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Review

Metabolomics of carotenoids: The challenges and prospects – A review

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Considerable progress in carotenoids research has been made to understand the carotenoid metabolism in animals including human. Epidemiological and clinical studies have correlated with dietary intake of carotenoids on reduction of vitamin A deficiency, age-related macular degeneration, cancer and cardiovascular diseases. Recent findings demonstrate the existence of carotenoid metabolites *in vivo* and their efficacy have made greater insight on prospecting carotenoid metabolites. Owing to their biological activity, exploration of analytical methods for the characterization of carotenoid metabolites is considered to be important before addressing the stability and bioactivity. Although few studies are available on carotenoid metabolites, their structural characterization in biological samples require a substantial refining of analytical protocols like isolation, purification, prerequisite of equipment parameters and robustness in hyphenated techniques. Recently, researchers have focused on biotransformation of carotenoids and made an attempt to screen their metabolites by high-throughput analytical strategies. However, till date there is no detailed analytical techniques available to fingerprint carotenoid metabolites, due to interference with complex biological matrices. This review highlights the carotenoid metabolism, possible bioconversion and available bio-analytical techniques to characterize metabolites *in vivo*. Further, advancement in sensitivity, mode of ionization and fragmentation patterns of metabolites were also discussed. The identification of carotenoid metabolites in system specific will have further insight in the emerging field of nutritional metabolomics.

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Introduction

Carotenoids are pigments with a wide range of spectrum consisting of C_{40} backbone (Britton *et al.*, 1997; Polívka & Frank, 2010). Fruits and vegetables (Sommerburg, Keunen, Bird, & van Kuijk, 1998; Raju, Varakumar, Lakshminarayana, Krishnakantha, & Baskaran, 2007), animal products, marine algae, and certain seaweeds are good sources of carotenoids (Britton, Liaaen-Jensen, & Pfander, 2009). Generally, carotenoids are classified as carotenes (carotenoid hydrocarbons) composed of only carbon and hydrogen (β-carotene and lycopene) and xanthophylls (oxygenated carotenoids), which contain an epoxy- (violaxanthin, neoxanthin, fucoxanthin), hydroxy- (lutein and zeaxanthin), keto- (astaxanthin, canthaxanthin) and methoxy- (spirilloxanthin) functional groups. In case of nutritional approach, carotenoids are considered to be an important bioactive compounds, and dietary ingestion is the only source to meet their requirement in humans and animals. Approximately, 750 carotenoids have been described in nature, 50 of them are identified in the human diet and more than 20 are detected in the human blood and tissues (Khachik, 2006). Among carotenoids, β-carotene, α-carotene, lycopene, lutein, zeaxanthin and β-cryptoxanthin are the most common carotenoids found in the human diet, blood and tissues (Khachik, Spangler, & Cecil Smith, 1997). Several epidemiological and clinical trials have well correlated the consumption of dietary carotenoids with decreased risk of vitamin A deficiency, cancer, cardiovascular diseases (CVD) and age-related macular degeneration (AMD) (Díaz, de las Cagigas, & Rodriguez, 2003; Rock, 2009; Kohlmeier & Hastings, 1995; Bone,

Landrum, Guerra, & Ruiz, 2003). The α-carotene, β-carotene and β-cryptoxanthin are known precursors of vitamin A (Bendich & Olson, 1989). The other non-pro vitamin A carotenoid such as lycopene has gained special attention, and many studies have correlated its role in the reduction of prostate cancers (Giovannucci, 2005; Talvas *et al*., 2010). Lutein and zeaxanthin are considered as a vital macular pigments located in central region of retina, acts as filter of high-energy blue light, quenchers of singlet oxygen, chain-breaking antioxidants and involve in the reduction of phototoxic damage to the eye (Bone et al., 2003). Astaxanthin, fucoxanthin, siphonaxanthin are other promising marine carotenoids combat with health associated benefits as bioactive molecules against diabetes, obesity, angiogenesis (Higuera-Ciapara, Felix-Valenzuela, & Goycoolea, 2006; Sachindra *et al*., 2007; Kim, Heo, Kang, Ahn, & Jeon, 2010; Woo *et al*., 2009; Ganesan *et al*., 2011). Although bioavailability and functionality of carotenoids are addressed knowledge on metabolomics of carotenoids and bioefficacy of those metabolites are not detailed. Therefore, characterization of carotenoid metabolites provide a broad scope for molecular nutrition. Further, improvement in analytical techniques to resolve the unexplored carotenoid metabolites *in vivo* requires a sensitive and rapid analysis. Carotenoid metabolites may involve in a specific intrinsic biological activity. Hence, currently metabolomics is preferably positioned in many areas of food science and nutrition research, especially consumption and physiological monitoring of carotenoids in interventional studies. Owing to the necessity for the improved analytical techniques to characterize carotenoid and their metabolites, hyphenated techniques were used to analyze biological and physiological samples. Currently, such methods are considered to be most promising tools in carotenoids biochemistry (Dueker, Jones, Smith, & Clifford, 1994; Hagiwara, Yasuno, Funayama, & Suzuki, 1998; Fraser, Enfissi, Goodfellow, Eguchi, & Bramley, 2007). Developing such advanced techniques is essential, since the emerging metabolomics concept in many natural components is the integrated part of proteomics, genomics, and lipidomics. Under the umbrella of lipidomics, here we have attempted to build the knowledge on "metabolomics". Therefore, this review highlights the profiling of carotenoids metabolism in animals and related hyphenated analytical techniques are discussed.

Trends in carotenoids metabolism and profiling of their metabolites in animals and human

Knowledge on carotenoid metabolism in humans and their biological significance has become important in nutrition and pharmacological research. The characterization of centric/eccentric cleavages, the formation of apocarotenoids, and oxidative products of pro-vitamin and non-provitamin A carotenoids is given a platform for metabolomics. As evident from available research reports, carotenoids are biologically converted into metabolites by enzymatic cleavages, oxidation, reduction, hydrolysis and by active reaction with free radicals (Olson, 1994; Nagao, 2004; Lakshminarayana *et al*., 2008) (Fig. 1). This raised an important question, whether intact carotenoids are involved in diverse cellular functions or its metabolites. Many studies, including our own, support the concept of biological functions are mediated by carotenoid metabolites (Stahl, von Laar, Martin, Emmerich, & Sies, 2000; Lian, Smith, Ernst, Russell, & Wang, 2007; Nidhi, Sharavana, Ramaprasad, & Baskaran, 2014; Lakshminarayana, Aruna, Sathisha, Dharmesh, & Baskaran, 2013). However, there are evidence that reveal, cleavage products of β-carotene is harmful to smokers when supplemented with high doses (Omenn, Goodman, & Thomquist, 1996). Hence, occurrence and assessment of carotenoids cleavage products is crucial to conclude their bioactivity. The carotenoids level in the biological samples are detected below *nanomolar* concentration, analyzed routinely by HPLC (Khachik *et al*.,1997; Stahl *et al*., 2000). Further, determination of carotenoid metabolites that exist at *picomolar* concentration is really a major task. In this context, several studies have attempted for the detection of major carotenoid metabolites *in vivo* by using different hyphenated analytical methods Table 1. The formation, occurrence and understanding of specific bioactivity of carotenoid metabolites are exciting and challenging. Hence, this section illustrates the possible metabolism of major carotenoids.

To increase our understanding of the above aspects and also to know the key role of carotenoids metabolites, we need greater insight on their bioavailability. However, many of the bioavailability studies are based on the measurement of intact carotenoids either in the blood or tissues or excretory products but not their metabolites, except for β-carotene. Hence, characterization and quantification of carotenoid metabolites is also necessary to estimate the real levels of carotenoid bioavailability. Carotenoid isomers/oxidative products/metabolites possibly form during food processing, digestion, absorption, transportation and the site of accumulations. The detailed possible stages of degradation, biotransformation and biological functions of carotenoids are hypothetically described under the concept of metabolomics (Fig. 1).

Metabolism of hydrocarbon carotenes

In animals, β-carotene conversion to vitamin A was first reported in the rat model (Moore, 1930). Later, the structure was elucidated and demonstrated the central cleavage of β-carotene at the central carbon double bond (15, 15′) leads to the retinal formation (Olson, 1994). Harrison, Dela Sena, Eroglu, and Fleshman (2012) reported eccentric cleavage of β-carotene to retinoic acid via β-apo-carotenoic acid. Eroglu and Harrison (2013) have detailed the pathways of centric and eccentric cleavages of β-carotene in vertebrates.

Central cleavage of β-carotene yields two molecules of retinal followed by oxidation to form retinoic acid. In case of eccentric cleavage of β-carotene, β-apocarotenals

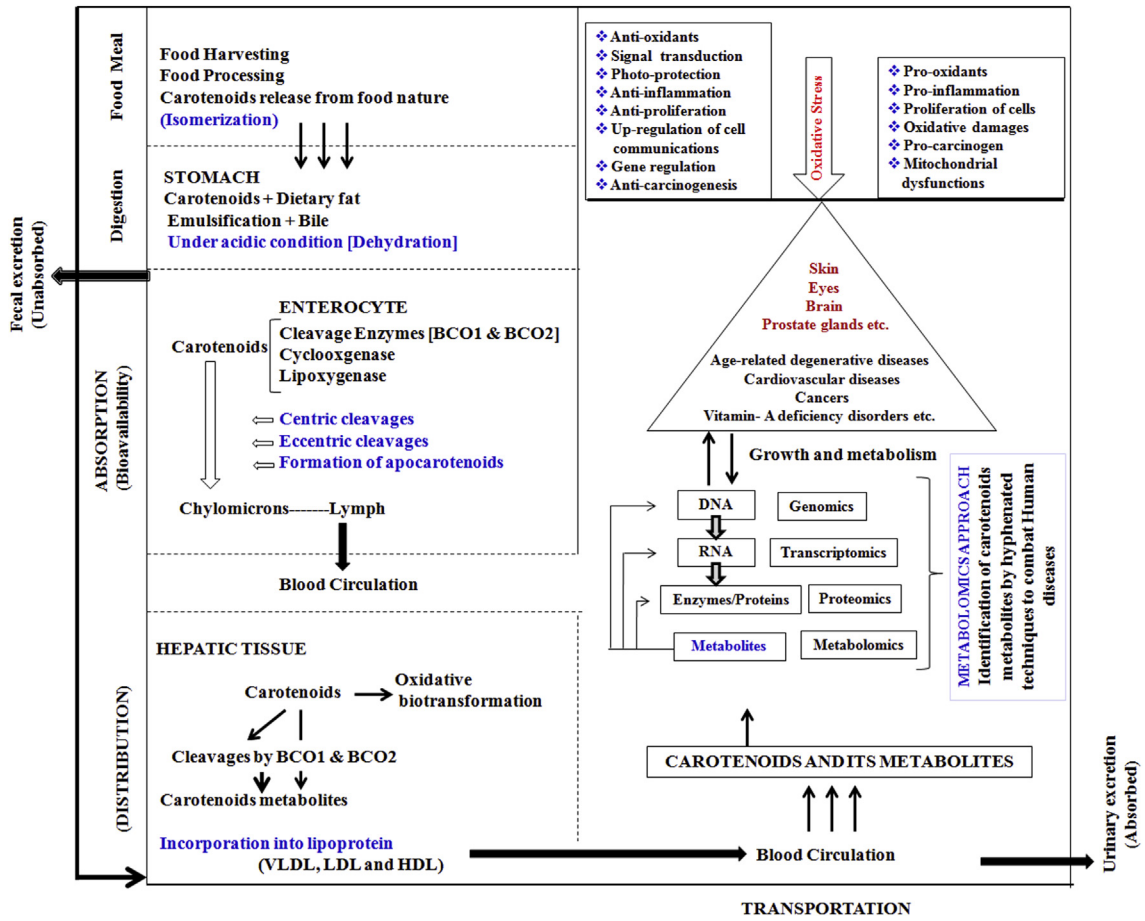

Fig. 1. Overview of carotenoid metabolism and hypothetical vision of metabolomics approach.

and β-apocarotenones are formed. Classical studies have focused on the responsible enzyme for the central cleavage of β-carotene, α-carotene, and β-cryptoxanthin (Olson, 1989; van Vliet, van Schaik, Schreurs, & van Den, 1996). Later, Lindquist and Anderson (2002) have shown β-carotene mammalian β-carotene-15,15′-oxygenase (BCMO1) enzyme acts at 15,15′ double bond of provitamin A carotenoids in mammals. Further, they have demonstrated that BCMO1 requires at least one nonsubstituted β-ionone ring to yield retinal. In addition, peroxidases and lipoxygenases are also reported to form apocarotenoids (Yeum *et al.*, 1995). Other than the enzymatic cleavages, non-enzymatic processes can also cause oxidative cleavages of carotenoids to form various oxidative products (Nagao, 2004).

The enzymatic conversions of non-provitamin A carotenoids in humans and animals are still poorly understood. Lycopene, a major non-provitamin A carotenoid has been detected in human plasma and tissues. The unique chemical property of lycopene is very sensitive to oxidative modification/degradation *in vivo* (Britton, 1995; Siems, Sommerbrug, & Van Kuijk, 1999). Understanding the chemical structures of enzymatic/oxidative degradation products of lycopene may help in ascertaining their health benefits, which is yet to be addressed. In this context, the possible formation of lycopene metabolites, also known as lycopenoids are discussed. Studies have claimed the presence of lycopene metabolites in serum and tissues at physiologically significant levels in contrast to retinoids (Nikki Ford & Erdman, 2012; Kopec *et al.*, 2010). This shows that the lycopene metabolites may be biologically active, hence it is interesting to elucidate further the metabolites in humans and animals. Nevertheless, the identification of enzymatic metabolites of lycopene in biological fluids is highly critical. Breakdown of lycopene occurs through isomerization followed by oxidation to form epoxides or undergoes eccentric cleavage by carotene-9′,10′-monooxygenase (CMO-II) to form apo-lycopenals (Hu *et al.*, 2006; Wang, 2012). Earlier, Khachik *et al.* (1997) identified lycopene oxidative product 2,6-cyclolycopene-1,5-diol, in human serum and breast milk. Hu *et al.* (2006) have demonstrated the formation of apo-10′-lycopenal in the ferret. Further, they have confirmed that apo-10′-lycopenal was converted into apo-10′-lycopenoic acid in the presence of cofactor NAD^+. Similarly, in the presence of NADH lycopene was converted into apo-10′-lycopenol and apo-10′-lycopenoic acid in ferret liver. Consequently, Gajic, Zaripheh, Sun, and Erdman (2006) have determined the existence of apo-8′- and apo-12′-lycopenal as well as

Table 1. Analytical techniques used for detection of carotenoid metabolites in biological samples.

Carotenoids	Metabolites identified[a]	Biological samples	Analytical method	References
β-carotene	β-apo-13-carotenone, β-apo-14′-carotenals & retinoids	Humans, monkey, ferret and rat tissues	HPLC-UV-VIS	Tang, Wang, Russell, and Krinsky (1991); Wang, Tang, Fox, Krinsky, and Russell (1991)
	Retinoic acid	Ferret intestine	HPLC-UV and GC/MS	Wang and Krinsky (1997)
	Retinol	Human blood	LC-MS ($APCI^{+ve}$)	Wang *et al.* (2000)
	(14) C-retinol,(14) C-β-apo-8′-carotene	Human plasma	HPLC & accelerator MS.	Ho, de Moura, Kim, and Clifford (2007)
	Vitamin A	Human plasma	LC/MS/MS	Oxley *et al.* (2014)
Lycopene	Epimeric 2,6-cyclolycopene-1,5-diols	Human serum and breast milk	HPLC-UV/visible-MS & -NMR	Khachik *et al.* (1997)
	Acycloretinoic acid	Pig liver homogenates	HPLC with GC–MS analysis	Kim *et al.* (2001)
	Apo-10-lycopenoids	Ferrets	RP-HPLC (C18 and C30)	Hu *et al.* (2006)
	Apo-8′-lycopenal and Apo-12′-lycopenal	F344 rat liver	HPLC-UV/VIS and ESI^{+ve} mode	Gajic *et al.* (2006)
	Apo-6′-, Apo-8′-, Apo-10′-, Apo-12′- & Apo-14′-lycopenals	Food sample and human plasma	HPLC-MS/MS ($APCI^{+ve}$)	Kopec *et al.* (2010)
Lutein and Zeaxanthin	Geometric isomers of Lutein and Zeaxanthin. 3-hydroxy-β, ε-caroten-3′-one, 3′-epilutein, ε,ε-carotene-3,3′-diol, ε,ε-carotene-3, 3′-dione, 3′-hydroxy-ε,ε-caroten-3-one, etc.	Human & Monkey retina's	HPLC (UV/Vis)-MS	Khachik *et al.* (1997)
	Lutein and zeaxanthin stereoisomer's	Retina	HPLC-LC and HP LC-NMR	Dachtler *et al.* (2001)
	Lutein sterioisomers, 3-hydroxy-β, ε-carotene-3′-one.	Human plasma, liver & other ocular tissues/ Amphibians ocular tissues	HPLC	Khachik *et al.* (2002)
	(3R, 3′S, 6′R)-lutein (3′-epilutein)	Human serum, milk & ocular tissues	HPLC-MS, ^1H NMR	Khachik *et al.* (1997); Khachik (2003)
	3-Hydroxy-β,ε-carotene-3′-one (3′-oxolutein)	Human retina	LC-MS ($APCI^{+ve}$)	Bhosale and Bernstein (2005)
	Ethyl oximes of zeaxanthin oxidation products	Eye samples	HPLC-ESI-MS/MS MRM	Prasain *et al.* (2005)
	meso-Zeaxanthin, and 3′-oxolutein	Human macula	HPLC & MS ($APCI^{+ve}$)	Bhosale *et al.* (2007)
	Lutein sterioisomers, 3′-oxolutein, lutein epoxides, and anhydrolutein	Rats plasma, liver and eyes	HPLC and LC-MS ($APCI^{+ve}$)	Lakshminarayana *et al.* (2008)
	3′-hydroxy-ε,ε-caroten-3-one, ε,ε-carotene-3,3′-dione	Plasma and tissues of ICR mice	HPLC-MS (APCI)	Yonekura *et al.* (2010)
Astaxanthin	3-hydroxy-4-oxo-β-ion and 3-hydroxy-4-oxo-7,8-dihydro-β-ionone	Primary cultures of rat hepatocytes	HPLC and GC–MS	Wolz *et al.* (1999)
	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-ionon	Humans plasma	HPLC and GC–MS	Kistler *et al.* (2002)
Fucoxanthin	Fucoxanthinol and other metabolites	White leghorn laying hens	HPLC (VIS), MS, ^1H NMR	Strand *et al.* (1998)
	Fucoxanthinol	Male ICR mice plasma	HPLC	Sugawara *et al.* (2002)
	Fucoxanthinol and amarouciaxanthin A	Mice plasma and liver	LC/MS with LCQ-MS	Asai *et al.* (2004)
	Fucoxanthinol, amarouciaxanthin A, deacetylated, hydrolyzed & demethylated fucoxanthin	Rats plasma	HPLC and LC-MS (APCI)	Sangeetha and Baskaran (2010)
	Fucoxanthinol and amarouciaxanthin A	Plasma, liver, kidney and adipose tissue of ICR mice	HPLC and LC-MS (APCI)	Yonekura *et al.* (2010)

[a] For structures of metabolites refer Figs. 2 and 3.

other polar metabolites of lycopene in rats. Kopec *et al.* (2010) have shown a series of apo-lycopenals formation in human plasma and concluded, metabolites are formed either by enzymatic or chemical oxidative cleavage. Interestingly, a recent study indicated that apo-10′- and apo-14′-lycopenoic acid have a remarkable ability to upregulate BCO2 expression (Reynuld, Aydemir, Ruhl, Dagles, & Caris-Veyret, 2011). The chemical structures of major

oxygenated carotenoids and their metabolites are shown in Fig. 2.

Metabolism of oxygenated carotenoids

Lutein and zeaxanthin are two major oxygenated carotenoids found in plasma and tissues, and they are recognized as macular pigments. Consumption of green leafy vegetables rich in lutein and zeaxanthin is reported to involve in the delayed progression of age-related macular degeneration and cataract (Bone et al., 2003). Krinsky, Landrum, and Bone (2003) have postulated that macular pigments improve acuity through amelioration effects of chronic aberration and protect the central retina. Previously, studies have shown the intestinal absorption and possible biotransformation of lutein and zeaxanthin *in vivo* (Bone, Landrum, Hime, Cains, & Zamor, 1993; Khachik, de Moura, Zhao, Aebischer, & Bernstein, 2002; Lakshminarayana, Raju, Krishnakantha, & Baskaran, 2006).

Apart from the dietary lutein and zeaxanthin, 3′-epilutein, 3′-dehydrolutein, (3R,3′S)-meso-zeaxanthin, 3′-oxolutein, 3-methoxy-zeaxanthin and other isomers/metabolites have also been reported in human serum, milk and ocular tissues (Khachik *et al*., 1997; Krinsky *et al*., 2003; Bernstein *et al*., 2001; Bhosale, Zhao, Serban, & Bernstein, 2007; Khachik, 1997; Bhosale & Bernstein, 2005). The Presence of these molecules indicates that lutein/zeaxanthin undergoes various modifications under *in vivo* conditions. Khachik *et al*. (1997) reported for the first time and explained the formation of oxidation products of lutein and zeaxanthin in human and monkey retinas. The *meso-zeaxanthin* detected in human and monkey retina, might be formed by double bond migration from lutein (Bone *et al*., 1993). The dehydration products of lutein such as, 3-hydroxy-3′, 4′-didehydro-β,γ-carotene and 3-hydroxy-2′,3′-didehydro-β,ε-carotene were thought to be formed non-enzymatically in stomach under acidic conditions (Khachik, Beecher, & Smith, 1995; Khachik, 2003). In human tissues, metabolites of lutein 3′-epilutein might be formed by a back reduction of 3′-oxolutein that was produced from lutein (Bone *et al*., 1993). Later, Yonekura, Kobayashi, Terasaki, and Nagao (2010) have demonstrated the conversion of lutein to keto-carotenoid 3′-hydroxy-ε,ε-carotene-3-one by oxidizing the secondary hydroxyl group in mice model. It is hypothesized that lutein oxidized products may be involved in the protection of eyes from ultraviolet rays. Epidemiological studies suggest that the complete characterization of carotenoids and their metabolites in the retina may help in understanding their functional properties (Khachik, 1997).

Astaxanthin a keto-carotenoid, red fat-soluble pigment present in various microalgae, certain microorganisms and marine animals (Guerin, Huntley, & Olaizola, 2003). Astaxanthin is considered as potent antioxidant molecule-involves in the reduction of various diseases in animals and humans (Higuera-Ciapara *et al*., 2006). Wolz, Liechti, Notter, Oesterhelt, and Kistler (1999) demonstrated that astaxanthin was metabolized into (rac)-3- hydroxy-4-oxo-β-ionone and its reduced form (rac)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone independent of the xenobiotic-metabolizing enzymes induced by astaxanthin in primary cultures of rat hepatocytes. Kistler *et al*. (2002) reported four radiolabeled metabolites 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. Sangeetha and Baskaran (2010) hypothesized that "non-provitamin A carotenoids may yield retinol and confirmed that astaxanthin is converted into retinol via β-carotene in retinol-deficient rats". However, responsible enzymes for the formation of astaxanthin metabolites and their biological functions have not been elucidated. Canthaxanthinis another keto-carotenoid pigment found in green algae, bacteria, crustaceans and fish (Schwartzel & Cooney, 1972). Tyczkowski, Yagen, and Hamilton (1988) revealed that a portion of canthaxanthin was reduced to 4-hydroxyechinenone (4-hydroxy-4′-keto-beta, beta-carotene) that in turn was reduced in part to isozeaxanthin (4,4′-dihydroxy-beta,beta-carotene) was confirmed by high performance liquid chromatography analysis of tissues from chicks. Bausch, Liechti, Oesterhelt, and Kistler (1999) administered the radiolabeled non-provitamin A carotenoid canthaxanthin and studied the urinary metabolic pattern in rats, purification of the metabolite with HPLC, GC–MS and NMR revealed that it was 3-hydroxy-4-oxo-7,8-dihydro-beta-ionone.

Fucoxanthin a marine carotenoid found in brown seaweeds has notable biological properties. Several reports have shown the potential role of fucoxanthin in human health, such as antioxidant, anti-inflammatory, anti-cancer, anti-obese, anti-diabetic, anti-angiogenic and anti-malarial activities (Sachindra *et al*., 2007; Kim *et al*., 2010; Woo *et al*., 2009). Reports have shown the formation of fucoxanthin metabolites and demonstrated the possible biological activity *in vitro* and *in vivo*. It has been reported that fucoxanthinol was detected in egg yolk of hens fed with brown seaweeds (*Fucus serratus and Fucus vesiculosus)* (Strand, Herstand, & Linaen-Jensen, 1998). Sugawara, Baskaran, Tuzuki, and Nagao (2002) have shown the formation of fucoxanthinol in the intestinal tract and demonstrated the dietary fucoxanthin was deacetylated by lipase and esterase from the pancreas or in intestinal cells. Asai, Sugawara, Ono, and Nagao (2004) reported biotransformation of fucoxanthinol in ICR mice and HepG2 cells. In addition, they also showed the bioconversion of fucoxanthinol into amarouciaxanthin-A through dehydrogenation/isomerization. It was found that fucoxanthinol and amarouciaxanthin-A were detected in plasma and tissue of ICR mice and retinol deficient rats fed with fucoxanthin, whereas no fucoxanthin was detected (Hashimoto *et al*., 2009; Sangeetha, Bhaskar, Divakar, & Baskaran, 2009b). Sangeetha, Bhaskar, and Baskaran (2009) reported various metabolites of fucoxanthin including fucoxanthinol and

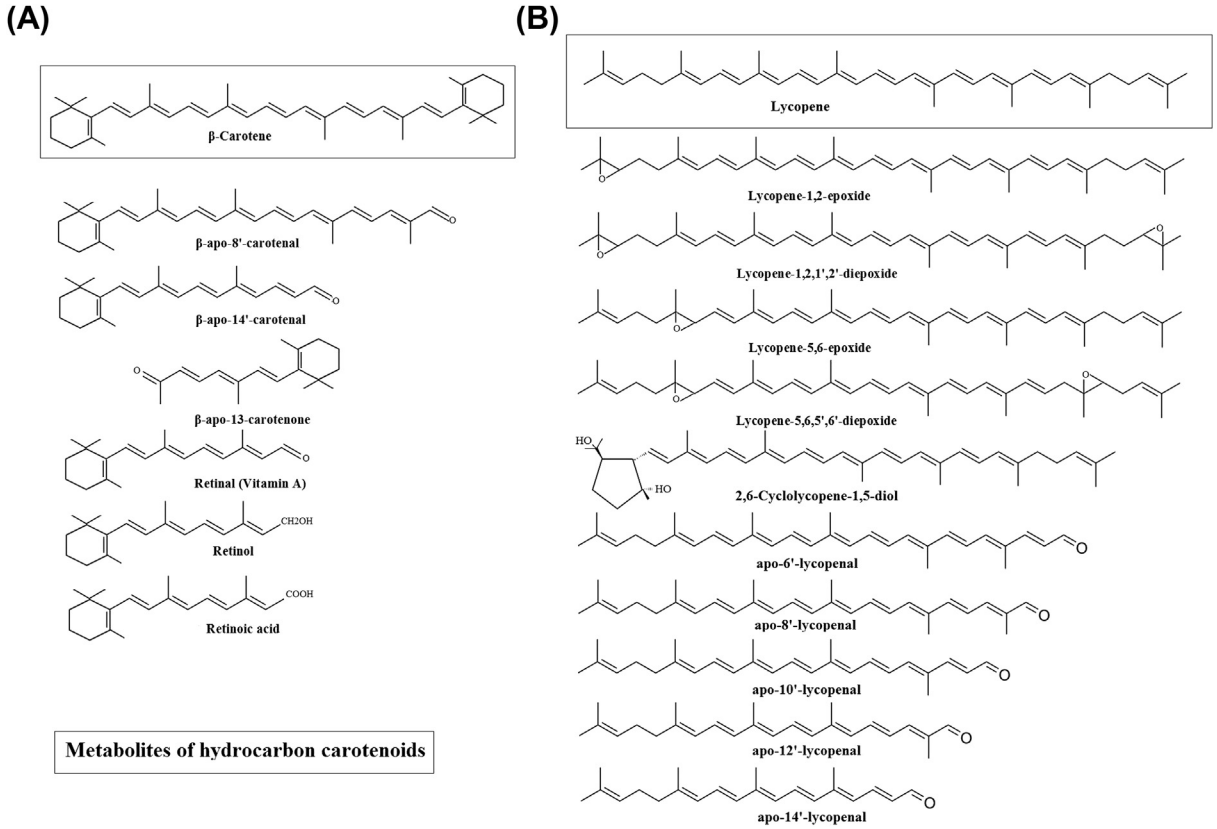

Fig. 2. Chemical structure of hydrocarbon carotenoid. β-carotene (A) and lycopene (B) metabolites. Refer Table 1 for the details of biological samples and analytical techniques used for their characterization.

amarouciaxanthin and proposed a possible metabolic pathway of fucoxanthin in the plasma and liver of rats. In addition, they have speculated the possible formation of fucoxanthin metabolites through various enzymatic and non-enzymatic reactions. Konishi, Hosokawa, Sashima, Kobayashi, and Miyashita (2006) demonstrated the fucoxanthinol was further metabolized into halocynthiaxanthin in sea squirt (*Halocynthia roretzi*). The chemical structures of major hydrocarbon carotenoids and their metabolites are shown in Fig. 3.

Although, *in vitro* studies demonstrated that carotenoid oxidation products exert effects that are either potentially beneficial or detrimental to human health (Khachik *et al*., 1995; Lakshminarayana *et al*., 2013; Zhang, Kotake-Nara, Ono, & Nagao, 2003; Alija, Bresgen, Sommerburg, Siems, & Eck, 2004; Hurst, Saini, Jin, Awasthi, & van Kuijk, 2005). Further, their active involvement in genetic regulation and expression with respect to specific functions is deserved to address health associated and nutrition-related disorders. Hence, it is vital to assess the formation of carotenoids metabolites/oxidative products and their cleavage/fragmentation pattern before understanding their biological function. Therefore, optimization and validation of sensitive and specific analytical methods are essential to characterize carotenoid metabolites (Bhosale & Bernstein, 2005; Sowmya, Arathi, Vijay, Baskaran, & Lakshminarayana, 2014)

Techniques to characterize carotenoids and their metabolites

Metabolomics are categorized into three approaches: fingerprinting, profiling and targeted analysis. Fingerprinting compares the patterns of metabolites that change in reaction to a specific function. Profiling focuses on the analysis of a group of selected metabolites of interest. The third approach would be targeted analysis of known compounds such as carotenoids and their profile in different groups of samples.

The diversified structural characteristic of dietary carotenoids requires an accurate method for their separation and identification. Although, many chromatography techniques are available for the determination of carotenoids in food sources, and characterization is more challenging due to similar molecular mass (structural isomers or geometrical isomers) and other derivatives. Standardization of improved chromatography techniques is evolved by their robustness, speed of analysis and resolution. Henceforth, advancement in mass spectrometry and revolution in liquid chromatography with different stationary phase made it possible to assess diversified carotenoids in a single platform. Analytical sensitivity and data processing for liquid chromatography/mass spectrometry (LC/MS) have been tremendously improved during last decade (Clarke, Rindgen, Korfmacher, & Cox, 2001; Rivera, Vilaró, &

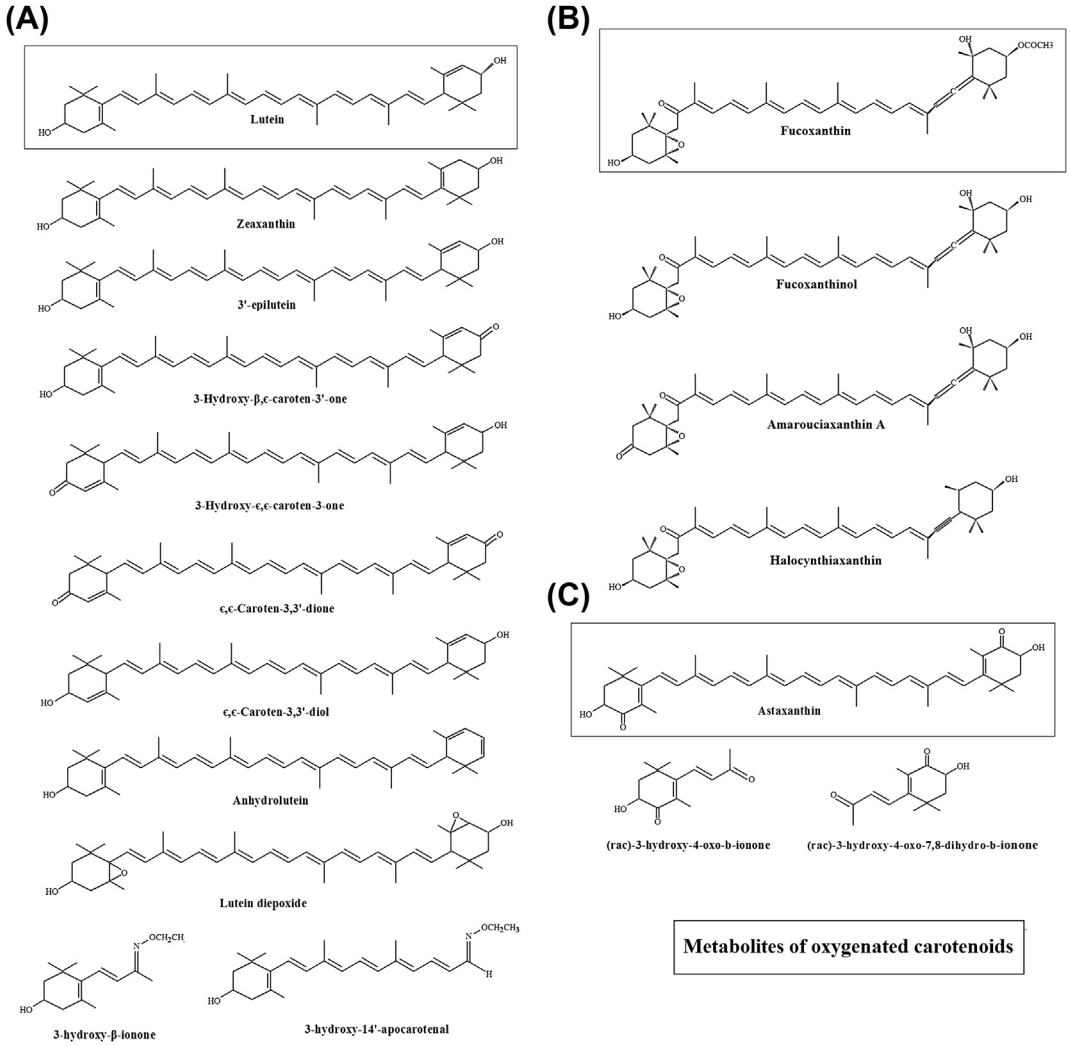

Fig. 3. Chemical structure of oxygenated carotenoid. Lutein (A) fucoxanthin (B) and astaxanthin (C) metabolites. Refer Table 1 for the details of biological samples and analytical techniques used for their characterization.

Canela, 2011). Even though, detection and identification of carotenoid metabolites in complex biological fluids and tissues continue to be a major task. Hence, we present the recent advancement of chromatography, mass spectrometry and their hyphenation technologies for carotenoid metabolomics (Fig. 4).

Classically, TLC was used as a separation technique for carotenoids with advantages of being fast, simple and low-cost. There are two significant improvements in TLC, HPTLC (reduction of the particle size and thickness) and 2D TLC (separation of samples in two orthogonal directions) to provide higher resolution (Rodić, Simonovska, Albreht, & Vovk, 2012). However, the major limitation of TLC is constrained by lower resolution, which considerably hinders its applications. In addition, detection of carotenoids on TLC requires higher sample concentration. Since, TLC operates in an open system, the compound is prone to oxidation or degradation and hence, needs inert chamber conditions. Further, separation of closely related carotenoids needs more standardization of solvent ratios for better separation and is time-consuming.

Routinely, HPLC with UV–Vis upgraded with PDA/DAD are used for the separation and quantification of carotenoids. These procedures made a provision to confirm the existence of unknown carotenoids in a single run by HPLC (normal or reversed-phase) systems under isocratic or gradient elution. Previously, chromatographic separation of carotenoids was based on HPLC analysis using C_{18} and C_{30} columns. In general, C_{30} and C_{18} stationary phases are extremely employed for the separation of geometrical isomers (Bijttebier *et al.*, 2014; Liu, Lee, Garofalo, Jenkins, & EliSohemy, 2011). Advancement in Liquid chromatography for separation of carotenoids and metabolites using ultra high-performance liquid chromatography (UPLC) are of current interest. Recently, UPLC-MS approaches have been developed for the analysis of carotenoids in foods and biological samples (Arathi *et al.*, 2014; Chauveau-Duriot, Doreau, Nozière, & Graulet, 2010;

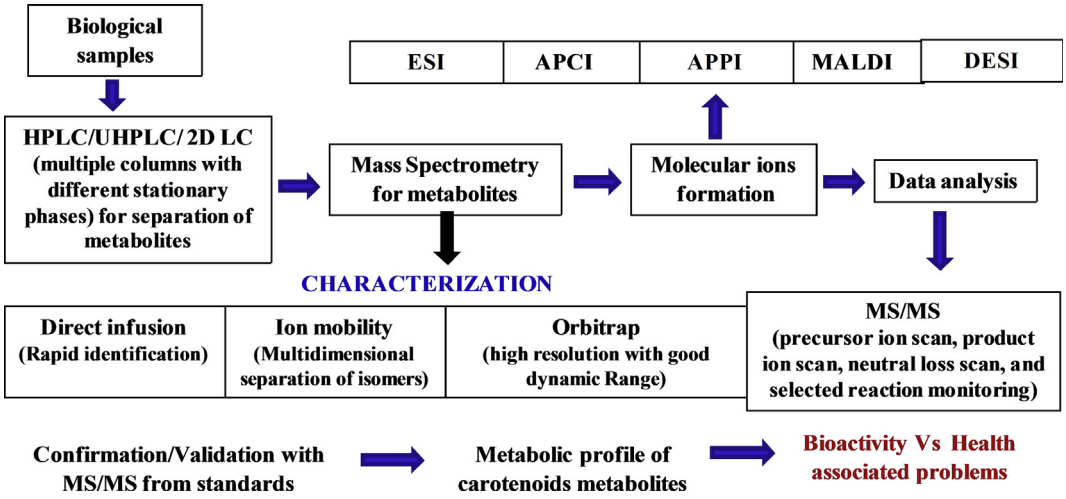

Fig. 4. Strategies for detection and characterization of carotenoid metabolites using hyphenated techniques.

Guzman, Gad, & Allan, 2012; Delpino-Rius *et al*., 2014). The UPLC stationary phase consist of narrow-bore columns packed with very small particles below 2 um and mobile phase delivery systems operating at high backpressures (103.5 MPa), While in conventional HPLC, the maximum back-pressure is 35–40 MPa. Thus, UPLC offers several advantages over conventional HPLC, such as rapid analysis with shorter retention time, narrower peaks giving increased signal-to-noise ratio with high resolution and sensitivity. However, the drawback of UHPLC is currently there is no C_{30} stationary phase, which is considered as a 'golden standard' in the chromatographic separation of carotenoids (Bijttebier et al., 2014).

Generally, closely related carotenoids and their metabolites co-elute in various analytical methods. Although, spectral confirmation of these carotenoids are based on characteristic UV–Vis spectra obtained by PDA/DAD and further characterization is required by MS analysis (Frenich, Torres, Vega, Vidal, & Bolanos, 2005).

Directinfusion of carotenoids to MS do not require any previous chromatographic separations. The method is accurate, reproducible, highly sensitive and less time consumption. But the limitation of this approach is ion suppression, co-elution and formation of complex spectra of metabolites that yield mass to charge ratio (*m/z)* (Draper, Lloyd, Goodacre, & Beckmann, 2013).

GC–MS is another promising technique used to identify the volatile compounds formed by enzymatic and non enzymatic oxidative degradation of carotenoids. However, there are only a few reports on the analysis of carotenoids and their metabolites by GC–MS. Novotny, Dueker, Zech, and Clifford (1995) determined the metabolism of oral fed β-carotene-d8 in plasma by HPLC, GCMS and measured β-carotene-d8 and retinol-d4 concentration–time curves in humans. Wolz *et al*. (1999) investigated the astaxanthin metabolites in primary cultures of rat hepatocytes by GC–MS. Kim, Nara, Kobayashi, Terao, and Nagao (2001) analyzed the acycloretinoic acid formed by autoxidation of lycopene in toluene by GC–MS. Aust *et al*. (2003) has reported the biologically active lycopene oxidative product 2,7,11-trimethyltetradecahexaene-1,14-dial by using GC–MS. The limitations of carotenoid analysis by GC–MS are degradation under the high temperature, and complex derivatization. The derivatization process may significantly eliminate structural information of molecular species.

There are remarkable milestones in the development of analytical methods for qualitative and quantitative analysis of carotenoids by LC-MS. Advancement of different ionization modes [EI; electron impact, FAB; fast atom bombardment, MALDI; matrix-assisted laser desorption/ionization, ESI; electrospray, APCI; atmospheric pressure chemical ionization, APPI; atmospheric pressure photoionization and ASAP; atmospheric pressure solids analysis probe] in mass spectrometry are notable in carotenoids biochemistry studies (Fraser *et al*., 2007; Kaufmann, Wingerath, Kirsch, Stahl, & Sies, 1996; van Breemen, 1997; Crupi, Milella, & Antonacci, 2010; Van Breemen, Dong, & Pajkovic, 2012). Most of the spectrometry analysis of carotenoids is acquired using positive ion mode. APCI has become the most widely used ionization technique for various carotenoids because of high sensitivity (Rivera *et al*., 2011). Several metabolites of carotenoids were identified and characterized by using LC-MS in APCI ionization mode (Lakshminarayana *et al*., 2008; Bhosale *et al*., 2007; Yonekura *et al*., 2010; Asai *et al*., 2004; Sangeetha *et al*., 2009a,b; Sicilia *et al*., 2005) (Table 1). The main drawback of LC-MS is closely eluting isobars, and isomers are indistinguishable by using this method. Further, MSMS is a more reliable and selective method for analysis of carotenoids. LC-MS/MS offers minimal sample size and distinguishes between co-eluted carotenoids and their metabolites, provides information about structural isomers. MSMS is used in order to find out the

characteristic/typical fragmentation pattern by collision-induced dissociation (CID) of carotenoids. Fang, Pajkovic, Wan, Gu, and van Breemen (2003) observed that lycopene, β-carotene, and α-carotene produced molecular ions of *m*/*z* 536 by LC–MS using APCI^{-ve} analysis. Prasain, Moore, Hurst, Barnes, and van Kuijk (2005) developed a sensitive and specific ESI-MSMS method for the identification of two oxime derivatives of 3-hydroxy-β-ionone (*m*/*z* 252) and 3-hydroxy-14′-apocarotenal (*m*/*z* 370) with protonated molecules in a human eye sample. Van Breemen *et al.* (2012) identified the characteristic fragment ions of carotenes and selected xanthophylls using positive and negative ions in APCI-MSMS, to distinguish between carotenoids. Rivera, Christou, and Canela-Garayoa (2014) determined the carotenoid content in transgenic maize seeds and callus using UHPLC-APCI^{+ve}-MSMS. However, MSMS produces identical fragmentation pattern of *cis*/*trans* isomers of carotenoids due to switching of *trans to cis* isomer under high probe temperature, hence a more sensitive technique like ion mobility studies may help in further differentiation of c*is*/*trans* isomers.

Ion mobility mass spectrometry (IMS) is the gas-phase separation of ions in an electric field on the basis of size and shape (Eiceman & Karpas, 2005). Due to thermal instability, carotenoids are isomerized during the ionization process and IM-MS/MS separates *cis*/*trans* isomers. This technique provides an opportunity to determine carotenoid geometrical isomers based on its fragmentation pattern formed during CID. Dong *et al.* (2010) characterized the geometrical carotenoid isomers using travelling-wave ion mobility spectrometry on a quadrupole time-of-flight mass spectrometer. However, various cis isomers cannot be resolved by using IMS.

Orbitrap MS is considered as a new tool, high-resolution mass spectrometry for carotenoids analysis. Orbitrap detectors are molecule screening detectors that can routinely 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). Whereas, high mass resolution and exact mass screening detectors enable the calculation of the most probable molecular formulae of the ions and fragments (Kind & Fiehn, 2006). This facilitates identification of unknown compounds and helps in the elucidation of fragmentation pattern. Bijttebier *et al.* (2014) reported for the first time using orbitrap technology for the elucidation of fragmentation pathways of carotenoids. More recently, Van Meulebroek, Vanden Bussche, Steppe, and Vanhaecke (2014) developed a full-scan high-resolution metabolomic screening for carotenoids in tomato fruit by using orbitrap-mass spectrometry.

One-dimensional LC is unsatisfactory when challenged with highly complex samples, hence 2D LC has emerged as a useful tool for carotenoid analysis. This provides an extraordinary separation and resolution for analysis of complex matrices. Carotenoids are analyzed in 2D by orthogonal dimension with reduced preparation steps when compared to traditional HPLC methods. Herrero, Ibáñez, Cifuentes, and Bernal (2009) highlighted multidimensional chromatographic techniques and their main applications in food analysis. 2D LC-MS may be considered as an useful technique for carotenoid analysis, especially comprehensive profiling of complex biological samples owing to its predominant, sensitive and excellent resolution. Cacciola *et al.* (2012) developed a comprehensive normal and reversed-phase liquid chromatography and applied for analysis of the intact carotenoid composition of red chili peppers, coupled with PDA and mass spectrometry. However, the drawbacks of 2D LC are solvent incompatibilities and immiscibility issues due to coupling of two different columns.

Desorption electrospray ionization (DESI) and laser ablation electrospray ionization (LAESI) are other two novel ionization techniques that can be employed to study carotenoid biochemistry. In comparison with ESI, DESI can be used to determine biological samples directly without complex sample pretreatment. This capability can enhance the application field of DESI-MS in the analysis of carotenoid. LAESI ionization technology was derived from ESI, exciting the OH vibrations in water molecules with a focused mid-IR laser beam in biological samples. Takáts, Wiseman, Gologan and Cookes (2004) observed working under ambient conditions, DESI can be used for the spatial analysis of native surfaces, such as plant or animal tissues. The DESI spectrum collected from tomato skin also indicates the localization of characteristic compounds, including lycopene at *m*/*z* 536.

LC-NMR is a pioneering technique that offers 1-D and 2-D NMR spectra for the components separated by HPLC. Recently, LC-NMR is widely 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 (Tode, Maoka, & Sugiura, 2009). Dachtler, Glaser, Kohler, and Abert (2001) employed on-line HPLC-NMR coupling for the unambiguous structural elucidation of lutein and zeaxanthin isomers at low-*nano moles* range. NMR studies of the carotenoid metabolites are difficult to be carried out due to the quantitative limitation.

In spite of advancement in hyphenated techniques, inconsistency of analytical results are possibly encountered by several artifacts during the analysis of carotenoids and their metabolites. The known pre-chromatographic errors are due to types of samples or tissues, the nature of carotenoids, sample preparation, incomplete extraction, solvent incompatibility, isomerization/oxidation, physical losses of carotenoids/metabolites and its accountability. In LC-MS or MS/MS analysis, the inaccuracy may arise due to low recovery, less stability, inaccurate method validation, co-elution, unavailability of standards, selection of improper mode of ionization, carotenoid/metabolites with

same molecular mass. Other than the sample preparation and optimization of analytical procedure/techniques, adequate laboratory conditions such as protection from light, circumvent high temperatures, avoid sample exposure to atmospheric oxygen, preferably recommended to use high purity solvents, proper storage under inert conditions or it is recommended to analyze the samples immediately, addition of antioxidants and completion of analysis within shortest run time must be employed along with sophisticated instruments (Rodriguez-Amaya, 2010).

Proposed future line of research

Till now, carotenoid research has typically focused on characterization of carotenoids in food and various natural sources. Currently, hyphenated analytical tools are also applied for the identification of carotenoids and their metabolites in body fluids and biological tissues. The comprehensive investigation of carotenoid metabolites drives further progress to develop a novel or advance analytical techniques for versatile analysis. The development of metabolomics database and its integration into genomics, transcriptomics, and proteomics have made links between different levels of biological systems leading to understanding systems biology. Furthermore, concept of metabolomics helps to explore thousands of new components with beneficial or harmful effects to humans. Notably, carotenoids are rarely absorbed and excreted in their ingested forms, but extensively metabolized in the body. The fate of metabolites and its role in comparison with intact carotenoids related to their health benefits needs to be addressed. Although, sensitive analytical techniques are used for carotenoids analysis, further these techniques could also be explored for metabolites too. Most of the bioactive degraded/oxidized/metabolites of carotenoid products may likely to have lesser life span, determination of such components is really challenging. The acquiring of entire spectrum of metabolites and their characterization helps to understand its structure-function relation. Hence, exploration of a suitable analytical method with optimization, validation and reproducibility is required to reveal unknown carotenoids and their metabolites.

Conclusion

Highly sensitive techniques are required to fingerprint small molecules having superior biological significance. The oxidative or cleavage products exist at *picomolar* range and may be less stable with a shorter life. Hence, it is important to analyze carotenoid metabolites quickly without laborious preparation steps. HPLC, LC-MS and NMR are widely used analytical techniques for the detection and quantification of carotenoids. Advancement in hyphenated techniques is applied to understand the metabolism of carotenoids and identify its role in human health and nutrition. Ongoing advances in techniques are crucial for characterization and quantification of novel carotenoids in plants, microorganisms, seaweeds for nutritional and pharmaceutical purpose. Characterization of food metabolomes leads to the discovery of food-specific biomarkers, which are indicators of diet exposure and food consumption. The dietary components influences on carotenoids/metabolites bioavailability and its disposition at ultra trace level with targeted bioactivity need to be addressed by using appropriate analytical methods.

Advancement in chromatography, mass spectrometry and their hyphenation will help the scientists to gain a profound insight into carotenoid biochemistry. Further, limited dynamic range, lack of comprehensive coverage, and limited annotations of carotenoid metabolites needs to be addressed. The rapid analysis and minimized sample requirement will gain the attention of researchers. Despite the analytical advances, there is no single platform for the analysis of carotenoids and their metabolites. The available data indicates that metabolomic studies require a universal protocol. Thus, for further approach a substantial need for improvement to above limitations in carotenoid metabolomics are warranted.

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