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MS tools for a systematic approach in survey for carotenoids and their common metabolites

Pérez-Gálvez, A.[1]*, Sánchez-García, A.[2], Garrido-Fernández, J.[1], Ríos, J.J.[2]

[1]Food Phytochemistry Department, Instituto de la Grasa (CSIC), Sevilla, Spain.
[2]Laboratorio de Espectrometría de Masas, Instituto de la Grasa (CSIC), Sevilla, Spain.
*author to whom correspondence should be addressed. Email:aperez@ig.csic.es.

Abstract

Metabolism of the carotenoids in humans is often reserved to bioconversion of provitamin A carotenoids in retinoids and oxidative/degradative products arising from enzymatic eccentric cleavage, yielding apo-carotenoids. Nevertheless, additional metabolic routes should be available, and some of the structures of those metabolites have only been anticipated. The identification of carotenoids and their metabolites is commonly performed by HPLC coupled to MS. The acquisition of MS data in different instrumental modes and hardware configurations allows the detailed structural description of the target compound. Indeed, the MS data of carotenoids and their metabolites available in literature could be collected in a database to automatically perform a screening of the theoretical MS data included in the database with the MS experimental values. This review describes the required fundamentals for a systematic pursuit of carotenoids and their metabolites. Highlights on the use of appropriate hyphenated HPLC-MS systems and the requirements for the identification are discussed, while the application of software tools to apply filtering rules and the implementation of post-processing workflow, which uses two orthogonal criteria for the identification from the mass spectra data, are described.

1. Introduction

Observational epidemiologic studies provide evidence of the inverse association between carotenoid-containing fruits and vegetables or serum carotenoid levels with risk for various chronic diseases, including lung, gastrointestinal tract, pancreas, breast and prostate cancers, heart disease, and age-related macular degeneration [1-7]. Actually, a causal association has been examined by a variety of meta-analysis studies and intervention trials performed with specific carotenoids, including β-carotene, lycopene, lutein and zeaxanthin [8-10]. The quest for that causal association is sustained by some reasons. Around 700 carotenoids have been identified in nature, but the diet provides us with 40 [11] and about 90% of the dietary carotenoid content is represented by the carotenoids mentioned above, including α-carotene and β-cryptoxanthin [12]. The carotenoid pool in serum is mostly characterized by that same group of carotenoids, and indeed some tissues accumulate an even more limited group of carotenoids. This is the case of prostate and *macula lutea* that selectively accumulate lycopene or lutein/zeaxanthin, respectively. However, the results obtained from clinical trials failed to find direct evidence between the intakes of such carotenoids and a protective effect [13]. Even negative outcomes arose in those intervention trials involving smokers where the carotenoids were administered individually in the form of supplements at the pharmacological range [14]. This paradigm questioned the design of the studies that did not consider the synergistic effects of carotenoids with complementary compounds like vitamin C and E, which are a key issue for an appropriate mode of action, and the toxic response of smoking in combination with high doses of carotenoids. Hence, some factors that also have an influence on the observed results are the prooxidant function of carotenoids at the provided doses, the health and nutrient status of the participants, and the stage of the disease at which the carotenoids may impact its development and progression [15,16].

While conclusive clinical evidences are presented through human intervention studies that integrate a holistic view of both the disease and the dietary, genetic, epigenetic and environmental factors that contribute to its prevention, and to its progress or delay [13,15], it is necessary to advance in the knowledge of the mechanisms at the molecular level by which carotenoids exert their actions. This work should not be limited to the carotenoids, but also to their metabolites. Significant progress has been made with the mechanisms and enzymes involved in the symmetric/asymmetric cleavage of carotenoids that undergo *in vivo* [17]. However, scarce information is known about the biological significance of the metabolic

products arising from these and other oxidative processes, although they are active in several cellular processes [18,19]. Indeed, the structural characterization of the metabolites may provide important insights into the complete understanding of the different mode of actions of the carotenoids, as well as the emerging functions of their products.

Considering the biochemical processes that originate the different metabolites, these could be classified in four groups. Retinoids, the group of carotenoid metabolites arising from the activity of β,β-carotene-15,15'-oxygenase 1 (BCO1) that converts the provitamin A carotenoids to retinaldehyde. Apo-lycopenoids, the metabolites arising from the activity of β,β-carotene-9,10-oxygenase 2 (BCO2) that cleaves lycopene eccentrically, yielding a variety of lycopene-derived products [20]. β-Apo-carotenoids, the metabolites arising from the activity of BCO2 on either provitamin A and non-provitamin A carotenoids with at least one β-type ring. It has been demonstrated that the asymmetric cleavage catalyzed by the BCO2 enzyme is not only active to convert carotenoids with at least one β-ionone type ring [17] but also xanthophylls, like zeaxanthin and lutein [21] showing that BCO2 displays broad substrate specificity. Finally, the group of carotenoid oxidative products arising from reaction with reactive oxygen species or with enzyme(s) not related with the carotenoid oxygenase family. This group includes several oxidized derivatives identified *in vivo*, such as the 3-oxo carotenoids [22] produced from lutein and zeaxanthin, or the oxidized products from fucoxanthin [23], β-apo-carotenoids [24] and apo-lycopenoids that have been the focus of recent studies regarding their antioxidant activities and their presence in human tissues and foods [25,26]. Subsequent breakdown processes, either chemical or enzymatic, could cleave these metabolites to unexplored oxidized derivatives that also cause significant biological activities *in vitro* through mechanisms of action only partially understood [27]. It is important to note that several carotenoid metabolites arising from enzymatic processes (even from the carotenoid oxygenase family) are also produced by chemical reactions, so that the origin of the metabolic products is difficult to determine, if the appropriate experimental conditions are not applied. Another kind of carotenoid metabolites that should be considered in this group is the xanthophyll esters. Their significant presence in the carotenoid profile of human colostrum [28] implies the existence of an active fatty acid acylation pathway esterifying the xanthophylls integrated in the cellular membrane of the mammary epithelium. Trace amounts of xanthophyll esters have been also described in human serum and skin [29,30], and in bare skin of the tropical bat *Ectophylla alba* [31]. Finally, different *in vitro* chemical autoxidation processes have been applied to produce oxidation products of carotenoids [32,33,34].

Although most of these products have not been detected *in vivo* their structural features are indicative of the available reaction mechanisms.

The significant advancements attained in the knowledge of carotenoid metabolites make conceivable to comprehensively determine them in each biological tissue, i.e. to perform targeted metabolomics of carotenoids [2]. Thus, the chemical information of the already known carotenoid metabolites could be integrated in a specialized target library to search systematically the complete array of metabolites. This analytical challenge is feasible considering the current hyphenated techniques available, which provide the required parameters of sensitivity and selectivity, in combination with modern software tools. These tools allow a workflow model for the efficient processing of experimental data, and the implementation of several restriction rules, based on combined chemical characteristics, for validating the metabolite identifications.

Therefore, the aim of this review is to describe the required fundamentals for a systematic pursuit of the main dietary carotenoids and their common metabolites described *in vivo*. Highlights on the use of appropriate hyphenated HPLC-MS systems and the requirements for the identification are discussed, while the application of software tools to apply filtering rules and the implementation of post-processing workflow, which uses two orthogonal criteria for the identification from the mass spectra data, are described.

2. Basics of sample preparation

Sample preparation aims to enrich the final extract in the targeted metabolites and remove potential interfering compounds. Although raw materials with different features are the starting point for selective isolation of carotenoids and carotenoid metabolites (fruit and vegetables, animal tissues, plasma/serum and red blood cells, cell cultures, micelles, reaction media), the steps for extract preparation are generic and they are based on the solubility of carotenoids and their metabolites (with few exceptions) in non-polar solvents. Common protocols applied for extraction from foods and biological samples have been compiled in guides and revision papers [35-37]. Pre-treatment of sample (freezing, grinding, reconstitution with organic solvent, filtering, precipitation of protein materials, centrifugation, ultrasonic/buffer-based lysis) are often applied before solvent extraction to increase quantitative recovery of the target compounds. All the steps should be carried out very quickly and under those experimental conditions that minimize degradation with light, oxygen, temperature and any other setting that lead to metabolite conversion and to avoid the

production of artifacts. This issue should be extremely considered to avoid the production of artifacts during sample preparation for analysis. Solvent extraction, either with pure solvent or mixtures, then extract cleanup (SPE, ion exchange, ultrafiltration) and concentration of the extract under vacuum or with N_2 gas are briefly the subsequent steps to obtain the final extract. Chemical derivatization might be performed on the extract to confirm the presence or absence of functional group(s) by changing the chromatographic behavior and the features of the UV-vis spectrum of the compound [36]. Chemical hydrolysis is routinely done to remove lipids (chlorophylls, triacylglycerides) that interfere in the analysis of the sample, and to transform the xanthophyll esters into the corresponding free xanthophyll form. However, if the presence and/or identification of xanthophyll esters is the subject of the analysis, then direct analysis of the extract should be carried out with the intact xanthophyll esters but still the interfering lipids must be removed. Hence, cleanup protocols of the extract [28,38,39] or the use of two-dimensional comprehensive LC-MS [40,41] have been proposed to identify the native composition in xanthophyll esters of the sample, decreasing significantly the interfering effect.

3. Liquid chromatography-mass spectrometry (LC-MS)

LC is the universal analytical technique applied to accomplish separation of the different carotenoids and/or their metabolites, although methods applying supercritical fluid chromatography are gaining recent attention [42]. The manifold alternatives and combinations of elution systems (isocratic or gradient elution) with normal- or reversed-phase modes arranged by a wide range of packing materials currently available provide solutions to face the separation of these substances, particularly when isomeric forms or structurally related compounds are present in the mixture, to diminish the interference of co-eluting lipophilic compounds, and to improve the sensitivity of the UV-vis/MS spectra. Additional solutions to improve the separation include the change of temperature column and the application of derivatization reactions *prior* to the LC analysis, to change the chemical functionality of the target compounds and consequently their chromatographic behavior. These issues have been discussed in recent reviews focused in the analysis of carotenoids [43,44]. Table 1 contains a brief description of some representative LC methodologies with the detection and identification systems applied in the analysis of carotenoids and carotenoid metabolites.

Table 1

Analytical methods applied for detection of carotenoids and their metabolites in biological samples.

Normal- and reversed-phase conditions and columns with different packing materials have been successfully applied for the analysis of the extracts that contain a complex mixture of carotenoid metabolites, sometimes mixed with their parent compounds. Although the C18 columns provide an efficient resolution for the apo-carotenoids and low-weight metabolites, the C30 columns with gradient elution are the best option for the separation of the wide polarity range of these compounds, particularly when geometric isomers of apolar carotenoids are present, as well as for long chain products like xanthophyll esters. Moreover, they provide higher sensitivity as the interfering effect of the matrix components is reduced [45,46].

3.1. Detection instruments

Photodiode-array or UV-vis detectors are the common instruments coupled to the LC system for qualitative and quantitative characterization of the carotenoids and their metabolites. Although some structural information is obtained from the UV-vis spectrum of carotenoids [47], it is impossible to achieve a complete characterization exclusively on the basis of UV-vis spectrum. The oxygenated functions located at the end-groups of the molecule are not essential modifiers of the spectrum, so that the spectra of many carotenoids are very similar. Additionally, it is very common that structurally-related compounds appear in the same chromatographic region that depreciates the quality of the UV-vis spectrum. Consequently, the use of MS is crucial for acquisition of structural information regarding the elemental composition and how the functional groups are arranged in the target compound, through the study of characteristic product ions. Moreover, MS overcome the problems in the identification of co-eluting individual carotenoids or in complex mixtures where UV-vis spectra are not well resolved due to the low sample amount or to the interference of the matrix. Most of the ionization strategies that became available with the development of the MS technique have been applied to the analysis of carotenoids. Thus, electron impact, fast atom bombardment and matrix-assisted laser desorption/ionization were used for direct analysis of the compound(s) [44,47-51] while atmospheric pressure ionization methods (electrospray ionization, ESI, and atmospheric pressure chemical ionization, APCI) are the current choice for LC-MS coupling [52]. These ionization methods display a high-mass capability extremely useful for the analysis of large, non-volatile, chargeable molecules,

which are shifted from the condensed phase into the state of isolated gas-phase ions starting at atmospheric pressure to subsequently move to the high vacuum operative conditions of the mass analyzer [53].

ESI has been successfully applied to the ionization of polar carotenoids. This ionization strategy requires small amounts of sample and the problems of thermal instability of some carotenoids are avoided [54,55]. Nevertheless, it is frequent the incorporation of additives to the mobile phase, including ferrocene-based derivatives, mildly acidic salts, weakly organic acids, halogen salts, trimethylamine or ammonium acetate [43,44,54-59], to enhance the efficiency of the ionization process, a significant advantage in the analysis of carotenes. 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, including lower ranges of apolar compounds, from carotenes to xanthophyll esters [38,49,52,60-63]. Thus, APCI is the choice for identification of complex mixtures of carotenoids/metabolites, the common task in MS of biological samples. In this ionization mode, the aerosol coming from the liquid flow, which it is higher than in ESI mode, is transformed into a vapor in a heating unit set up at high temperatures what may cause thermal instability to the protonated ions. However, the transfer of the vapor is very fast, so that the ions do not necessary reach the high temperature conditions, while the soft collisions between ions that initiate at this stage spend part of the excess energy, although in the case of hydroxylated carotenoids it is very common that the protonated molecular ion directly losses a water molecule even during the acquisition of MS spectra. 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 select fragmentation pathways [52].

The final link in the instrumentation of LC-MS is the mass analyzer, which may present different configurations according to the kind of ion acceleration and detection systems. Each configuration performs diverse intrinsic parameters (mass resolving power, mass accuracy, linear dynamic range and sensitivity) at distinct levels. Thus, the hardware adjustments are in a constant developing process, together with the software capabilities, to meet demanded robustness, reproducibility and efficiency of the analyses. The literature of MS analysis of carotenoids reports the use of ion trap (IT) [21,39,64-66] and quadrupole (Q) [67-70] instruments that offer good sensitivity but limited resolving power [71]. The arrangement of these analyzers in a tandem configuration to structure hybrid mass spectrometers (Q-time-of-flight, Q-IT, triple Q-Orbitrap) [26,28,31,52,54,72-74] allows the achievement of higher performance by increasing the mass accuracy level and the resolving

power of the instrument. 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 identification of the compounds, as well as to enhance the rigor and strength of the peak assignment. Most of the data available to date regarding the identification of carotenoids and their metabolites have been obtained from single Q or IT instruments, providing *m/z* values of the molecular ion with mass uncertainty at the part-per-thousand level. The acquisition of *m/z* values is combined with broad band collision-induced dissociation (bbCID) analyses to obtain information of the structural features about the fragmented ion. In such experiments, the production of the molecular ion and the subsequent application of kinetic energy for ion fragmentation are events separated in time rather than spatially with longer time period for reaction and dissociation. This fact increases the possibility for extensive resonance stabilization of a wide variety of product ions arising from low-energy fragmentation pathways [75], so that the bbCID spectra of compounds having different functional groups usually contain a high amount of information 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 and their metabolic products [43,48,51,52].

While this information is enough for identification of several carotenoids, those with similar structure and the isomers require complementary information for characterization of their structural arrangements. Isomers of carotenoid metabolites have been described as well, like for the apo-carotenoids and retinoids. Thus, the comparisons of mass spectra by positive and negative ion modes [76,77], 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. Still, the assignment of peaks from experimental data means an intensive manual labor, and the implementation of modern software tools to assist in the production of a positive list of identified compounds in each sample is not underlying. However, with the available information regarding the MS characteristics of both carotenoids and their metabolites, it is possible to automatically obtain a report of compounds (carotenoids/metabolites) from an in-house mass database created *ex profeso* that matches the experimental *m/z* signals with two selected orthogonal characteristics of the former as detailed below.

3.2. Metabolite identification

Data analysis for metabolite identification is the last step of untargeted metabolomics. The extraction of meaningful biological perspective from the data depends on the confident and rigorous identification of the metabolites. There is not a single standardized approach for identification, because the strategy applied is related with the chemical class of metabolic compounds and the instrumental techniques used for the analysis, so that different workflow models are possible. However, some rules and recommendations for validating the identification should be followed. The key is that identification should not only be based on 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 [78] to match with the compounds analyzed in the sample. In the case of LC-MS analysis applied for metabolomics, several pairs of independent and complementary physicochemical properties are available (retention time and mass spectrum, accurate mass and tandem MS, accurate mass and isotopic pattern) for matching between the compounds of the sample profile and the standards, so that selecting more than one pair to perform the identification, complementary confidence is provided. Additionally, the selection of these pairs of orthogonal data implies the following advantage. The process of matching the experimental and the theoretical values of the selected criteria of the target compounds compiled in a selected database could be automated for reporting of findings by means of post-processing software tools [80,81]. Among the different pairs of available orthogonal data, accurate mass and isotopic pattern are widely accepted to successfully characterize the elemental composition of target compounds in metabolomics by MS. Accurate mass alone is insufficient to exclude an enough number of potential candidate compounds, particularly at high mass regions (>300 Da), but when combined with the isotopic pattern resulting from natural isotope abundance and elemental composition [82] the number of potential candidates is significantly reduced. Therefore, the first screening in the MS data that could be automatically performed is to match the predicted pairs of accurate mass and isotopic pattern of the protonated molecular ions in a target database *vs*. the experimental values. This first screening yields a list of tentative positive candidates. Subsequently, the tentative list of positive findings is processed performing a second screening, matching the theoretical characteristic product ions of the tentative positive candidates with the experimental MS^2 values.

Fig. 1. Workflow for the identification of carotenoids and their metabolites using two pairs of orthogonal data from MS in bbCID mode analysis and filtering rules.

The workflow we describe here is summarized in Figure 1. It starts with the creation of an *in-house* database with the data reported in literature regarding chromatographic behavior, features of the UV-vis spectrum, elemental composition of known carotenoids and their metabolites and of their characteristic product ions. The theoretical monoisotopic exact mass values and isotopic patterns for each target compound and for their characteristic product ions are calculated with the utility software IsotopePattern (Bruker Compass Utilities version 2.0). The data contained in the database are checked experimentally with authentic carotenoid standards isolated from their natural sources (astaxanthin, canthaxanthin, capsanthin, capsorubin, α- and β-carotene, β-cryptoxanthin, echinenone, fucoxanthin, lutein, lycopene, neoxanthin, phytoene, phytofluene, violaxanthin, zeaxanthin; xanthophyll esters), or in the case of standards of carotenoid metabolites analyzed in authentic samples including serum, red blood cells, human colostrum and human mature milk, micellar fractions from *in vitro* digestion of carotenoid standards, and *in vitro* incubations with subcellular organelles obtained during the development of our experimental research (β-apo-carotenoids, apo-lycopenoids, xanthophyll esters). We also reproduced some of the experimental protocols already published in literature to obtain experimental data of the corresponding carotenoid metabolites. Other products are commercially available. Some of the carotenoid metabolite standards were not available in our laboratory, so that the identification with our target database reaches the level of putatively annotated compounds. Table 2 contains the list of the carotenoid metabolites described in biological samples and from which we have enough experimental data regarding their chromatographic behavior, UV-visible features and MS pattern to be included in the database.

Table 2

Carotenoid metabolites described in biological samples.

The acquisition of the mass spectra in the problem samples is performed using a micrOTOF-QII high resolution TOF mass spectrometer (UHR-TOF) with Qq-TOF geometry (Bruker Daltonics, Bremen, Germany) equipped with an APCI source. The instrument is operated in positive ion mode using a scan range of *m/z* 50-1200 Da. Mass spectra are acquired using bbCID mode, providing MS and MS/MS spectra simultaneously. Instrument

calibration is performed externally prior to each analysis, and an automated post-run internal mass scale calibration of each sample is enabled by injecting the same calibrating solution. Once the experimental MS data have been acquired the automated post-processing routine is applied. Raw data are analyzed with the TargetAnalysis™ (version 1.2) software that automatically performs mass scale calibration, and searches the experimental extracted ion chromatograms (EIC) of each compound included in the database ($[M+H]^+$) in the raw data by matching the theoretical accurate mass values *vs*. the experimental ones. Only those matched pairs of theoretical and experimental accurate mass values that fulfil the filtering rule of mass error below 5 ppm are pre-selected. The mass error (me) is calculated from the measured monoisotopic mass (m_{meas}) and the theoretical monoisotopic mass (m_{theo}) values with the following equation:

$$m_e\ (ppm) = \frac{monoisotopic\ m_{meas} - monoisotopic\ m_{theo}}{monoisotopic\ m_{theo}} \times 10^6$$

Now, the mSigma value for each pre-selected matched pair is computed with the SigmaFit™ utility software (Bruker Compass DataAnalysis version 4.1). It is an algorithm [79] for calculating isotope distributions that retrieves a match factor between the theoretical and experimental isotopic patterns. The filtering rule of the mSigma values is set at 50. Only the experimental EICs that present mass error and mSigma value below the tolerance limits are included in a first list of tentatively identified carotenoid/carotenoid metabolites, which is summarized in an Excel-based results report of positive findings. Subsequently, the information supplied by the MS^2 spectra is automatically analyzed for each carotenoid/metabolite included in the first list by an algorithm (SmartFormula3D™ version) that estimates whether a formula for a product ion is a subset of a formula for the precursor applying the same orthogonal criteria as in the first step, i.e., mass accuracy and isotopic pattern with the same filtering rules (mass error below 5 ppm and mSigma value below 50). This second post-processing routine allows the differentiation of those carotenoids/metabolites with the same elemental composition but with different functional groups that yield characteristic product ions included in the database, as it has been shown for structural isomers [51,52,83]. Finally, the complementary data included in the database regarding chromatographic behavior, UV-vis features and intensity of mass signals are still need for the differentiation of geometrical isomers that might be present in the analyzed

sample. Similar software tools are available from diverse MS instrument producers and even available to download from the internet.

This strategy has been successfully applied for identification of carotenoids and xanthophyll esters in human colostrum and mature milk, and in the bare skin of the tropical bat *Ectophylla alba* [28,31]. Particularly, the human colostrum could be considered a suitable source of carotenoids and their metabolites because the accepted pathway for accumulation of lipids (including carotenoids from diet) in the human mammary gland is compatible with several metabolic routes that might produce some of the carotenoid metabolites described so far, and it has been demonstrated, xanthophyll esters are among them [31]. The workflow we describe here is feasible with those experimental conditions described in literature regarding the chromatographic conditions more suitable to the identification of different carotenoid metabolites (Table 1) that with our post-processing routine will increase the high-throughput screening.

4. Conclusion

Although more than 700 carotenoids have been identified in Nature, a reduced group of 40 is available through diet and therefore could incorporate to the pool of circulating carotenoids, accumulate in different tissues, and finally be subjected to metabolic conversions. The latter processes have been extensively studied and the structure of several metabolic products of carotenoids is now characterized. The advancements in knowledge of carotenoid metabolism have been challenging because of the products accumulate in low amounts and present a short life with unanticipated structural arrangements. Therefore, the acquisition of this knowledge has required the application of hyphenated analytical techniques, mainly HPLC coupled to MS, to obtain the accurate information for the correct identification of the metabolites and finally provide the right biological meaning. Now, the information regarding both the structural features and MS behavior of carotenoids and their metabolites and the developments in the analytical techniques could be complemented with the available software tools to build a workflow that allows a systematic high-throughput screening of carotenoids and their metabolites in biological tissues.

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