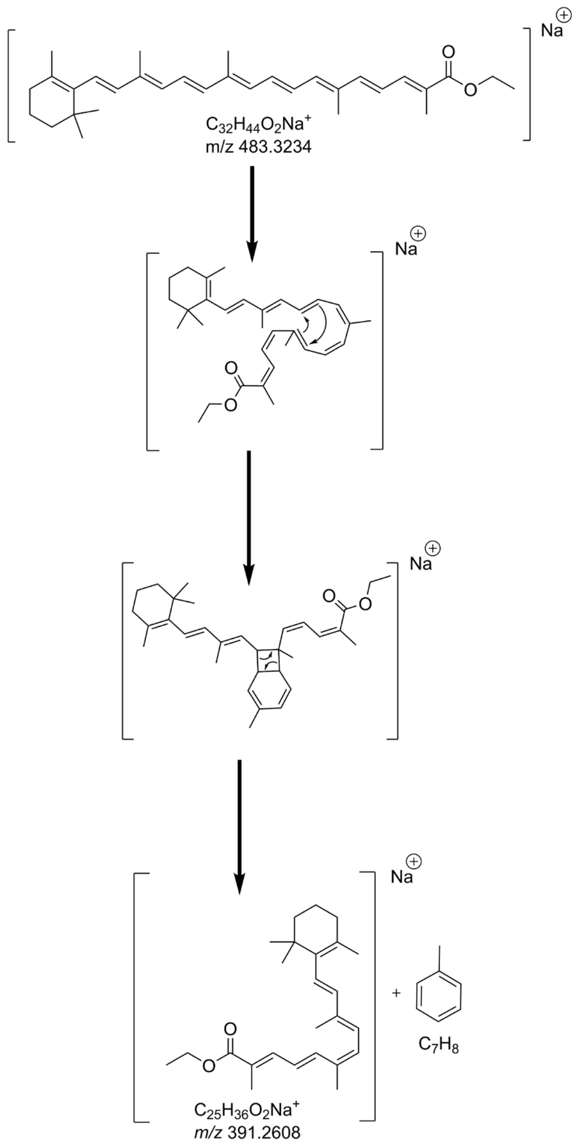

Figure 4. Proposed neutral losses of toluene from sodiated ethyl-8'-apo-β-carotene-8'-oate adduct.

by a sequential $8\pi/6\pi$ electron electrocyclization, required the correct orbital orientation and γ-hydrogen distribution, achieved through carbocation formation and cis-trans π-system conversion.[13]

According to the results, the coordination between sodium and the carotenoids suggested that the characteristically distorted tetrahedral geometry favoured the electrocyclic elimination, e.g., the spatial requirements to the symmetry allowed disrotatory ring-closure of the cyclo-octatetraene observed in the electrocyclization followed by formation of a four-membered ring intermediate.[13, 22]

Additionally, previous studies indicated that an increase in the metal ionic radius led to decrease in metal electronegativity and, consequently, an increase in the metal-ligand average distance. In case of $[M+Na]^+$, the sodium's eletronegativity and ionic radium permits the attraction of the electrons to start the electrocyclic elimination, but not to produced significantly more fragment ions through retro-ene eliminations of the polyene chain.

4. CONCLUSIONS

This study described the ionization and fragmentation of carotenoids standards in FTICR MS, showing that sodium adduct parent masses $[M+Na]^+$ gave much simpler MS/MS spectra than $[M]^{+\bullet}$ and $[M+H]^+$ parents. Coordination with sodium resulted in diagnostic aromatic ring eliminations from polyene chain, whereas protonated molecules generated a multitude of fragment ions, mainly direct cleavages of the conjugated π-system by retro-ene and vinyl-allyl reactions. These findings indicated that sodium ionic radius and electronegativity are related to the intensity and number of fragment ions and demonstrate its ability to induce electrocyclic aromatic ring elimination by assisting the correct orbital conformation of the polyene chain, giving clearly fragments to the unambiguous determination of carotenoids in biological samples. The results suggested that Na^+ promoted electrocyclic aromatic ring elimination by assisting the correct orbital conformation of the polyene chain, giving clearly fragments to the unambiguous determination of carotenoids in biological samples.

5. ACKNOWLEDGEMENTS

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