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Review

Carotenoid extraction methods: A review of recent developments

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ABSTRACT

The versatile use of carotenoids in feed, food, cosmetic and pharmaceutical industries has emphasized the optimization of extraction methods to obtain the highest recovery. The choice of method for carotenoid extraction from food matrices is crucial, owing to the presence of diverse carotenoids with varied levels of polarity, and the presence of various physical and chemical barriers in the food matrices. This review highlights the theoretical aspects and recent developments of various conventional and nonconventional methods used for the extraction of carotenoids, including ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE), and supercritical fluid extraction (SFE). Recent applications of non-toxic and environmentally safe solvents (green solvents) and ionic liquids (IL) for carotenoid extraction are also described. Additionally, future research challenges in the context of carotenoids extractions are also identified.

1. Introduction

Carotenoids are the most widespread class of isoprenoid pigments that are synthesized by photosynthetic organisms (including plants), some non-photosynthetic bacteria and fungi. Since animals (except some species of aphids) are unable to synthesize carotenoids (Moran & Jarvik, 2010), they need to be obtained from food. In animals, carotenoids play significant roles, including: i) ornamentations (e.g., in flamingos and salmon); ii) protection against lung, head, neck and prostate cancer, most likely due to their potent antioxidant properties mediated by oxidizing the superoxide radical anion; iii) in the modulation of the immune system, growth factors and intracellular signaling pathways (gap junction communication); iv) the regulation of cell differentiation, cell cycle and apoptosis; v) photoprotection against UV radiation; and vi) as precursors for the visual pigment retinol (Vitamin A) (Saini, Nile, & Park, 2015). Carotenoids are also widely used in cosmetic products due to their photoprotection properties against UV radiation. There are over 600 known carotenoids which can be classified into two groups; i) Xanthophylls (originally known as phylloxanthins), containing oxygen as a functional group, including astaxanthin, lutein and zeaxanthin, and ii) Carotenes, which contain only a hydrocarbon chain without any functional group, including β-carotene and lycopene. In addition to antioxidant, photoprotection and provitamin A activity, use of carotenoids to improve the commercial value of animals and animal products has also been explored. For instance, dietary astaxanthin is used commercially in the natural colouration of high-value ornamental fishes (Ambati, Phang, Ravi, & Aswathanarayana, 2014; Gouveia, Rema, Pereira, & Empis, 2003), and for the improved pigmentation of egg yolks (Shah, Liang, Cheng, & Daroch, 2016).

Over the last few decades, there have been concerted efforts for the development of improved extraction methods for carotenoids. However, the recovery from complex food matrices remains low, as various physical and chemical barriers present in the food matrix prevent the mass transfer of carotenoids during extraction. Presence of diverse sets of carotenoids with varied levels of polarity also makes their simultaneous extraction difficult. Additionally, the oxidative property of carotenoids limits the exposure to excess heat, light, acids, and long extraction times. Owing to their hydrophobic nature, carotenoids are conventionally extracted using organic solvents. Usually, non-polar solvents, such as hexane, petroleum ether or tetrahydrofuran (THF), are an excellent choice for extraction of non-polar carotenes or esterified xanthophylls, whereas polar solvents such as acetone, ethanol, and ethyl acetate are more appropriate for extraction of polar carotenoids. The different methods used for the extraction of carotenoids from natural sources can be classified into five main categories: i) the atmospheric liquid extraction with Soxhlet, maceration, microwave (MAE: microwave-assisted extraction) or ultrasound (UAE: ultrasound-assisted extraction); ii) the accelerated solvent extraction (ASE), also known as pressurized liquid extraction (PLE); iii) pulsed electric field (PEF) assisted extraction; iv) the supercritical fluid extraction (SFE), which is often based on the use of supercritical CO_2 (SC-CO_2) as a solvent, with minimal use of organic co-solvent such as ethanol; and v) the enzyme-assisted extraction (EAE). These above-listed extraction methods differ in the mode of cell disintegration, and

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degree of applied temperature and pressure for the extraction of carotenoids. For instance, Soxhlet extraction utilizes solvents at boiling temperature and ambient pressure, while, ASE and SFE are operated at low temperature and high pressure. In contrast, UAE, PEF and EAE utilize ultrasound waves, high voltage pulses and cellulolytic enzymes, respectively for the disintegration of cells, which facilitates the release of intracellular carotenoids.

Multiple reviews have been published on various aspects of carotenoid extraction (Grosso, Valentão, Ferreres, & Andrade, 2015; Mäki-Arvela, Hachemi, & Murzin, 2014; Singh, Ahmad, & Ahmad, 2015; Sowbhagya & Chitra, 2010). Grosso et al. (2015) compared different traditional and advanced methods for efficient extraction of marine bioactive compounds, including carotenoids. Mäki-Arvela et al. (2014) reviewed the carotenoid extraction methods from algae, with modeling of extraction kinetics. The authors discussed the carotenoid availability in microalgae, sample pre-treatment methods, saponification, antioxidant and antimicrobial properties, and downstream processing of the algal carotenoid extract. Sowbhagya and Chitra (2010) reviewed the enzymes-assisted extraction methods of carotenoids from alfalfa, chili, safflower, marigold, strawberry and tomato products using cellulase, hemicellulase, pectinase, and glycosidase enzymes. In recent years, the extraction methods for carotenoids have been evolving quicklyly, with improved solvents and techniques aimed at rapid, cost-effective and efficient extraction. This review compiles and highlights the recent developments in various techniques used for the extraction of carotenoids. In contrast to conventional organic solvents, extraction using environmentally safe and nontoxic solvents (termed as green solvents) has also gained significant attention in recent years. Extractions using these solvents are termed “Green extraction”; these methods are also included and discussed in this review.

2. Chemistry, classification and occurrence of carotenoids

The majority of the carotenoids are derivatives of the C40 tetraterpenoid pigment phytoene, and are biosynthesized by the head to head condensation of two C20 geranylgeranyl diphosphate molecules. The remainder of the carotenoids are derived from a phytoene molecule, by undergoing various enzymatic reactions. The carotenoids are divided into two major classes: i) hydrocarbon carotenoids are referred to as carotenes, such as α-carotene, β-carotene and lycopene; ii) oxygenated derivatives of hydrocarbon carotenoids, which are known as xanthophylls. In xanthophylls, the oxygen atom can be present in the form of an alcohol (−OH) (e.g., lutein), a ketone (=O) (e.g., canthaxanthin), a combination of alcohol and ketone (e.g., astaxanthin) or as esters of alcohol (e.g., fucoxanthin) (Fig. 1). Apart from these two classes, apocarotenoids and C50 carotenoids are other categories of carotenoids. Apocarotenoids are the oxidative cleavage products of carotenoids, catalyzed by a family of carotenoid cleavage dioxygenases (CCDs). Biologically and commercially important apocarotenoids include annatto pigment bixin, aromatic volatile aroma compounds β-ionone and α-ionone, plant hormone abscisic acid and strigolactone, and vitamin A (Saini et al., 2015). Microbes such as *Corynebacterium glutamicum* (soil bacterium) are unique in producing the C50 carotenoid decaprenoxanthin and its glycosylated forms, which are principally used in cosmetic products, owning to its UV light-protecting properties (Saini & Keum, 2017). Carotenoids can be further classified into provitamin A carotenoids (e.g., β-carotene, β-cryptoxanthin and mutatochrome), and the non–provitamin A carotenoids, which cannot be converted to retinal (e.g., lutein and lycopene).

Dark green leafy vegetables, coloured fruits and unicellular microalgae are the major sources of natural carotenoids (U.S. Department of Agriculture, 2016). Foods rich in β-carotene, lutein, zeaxanthin and lycopene [as listed in United States Department of Agriculture (USDA) nutrient database] are given in Table 1. The data show dehydrated peppers are the richest source of β-carotene, followed by carrot and grape leaves. The fresh leaves of sweet potato, spinach, dandelion green and turnip green are rich sources of lutein and zeaxanthin, whereas tomato is the most abundant source of lycopene (45.9 mg/100 g DW) (Table 1). In addition to the fruits and vegetables listed in the USDA database, there are many other rich sources of carotenoids. For instance, the seed arils of Gac fruit (*Momordica cochinchinensi* Spreng.) and the deep red fruits of *Tinospora cordifolia* (willd.) are established as a rich source of lycopene, containing 408 μg/g and 508 μg/g FW of lycopene, respectively (Khan, Sri Harsha, Giridhar, & Ravishankar, 2011a, 2011b; Vuong, Franke, Custer, & Murphy, 2006). Similarly, the seed arils of bitter melon (*Momordica charantia* L.) fruits have recently been characterized with a high content of lycopene (273 μg/g FW) (Saini, Assefa, & Keum, 2017). The green foliage of the *Moringa oleifera* tree is also established as a traditional and affordable source of carotenoids, containing 182.7 and 368.8 μg/g (FW) of β-carotene and lutein, respectively (Saini, Shetty, & Giridhar, 2014). Unicellular microorganisms are also rich sources of commercially vital carotenoids. The unicellular microalgae *Dunaliella salina* and *Dunalielle bardawil* are the most common source for natural production of β-carotene, producing up to 10–12% β-carotene from dry cell weight (DCW) (Saini & Keum, 2017). Similarly, *Haematococcus haematocysts* produce 3–5% astaxanthin from DCW (Ambati et al., 2014). To extract carotenoids from these natural sources (for analysis and commercial application), a simple, rapid, and inexpensive extraction method is the prime requirement. Moreover, a rapid and simplified protocol would help to efficiently examine the dynamics of carotenoid biosynthesis and accumulation. In the upcoming sections, we discuss the various conventional and nonconventional methods employed for the extraction of carotenoids.

3. Extraction of carotenoids: points to consider

The various steps involved in the extraction and analysis of carotenoids from natural sources are summarized in Fig. 2. The high water content of fruits and algal cells is generally considered unfavorable for the efficient extraction of carotenoids, in particular with SFE, owing to the hydrophobic nature of carotenoids and extracting solvents (Durante, Lenucci, & Mita, 2014). However, commonly used thermal based dehydration methods (e.g., oven- and microwave drying) causes thermal degradation and isomerization of carotenoids (Saini, Shetty, Prakash, & Giridhar, 2014). Thus, to protect the carotenoids from high temperature mediated degradation, food samples are dehydrated using lyophilization (freeze-drying). However, it significantly increases the extraction time and cost. Given these factors, dehydration is considered as an optional step for the samples containing small amounts of water. The disruption of the cellular system during execution also causes rapid degradation of carotenoids, even at low temperature. In general, the following five points should be considered to minimize the degradation of carotenoids during extraction, storage and analysis i) a neutralizer, such as calcium carbonate ($CaCO_3$), sodium bicarbonate ($NaHCO_3$) or magnesium carbonate ($MgCO_3$), to be added during the extraction to neutralize acids liberated from the plant samples, as the acids can cause potential isomerization and rearrangement of 5,6-epoxy- to 5,8-epoxycarotenoids (e.g., in violaxanthin and neoxanthin); ii) an antioxidant, such as tert-butlylhydroqinone (TBHQ), butylated hydroxytoluene (BHT), pyrogallol or ascorbyl palmitate, added to extraction solvents at concentrations of ≈0.1% (w/v) (Cernelic et al., 2013); iii) the time lag between sample maceration and extraction are minimized to prevent enzymatic oxidation; also, a short extraction time with appropriate temperature is recommended for viable extraction; iv) samples are protected from direct exposure to UV light, as this can promote *trans-cis* photoisomerization and photodestruction; and v) sample tubes during extraction are flushed with N_2 to eliminate oxygen and provide an inert environment.

Carotenoid extraction occasionally involves pre-treatment steps, which help in the breakdown of the cell wall and other physical barriers present in the sample. For example, diatoms (a major group of

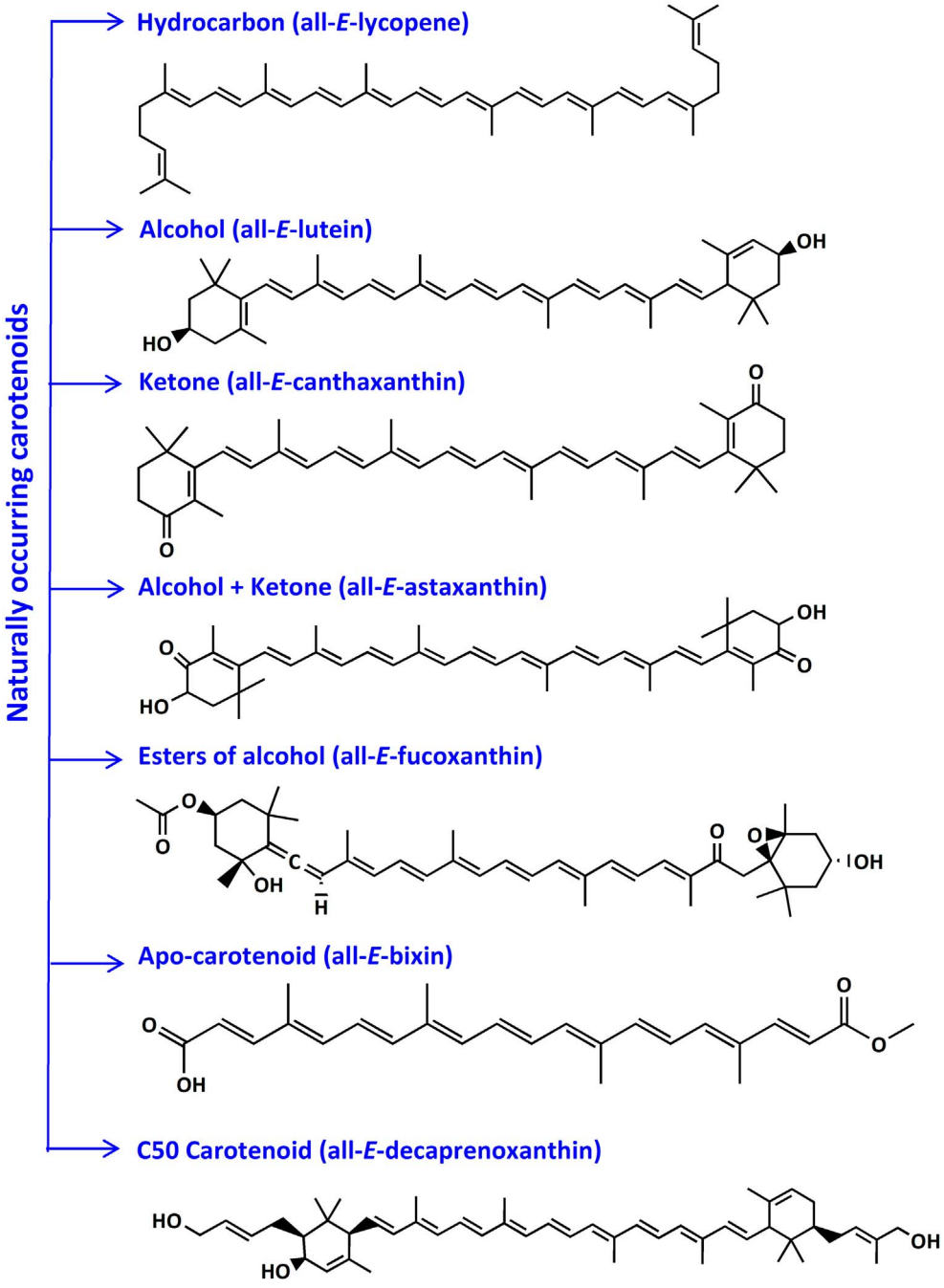

Fig. 1. The structures of various naturally occurring carotenoids.

unicellular algae) contain an external hard silica layer (frustule), which acts as a mechanical barrier to pigment extraction (Pasquet et al., 2011). Thus, various chemical and physical pre-treatments are applied to facilitate the release of carotenoids from complex food matrices. In the upcoming section, we have discussed the major pre-treatment methods applied for the extraction of carotenoids.

4. Pre-treatments before extraction

Plant and microalgal cells are composed of dynamic, complex, and rigid cell walls, which constitute an obstacle to the entry of solvents into the cell. Additionally, the firm association between carotenoids with other macromolecules, such as proteins and fatty acids prevent the mass transfer of carotenoids during extraction. Thus, in the first step of extraction, these barriers are disrupted by physical (cooking, drying, osmotic shock, freeze–thaw, cryogenic grinding), chemical (acid, base, surfactants), enzymatic or biological means to facilitate the efficient extraction of carotenoids. Selection of appropriate cell disruption methods is fundamentally dependent on cellular matrix and cell-wall characteristics. For instance, the robust structure of trilayered cell wall in *Haematococcus pluvialis* commonly requires intense methods for efficient disruption. Mezzomo, Maestri, dos Santos, Maraschin, and Ferreira (2011) studied various pre-treatment methods, followed by extraction of astaxanthin and total carotenoids from the pink shrimp (*Penaeus brasiliensis* and *P. paulensis*) residue, using conventional and advanced methods, and employing different solvents. Cooking was found to be the most useful to obtain the highest yield of carotenoids, compared to dehydration and milling procedures. This could probably be due to the cooking mediated disintegration of carotenoid-protein complex from the sample, thus increasing the carotenoids yield. The pre-treatment by cryogenic grinding, composed of precooling (2 min with 5/s frequency), grinding (3 min, 20/s), and intermediate cooling

Table 1
Carotenoid-rich fruits and vegetables listed in the United States Department of Agriculture (USDA) nutrient database.

Source	β-Carotene	Lutein + zeaxanthin	Lycopene
Peppers, sweet, red, freeze-dried	42,891	5,799	n.d.
Carrot, dehydrated	33,954	1,051	3
Carrots, raw	8,285	256	1
Grape leaves, raw	16,194	1,747	n.d.
Spices, chili powder	15,000	310	21
Sweet potato leaves, raw	2,217	14,720	n.d.
Dandelion greens, raw	5,854	13,610	n.d.
Turnip greens, raw	6,952	12,825	n.d.
Spinach, raw	5,626	12,198	n.d.
Tomatoes, sun-dried	524	1,419	45,902
Rose Hips, wild (Northern Plains Indians)	2,350	2,001	6,800
Guavas, common, raw	374	n.d.	5,204
Watermelon, raw	303	8	4,532
Tomatoes, red, ripe, raw, year round average	449	123	2,573

Values are in μg/100 g, n.d. – Not detected. Source – (U.S. Department of Agriculture, 2016).

(1 min, 5/s), was found to be the most efficient for cell wall lysis of the microalga *Neochloris oleoabundans,* compared to freeze–thaw (−20 °C), acid hydrolysis (0.1 N HCl) and alkaline hydrolysis (0.1 M NaOH) pre-treatments (Castro-Puyana et al., 2013). Microemulsion techniques using surfactants were effectively employed for the extraction of carotenoids (Amiri-Rigi & Abbasi, 2016). Surfactants form microemulsions by reducing the surface tension, and converting the hydrophobic molecules to polar, thus improving the extraction yield. Amiri-Rigi and Abbasi (2016) studied the comparative efficacy of eight different surfactants (Span 20, Tween 20, Tween 60, Tween 80, saponin, rhamnolipid, sucrose mono palmitate and lecithin), four co-surfactants (glycerol, propylene glycol, 1-propanol, and ethanol), ultrasound and enzyme pre-treatments for lycopene extraction from tomato pomace. The highest extractability of lycopene (35%) was obtained by a combination of ultrasound, enzyme pre-treatments, saponin as surfactant, and glycerol as co-surfactant. Co-surfactant plays a crucial role in stabilizing the microemulsion formed by surfactant, resulting in improved extraction of carotenoids. Use of saponin is promising for large-scale industrial extraction, as saponin is a natural glycoside found in a wide variety of plants, which can be produced with low-cost. Moreover, saponin is recognized as safe for use in foods and drinks by the FDA (Agency Response Letter GRAS Notice No. GRN 000165). Above all, lycopene-containing microemulsions (formed with saponin) are water soluble, which offers significant advantages for food application.

The maceration of freeze dried yeast (*Phaffia rhodozyma*) with diatomaceous earth resulted in the highest recovery of carotenoids, compared to ultrasonic treatment, abrasion with glass pearls and immersion in liquid nitrogen (Michelon, Borba, Rafael, Burkert, & Burkert, 2012). Treatment with dimethyl sulfoxide (DMSO) gave the highest yield of carotenoids, compared to lactic acid, acetic acid, hydrochloric acid (HCl) and sodium bicarbonate. However, for industrial production, the use of DMSO is not advisable, owing to the toxicity of its derivatives. The enzymatic lysis of yeast cells with Glucanex® was also found to be beneficial for the improved extraction of carotenoids. The authors obtained the highest contents of carotenoids by combining the techniques of lyophilized biomass maceration using diatomaceous earth and enzymatic lysis with Glucanex®; this enabled the highest recovery of

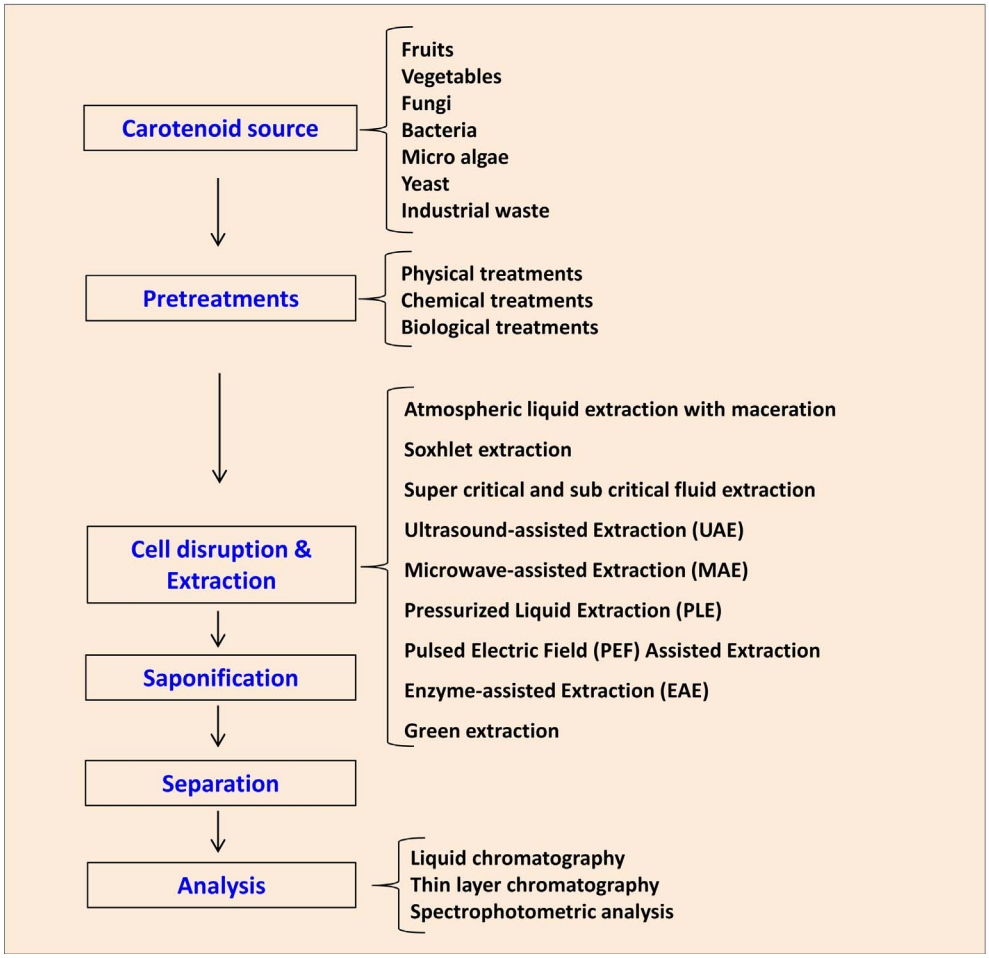

Fig. 2. Different steps involved in the extraction and analysis of carotenoids from natural sources.

carotenoids without using DMSO. Singh, Gupta, et al. (2015) compared the various physical, chemical and mechanical methods for the extraction of astaxanthin from thraustochytrids. The treatment with DMSO before solvent extraction improved the astaxanthin yield by 2.5-fold, compared with direct extraction. Among the different organic and inorganic acids, yield was higher for inorganic acids; the highest astaxanthin yield was obtained using HCl. Authors also revealed that the astaxanthin yield is directly proportional to the concentration of acid. However, it also caused degradation of astaxanthin. Among the different mechanical cell disruption methods, ultrasonication (30 min, at 20 kHz for 50 cycles) and bead beating (25 mg/500 μL beads for 30 min, 0.5 μm size) resulted in highest astaxanthin yield (6 and 5-fold, respectively), compared to homogenization (10,500 rpm for 30 min) and maceration with mortar and pestle. High-pressure homogenization (HPH) was also found useful to disrupt lutein-rich thermo-tolerant microalga *Desmodesmus* sp. with high efficiency, compared to autoclaving, microwaving, osmotic shock and sonication (Xie et al., 2016). In HPH, the disruption efficiency improved with increasing the homogenization pressure (600–2700 bar) and cycle number (1–4), irrespective of cell density, in the range of 2.0–90g/L. Authors also revealed that the operating pressure was more crucial than cycle number. Owing to low heat formation, HPH is an efficient technology for cell disruption and recovery of thermolabile compounds such as carotenoids. Additionally, due to minimum dead volume and low energy requirement (41.4 kJ/g for *Saccharomyces cerevisiae* cells and 82.7 kJ/g for *Desmodesmus* sp), this process is economically suitable for industrial scale-up.

Praveenkumar, Lee, Lee, and Oh (2015) exploited a biological means of utilizing a short-period germination (breaking dormancy) of *H. pluvialis* cyst cell (aplanospores) for effective penetration of ionic liquids (IL; green solvent). The natural germination of aplanospores causes weakening of the strength and rigidity of the cell walls, which facilitates IL-mediated extraction of astaxanthin. In this study, time of extraction after germination of cyst cell was most crucial, as astaxanthin contents were highest during 12 to 18 h of germination, whereas at 24 h, the astaxanthin contents were reduced by 30%. The significant decrease in astaxanthin content was possibly due to bioconversion of astaxanthin (secondary carotenoid) into photosynthetic pigments (primary carotenoids) for photosynthesis during active cell division and growth. Remarkably, the environmental stress factors responsible for astaxanthin accumulation in *H. pluvialis* are well characterized, and inhibition of cell division (mainly by physiological stress) is established as a key factor for enhanced accumulation of astaxanthin (Boussiba, 2000). The breaking of cyst cell dormancy is a simple, cost effective and a green pre-treatment method for improved extraction of commercially valuable astaxanthin. However, the pattern of accumulation and degradation of astaxanthin during germination should be investigated accurately to use during large scale extraction.

Kim et al. (2016) reviewed the various cell wall disruption and extraction methods for the extraction of lipids and astaxanthin from the microalgae *Chlorella* and *Haematococcus*. They concluded that although various cell-wall disruption techniques utilizing physical and chemical means are available, many critical issues, such as energy consumption, toxicity, metabolite stabilities and scale-up, still need to be investigated. A flawlessly optimized extraction process can help in the cost effective production of natural carotenoids.

5. Selection of appropriate solvent

Conventionally, carotenoids are extracted using organic solvents, such as acetone, chloroform, hexane, isopropanol, methanol, methylene chloride and diethyl ether. A wide variety of solvent combinations have also been used, which provides a synergistic effect on extraction of carotenoids. The choice of appropriate solvent or solvent combination is one of the most critical factors for efficient extraction of carotenoids. However, selection of the appropriate one is not always easy, as the functional group (polarity) and chain length of the existing carotenoids, the sample matrix and its components, and moisture content play important roles. Lycopene and β-carotene are highly lipophilic non-polar carotenoids due to their conjugated hydrocarbon structure lacking polar functional groups. The addition of polar functional groups, such as epoxy (violaxanthin and neoxanthin), hydroxyl (lutein and zeaxanthin), keto (canthaxanthin) or keto with hydroxy (astaxanthin) groups, increases the polarity of carotenoids. Usually, acetone and hexane are frequently selected for extraction of polar and nonpolar carotenoids, respectively. On the other hand, a mixture of acetone/ethanol/hexane is most frequently applied for the simultaneous extraction of polar and nonpolar carotenoids. Owning to water-miscible properties, acetone and ethanol are preferred for efficient extraction of carotenoids from plant material containing a high amount of moisture. Amorim-Carrilho, Cepeda, Fente, and Regal (2014) conducted a literature survey on carotenoid extraction methods and concluded that hexane, acetone and ethanol/hexane (4:3) are the solvents most commonly used for plant derived samples.

Most of the solvents (including, ethanol, hexane and acetone) used for extraction possess environmental (air, water and persistency), health (chronic and acute toxicity and irritation) and safety (decomposition, explosion) hazards. However, on the basis of environmental and health and safety issues, ethanol and acetone are preferred solvents, compared to hexane, diethyl ether, dichloromethane and chloroform, which are generally used for extraction of carotenoids (Alfonsi et al., 2008; Capello, Fischer, & Hungerbühler, 2007). To improve sustainability, more environmentally friendly green solvents and ionic liquids are explored for the extraction of carotenoids and other bioactive compounds, which have been discussed in subsequent sections.

6. Carotenoid extraction methods

6.1. Atmospheric liquid extraction with maceration

Effective cell disruption, either by physical, chemical or mechanical means, is a prerequisite for the efficient extraction of intracellular carotenoids. Cell wall disruption facilitates the entry of solvents into the cell to solubilize intracellular carotenoids. Effective cell wall disruption improves the carotenoid yield by 8–10-fold (Michelon et al., 2012; Uquiche, Antilaf, & Millao, 2016). Using a full factorial design (FFD), extraction temperature of 20 °C for 40 min, a solvent mixture of acetone/*n*-hexane (1:3, v/v), and solvent volume of 40 ml/g, was optimized for the highest recovery of total lycopene (94.7%), having a maximum purity (98.3%) for all-*E*-lycopene from tomato pulp waste (Poojary & Passamonti, 2015). Among the extraction methods, the highest yield of total carotenoids from pink shrimp (*P. brasiliensis* and *P. paulensis*) was obtained by maceration using acetone, followed by Soxhlet extraction with hexane/isopropanol, as compared to ultrasound-assisted extraction (Mezzomo et al., 2011). The authors hypothesized that maceration was superior to other methods, probably due to the non-requirement of a heating system during extraction, which avoids the thermal degradation of carotenoids, and minimizes the contact time between solvent and raw material. Subsequently, Soxhlet also presented a satisfactory yield of carotenoids, probably due to the low viscosity and surface tension at boiling temperature, which improves the diffusion and solubilization of carotenoids. In a comparative study, the highest yield of the xanthophyll-rich extract from brown macroalgae *Fucus serratus* was obtained using solvent extraction (hexane/acetone, 7:3 at 50 °C for 24 h), compared to SFE using CO_2, and SFE with CO_2 and ethanol as co-solvent (Heffernan et al., 2016). In contrast, SFE using CO_2 at 50 °C, 304 bar pressure and extraction time of 60 min, was suitable for the extraction of carotenoids, yielding a higher purity fucoxanthin. The comparative results show that solvent extraction delivers the highest total yield of the target carotenoids, while SFE led to higher purity. The use of a mixture of polar (ethyl acetate) and non-polar (hexane) solvents resulted in the highest yield of

carotenoids from tomato waste, compared to the other solvent combinations. The highest yields of carotenoids were obtained with 45% hexane in the solvent mixture, with the solvent to waste ratio of 9.1:1 (v/w) (particle size 0.56 mm) (Strati & Oreopoulou, 2011). A mixture of chloroform and methanol (2:1) was found to be the most efficient for the extraction of carotenoids from the seed arils of Gac fruit, compared to petroleum ether and hexane (Kubola, Meeso, & Siriamornpun, 2013). However, the use chloroform limits its application due to environmental and health and safety issues. In a recent study, the highest total carotenoids and antioxidant potential from the lyophilized peel of Gac fruit were obtained using ethyl acetate compared to acetone, ethanol and hexane (Chuyen, Roach, Golding, Parks, & Nguyen, 2017). With increasing evidence of the carotenoid composition of Gac fruit peel (containing 54, 31 and 13% of β-carotene, lycopene and lutein respectively), the higher yield of total carotenoids obtained by ethyl acetate compared to hexane might be due to the higher polarity of ethyl acetate, which can extract both carotene and xanthophylls simultaneously. In this study, the authors have not investigated the extraction efficiency of hexane and ethyl acetate mixture, which can possibly yield higher carotenoids, as described by Strati and Oreopoulou (2011) for highest extraction of carotenoids from tomato waste.

Carotenoid extraction with organic solvents thus provides good extraction yields without utilizing sophisticated instruments. However, owing to the cost, toxic nature and the residue contamination of final products, there are limitations for its application in food and feed products.

6.2. Soxhlet extraction

Soxhlet extraction is a type of atmospheric liquid extraction, utilizing solvents at boiling temperature and low pressures (ambient pressure), for the selective extraction of targeted compounds. Soxhlet extraction is a conventional technique providing the highest recovery of carotenoids. Thus, it is commonly used for evaluating the performance of other methods (Macías-Sánchez, Fernandez-Sevilla, Fernández, García, & Grima, 2010). However, it's time consuming, and also uses significant amounts of solvents, thus increasing the cost of extraction. Additionally, the high temperature and long extraction time increases the possibilities of thermal degradation and *cis-trans* isomerization of carotenoids. Cardenas-Toro et al. (2015) recorded the highest yield of lipophilic extract from pressed palm fiber (a residue obtained from the oil palm industry) using soxhlet extraction (6 h), compared to percolation (35 °C, 2.4 g/min) and ASE (55 °C, 40 bar, ethanol flow rate of 2.4 g/min) under the optimized conditions (solvent/sample ratio of 20 ml/g). However, the selectivity for carotenoids was higher in ASE, giving the highest yield of carotenoid, compared to other methods. Additionally, the cost of production was lower with ASE (US$39.1/kg carotenoid), compared to Soxhlet (US$ 98.1/kg) and percolation (US$ 48.9/kg), for a 0.5 m^3 vessel capacity. Thus, the authors suggested that ASE can replace the time-consuming Soxhlet extraction for industrial scale extraction.

6.3. Microwave-assisted extraction (MAE)

Microwave-assisted extraction (MAE) is a simple, rapid and economic method for extraction of carotenoids, requiring a very short extraction time with low amount of solvents (Hiranvarachat & Devahastin, 2014). Ho, Ferruzzi, Liceaga, and San Martín-González (2015) optimized the MAE of all-*E*-lycopene from tomato peels using ethyl acetate in the solid to liquid ratio of 1:20 (w/v), and 400 W power (24 kJ equivalents) for 60 s. Interestingly, all-*Z*-lycopene yields increased, as the solid to liquid ratio was decreased and the ethyl acetate proportion was increased compared to hexane. Overall, ethyl acetate was suggested to be a better MAE solvent for highest recovery of all-*E*-lycopene, compared to hexane. Although MAE involved a significantly shorter extraction time, the carotenoid yield was lower than Soxhlet extraction. Extending the extraction time in MAE causes the thermal degradation of carotenoids. Hiranvarachat and Devahastin (2014) used an alternative procedure of intermittent microwave radiation to enhance the yield of β-carotene and carotenoids from carrot waste, without causing excessive thermal degradation, by minimizing the high-temperature environment due to the presence of the off period. The fraction of the radiation time to the total processing time in one cycle is designated by intermittency ratio (α), such as α = 1/2, 1/3 or 1/4. Combined use of lower microwave power (180 W) and solvent volume (75 ml), or higher microwave power (300 W) and solvent volume (150 ml), along with a lower intermittency ratio (α = 1/4), resulted in higher contents of β-carotene and total carotenoids, compared to the continuous MAE under similar conditions. Additionally, prolonging the off period (α = 1/4, compared to 1/2 and 1/3) resulted in higher antioxidant activity of the extracts.

The structure of the material is the key factor influencing the extraction efficiency of carotenoids. Certain pre-treatments of the sample before MAE were found to be beneficial in improving the yield of carotenoids (Hiranvarachat, Devahastin, Chiewchan, & Vijaya Raghavan, 2013; Pasquet et al., 2011). Hiranvarachat et al. (2013) investigated the various influences of sample pre-treatment prior to extraction, on the sample structure and subsequent microwave-assisted extraction (MAE) of carotenoids from carrot. Results show that carotenoid recovery and antioxidant activity from carrots blanched in water and citric acid were significantly higher, when compared to untreated carrots. These pre-treatments damaged and softened the sample structure, possibly by heat mediated solubilization of pectin. Pasquet et al. (2011) compared the efficiency of MAE and Vacuum-MAE (VMAE) to conventional processes (cold and hot soaking and ASE), to extract the carotenoids from frustulated (*Cylindrotheca closterium*) and unfrustulated (*Dunaliella tertiolecta*) microalgae. A frustule is the external hard silica layer of diatoms, which acts as a mechanical barrier to pigment extraction. VMAE facilitates an efficient extraction of chlorophyll "a" and fucoxanthin from *C. closterium*, at moderate irradiation power (75 W). However, the extraction yield was higher with MAE; also, VMAE is technically more complex due to the requirement of vacuum. Cold soaking was an efficient, simple and rapid method to extract pigments from *D. tertiolecta*, as the absence of frustule allows easy penetration of solvents. However, cold soaking was not efficient for extracting carotenoids from frustulated microalgae. The authors further demonstrated that temperature is a crucial parameter determining the yield of microalgal carotenoids. Thus, it is important to accurately define the highest extraction temperature for rapid extraction for highest yields, without damaging the thermolabile carotenoids. In this regard, MAE at 60 °C was found to be optimum for the extraction of carotenoids in non-denaturating conditions. The authors used 100% acetone for the pigment extraction, which helps in minimizing 'chlorophyll a' hydrolysis by the chlorophyllase enzyme, as this enzyme will actively degrade 'chlorophyll a' in aqueous acetone or methanol. The authors also suggested that acetone is an efficient solvent for the extraction of major and minor pigments of different polarities from the studied microalgae species. However, the authors have not studied the comparative efficiency of acetone with other solvents (e.g., hexane and ethyl acetate), which possibly can yield higher carotenoids.

MAE is a simple and economical method for a large variety of samples. However, thermal degradation and *cis-trans* isomerization cannot be ruled out. In this regard, intermittent radiation has proven efficient for the higher recovery of carotenoids and improved antioxidant activities, minimizing the thermal degradation. Thus, the MAE procedure can be further optimized with various levels of microwave power, solvent volume and intermittency ratio.

6.4. Ultrasound-assisted extraction (UAE)

An effective technique of food processing, ultrasound can be applied to many other processes, including intracellular metabolite extraction and microbial inactivation. The major impact of ultrasound in a liquid

medium is attributed to the acoustic cavitation, leading to cell rupture that enhances the mass-transfer of extractants. The ultrasonic power, intensity, temperature and density (sample to solvent ratio) are factors required to be optimized for efficient extraction of metabolites. Using response surface methodology (RSM), solvent (acetone)/CDW ratio of 67.38 µl/mg, power 27.82% (total power 500 W), pulse length of 19.7 s and extraction time of 13.48 min were optimized to obtain the highest yield of zeaxanthin (11.2 mg/g) and β-carotene (4.98 mg/g) from the green microalgae *Chlorella saccharophila* (Singh, Barrow, Mathur, Tuli, & Puri, 2015). In another experiment, comparison among five different solvents yielded highest carotenoid content (79.61%) from Rapeseed (*Brassica napus* L.) meal, using petroleum ether/acetone (v/v = 1/1), when compared to petroleum ether, acetone, chloroform and methanol (Yan, Fan, He, & Gao, 2015). Additionally, an extraction temperature of 49.6 °C, ultrasonic power 252.9 W, liquid to material ratio 41.4 ml/g and duration of 48.5 min, was optimized using RSM for the highest recovery of carotenoids. The use of an optimized range of ultrasound intensity is a crucial parameter to obtain the highest yield of carotenoids in UAE. Above the optimum range, the higher ultrasound intensity can lead to formation and accumulation of OH and H radicals during the cavitation process, which can lead to the significant degradation of the antioxidant compounds including carotenoids (Pingret, Fabiano-Tixier, & Chemat, 2013). Jaeschke, Rech, Marczak, and Mercali (2017) obtained the highest recovery of carotenoids (80%) from the microalgae *Heterochlorella luteoviridis* using 50% ultrasound intensity (total power 664 W), at 30 °C temperature and 75% of ethanol concentration. The authors observed a significant decrease (59%) in the extraction yield at an ultrasound intensity of 100%. Using UAE, the highest extractability of xanthophylls from lyophilized *Aristeus antennatus* shrimp was achieved using acetone as a solvent (10 ml/g), compared to N,N-dimethylformamide (DMF), isopropanol/*n*-hexane (1:1 v/v) petroleum ether/acetone (1:1 v/v) and petroleum ether/acetone/ethanol mixture (2:1:1 v/v/v) (Tsiaka et al., 2015). Interestingly, *n*-hexane/acetone/ethanol mixture (2:1:1) with a liquid to material ratio of 20 ml/g, was the most suitable solvent for MAE of xanthophylls from the same samples. These results can be explained by the heating efficiency of solvents that absorb microwaves and provide high extraction yields. Other UAE (5 min, 600 W), and MAE (7 min, 30 W) parameters were also optimized using RSM for the highest yields of 60% and 105% recovery of carotenoids, respectively. The power 27.82% (total power 500 W), solvent/CDW ratio of 67.38 ml/mg, a pulse length of 19.7 s for 13.48 min, were found to be optimum ASE conditions for the highest yield of zeaxanthin and β-carotene from *Chlorella Saccharophila* (Singh, Gupta, et al., 2015).

6.5. Accelerated solvent extraction (ASE)

Accelerated solvent extraction (ASE), also known as pressurized liquid extraction (PLE), involves extraction at constant high pressure, facilitating improved cell permeability, intermolecular physical interactions and penetration of the extracting solvent(s), and thus improves the mass transfer of carotenoids. High-pressure denaturation of the carotenoid-binding protein induced by pressure improves the extraction of carotenoids (Strati, Gogou, & Oreopoulou, 2015). Higher temperature also helps in reducing the viscosity of the solvent(s), which facilitates the diffusion of solvent into sample matrix. Automated equipment are available, which allow a precise control of temperature, pressure and time of extraction, with amenities for simultaneous extraction of a large number of samples, protected from light and oxygen. ASE at 40 °C, using methanol/tetrahydrofuran (2:8, v/v), 103 bar pressure and 5 min of extraction time, were found as optimum conditions to extract the carotenoids of different polarities from lyophilized apricot (*Prunus armeniaca*), peach (*Prunus persica* L.) and Tunisian Kaki (*Diospyros kaki*) fruits (Zaghdoudi et al., 2015). Using these ASE conditions, high recovery of less polar carotenoids, β-carotene (101%) and β-cryptoxanthin (84%) were obtained in a short extraction time (5 min), with an acceptable recovery of polar carotenoids, lutein and zeaxanthin (79% and 71%). The average extraction yield quantified from the three fruits was 87%. ASE conditions of 100 °C temperature, extraction time of 20 min, extraction pressure of 103 bar and 100% ethanol as the extraction solvent, were optimized for the highest recovery of carotenoids from the cryogenic ground biomass of the microalga *Neochloris oleoabundans* (Castro-Puyana et al., 2013). Saha, Walia, Kundu, Sharma, and Paul (2015) utilized the Hildebrand solubility parameter (δ) to predict the extraction yield of carotenoids in ASE. Hildebrand solubility parameter (δ) is a statistical estimate of interaction between materials, which depicts "strength" of the solvent. This theory predicts that the δ value of solute (target compound) and solvent should be close to achieve the highest extraction efficiency. Lutein and β-carotene have a δ value of 10.01 and 8.71 $cal^{1/2}\ cm^{3/2}$, respectively. Thus, maximum extraction efficiency of lutein and β-carotene from the orange carrot was achieved using a mixture of acetone/ethanol/hexane (1:2:3, v/v/v: δ value of 9.56 $cal^{1/2}\ cm^{3/2}$), compared to acetone/hexane (3:5, v/v), ethanol/hexane (4:3, v/v), acetone/ethanol/hexane/ (3:2:1, v/v/v). In addition to the solvent composition, extraction time and temperature also had a significant effect on the yield of carotenoids, and the highest yield was obtained at 60 °C (103 bar pressure) with 15 min extraction in three cycles. ASE (at 7000 bar) led to higher (64%) extraction yields from tomato waste, in a short time (10 min), compared to conventional solvent extraction process of 30 min (Strati et al., 2015). Additionally, ASE utilizes lower quantities of solvents (6 ml/g, w/v) compared to solvent extraction (10 ml/g), without affecting the extraction yields. Jaime et al. (2010) optimized the PLE of carotenoids from green vegetative cells and red cysts of *Haematococcus pluvialis* microalga using hexane and ethanol as extracting solvents. The extraction temperature correlated positively with the extraction yield, and negatively with the antioxidant activity, assessed by the Trolox Equivalent Antioxidant Capacity (TEAC). Moreover, the extraction yield was higher with the polar solvent ethanol, when compared to hexane.

6.6. Pulsed electric field (PEF) and moderate electric field (MEF) assisted extraction

Pulsed electric field (PEF) technology is a non-thermal method involving the application of repetitive short high voltage pulses of less than a few milliseconds (ms) to a biological material (plant, animal or microbial cells) placed between two electrodes. This process enhances the permeability of the cytoplasmic membrane, called "electroporation," which enables the extraction of intracellular carotenoids. The permeability may be transient (reversible electroporation) or permanent (irreversible electroporation), depending on the applied electric field strengths. Luengo et al. (2015) studied the influence of the pulse duration of milliseconds (ms) and microseconds (µs) at different electric field strengths, for the permeabilization and extraction of pigments from *Chlorella vulgaris*. In the ms range, reversible electroporation was detected with 20 pulses at electric field strengths of 3.5 to 5 kV/cm, whereas and it was irreversible above 4–5 kV/cm. In the µs range, shorter treatment times (75 µs, 25 pulses) with higher electric field strength (15 kV/cm) were required for irreversible electroporation. When comparing the specific energy delivered for similar levels of extraction (electroporation of 90% of the population), pulses in the µs range were more energy efficient. Jaeschke, Menegol, Rech, Mercali, and Marczak (2016) used a moderate electric field (MEF), and ethanol for the extraction of carotenoid and lipid from a microalgae *Heterochlorella luteoviridis*. They observed a positive correlation of carotenoid yield with electrical field strength and ethanol concentration. The highest yield, up to 73% of carotenoid and 83% lipids, was obtained using application of MEF (180 V), with ethanol as solvent.

The PEF has shown promising results, in terms of non-thermal and efficient extraction of carotenoids, with low energy usage. However, the pulse duration and electric field strength intensity require optimization for each type of sample matrix, as these parameters may change with a

change in the electrical conductivity and texture of tissue (Vito, Ferrari, Lebovka, Shynkaryk, & Vorobiev, 2008).

6.7. Supercritical fluid extraction

Supercritical Fluid Extraction (SFE) is an efficient method for extraction of thermolabile compounds such as carotenoids, from a solid or liquid matrix, using supercritical CO_2 (SC-CO_2) as the extracting solvent. The use of SC-CO_2 offers many advantages over conventional methods. The higher diffusion coefficient and lower viscosity of SC-CO_2 allows for rapid penetration into the pores of complex matrices, thus enhancing extraction efficiencies. Additionally, the extracts obtained after using SFE are highly concentrated, as the process of depressurization readily separates the CO_2, thus leaving no trace of toxic organic solvents in the final product. Furthermore, since the CO_2 gas stream can be recycled, SC-CO_2 extraction is regarded as an environmentally friendly (Green extraction) process. Cell disruption using rapid depressurization treatment can be carried out in SFE equipment, which improves extraction with the reduction of time and labor requirements (Uquiche et al., 2016). The major advantages and disadvantages of various extraction methods are described in Table 2.

In general, extraction temperature (40-60 °C), pressure (300–400 bar), time (30–120 min), CO_2 density (solvent power), CO_2 flow rate (1–5 ml min^{-1}) and entrainers concentration (5–25% v/v) are the five most critical parameters for SFE extraction of carotenoids (Zaghdoudi et al., 2016). The optimized conditions of these parameters for the extraction of carotenoids are listed in Table 3. In a comparative study, SFE with CO_2 was comparable to UAE with N, N′-dimethylformamide, and using methanol as a solvent for the extraction of carotenoids from the microalgae *Dunaliella salina* (Macías-Sánchez et al., 2009). In SFE, the highest extraction yield of total carotenoids was obtained at the operating temperature of 60 °C and 400 bar pressure. For chlorophylls, the highest yield was achieved at 60 °C and 500 bar pressure. It was concluded that SFE is more selective than the UAE for the extraction and separation of less polar carotenoids from samples with high chlorophyll content (polar pigments) such as *D. salina*, owing to the differential polarity of CO_2 under varied conditions of temperature and pressure. Macías-Sánchez et al. (2010) obtained the highest extraction yield of carotenoids (50% recovery), at 400 bar and a temperature of 60 °C, from freeze-dried powder of the marine microalga, *S. almeriensis*. Pour Hosseini, Tavakoli, and Sarrafzadeh (2017) recovered 47% of *Dunaliella salina* carotenoids using SC-CO_2 extraction (400 bar and 55 °C), compared to the Soxhlet extraction for 8 h using methanol. However, a high carotenoids/chlorophylls ratio (11.09) was obtained using SFE extraction at 300 bar and 30 °C, which proves the higher selectivity, compared to Soxhlet extraction (0.268).

SFE extraction is a green method for the extraction of high purity thermolabile compounds, such as carotenoids. However, the yield of polar carotenoids (xanthophylls) is often low (Pour Hosseini et al., 2017). By optimizing the key parameters, and using an organic modifier (called co-solvent or entrainers), such as ethanol, the extraction efficiency can be significantly improved by increasing the solubility of the analytes, and by reducing their interaction with the sample matrix, or by inducing matrix modification, resulting in the release of the analytes from the matrix. A considerable increase in the extraction efficiency of astaxanthin from *Haematococcus pluvialis* was observed, following the addition of ethanol as an organic modifier (Machmudah, Shotipruk, Goto, Sasaki, & Hirose, 2006). The addition of ethanol enhances the solvent power of SC-CO_2, resulting in swelling of the matrix, increasing the internal volume and the surface area, and also decomposing the cellular wall, thereby possibly improving the bioavailability of astaxanthin for extraction.

In the supercritical state, the polarity of CO_2 positively correlates with pressure and temperature. Hence, the polarity of CO_2 can be adjusted to match the polarity of the targeted carotenoid, resulting in a higher extraction yield. Additionally, an increase in the CO_2 flow rate can increase the mass transfer of carotenoids. Mezzomo, Martínez, Maraschin, and Ferreira (2013) evaluated the SC-CO_2 extraction of astaxanthin and total carotenoids from Pink shrimp residues, under varying conditions of moisture content in the raw material, solvent flow rate, varied levels of temperature and pressure, and the use of co-solvents (hexane/isopropanol, 1:1 v/v, and sunflower oil) at 2% and 5% concentrations. Increasing CO_2 flow rate (8.3 g/min to 13.3 g/min) and pressure (at constant temperature), and decreasing moisture content (to 11.210%) in the raw material, enhanced the carotenoid yield. The highest astaxanthin yield was obtained by SC-CO_2 at 300 bar pressure

Table 2
Advantages and disadvantages of various carotenoid extraction methods.

Extraction Method	Advantages	Disadvantages
Atmospheric liquid extraction with maceration	• High extraction yields without utilizing sophisticated instruments	• Requires large amounts of toxic solvents, thus increasing the cost of production
Soxhlet extraction	• Simple and conventional method providing the highest recovery of carotenoids • No sophisticated instruments required	• Time-consuming and also uses large amounts of solvents, which increases the cost of extraction • Can cause thermal degradation and *cis-trans* isomerization of carotenoids
Microwave-assisted extraction (MAE)	• Simple, fast and economical method	• Can cause thermal degradation and *cis-trans* isomerization of carotenoids
Ultrasound-assisted extraction (UAE)	• Rapid, non-thermal and efficient extraction	• Aging of the ultrasonic probe surface can change the extraction efficiency • Small particle size (≈50 μm) is required to achieve good extraction
Pressurized liquid extraction (PLE)	• Fast (few minutes), requires minimum amount of organic solvent • Highly applicable to a laboratory-scale context	• Difficult to apply to large volumes due to clogging caused by sugars and pectins of plant matrices
Pulsed electric field (PEF) extraction	• High extraction yield • Non-thermal process • Low energy usage	• High costs of instrumentation • Bubbles in the samples may cause technical problems • PEF parameters may differ with change in electrical conductivity of the sample
Supercritical fluid extraction (SFE)	• Uses non-flammable, non-toxic and recyclable solvent (CO2 and ethanol) • Continuous extraction process instead of batch processing • Useful for extraction of thermolabile compounds • Provide carotenoids with high purity	• Not suitable for samples containing high amounts of water • Low yield of polar carotenoids • High cost of instrumentation
Enzyme-assisted extraction (EAE)	• Rapid and efficient extraction with minimal usage of solvents	• High cost of the enzymes

Table 3
The optimized conditions of extraction temperature, pressure, CO_2 flow rate and entrainer concentration for supercritical fluid extraction (SFE) of carotenoids.

S/No.	Sample	% Moisture	Extracted carotenoids	Optimized conditions (Pressure, temperature, time, entrainers and CO_2 flow rate	Reference
1.	Tomato by-products	Dehydrated#	Lycopene and β-carotene	400 bar, 80 °C, 4 g min^{-1}	(Kehili et al., 2017)
2.	*Dunaliella salina*	0.93	Carotenoids	400 bar, 55 °C	(Pour Hosseini et al., 2017)
3.	Pumpkin	9	β-carotene	300 bar, 47.75 °C,	(Wang et al., 2017)
4.	Persimmon (*Diospyros kaki* L.) fruits	13.67	Xanthophylls	300 bar, 60 °C, 30 min, 25% ethanol, 3 ml min^{-1}	(Zaghdoudi et al., 2016)
5.	Persimmon (*Diospyros kaki* L.) fruits	13.67	Carotenes	100 bar, 40 °C, 30 min, 25% ethanol, min	(Zaghdoudi et al., 2016)
6.	*Undaria pinnatifida*	Dehydrated#	Fucoxanthin	400 bar, 60 °C, 150 min, 5% ethanol	(Goto, Kanda, Wahyudiono, & Machmudah, 2015)
7.	Pink shrimp (*Penaeus brasiliensis*and *P. paulensis*) waste	11.2	Astaxanthin	300 bar, 60 °C, 13.3 g min^{-1}	(Mezzomo et al., 2013)
8.	*Scenedesmus almeriensis*	Dehydrated#	Lutein and β-carotene	400 bar, 60 °C	(Macías-Sánchez et al., 2010)
9.	Pumpkin (*Curcurbita moschata*)	10	Carotenoids	350 bar, 70 °C	(Shi et al., 2010)
10.	Pumpkin (*C. moschata*)	10	β-carotene	250 and 350 bar, 40 °C	(Shi et al., 2010)
11.	Pumpkin (*C. moschata*)	Dehydrated#	Carotenoids	50 °C, 10% ethanol + 10% olive oil	(Shi et al., 2013)
12.	*Haematococcus pluwialis*	Dehydrated#	Astaxanthin	400 bar, 40 °C, 1.67% ethanol, 3 ml min^{-1}	(Machmudah et al., 2006)

#Moisture content is not specified.

and 333.15 K temperature. The use of hexane/isopropanol was not favorable in enhancing carotenoid yields, probably due to the high lipid content of the raw material, which changes the system selectivity toward the lipid components, resulting in reduced carotenoid yields. The authors hypothesized that the higher CO_2 flow rate enhances the solvent velocity and availability, increasing the concentration gradient between sample and solvent phases, and thereby improving the mass transfer rates following the convection mechanism.

SFE extractions of polar (e.g., xanthophylls) and non-polar (e.g., β-carotene) carotenoids require different levels of pressure, temperature, CO_2 density and flow rate. SC-CO_2 with ethanol as co-solvent, at the pressure of 300 bar, 60 °C temperature, 25% (w/w) ethanol as co-solvent, and 3 ml/min flow rate for 30 min, was optimal for the highest yield of xanthophyll, composed of lutein, zeaxanthin and β-cryptoxanthin (Zaghdoudi et al., 2016). However, a non-oxygenated carotenoid (β-carotene) was optimally extracted with moderate CO_2 density (0.558 g/ml) at 100 bar pressure, 40 °C temperature, 25% ethanol (w/w) and 30 min extraction, with the flow rate of 1 ml/min. Surprisingly, the recovery of carotenoids using SC-CO_2 ranged from 128% to 135%, compared to Soxhlet extraction using methanol/THF (1:1, v/v). Taking into account the extraction time (30 min) and solvent volume (15 ml ethanol), the authors conclude that SC-CO_2 is far more efficient than Soxhlet extraction, which requires a 6 h extraction time and 250 ml of methanol/THF (1:1, v/v), to obtain an equivalent yield of β-carotene.

In addition to ethanol, other organic modifiers such as acetone, methanol, propane, methylene chloride or ethyl acetate, can also be used in sub- or supercritical fluid extraction. Lu, Feng, Han, and Xue (2014) obtained a satisfactory yield of carotenoids and chlorophylls from *Laminaria japonica* Aresch (seaweed) using ethanol-modified subcritical 1,1,1,2-tetrafluoroethane (R134a), at 56 °C temperature, 170 bar pressure, and 4.73% co-solvent amount. R134 has critical properties at 100.9 °C (374.1 K), and 40.6 bar (4.06 Mpa), and a permanent dipole moment of 2.05 D, which can be used as an alternative to supercritical CO_2 for the extraction of more polar components. Additionally, high volatility and boiling point at −246.8 K (-550 °C), allows the target material to be easily separated via depressurization of the solvent.

The extraction using supercritical CO_2 usually requires a drying and grinding process. This can be eliminated by using water-miscible solvents, such as dimethyl ether (subcritical), which can eliminate the needs of sample dehydration, cell disruption and solvent evaporation (Goto, Kanda, Wahyudiono, & Machmudah, 2015). Singh, Ahmad, et al. (2015) reviewed the various conventional and non-conventional extraction methods of carotenoids, and concluded that among all the extraction methods employed for carotenoids, SC-CO_2 is the best method under optimized conditions, leading to optimum yield with the highest purity, and without using any environmentally toxic solvents.

Most of the analytical procedures comprise isolated sample preparation (extraction) and separation (analysis) techniques. In recent years, significant efforts have been made towards automation, with the online coupling of sample preparation with analytical techniques. More specifically, SFE coupled with chromatographic techniques such as liquid chromatography (LC), gas chromatography (GC) and supercritical fluid chromatography (SFC) are successfully utilized for simultaneous extraction and analysis of phytoconstituents (Pan, Zhang, Zhang, & Li, 2014; Pól, Hyötyläinen, Ranta-Aho, & Riekkola, 2004). On-line coupling of extraction and separation techniques offers several advantages, including improved sensitivity, reliability and repeatability, minimization of operation time, cost and solvent consumption (Sánchez-Camargo, Parada-Alfonso, Ibáñez, & Cifuentes, 2016). Pól et al. (2004) employed SFE coupled high-performance liquid chromatography (HPLC) for analysis of lycopene from fresh fruits and processed products including tomato, grapefruit, guava, watermelon, tomato paste and rosehip paste. Authors obtained 100% recovery of lycopene from the studied samples in a short time (35 min.) using optimized conditions of SFE (90 °C temperature, 400 bar pressure and 1.5 ml min^{-1} CO_2 flow rate) and HPLC (single monolithic column, 90% acetonitrile and 10% methyl-tert-butyl ether as mobile phase with a flow rate of 1 ml min^{-1}). In addition to the high recovery, atmospheric oxygen and UV light-mediated isomerization of lycopene (*trans*- to *cis*-) were avoided, as the whole analysis takes place in a closed system. The online coupling of SFE with LC offers several advantages for fast and accurate analysis of carotenoids and other thermo-liable metabolites. However, only limited studies are available in these aspects. In future, the online coupling of SFE with analytical techniques can be optimized for precise analyses of carotenoids from diverse samples.

6.8. Enzyme-assisted extraction (EAE)

Enzyme-assisted extraction (EAE) methods utilize hydrolytic enzymes to break down the structural integrity of cell walls to expose intracellular materials for improved extraction yield. EAE of carotenoids from plant products appears to have a high potential, and is promising for commercial applications (Sowbhagya & Chitra, 2010).

Cellulase and pectinase enzymes are generally employed in a pre-treatment step before solvent extraction. Cellulase hydrolyzes the 1,4-β-d-glycosidic linkages of the cellulose present in the primary cell wall of plant cells. Similarly, pectinase breaks down the pectic substances and pectin found in the middle lamella and primary cell walls (Strati et al., 2015). From tomato waste, a 6-fold and 10-fold higher yield of total carotenoid and lycopene were obtained in samples treated with cellulase and pectinase (70 and 122.5 U/g, respectively), followed by extraction using ethyl lactate (solvent to sample ratio of 10:1, v/w), compared to non-enzyme-treated samples (Strati et al., 2015). The cellulase was found more efficient than pectinase for the enzymatic treatment to obtain a higher yield of lycopene, possibly due to the high contents of polysaccharides (cellulose, hemicelluloses, etc.,) in tomato fruits. In the samples extracted after enzyme pre-treatment or ASE, total carotenoids and lycopene yield was higher in samples extracted with ethyl lactate, compared to acetone, ethanol, ethyl lactate/hexane and ethyl acetate/hexane. Ethyl lactate is a water miscible solvent that penetrates efficiently into the wet material, thus extracting higher amounts of lycopene. For the cell wall disruption and extraction of astaxanthin from the microalgae *Haematococcus pluviali,* Machado et al. (2016) studied the effectiveness of three different enzymatic formulations: i) Driselase® containing β-1,3-glucanase and xylanase from the fungus *Basidiomycetes sp*; ii) Glucanex® containing β-1,3-glucanase and protease from the fungus *Trichodermaharzianum*); and iii) Lyticase® containing β-1,3-glucanase and protease from the bacteria *Arthrobacter luteus*. The highest extractability of astaxanthin (83.90%) was obtained using Glucanex®, assisted by ultrasound treatment, without freezing of *H. pluvialis* biomass.

6.9. Carotenoid extraction using green solvents

The solvents utilized in the extraction processes are generally obtained from non-renewable resources. These solvents are highly flammable, volatile and often toxic, causing environmental pollution and the greenhouse effect. Therefore, industries are moving towards more environmentally friendly green solvents, commonly produced from renewable resources of biomass feedstock (e.g., wood, starch, fruits and vegetable oils) or from petrochemical products that are non-toxic and biodegradable (Yara-Varón et al., 2016). Yara-Varón et al., 2016 evaluated five green solvents, cyclopentyl methyl ether (CPME), dimethyl carbonate (DMC), ethyl acetate (EA), isopropyl alcohol (IPA), and 2-methyltetrahydrofuran (2-MeTHF), for the substitution of *n*-hexane in the extraction of carotenoids from dehydrated carrots. The solvents were selected on the basis of the statistical thermodynamics approach to predict solute-solvent solubility using Hansen Solubility Parameters (HSPs) and Conductor-like Screening Model for Realistic Solvation (COSMO-RS). In accordance with COSMO-RS predictions, the highest yield of carotenoids was obtained with CPME and 2-MeTHF using the conventional solid–liquid extraction by maceration. Razi Parjikolaei, Bahij El-Houri, Fretté, and Christensen (2015) obtained 80% and 60% recovery of astaxanthin from shrimp (*Pandalus borealis*) processing waste using methyl ester of sunflower oil and sunflower oil, respectively, as the green extraction solvents. Additionally, extraction temperatures of 70 °C, a solvent/waste ratio of 9 ml/g, stirrer speed of 400 rpm, sample particle size of 0.6 mm, and moisture content of 86.8%, were found optimum to obtain the highest yield of astaxanthin. The authors suggested that the carotenoid-saturated vegetable oil product could be used directly in the diet.

Recently, Goula, Ververi, Adamopoulou, and Kaderides (2017) developed a process for ultrasound-assisted extraction of carotenoids from pomegranate peels (a by-product of fruit juice industry) using vegetable oils (sunflower oil and soy oil) as solvents. The most efficient extraction was achieved using sunflower oil (applied as a substitute to organic solvents) with the extraction time of 30 min, 51.5 °C temperature, solvent/peels ratio of 10 ml g^{-1} and the amplitude level of 58.8. The ultrasound helps in the improved diffusivity of carotenoids which is the major limitation for vegetable oil mediated extraction of carotenoids owing to the higher viscosity. The carotenoid extractions using vegetable oils offers clear advantages for food application over traditional ones (solvents based), as the carotenoids rich vegetable oil fraction can be directly used in food formulations. However, substantial economic studies are not available for vegetable oil assisted carotenoid extraction at an industrial scale. The cost of manufacturing (economic feasibility) of various natural carotenoids using green extraction methods should be investigated under optimized conditions, to identify the most viable techniques for industries.

In recent years, ionic liquids (ILs) were also successfully utilized for green extraction of carotenoids. ILs are non-flammable, non-volatile versatile salts composed of loosely held anions (imidazolium, pyridinium, and ammonium) and cations, such as Br^-, Cl^-, tetrafluoroborate ($BF4^-$) and methylsulfate (MS^-). Most ILs are environmentally friendly and can be designed as task specific by selecting the cationic or the anionic constituents. Ionic liquids have been widely studied for the extraction of bioactive compounds, including carotenoids (Grosso et al., 2015). In a recent study, Desai, Streefland, Wijffels, and Eppink (2016) exploited ILs as a promising pre-treatment step to the *Haematococcal* cyst cell at mild temperatures (45 °C) for efficient extraction of astaxanthin. The authors hypothesize that the ILs helps in permeabilisation of the cell wall by disrupting the hydrogen bond network of cellulose. Among the several classes of the ionic liquids tested, imidazolium-based ionic liquids were found to be most efficient to extract astaxanthin, compared to phosphonium and ammonium based ILs. Bi, Tian, Zhou, and Row (2010) investigated the comparative efficiency of seven task-specific imidazolium-based ionic liquids with combinations of diverse cations and anions for extraction of astaxanthin from shrimp waste. It was found that ethanol containing 1-*n*-butyl-3-methylimidazolium ILs (0.50 mol L^{-1}) with Br− was found most efficient for the extraction of astaxanthin, compared to Br^-, Cl^-, $BF4^-$ and MS^-. The authors also demonstrated that the strong dissolving power of ILs and their charged environment protect astaxanthin from oxidation. The exploration of ILs for extraction of carotenoids has shown great promise in improving yield and selectivity. However, only a few reports are available regarding these aspects. Thus, in future, extraction utilizing ILs with combinations of different anions and cations can be investigated for extraction of various carotenoids from diverse sources. Also, chemical properties, cytotoxicity and environmental impact of ILs should be examined to make appropriate safety recommendations.

The ripening fruit generally contains a high proportion of esterified xanthophylls and lipids. Similarly, green leafy vegetables contain a significant amount of chlorophylls which may interfere during chromatographic analysis. Thus, these chlorophylls and lipids are removed by saponification. Additionally, this step helps in the release and removal of the lipid molecules from carotenoids, thus aiding in a clean preparation for analysis, improved chromatographic separation and accurate identification of carotenoids. The identification of esterified xanthophyll is a difficult task, as a single xanthophyll can be esterified with numerous different fatty acids, generating a large number of different compounds with similar UV/Vis spectral characteristics (Mercadante, Rodrigues, Petry, & Mariutti, 2016), eluting at different retention times in Liquid Chromatography (LC) analysis. In the upcoming section, we have discussed the primary saponification parameters, which influence the extraction yield of carotenoids.

7. Saponification

Carotenes are found in the free form, whereas xanthophyll can be found in either the free or more stable fatty acid esterified forms. In most fruits and flowers, xanthophylls are acylated with saturated and unsaturated fatty acids like lauric ($C_{12:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1n9c}$), linoleic ($C_{18:2n6c}$) or α-linolenic ($C_{18:3n3}$) acids (Mercadante et al., 2016). The esterified xanthophylls

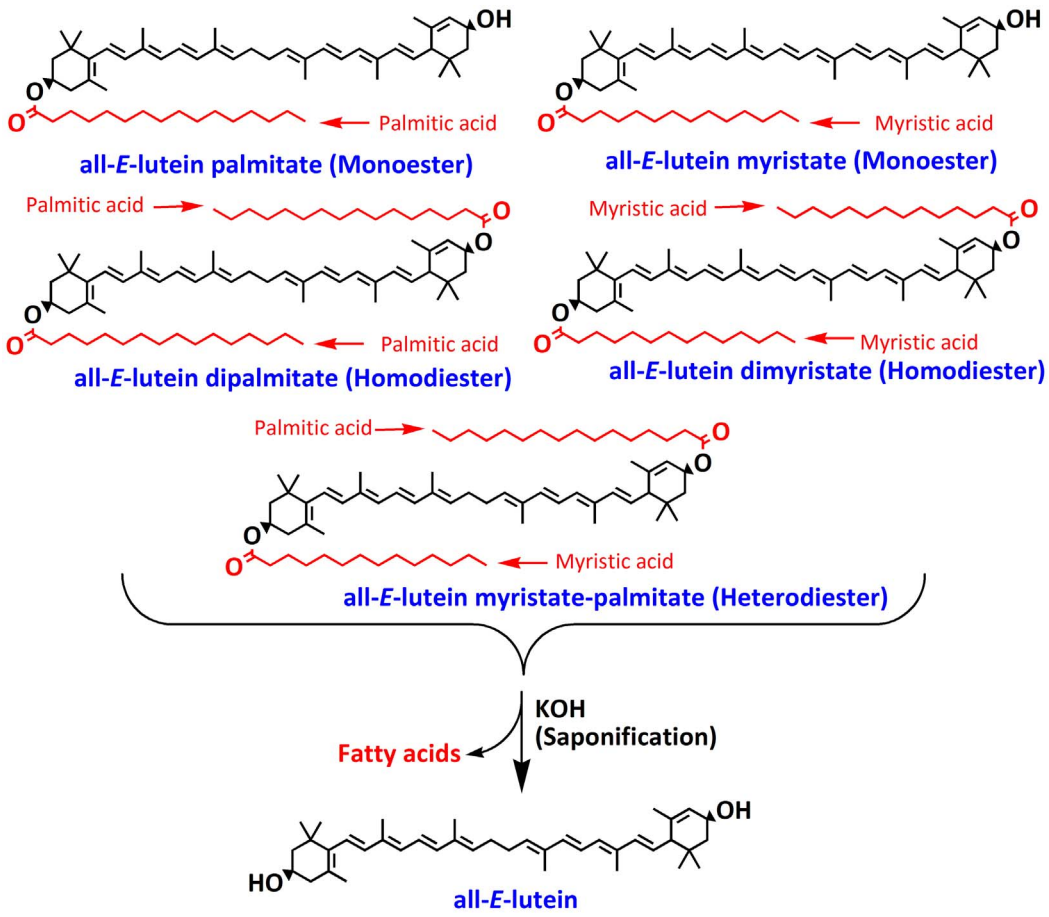

Fig. 3. Representative chemical structures of lutein monoester and diester (homodiester and hetrodiester). The successive removal of fatty acid during saponification is also shown.

can exist as either monoesters or diesters. The esterification of the same fatty acid on both sides of the xanthophyll molecule gives rise to a homodiester, whereas the esterification with two different kinds of fatty acids forms a heterodiester (Petry & Mercadante, 2016) (Fig. 3). During the extraction, these esterified xanthophylls, along with chlorophyll and lipids, are generally removed by saponification, which may interfere with chromatographic analysis. However, saponification is unnecessary for the samples containing low-lipid materials and are almost free of xanthophyll esters, such as leafy vegetables. Saponification is also a source of quantitative losses, artifact formation, and degradation of carotenoids. Mostly, saponification is carried out as a separate step after the extraction of carotenoids, as the simultaneous process can reduce the yield. Inbaraj et al. (2008) reported that simultaneous extraction and saponification was not advisable for the extraction of free carotenoids in *L. barbarum* fruits. Thus, the extraction and saponification steps should be carried out separately. The carotenoid esters were completely saponified after 6 h incubation with 2% methanolic KOH (w/v), and further prolonging the saponification time (8 h) caused the degradation of carotenoids.

The magnitude of qualitative and quantitative losses of carotenoids during saponification depends on the conditions used, such as temperature, hydrolysis time, potassium hydroxide (KOH) concentration and volume for partition and washing (Granado, Olmedilla, Gil-Martinez, & Blanco, 2001). A large number studies have been conducted to optimize these conditions to reduce the time and cost, and to improve the carotenoid extraction. Both hot saponification (56 °C, 20 min) and cold saponification (25 °C, 16 h) methods have been recommended for extraction of free carotenoids (Inbaraj et al., 2008). However, both the methods have their advantages and disadvantages. For example, the high temperature in hot saponification causes isomerization and degradation of carotenoids, whereas cold saponification is time-consuming. Granado et al. (2001) developed the rapid centrifugation protocol to hydrolyze food extracts, using small sample volumes (0.5 ml) in excess of KOH (final concentration 20%), and high-speed mixing (overtaxing, 3–5 min, room temperature) to speed up the reaction, followed by quick partitioning in hexane and/or hexane: methylene chloride (5: 1 v/v). They concluded that the procedure is a feasible alternative to hydrolyze > 95% of the carotenoid esters present in the samples studied (Certified Reference Material 485), in a very short time of 3–5 min without loss of accuracy and precision. In addition to the treatment time, the concentration of KOH or NaOH is also very crucial in saponification of carotenoids. Cerón et al. (2008) studied the carotenoid extraction method from the microalgae *Scenedesmus almeriensis,* and established that 2% KOH is not sufficient for complete saponification. The highest recovery of carotenoids was obtained with 4% KOH in MeOH. In the study by Divya, Puthusseri, and Neelwarne (2012), 20 to 30% of β-carotene and 50% of xanthophylls (neoxanthin, violaxanthin and lutein), were lost after saponification with 10% KOH. In contrast, no significant differences were observed for the contents of carotenoid in saponified and nonsaponified samples of orange-coloured Chinese cabbage (Watanabe, Musumi, & Ayugase, 2011). For the extraction of carotenoids from avian integument, the best results were obtained with 1.1% KOH, incubated overnight at room temperature. Higher or lower KOH concentrations (0.11% and 5%, respectively) were found to decrease the yield of free carotenoids. In contrast, for the extraction of carotenoids from the microalgae *Scenedesmus obliquus*, saponification with 2.5% (w/v) KOH for 40 min at 40 °C in a hot water bath, resulted in a nearly 2-fold increase in the extracted lutein content, compared with the nonsaponified sample (Chan et al., 2013). Further increase in KOH, from 2.5% to 80%, did not significantly affect the extracted lutein content. Also, prolonged overnight incubation at 4 °C was not beneficial in improving the contents of free lutein, indicating

that the previous step of the hot water bath (40 °C for 40 min) is sufficient to provide the required energy for complete saponification.

In progression to the conventional saponification procedures, Larsen and Christensen (2005) used basic resin (Ambersep 900 OH) for selective removal of chlorophylls and fatty acids esters from the acetone extract of commonly used vegetables, including beans, broccoli, green bell pepper, chive, lettuce, parsley, peas and spinach. The hydroxide ion ($-OH^-$) coordinates to the resin, reacts with the carboxylic ester (electrophilic C of the ester C=O) of the chlorophyll molecule in a nucleophilic substitution reaction (ester hydrolysis), thus immobilizing the chlorophyll as a salt to the basic resin, and releasing phytol. A similar hydrolysis reaction occurs with any other carboxylic ester, such as the triglycerides of fatty acids and carotenoid esters. The authors revealed that this method of saponification is only suitable for samples lacking carotenoid esters, such as green vegetables, since a mixture of hexane-acetone is required for complete extraction of carotenoid esters, and hexane and other solvents are not compatible with this procedure.

8. Conclusion

The characteristics of the food matrix (e.g., moisture content, the presence of rigid cell wall and carotenoid composition) and the extraction parameters (e.g., solvent, pressure and temperature) are the most vital factors influencing the efficient extraction of carotenoids. The sample pre-treatment with physical and chemical approaches, such as cooking, cryogenic grinding, osmotic shock, microemulsion with surfactants, bead-beating and high-pressure homogenization (HPH) is effective in releasing the carotenoids from a complex matrix, thus significantly improving the extraction yield. The choice of solvent is the most critical factor for efficient extraction of carotenoids, and mainly depends on the carotenoid composition of the food. In general, acetone and hexane are frequently selected for extraction of xanthophyll (polar), and carotenes (nonpolar), respectively. On the other hand, a mixture of acetone/ethanol/hexane is most frequently applied for the simultaneous extraction of polar and nonpolar carotenoids. Moreover, the water miscible properties of acetone and ethanol helps in the efficient extraction of carotenoids from wet tissue. A large number of studies have been conducted to evaluate the efficiency of various solvents for the extraction of various carotenoids from diverse samples of plant, animal and microbial origin. However, no precise recommendations can be made to use a specific solvent or solvent mixture for a particular sample. The use of mathematical modeling (e.g., Hildebrand solubility parameter) to predict the interactions between solute (target compound) and solvent, has shown promising results to select a particular solvent for higher extraction yield of carotenoids. These parameters can be further studied in detail to minimize the efforts required for optimization of solvents for each kind of sample matrix.

Soxhlet extraction is a conventional technique providing the highest recovery of carotenoids. Nevertheless, it's a time consuming process, and also uses significant amounts of solvents, thereby increasing the cost of extraction. SFE using CO_2 as a solvent and ethanol as a co-solvent, has been documented as a superior "green" and non-thermal method for the efficient and selective extraction of carotenoids. However, the process requires improvement to obtain a higher recovery of xanthophylls (polar carotenoids). The ultrasound assisted extraction (UAE), pressurized liquid extraction, pulsed electric field (PEF) extraction and enzyme assisted extraction (EAE), are the other evolving non-thermal methods for the rapid extraction of carotenoids. However, detailed analysis of cost, environmental safety, efficiency and reproducibility of these methods need to be further evaluated. Additionally, automation of these methods can help for large scale industrial applications. The carotenoid extraction using nontoxic solvents offers several advantages over conventional solvents, in terms of environmental safety and also its application in food and cosmetics. Thus, methodological and technological advancements in these fields are desirable.

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Conflict of interest

The authors have declared that there is no conflict of interest.

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