Chapter 2

Recent Developments in the Analysis of Carotenoids by Mass Spectrometry

Antonio Pérez-Gálvez and María Roca

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Abstract

Mass spectrometry has become an indispensable tool for the analysis of carotenoids in photosynthetic tissues, foods, and biological materials from different sources to accurately establish their pigment profile, to provide evidences to distinguish the different structural arrangements, and to obtain biological meaning from metabolic processes where carotenoids participate during the development of their natural functions and activities. The recent progresses in the hyphenated HPLC systems with hybrid mass spectrometers, which enhance the acquisition of independent and complementary physicochemical properties for the identification of carotenoids, are detailed in this chapter. A reasonable guide for the implementation of post-processing routines, assisted by modern software tools, and the key issues for the analysis of the characteristic product ions are also defined in this contribution to help the readers in the understanding of the potential capabilities of mass spectrometry in the field of carotenoid pigments.

Keywords: extraction methods of carotenoids, liquid chromatography, ionization procedures, mass spectrometry, metabolomics, fragmentation pathways and carotenoid identification, data analysis from mass spectra, software tools

1. Introduction

The research interest in the characterization of the carotenoid content does not exclusively aim to determine the identity or taxa of the species, or to perform the screening of complex and intriguing carotenoid structures that are consequence of the variable gene expression, but also to find and/or select those species that may fulfill the claim in an applicative field. The analytical technique(s) applied to unequivocally identify the individual carotenoids must

provide solutions to face several challenges, because the carotenoid profile usually contains a complex mixture of pigments, with a wide polarity range where the presence of isomeric forms or structurally related compounds like xanthophyll esters is frequent, as well as interfering compounds (chlorophylls and triacylglycerides) in the analysis of the sample. In some biological samples, as serum or animal tissues, the carotenoid concentration could be very low, even below the nanomolar concentration [1]. In addition, carotenoids are unstable in the presence of light, oxygen, or heat [2]. Such lability to high temperatures has limited the application of gas chromatography mass spectrometry (GC-MS) in the carotenoid analysis, in combination with the complexity of the derivatization reactions. However, GC-MS has been applied to report the presence of different low-molecular weight compounds from the metabolism of several carotenoids in biological studies [3–6] with interesting results.

Historically, carotenoids have been analyzed by thin layer chromatography (TLC), a simple and fast technique to separate carotenoids, which allowed their characterization during the end of the last century [7]. But the need of high concentration of samples, the open system feature, and the low resolution of this technique induced its replacement by analytical techniques with higher sensitivity and superior selectivity. Consequently, high-performance liquid chromatography (HPLC) coupled to photodiode-array or UV-visible detectors is the common analytical technique currently applied to achieve the separation of carotenoids and their subsequent identification (in most cases) and quantification considering the features of the absorption spectrum, the location of the main absorption bands and its shape or fine structure, and the chromatographic behavior. The UV-visible spectrum of carotenoids provides some structural information, but the complete characterization is not feasible exclusively in the basis of the spectroscopic properties. Many carotenoids share the same spectrum and when the structures are closely related, even the chromatographic behavior is similar. Hence, the hyphenation of HPLC with mass spectrometry (MS) has become the ideal analytical technique because MS allows the acquisition of the elemental composition and structural arrangement of the functional groups in each individual carotenoid, overcomes the problems of resolution and sensitivity when the amount of sample/compound is low, and reduces the effect of interfering components. A diverse collection of ionization strategies and configurations of the mass analyzer is feasible, and each one performs diverse intrinsic parameters (mass resolving power, mass accuracy, linear dynamic range and sensitivity) at different levels. While HPLC-MS has been successfully applied to the qualitative and quantitative analysis of carotenoids, and there is an abundant available information regarding the MS characteristics of carotenoids under different MS acquisition modes, the assignment of peaks from experimental data still requires an intensive manual labor. Conversely, the implementation of software tools to assist in the production of a positive list of identified carotenoids is still an occasional practice. In reference [8], the process to build a database with 22 standard pigments (carotenoids and chlorophylls) including their retention time and the theoretical *m*/*z* values of both the parent and most intense product ions is described. The experimental retention time and *m*/*z* values acquired from the MS analysis of ethanol extracts of *Porphyridium purpureum* were compared with the values in the database, and when the mass errors were below 5 ppm, the presence of the target compound was confirmed. However, it would be encouraged to follow the rules for validating the identification, not only considering a single physicochemical feature (chromatographic behavior, UV-vis spectrum, *m*/*z*…) but on a minimum of two independent and orthogonal data relative

to an authentic compound (standard) analyzed under identical experimental conditions [9] to match with the compounds analyzed in the sample (see Section 3.2). Indeed, complementary confidence is provided when more than one pair of orthogonal data are selected to perform the identification. In the case of HPLC-MS analysis, different pairs of independent and complementary physicochemical properties are available (retention time and mass spectrum, accurate mass and tandem MS, and accurate mass and isotopic pattern). With the available information regarding the MS characteristics of carotenoids, it is possible to automatically obtain a report of carotenoids from an in-house mass database created *ex profeso* that matches the experimental *m*/*z* values with the selected orthogonal characteristics of the former. These MS criteria are complemented with additional matching data (retention time and UV-visible spectrum) to ensure the identification. Indeed, it is possible to perform a screening for unknown carotenoids or those not included in the database by the molecular feature extraction algorithms and subsequently apply the same pairs of orthogonal data to the new *m*/*z* value(s).

Alternatively, analysis of carotenoids by direct infusion without the necessity of a previous chromatographic separation step has been also described. It is possible to distinguish the *cis-trans* isomers of lycopene and β-carotene by their fragmentation patterns with the use of electrospray ion mobility mass spectrometry, for example see [10]. In a similar fashion, reference [11] describes the isolation of zeaxanthin and its glucoside fatty acid esters (thermozeaxanthins and thermo-*bis*-zeaxanthins) to characterize mutants in the genes, which encode the enzymes performing the final carotenoid glycosylation and acylation steps. Nevertheless, the flow infusion mass spectrometry has some limitations [12]. In this sense, the combination of a multidimensional analytical approach based on UPLC-UV-ion mobility-MS proposed for the screening of natural pigments could be the last novelty [13]. The authors described this novel approach with the aim to increase the selectivity and specificity in the analysis, and to improve the characterization of carotenoids and chlorophylls in complex biological matrices.

2. Extraction of carotenoids and sample preparation for analysis

It is challenging to define a general and common method for carotenoid extraction. The optimal extraction depends not only on the polarity of the carotenoids, but also on the characteristics of the matrix (moisture content, presence of cell wall, etc.). Additional parameters to take into consideration are the environmental and health hazards issues (with some solvents), time consuming techniques, costs, or even feasibility. In any case, the analytical parameters that should be determined for the development of an analytical method are precision, accuracy, specificity, linearity, and limits of detection and quantification. The present section discusses in detail the advantages or disadvantages of each method and its applicability.

Liquid-liquid extraction or solvent extraction, and partitioning method is the more widespread technique applied for carotenoid extraction. The selection of the organic solvent nature is not a simple task. The polarity of the solvent and the characteristics of the matrix are determining parameters for the efficiency of the extraction. Typically, nonpolar solvents are used for nonpolar carotenoids (hexane for β-carotene extraction), while polar solvents are suitable for polar carotenoids (acetone for lutein extraction). However, the selection of the

proper solvent or mixture of solvent will subsequently require the optimization for each specific matrix. Thus, the hexane and the ethanol/hexane (4:3) are the solvents most frequently applied, although numerous alternatives have been assayed: acetone, ethanol, petroleum ether, tetrahydrofuran, diethyl ether, methanol, etc. The solvent or solvents mixture could be supplemented with antioxidants to avoid degradation and transformation of carotenoids during extraction. Butylated hydroxytoluene is the most well-known, although other antioxidants, such as pyrogallol and ascorbic acid, are also utilized.

Recent reviews have recapitulated the different extraction methods postulated for carotenoids [14–16]. One specific case is the esterified carotenoids [15]. Chemical hydrolysis cannot be applied if the aim is to determine the nature of fatty acids involved in the esterification, while the presence of triacylglycerides in the sample produces interferences in the subsequent chromatographic analysis. Therefore, the extraction methods mean the application of similar protocols applied for the extraction of free carotenoids, but the oily-dense nature of some matrices containing carotenoid esters requires clean-up steps. Different approaches have been set up, including the utilization of open column chromatography on silica gel [17] or magnesium oxide [18], enzymatic hydrolysis [19], solid-phase extraction [20], or even the use of semi-preparative isolation on C18 column and subsequent identification [21].

Although the most common method for the isolation of the carotenoid profile is solvent extraction, during the last decade, new techniques have raised becoming popular because of their *green* features and environmentally friendly practices. They include the microwaved-assisted extraction (MAE), ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE), pulse electric field-assisted extraction (PEF), supercritical fluid extraction (SFE), and enzyme-assisted extraction (EAE). The development of these techniques is mainly motivated by the specific difficulties for the extraction of lipophilic content from microalgae and seaweeds species. The cell walls and the extracellular material with different polysaccharide distributions, such as agars, carrageenans, fucoidans, and alginates, limit the mass transfer of carotenoids during conventional extraction processes. The biological characteristics of those species have aimed the development of alternative methodologies to optimize the efficiency of the extraction process.

The development of MAE was first reported in 1986 [22]. During the MAE, the nonionizing electromagnetic radiations heat the extraction medium, transferring the analytes from their cellular environment. In addition, the heat evaporates the water inside the cells, increasing the pressure that increases the cell porosity and breaks cell membranes, to finally improve the solvent penetration [23]. MAE can be developed in open vessels at low temperatures, or in closed systems at high-temperature extractions. The advantage of the microwave is that heating is simultaneously produced in the whole sample, unlike classical conductive heating methods [24]. To achieve an efficient extraction, MAE requires an optimization process in function of the matrix properties, being significant the determination of the solvent-sample ratio, solid-liquid ratios, microwave power, and delivered energy equivalents [25]. This optimization process is performed by the application of experimental design with response surface methodology. MAE has been satisfactorily applied in the extraction of carotenoids from fruits [26], wastes, and by-products [27] or seaweeds [23].

UAE is recognized as the lowest effective method among the nonconventional methods for carotenoid extraction. Consequently, the trend is to combine several *green* methodologies as

MAE and UAE, or to join UAE with solvent extraction methods to enhance the efficiency of the conventional system [28]. UAE utilizes wave sounds of 20 kHz–100 MHz, generating cavitation in the medium through compression and expansion cycles, and consequently, the production of bubbles. The collapse of the bubbles induces disruption of the cell walls and the release of the cellular content. The main benefit of this technique is the extraction at low temperatures.

PLE was developed first time in 1996 [29] and since then it has been successfully applied to different matrices. This methodology utilizes solvents at high temperature and pressure in the ranges of 50–200°C and 35–200 bar, respectively [23]. The high temperatures help in the cell wall disruption process, while the high pressure forces the contact between the solvent and the disrupted matrix [30]. A recent modification introduced in PLE technique is the pressurized hot water extraction (PHWE) allowing the claim of *green* recovery of bioactive compounds from leaves of *Stevia rebaudiana* (Bertoni), with positive results for carotenoids. Nevertheless, the application of different processing conditions including different temperatures (100, 130, and 160°C), static extraction times (5 and 10 min), and cycle numbers (1, 2, and 3) did not improve the carotenoid recuperation [31].

The strategy followed by the PEF is to locate the sample matrix between two electrodes and apply intermittent high electric frequencies with intense electric fields during nanoseconds or milliseconds periods of time. When the transmembrane potential exceeds 1 V, the charged molecules initiate the repulsion, generating pores in the membranes [28], process that could be reversible or permanent. Recently, PEF has been validated as an enhancer of the carotenoid bioaccessibility [32], setting the processing parameters to increase the carotenoid amount incorporated into the micellar phase from tomato fruits.

Since its discovery in 1879 [33], SFE has been applied with a great success mostly at industrial scale. The basis of this technique consists on to leave the substance at temperature and pressure conditions beyond its critical point. At this stage, the supercritical fluid exhibits high diffusivity and low viscosity, features of the gas phase, but develops a high solvation power, a feature characteristic of the liquid phase. Usually, the solvent is carbon dioxide as the critical temperature and pressure are relatively low. SFE can be applied as the traditional *off-line* mode, which means that the extracted analyte is collected in an external collector or in the more recent *on-line* mode, when the extracted analyte is directly transferred to the chromatographic column [34]. Following this approach, it is possible to efficiently extract the targeted carotenoids from red Habanero peppers (*Capsicum chinense* Jacq.) [35]. In comparison with the *off-line* systems, the *on-line* approach seems to improve the run-to-run precision, enables the setting of batch-type applications, and reduces the risk of sample contamination.

The EAE performs the disruption of the cell walls by the activity of different enzymes as pectinases, cellulose, or hemicellulose. Two different strategies are applied: the cell walls and the polysaccharide-protein colloids are the targets of the enzymatic activity of the aqueous-EAE. The alternative method is the cold-EAE, where the enzymes only hydrolyze the cell walls [30].

Independently of the extraction method, sometimes the matrix required a saponification step prior the analysis. Such reaction should be considered as a clean-up process, which eliminates lipids and chlorophylls, facilitating the separation in the HPLC column. On the contrary, if saponification is necessary, it should proceed with caution as several carotenoids are prone to

modification, transformation, or degradation in the presence of KOH. In any case, recently, it has been highlighted the importance of the gap between comprehensive extraction protocols in plant metabolomics studies and method validation [36]. Classically, the determination of specific secondary metabolites has used targeted methods for very specific purposes. But modern metabolomics consist on a data-driven approach with predictive power to determine all measurable metabolites without any preselection. Consequently, classical extraction methods cannot be strictly applied in metabolomics studies and requires validation to new purposes.

3. Liquid chromatography-mass spectrometry

Old fashion methods formerly applied in the analysis of carotenoid, as open column chromatography or TLC are now considered purification techniques, due to its low cost and simplicity. Thus, unambiguously, the HPLC is the most widely used technique in carotenoid analysis currently. Several reviews have been published compiling different HPLC methods for carotenes and xanthophylls [14, 16, 37–40] and specifically for carotenoids esters [15]. Therefore, our aim is to highlight the advantages and limitations of the different alternatives that are available, allowing the reader to evaluate them and select the best option.

Three main sorts of chromatographic columns are applied in the carotenoid analysis: octyl (C_8), octadecyl (C_{18}), and triacontyl (C_{30}) bonded phases. In [41], a high-performance liquid chromatographic method was developed with a reversed phase C_8 column and pyridine-containing mobile phases for the simultaneous separation of chlorophylls and carotenoids. The proposed method also allows the separation of taxon-specific carotenoids belonging to eight algal classes, including some critical pigment pairs for previous HPLC methods. The method works with a binary gradient, so that it can be implemented with both low-pressure and high-pressure mixing instruments. In general, C_{18} columns are used for the analysis of carotenoids with a wide range of polarity as the gradient only requires 10–30 min. To separate and identify geometrical isomers of carotenoids, a C_{18} Vydac 201TP54, one of the more popular columns, is combined with a ternary mobile phase, methanol mixture (0.1 M ammonium acetate), tert-butyl methyl ether, and water [42]. The proposed method allows the separation of 17 different carotenoids, including *cis* and *trans* isomers. Nevertheless, it is generally assumed that C_{30} columns exhibit better resolution for chromatographic separation of the geometric isomers, although the use of this column means more run time (60–100 min) to complete the analysis [43, 44]. Specifically, to control the carotenoids used as food colorants (norbixin, bixin, capsanthin, lutein, canthaxanthin, β-apo-80-carotenal, β-apo-8′-carotenoic acid ethyl ester, β-carotene, and lycopene), there is a method, where the resolution of C_{30} columns allows the intricate chromatographic isolation of bixin and norbixin due to their acidic character [45] in combination with the optimization of accelerated solvent extraction applied to different food matrices. Alternatively, the use of C_{34} columns has also been evaluated, but at least with similar results as with a C_{30} column [46].

The particle size of the stationary phases of HPLC columns commonly used for carotenoid analysis has evolved from the classical 5 μm, through the common 3 μm, finally to reach the more recent sub-2-micron particles. This new technology, known as ultra-high-performance liquid chromatography (UPLC), means that the delivery system of the mobile phases operates

at high back pressure (as an example, HPLC works at 35–40 MPa, while UPLC can reach more than 100 mPa). The advantages of the new UPLC systems are the higher sensitivity, lower run times, and lower consumption of the mobile phases. Years ago, the commercially available columns for UPLC were packed with C_{18}, but in the last 5 years also C_{30} columns with less than 2 μm of particle size are accessible. In Ref. [47], the performance of a conventional C_{30} (3 μm particle size) column with the novel sub-2-micron C_{30} was compared in the analysis of 31 different carotenoids. The results have showed a better compound resolution and shorter analysis time with the novel particle size.

Mono-dimensional (single-column or one-dimensional) chromatography can be considered the most common methodology used for carotenoid analysis. However, a recent development [48] was the application of two-dimensional chromatography to elucidate the carotenoid pattern in citrus. In this technique, the carotenoids are analyzed by two *on-line* chromatographic systems, increasing the separation power and peak capacity [49]. This modality of chromatography should be applied in the analysis of complex matrices or when the carotenoid profile is intricate. The best performance is achieved when both systems operate with complementary selectivity, that is, normal-phase × reversed-phase. A recent optimization implements UPLC in the second-dimensional chromatography increasing the resolution [50]. Even so, the practical performance of this methodology must resolve different challenges including solvent immiscibility or the peak focusing at the head of the second column [49]. To overcome these drawbacks, the normal phase applied in the first-dimensional chromatography could be replaced by supercritical fluid chromatography [35] increasing the identification power. In this sense, a rapid ultra-performance convergence chromatography (UPC^2) method for the determination of seven fat-soluble vitamins (vitamin A: retinol, retinyl acetate; vitamin D: ergocalciferol, cholecalciferol; vitamin E: α-tocopherol; and vitamin K: phylloquinone, menaquinone) and three carotenoids (lutein, lycopene, and β-carotene) was developed [51]. Carotenoids were separated within 3 min with an HSS C-18 SB column (3.0 × 100 mm, 1.8 μm) under isocratic conditions with a mobile phase of carbon dioxide and ethanol (75:25, v/v).

Another parameter to take note is the influence of the injection conditions in the resolution of the chromatography [52]. The authors proposed that the nature of the injection solvent could be responsible of distorted or false peaks. Parameters as the polarity index, solvent strength, injection volume, solute concentration, or solvent selectivity should be optimized before a chromatographic method is developed.

The main solvents utilized as mobile phases in the carotenoid separation are acetonitrile and methanol. The first solvent presents low absorbance in the range of UV light and low back pressure in the column. On the contrary, methanol is less expensive and less toxic [14]. In addition, those solvents are commonly mixed with less polar solvents as dichloromethane, ethyl acetate, tetrahydrofuran, etc., to improve the resolution power. Some methods also include ammonium acetate or trimethylamine to enhance the recovery. Mixtures of those solvents can be applied in isocratic mode, allowing reproducible retention times and relatively stable baselines [39]. As an example, the isocratic HPLC method with a resolution time of 12 min has been developed for the simultaneous detection of eight xanthophylls: 13-*cis*-lutein, 13′-*cis*-lutein, 13-*cis*-zeaxanthin, all-*trans*-lutein, all-*trans*-zeaxanthin, all-*trans*-canthaxanthin, all-*trans*-β-apo-8′-carotenoic acid ethyl ester, and all-*trans*-β-apo-8′-carotenal [53]. The separation

is performed in a YMC C-30 reversed phase column with as a solvent system containing methanol/tert-butyl methyl ether/water. The method can additionally resolve the identification of lutein and zeaxanthin stereoisomers. When isocratic separations are not enough to achieve the separation, then gradient mode elution should be applied. This operation mode improves the sensitivity, increases the resolving power, and allows the elution of retained compounds. On the contrary, the application of gradient mode elution could need the re-equilibration of the column and could give high differences of the detector response [39].

Carotenoids are universally separated through reverse phase HPLC, while scarce methods imply the utilization of normal phase chromatography, and usually associated with the simultaneously detection of compounds of different nature. Thus, a normal phase high performance liquid chromatography method was developed to simultaneously quantify several bioactive compounds in canola oil as α-tocopherol, γ-tocopherol, δ-tocopherol, β-carotene, lutein, β-sitosterol, campesterol, and brassicasterol [54]. The method claims a higher analytical selectivity in less run time that other reported methods.

An additional approach [55] has been the application of software (DryLab) to help in the development of an HPLC method for enhancing the pigments separation. The method consists of the record of retention times of specific carotenoids in four chromatographic conditions, with the aim to predict the retention time in the hypothetical chromatographic method developed by the user (using variables as gradient time, column, column dimensions, and flow rate). With this computer-assistance procedure, the user finally selects the suitable chromatographic conditions to apply. This methodology is highly advisable for the separation of complex carotenoid profiles that phytoplankton species present.

In addition, more than 20 different carotenoids have been identified in blood and animal tissues. The existence of a carotenoid metabolism in animals generates a high diversity of metabolites in the biological samples [38, 40]. Consequently, it is extremely important for the metabolomics studies of the application of hyphenated techniques that could perform the detection of carotenoid metabolites. Recently, it reviewed the analytical methods developed for the identification of carotenoid metabolites in biological samples. Those metabolites are mainly derived from β-carotene, lycopene, lutein, zeaxanthin, astaxanthin, and fucoxanthin [38, 40].

3.1. Mass spectrometry: strategies for ionization and acquisition of mass data

Hyphenated LC-UV-visible-MS instruments have become the standard choice for carotenoid analysis for several reasons. The acquisition of MS data provides structural information that with the sole use of UV-visible detection could not be concluded. Thus, elemental composition can be determined from the measured mass of the molecular/protonated molecular ion with a high degree of accuracy (high-resolution mass or mil mass measurement) to discard alternative compounds that show close values of mass to our target compound, but different elemental composition. Isotopic pattern is a second dimension that can be obtained from the MS data, which may apply as a filtering rule to reject alternative compounds with similar mass but different elemental composition. With both MS data, the number of potential elemental compositions that may fit for each measured molecular/protonated molecular ion is considerably reduced. However, the elemental composition is not enough for the characterization of

carotenoids because several of them share the same elemental composition with different structural arrangements. MS offers a second experimental approach, the tandem MS to obtain fragmentation spectrum with the different product ions that arise from the parent compound. A detailed analysis of the product ions and the operating fragmentation pathways afford the acquisition of crucial evidence for identification.

In addition to the information that MS data produces several drawbacks commonly observed with the application of LC-UV-visible are overcome. MS relieves the identification of co-eluting individual carotenoids or when complex profiles where the UV-visible spectra are not completely resolved by the presence of interfering compounds or when the concentration of the compounds in the sample is low. Another benefit is the multiplicity of instrument configurations (ionization methods and mass analyzers) in combination with different ionization and MS-acquisition modes, which are described below, offering several alternatives to obtain spectra from diverse MS approaches. **Table 1** contains a brief description of several chromatographic methods applied to the analysis of carotenoids in foods and human samples, where the detection system included mass spectrometry.

3.1.1. Ionization methods

The analysis of the target compounds in a mass spectrometer is based on the magnetic and electric fields to apply forces on ions in a vacuum environment. Consequently, a method to provide charged or ionized compounds must be applied to the sample and subsequently produce desorption of those ions into the vacuum system of the mass analyzer. Different ionization methods are available and the main factors to select one of them are the nature of the sample and the aimed information from the analysis. Indeed, some ionization methods are conveniently hyphenated with liquid chromatography, so that the experimental approach and high-throughput screening of complex mixtures are more feasible. Carotenoids are thermally labile compounds and the application of electron impact (EI) and negative ion chemical ionization (CI), which requires vaporization of the sample before the ionization process, presents therefore a main drawback. However, the initial studies in MS of carotenoids applied EI or CI giving the first approaches to the behavior of these compounds under the experimental conditions required for ionization and MS analysis [14, 56–60]. Fast atom bombardment (FAB) is a modification of the EI ion source where a fast atom beam (particles with high kinetic energy) impacts on the target compound dissolved in a liquid matrix (glycerol, thioglycerol, m-nitrobenzyl alcohol, and diethanolamine) yielding molecular ions and fragments. The target compound is maintained at the same temperature of the ion source, usually ambient temperature and therefore, this method widely displaced the EI and CI ionization techniques for the analysis of nonvolatile and/thermally labile compounds. The characteristics of the MS spectra after FAB ionization are related with the properties of the liquid matrix and indeed it produces a chemical background that may interfere with the ions of interest. The successful application of this method hyphenated with liquid chromatography to the MS analysis of carotenoids was reported in [61]. This ionization strategy yields abundant signal of the molecular ion, while scarce fragmentation processes are observed. The use of FAB MS/MS with high-energy collision-assisted dissociation allows the study of the most abundant product ions that contain structural information to confirm the presence of functional groups and the extent of

Sample	Carotenoids determined	Chromatographic conditions	Detection system	Refs.
Serum	Dietary carotenoids	Gradient mode: (A) MeOH/ACN/water 84:14:4 (B) DCM; C30 column (3 μm, 4.6 × 150 mm), flow rate 1 mL/min	PDA/APCI(+)/ Quadrupole	[80]
Fruits and vegetables	Dietary carotenoids and xanthophyll esters	Gradient mode: (A) MeOH/MTBE/water 81:15:4 (B) MeOH/MTBE/water 16:80.4:3.6; C30 column (5 μm, 4.6 × 250 mm), flow rate 1 mL/min	PDA/APCI(+)/ Ion trap	[18]
Human colostrum and mature milk	Dietary carotenoids and xanthophyll esters from MFGM	Gradient mode: (A) MeOH/MTBE/water 81:15:4 (B) MeOH/MTBE/water 7:90:3; C30 column (3 μm, 4.6 × 250 mm), flow rate 1 mL/min	PDA/APCI(+)/ Quadrupole-time-of-flight	[20]
Red blood cells	Lutein, zeaxanthin, α-and β-carotene, β-cryptoxanthin, and lycopene	Gradient mode: (A) MeOH/MTBE/water-3.9 mM ammonium acetate 83:15:2 (B) MeOH/MTBE/ water 8:90:2–2.6 mM ammonium acetate; C30 column (5 μm, 4.6 × 250 mm), flow rate 1 mL/ min	PDA/APCI(+)/ Quadrupole	[102]
Fruits, vegetables, and human plasma	Apo-lycopenals	Gradient mode: (A) MeOH/0.1% aqueous formic solution 80:20 (B) MTBE/MeOH/0.1% aqueous formic solution 78:20:2; C30 column (5 μm, 4.96 × 150 mm), flow rate 1.3 mL/min	PDA/APCI(−)/ Quadrupole-time-of-flight	[83]
Enriched fruit juices	Retinol, retinyl acetate, retinyl palmitate, and β-carotene	Gradient mode: (A) MeOH (B) water; C18 column (5 μm, 4.6 × 150 mm), flow rate 0.9 mL/ min	PDA/APCI(+)/ Ion trap	[103]
Capsicum fruits	carotenes, xanthophylls, and xanthophyll esters	Gradient mode: (A) MeOH/water 82:16:2 (B) MeOH/water 10:88:2; C30 column (5 μm, 4.6 × 250 mm), flow rate 0.8 mL/min	PDA/APCI(±)/ Ion trap-time-of-flight	[104]
Tamarillo fruits	Apo-carotenoids, carotenes, xanthophylls, and xanthophyll esters	Supercritical fluid chromatography: (A) CO_2 (B) CH_3OH; C30 column (2.7 μm, 4.6 × 150 mm), flow rate 2 mL/min and 0.5 mL/min for make-up solvent	PDA/APCI(±)/ Triple quadrupole	[47]
Whole grain flours	Lutein and lutein esters	Gradient mode: (A) MeOH/MTBE/water-10 g/L ammonium acetate 80:18:2 (B) MeOH/MTBE/ water-10 g/L ammonium acetate 6:92:2; C30 column (3 μm, 3 × 150 mm), flow rate 0.42 mL/ min	PDA/APCI(±)/ Ion trap	[21]
Ketchups and Mediterranean soup	Lutein, α- and β-carotene, lycopene, and *cis* isomers of lycopene	Gradient mode: (A) water (B) MeOH (C) MTBE; C30 column (5 μm, 4.6 × 250 mm), flow rate 1 mL/min	PDA/ESI(Li+)/ Triple quadrupole	[65]
Red orange essential oil	Carotenes, xanthophylls, and xanthophyll esters	Two-dimensional liquid chromatography; first dimension, gradient mode: (A) hexane/ butyl acetate/acetone 80:15:5 (B) hexane; cyanopropylsilane column (5 μm, 1 × 250 mm), flow rate 10 μL/min; second dimension, gradient mode: (A) 2-propanol (B) ACN-20% water; C18 column (5 μm, 4.6 × 100 mm), flow rate 5 mL/min	PDA/APCI(±)/ Ion trap-triple quadrupole	[105]

Table 1. Chromatographic methods applied to the analysis of carotenoids in foods and human samples with mass spectrometry detection.

conjugation of the polyene chain [62]. Nevertheless, the group of atmospheric pressure ionization methods (API) has replaced the FAB technique because they show a high versatility and convenience for hyphenation with liquid chromatography. Thus, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the ionization methods widely used in the LC-MS analysis of carotenoids [58, 60]. The approach of the API methods consists of a solution of the compounds in a volatile solvent. In the case of ESI, the application of electric potential to the sample in solution transforms it in an electro-sprayed aerosol. Then, a stream of hot gas evaporates the solvent at a high rate to produce gas-phase ions. In APCI, the sample in solution is sprayed at a higher liquid flow than in ESI through a heated vaporizer. The ionization is produced by a corona discharge with ion-molecule reactions taking place at atmospheric pressure. In both cases, the ions are incrementally transferred from the initial atmospheric pressure conditions into the high vacuum at the mass analyzer [63]. This design of the API methods provides the softness feature to the ionization process, increasing the range of compounds to which application of these techniques is possible.

Specifically, ESI requires small amounts of sample and the problems of thermal instability of some carotenoids are avoided [64, 65]. To enhance the efficiency of the ionization process, which takes place in the aerosol, it is frequent that the incorporation of additives to the mobile phase of the LC system, including ferrocene-based derivatives, mildly acidic salts, weakly organic acids, halogen salts, trimethylamine, or ammonium acetate [14, 64–70]. This strategy means a significant advantage in the analysis of carotenes and xanthophyll esters, considering their lower polarity in comparison with the un-esterified xanthophylls. However, the use of APCI for ionization in LC-MS analysis of carotenoids has become general because of its ability over ESI to generate ions from neutrals, that is, carotenes and xanthophyll esters [2, 17, 71–74]. The application of high temperatures in the heated vaporizer may cause thermal degradation of the carotenoids in the source. However, the transfer of the vapor is very fast, so that the sample compounds do not necessarily reach the high-temperature conditions, while the soft collisions that initiate at this stage spend part of the excess energy. APCI allows working in positive ion or negative ion mode, which has demonstrated to be useful to distinguish between some isomeric carotenoids as the deprotonated molecular ions follow specific fragmentation routes [2].

It is relevant to note that the kind of ions produced is related with the ionization method applied. EI and FAB methods yields abundant molecular ions, a quality that is useful for the measurement of the molecular weight. In the case of API methods, positive or negative ions (depending on the ionization mode) are formed, while the characteristics of the solvent and the presence of alkali metal ions trend to produce clusters and adducts. The production of radicals is not frequent in ESI while the APCI method may yield them depending on the actual conditions and the structural arrangement of the analyzed compound.

3.1.2. Mass spectrometers

The mass spectrometer is the final bond in the instrumentation of LC-MS, which may present diverse configurations according to the technology applied for ion acceleration and detection systems. Each configuration performs diverse intrinsic parameters (mass resolving power, mass accuracy, linear dynamic range, and sensitivity) at distinct levels. Consequently, the

hardware is subjected to a constant developing process, in addition to the software capabilities, meeting demanded robustness, reproducibility, and efficiency of the analyses. Accordingly, the experimental parameters associated with these various instrument configurations are related to the capability range that could be reached with the mass analyzer. Indeed, the performance of the instrument is particularly significant to select the suitable strategy for the identification of compounds, as well as to enhance the rigor and strength of the peak assignment. Different mass spectrometers have been applied in the MS analysis of carotenoids, including the ion trap (IT) [18, 75–78] and quadrupole (Q) [79–82] instruments that offer good sensitivity but limited resolving power [63]. These instruments provide mass-to-charge ratio (m/z) values of the molecular ion with mass uncertainty at the part-per-thousand level. The introduction of mass spectrometers with higher peak resolution enhances the rate of mass accuracy measurements and the resolving power of the instrument, allowing the delivering of molecular formula with a lower uncertainty (part-per-million level) as described below. Another significant improvement in the hardware configuration of the mass spectrometer is the incorporation of a second stage of m/z analysis to obtain the mass spectra of selected ions. Thus, the arrangement of mass spectrometers in a tandem configuration to structure hybrid mass spectrometers (Q-time-of-flight, Q-IT, and triple Q-Orbitrap) [2, 20, 64, 83–87] allows the achievement of higher performance by increasing the mass accuracy level and the record of mass spectrum of a precursor ion to generate product ions. This configuration is essential when soft ionization methods are applied because they barely produce fragment ions and the experimental information from the MS analysis would limit to m/z values and isotopic pattern. The application of tandem MS generates a second dimension of data related with the structural arrangements featured in the selected precursor ion for MS/MS analysis.

3.1.3. Experimental information acquired from MS analysis

The primary piece of information that contains a mass spectrum is the m/z values of the ions produced at the ionization source. A pure compound would yield basically only one m/z value corresponding to the molecular/protonated molecular ion (depending on the ionization method applied). The intensity of the signal associated to each m/z value correlates to the abundance of each ion. Alternatively, some product ions could appear at the mass spectrum with smaller m/z values than the parent ion from which they are produced. We have mentioned that this is not usual when soft ionization methods are used, but product ions are necessarily generated in tandem MS equipment, so that a significant increment in the number of m/z signals is observed in the MS spectra generated in such hardware configuration. Hence, the highest m/z value would correspond to the molecular/protonated molecular ion while the rest m/z values are fragments ions derived from the former. As mentioned above, the measured m/z value delivers molecular formula and the higher the accurate level of the acquisition, the lower number of molecular formula corresponding to the m/z value. In the case of carotenoids that usually show m/z values in the range of 450–650 Da, the unequivocal assignment of formula by the measurement of accurate mass is not possible and restrictions, in addition to complementary information from the MS data are required [40]. That information is contained in the isotopic pattern, the second piece of information that should be obtained from MS data, which results from the natural isotope abundance and elemental composition

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[88]. The comparison of the theoretical isotopic pattern and the experimental one is a powerful additional constrain for removing molecular formula candidates that initially fitted with a determined *m*/*z* value. To obtain a notion over the matching result of both pieces of analytical information, **Figure 1** depicts the calculated monoisotopic mass, that is, the exact mass of the most abundant isotope, of the protonated molecular ion $[C_{40}H_{56} + H]^+$ (*m*/*z* = 537.4455 Da), one of the possible *m*/*z* values for carotenoids. The experimental MS spectrum in the analysis of a compound yields the *m*/*z* value equal to 537.4462 Da. The application of filtering rules to the mass error and isotopic pattern (error below 3 ppm and isotopic matching value below 50) with a software tool assures the assignment of the $C_{40}H_{57}$ ion formula to the experimental *m*/*z* value, which indeed fits with the starting proposal (see Section 3.2.2).

Although MS data is enough for the identification of several carotenoids without considering additional analytical information (chromatographic behavior, UV-visible spectrum), those with the same elemental composition but different constitution (β-carotene and lycopene), and the isomers require complementary information for the characterization of their structural arrangements because the *m*/*z* values and the isotopic pattern are the same. **Table 2** contains the calculated monoisotopic mass of protonated molecular ions and product ions observed in the analysis of common carotenoids. The aim of **Table 2** is not to compile comprehensive information of the MS behavior of carotenoids, but to remark the reader that different structures share the same molecular weight and fragmentation pathways. Thus, the list of carotenoids with a calculated *m*/*z* equal to 537.4455 Da includes 12 compounds [89]. The comparisons of mass spectra by positive and negative ion modes [15, 90], the possibility of performing additional MS^n experiments of isolated product ions, and even the differences in the intensity of the MS signals are existing alternatives that can aid identification. Among those alternatives, the application of techniques to select *m*/*z* ions, fragment them,

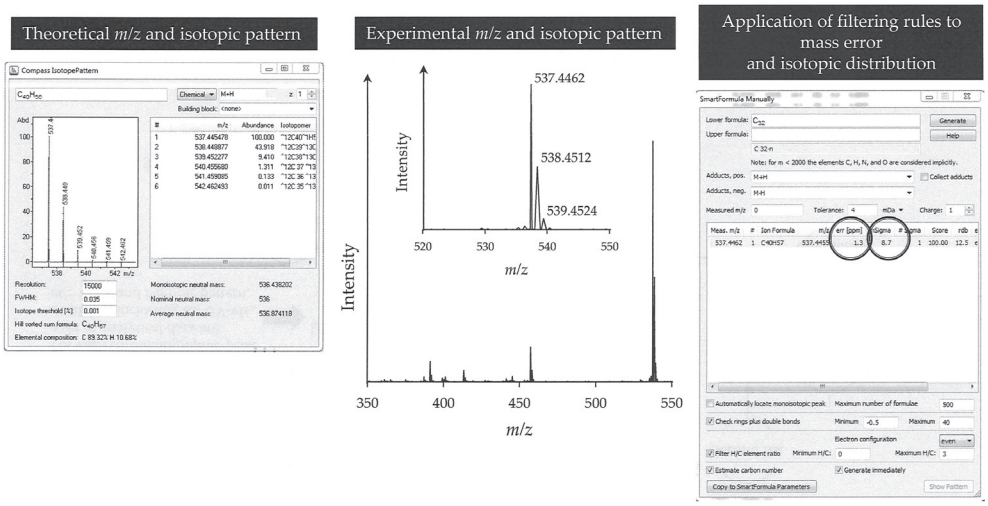

Figure 1. Matching the theoretical *m*/*z* and isotopic pattern with the experimental values and the application of filtering rules for the acquisition of mass ion formula [91, 92].

Carotenoid	Formula	$[M + H]^+$	Product ions		
			In-chain units	Loss from end groups	De-cyclization
α-Carotene	$C_{40}H_{56}$	537.4455	457.3829; 445.3829	413.3203	481.3829
β-Carotene	$C_{40}H_{56}$	537.4455	457.3829; 445.3829	413.3203	—
Lycopene	$C_{40}H_{56}$	537.4455	457.3829; 445.3829	413.3203	467.3673[a]
α-Cryptoxanthin	$C_{40}H_{56}O$	553.4404	461.3778	535.4298	479.3673
β-Cryptoxanthin	$C_{40}H_{56}O$	553.4404	473.3778; 461.3778	535.4298	495.3985
Echinenone	$C_{40}H_{54}O$	551.4247	471.3621; 459.3621	533.4141	495.3621
Lutein	$C_{40}H_{56}O_2$	569.4353	477.3727; 463.3570	551.4247; 533.4141	495.3622
Zeaxanthin	$C_{40}H_{56}O_2$	569.4353	489.3727; 477.3727; 463.3570	551.4247; 533.4141	—
Astaxanthin	$C_{40}H_{52}O_4$	597.3939	505.3313	579.3833; 561.3727	—
Neoxanthin	$C_{40}H_{56}O_4$	601.4251	509.3625	583.4145; 565.4039	—
Violaxanthin	$C_{40}H_{56}O_4$	601.4251	509.3625	583.4145; 565.4039	—

Product ions have been described in [2, 57, 60, 73].

[a]De-cyclization reaction means loss of the acyclic ψ end-group of lycopene.

Table 2. Calculated monoisotopic mass (Da) of protonated molecular ion and product ions observed in the analysis of carotenoids by APCI-MS.

and re-analyze the MS spectrum derive significant information regarding the operative fragmentation pathways while the subsequent analysis of the product ions gives a high amount of data that facilitates the peak assignment and structural characterization. Hence, the fragmentation pathways operating at the molecular ion yield characteristic product ions that allow the identification and location of functional groups, configuration of end-rings, chromophore length, and other key structural features of the carotenoids [2, 57, 60, 66].

3.2. Data analysis and identification

The high amount of analytical information obtained from MS must be conveniently processed to give results with a meaningful biological context, and enough confidence and rigor is demanded when identification is the aim. There is not a single standardized approach for data analysis and identification because the procedure applied to study the MS spectra depends on the chemical class of the analytical compounds and the instrumental techniques used to obtain the data, so that diverse workflow models are possible. The acquisition of experimental data from hyphenated LC-UV-visible-MS derives a minimum of three dimensions on the information: chromatographic behavior or retention time, UV-visible spectrum, and MS spectrum of each chromatographic peak that should correspond to a molecular/protonated molecular ion. An additional dimension of information is obtained when tandem MS experiments are performed with the selected precursor ions. Therefore, several independent and complementary physicochemical properties are available (chromatographic behavior,

UV-vis spectrum, *m*/*z*…). The key in the data analysis of LC-UV-visible-MS data is to organize the physicochemical features on pairs of independent and orthogonal data (retention time and mass spectrum, accurate mass and tandem MS, and accurate mass and isotopic pattern). Once, the information is organized, this could be compared with the same analytical pairs of data relative to authentic compounds (standards) analyzed with the same identical experimental conditions [9] to match the data in the sample with those of the standards. This strategy is summarized in **Figure 2** and involves the following advantage. The process of matching the experimental data of the sample and those of the standards for the selected orthogonal criteria could be automated for reporting of findings by means of post-processing software tools [91, 92]. Among the different pairs of possible orthogonal data, accurate mass and isotopic pattern are widely accepted to successfully characterize the elemental composition of compounds in combination with orthogonal data related with the chromatographic behavior and UV-visible features. Thus, in the analysis of a complex mixture of carotenoids by LC-UV-visible-MS, a list of compounds with different *m*/*z* values and isotopic pattern is obtained, and a first matching process among the experimental data and the standards could be performed. This first screening is not unequivocal, because for each single pair of *m*/*z*

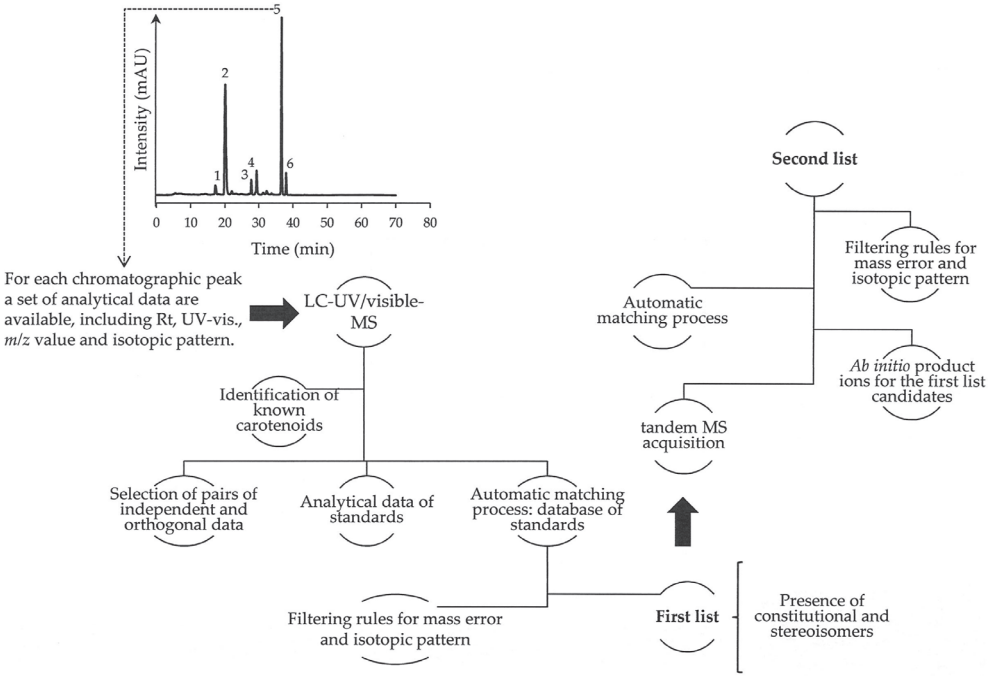

Figure 2. Overview of the workflow model for data analysis and identification of carotenoids. The selected pairs of independent and orthogonal data are matched with the corresponding experimental values of standards acquired with the same conditions. A first list of tentative identified carotenoids is obtained. This list is not unequivocal, and subsequently tandem MS data are obtained to match the experimental values of product ions with the *ab initio* product ions produced with the assistance of predictive software tools. The second list of identified carotenoids fulfills the rules and requirements for validating the identification [9, 40].

and isotopic pattern values a second reduced list of alternatives is possible due to the possible presence of constitutional and stereoisomers compounds in the sample (**Figure 2**). For example, it is not possible to distinguish β-carotene from lycopene or lutein from zeaxanthin exclusively with the *m*/*z* and isotopic pattern data (**Table 2**). In the first case, the assignment could be constrained considering the chromatographic behavior and UV-visible spectrum, but still the assignment is not explicit and for stereoisomers further information is needed. Here is where the third dimension of information plays a key role. The tandem MS data may contain characteristic product ions that arise from a fragmentation route available for one of the isomers and not for the others. Therefore, a list of potential "qualifiers" product ions could be built *ab initio*, which contains their *m*/*z* and isotopic pattern pair of values. This database is matched with the experimental tandem MS data acquired for the target compound to screen whether the "qualifiers" product ions are present or not (**Figure 2**). The search for these "qualifiers" product ions starts not only with the acquisition of tandem MS spectra, but also with the application of software tools to predict their structure considering the knowledge of the fragmentation rules that may be applied for each compound (see Section 3.2.2).

3.2.1. Common fragmentation pathways of carotenoids

The acquisition of tandem MS spectra encompasses a two-stage mechanism with a single aim, to produce an ion with enough internal energy (first stage) that causes it to fragment and generate a mass spectrum (second stage). The first stage starts with the ions produced at the ionization source, which are activated by collision with a neutral species. The transfer of energy from the neutral species may result from different processes [93] that are an extensive field of study regarding the characterization of the collisional-activated ion structure [94]. The second step consists on the competitive unimolecular dissociation routes that the collisional-activated ion experiments. According to the theory of unimolecular reactions, the Rice-Ramsperger-Kassel-Marcus theory, the dissociation of a sufficiently large and suitably energized molecule is basically statistical, that is, the internal energy of the activated ion is statistically distributed among the internal degrees of freedom [95]. Consequently, different energetically accessible dissociation/fragmentation pathways start to compete yielding a mass spectrum containing different product ions. The key in this second step is that once the structure of the collisional-activated ion is known, the sequence of fragmentation pathways can be modeled, and the structure of the product ions are predicted [96, 97]. This results in a significant advantage for the study of tandem MS spectra because, as it was mentioned above, the product ions from a selected molecular/protonated molecular ion could be known *ab initio* and the screening in an experimental tandem MS spectrum could be automatically performed with the aid of the appropriate software tool (see Section 3.2.2).

The carotenoids follow three major fragmentation processes: elimination of in-chain units, elimination of terminal groups, and *retro*-Diels-Alder fragmentation [56] (**Table 2**). Indeed, several mechanisms are possible within each of the noted fragmentation schemes, because the different localization of functional groups in the structure of carotenoids, and the frequent stereochemical arrangements introduce alternative events in the progress of the fragmentation process. These mechanisms have been extensively reviewed in [2, 56, 57, 60] with tabulated data regarding the main product ions and the structural significance of the observed

losses and the intensity of the signal in the parent compound. The ionization technique and the composition of the mobile phase are factors that may modulate the fragmentation pattern, but the characteristic product ions are observed under different experimental conditions and instrumental configurations.

The elimination of in-chain units follows the Woodward-Hoffmann rules and the process yields to the losses of toluene (92 Da) and xylene (106 Da) from the polyene chain. Both losses are almost ubiquitous in the MS spectra of carotenoids, and the intensity ratio for the corresponding peaks is inversely related with the number of conjugated double bonds, providing information about the nature of the polyenoic chain [98]. Indeed, the kinetics of the reactions leading to both fragments is modified not only by the amount of double bonds of the chromophore system, but also by the cyclic/acyclic conformation of the end groups and the presence of oxygenated functions. Moreover, the ionization method applied changes the abundance of the signals as it is the case of APCI [60, 66]. Therefore, the information obtained from the intensity ratio should be carefully analyzed as the influencing factors are multiple. Other reactions lead to alternative product ions by the loss of 78, 79, and 80 Da from the parent compound, involving functional groups close to the polyenoic system like the 5,6- and 5,8-epoxides. The analysis of the product ions arising for the elimination of terminal groups represents a helpful diagnostic strategy to establish the structural arrangements and functional groups that feature the carotenoid conformation. The loss of 69 Da indicates the presence of at least one linear ψ end group [73, 99]. The loss of 18 Da points to the presence of hydroxyl group(s), while the loss of fatty acid(s) shows the occurrence of xanthophyll esterification [15, 17, 73]. Some of these product ions reveal further structural information when the signal intensity is accounted. Thus, the loss of 18 Da detected in the MS spectrum of lutein and zeaxanthin shows different abundance (higher for lutein, lower for zeaxanthin) in comparison with the signal of the molecular/protonated molecular ion [60, 73, 100]. A similar behavior displays the pairs of structurally related carotenoids like α-cryptoxanthin and zeinoxanthin or lutein epoxide and antheraxanthin for the same signal (loss of 18 Da) in comparison with the intensity of the molecular/protonated molecular ion [73, 101]. The reactions based on the *retro*-Diels-Alder fragmentation are the third general scheme to follow the interpretation of tandem MS of carotenoids. The reaction may proceed in a single step or by a double α-cleavage starting at the allylic position from where subsequently the fragmentation is produced. This process yields characteristic product ions that can be used to distinguish isomeric α-carotene, β-carotene, γ-carotene, and lycopene, in combination with other characteristic product ions arising from alternative fragmentation pathways. Hence, the *retro*-Diels-Alder fragmentation at the ε-ring of α-carotene generates the loss of 56 Da, so that its presence or absence is indicative of the configuration at least in one of the cyclic end groups [60, 73]. The same applies to distinguish lutein, which presents one hydroxylated-ε-ring and one hydroxylated-β-ring, and zeaxanthin that includes two hydroxylated-β-rings. The product ion at 495 Da that generates from the *retro*-Diels-Alder fragmentation of the ε-ring (56 Da) and subsequent water loss (18 Da) is observed in lutein but not in zeaxanthin [2, 62, 82].

The occurrence of these fragmentation processes with different prevalence (some product ions may not appear, while other could constitute the base peak of the MS pattern) produces a fragment MS spectrum that should provide enough information for the characterization of the different carotenoids present in the sample. The application of published data on the

characteristic MS spectrum of carotenoids and the search in databases on the Internet is helpful for interpretation, but they should be applied with caution considering the instrumental configuration and conditions for acquisition of the MS that were used in the references, to avoid confusing or incorrect identification. The use of experimental data from authentic standards analyzed with the same MS acquisition settings used for the sample, as well as any complementary source of data should be considered a golden rule for identification.

3.2.2. Software tools

The implementation of software tools to assist in the production of a positive list of identified compounds in sample is one of the significant advances that has been introduced in the capabilities of MS. The analysis of MS spectra for the identification of the molecular/protonated molecular ions, the application of filtering rules based on the mass accuracy error and isotopic pattern, and the study of product ions in tandem MS means an intensive manual labor that could be released with the aid of suitable software. These tools may be embedded as a module or option in the main software distributed with the MS instrument, or may be available to download from the Internet. Different software utilities are designed to resolve each of the steps that lead to obtain the correct elemental composition and to establish the main structural features.

To achieve that aim, the application of filtering rules to the measured mass, regarding the mass error and isotopic pattern distribution, is the main approach to constrain the number of possible candidate structures. Thus, for a given molecular/protonated molecular ion with an experimentally measured *m*/*z* value, a set of elemental compositions is possible (see Section 3.1.3 and **Figure 2**). Increasing the mass accuracy, the number of potential candidates is reduced. The software tools to perform this first screening automatically, the operator should only establish the mass error value to allow an elemental composition to enter in the positive list or to be excluded. The second filtering rule involves isotopic pattern. The software tools compare the isotopic distribution of the experimental *m*/*z* value with the theoretical ones of the potential candidates that fulfilled the first filter (mass error) yielding a correlation value for each candidate. The operator should only establish the threshold for that correlation value that makes an elemental composition to pass this second screening. Free software to complete both screening steps is available on the Internet (http://tarc.chemistry.dal.ca/soft_down.htm; https://omics.pnl.gov/software/molecular-weight-calculator). Regarding the evaluation of data from tandem MS spectra, the same programs may apply to each of the characteristic product ions. The filtering rules process should be reliably accomplished, both in the parent compound and in its product ions. Thus, the consistency of the elemental composition and isotopic pattern of the product ions with the structural features of their parent compound is assured [91, 92]. To perform this step and the subsequent study of the contribution of each characteristic product ion to the structure of the molecular/protonated molecular ion, we should be able to predict product ions from the candidate structures ascertained in the first screening process. As it was noted in Section 3.2.1, the product ions generated in a tandem MS spectrum could be modeled by the application of the general fragmentation rules. Predictive software tools (Mass Frontier, HighChem, Mass Fragmenter, Advanced Chemistry Development, and EPIC) evaluate the starting structure of the parent compound and generate a set of hypothetical product ions that may arise from fragmentation. This list of candidate product ions could be matched with the list of experimental

product ions observed in the tandem MS spectrum. Once the annotated product ions are tabulated, the filtering rules regarding mass error and isotopic pattern assure that the assignation of product ions is unambiguous and related to the parent compound. Indeed, this second screening process contributes to discard some of the candidate structures or even to point only one of them as the assigned structure for a given molecular/protonated molecular ion.

4. Conclusions

The advance in knowledge of carotenoids occurrence in nature has required the application of different analytical techniques to characterize the structure of this family of pigments, how they accumulate in different tissues and the available metabolic conversions they experiment either in their natural cellular surrounding or in the tissues they are incorporated. Classical procedures for the extraction of carotenoids from raw sources and different biological materials have evolved with the inclusion of *green* technologies and environmentally friendly practices, which are summarized in this chapter. Indeed, the development of HPLC methods has introduced the new generation of stationary phases that shortens the run time and solvent expenditure. With the application of UPLC and two-dimensional liquid chromatography, the challenge of the characterization of intricate carotenoid profiles has been accomplished, and it is possible to select almost tailored chromatographic conditions to the target sample matrix containing the carotenoids. Thus, a relevant improvement has been the analysis of xanthophyll esters, which was a difficult approach few years ago. The current accessibility to mass spectrometry detectors has overcome several of the drawbacks that the traditional detection/identification of carotenoids by the UV-visible spectrum. This has been possible due to the improvements in the ionization strategies and equipment applied to yield ions mass, as well as the use of mass spectrometers with high capabilities in terms of mass resolving power, mass accuracy, linear dynamic range, and sensitivity. Thus, the acquisition of full detailed MS data in conjunction with the information obtained from the chromatographic behavior and UV-visible features produce the accurate evidences for the correct identification and provide the appropriate biological meaning to the research issue. Finally, the practice in software tools implemented in the workflow of MS data analysis alleviates the manual labor of data processing and allows the systematic high-throughput screening of carotenoids and their metabolites.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Antonio Pérez-Gálvez* and María Roca

*Address all correspondence to: aperez@ig.csic.es

Food Phytochemistry Department, Instituto de la Grasa (CSIC), Campus Universitario, Sevilla, Spain

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