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Biofunctionality of Carotenoid Metabolites: An Insight into Qualitative and Quantitative Analysis

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Abstract

Epidemiological and clinical studies have shown that dietary intake of carotenoid-rich fruits and vegetables is positively correlated with reduction in age-related eye diseases, atherosclerosis, certain cancers and chronic diseases. Carotenoids consist of unique chemical characteristics and are highly vulnerable to structural modifications, leading to the formation of various derivatives under physiological conditions. The identification of these molecules is necessary before addressing their biological functions. Carotenoid metabolomics is believed to be highly complex to fingerprint due to instability and interference with complex biological matrices. Noteworthy, progress has been made in understanding carotenoid metabolism or its biotransformation in biological samples. In this regard, the chapter highlights the concept of metabolomics and their related bio-analytical techniques pertaining to the detection of carotenoids and their derived products to elucidate their bio-transformation on targeted biological functions. Further, this chapter highlights the various hyphenated analytical tools and their optimization.

Keywords: metabolomics, hyphenated technique, bio-functionality, metabolites

1. Introduction

Considerable research has made in understanding the potential role of carotenoids in plants and animals. Carotenoids are tetra-terpenoids, a group of natural compounds, found

predominantly in photosynthesizing organisms such as green plants, algae and some bacteria [1]. There are over 750 carotenoids identified in nature; among them, major carotenoids like α -carotene, β -carotene, lycopene, lutein, zeaxanthin, astaxanthin, fucoxanthin and β -cryptoxanthin are well explored. In plants, carotenoids and their derivatives (apo-carotenoids) are believed to involve in photosynthesis, photo-protection, the precursor for hormones and production of aroma and flavor [2]. The diversity of carotenoids in nature is formed from the common biosynthetic pathway. The exploration of each distinctive carotenoid pigment from various dietary sources or non-dietary sources is the milestone in carotenoids biochemistry and metabolism. In animals and human, dietary ingestion is the only source of carotenoids obtained from plants (vegetables and fruits) or animals (meat, fish and poultry products) or other nutraceuticals sources. They are categorized based on biological functions as provitamin A or non-provitamin A carotenoids. Further, they classified as carotenes (β -carotene and lycopene) and xanthophylls with epoxy- (violaxanthin, neoxanthin, fucoxanthin), hydroxy- (lutein and zeaxanthin), keto- (astaxanthin and canthaxanthin) and methoxy- (spirilloxanthin) functional groups. In plants and certain microorganisms, under various environmental conditions, the biotransformation of carotenoids occurs as a necessary adaptation. Carotenoids research is an active area of research due to the characteristics of different chemical nature with unique biological function [3]. Epidemiological and clinical trials have correlated that consumption of carotenoids rich fruits and green vegetables decreases vitamin A deficiency problems, cancer, cardiovascular diseases and age-related macular degeneration [4]. Although diversified carotenoids with unique characteristics structures exist in nature, the relation between structure function needs to explore by developing appropriate analytical protocols and techniques. Only a few carotenoids are studied and detailed from past three decades, due to their presence in common dietary sources and human serum and tissues. Many of the bioactive compounds execute their function either by intact or by its derivatives [5, 6]. In many cases, carotenoids daughter molecules exist in nature as a secondary compound, or they possibly formed over series of reaction that occurs in physiological condition [7]. Carotenoids consist of long polyene chain with series of conjugated double bonds and functional groups that make them highly reactive. Thus, carotenoids are more prone to various modifications such as hydrogenation, dehydrogenation, double-bond migration, chain shortening or extension, rearrangement, isomerization, oxidation or combinations of these processes under different conditions. Apart from these, carotenoid metabolites may also form due to the presence of enzymes monooxygenase, cyclooxygenase and dioxygenase [8]. Investigation on β -carotene metabolism and their conversion to vital active molecules, that is, retinyl palmitate, retinal, retinoic acids and apo-carotenoids, has made a greater contribution to the concept of metabolomics. Further, these molecules augmented with cellular function on vision, growth, immune response and related chronic health problems. With this discovery, subsequently many notable researchers worked on other non-provitamin A carotenoids and succeeded partly in the identification of bioactive metabolites in vivo. Therefore, metabolomics has attracted and motivated in an identification of carotenoid bioactive metabolites. In continuation, several studies revealed the potential role of carotenoid metabolite or oxidative products in vitro and in vivo and supported the concept that biological functions mediated through their metabolites [6–9]. With this background, we highlight the possible biotransformation of

carotenoids (β -carotene, lycopene, lutein, astaxanthin, fucoxanthin and other carotenoids) and the related analytical techniques used for their determination. Also, biofunctionality of carotenoids metabolites discussed. Further, this chapter details the improvement of analytical techniques and their hyphenation for evaluation of carotenoids metabolite (beneficial or harmful effect). The advancement in analytical tools and the discovery of unknown carotenoids metabolites broaden the scope of carotenoids research. Hence, omics instruments and their methods perform untargeted and targeted profiling of carotenoids in foods and human samples. Apart from these, this chapter also emphasizes on the suitability and optimization of analytical methods to determine carotenoid metabolites.

2. Biotransformation of carotenoids

Even though more than 750 carotenoids are identified and predicted in the natural source, only a few carotenoids (β -carotene, lycopene, lutein, astaxanthin, fucoxanthin, canthaxanthin) addressed by using routine analytical techniques. Presently, carotenoids research is mainly focused on the analysis of dietary carotenoids and linked to biological functions. Since carotenoids are unstable molecules, it undergoes various modifications (discussed elsewhere), and hence, it is challenging to analyze such carotenoids or its metabolites. β -Carotene metabolites such as retinol, retinal and retinoic acid are studied extensively due to its significance in human health. Therefore, creating an awareness to study the metabolism of carotenoids and their bio-functions is currently warranted. The carotenoid metabolites may be involved in differential gene expression, cell-to-cell communication and cell differentiation. Furthermore, it is interesting to address the molecular interaction of carotenoids metabolites with free radicals, protein and lipids at cellular levels. Studies also show that carotenoids oxidative products may be involved in oxidative stress and act as pro-oxidant [10]. The identification of oxidative metabolites is considered to be more important before addressing their biological activity. Hence, rapid and sensitive analytical techniques are given priority. The bioconversion of major carotenoids such as β -carotene, lycopene, lutein, astaxanthin and fucoxanthin is discussed in this section.

2.1. Hydrocarbon carotenoids

β -Carotene is the most abundant provitamin A carotenoid found in human diet and tissues. It exerts a beneficial function in mammals, including humans, due to its ability to convert to vitamin A. Even though β -carotene considered as a safer form of vitamin A, under circumstances detrimental effects also ascribed [11]. A better understanding metabolism of the β -carotene and their derivatives of (retinoids) are still needed to unequivocally discriminate the beneficial or detrimental effects under various physiological conditions and thus enable the formulation of adequate dietary recommendations for different age groups of individuals. Symmetric oxidative cleavage of β -carotene by the enzyme β -carotene-15, 15'-monooxygenase (BCMO1) generates two molecules of retinaldehyde, and these molecules further oxidized to form retinoic acids by aldehyde dehydrogenase. The oxidation of retinoic acid conversion into more polar compound 4-oxo retinoic acid by cytochrome P450 enzymes is believed to be

transcriptionally inactive. Alternatively, different forms of alcohol dehydrogenase and a variety of retinol dehydrogenases reduce retinaldehyde to retinol. Subsequently, these molecules further get esterified into retinyl esters by the enzyme lecithin retinol acyltransferase. Also, apo-carotenals can be generated by eccentric cleavage of β -carotene. The cleavage at the 9', 10' double bond is catalyzed by β -carotene 9', 10'-oxygenase 2 (CMO II) and leads to the formation of β -apo-10'-carotenal and β -ionone. Apo-carotenals are ultimately converted into one molecule of retinaldehyde, and the mechanism of this conversion is not completely elucidated [12].

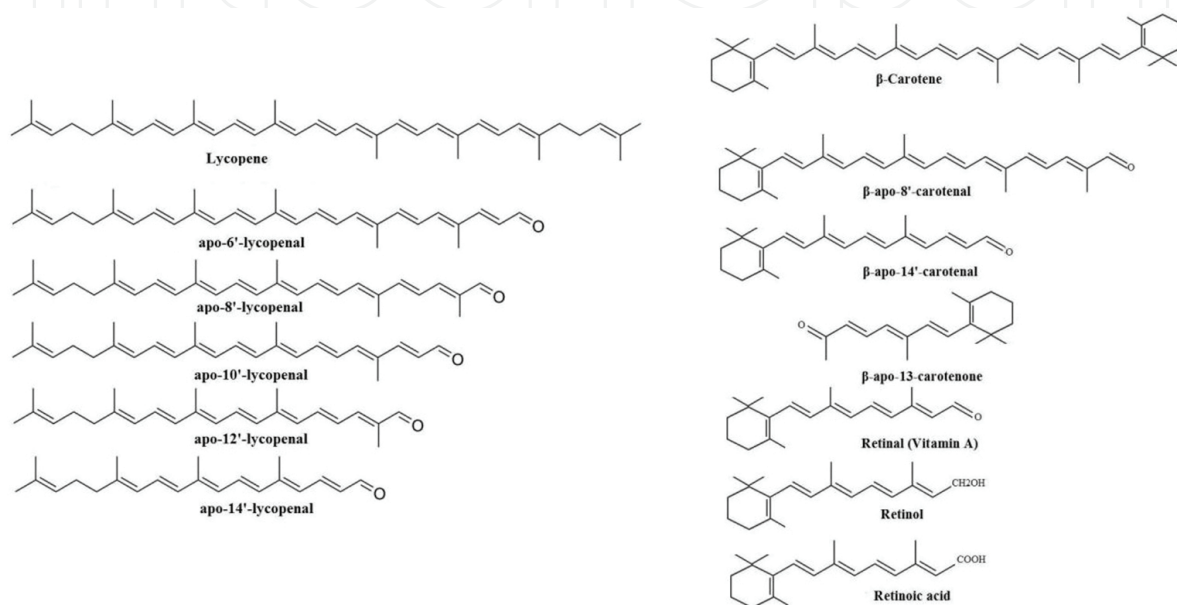


Figure 1. Molecular structures of hydrocarbon carotenoids and their metabolites.

Lycopene is another major hydrocarbon carotenoid extensively studied due to its potential role in the reduction in certain chronic diseases including cancer and cardiovascular disease. Recently, metabolism of lycopene has made a greater insight into the biological role of its derivatives. These observations raised an important question about the effect of lycopene on various cellular functions and signaling pathways are a result of the direct actions of intact lycopene or its derivatives. Considerable efforts have been expended to identify its biological and physiochemical properties. β -Carotene and lycopene have the same molecular mass and chemical formula, yet lycopene is an open-polyene chain lacking the β -ionone ring structure. The metabolism of β -carotene studied extensively, but biological activities of lycopene are not detailed. Derivates of lycopene formed due to shortening the carbon chain by removal of fragments from one or both the ends. Recent studies have shown that BCMO II enzyme catalyzes lycopene eccentrically to form apo-10-carotenoids. Cleavage of cis-lycopene by BCO II may occur at either 9, 10 or 9', 10' double bond to produce apo-10'-lycopenal, which can be oxidized to apo-10'-lycopenoic acid or reduced to apo-10'-lycopenol. Lycopene metabolites, including apo-6-, apo-8-, apo-10-, apo-12- and apo-14'-lycopenal, were detected in the plasma of humans [13]. However, the identification of cleaved lycopene metabolites in vivo is

challenging. Previously, Khachik et al. [9] identified a group of lycopene oxidative products, 2,6-cyclolycopene-1,5-diol in human serum and tissues. In animal studies, several metabolites were detected in lung tissue of lycopene-supplemented ferrets. The intermediate primary cleavage product apo-10'-lycopenal could be either reduced apo-10'-lycopenol or oxidized to apo-10'-lycopenoic acid. The apo-10'-lycopenal was further converted into apo-10'-lycopenoic acid in the presence of NAD⁺. Similarly in presence of NADH lycopene was converted into both apo-10'-lycopenoic acid and apo-10'-lycopenol [14]. Gajic et al. [15] reported apo-8'- and apo-12'-lycopenal as well as other unidentified polar metabolites of lycopene in the liver of rats when supplemented with lycopene-rich diet. Interestingly, a recent study indicated that apo-10'- and apo-14'-lycopenoic acid have a remarkable ability to upregulate BCO II expression [16]. These results showed that lycopene converted to apo-10'-lycopenoids in mammalian tissues both in vitro and in vivo. These observations presumed that lycopene metabolites play an important biological functions related to human health. The molecular structures of hydrocarbon carotenoids and their metabolites are shown in **Figure 1**.

2.2. Hydroxyl carotenoids

Lutein is one of the major xanthophylls present in green leafy vegetables. Lutein and its isomer zeaxanthin selectively accumulated in the macula of the human retina. They are recognized as antioxidants and as blue light filters [17] to protect the eyes from sunlight exposure and other lifestyle-related oxidative stress, which can lead to age-related macular degeneration and cataracts. Khachik et al. [9] identified lutein metabolites in human tissues such as plasma, milk, liver and retina. Yonekura et al. [18] observed remarkable accumulation of lutein metabolites, 3'-Hydroxy- ϵ,ϵ -caroten-3-one along with ϵ,ϵ -carotene-3,3'-dione in the plasma, liver, kidney and adipose tissues of mice fed with lutein. Further, findings indicated that mice actively convert lutein to keto-carotenoids by oxidizing the secondary hydroxyl group. 3'-oxolutein is the metabolite of lutein detected in human plasma and retina [19]. Similarly, 3'-epilutein identified in human retina and presumed that this product might form by a reduction of 3'-oxolutein from lutein. Other lutein derivatives like meso-zeaxanthin detected only in the retina and formed due to double-bond migration from lutein [20]. The dehydration products of lutein such as, 3-hydroxy-3', 4'-didehydro- β,γ -carotene and 3-hydroxy-2',3'-didehydro- β,ϵ -carotene may be formed non-enzymatically in stomach under acidic conditions [9]. Also, studies demonstrated and indicated the cleavage reaction of xanthophylls occurred in mammals by BCO II by cleaving double bond at C-9' and C-10' of xanthophylls [21].

2.3. Epoxy- and keto-carotenoids

The fucoxanthin is a marine carotenoid found in brown seaweeds, macroalgae and diatoms, with biological properties. The bioconversion of fucoxanthin to fucoxanthinol and amarouciaxanthin A was found in the plasma and liver of mice fed with fucoxanthin, whereas fucoxanthin was not detected [22]. Fucoxanthinol hydrolyzed from fucoxanthin in the intestinal tract, circulated in the body and then oxidatively converted into amarouciaxanthin A. The conversion of fucoxanthinol into amarouciaxanthin A found in human hepatoma HepG2 cells. Moreover, oxidative conversion of xanthophylls in mouse liver microsomal

fractions required NAD^+ as a co-factor, and this result demonstrates the metabolic conversion of the 3-hydroxyl end group at the level of enzyme reaction [23].

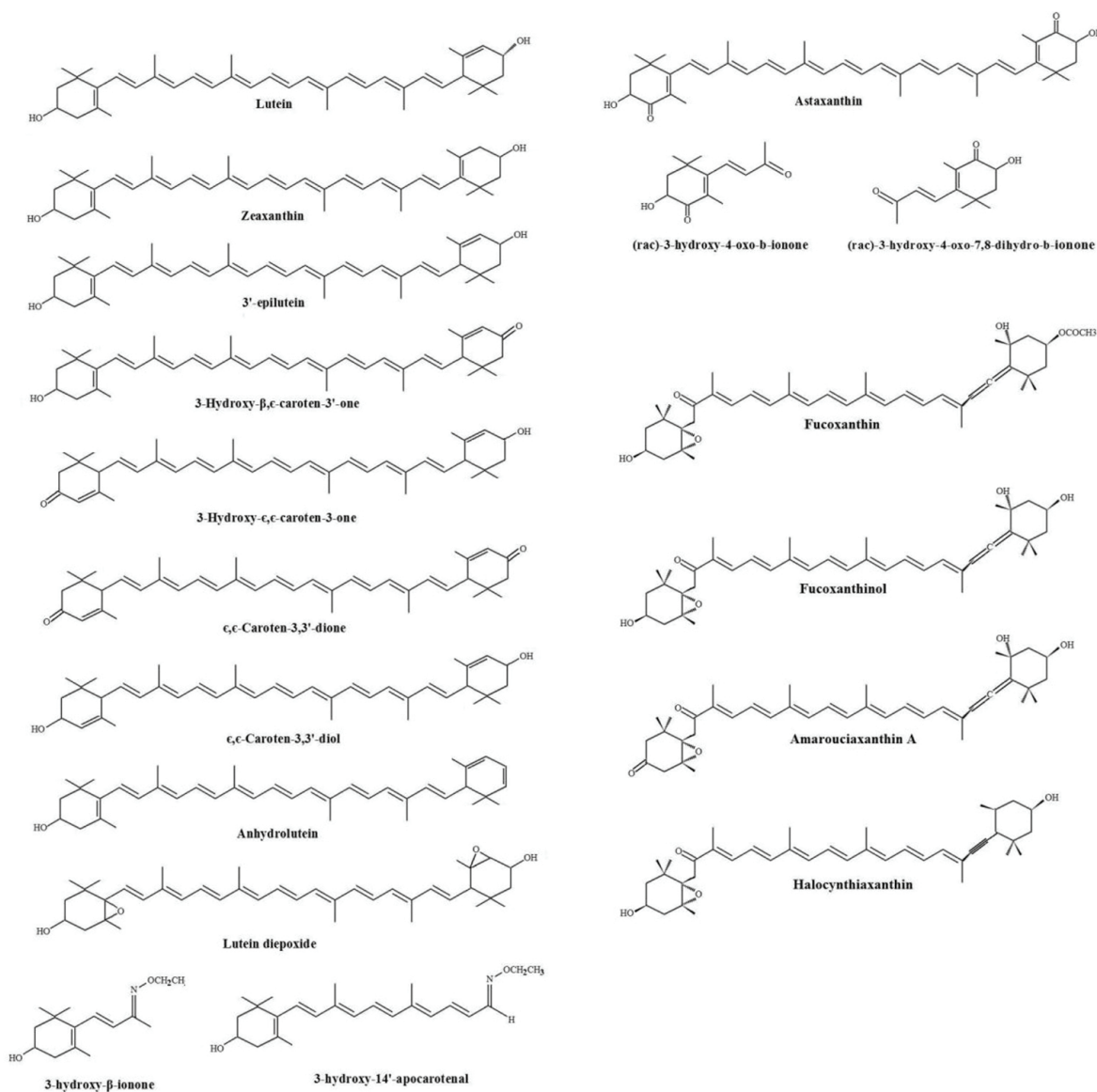


Figure 2. Molecular structures of hydroxyl-, epoxy- and keto-carotenoids and their metabolites.

Astaxanthin (ASTX) is a major keto-carotenoid metabolized into 3-hydroxy-4-oxo- β -ionone and 3-hydroxy-4-oxo-7,8-dihydro- β -ionone in primary rat hepatocytes [24]. However, enzymes involved in the synthesis of these metabolites and their potential biological functions are not elucidated. However, ASTX incubated with microsomes containing cytochrome P450 (CYPs) did not generate ASTX metabolites and induction of CYP activity in hepatocytes [25]. Therefore, the CYPs enzymes are not responsible for the production of ASTX metabolites. Hence, investigation on ASTX metabolites and their metabolism needs to explore. However, there are no detailed *in vivo* studies available on astaxanthin metabolites except isomers detected in human serum samples [26]. Canthaxanthin, (4,4'-diketo- β,β -carotene), is a keto-

carotenoid found in green algae, crustaceans and certain microorganism. This pigment also used as poultry feed to extend the dominant pigments in the skin and egg yolk of chickens. Analysis of tissues from chicks revealed that a portion of canthaxanthin reduced to 4-hydroxy-yechinenone (4-hydroxy-4'-keto- β,β -carotene) and iso-zeaxanthin (4,4'-dihydroxy- β,β -carotene). Ratio of canthaxanthin to metabolites depends on type of tissue, but in general metabolites concentrated in the skin [27]. The molecular structure of hydroxyl-, epoxy- and keto-carotenoids and their metabolites is shown in **Figure 2**.

3. Sample preparation for carotenoids and their metabolites

Biological samples used for analysis of carotenoid and their metabolites need to store in -80°C under argon or nitrogen gas to prevent carotenoids deterioration. Before analysis, the tissues samples thawed at room temperature before homogenization using suitable solvents, and then, organic phase collected after vortexing and centrifugation. To this appropriate amount of anhydrous, sodium sulfate should be added to remove traces of moisture. The organic phase evaporated with argon or nitrogen gas, and the extract was reconstituted in mobile phase and injected into the LC/MS/MS equipment. Other than the sample preparation adequate care should be taken to avoid light, heat and exposure to atmospheric oxygen, and the high quality solvents used for extraction and analysis, proper storage of samples under inert conditions, addition of antioxidants and completion of analysis within short run time applied with sophisticated instruments.

4. Analysis of carotenoids and their isomers, cleavage products/oxidation products or metabolites in food and biological samples

HPLC with C18 and C30 stationary phases either with reverse phase or normal phase used extensively for the analysis of a diverse group of carotenoids in various natural sources including food and biological samples [28, 29]. In general, the separation and resolution of carotenoids are better with gradient than isocratic solvents system. However, these methods require higher analysis time and solvent consumption. Further, these conditions and stationary phase were successfully employed in the analysis of carotenoids and their isomers or/and related derived products in biological fluids and tissues samples [30, 31]. Others have attempted and identified several types of carotenoids and their metabolites/oxidative products by employing gas chromatography and mass spectrometry. Stratton et al. [32] determined β -carotene oxidation products as β -ionone, β -apo-14'-carotenal, β -apo-10'-carotenal, β -apo-8'-carotenal and β -carotene 5,8-endoperoxide by using RP-HPLC and GC-MS. Wyss and Bucheli [33] developed an HPLC method with automated column switching for the simultaneous determination of endogenous levels of 13-cis-retinoic acid, all-trans-retinoic acid and their 4-oxo metabolites in human and animals tissue samples. Khachik et al. [9] separated, identified, quantified and compared 34 carotenoids, including 13 geometrical isomers and 8 metabolites in breast milk and serum of lactating mothers by HPLC-

PDA-MS. Wolz et al. [24] investigated the astaxanthin metabolites in primary cultures of rat hepatocytes by GC-MS. Siems et al. [34] demonstrated the oxidation products of β -carotene by using capillary gas-liquid chromatography and HPLC. The method developed was linear in the range of 0.3–100 ng/mL with a lower quantification limit. Kim et al. [35] isolated and analyzed auto-oxidation products of lycopene by GC-MS. Subsequently, Bernstein et al. [36] identified and quantified the dietary lutein and their geometrical (E/Z) isomers and related metabolites by HPLC in tissues of the human eye. Dachtler et al. [37] identified carotenoid stereoisomers in spinach and human retina samples by using HPLC online coupled to mass spectrometry and nuclear magnetic resonance spectroscopy. Aust et al. [38] have reported lycopene oxidative product 2,7,11-trimethyltetradecaheptaene-1,14-dial by using GC-MS. Sommerburg et al. [39] separated and identified 5,6-epoxy- β -ionone, ionene, β -cyclocitral, β -ionone, dihydroactinidiolide and 4-oxo- β -ionone as major cleavage products of β -carotene mediated by hypochlorous acid using GC-MS.

Simultaneously from the late 2000 s, spectroscopic and mass spectrometric techniques have been used for qualitative and quantitative analysis for structurally different carotenoids and their derived products. The carotenoid analysis is done by using different ionization modes such as matrix-assisted laser desorption/ionization (MALDI), electrospray (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). These methods are employed successfully to target the mass with better ionization and high resolution for the identification of broad range of natural organic molecules including carotenoids [40–42]. Further, this application is extended to explore several metabolites and oxidative products of carotenoids in rodents and human biological samples [18, 43–45]. Initially, van Breemen [46] developed an APCI-LC-MS method and analyzed all-trans-retinol and all-trans-retinyl palmitate with the lower limit of detection (LOD) of 34 fmoles/ μ L and 36 fmoles/ μ L (on-column), and limit of quantitation (LOQ) was 500 fmoles/ μ L and 250 fmoles/ μ L (on-column), respectively. Later, Zhu et al. [47] developed LC/APCI-MS a negative ion mode for the measurement of labeled and unlabeled β -carotene in human serum and feces to demonstrate bioefficacy of orally administered β -carotene, and the limit of detection of 0.25 pmol (on injection of 20 μ L of 0.0125 μ M β -carotene) and LOQ was 1.0 pmol (on the injection of 20 μ L of 0.050 μ M β -carotene) with the linear range of 1.1–2179 pmol on-column. Further, they believed that linear range with low LOD and LOQ facilitated sensitive and selective analysis of provitamin A carotenoids. Fraser et al. [40] used MALDI/TOF-MS to detect and quantify plant carotenoids, and other metabolites (m/z) in complex biological systems. Schäffer et al. [48] validated RP-HPLC-DAD method for simultaneous quantification of retinol, retinyl esters, tocopherols and selected carotenoids in the lung, liver and plasma of various animal samples. Würtinger and Oberacher [49] demonstrated influence of analytical parameters like constituents of mobile phase, including the modifiers added (acids, bases, dopants, metals and salts) and other experimental conditions (collision energy, flow collision gas, temperatures, etc.) on the ionization of the analyses (e.g., ESI, APCI, APPI, FAB). Consequently, Giuffrida et al. [50] for the first time analyzed and identified 52 carotenoids among the various cultivars of *Capsicum* using HPLC-DAD-APCI-MS. Further, Rivera et al. [51] studied the effect of ionization of carotenes, oxygenated carotenoids using ESI, APCI and APPI and reported that 12 of the 16 carotenoids exhibited strongest signal strength with APCI.

Furthermore, MS/MS is considered to be an efficient method for carotenoids identification by the use of transitions through precursor and daughter ions. This approach is also suitable for the determination of carotenoids with the same molecular mass such as structural isomers. Prasain et al. [52] developed a sensitive and specific ESI-MS/MS method using MRM for the detection of carotenoid oximes (zeaxanthin oxidation products) in a human eye sample. Further, this provides invaluable information on the characterization and quantification of carotenoids and their oxidized products formed during *in vitro* and *in vivo* studies. Crupi et al. [41] investigated carotenoids in a typical wine grape variety by using RP-HPLC-DAD-MS (ESI⁺) method. Due to an unusual ionization process, their mass spectra of carotenoids comprised both protonated molecules and molecular ion radicals. Further, they were subjected for the selective collision-induced dissociation (CID) to differentiate structural and geometrical isomers such as lutein isomers (zeaxanthin, 9Z and 9'Z-lutein) and a cis-isomer of β -carotene (9Z- β -carotene), 5,6-epoxy xanthophylls, violaxanthin, (9'Z)-neoxanthin, lutein-5,6-epoxide and 5,8-epoxy xanthophylls diastereoisomers (neochrome, auroxanthin, luteoxanthin, flavoxanthin, chrysanthemaxanthin). Kopec et al. [13] performed HPLC-MS/MS using APCI^{-ve} mode to separate and detect the apo-6'-, apo-8'-, apo-10'-, apo-12'-, apo-14'- and apo-15'-lycopenal products formed by *in vitro* oxidation reaction. The quantitative analysis of carotenoids and other fat-soluble compounds performed simultaneously on a C30 column and detected by APCI-MS/MS with operation of selected reaction monitoring (SRM) mode. Also, this method calibrated to shown a less variability in intra- and inter-day precision analysis. Recently, ultrahigh performance liquid chromatography (UPLC/UHPLC) has attracted much attention due to faster analysis and higher sensitivity with 2 μ m particle size stationary phases, thereby increasing column efficiency, decreasing band broadening, and increasing resolution [4]. Granado-Lorencio et al. [53] assessed the suitability of UHPLC for the simultaneous determination of biomarkers of vitamins including vitamin A (retinol, retinyl esters) and major carotenoids in human serum. This method allowed a better resolution for carotenes and xanthophylls isomers provides better sensitivity and reproducibility in peak area and retention time than the HPLC, with mean RSDs. Rivera et al. [51] analyzed various carotenoids by UHPLC-MS/MS detection. Further, they compared three different ionization techniques (ESI, APCI and APPI) to ionize the carotenoids and concluded that APCI has a powerful technique to ionize carotenoids. They also used dopants (acetone, toluene, anisole and chlorobenzene) that allowed the enhancement of the carotenoid signals strength up to 178-fold. Delpino-Rius et al. [54] analyzed simultaneously epoxy carotenoids, hydroxyl carotenoids and carotenes in fresh homemade and industrially processed fruit products by UPLC. They identified 27 carotenoids eluted within 17 min; furthermore, this method allowed to differentiate the carotenoid profiles and 5,6- to 5,8-epoxycarotenoids. Separation of carotenoids on UHPLC columns illustrates less analysis time compared to HPLC C30 column; however, the separation is better in C30 column. Hence, there is a requirement for appropriate UHPLC column for rapid and sensitive analysis of carotenoids with better separation. Zhao et al. [55] developed a quick and simple ultrahigh performance supercritical fluid chromatography-photodiode array detector (UHPSFC-PDA) method and validated the determination of carotenoids in dietary supplements using Acquity UPC2HSS column by gradient elution with carbon dioxide and sol-

vent system. We have developed a rapid UPLC-MS/MS method for analysis of lycopene isomers and their fragmentation pattern with CID and demonstrated the importance of ion mobility to differentiate carotenoids geometrical isomers [56]. Dong et al. [57] characterized and distinguished geometrical isomers of lycopene and β -carotene using ion mobility spectrometry. Raphael et al. [58] identified canthaxanthin oxidation products and compared the similarity of β -carotene like oxidation products by using both LC-MS and GC-MS chromatograms.

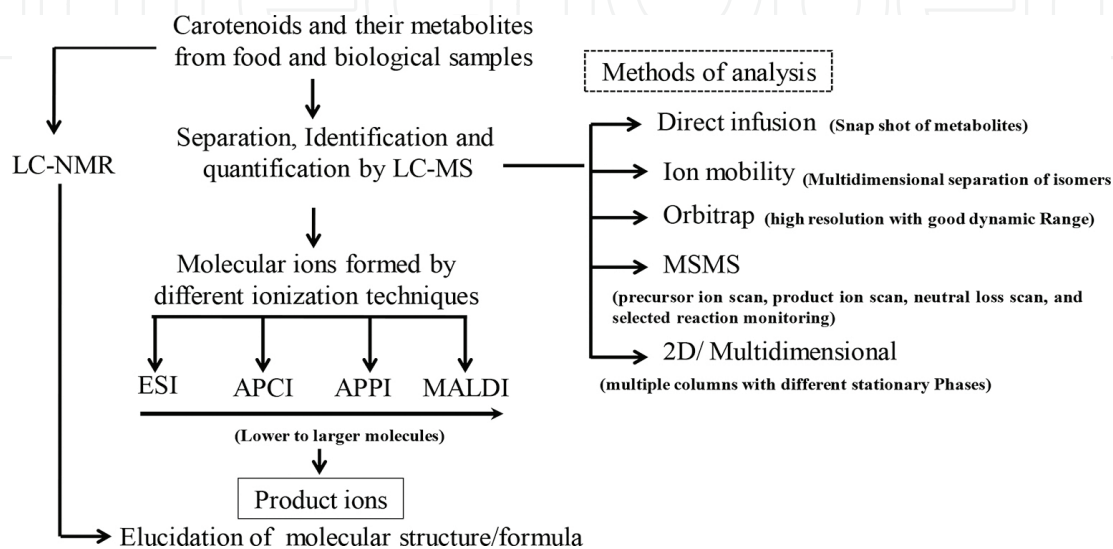


Figure 3. Hyphenated analytical techniques for the characterization of carotenoids and their metabolites. The details of these techniques are discussed in Section 4.

Apart from these, Orbitrap MS a high-resolution mass spectrometry was also exploited for carotenoids analysis to generate mass spectra with a resolving power up to 100,000 at full-width half-maximum and mass accuracies within two parts per million (ppm). Due to its high mass resolution and exact mass screening detectors, probable molecular formulae of the ions and fragments were elucidated [59]. In continuation, Van Meulebroek et al. [60] developed a full-scan high-resolution Orbitrap MS method enabling the metabolomic screening for carotenoids in tomato fruit tissue. The validation demonstrated the excellent performance in terms of linearity, repeatability and higher range of mean corrected recovery. Additionally, a well-established detection technique, that is, MS/MS and ultraviolet-visible spectroscopy photodiode array, indicated superior performance of high-resolution Orbitrap MS (with limits of detection ranging from 1.0 to 3.8 $\text{pg } \mu\text{L}^{-1}$). Contemporarily, 2D-LC and multi-dimensional chromatography have emerged as a tool for carotenoid analysis. This provides an excellent separation and resolution for analysis of complex matrices. In this regard, Cacciola, et al. [61] developed and applied a comprehensive normal-phase \times reversed-phase liquid chromatography (NP-LC \times RP-LC) system for analysis of the intact carotenoid composition of red chili peppers, with photodiode array and mass spectrometry detection. A total of 33 compounds separated into 10 diverse chemical classes in the 2-D space and identified by accurate IT-TOF (ion trap-time of flight) MS. Apart from these, the robust technique LC-NMR offers 1-D and

2-D NMR spectra for the components separated by HPLC. Recently, LC-NMR is used because of improved sensitivity due to higher magnetic fields. Further, NMR provides information about conformational geometry for structural elucidation. LC-NMR is established as a method of analyzing major carotenoids and metabolites in food and biological samples [62]. The NMR studies on carotenoids metabolites are scanty due to quantitative limitations. Therefore, LC-MS-APCI studies are widely used in the characterization of carotenoids metabolites or oxidative cleavage products. The approach of LC-MS techniques and ionization modes for characterization of carotenoids and their metabolites are shown in **Figure 3**.

Even advancement in hyphenated analytical techniques and inconsistency of results may arise due to several pre-chromatographic (samples or tissues, the nature of carotenoids, sample preparation, incomplete extraction, solvent incompatibility, isomerization/oxidation, physical losses of carotenoids/metabolites and its accountability) and post-chromatographic (low recovery, less stability, inaccurate method validation, co-elution, unavailability of standards, selection of improper mode of ionization, carotenoid/metabolites with same molecular mass) errors in the carotenoids and their metabolites analysis [4]. The qualitative and quantitative analyses of carotenoids need a proper method validation as per the ICH (International Council on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) and IUPAC (International Union of Pure and Applied Chemistry) guidelines. The method validation mainly comprises of precision, specificity, LOD, LOQ, linearity, sensitivity, range and robustness. Carotenoid standard curves prepared for subsequent quantitation by HPLC-PDA. The amounts of carotenoids calculated based on the regression equations. The LOD and LOQ calculated for each standard on the basis of the signal-to-noise ratio (3:1 for LOD and 10:1 for LOQ). LOD is the amount that resulted in a peak with a height three times that of the baseline noise, respectively, and the LOQ determined as lowest injected amount which could be quantifiable reproducibility (RSD \leq 5%). The precision evaluated by the relative coefficient of variation (%). The inter-and intra-day relative standard deviations (RSD) for retention times of individual carotenoid are considered for standard concentration to check the reproducibility of the method. The accuracy of the extraction method is assessed by determining recovery of carotenoids with a mean value, respectively.

5. Biofunctionality of hydrocarbon carotenoid metabolites

β -Carotene is one of the most potent vitamin A precursor among other provitamin A carotenoids (α -carotene, β -cryptoxanthin and γ -carotene). Chemically, vitamin A refers to all isoprenoid compounds that possess the biological activity of all-trans-retinol. The parent structure of most retinoids contains a substituted β -ionone ring with a side chain of three isoprenoid units linked at the 6-position of the β -ionone ring. Retinol plays a role in vision, differentiation and proliferation of a wide range of epithelial cells, bone growth, reproduction and embryonic development. β -Carotene conversion to retinal, retinoic acid and other active forms of the vitamin A family is well documented. Retinoic acids serve as ligands for nuclear retinoic acid receptors (RARs), namely RXR and RAR, mediate vitamin A dependent activities. Several studies have reported the biological properties of β -apocarotenoids. Zile et al. [63]

demonstrated that retinol β -D-glucuronide (a metabolite of vitamin A) could arrest HL-60 cell proliferation and induces differentiation into mature granulocytes; it may act by itself or by being hydrolyzed to retinoic acid. Others have reported that β -carotene cleavage products, such as β -apo-14'-carotenoic acid, stimulate the differentiation of U937 leukemia cells, and β -apo-12'-carotenoic acid inhibits the proliferation of HL-60 cells [64, 65]. Hu et al. [66] characterized a polar oxidation product of β -carotene 5,8-endoperoxy-2,3-dihydro- β -apocarotene-13-one and demonstrated the inhibition of cell growth and cholesterol synthesis in MCF-7 cells. Kawada et al. [67] suggested that the inhibitory action of adipocyte differentiation by carotenoids and retinoids exhibited through the RAR upregulation and the suppression of PPAR γ . Tibaduiza et al. [68] tested the effect of synthetic eccentric cleavage products of β -carotene such as β -apocarotenoid acids, including β -apo-8'-, β -apo-10'-, β -apo-12'- and β -apo-14'-carotenoic acid on the growth of the human breast cancer cells. Further, they observed β -apo-14' and β -apo-12'-carotenoic acid significantly inhibited MCF-7 growth, and only β -apo-14'-carotenoic acid inhibited Hs578T growth. Further, they observed none of these treatments inhibited the growth of MDA-MB-231 cells. Also, authors concluded that apocarotenoid acids exhibit antiproliferative effects by downregulation of cell cycle regulatory proteins and inhibition of AP-1 transcriptional activity. Rühl et al. [69] also reported that β -carotene is involved in activation of human pregnane receptor (PXR) a ligand-activated transcription factors involved in xenobiotic detoxification in the liver. Ziouzenkova et al. [70] demonstrated that β -apo-14'-carotenal, but not other structurally related apo-carotenals, represses peroxisome proliferator-activated receptors (PPAR) and RXR activation. Eroglu et al. [71] demonstrated that none of the β -apocarotenoids significantly activated RARs. However, β -apo-14'-carotenal, β -apo-14'-carotenoic acid and β -apo-13-carotenone antagonized ATRA-induced transactivation of RARs.

Lycopene is another non-provitamin A hydrocarbon carotenoid present in human serum and tissues. Recently, lycopene has attracted much attention due to its association with a decreased risk of certain cancers, cardiovascular diseases and other chronic problems [72]. Several studies from *in vivo* and *in vitro* suggest that lycopene induces apoptosis in cancer cells [73–75]. Structurally, lycopene consists of open-polyene chain lacking the β -ionone ring compared to β -carotene and share a same molecular mass and chemical formula. Even though the metabolism of β -carotene extensively studied, the metabolism and biological activities of lycopene are not detailed. Several studies support the concept that biological activities of lycopene are mediated by its oxidative products/cleavage products [76–78]. The characterization of BCO II enzyme has demonstrated that this enzyme can catalyze the eccentric cleavage of lycopene to form apo-10'-lycopenoids [14]. Several lycopene metabolites have been identified *in vivo* and *in vitro* [9, 13, 15, 35, 79], but their biological activities and mechanism of action need elucidation. The discovery of various oxidation products or metabolites of carotenoids has questioned the active role of them compared to intact carotenoid molecules in combating various diseases. However, metabolites or oxidative products of LYC on health benefits are warranted. Nara et al. [80] have demonstrated that autoxidation mixtures of LYC inhibited the HL-60 cell growth effectively than LYC. Similarly, Zhang et al. [81] identified a cleavage product of LYC (E, E, E)-4-methyl-8-oxo-2,4,6-nonatrienal and evaluated its apoptosis-inducing activity in HL-60 cells. Consequently, Aust et al. [38] reported the role of LYC degraded products in enhancing cell communication and cell signaling. Lian et al. [5] demonstrated that apo-10'-lycopenoid

acid is a biologically active metabolite of lycopene with potential chemopreventive agent against lung tumorigenesis. Ford et al. [82] demonstrated that lycopene and apo-12'-lycopenal reduce the proliferation of prostate cancer cells. In the recent study, apo-10'-lycopenoic acid led to the increase in SIRT1 (Sirtuin 1) enzyme activity by treatment with this metabolite in mice, resulting in prevention of fatty liver [83]. Due to its high antioxidant activity, lycopene is more unstable and needs more advanced analytical techniques to identify and characterize lycopene metabolites and their biological activities.

6. Biofunctionality of oxygenated carotenoid metabolites

Lutein and its isomer zeaxanthin are the two major carotenoids found in the human eye associated with vision protective properties. Dietary consumption of lutein-rich fruits and vegetables positively associated in decreasing AMD and cataract [8, 17]. Although conversions of β -carotene to retinoids documented in animals and humans, only little is known about the metabolism of xanthophyll carotenoids. In general, carotenoid metabolites are involved in chemoprevention of cancer [6]. Lutein metabolites such as 3'-epilutein, 3'-oxo-lutein, 3'-dehydrolutein, meso-zeaxanthin, methoxy-zeaxanthin, oxime derivatives of 3-hydroxy- β -ionone and 3-hydroxy-14-apocarotenal reported in human tissues and serum [9, 43, 52]. Characterization of these lutein and zeaxanthin metabolites in vitro and in vivo is warranted to address their biological functions. The health benefit of these metabolites is not detailed. The oxidized lutein may be highly reactive when combined with similar reactive oxygen species and presumed to enhance antioxidant property [6], but the mechanism of action of these xanthophylls (lutein) metabolites remains unanswered. Several noteworthy studies have explored lutein metabolites in vivo [9, 18, 43, 44, 52]. However, mechanism of action and other functional aspects of these metabolites/oxidative cleavage products need further research. We reported the possible protective effect of lutein oxidation products in cervical cancer cell lines [84]. Further, we also elucidated the formation of lutein oxidation products mediated through peroxy radicals and screened the antioxidant and cytotoxic effects of oxidized lutein on HeLa cancer cells [6]. Previously, we identified the apocarotenals, diepoxides and other oxidative degradation products of lutein in liver. Further, we presumed that these products are formed due to the peroxy radical-mediated oxidation in the body [6, 44]. These results are significant in chain breaking peroxy radical or quenching of singlet oxygen. Furthermore, the existence of these oxidized molecules in vivo is important in free radical chemistry and oxidative stress [85]. Possibly, oxidized lutein may reduce the cancer cells viability through induction of apoptosis. Further, result from our study demonstrated the inhibitory effect of these compounds on cancer cell growth, which may be due to the effect on signaling pathway involved in apoptosis. The biological activity of intact lutein may be different than its metabolites. Hence, it is important to address that the beneficial role of lutein and zeaxanthin in delaying and possibly protecting against ascribed chronic diseases may be due to their metabolites. In the case of other oxygenated carotenoids, such as astaxanthin, Wolz et al. [24] identified the metabolized products such as 3-hydroxy-4-oxo- β -ionone and 3-hydroxy-4-oxo-7,8-dihydro-

β -ionone in primary rat hepatocytes. Later, Kistler et al. [25] reported four radiolabeled metabolites of astaxanthin including 3-hydroxy-4-oxo- β -ionol, 3-hydroxy-4-oxo- β -ionone, 3-hydroxy-4-oxo-7,8-dihydro- β -ionol and 3-hydroxy-4-oxo-7,8-dihydro- β -ionone in human and primary human hepatocytes. Further, they demonstrated that incubation of astaxanthin with microsomes from liver containing detoxifying enzymes did not generate astaxanthin metabolites. Sangeetha and Baskaran [86] hypothesized that astaxanthin might be converted into retinol via β -carotene in retinol-deficient rats. However, the formation of astaxanthin metabolites and their biological functions is not detailed. Further, studies related to astaxanthin metabolites and their biological functions are not detailed and explored.

Fucoxanthin is a major epoxy carotenoid found in the marine source (seaweeds) and explored for its anticancer, anti-allergic and anti-obese activities [87, 88]. Dietary fucoxanthin is metabolized to fucoxanthinol in the gastrointestinal tract by digestive enzymes, and further, it is converted to amarouciaxanthin in liver [23]. The bioactivity of fucoxanthin is attributed to its metabolites fucoxanthinol and amarouciaxanthin A. There are reports demonstrated that fucoxanthinol is effective and plays an important role in health benefits than intact fucoxanthin [89, 90]. Anti-proliferative and cancer preventing influences of fucoxanthin and fucoxanthinol are mediated through different signaling pathways [90]. Also, others have reported fucoxanthin, and its metabolites regulate adipogenic gene expression and inhibit the adipocyte differentiation of 3T3-L1 cells through downregulation of PPAR γ . The suppressive effect of fucoxanthinol is superior on adipocyte differentiation than its parent molecule [91]. In MDA-MB-231 cells, fucoxanthinol reduced nuclear levels of NF- κ B members and indicated an effective for the treatment and/or prevention of breast cancer [89]. However, there are no much reports on epoxy carotenoids identified in blood and tissues and may be due to the less dietary importance. This is an active area of research and deserves further study. The overview of biofunctionality of carotenoid cleavage products/metabolites is shown in **Table 1**.

Carotenoid	Metabolites identified	Bio-functionality of carotenoid metabolites	References
Lycopene	Oxidation products of lycopene	Inhibits the growth of HL-60 human promyelocytic leukemia cells	[80]
	Acyclo retinoic acid	Activates RAR to inhibit mammary cancer cells growth	[92]
	2,7,11-Trimethyl-tetradecaheptaene-1,14-dial	Enhance gap-junction communication	[38]
	(E,E,E)-4-methyl-8-oxo-2,4,6-nonatrienal (MON)	Induce apoptosis by downregulation of Bcl-2 and Bcl-XL, and activated caspase cascades in HL-60 cells	[81]
	Apo-10'-lycopenoic acid	Chemopreventive effect on lung carcinogenesis	[5]
	Apo-12'-lycopenal	Reduced androgen-independent prostate cancer cells	[82]
	Apo-10'-lycopenoic acid	Influence on SIRT1 enzyme activity and prevention of fatty liver	[83]

Carotenoid	Metabolites identified	Bio-functionality of carotenoid metabolites	References
β-Carotene	5,8-Endoperoxy-2,3-dihydro-β-apocarotene-13-one	Inhibits cell growth and cholesterol synthesis in MCF-7 cells	[66]
	5,6-Monoepoxide	Greater differentiation-inducing activity than β-carotene toward NB4 human leukemia cells	[93]
	Retinoyl β-D-glucuronide	Arrest HL-60 cell proliferation and induce their differentiation into mature granulocytes	[63]
	β-Apo-14'-carotenoic acid	Stimulates the differentiation of U937 leukemia cells	[64]
	β-Apo-12'-carotenoic acid	Inhibits the proliferation of HL-60 cells	[65]
	Retinoids	Inhibits adipocyte differentiation through RAR upregulation and suppression of PPARγ	[67]
	β-Apo-14' and β-apo-12'-carotenoic acid	Inhibits MCF-7 growth, and Hs578T growth	[68]
	β-Apo-14'-carotenal	Represses PPAR and RXR activation	[70]
	β-Apo-14'-carotenal, β-apo-14'-carotenoic acid, and β-apo-13-carotenone	Antagonized ATRA-induced transactivation of RARs	[71]
Lutein	Photodegraded and peroxy mediated lutein oxidized products	Higher antioxidant and cytotoxic effects in HeLa cells	[6, 84]
Fucoxanthin	Fucoxanthinol and amarouciaxanthin A	Anti-proliferative effect on human prostate cancer cells MCF-7 and MDA-MB-231 cells	[23] [89]
	Halocynthiaxanthin and fucoxanthinol	Induces apoptosis in human leukemia, breast and colon cancer cells	[94]
	Fucoxanthinol	Fucoxanthin metabolites reduced the growth of primary effusion lymphoma tumor cells	[95]
Canthaxanthin	4-Oxoretinoic acid	Induction of gap junctional communication in murine fibroblasts	[96]

Table 1. Biofunctionality of carotenoid cleavage products/metabolites.

7. Conclusion

In concern to the application of analytical techniques in carotenoid, research needs a more sophisticated instrumentation to increase sensitivity, precision, specificity and speed of analysis. The increase in analytical hyphenation considered as cutting edge with multi-dimensional or techniques that may support to decipher the carotenoids found in natural sources and their effects on human health. Further, metabolomics corresponds to prevailing analytical platforms to obtain detailed and complete information on the composition of food components and their existence in biological entities. Further, the target in carotenoid/

metabolites analysis is to understand the role of these compounds at the molecular levels (i.e., their interaction with genes and their subsequent effect on proteins and metabolites). This information will provide a rational design of strategies to manipulate cell functions through diet/nutraceuticals, which is expected to have an extraordinary impact on human health. The development of the framework in genomics, transcriptomics, proteomics and metabolomics has given rise to opportunities for increasing our understanding of different issues that can be addressed by profiling carotenoid metabolites.

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