Title: Identification of carotenoids using mass spectrometry in positive ion mode

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Identification of carotenoids using mass spectrometry in positive ion mode

The present review compiles positive MS fragmentation data of selected carotenoids obtained using various ionization techniques and matrices. In addition, new experimental data from the analysis of carotenoids in transgenic maize and rice callus are provided. Several carotenes and oxygen-functionalized carotenoids containing epoxy, hydroxyl and ketone groups were ionized by atmospheric pressure chemical ionization (APCI)-tandem mass spectrometry (MS/MS) in positive ion mode. Thus, on the basis of the information obtained from the literature and our own experiments, we identified characteristic carotenoid ions that can be associated to functional groups in the structures of these compounds. In addition, pigments with a very similar structure were differentiated through comparison of the intensities of their fragments. The data provide a basis for the structural elucidation of carotenoids by mass spectrometry (MS).

Keywords: mass spectrometry (MS); tandem mass spectrometry (MS/MS); fragmentation; carotenoids

I. INTRODUCTION

Carotenoids are natural pigments that serve a multitude of functions (Walter & Strack, 2011). They are synthesized by a huge range of organisms within archaea and eubacteria (including cyanobacteria), as well as eukaryotes (algae, fungi and plants). They are found in a large number of fruits and vegetables (oranges, tomatoes, carrots, spinach, sweet potatoes, pumpkins), spices (paprika), some animal products (eggs, butter, milk) and seafood (salmon, shrimp, trout, mollusk, etc.) (Lesellier et al., 1993). Carotenoids are required for the correct assembly of photosystems (Matthews, Luo & Wurtzel, 2003) in plants. These pigments absorb light across a broader range of the spectral region in which the sun irradiates maximally and they transfer the energy to chlorophyll, thereby initiating the photochemical events of photosynthesis (Polívka & Frank, 2010). In addition, carotenoids serve as precursors for the hormones abscisic acid (ABA) and strigolactones (Zhu et al., 2010). They also act as attractants for animals, such as pollinating insects and seed-disbursing herbivores (Zhu et al., 2009). Carotenoids also have a number of functions in animals. For example, they boost the immune system and promote general health. These pigments are critical in determining sexual behavior and reproduction processes. They also help to prevent predation and parasitism (Cazzonelli, 2011). In humans, carotenoids are precursors of vitamin A, this being one of their

most important physiological functions (Vílchez et al., 2011). These pigments are also known to contribute to the prevention of and protection against serious health disorders such as cancer, heart disease, and macular degeneration (Rivera & Canela-Garayoa, 2012; Fraser & Bramley, 2004). Carotenoids are used in industry: (a) in nutrient supplementation; (b) for pharmaceutical purposes; (c) in animal feed; (d) as food colorants (such as bixin and crocetin, found in annatto seeds and saffron respectively); and (e) in fragrances (such as ionones, damascones, and damascenones) (Rivera & Canela-Garayoa, 2012; Zhu et al., 2010). Consequently, these pigments have been extensively studied by organic and food chemists, biologists, physiologists, medical doctors, and recently also by environmental scientists. The widespread interest in carotenoids has led to an increased demand for reliable analytical methodologies. Among these, mass spectrometry (MS) is a powerful technique for the identification of these compounds.

The application of MS to carotenoid analysis has been a significant step forward with regards to the classical carotenoid analysis based on the use of spectrophotometric ultraviolet-visible (UV-vis) techniques. The usefulness of mass spectrometry as an aid to carotenoid analysis and identification has been proved since the pioneering work carried out by Schwieter et al., 1965. However, the wide-spread use of LC-MS in the current century has led to considerable new advances and begun yet another phase in the development of carotenoid chemistry which allows the use of smaller samples and provides data on isomerism which was not previously available. MS allows us to distinguish between co-eluting carotenoids and to determine molecular weights. This technique has also been used to assess the presence of functional and end groups in carotenoid structures , to gain a rapid overview of the carotenoids present in a sample , and to classify samples . Several ionization techniques have been reported for MS analysis of carotenoids, including electron impact (EI), fast atom bombardment (FAB), matrix-assisted laser desorption/ionization (MALDI), electrospray (ESI), atmospheric pressure chemical ionization (APCI) and, more recently, atmospheric pressure photoionization (APPI) and atmospheric pressure solids analysis probe (ASAP) . APCI has become the most widely used ionization technique for carotenoids because of its high sensitivity . Hao et al. reported that the detection of *β*-carotene, *β*-cryptoxanthin, lutein and zeaxanthin in botanical samples is 100-fold more sensitive using APCI than ESI . Similarly, Rivera et al. compared the effect of ionizing four carotenes and twelve oxygen-functionalized carotenoids using ESI, APCI, and APPI. They reported that twelve of the sixteen carotenoids studied exhibited the strongest signal strength with APCI . Although most mass spectra of carotenoids have been acquired using positive ion mode, negative ion mode has also been reported . An interesting article about negative ion tandem mass spectra of carotenoids as well as their APCI pathways has been recently published by van Breemen et al. .

This review compiles positive MS fragmentation data of selected carotenoids in order to provide a basis for their structural elucidation, using mainly liquid chromatography (LC)-MS techniques. In addition, the mechanisms through which significant carotenoid fragment ions form are also discussed.

II. EXPERIMENTAL PART

On the basis of our previous work and information from the literature about characteristic carotenoid ions , we monitored the presence of these *m/z* ions in samples derived from transgenic maize seeds and rice callus. Samples were analyzed using an UHPLC-APCI-MS/MS system and carotenoid standards.

A. Materials and methods

Chemicals

β-Carotene, lycopene, lutein, *β*-cryptoxanthin, astaxanthin, *β*-apo-8′-carotenal were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Canthaxanthin and zeaxanthin were acquired from Fluka (Buchs SG, Switzerland). Phytoene, violaxanthin, neoxanthin, and

antheraxanthin were purchased from Carotenature (Lupsingen, Switzerland). Methanol, ethyl acetate, ethyl eter, *tert*-butyl methyl ether, acetonitrile and acetone (HPLC grade purity) were acquired from J.T. Baker (Deventer, The Netherlands). Water was prepared using a Milli-Q reagent water system.

Plant material

Transgenic maize and rice callus, expressing several carotenogenic genes, were selected to carry out this experiment. The transgenic maize seeds and rice callus were generated by combinatorial nuclear transformation as reported in Zhu et al. . The extractions were performed as described by Rivera et al. .

UHPLC-APCI-MS/MS analysis

UHPLC analysis was carried out using an ACQUITY Ultra Performance LC™ system. Mass detection was carried out using an Acquity™ TQD tandem-quadrupole MS equipped with a Z-spray electrospray interface (Manchester, UK). MassLynx™ software version 4.1 (Waters, Milford, MA, USA) was used to control the instruments, and also for data acquisition and processing. UHPLC chromatographic separations were performed on reversed-phase column ACQUITY UPLC® C18 BEH 130Å, 1.7 μm, 2.1×100 mm (Waters, Milford, MA). Mobile phase consisted of solvent A: ACN: MeOH 7:3, v/v and solvent B: water 100%. The gradient program used is shown in Table 1. The column and sample temperatures were set at 32 °C and 25 °C respectively. Injection volume was 5 μL. Optimized MS conditions are listed in Table 2.

TABLE 1. Gradient profile used in the separation of carotenoids by UHPLC. Linear gradient

Time[a] (min)	Flow rate (mL/min)	A (%, v/v)	B (%, v/v)
Initial	0.4	80	20
2.0	0.4	80	20
3.0	0.4	100	0
7.0	0.4	100	0
8.0	0.6	100	0
11.6	0.6	100	0
12.6	0.4	80	20

[a] After this time, the system was left 2 min more to reach its re-equilibration before injecting a new sample.

Each sample extract for LC analysis was dissolved in 300 μL and 1000 μL (for light and dark color extract respectively) of the injection solvent [ACN: MeOH 7:3, v/v]: acetone 6.7:3.3, v/v. Before use, all solutions were filtered through Millex 0.2 μm nylon membrane syringe filters (Millipore, Bedford, MA, USA).

TABLE 2. MS conditions

MS conditions	APCI
Polarity	Positive
Corona (kV)	4.0
Cone (V)	30
Extractor (V)	3
RF (V)	0.1
Source temperature (°C)	150
Probe temperature (°C)	450
Cone gas flow (L/h)	10
Desolvation gas flow (L/h)	150
Collision gas flow (mL/min)	0.15

III. IONIZATION TECHNIQUES USED FOR CAROTENOID DETECTION

Fragmentation patterns are strongly dependent on the chemical and physical properties of the analytes and the ionization technique used . In addition, when using soft ionization (e.g. ESI,

APCI, APPI, FAB), factors such as the mobile phase, the modifiers added (acids, bases, dopants, metals and salts), the experimental conditions (collision energy, flow collision gas, temperatures, etc.), and the instrument design also affect the ionization of the analytes . Therefore, the mass spectrum of a given compound should not be expected to exactly match a published spectrum.

We conducted a literature survey to collect fragments of 21 carotenoids obtained with various ionization techniques (EI, ESI, APCI, APPI and FAB). The samples were obtained from diverse materials. Spectra information comes from publications on applications of MS, MS/MS, LC/MS and LC/MS/MS for carotenoid detection in positive ion mode. Thus, Table 3 summarizes two types of ions: i) those based on ions resulting from the initial ionization of the whole molecule and ii) those ions arising directly from the precursor ions, which produce a fingerprint pattern specific to the compound under investigation. For example, van Breemen et al. observed that lycopene, γ-carotene, β-carotene, and α-carotene produced the molecular radical ion at m/z 536 during FAB ionization in positive ion mode. However, during collisionally activated dissociation (CAD) only the molecular ion of α-carotene formed unique fragment ions at m/z 388 and 480, corresponding to $[M-148]^{+\bullet}$ and $[M-56]^{+\bullet}$ respectively . In many cases, identical carotenoid fragment ions were observed in all techniques such as the ion at *m/z* 221 for violaxanthin and auroxanthin; *m/z* 205 for 5,6-epoxy-β-carotene; *m/z* 69 for echinenone; *m/z* 119 for β-apo-8'-carotenal; and *m/z* $[M-92]^{+\bullet}$ or/and $[M+H-92]^{+}$ for 5,6-epoxy-β-carotene, α-cryptoxanthin, zeaxanthin, β-cryptoxanthin, γ-carotene, α-carotene and β-carotene. However, differences among the techniques were also observed. All carotenoids ionized by APCI showed the protonated molecule $[M+H]^{+}$ whereas those ionized by EI and FAB showed the molecular ion $[M]^{+\bullet}$, as expected considering the ionization method. For ESI and APPI, the molecular ion species observed was either the protonated molecule $[M+H]^{+}$, the molecular ion $[M]^{+\bullet}$, or both (see Table 3). The high polyene conjugation and the presence of oxygen in these molecules, as well as the solvent system , have a significant influence on the stability and formation of molecular and protonated ion species. ESI analysis of violaxanthin in MeOH:H_2O (20:80, v/v) solution showed a predominant molecular ion $[M]^{+\bullet}$, whereas the use of H_2O/MeOH/TBME (4:61:35, v/v/v) as mobile phase led to the predominance of the protonated molecule in the MS spectrum of this oxygen-functionalized carotenoid . The effect of the mobile phase composition on LC-APPI-MS has also been described . Generally, polar solvents, such as alcohols, lead to an increased abundance of protonated carotenoids, while less polar solvents, such as *tert*-butyl methyl ether, facilitate the formation of more abundant molecular ions .

Another technique-dependent difference was the absence or presence of particular fragment ions. For example, the *m/z* $[M-80]^{+\bullet}$ fragment was detected for epoxy carotenoids ionized by EI or FAB. In contrast, the equivalent fragment was not found using APCI and ESI (see Table 3). Table 3 shows that the loss of 18 mass units in hydroxycarotenoids, $[M+H-18]^{+}$ or $[M-18]^{+\bullet}$ was usually observed in all the ionization techniques but not using FAB . In addition, Azevedo et al. reported that the $[M-18]^{+\bullet}$ fragment was not present in the EI-MS spectrum of zeaxanthin or β-cryptoxanthin . This finding contrasts with other reports in the literature utilizing the same technique but different instruments . Thus, even when using the same ionization technique, differences can be observed in the intensities or presence/absence of fragment ions. Of note, in addition to the factors mentioned above, the matrices in which the analytes are detected might also affect the ionization process and lead to inaccurate quantitation (Chiu et al., 2010).

Finally, the reader should be aware that soft ionization techniques used in LC-MS analysis such as ESI, APCI and APPI lead to positive ions based on the protonation of the corresponding molecules. In turn, these non radical ions can lead to even mass ions. Those ions must be odd-electron species. In contrast, uneven mass ions provided by the same protoned molecules must be paired-electron species. These behavior are the opposite resulting from the molecular radical ion, the one usually obtained using EI or FAB ionization techniques.

195 **TABLE 3.** Carotenoid fragments obtained with distinct ionization techniques

Neoxanthin (epoxycarotenoid)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	600	584 [M-16], 582 [M-18], 566 [M-16-18], 564 [M-18-18], 520 [M-80], 508 [M-92], 502 [M-18-80], 221,181	
EI	$[M]^{+\bullet}$	600	582 [M-18], 564 [M-18-18], 520 [M-80], 502 [M-18-80], 352, 221,181, 105	
EI	$[M]^{+\bullet}$	600	221,181, 172	
EI	$[M]^{+\bullet}$	600	520 [M-80], 419 [M-181], 352, 221, 181, 172	
FAB	$[M]^{+\bullet}$	600	449, 393, 391, 339, 337, 313, 171, 69	
ESI	$[M+H]^{+}$	601	583 [M+H-18], 565 [M+H-18-18], 547 [M+H-18-18-18], 509 [M+H-92], 491 [M+H-18-92], 393, 221	
ESI	$[M+H]^{+}$	601	583 [M+H-18], 565 [M+H-18-18], 221, 181	
ESI	$[M+H]^{+}$ $[M]^{+\bullet}$	601.5 600.1	583.2 [M+H-18], 565.3 [M+H-18-18], 509.5 [M+H-92], 221.1	
ESI	$[M+H]^{+}$	601.4	167.3, 105.3	
APCI	$[M+H]^{+}$	601	583 [M+H-18], 565 [M+H-18-18], 547 [M + H-18-18-18], 509 [M+H-92], 491 [M+H-18-92], 393, 221	
APCI	$[M+H]^{+}$	601	583 [M+H-18], 565 [M+H-18-18], 547 [M+H-18-18-18], 509 [M+H-92], 221	
APCI	$[M+H]^{+}$	601	583 [M+H-18], 565 [M+H-18-18], 547 [M+H-18-18-18], 509 [M+H-92], 491 [M+H-18-92], 221	
APCI	$[M+H]^{+}$	601.4	583.4 [M+H-18], 167.3	
APPI	$[M+H]^{+}$	601.4	167.3, 105.3	

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Auroxanthin (furanoid oxide)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
FAB	$[M]^{+\bullet}$	600	582 [M-18], 520 [M-80], 419, 379, 352, 287, 247, 221, 181	
ESI	$[M+H]^{+}$ $[M]^{+\bullet}$	601.5 600.1	583.5 [M+H-18], 565.5 [M+H-18-18], 509.5 [M+H-92], 491.5 [M+H-18-92], 221.1	
APCI	$[M+H]^{+}$	601	583 [M+H-18], 565 [M+H-18-18], 547 [M+H-18-18-18], 509 [M+H-92], 491 [M+H-18-92], 221	

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Violaxanthin (epoxycarotenoid)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	600	520 [M-80], 440 [M-80-80], 352,	

			221,181, 105	
EI	$[M]^{+\bullet}$	600	520 [M-80], 352, 221, 181	
EI	$[M]^{+\bullet}$	600	520 [M-80], 502 [M-80-18], 352, 221, 181, 172	
FAB	$[M]^{+\bullet}$	600	582 [M–18], 520 [M–80], 419, 352, 221	
ESI	$[M+H]^{+}$	601	583 [M+H-18], 565 [M+H-18-18], 547, 509 [M+H-92], 491 [M+H-18-92], 221	
ESI	$[M+H]^{+}$	601	583 [M+H-18], 565 [M+H-18-18], 221, 181	
ESI	$[M+H]^{+}$ $[M]^{+\bullet}$	601.5 600.1	583.5 [M+H-18], 565.5 [M+H-18-18], 509.5 [M+H-92], 491.5 [M+H-18-92], 221.1	
ESI	$[M+H]^{+}$	601.3	221.3, 93.1	
APCI	$[M+H]^{+}$	601	583 [M+H-18], 565 [M+H-18-18], 509 [M+H-92], 491 [M+H-18-92], 221	
APCI	$[M+H]^{+}$	601	583 [M+H-18], 565 [M+H-18-18], 509 [M+H-92], 491 [M+H-18-92], 221	
APCI	$[M+H]^{+}$	601	583 [M+H-18], 565 [M+H-18-18], 547 [M+H-18-18-18], 491 [M+H-18-92], 221	
APCI	$[M+H]^{+}$	601.3	133.3, 93.1	
APPI	$[M+H]^{+}$	601.3	221.3, 93.1	

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Antheraxanthin (epoxycarotenoid)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	584	566 [M–18], 504 [M-80], 492 [M–92], 352, 221	
FAB	$[M]^{+\bullet}$	584	566 [M–18], 504 [M–80], 492 [M–92], 404, 352, 221, 181	
ESI	$[M+H]^{+}$	585	567 [M+H-18], 549 [M+H-18-18], 493 [M+H-92], 475 [M-92-18], 221	
ESI	$[M+H]^{+}$	585.3	105.2, 93.1	
APCI	$[M+H]^{+}$	585	567 [M+H-18], 549 [M+H-18-18], 493 [M+H-92], 221	
APCI	$[M+H]^{+}$	585	567 [M+H-18], 549 [M+H-18-18], 221	
APCI	$[M+H]^{+}$	585.3	105.2, 93.1	
APPI	$[M+H^{+}$	585.3	145.2, 119.1	

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5,6-Epoxy-*β*-carotene (epoxycarotenoid)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	552	472 [M-80], 460 [M-92], 336, 236, 205, 135, 121	
EI	$[M]^{+\bullet}$	552	205, 165	
ESI	$[M+H]^{+}$	553	535 [M+H-18], 461 [M+H-92], 205	
APCI	$[M+H]^{+}$	553	535 [M+H-18], 461 [M+H-92], 205	

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Zeaxanthin (hydroxycarotenoid)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	568	550 [M-18], 476 [M-92], 462 [M-106], 133, 109, 91, 69, 43	
EI	$[M]^{+\bullet}$	568	462 [M-106], 153	
FAB	$[M]^{+\bullet}$	568	553, 551 [M-17], 489 [M-79], 476 [M-92], 445, 415 [M-153]	
ESI	$[M+H]^{+}$	569	551, 533 [M+H−18−18], 463 [M+H-106]	
ESI	$[M+H]^{+}$ $[M]^{+\bullet}$	568.9 567.9	550.9 [M+H−18], 532.9 [M+H−18−18], 476.4 [M+H–92]	
ESI	$[M]^{+\bullet}$	568	476.6 [M–92], 283.2	
APCI	$[M+H]^{+}$	569	551 [M+H-18], 533 [M+H-18-18], 463 [M+H-106]	
APCI	$[M+H]^{+}$	569.4	551.5 [M+H–18], 135	
APCI	$[M+H]^{+}$	569.4	551.4 [M+H−18], 477.4 [M+H–92], 459.4 [M+H-18-92], 416.3 [M+H-153], 175.2, 135.1, 119.1	
APCI	$[M+H]^{+}$	569	551 [M+H−18], 533 [M+H−18−18], 459 [M+H-18-92], 416 [M+H-153], 175	
APPI	$[M+H]^{+}$	569.4	477.6 [M+H–92], 135	

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α-Cryptoxanthin (hydroxycarotenoid)

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β-Cryptoxanthin (hydroxycarotenoid)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	552	534 [M-18], 460 [M–92], 442, 105	
EI	$[M]^{+\bullet}$	552	460 [M–92], 446 [M-106], 399 [M-153]	
FAB	$[M]^{+\bullet}$	552	537, 535 [M-17], 460 [M-92], 445	
ESI	$[M+H]^{+}$	553	535 [M+H−18], 497, 461 [M+H–92]	
ESI	$[M+H]^{+}$	553.6	461.6 [M+H-92], 119	
APCI	$[M+H]^{+}$	553	535 [M+H-18], 495, 461 [M+H-92]	
APCI	$[M+H]^{+}$	553	535 [M+H-18], 495, 461 [M+H-92]	
APCI	$[M+H]^{+}$	553	535 [M+H-18]	
APCI	$[M+H]^{+}$	553.6	461.6 [M+H-92], 119	
APCI	$[M+H]^{+}$	553.4	535.4 [M+H−18], 461.4 [M+H–92], 400.3 [M+H-153], 177.2, 135.1, 119.1	
APPI	$[M]^{+\bullet}$	552.6	460.6 [M-92], 119	

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Lutein (hydroxycarotenoid)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	568	550 [M-18], 512 [M-56], 476 [M-92], 462 [M-106], 458 [M-92-18], 415 [M-153], 392, 324, 133, 109, 91, 83, 69, 43	
EI	$[M]^{+\bullet}$	568	550 [M-18], 476 [M-92], 193, 153	
EI	$[M]^{+\bullet}$	568	550 [M-18], 476 [M-92], 415 [M-153], 193, 153	
FAB	$[M]^{+\bullet}$	568	476 [M-92], 428	
ESI	$[M+H]^{+}$	569	551 [M+H−18], 533 [M+H−18−18], 463 [M+H-106]	
ESI	$[M+H]^{+}$	569	551 [M+H-18], 533 [M+H-18-18], 416 [M+H-153], 376	
ESI	$[M+H]^{+}$ $[M]^{+\bullet}$	568.9 567.9	550.9 [M−18], 532.9 [M−18−18], 476.4 4 [M–92], 429.4	
ESI	$[M]^{+\bullet}$	568	476.6, 283.2	
ESI	$[M]^{+\bullet}$	568.5	550.5 [M−18], 476.5, 429.5	
APCI	$[M+H]^{+}$	569	551 [M+ H-18], 533 [M+H−18−18], 477 [M+H-92], 463 [M+H-106], 459 [M+H-18-92]	
APCI	$[M+H]^{+}$	569.4	551.5, 135	
APCI	$[M+H]^{+}$	569.4	551.4 [M+H−18], 495.4 [M+H−18−56], 477.4 [M+H–92], 459.4 [M+H−18−92], 430.3, 416.3 [M+H-153], 175.2, 119.1, 135.1	
APCI	nd	nd	551 [M+H−18], 533 [M+H−18−18], 495 [M+H−18−56], 459 [M+H−18−92], 430, 175	
APPI	$[M+H]^{+}$ $[M]^{+\bullet}$	569.4 568	338.5, 135	

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Astaxanthin (ketocarotenoid)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	596	580, 564 [M-16-16], 504 [M-92], 490 [M-106], 445, 133, 109, 91, 83, 69, 59, 43	
FAB	$[M]^{+\bullet}$	596	579 [M-17], 472 [M-92], 550, 540, 443, 429	
ESI	$[M+H]^{+}$	597.6	505.4 [M+H-92], 147.1	
APCI	$[M+H]^{+}$	597.6	579.6 [M-18], 147.1	
APCI	$[M+H]^{+}$	597.4	579.4 [M+H−18], 379.3, 285.2, 201.1, 173.1, 147.1	
APCI	$[M+H]^{+}$	597	579 [M+H−18], 561 [M-18-18], 505 [M-92], 379, 285	
APPI	$[M+H]^{+}$	597.6	579.6 [M-18], 147.1	
Canthaxanthin (ketocarotenoid)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	564	549 [M-15], 508, 472 [M-92], 485, 458 [M-106], 426, 406, 361, 133, 109, 91, 83, 69, 43	
FAB	$[M]^{+\bullet}$	564	472 [M-92], 430, 363	
APCI	$[M+H]^{+}$	565	nd	

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Echinenone (ketocarotenoid)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	550	535 [M-15] 558 [M-92], 471, 444 [M-106], 392, 347, 133, 91, 69	
EI	$[M]^{+\bullet}$	550	558 [M-92], 444 [M-106]	
ESI	$[M+H]^{+}$	551.6	93, 69	
APCI	$[M+H]^{+}$	551.6	93, 69	
APCI	$[M+H]^{+}$	551.4	471.4 [M+H–80], 536.4, 495.4, 459.4 [M+H−92], 255.2, 203.1, 133.1	
APPI	$[M]^{+\bullet}$	550.6	93, 69	

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β-Apo-8'-carotenal (oxocarotenoid)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	416	324 [M-92], 310 [M-106], 309, 281, 267, 251, 239, 209, 195, 183, 166, 157, 145, 133, 119, 105, 91, 82, 69, 55, 41	
FAB	$[M]^{+\bullet}$	416	360, 347, 337 [M-79], 333, 279, 267, 143, 119, 105	
APCI	$[M+H]^{+}$	417.5	325.3 M+H-92], 94.9	
APCI	$[M+H]^{+}$	417.3	399.3 [M+H-18], 389.3, 361.3, 338.3, 325.3 [M+H-92], 293.2, 157.1, 119.1, 95.1	
APPI	$[M+H]^{+}$	417.5	145.2, 119	

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Lycopene (carotene)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	536	467 [M-69], 444 [M-92], 430 [M-106], 133, 109, 91, 83, 69, 43	
EI	$[M]^{+\bullet}$	536	467 [M-69], 444 [M-92], 430 [M-106], 69	
EI	$[M]^{+\bullet}$	536	467 [M-69], 444 [M-92], 430 [M-106]	
FAB	$[M]^{+\bullet}$	536	467 [M-69], 444 [M-92], 209, 185, 183, 171, 169, 157, 145, 143, 131, 119, 105, 91	
ESI	$[M]^{+\bullet}$	536.7	444.7 [M-92], 69	
ESI	$[M]^{+\bullet}$	536.4	467.4 [M-69] (80), 444.4 [M-92]	
APCI	$[M+H]^{+}$	537	467 [M-69], 444 [M-92]	
APCI	$[M+H]^{+}$	537.7	93, 69	
APCI	$[M+H]^{+}$	537.4	457 [M+H–80], 413.3, 177.2, 137.1, 121.1	
APPI	$[M]^{+\bullet}$	536.7	93, 69	

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Phytofluene (carotene)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	542	405 [M-137], 337 [M-205]	
EI	$[M]^{+\bullet}$	542	405 [M-137], 337 [M-205], 137 [M-405], 69	
FAB	$[M]^{+\bullet}$	542	337 [M-205], 448 [M-94], 405 [M-137]	

ESI	$[M+H]^+$	543	406 [M+H-137], 338 [M+H-205]	
ESI	$[M]^{+\bullet}$	542.5	93, 69	
APCI	$[M+H]^+$	543	461, 406 [M+H-137], 338 [M+H-205]	
APCI	$[M+H]^+$	543	461, 406 [M+H-137], 338 [M+H-205]	
APCI	$[M+H]^+$	543.5	93, 69	
APPI	$[M]^{+\bullet}$	542.5	93, 69	

Phytoene (carotene)				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	544	450 [M-94], 339 [M-205]	
EI	$[M]^{+\bullet}$	544	339 [M-205], 137 [M-407], 69	
FAB	$[M]^{+\bullet}$	544	339 [M-205], 450 [M-94], 407 [M-137]	
ESI	$[M+H]^+$	545	339 [M-205]	
ESI	$[M]^{+\bullet}$	544.5	81, 69	
APCI	$[M+H]^+$	545	489, 339 [M-205], 435	
APCI	$[M+H]^+$	545.5	81, 69	
APPI	$[M]^{+\bullet}$	544.5	81, 69	

***β*-Zeacarotene (carotene)**				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	538	446 [M-92], 401 [M-137]	
EI	$[M]^{+\bullet}$	538	401 [M-137], 309 [M-137-92]	
APCI	$[M+H]^+$	539	455, 402 [M+H-137], 310 [M+H-137-92]	

***γ*-Carotene (carotene)**				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	536	467 [M-69], 444 [M-92], 375 [M-92-69], 361 [M-106-69], 430 [M-106]	
EI	$[M]^{+\bullet}$	536	467 [M-69], 444 [M-92], 430 [M-106]	
FAB	$[M]^{+\bullet}$	536	467 [M-69], 444 [M-92]	
APCI	$[M+H]^+$	537	467 [M-69], 444 [M-92]	
APCI	$[M+H]^+$	537.4	457.4 [M+H–80], 399.3, 269.2, 255.2, 177.2, 145.1, 137.1 [M+H-400], 119.1	

***δ*-Carotene (carotene)**				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	536	480 [M-56], 467 [M-69], 444 [M-92], 430 [M-106], 388, 375, 361	
APCI	$[M+H]^+$	537	481 [M+H-56], 444 [M-92]	

***α*-Carotene (carotene)**

Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	536	480 [M-56], 444 [M-92], 430 [M-106], 388, 378, 374	
EI	$[M]^{+\bullet}$	536	444 [M-92]	
FAB	$[M]^{+\bullet}$	536	521 [M-15], 480 [M-56], 444 [M-92], 388 [M-56-92]	
ESI	$[M]^{+\bullet}$	536.4	536.4, 444.4 [M-92], 177.4, 137.4 [M-399]	
APCI	$[M+H]^{+}$	537	481 [M+H-56], 444 [M-92]	
APCI	$[M+H]^{+}$	537	481 [M+H-56], 444 [M-92]	
APCI	$[M+H]^{+}$	537	481 [M+H-56], 444 [M-92]	
APCI	$[M+H]^{+}$	537.4	457.4 [M+H−80], 413.3, 177.2, 137.1, 123.1	

***β*-Carotene (carotene)**				
Ionization technique	Molecular ion species		Fragments (*m*/*z*)	Ref.
EI	$[M]^{+\bullet}$	536	444 [M-92], 430 [M-106], 119, 109, 91, 83, 69, 43	
EI	$[M]^{+\bullet}$	536	444 [M-92], 430 [M-106], 399 [M-137], 137 [M-399], 105	
EI	$[M]^{+\bullet}$	536	444 [M-92], 430 [M-106], 399 [M-137]	
FAB	$[M]^{+\bullet}$	536	521 [M-15], 489, 444 [M-92], 429 [M-92-15], 399 [M-137], 157, 143, 119, 105, 91, 69, 55	
ESI	$[M+H]^{+}$	537	444 [M-92]	
ESI	$[M+H]^{+}$	537	445 [M-92], 203, 177, 149, 137 [M+H-400], 123	
ESI	$[M+H]^{+}$ $[M]^{+\bullet}$	536.9 535.9	444.2 [M-92], 430.3 [M-106], 399.3 [M-137]	
ESI	$[M]^{+\bullet}$	536.7	444.7 [M-92], 69	
ESI	$[M]^{+\bullet}$	536.4	444.4 [M-92], 177.4, 137.4 [M-399]	
APCI	$[M+H]^{+}$	537	444 [M-92]	
APCI	$[M+H]^{+}$	537.7	445.7 [M-92], 119	
APCI	$[M+H]^{+}$	537.4	457.4 [M+H−80], 445.4 [M+H–92], 413.3, 400.3 [M+H-137], 269.2, 177.2, 137.1 [M+H-400]	
APCI	$[M+H]^{+}$	537	444 [M-92], 413 [M-124], 400 [M-137], 269, 177	
APPI	$[M]^{+\bullet}$	536.7	444.7 [M-92], 119	

nd: not detected

IV. CHARACTERISTIC CAROTENOID FRAGMENTATION PATTERNS

A. Improvements in the detection of carotenoids using APCI-MS/MS

Since MS/MS can provide high sensitivity and selectivity for the identification and quantitative analysis of carotenoids in biological samples, we evaluated several characteristic transitions to analyze these molecules. Transitions to distinguish between carotenoids were monitored considering the *m*/*z* value of protonated molecules as precursor ions, and the *m*/*z* value of ions reported in the literature as product ions. All analyses were carried out in positive ion mode. Table 4 summarizes the best transitions encountered to identify

carotenoids. Figure 1 shows the structures of the carotenoids indicated in Table 4. Common carotenoid product ions among pigments bearing the same functional group were observed (see Table 4). The *m/z* 147 product ion was observed in ketocarotenoids containing a hydroxyl group in carbon 3, (3') and a keto group in carbon 4, (4') in the *β*-ring. In addition, the transition obtained with this ion corresponded to the strongest signal strength for these compounds. The *m/z* 203.1 product ion was given by echinenone, canthaxanthin and adonirubin. This signal is characteristic of carotenoids containing a keto group (as the only substituent on the *β*-ring) conjugated to the polyene chain. The *m/z* 135.1 product ion was characteristic of hydroxylated carotenoids, such as adonixanthin, lutein, zeaxanthin and *β*-cryptoxanthin. Other characteristic fragment ions reported in the literature (see Table 3) were also monitored to determine possible useful transitions. For example, the $[M+H-92]^+$ product ion was detected in *α*-cryptoxanthin and *β*-zeacarotene. The loss of a water molecule $[M+H-18]^+$ was diagnostic for pigments such as neoxanthin and astaxanthin.

TABLE 4. Precursor and product ions obtained by APCI, in positive ion mode, for carotenoids exhibiting distinct functional groups

Funtional group	Carotenoid	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision Energy (V)
Epoxycarotenoids	Neoxanthin	601.4	167.2(Q2)	20
	Neoxanthin	601.4	583.4(Q1)	10
	Violaxanthin	601.4	93(Q1)	45
	Violaxanthin	601.4	133.3(Q2)	40
	Antheraxanthin	585.3	93.1(Q1)	55
	Antheraxanthin	585.3	105.2(Q2)	45
Ketocarotenoids	Astaxanthin	597.6	**147**[a](Q1)	40
	Astaxanthin	597.6	579.6(Q2)	15
	Adonixanthin	583.4	**135.1**[a](Q2)	40
	Adonixanthin	583.4	**147**[a](Q1)	40
	Adonirubin	581.5	**147**[a](Q1)	40
	Adonirubin	581.5	**203.1**[a](Q2)	40
	Canthaxanthin	565.9	69(Q2)	40
	Canthaxanthin	565.9	**203.1**[a](Q1)	40
	3-Hydroxyechinenone	567.3	93(Q2)	50
	3-Hydroxyechinenone	567.3	**147**[a](Q1)	40
	Echinenone	551.6	69(Q1)	45
	Echinenone	551.6	93(Q2)	35
	Echinenone	551.6	**203.1**[a](Q3)	40
Hydroxycarotenoids	Lutein	569.4	69(Q1)	40
	Lutein	569.4	**135.1**[a](Q2)	30
	Zeaxanthin	569.4	93(Q2)	40
	Zeaxanthin	569.4	**135.1**[a](Q1)	30
	α-Cryptoxanthin	553.6	119(Q2)	35
	α-Cryptoxanthin	553.6	461.6(Q1)	15
	β-Cryptoxanthin	553.6	119(Q1)	35
	β-Cryptoxanthin	553.6	**135.1**[a](Q2)	30
Carotenes	Lycopene	537.7	69(Q1)	40
	Lycopene	537.7	93(Q2)	50
	β-Zeacarotene	539.6	69.3(Q1)	35
	β-Zeacarotene	539.6	447(Q2)	10
	α-Carotene	537.6	95.1(Q2)	35
	α-Carotene	537.6	123.1(Q1)	40
	β-Carotene	537.6	68,9(Q2)	40

	β-Carotene	537.6	95.1(Q1)	35
	phytofluene	543.5	69(Q1)	45
	phytofluene	543.5	93(Q2)	45
	Phytoene	545.5	69(Q2)	35
	Phytoene	545.5	81(Q1)	35

[a] Fragments in bold are common product ions among pigments bearing the same functional group

B. Usefulness of intensity fragment ratios to distinguish carotenoids in APCI-MS

Carotenoids with a very similar structure can be differentiated by comparing the intensities of specific fragments. Figure 2A shows that in the MS spectrum of lutein, the fragment $[M+H-18]^+$ at *m/z* 551 is a much more abundant ion than the corresponding protonated molecule (*m/z* 569). However, zeaxanthin exhibits the opposite behavior. Lutein and zeaxanthin are isomers, which differ in the location of the double bond in one of the end rings. This difference gives lutein three chiral centers whereas zeaxanthin has two. In addition, this difference causes the OH functional group to be located in the allylic position of the *ε*-ring in lutein, while in zeaxanthin it is located in the *β*-ring and thus not in an allylic position (see Figure 1). The loss of water as a result of the presence of the hydroxyl group in an allylic position (a hydroxyl group located in *ε*-ring) produces the $[M+H-18]^+$ ion, which is stabilized by mesomeric effects (see Figure 2A). Consequently, this ion should be more stable than that formed by the loss of water caused by the presence of a non-allylic hydroxyl group located in the *β*-ring. The increase in ion stability promotes the loss of water and enhances the corresponding fragment ion. This mass spectrometric behavior was used to confirm the identity of carotenoids such as lutein epoxide and antheraxanthin , and zeinoxanthin and *α*-cryptoxanthin .

Similarly, Figure 2B indicates that *α*- and *β*-carotene exhibit equivalent transitions in the positive ion mode (e.g., 537.6>445.6, 537.6>123.1, 537.6>119 and 537.6>95.1). However, the most intense transition for *α*-carotene corresponded to the transition 537.6>123.1, which was also observed in *β*-carotene but with a lower intensity. *α*-Carotene differs from *β*-carotene only in the position of a double bond in one of the terminal rings, an *α*-ionone moiety (see Figure 1). The formation of the ion at *m/z* 123.1 was facilitated by the position of the double bond in the terminal ring in *α*-carotene, which enhances the stabilization of the resulting carbocation (see Figure 2B). Comparison of the intensities of these ions might allow the identification of those carotenoid structures with an *ε*-ring. Although some carotenoids show the same or a very similar fragmentation pattern (meaning that their structures are similar and therefore they might co-elute), differences between the intensities of their fragments can be used to distinguish these molecules. Moreover, these differences can provide an insight into the predominant carotenoid when co-elution occurs.

Considering the above, in addition to the published MS spectra of known carotenoids (see Table 3), as well as the new information generated as a result of our own experiments the following fragments are put forward to facilitate the structural elucidation of these compounds.

C. Hydroxycarotenoids

Figure 3 illustrates the characteristic fragment ions given by hydroxycarotenoids.

Presence of a hydroxyl group:

The typical fragment ion given by a compound bearing a hydroxyl group is the loss of a molecule of water or a hydroxyl group $[M+H-18]^+$, $[M-18]^{+\bullet}$, or $[M-17]^+$. These fragments ion were observed for astaxanthin, zeaxanthin, auroxanthin, neoxanthin, violaxanthin, antheraxanthin, *β*-cryptoxanthin, *α*-cryptoxanthin and lutein (see Table 3) and has been obtained using EI , FAB , ESI and APCI . As discussed earlier, the *m/z* 135.1 product ion was also characteristic of hydroxycarotenoids and corresponds to the dehydrated terminal ring

with cleavage between carbons 7 and 8 . In addition, lutein, zeaxanthin and *β*-cryptoxanthin exhibited a loss of 153 units (see Table 3), indicating pigments containing a *β*-ring containing a hydroxyl group (cleavage between carbons 7 and 8 of the polyene chain) . The $[M-153]^+$ and/or $[M+H-153]^{+\bullet}$ ion were detected using EI , ESI , FAB and APCI .

Presence of a hydroxyl group in an allylic position:

For hydroxycarotenoids with a hydroxyl group in an allylic position, the loss of a water molecule results in a fragment ion that is commonly the base peak in the APCI-MS spectrum .

Several authors concluded that the fragment ions at *m/z* 428, 429 and/or 430 observed in the MS/MS spectrum of lutein can be used to distinguish this pigment from its isomer zeaxanthin. These ions have been obtained using ESI , APCI and FAB , in both negative and positive ion mode. These fragments are generated by the loss of the terminal ring containing the unconjugated carbon-carbon double bond present in lutein .

D. Epoxycarotenoids and furanoid oxides

Presence of an oxirane or oxolane fused to an end ring:

EI and FAB are very useful for the characterization of carotenoids containing an oxirane or oxolane fused to an end ring. The mass spectra of carotenoids containing both 5,6-epoxy and 5,8-furanoid groups exhibited characteristic fragment ions at *m*/*z* $[M-80]^{+\bullet}$ or $[M-80-80]^{+\bullet}$, corresponding to the loss of one or two dimethylcyclobutadienes, respectively. Figure 4 shows the proposed mechanism for the formation of the ion at *m*/*z* $[M-80]^{+\bullet}$ from both an epoxy carotenoid and a furanoid oxide.
Mass fragments at *m*/*z* 165 (EI) and 205 (EI, ESI and APCI) indicate the presence of an epoxy group as the only substituent on the *β*-ring. The cleavage between carbons 8 and 9, and 10 and 11, respectively leads to these two fragment ions .

Presence of an oxirane or oxolane fused to a hydroxylated end ring:

In addition to the fragment indicated above, ions at *m*/*z* 352 and 181 indicate that the epoxide is located in a ring with a hydroxyl substituent. These ions correspond to cleavages between carbons 12’ and 13’, and 8 and 9 of the polyene chain respectively, . These ions were observed using EI and FAB. The ion at *m*/*z* 181 was also confirmed by ESI (see Table 3). In contrast, the ion at *m*/*z* 221 was observed in all the techniques (see Table 3) and corresponds to the oxocycle fused to the 3-hydroxy-*β*-ring. This fragment is produced by cleavage between carbons 10 and 11 (or 10’ and 11’) (see Figure 5). In addition to these ions, neoxanthin gives a fragment at *m*/*z* 393, resulting from the cleavage of the double bond allylic to the allenic carbon . Thus, this diagnostic ion can be used to distinguish neoxanthin from its structural isomer violaxanthin. This fragment has been obtained using ESI , APCI and FAB .

E. Ketocarotenoids

Figure 6 shows the most representative fragment ions for ketocarotenoids.

Presence of an oxo group in an end ring

The *m/z* 203.1 ion is characteristic of carotenoids containing a keto group as the only substituent on the *β*-ring (see Table 4). van Breemen et al. proposed that this ion is formed by cleavage between carbons 10 and 11 with the positive charge remaining at the ketone moiety .

Presence of an oxo group in a hydroxylated end ring

The ion at *m/z* 147 is typical of ketocarotenoids containing a hydroxyl group at carbon 3, (3’) and a keto group at carbon 4, (4’) in the same *β*-ring (see Table 4). van Breemen et al. suggested that the fragment at *m/z* 147 corresponds to a dehydrated terminal ring with cleavage between carbons 7 and 8 .

F. Polyene chain

Presence of conjugation within the molecule

Table 3 shows that most of the carotenoids produce the fragment ion at *m/z* $[M-92]^{+\bullet}$ and/or $[M+H-92]^{+}$, which corresponds to loss of toluene. This fragmentation indicates the presence of extensive conjugation within the molecule. In contrast, the fragment ion at *m/z* $[M-94]^{+\bullet}$ was detected only in the mass spectra of phytoene and phytofluene (see Table 3). This finding therefore suggests that the extent of conjugation in these compounds is much lower than in lycopene or *β*-carotene . Similarly, loss of xylene, $[M-106]^{+\bullet}$ and/or $[M+H-106]^{+}$ is also indicative of the cyclization of fragments of the polyene chain, without the involvement of the *β*-ionone ring. These ions have been detected using EI ; , FAB , ESI , APPI and APCI . Using EI, the intensities of the peaks at *m/z* $[M-92]^{+\bullet}$ and $[M-106]^{+\bullet}$ have been related to the number of conjugated double bonds in the acyclic polyene chain. The $[M-92]^{+\bullet}/[M-106]^{+\bullet}$ ratio has been proposed as a reliable indicator of the chain length of carotenoids containing no more than one oxygen substituent in each end group . Figure 7 shows the proposed mechanisms for the formation of several fragments related to the presence of extensive conjugation within the carotenoid structure.

The fragment ion at *m/z* 119 was observed in antheraxanthin, lutein, *α*-cryptoxanthin, zeaxanthin, *β*-cryptoxanthin, lycopene, *β*-apo-8'-carotenal, *γ*-carotene, and *β*-carotene using a number of ionization techniques (see Tables 3 and 4). Moreover, the fragment ions at *m/z* 133, 93 and 69 were also encountered in several carotenoids exhibiting distinct end groups (see Tables 3 and 4). Consequently, these ions should also be the result of the elimination of part of the central acyclic chain of the carotenoid skeleton. Fu et al. proposed that the ion at *m/z* 119 is produced by cleavage between carbons 9 and 10, and 13' and 14' (see Figure 7). Here we propose a mechanism for the formation of this ion from $[M]^{+\bullet}$ and $[M+H]^{+}$ molecular ion species (see Figure 7). The fragment at *m/z* $[M-205]^{+}$ and/or $[M+H-205]^{+\bullet}$ shown by phytoene and phytofluene (see Table 3) is the result of the bis-allylic cleavage of the C11-C12 single bond .

G. End groups

Presence of a Ψ end group*:*

Most of the end groups show characteristic signal peaks that are useful for their identification. Fragment ion at *m/z* $[M-69]^{+}$ encountered in compounds such as lycopene, *γ*-carotene, and *δ*-carotene is characteristic of the *Ψ* end group (see Table 3). This ion was detected using EI , ESI , FAB and APCI . Figure 8 shows the mechanism proposed for the formation of this fragment as a result of cleavage on the bisallylic 3,4-bond.

Presence of an ε-ring end group*:*

The presence of an *ε*-ring end group in the corresponding carotenoid is confirmed by the ion $[M-56]^{+\bullet}$ and/or $[M+H-56]^{+}$. This fragment has been observed using EI , FAB and APCI . Figure 9 illustrates two proposed mechanisms based on a retro-Diels-Alder fragmentation that lead to the loss of 56 units from both $[M+H]^{+}$ and $[M]^{+\bullet}$ ions.

The presence of these diagnostic ions alone or in combination with other characteristic fragments, such as $[M-92-69]^{+}$, $[M-106-69]^{+}$, $[M-56-92]^{+\bullet}$ or/and $[M+H-18-56]^{+}$, can be used to distinguish between structural isomers .

Presence of a β-ring end group*:*

The fragment at *m/z* $[M-137]^{+}$ and/or $[M+H-137]^{+\bullet}$ (see Table 3) was observed in carotenoids containing a *β*-ring end group. This fragment has been assigned to cleavage between carbons 7 and 8 , resulting in the loss of a terminal ring plus a methylene group (see Figure 10). However, acyclic carotenes such as phytofluene and phytoene also showed this fragment and its counter ion at *m/z* 137 (see Table 3), both resulting from cleavage of the polyene chain . Figure 10C shows the proposed mechanism for the formation of the ion at *m/z* $[M+H-137]^{+\bullet}$ from $[M+H]^{+}$ ion.

V. IMPROVING CAROTENOID IONIZATION

Carotenoid ionization can be improved by adding chemical compounds to favor the formation of ions and consequently, enhance the signal of analytes. Examples of such compounds are: (a) ammonium acetate, used to increase the abundance of deprotonated xanthophyll molecules using ESI in negative ion mode; (b) acetic acid, to increase the abundance of protonated xanthophyll molecules using ESI in positive ion mode; (c) halogen-containing eluents, used to increase the molecular ions of xanthophylls and carotenes using ESI in positive ion mode ; (d) dopants, such as toluene, anisole and chlorobenzene, employed in APPI to improve carotenoid signal strength and (e) sodium, silver or lithium salts, which have promoted the cationization of these pigments using ESI by intense formation of carotenoid-adducts . The ionization of several xanthophylls and carotenes is greatly enhanced by the addition of $AgClO_4$. In that experiment, the mass spectra of the peaks revealed that the silver adducts $[M+Ag]^+$ were more abundant than the molecular ions $[M]^{+\bullet}$. In addition, the characteristic loss of silver (with a relative intensity of almost 60%) from adducts permitted the use of multiple reaction monitoring (MRM) techniques and therefore improve detection limits. van Breemen observed that abundant $[M+Na]^+$ ions for xanthophylls, such as astaxanthin, were detected using ESI when sodium acetate was added to the mobile phase . In this case, the protonated molecule or molecular ion were not produced. Therefore, some carotenoids can be detected using this adduct since a higher intensity signal is obtained. Triethylamine (TEA) is a common modifier added to the mobile phase when analyzing carotenoids since it minimizes the effects of acidity generated by the free silanol groups present on the silica support and therefore increases carotenoid recovery from the column . However, attention should be paid when TEA is used for sample detection by MS because this base shows high proton affinity (more easily ionized in the APCI source) and as a result the carotenoid ion signals may decrease .

VI. CONCLUSIONS

The great diversity and number of carotenoids present in the transgenic rice callus and maize seeds allowed us to test and confirm the utility of several fragment ions to identify these compounds. Since the information available for the analysis of ketocarotenoids by MS is scarce, here we analyzed several ketocarotenoids, including astaxanthin, adonixanthin, adonirubin, canthaxanthin, 3-hydroxyechinenone and echinenone by UHPLC-APCI-MS/MS in positive ion mode. Thus, significant carotenoid fragment ions were identified that can be related to the presence of ketone, epoxide, alcohols, rings in the corresponding carotenoid structure, and the extent of conjugation of the polyene chain. In addition, new mechanisms are proposed for the formation of these significant ions. Although many of the diagnostic ions do not form the base peak of the MS spectrum, their presence or absence and the differences between the intensities of the fragments can be used to distinguish carotenoids. Finally, the comparison of the ionization methods described in this review shows that some positive fragment ions are observed only in certain techniques, such as the ion at *m*/*z* $[M-80]^{+\bullet}$, which was obtained solely through EI and FAB.

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REFERENCES

Aman R, Biehl J, Carle R, Conrad J, Beifuss U, Schieber A. 2005. Application of HPLC coupled with DAD, APcI-MS and NMR to the analysis of lutein and zeaxanthin

443 stereoisomers in thermally processed vegetables. Food Chem 92:753-763.

444 Azevedo-Meleiro CH, Rodriguez-Amaya DB. 2004. Confirmation of the identity of the
445 carotenoids of tropical fruits by HPLC-DAD and HPLC-MS. J Food Comp Anal
446 17:385-396.

447 Azevedo-Meleiro CH, Rodriguez-Amaya DB. 2007. Qualitative and quantitative differences
448 in carotenoid composition among Cucurbita moschata, Cucurbita maxima, and
449 Cucurbita pepo. J Agricult Food Chem 55:4027-4033.

450 Berger R, Liaaen-Jensen S, McAlister V, Guillard RRL. 1977. Carotenoids of
451 Prymnesiophyceae (Haptophyceae). Biochem Sys Ecol 5:71-75.

452 Bjørnland T. 1982. Chlorophylls and carotenoids of the marine alga Eutreptiella gymnastica.
453 Phytochemistry 21:1715-1719.

454 Bjørnland T, Borch G, Liaaen-Jensen S. 1984. Configurational studies on red algae
455 carotenoids. Phytochemistry 23:1711-1715.

456 Britton G, Brown DJ, Goodwin TW, Leuenberger FJ, Schocher AJ. 1977. The carotenoids of
457 Flavobacterium strain R1560. Arch Microbiol 113:33-37.

458 Britton G, Goodwin TW, Harriman GE, Lockley WJS. 1977. Carotenoids of the ladybird
459 beetle, Coccinella septempunctata. Insect Biochem 7:337-345.

460 Britton G, Liaaen-Jensen S, Pfander H. 2004. Carotenoids Handbook, 1st ed. Basel,
461 Switzerland: Birkhäuser. 3-482 p.

462 Caccamese S, Garozzo D. 1990. Odd-electron molecular ion and loss of toluene in fast atom
463 bombardment mass spectra of some carotenoids. Org mass spectrom 25:137-140.

464 Cazzonelli CI. 2011. Carotenoids in nature: Insights from plants and beyond. Funct Plant Biol
465 38:833-847.

466 Clarke PA, Barnes KA, Startin JR, Ibe FI, Shepherd MJ. 1996. High Performance Liquid
467 Chromatography/Atmospheric Pressure Chemical Ionization-Mass Spectrometry for
468 the Determination of Carotenoids. Rapid Commun Mass Spectrom 10:1781-1785.

469 Chiu ML, Lawi W, Snyder ST, Wong PK, Liao JC, Gau V. 2010. Matrix Effect-A Challenge
470 Toward Automation of Molecular Analysis. J Assoc Lab Automation 15:233-242.

471 Crupi P, Milella RA, Antonacci D. 2010. Simultaneous HPLC-DAD-MS (ESI+)
472 determination of structural and geometrical isomers of carotenoids in mature grapes. J
473 Mass Spectrom 45:971-980.

474 de Faria A, de Rosso V, Mercadante A. 2009. Carotenoid composition of jackfruit
475 (*Artocarpus heterophyllus*), determined by HPLC-PDA-MS/MS. Plant Foods Hum
476 Nutr 64:108-115.

477 de Rosso VV, Mercadante AZ. 2007. Identification and quantification of carotenoids, by
478 HPLC-PDA-MS/MS, from Amazonian fruits. J Agr Food Chem 55:5062-5072.

479 de Rosso VV, Mercadante AZ. 2007. HPLC-PDA-MS/MS of anthocyanins and carotenoids
480 from dovyalis and tamarillo fruits. J Agr Food Chem 55:9135-9141.

481 Enzell C, Francis G, Liaaen-Jensen S. 1968. Mass spectrometric studies of carotenoids. I.
482 Occurrence and intensity ratios of M--92 and M--106 peaks. Acta Chem Scand
483 22:1054.

484 Enzell CR. 1969. Mass spectrometric studies of carotenoids. Pure Appl Chem 20:497-515.

485 Enzell C, Francis G, Liaaen-Jensen S. 1969. Mass spectrometric studies of carotenoids. 2. A
486 survey of fragmentation reactions. Acta Chem Scand 23:727.

487 Fang L, Pajkovic N, Wang Y, Gu C, van Breemen RB. 2003. Quantitative analysis of

lycopene isomers in human plasma using high-performance liquid chromatography-tandem mass spectrometry. Anal Chem 75:812-817.

Feltl L, Pacakova V, Stulik K, Volka K. 2005. Reliability of carotenoid analyses: a review. Curr Anal Chem 1:93-102.

Fraser PD, Bramley PM. 2004. The biosynthesis and nutritional uses of carotenoids. Prog Lipid Res 43:228-65.

Fraser PD, Enfissi EMA, Goodfellow M, Eguchi T, Bramley PM. 2007. Metabolite profiling of plant carotenoids using the matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Plant J 49:552-564.

Fu W, Magnúsdóttir M, Brynjólfson S, Palsson BO, Paglia G. 2012. UPLC-UV-MS^{E} analysis for quantification and identification of major carotenoid and chlorophyll species in algae. Anal Bioanal Chem 10:3145-3154.

Gao S, Zhang ZP, Karnes HT. 2005. Sensitivity enhancement in liquid chromatography/atmospheric pressure ionization mass spectrometry using derivatization and mobile phase additives. J Chromatogr B Analyt Technol Biomed Life Sci 2:98-110.

Gross J, Stroz RJ, Britton G. 1975. The Carotenoid Hydrocarbons of Euglena gracilis and Derived Mutants. Plant Physiol 55:175-177.

Guaratini T, Vessecchi R, Pinto E, Colepicolo P, Lopes NP. 2005. Balance of xanthophylls molecular and protonated molecular ions in electrospray ionization. J Mass Spectrom 40:963-968.

Guaratini T, Gates PJ, Pinto E, Colepicolo P, Lopes NP. 2007. Differential ionisation of natural antioxidant polyenes in electrospray and nanospray mass spectrometry. Rapid Commun Mass Spectrom 21:3842-3848.

Hao Z, Parker B, Knapp M, Yu L. 2005. Simultaneous quantification of α-tocopherol and four major carotenoids in botanical materials by normal phase liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry. J Chromatogr A 1094:83-90.

Kurz C, Carle R, Schieber A. 2008. HPLC-DAD-MSn characterisation of carotenoids from apricots and pumpkins for the evaluation of fruit product authenticity. Food Chem 110:522-530.

Lacker T, Strohschein S, Albert K. 1999. Separation and identification of various carotenoids by C30 reversed-phase high-performance liquid chromatography coupled to UV and atmospheric pressure chemical ionization mass spectrometric detection. J Chromatogr A 854:37-44.

Lesellier E, Tchapla A, Marty C, Lebert A. 1993. Analysis of carotenoids by high-performance liquid chromatography and supercritical fluid chromatography. J Chromatogr A.633:9-23.

Li H, Tyndale ST, Heath DD, Letcher RJ. 2005. Determination of carotenoids and all-trans-retinol in fish eggs by liquid chromatography-electrospray ionization-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 816:49-56.

Maoka T, Fujiwara Y, Hashimoto K, Akimoto N. 2004. Characterization of epoxy carotenoids by fast atom bombardment collision-induced dissociation MS/MS. Lipids 39:179-183.

Mariutti LRB, Pereira DM, Mercadante AZ, Valentão P, Teixeira N, Andrade PB. 2012. Further insights on the carotenoid profile of the echinoderm Marthasterias glacialis L. Mar Drugs 10:1498-1510.

Matsumoto H, Ikoma Y, Kato M, Kuniga T, Nakajima N, Yoshida T. 2007. Quantification of carotenoids in citrus fruit by LC-MS and comparison of patterns of seasonal changes for carotenoids among citrus varieties. J Agr Food Chem 55:2356-2368.

Matthews PD, Luo R, Wurtzel ET. 2003. Maize phytoene desaturase and ζ-carotene desaturase catalyse a poly-Z desaturation pathway: Implications for genetic engineering of carotenoid content among cereal crops. J Exp Bot 54:2215-2230.

McEwen CN, McKay RG, Larsen BS. 2005. Analysis of solids, liquids, and biological tissues using solids probe introduction at atmospheric pressure on commercial LC/MS instruments. Anal Chem 77:7826-7831.

Meléndez-Martínez AJ, Britton G, Vicario IM, Heredia FJ. 2005. Identification of isolutein (lutein epoxide) as cis-antheraxanthin in orange juice. J Agr Food Chem 53:9369-9373.

Meléndez-Martínez AJ, Vicario IM, Heredia FJ. 2007. Geometrical isomers of violaxanthin in orange juice. Food Chem 104:169-175.

Meléndez-Martínez AJ, Vicario IM, Heredia FJ. 2007. Review: Analysis of carotenoids in orange juice. J Food Comp Anal 20:638-649.

Mercadante AZ, Britton G, Rodriguez-Amaya DB. 1998. Carotenoids from Yellow Passion Fruit (*Passiflora edulis*). J Agr Food Chem 46:4102-4106.

Polívka T, Frank HA. 2010. Molecular factors controlling photosynthetic light harvesting by carotenoids. Acc Chem Res 43:1125-1134.

Raffaelli A, Saba A. 2003. Atmospheric pressure photoionization mass spectrometry. Mass spectrom Rev 22:318-331.

Ren D, Zhang S. 2008. Separation and identification of the yellow carotenoids in *Potamogeton crispus* L. Food Chem 106:410-414.

Rentel C, Strohschein S, Albert K, Bayer E. 1998. Silver-plated vitamins: A method of detecting tocopherols and carotenoids in LC/ESI-MS coupling. Anal Chem 70:4394-4400.

Řezanka T, Olšovská J, Sobotka M, Sigler K. 2009. The use of APCI-MS with HPLC and other separation techniques for identification of carotenoids and related compounds. Curr Anal Chem 5:1-25.

Rivera SM, Canela-Garayoa R. 2012. Analytical tools for the analysis of carotenoids in diverse materials. J Chromatogr A 1224:1-10.

Rivera S, Canela R. 2012. Influence of sample processing on the analysis of carotenoids in Maize. Molecules 17:11255-11268.

Rivera S, Vilaró F, Canela R. 2011. Determination of carotenoids by liquid chromatography/mass spectrometry: Effect of several dopants. Anal Bioanal Chem 400:1339-1346.

Schwieter U, Bolliger HR, Chopard-Dit-Jean LH, Englert G, Kofler M, Koenig A, Planta Cv, Ruegg R, Vetter W, Isler O. 1965. Physical properties of carotenes. Chimia 19: 294-302.

Schwieter U, Englert G, Rigassi N, Vetter W. 1969. Physical organic methods in carotenoid research. Pure Appl Chem 20:365-420.

Tian Q, Duncan CJG, Schwartz SJ. 2003. Atmospheric pressure chemical ionization mass spectrometry and in-source fragmentation of lutein esters. J Mass Spectrom 38:990-995.

Tonhosolo R, D'Alexandri FL, de Rosso VV, Gazarin ML, Matsumura MY, Peres VJ, Merino EF, Carlton JM, Wunderlich G, Mercadante AZ, Kimura EA, Katzin AM. 2009.

581 Carotenoid biosynthesis in intraerythrocytic stages of Plasmodium falciparum. J Biol
582 Chem 284:9974-9985.

583 Vallverdú-Queralt A, Martínez-Huélamo M, Arranz-Martinez S, Miralles E, Lamuela-
584 Raventós RM. 2012. Differences in the carotenoid content of ketchups and gazpachos
585 through HPLC/ESI(Li+)-MS/MS correlated with their antioxidant capacity. J Sci Food
586 Agr 92:2043-2049.

587 van Breemen RB. 1995. Electrospray liquid chromatography-mass spectrometry of
588 carotenoids. Anal Chem 67:2004-2009.

589 van Breemen RB, Schmitz HH, Schwartz SJ. 1995. Fast atom bombardment tandem mass
590 spectrometry of carotenoids. J Agr Food Chem 43:384-389.

591 van Breemen RB, Huang CR, Tan Y, Sander LC, Schilling AB. 1996. Liquid
592 chromatography/mass spectrometry of carotenoids using atmospheric pressure
593 chemical ionization. J Mass Spectrom 31:975-981.

594 van Breemen RB. 2001. Unit F2.4 Mass Spectrometry of Carotenoids. Current Protocols in
595 Food Analytical Chemistry. New York: John Wiley & Sons. p F2.4.6-F2.4.12.

596 van Breemen RB, Dong L, Pajkovic ND. 2012. Atmospheric pressure chemical ionization
597 tandem mass spectrometry of carotenoids. Int J Mass Spectrom 312:163-172.

598 Vílchez C, Forján E, Cuaresma M, Bédmar F, Garbayo I, Vega JM. 2011. Marine
599 carotenoids: Biological functions and commercial applications. Mar Drugs 9:319-333.

600 Walter MH, Strack D. 2011. Carotenoids and their cleavage products: Biosynthesis and
601 functions. Nat Prod Rep 28:663-692.

602 Würtinger P, Oberacher H. 2012. Evaluation of the performance of a tandem mass spectral
603 library with mass spectral data extracted from literature. Drug Test Anal 4:235-241.

604 Zepka LQ, Mercadante AZ. 2009. Degradation compounds of carotenoids formed during
605 heating of a simulated cashew apple juice. Food Chem 117:28-34.

606 Zhu C, Naqvi S, Breitenbach J, Sandmann G, Christou P, Capell T. 2008. Combinatorial
607 genetic transformation generates a library of metabolic phenotypes for the carotenoid
608 pathway in maize. PNAS 105:18232-18237.

609 Zhu C, Naqvi S, Capell T, Christou P. 2009. Metabolic engineering of ketocarotenoid
610 biosynthesis in higher plants. Arch Biochem Biophys 483:182-190.

611 Zhu C, Bai C, Sanahuja G, Yuan D, Farré G, Naqvi S, Shi L, Capell T, Christou P. 2010. The
612 regulation of carotenoid pigmentation in flowers. Arch Biochem Biophys 504:132-141.

Figure captions

FIGURE 1. Carotenoid structures. The compounds are clustered on the basis of the main functional group(s) present in their structures: A) hydroxycarotenoids; B) epoxycarotenoids; C) carotenes; and D) ketocarotenoids.

FIGURE 2. A) Positive ion APCI mass spectra of zeaxanthin and lutein standards and the formation of the $[M+H-18]^+$ fragment, and B) comparison of the intensities of the transitions between β-carotene and α-carotene and the formation of the ion at *m/z* 123.1.

FIGURE 3. Characteristic fragment ions that are related to the hydroxyl group present in the carotenoid structures.

FIGURE 4. Proposed mechanism for the formation of the ion at *m/z* $[M-80]^{+\bullet}$. Loss of 80 mass units from A) oxirane fused to an end ring, and B) oxolane fused to an end ring.

FIGURE 5. Characteristic fragment ions for 5,6-epoxy and 5,8-furanoid carotenoids.

FIGURE 6. Diagnostic ions for ketocarotenoids.

FIGURE 7. Characteristic fragments originated from the polyene chain.

FIGURE 8. Feature breaking of the Ψ end group.

FIGURE 9. Loss of 56 units from an ε-ring end group from $[M+H]^+$ and $[M]^{+\bullet}$ ions.

FIGURE 10. Loss of 137 units from A) cyclic, and B) and C) acyclic carotenes.