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1. ABSTRACT

Carotenoids 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, carotenoid 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 $[M]^\cdot^+$ and $[M+Na]^+$ of two natural xanthophylls (canthaxanthin and fucoxanthin) and the synthetic apo-$\beta$-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 cleav-
geages 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 con-
formation 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 mol-
cules are widely distributed in nature where
they play a key role as antioxidants, singlet
oxygen quenchers, membrane stabilisers and
light harvesters in photosynthetic organisms.

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 identi-
fication 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/ioniza-
tion (MALDI), electrospray (ESI), atmospheric
pressure chemical ionization (APCI).\(^{[9-12]}\)

Recently, it was reported the full frag-
mentation of 16 carotenes and xanthophylls
by electrospray ionization tandem mass spec-
trometry [ESI-CID-MS/MS] and nanospray
tandem mass spectrometry [nanoESI-CID-
MS/MS] where it was demonstrated that
fragmentation of protonated carotenoids pro-
duced 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 indi-
cated that carotenoids and xanthophylls can be
ionized by a radical process due to lower oxida-
tion potentials \(<1.0\ \text{V versus Ag/AgCl}_{\text{sat}}\)\),
forming molecular ion besides protonated mol-
ecule 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 elucida-
tion.\(^{[13]}\)

Despite all information regarding ca-
rotenoids 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 elimina-
tion of aromatic ring in carotenoids.

Generally, the presence of carotenoids
can be achieved based on the detection of tolu-
ene \(92\ \text{u}\) and xylene \(106\ \text{u}\) neutral losses.\(^{[13,}
\[17, 18]\) However, ionization of carotenes and
xanthophylls into \([M]^{+•}, [M+H]^{+}\) 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 rearrange-
ments, such as retro-ene eliminations.\(^{[13]}\)

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

Canthaxanthin and ethyl-8'-apo-β-carotene-8'-oate were obtained from CaroteNature (Lupingen, 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, 4600 V; 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⁺•, 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/AgClsat), 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.

Considering that carotenoids have the ca-
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 $M^{+}$, $[M+H]^{+}$, 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.\textsuperscript{10, 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 $\pi$-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.

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>observed m/z</th>
<th>Electrocyclic reaction$^{**}$</th>
<th>other abundant ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>565.4039</td>
<td>473, 459, 441</td>
<td>203, 215, 255, 269, 293</td>
</tr>
<tr>
<td>(M+Na)$^+$</td>
<td>587.3871</td>
<td>495, 481</td>
<td>277</td>
</tr>
<tr>
<td>2</td>
<td>659.4306</td>
<td>549, 531</td>
<td>391, 355, 275, 213, 193, 149</td>
</tr>
<tr>
<td>(M+Na)$^+$</td>
<td>681.4164</td>
<td>589</td>
<td>449, 357, 327, 221, 215, 175</td>
</tr>
<tr>
<td>3</td>
<td>461.3414</td>
<td>-</td>
<td>337, 291, 235, 221, 171, 157, 119</td>
</tr>
<tr>
<td>(M+Na)$^+$</td>
<td>483.3206</td>
<td>391</td>
<td>327, 295, 202, 189, 165, 152, 115</td>
</tr>
</tbody>
</table>

$^{**}$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 fragmentations 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.](image-url)
Similar results were obtained for fucoxanthin, a xanthophyll with an unusual allenic bond besides epoxyl, hydroxyl and carbonyl moieties. The major fragments of $[\text{M+H}]^+$ 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, $[\text{M+Na}]^+$ showed the neutral loss of 92 u ($m/z$ 589) although at low intensity, as indicated in figure 3.

Ethyl-$8'$-apo-$\beta$-carotene-$8'$-oate also generated different MS/MS spectra. For $[\text{M+H}]^+$ several fragments were observed; however none represented characteristic aromatic ring eliminations. In case of $[\text{M+Na}]^+$, 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.\textsuperscript{[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 3. Proposed neutral losses of toluene from sodiated fucoxanthin adduct.](image-url)
by a sequential $8\pi/6\pi$ electron electrocyclization, required the correct orbital orientation and \( \gamma \)-hydrogen distribution, achieved through carbocation formation and cis-trans \( \pi \)-system conversion.\textsuperscript{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 electrocyclication followed by formation of a four-membered ring intermediate.\textsuperscript{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 \([\text{M+Na}]^+\), the sodium’s electronegativity and ionic radius 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 \([\text{M+Na}]^+\) gave much simpler MS/MS spectra than \([\text{M}]^{+}\) and \([\text{M+H}]^{+}\) 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 \( \pi \)-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 \( \text{Na}^+ \) 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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