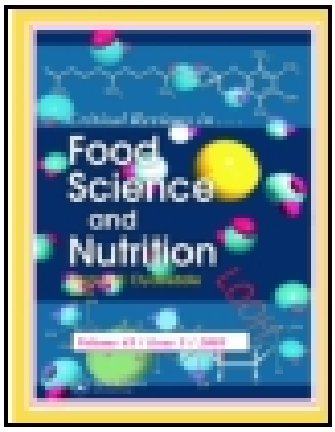

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Natural Origin Lycopene and Its 'Green' Downstream Processing

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Keywords

natural lycopene, downstream processing, food colorants, antioxidants, green processes, eco-friendly processes

Abstact

Lycopene is an abundant natural carotenoid pigment with several biological functions (well-known for its antioxidant properties) which is under intensive investigation in recent years. Lycopene chemistry, its natural distribution, bioavailability, biological significance, and toxicological effects are briefly outlined in the first part of this review. The second, major part, deals with various modern downstream processing techniques, which are assessed in order to identify promising approaches for the recovery of lycopene and of similar lipophilic compounds. Natural lycopene is synthesized in plants and by microorganisms, with main representatives of these two categories (for industrial production) tomato and its by-products and the fungus

Blakeslea trispora, respectively. Currently, there is a great deal of effort to develop efficient downstream processing for large scale production of natural-origin lycopene, with trends strongly indicating the necessity for ñgreenò and mild extraction conditions. In this review, emphasis is placed on final product safety and ecofriendly processing, which are expected to totally dominate in the field of natural-origin lycopene extraction and purification.

1. Introduction

Color is an important characteristic of everyday life, giving an appealing look to final products, also playing an important role in consumersô food choice. In the food industry, colorants are used for various reasons; i.e. to enhance an already existing color, to restore original appearance after processing, to ensure uniformity and to give color to otherwise uncolored food. In general, food colorants are divided into two groups, i.e. natural and synthetic (Toprak Aktas and Yildiz, 2011). Today, natural colorants are globally favored due to intense consumer demand for óall naturalô products (Toprak Aktas and Yildiz, 2011; Papaioannou and Liakopoulou-Kyriakides, 2012). The advantages of using natural colorants are manifold as they are ecofriendly, safe for humans, and harmonised with nature (Brian, 1998), obtained from renewable sources without chemical reactions. Furthermore, the use of natural colorants creates no significant disposal problems (Kumar and Sinha, 2004). The ordinary natural colorants sometimes function as a health cure like rathanjot, turmeric and annatto etc (Kumar and Sinha, 2004). Rathanjot (Khatoon and Shome, 1993), a red dye from the roots of various *Boraginaceous* plant species, is not only used for colouring food stuffs and oils but also as medicine to treat eye diseases, bronchitis, abdominal pains, anthelmintic, antipyretic and antiseptic properties. Yellow dye from rhizomes of turmeric (Shah, 1997) is traditionally used in medicine as an anti-inflammatory drug. Similarly, the dye obtained from annatto (Kanjilal and Singh, 1995) is used in traditional medicine as a purgative, astringent, antileprotic, antiemetic and to treat blood and kidney diseases. Natural origin food pigments do not need to be certified and can be listed in

food ingredients simply as ñcolorantsò (Çinar, 2004). However, the main drawbacks of natural colorants, that have to be overcome for their widspread usage, are their reduced stability and higher cost compared to synthetic ones.

Highly conjugated systems that absorb electromagnetic radiation at wavelengths from 400 to 800 nm appear to be colored (Brian, 1998). Carotenoids are brightly colored natural pigments in which the highly conjugated ´-electron system confers different colors like yellow, orange and red, especially in fruits and vegetables. Aside from plants, carotenoids are also biosynthesized by all photosynthetic bacteria, cyanobacteria, algae, and by some non-photosynthetic bacteria, fungi, and yeasts (Namitha and Negi, 2010; Jaswir et al., 2011). The restrictions on several certified food synthetic colors have stimulated the interest in commercial production and storage stability of these natural pigments. Moreover, they have attracted researchersô attention due to their commercially desirable properties, such as their natural origin, null toxicity and high versatility, providing both lipo- and hydro-soluble colorants (Çinar, 2004).

Carotenoids act in nature as photosensitisers in conjunction with chlorophyll, also protecting the plant cells acting as antioxidants by deactivating the singlet oxygen species produced when exposed to sunlight. Moreover, the carotenoids, either in isolation or jointly with other natural pigments such as chlorophylls and anthocyanins, are mainly responsible for many foods color (Choksi and Joshi, 2007). The carotenoids total production in nature has been estimated to be about 100 million tons annually (Zollinger, 1991), with more than 700 different carotenoids isolated and identified from natural sources so far (Jaswir et al., 2011; Britton et al., 2004; Isler, 1971; Ernst 2002). The characteristic color of many birds, insects, and marine invertebrates is due to the presence of carotenoids in their diet. Animals are not able to

synthesize carotenoids *de novo*. Commercially, carotenoids are used as colorants in food, in nutritional supplements, as feed additives (e.g. enhancing the pigmentation of fish, eggs etc), in pharmaceutical products (as antioxidants), and in the cosmetics industry (Namitha and Negi, 2010; Jaswir et al., 2011; Ishida and Bartley, 2005; Ishida and Chapman, 2009).

Of the more than 700 naturally occurring carotenoids, as many as 50 are present in the human diet that can be absorbed and metabolized, with only six (Ŭ-carotene, ɓ-carotene, lycopene, ɓ-cryptoxanthin, lutein and zeaxanthin; **Figure 1**) representing more than 95% of total blood carotenoids in people from different countries, associated with some health benefits (Maiani et al., 2009). Lycopene contributes to between 21% and 43% of total blood carotenoids (Choksi and Joshi, 2007). Lycopene (ɣ, ɣ-carotene, $C_{40}H_{56}$), a red lipophilic carotenoid hydrocarbon pigment, is a symmetrical tetraterpene assembled from 8 isoprene units (**Figure 1** iii).

Lycopene was first discovered in tomato by Millardet in 1876, as a red colored pigment and it was named later by Schunck ñlycopeneò (Vogele, 1937) from the scientific name of tomato ñ*Lycopersicon esculentum*ò. *In vitro* and *in vivo* studies have shown that lycopene has a beneficial role in chronic diseases such as cardiovascular disease, atherosclerosis, cancer (Abdalla et al., 2007; Montesano et al., 2008; Tucker, 2003) and neurodegenerative disorders, with few studies exhibiting contrasting outcomes (Abdalla et al., 2007; Tucker, 2003). Lycopene is in high demand not only by pharmaceutical companies but also by the food, feed, and cosmetics industries (Borguini and Torres, 2009). The USA Food and Drug Administration (FDA) has approved lycopene as *Generally Recognized as Safe (GRAS)* (Amarowicz, 2011). Today lycopene is available in different forms such as tablets, capsules, syrups and granulated

powders in multivitamin formulations (Kaur et al., 2011). Total world consumption of lycopene has tripled to 15,000 tonnes in 2004 (Choudhari and Singhal, 2008) compared to 5,000 tonnes in 1995. At present, commercial lycopene is available as standardized tomato extract or from chemical synthesis; there are also ongoing research efforts for its biotechnological production in large scale (Choksi and Joshi, 2007). The commercially available natural-origin product, however, is still very expensive and current production from whole tomato fruits is relatively small compared to the future demand projections. Thus, alternative sources for the production of natural lycopene (both of plant and microbial origin) are in great demand; in parallel, considerable attention is given to the development of efficient and cost-effective processes for the isolation and purification of such products (Bao et al., 2010). The lycopene isolation and purification steps followed usually comprise the main part of the high production cost, as is the case with the purification of many biotechnological products.

2. Lycopene physicochemical properties

Chemically, lycopene is an acyclic polyene tetraterpenic hydrocarbon with 13 carbon-carbon double bonds, 11 of which are conjugated in a linear array, thus rendering it the longer conjugated molecule among other natural carotenoids (Figure 1 iii). The extended conjugated double-bond system of carotenoids is an important feature of these compounds that constitutes the light absorbing chromophore; the greater the number of conjugated double bonds, the higher the observed wavelength value for maximum absorption (Kong et al., 2010). The existence of visible color in these compounds requires at least seven conjugated double bonds. The acyclic structure confers to lycopene no vitamin A activity (Preedy and Watson, 2008).

Lycopene may be expected to undergo two changes during processing and storage; i.e. isomerization from all-trans to mono-cis or poly-cis isomers and oxidation. The all-trans lycopene is the predominant geometrical isomer in fruits and vegetables (94-96% of total lycopene in red tomato fruits) and the most thermodynamically stable form. Seven out of the eleven lycopene double bonds could be isomerized to mono-cis or poly-cis different isomers due to exposure to high temperature, light, oxygen, acids, catalysts and metal ions (Cu^{2+}, Fe^{3+}) (Shi and Le Maguer, 2000).

All-trans-lycopene is a *lipophilic* compound with hydrophobic characteristics due to its acyclic structure that make it soluble in organic solvents such as chloroform, hexane, benzene, dichloromethane, acetone and petroleum ether (Preedy and Watson, 2008), whereas it is considered insoluble in water and slightly soluble in lower alcohols (e.g. methanol, ethanol) (Shi and Le Maguer, 2000). In aqueous systems, lycopene tends to aggregate and precipitate as crystals. In ripe tomato fruits, lycopene exists as elongated, needle-like crystals (Schweiggert et al., 2012) into chromoplast or deeply embedded into its composite polysaccharides membrane structure. Pure lycopene crystals are unstable when exposed to oxygen and light. To assure adequate stability, lycopene is stored under nitrogen or other inert gas in light-proof containers. cis-Lycopene isomers have distinct physical characteristics and chemical behavior compared to their all-trans counterpart, including decreased color intensity, lower melting points, greater polarity, lesser tendency for crystallization, and greater solubility in oil and hydrocarbon solvents (Preedy and Watson, 2008).

Lycopene, with its acyclic structure, large array of conjugated double bonds, and extreme hydrophobicity, exhibits many unique and distinct biological properties, with more important its

antioxidant function. Lycopene is among the most efficient singlet oxygen quenchers of the natural carotenoids. There are considerable differences in the quenching rate constants (K_q) for various carotenoid species (Edge et al., 1997). Comparison of the structures of lycopene, ↄ-carotene, ɓ-carotene reveals that the opening of the ɓ-ionone ring increases its quenching ability, mainly due to the higher number of linearly arrayed conjugated double bonds (Papaioannou et al., 2011). The antioxidant activities of lycopene and other carotenoids are highlighted by their singlet oxygen quenching properties and their ability to trap peroxyl radicals (Choe and Min, 2009). Taking into consideration an average concentration of lycopene in human plasma (0.7 mM), it is worth emphasizing the high $^1O_2$-quenching potency of this carotenoid (Kong et al., 2010). Lycopene lies parallel to cell membrane surface within the lipid bilayer. Thus, it is expected to be a poor hydrophilic antioxidant due to its limited interaction with aqueous phase radicals in the lipid bilayer, compared to more polar carotenoids such as zeaxanthin (**Figure 2**). However, the role of lycopene as a lipid phase antioxidant should not be overlooked. The combinations of lycopene and other antioxidants has been proven to lead to higher scavenging activity on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical than their individual antioxidant activities (Kong et al., 2010; Papaioannou et al., 2011), indicating a synergistic action among them.

3. Lycopene bioavailability, health benefits and toxicity

Intestinal absorption of dietary carotenoids with respect to the food matrix (Schweiggert et al., 2012; Yonekura and Nagao, 2007), dietary components, and carotenoid species, has been recently reviewed by Yonekura and Nagao (Yonekura and Nagao, 2007). In humans, lycopene

absorption from the diet is in the range of 10ï 30% of the intake amount, with the remaining being excreted. Daily lycopene intake is usually evaluated on the basis of food taken by a general society (its predominant source being tomato and tomato-based products) with an average daily human intake in the range 0.7 to 25.2 mg/day (Kaur et al., 2011). In a British study, the daily consumption of lycopene-rich food was equivalent to a lycopene intake of about 1.1 mg/day (Ajila et al., 2007) during summer and fall, which is significantly higher than in winter and spring. However, no significant seasonal variation was observed in lycopene serum levels. In another study from the United States, a higher daily lycopene intake of about 3.7 mg/day was reported, whereas a lower intake was determined in the Finnish Mobile Clinic Health Examination Survey with a mean intake of 0.7 mg/day for men and 0.9 mg/day for women (Abdalla et al., 2007). The mean human plasma lycopene levels vary in the range 0.22 to 1.06 nmol/mL (Brian, 1998). The tissue lycopene levels vary from 1 nmol/g wet adipose tissue up to 20 nmol/ g wet adrenals and testes. It has been suggested that an intake of 5-10 mg/ day would be required to provide a reasonable plasma level of lycopene (Tucker, 2003), whereas the therapeutic dosage of lycopene is proposed to be in the range of 6-60 mg daily (Kaur et al., 2011).

Many biological and lifestyle factors influence the absorption of dietary lycopene including age, gender, hormonal status, body mass and composition, blood lipid levels, smoking, alcohol, and the presence of other carotenoids in the food. Fasting serum lycopene levels were found to be higher and more reproducible than postprandial levels indicating that diet induced metabolic stress. Exposing fresh plasma under *in vitro* conditions to cigarette smoke resulted in the depletion of the lycopene and other lipid-soluble antioxidants. Alcohol consumption was also

shown to alter serum lycopene levels. Other factors that influence the bioavailability of lycopene are its release from the food matrix due to processing (Anese et al., 2013; Colle et al., 2013; Page et al., 2012), presence of dietary lipids (Garrido et al. 2012; Page et al., 2012), and heat-induced isomerization from the all-trans to cis conformation (Page et al., 2012). These factors enhance lycopene body absorption. Only about 5% of the carotenoids in whole, raw vegetables, for example, are absorbed by the intestine, whereas 50% or more of the carotenoids are absorbed from micellar solutions. Thus, the physical form in which the carotenoid is presented to intestinal mucosal cells is of crucial importance (Olson, 1994). Ingestion of cooked tomato juice in oil medium increased serum lycopene levels threefold, whereas consumption of an equal amount of unprocessed juice did not have any effect. It is generally believed that conversion of all-trans lycopene to its cis isomeric form enhances its absorption. The presence of ß-carotene was shown to increase the absorption of lycopene in some studies, while the presence of canthaxanthin appears to decrease lycopene absorption. Although the serum lycopene levels increased in a dose-dependent manner with dietary intake, biomarkers of lipid and protein oxidation did not differ significantly between the treatments. The absorption effciency of lycopene was observed to be greater at the lower levels of dietary intake (Olson, 1994).

Ingested lycopene is incorporated into dietary lipid micelles and chylomicrons, absorbed into the intestinal mucosal lining via passive diffusion, and released afterwards into the lymphatic system. Bile salts play an essential role in the absorption of carotenoids, and their concentration must be above their critical micelle concentration (Olson, 1994). Lycopene is transported by plasma for distribution to the different organs. Owing to the lipophilic nature of lycopene, it was found to concentrate in the LDL and very low density lipoprotein (VLDL)

fractions and not in the high density lipoprotein (HDL) fraction of the serum (Rao et al., 2006; Shi and Le Maguer, 2000). The incorporated into lipoproteins lycopene is decreasing the oxidation of cholesterol, thereby helping to prevent vascular damage (Ishida and Bartley, 2005). Lycopene in the blood has been shown to be inversely proportional to the incidence of prostate tumors. Research also shows that carotenoids may provide protection against damage from ultraviolet radiation from sunlight, the major cause of sunburn, photodamage, and nonmelanoma skin cancer (Ishida and Bartley, 2005).

In order to improve the delivery of bioactive compounds into foods several microencapsulation technologies have been developed in the food industry and shown promising results for the production of functional foods (Champagne and Fustier, 2007). These technologies could promote the successful delivery of bioactive ingredients to specific parts of gastrointestinal tract, safely from stomach acid degradation or isomerization. Future research is likely to focus on aspects of co-encapsulation methodologies, where two or more bioactive ingredients can be combined to have a synergistic effect (Champagne and Fustier, 2007).

Lycopene half-life in human plasma is about 2ï 3 days, present as an isomeric mixture containing 50% of the total lycopene as cis-isomer. When animals were fed lycopene containing predominantly the all-trans isomeric form, serum and tissue lycopene showed the presence of cis lycopene (Ishida and Bartley, 2005; Rao et al., 2006). There are also some indications of *in vivo* trans to cis isomerization reactions. Very little is known about the *in vivo* metabolism of lycopene. Recent data suggest that lycopene metabolites may possess specific biological activities on several important cellular signaling pathways and molecular targets. Carotenoid metabolites have been proved to possess more important biological roles than their parent

compounds in human health and disease (Kelkel et al., 2011; Mein et al., 2008). In a recent study, dos Anjos Ferreira *et al*. (2004) used the postmitochondrial fraction of rat intestinal mucosa to study lycopene metabolism. They identified two types of metabolic products of lycopene, i.e. cleavage products and oxidation products. It is possible that similar metabolites of lycopene are also found *in vivo* in the presence of lipoxygenase enzymes. Nagao (2004) on the other hand showed *in vitro* cleavage of lycopene to acycloretinal, acycloretinoic acid, and apolycopenals in a non enzymatic manner. They also showed that these cleaved products of lycopene induced apoptosis of HL-60 human promyelocytic leukemia cells. However, only a few metabolites, such as 5,6-dihydroxy-5,6-dihydro lycopene, have been detected in human plasma (Khachik et al., 2002). It is suggested that lycopene may undergo *in vivo* oxidation to form epoxides, which then may be converted to the polar 5,6-dihydoxy-5,6 dihydro-lycopene through metabolic reduction. Oxidised lycopene (i.e. a mixture of apolycopenones and diapocarotenedials) exhibited as well biological activity (Amarowicz, 2011). A controversy still exists on the biologically active form, i.e. the lycopene or its polar metabolites; thus further research is needed for clarifying this aspect.

Following absorption, lycopene is transported to various organs and accumulates in tissues. Distribution of lycopene in tissues varies considerably, suggesting unique biological effects on some tissues and not on others. The highest concentrations of lycopene are found in the testes, adrenal glands, liver, and prostate. Lycopene and its oxidation products are present in human milk and other body fluids. Human seminal plasma also contains lycopene and its levels were found to be lower in immunoinfertile men compared to normal individuals (Rao et al., 2006).

In addition to being a powerful antioxidant, lycopene is thought to possess some other important biological properties, including induction of apoptosis, inhibition of cell proliferation and increase in gap-junctional communication (Konwarh et al., 2012 ; Rao et al., 2006), which are constantly vouching for its inclusion in functional foods and nutraceuticals.

The recent search for new anti-cancer drugs focuses more on natural compounds from the regular human diet because these compounds rarely exhibit severe side-effects yet efficiently act on a wide range of molecular targets involved in carcinogenesis. One promising compound, which is now being tested in clinical studies, is the tomato-derived lycopene. Lycopene health benefits have been reviewed by Kelkel *et al*. (2011) about its cellular action and the molecular targets responsible for its remarkable chemopreventive and anti-proliferative activity.

Historically, lycopene-containing fruits and vegetables have been part of humansô diet for centuries without any safety problems. Several studies were undertaken to evaluate the safety of both natural and synthetic lycopene (Jonker et al., 2003; Matulka et al., 2004). The intake levels of lycopene were ingested in the form of tomato juice, tomato sauce, and nutritional supplement from 5 to 75 mg/day in healthy human subjects. In these studies no adverse effects of consuming lycopene were observed. In another study (Mellert et al., 2002) two synthetic crystalline lycopene sources, BASF lycopene 10 CWD and Lyco Vit 10%, each containing approximately 10% lycopene were tested in rats. After ingesting the test products for 13 weeks at intake levels of up to 3000 mg/kg body weight/day, no adverse effects were observed. Lycopene derived from fungus *Blakeslea trispora*, suspended in sunflower oil at a concentration of 20% w/w, was tested for subchronic toxicity at concentrations of 0%, 0.25%, 0.50%, and 1.0% in rats for 90 days (Jonker et al., 2003). No evidence of toxicity of lycopene at dietary intake levels up to 1.0% was

observed in this study. The authors (Mellert et al., 2002) suggest the non observed- effect level (NOEL) for this lycopene to be 1.0%, i.e. the highest dietary tested concentration. McClain and Bausch (2003) published a summary of additional safety studies using synthetic lycopene. No teratogenic effects were observed in the two-generation rat study. The deposition of lycopene in plasma, liver, and other tissues also had no adverse effects. In case of prolonged and excessive intake of tomato juices, the skin and liver becomes colored orange yellow and the lycopene level in the blood is elevated. After two or three weeks on a lycopene free diet, the skin color returned to its normal color. The skin coloration is known as lycopenodermia and is non toxic. The human data of Highest Observed Intake (HOI) for lycopene led to identification of a 75 mg/d, but the animal data risk assessment indicated that 270 mg/d should be safe for human adults (Hathcock and Kriengsinyos, 2011). However, tomato and tomato based products may be acidic and irritate stomach ulcers (Haber and Lu, 2002). No other adverse effects were associated with the skin coloration. In consideration of these studies establishing the safety of lycopene for human consumption, the USA FDA granted GRAS status to lycopene as a nutritional supplement (Rao et al., 2006).

4. Synthetic lycopene

Different chemical synthetic pathways for lycopene have been reviewed (Ernst 2002; Shen et al., 2011), but all cases seem to have disadvantages such as low yields, product instability, low product quality and economically unattractive processes (Naviglio et al., 2008; Wang et al. 2011), with sometimes toxic intermediate reagents (Shen et al., 2011).

The chemical synthesis of lycopene from smaller molecules generally involves three steps. In the first phase, a C15 compound is synthesized and then dissolved in methanol. In the second phase, a C10 compound is synthesised in crystalline form. The Wittig reaction has been used as a main shortcut for this purpose; the most representative reagents for lycopene **iii** synthesis based on the available C15 Wittig salts **2** and the C10-triene dialdehyde **3** building blocks are shown in **Figure 3**. The use of costly and toxic reagent triphenyl-phosphine will lead to the difficult post-handling and environmental pollution due to the formation of triphenyl-phosphine oxide as by-product. The Wittig-Horner reaction, as an improved method, in which C15 Wittig-Horner phosphonate was used as the synthon, can resolve the above problems **Figure 3**. In the final phase, these two intermediate compounds are mixed together at a high temperature, in the presence of a catalyst, and lycopene is formed. After cooling, the crude product is filtered out and washed repeatedly with water and methanol. The synthesised material is then further purified by washing with methanol. Following the final filtration, the product is dried using warm nitrogen.

Babler and Harvey (2000) described a four-step route for the conversion of pseudoionone to lycopene, in which allenic phosphonate reagent and allylicphosphonate were employed as two key intermediates instead of triphenyl-phosphine halide salts. But the reaction conditions for the synthesis of allenic phosphonate from 3-propargylic alcohol and dialkyl chlorophosphite was rigorous and subsequent partial hydrogenation of allenic phosphonate was difficult to finish with high selectivity. Shen *et al*. (2011) has recently reported a practical approach for lycopene synthesis by reacting C15-phophonate **2** and the known C10-triene dialdehyde **3**. The key intermediate C15-phophonate **2** could be synthesized in four steps by using 4,4-dimethoxy-3-

methylbutanal **4** as starting material **(Figure 4).** The Wittig-Horner reaction is adopted as an efficient approach to introduce new double bonds under mild reaction conditions. However, this new route for the total synthesis of all-trans-lycopene **iii** has a relatively low total yield of 26.9% (Shen et al., 2011).

5. Natural origin lycopene

Natural origin lycopene is produced by extraction from plant material and by biotechnological means. The extraction methods used today for lycopene isolation from such sources are expensive, resulting from its relatively low concentration in the fruit and from the rather complicated purification process due to the numerous compounds in the raw material. It is noted that marketing of chemically synthesized lycopene is currently not permitted in Europe, even though it has been categorized as ógRASôby US FDA. Moreover, the consumer perception that ñnatural is goodò has been motivating research for the development of efficient methods of natural-lycopene extraction/purification as well as microbial lycopene production. Indeed, biotechnology is currently considered as the most promising approach for natural origin lycopene future production (Kim et al., 2011).

5.1. Plant lycopene

Lycopene imparts red color to gac fruit, tomato, watermelon, apricot, papaya, rosehip, passion fruits, guava, pink grapefruit, persimmon etc. (Bao et al., 2010; Preedy and Watson, 2008; Tarazona-Díaz et al., 2011) (**Table 1**). Tomatoes (especially deep-red fresh tomato fruits) and tomato products (**Table 2**) are considered the most important source of lycopene in many

human diets; therefore, most of the research on lycopene focuses on tomato and tomato-based products (Preedy and Watson, 2008).

Tomatoes are globally among the most widely cultivated vegetable crops, used in the human diet either as raw fruits or as processed products. Tomato lycopene content varies considerably, reflecting the influence of a variety of (generally genetic) factors, maturity, and both agronomic and environmental conditions during plant growth (Kaur et al., 2008). At present, natural lycopene is mostly produced by extraction, concentration and purification from whole tomato fruits that have been grown specifically for this purpose. Concentration of lycopene in tomato vary from 8.8 to 200 mg/kg in fresh fruit and from 430 to 2950 mg/kg on a dry basis (Toprak Aktas and Yildiz, 2011; Ishida and Bartley, 2005; Bao et al., 2010; Rao et al., 2006; Saldaña et al., 2010; Machmudah et al. 2012). Lycopene represents more than 85% of the total tomato carotenoids. The tomato peel contains about five times more lycopene than tomato pulp, on wet basis (Machmudah et al. 2012; Naviglio et al., 2008). Lycopene is found predominantly in the chromoplast of plant tissues. In tomatoes, lycopene biosynthesis increases sharply during the ripening process, as chloroplast undergoes transformation to chromoplast (Ishida and Bartley, 2005). The highly hydrophobic carotenoids are biosynthetized and deposit into these specialized plant plastids (Schweiggert et al., 2012). Low amounts of other carotenoids such as Ŭ-, ɓ-, ɔ-, and Ů-carotenes, phytoene, phytofluene, neurosporene, and lutein are also present in tomatoes (Ishida and Bartley, 2005).

The watermelon is considered as the second important dietary source of lycopene, and is also widely cultivated around the world. There are several hundred watermelon cultivars used commercially, and these are either seeded or seedless (Perkins-Veazie et al., 2006). The lycopene

content of watermelons has been reported to be 11.6-112 ε g/g fresh weight (Tarazona-Díaz et al., 2011; Perkins-Veazie et al., 2001; Perkins-Veazie et al., 2006); moreover, it is the prevailing carotenoid in red-fleshed watermelons (70-90% of total carotenoids), while other carotenoids include phytofluene, phytoene, ϐ-carotene, lutein, neurosporene, and ε-carotene (Perkins-Veazie et al., 2006).

5.2. Plant by-products as natural lycopene source

Million tons of tomatoes are annually processed to produce juices, sauses, purees, pastes, and canned tomatoes, resulting in large amounts of tomato peel, pulp, and seed as industrial waste (called in general *tomato pomace*) that represent 10%ï 40% of the fruit weight (Machmudah et al. 2012; Strati and Oreopoulou, 2011a). A major problem of tomato industry is the accumulation, handling and disposal of processing wastes. Meanwhile, the price of raw materials, such as the whole tomato fruit, is increasing and their availability does not keep pace with the demand. Therefore, the recovery of useful compounds from the wastes is favored around the world, with increasing emphasis for their transformation into useful products; this trend is in accord with the desirable environmental balance and sustainable development (Laufenberg et al., 2003). Food processing by-products from orange, mango, guava, pomegranate, and also vegetables including tomato, and carrot are potential sources of functional food ingredients, which may have preventive effects against numerous diseases and are already intensively researched (Kong et al., 2010).

Tomato pomace is currently disposed of as a solid waste or used as animal feed, but the abundance of lycopene in the peel fraction of these wastes suggests the possibility of utilizing it

as a cheap source of lycopene, which offers at the same time a great advantage in the management of these wastes (Kaur et al., 2008; Strati and Oreopoulou, 2011a). Most of the lycopene is associated with the water-insoluble fraction and the peel (Kaur et al., 2008; Ranveer et al., 2013), but its high moisture level renders it quite problematic due to its susceptibility to microbial proliferation and spoilage during storage and processing. Therefore, peel must be preserved by drying before lycopene extraction or processed as soon as it is produced (Kaur et al., 2008). However, despite such high lycopene content, the available extraction technologies do not seem to allow an efficient lycopene recovery from this by-product. Indeed, lycopene extractability from tomato peels by conventional food-grade organic solvents is extremely low, at least under the conditions that normally preserve the activity of the carotenoid *in vivo*. The main reason is the cellular localization of lycopene into the peels, which is deeply embedded within the chromoplast complex membrane structure (Konwarh et al., 2012; Papaioannou and Karabelas, 2012; Ranveer et al., 2013). In addition, the progressive removal of water during the manufacturing process leads to a partial collapse of the polysaccharide material network, hindering solvent penetration and lycopene extraction. Although these limitations could be partly overcome by using more severe process conditions, the risk for lycopene to undergo degradation would proportionally increase (Papaioannou and Karabelas, 2012).

5.3. Microbial lycopene as natural source

Biotechnological lycopene production has been achieved, but in most cases at bench scale; to the authorsô knowledge, only one approach, that can be scaled up to industrial scale has been reported recently (Lopez-Nieto et al., 2004). Some microorganisms like *Streptomyces*

chrestomyceticus, *Candida utilis*, *Blakeslea trispora*, *Phycomyces blakesleeanus*, *Mucor circinelloides, Mycobacterium aurum* (Wang et al., 2011), *Rhodobacter sphaeroides*, *Rhodospirillum molischianum, Rhodopseudomonas palustris* (Bao et al., 2010) and genetically engineered *Escherichia coli* (Farmer and Liao, 2000), *Pichia pastoris* (Bhataya et al., 2009), and *Flavobacterium* species (Bao et al., 2010; Lopez-Nieto et al., 2004), with significant lycopene producing abilities have been reported. A comprehensive review has been presented by Schmidt-Dannert (2000) on carotenoids biosynthesis, gene combination and molecular breeding of biochemical pathways used to produce novel and rare high-value carotenoids. Important problems remaining to be solved are lycopene production in sufficient quantities for efficient industrial-scale production. Nevertheless, lycopene has been manufactured commercially already by biotechnological means by at least one European company (Vitatene, Leon, Spain) employing fungus *B. trispora* (Kim et al., 2011).

Blakeslea trispora, a microorganism used industrially for ɓ-carotene production, is a heterothallic zygomycete fungus with two sexual mating strains, plus (+) and minus (-). Lycopene, as well as other carotenes, is produced intracellularly by co-fermentation of two non-pathogenic and non-toxigenic strains (Bao et al., 2010; Papaioannou and Liakopoulou-Kyriakides, 2010). Lycopene biosynthesis in *B. trispora* could be stimulated by various accelerants, such as trisporic acid, non-ionic surfactant, abscisic acid, as well as vitamin A and antibiotics, oxygen-vectors, cyclase inhibitors, and ergosterol biosynthesis inhibitors (Shi et al., 2012b).

The maximum lycopene production of separate *B. trispora* strains NRRL 2895 (+) and 2896 (-) has been reported to be only 4.2 and 1.8 mg/L, respectively, whereas the highest

lycopene production of 92.2 mg/L, for the same conditions, was obtained under the best (+) to (-) strain ratio of 5:1 (Wang et al., 2011). However, Lopez et al. (2004) reported that the maximum production of lycopene was achieved using ratios from 1(+)/10(ī) to 1(+)/15(ī) strain ratio of *B. trispora* F-816 (+) and *B. trispora* F-744 (ī). The strains in that study were obtained from the Russian National Collection of Industrial Microorganisms. The difference in strain proportion might be attributed to the fact that the (-) strain was the main producer of carotenoid in such case. The accumulation of ɓ-carotene and ɔ-carotene reached its highest concentration according to this study when using 1(+)/15(ī) (Lopez-Nieto et al., 2004). The use of extreme proportions of both strains, i.e. higher than 1 (+)/15(ī) or lower than 1(+)/5(ī), led to a significant decrease in lycopene, ɓ-carotene, and ɔ-carotene production, probably due to a complete wash-out of one of the strains, avoiding carotenoid stimulation by mated culture (Lopez-Nieto et al., 2004). These results suggest that the best ratio of (+) to (-) strains for lycopene accumulation may be different among various lycopene-producing microorganisms.

Global demand for the development of sustainable processes for the production of chemicals and materials from renewable biomass, rather than from fossil fuel resources, has been increasing. Therefore, microorganisms have been employed for the production of various chemicals and materials, but their efficiencies are rather low when they are isolated from nature. During the past few decades, successful examples that have overcome this obstacle have combined traditionally employed methods of random mutation and selection with metabolic engineering to produce high-value chemicals with good yield (Lee et al., 2011). In this context, there are some attempts to develop a method for enhanced lycopene production by metabolically engineered cells (Bhataya et al., 2009; Jin and Stephanopoulos, 2007; Kim et al., 2009), but the

concentration and productivity of these methods are not currently competitive with those obtained from the carotenogenic fungus (Tereshina et al., 2010). The combination of carotenogenic genes from different organisms into new biosynthetic reaction sequences, and the in vitro evolution of new catalytic functions with visually detectable products have enabled the exploration of engineering strategies for generating chemical diversity. Moreover, many carotenoid enzymes are catalytically promiscuous and accept substrates other than their natural ones, enabling synthesis of even more diverse molecular architectures (Klein-Marcuschamer et al., 2007). In the case of *Escherichia coli* the simultaneous amplification of the identified target genes, 1-deoxyxylulose- 5-phosphate synthase (dxs), isopentenyl diphosphate isomerase (idi) and malate dehydrogenase (mdh) genes, has resulted in a 3.2-fold increase of lycopene content compared with the control strain (Lee et al., 2011). Recently Kim et al. (2011) reported 5.2- and 9.2-fold higher lycopene concentration and productivity than the highest previously reported using metabolically engineered *E. coli* (Kim et al., 2009). However, the highest reported specific lycopene content was 64.9 mg/ g cells in *B. trispora* (Choudhari et al., 2008), that is 2.0-fold higher than that obtained from the metabolically engineered *E. coli* (Kim et al., 2011). *B. trispora* produced 930 mg/L of lycopene over a 120-h period, thus achieving a productivity of 7.8 mg/L/h; these are also the highest reported concentration and productivity of lycopene (Tereshina et al., 2010). The concentration (1,350 mg/L) and productivity (40 mg/L/h) of lycopene observed with the metabolically engineered *E. coli* are 1.5- and 5.1- fold higher, respectively, than those with the carotenogenic fungi, *B. trispora* (Kim et al., 2011).

The increasing cost of energy and raw materials for building complex chemical structures, combined with the environmental concerns associated with conventional

manufacturing, mean that biosynthesis using engineered microbial cells will probably become a preferred route for obtaining valuable chemicals (Klein-Marcuschamer et al., 2007). However, it should be always kept in mind that still, even if one has completely redesigned a metabolic pathway, the problem for environmental release remains that of nesting stably such a route within the existing metabolic network of the host. One key aspect is the background metabolic complexity in which the implanted or rewired metabolic activities are to be implemented (de Lorenzo, 2008).

The biotechnological efforts are supported by the fact that the microorganisms compared with higher plants have the advantage of cultivation with relatively rapid growth rate, under controlled and repeatable conditions and on a large scale, which can provide a continuous and reliable source of lycopene. Moreover, unlike plant cells, in which lycopene is found in relatively low percentage into a complex carotenoid mixture, higher purity lycopene might be available into microorganisms cells cultured into media with appropriate composition (Lopez-Nieto et al., 2004; Choudhari et al., 2008).

6. Main Lycopene extraction processes

6.1 General considerations

Natural products in general could be broadly divided into two main categories of water-soluble (e.g., glycosides, phenolic compounds) and lypophilic, meaning water-insoluble (e.g., oils, carotenoids, lycopene) compounds. Considerable effort has been expended towards developing specific extraction methods for these two distinct categories. Solvent extraction and

supercritical fluid extraction are the two major technologies in use today that will be briefly outlined here and presented in more detail in following sections.

An environmentally friendly lycopene extraction process with minimal loss of its bioactivity is highly desirable. Solubility and stability of lycopene are the two main problems during its extraction. The extraction, storage, handling, and analysis of lycopene have to be carried out under controlled environmental conditions to minimize lycopene losses through oxidation or isomerization (Choksi and Joshi, 2007).

Most extraction methods follow a common path consisting of release of desired compounds from their natural matrices by disrupting the cells, followed by clarification to remove unwanted cellular components, and an initial liquidï liquid or liquidï solid extraction before final purification. The fundamental step for obtaining lycopene from a cell, e.g. from tomatoes, is breaking down the cell. If the breaking is not complete, only partial extraction will be achieved; thus, the number of extraction stages necessary to achieve a high extraction yield is proportional to cell breakage. As it is very difficult to break vegetable cells, especially those of tomatoes, the extraction is currently performed using a sequence of mechanical, physical and chemical actions (Montesano et al., 2008). Because many mechanical processes typically employed to disrupt cells (e.g., cutting, shearing, etc.) contribute to thermal or oxidative degradation of labile, biologically active molecules, alternative, gentler disrupting methods should be evaluated (e.g. enzymatic pretreatment) (Ishida and Chapman, 2012).

Solvent extraction has been intensively used for important natural compounds isolation and for the qualitative and quantitative analysis in various fields such as environmental analysis, food and agricultural analysis, drugs and herbal medicines. Extraction represents the primary

step in getting crude extract from plants. Conventional extraction techniques include soaking, maceration, percolation, Soxhlet extraction, etc (Chan et al., 2011). However, the ordinary extraction process utilized in such cases requires long times, consumes large amounts of organic solvents, uses heat treatment of the extract for solvent removal and may result in solvent residues in the final products (Saldaña et al., 2010; Puri et al., 2012).

An efficient extraction procedure can be achieved by optimizing the extraction conditions such as time, temperature, number of extraction cycles and the amount of extracting solvent (Dehghan-Shoar et al., 2011). The extraction largely depends on the type of solvent and the energy input, e.g. agitation to improve the efficiency of mass transfer. The solvent extraction methods used for bioactive compounds isolation from such materials are widely used because they are well established and easy to perform (Puri et al., 2012). One of the most important considerations in developing new processes and technology for lycopene extraction, and carotennoids in general, is also *the safety issue* of the final products when used as food or nutraceuticals. A critical and demanding step is the selection of appropriate and safe, food grade solvents, to avoid the elimination problems of trace residual solvents (Calvo et al., 2006). The most effective, usually applied solvents are toxic for human consumption, with only a few environmentally friendly solvents being available to extract carotenoids (Strati and Oreopoulou, 2011b). Most nonpolar solvents that have high extraction efficiencies are considered to be toxic (Ishida and Bartley, 2005).

Carotenoids due to their hydrophobic nature are generally extracted from plant and microbial cells, using organic solvents or their mixtures, such as hydrocarbons or chlorinated methane derivatives. However, these methods have some disadvantages; in particular, they are

usually time-consuming and laborious, they may involve rather large amounts of solvent wastage, poisonous residual solvents (chloroform, tetrahydrofuran (THF) and benzene, etc. (Periago et al., 2004) and entail high cost and low efficiency. Therefore, they are not generally considered efficient for large-scale industrial production (Liu et al., 2010). Additionally, there are several regulations to be satisfied in the use of appropriate solvents, if the products are to be used in foods.

On the other hand, *supercritical fluid extraction (SCFE)* commonly uses supercritical carbon dioxide (SC CO_2), which reduces the potential for oxidation of carotenoids and eliminates any harmful solvent residue in the products. In addition, the low critical temperature of CO_2 (31^oC) is beneficial for thermally labile carotenoids, resulting in minimal carotenoid degradation during extraction (Saldaña et al., 2010). However, SCFE equipment is more expensive and sophisticated (Eh and Teoh, 2012).

Over the past few decades, various alternative extraction techniques have been introduced and investigated, which were claimed to be better in terms of efficiency, extraction time and solvent consumption. These techniques include the microwave assisted extractions (MAE), pressurized solvent extraction (PSE) (Chan et al., 2011; Papaioannou and Karabelas, 2011), high pressure processing, ultrasound, electrical methods (Toprak Aktas and Yildiz, 2011), enzyme assisted extraction (Papaioannou and Karabelas, 2011) and surfactant assisted extraction (Papaioannou and Karabelas, 2011; Zhu et al., 2010; Fish, 2006). However, studies conducted via these techniques for lycopene recovery are rather limited. It will be added that ideally, extraction methods should be quantitative and time saving (Puri et al., 2012).

6.2 Solvent extraction

The lycopene extraction from natural origin sources is still commonly implemented via organic solvents, but in general tends to be time consuming and problematic due to oxidation and losses during processing. It is well known that light, heat, acids, etc. promote isomerization of carotenoids to the cis- forms. Oxidative degradation on the other hand, the principal cause of extensive losses of carotenoids, depends on the availability of oxygen and is stimulated by factors such as light (Calvo et al., 2006). The existing procedures for extracting lycopene from vegetal sources that use organic solvents present significant disadvantages: (1) some organic solvents are toxic, and must be completely removed from exhausted material, necessitating a complicated purification step before its disposal; (2) exhausted matrices must be treated as a special residue after extraction and cannot be reused, leading to large volumes of hazardous waste that are costly and must be disposed of under strict environmental guidelines (Fish et al., 2002); (3) organic solvents are not specific for lycopene, but at the same time other pigments or hydrophobic compounds (e.g., carotenes, xanthophylls, fats) present in the original natural material are simultaneously extracted (Naviglio et al., 2008).

Solvents proposed in various US patents for extracting lycopene include hexane, ethyl acetate, dichloromethane, methanol, ethanol, propanol, and acetone (Ishida and Chapman, 2009). The amount and the cost of organic solvents directly influence the total production cost of an acceptable extract/product. Moreover, when the final product is used as a food ingredient, it is absolutely necessary to remove all traces of potentially toxic solvents. Removing solvent from the residue in the various steps of extraction is normally carried out under vacuum filtration at room temperature and then the solvents are re-distilled at low temperature (~35^{o}C). Under these

conditions, the recovery of solvents is usually very high (over 80%) thus allowing cost reduction. Acetone and diethyl ether are volatile solvents, non-toxic and under the above-mentioned conditions are removed completely from the extract (Montesano et al., 2008).

Lycopene and carotenes, as lipophiles, are usually extracted with non-polar lipophilic organic solvents such as dichloromethane, hexane, THF, benzene and chloroform (Periago et al., 2004), most of which are not generally recognized as safe for human consumption (Vaughn Katherine et al., 2008). These solvents, therefore, are not acceptable for food applications. Chlorinated hydrocarbons belong to class I and II solvents of the International Conference of Harmonization (ICH) meaning that they are toxic and should not be used in such processes in addition to the environmental problems that they cause (Papaioannou et al., 2008). Dichloromethane is the most efficient solvent; however, because of its toxicity, it cannot be used for ingestable products. Lycopene extraction by absolute ethanol is 87-88% as effective as dichloromethane, but is more costly and more flammable. In addition, industrial ethanol is traditionally manufactured from ethylene that prohibits its use. Currently, ethyl acetate is the most commonly utilized solvent for extracting carotenoids which are going to be used in food products (Ishida and Chapman, 2009).

On the other hand, since samples of biological origin contain large amounts of water, water-miscible organic solvents, such as ethanol, acetone, etc. have to be used to facilitate lycopene diffusion from its natural matrix (Papaioannou et al., 2008). Furthermore, lycopene is considered to be soluble in acetone, while it is insoluble in water, and slightly soluble in methanol and ethanol (Shi and Le Maguer, 2000). The combination of polar solvents with the non polar hexane seems to enhance the solubilisation of the non polar carotenoids (lycopene),

whereas individual polar solvents (ethanol, acetone and ethyl acetate; Periago et al., 2004; Fish et al., 2002) enhance the solubilisation of the polar xanthophyls, e.g. lutein. This is possibly related to the relative solubility of lutein in ethanol, acetone and ethyl acetate which is 15ï 40 folds higher than that in hexane. Similarly, the relative solubility of ß-carotene in ethanol is 20 times smaller than in hexane (Craft and Soares, 1992). Although acetone and ethanol are both polar solvents, acetone tends to extract more effectively tomato carotenoids than ethanol; setting aside the higher solubility for apolar carotenoids, this is possibly due also to the better penetration of this solvent into plant cells, where carotenoids are enclosed (Strati and Oreopoulou, 2011a). Indeed, in addition to the solubilising capacity, penetration or diffusion of the solvents into the solid matrix apparently plays an important role in extraction efficiency. Acetone alone is a good solvent and a wetting material that penetrates easier in the solid matrix than the hexaneï acetone mixture. Therefore, the yield obtained by the mixture is lower than that of acetone, and almost in the middle between acetone and hexane, indicating a cumulative action and no syergistic effect of these solvents. Similar results were obtained by Lin and Chen (2003) who found that the acetone/hexane mixture was less efficient than a ethyl acetate/hexane mixture in extracting carotenoids from tomato juice, whereas the ethanol/hexane mixture was the most efficient, a fact that can be explained by the high water content of the tomato juice.

The optimum extraction conditions depend on the processing technique used to manufacture the food product. For example, longer extraction times and more extraction cycles are required to efficiently extract lycopene from raw tomato and tomato skin, compared to the tomato paste. In the unprocessed tomato, the lycopene is bound within the cellular structures, while the cells in the tomato paste have been weakened by processing, resulting in the higher

extraction efficiency of lycopene from the tomato paste (Papaioannou and Karabelas, 2012). Boiling into ethanol has been proposed for lycopene extraction from tomato. However, there are inadequate data concerning the influence of heat on the yield and on the isomerization and degradation of carotenoids during their extraction from tomato using ethanol or ethyl acetate. The concentration of lypidic extract and the yield of each carotene were noticeably higher in extractions performed with ethanol than with ethyl acetate. Taking into account that carotenoids are enclosed within cells, that the cell walls are of a complex composition, and that ethanol can break cell walls, the best yield obtained with ethanol may be due to better accessibility of this solvent to the carotenoids (Calvo et al., 2006). Low extraction lycopene efficiencies reported for the tomato peel case can be attributed to the difficulty for the solvent molecules to penetrate the compact tomato peel tissue and solubilize the lycopene, which is deeply embedded within the chromoplast membrane structures. Tomato peel is a highly structured plant material containing many different polysaccharide components, such as cellulose, hemicelluloses and pectins. In theory, the extraction effciency could be improved by using more severe extraction conditions, but the risk for lycopene to undergo oxidative degradation would proportionally increase (Xianquan et al., 2005).

According to Strati and Oreopoulou (2011a), the solvent mixture with ethyl acetate proved to be more efficient than other mixtures of hexane, adequate to extract non polar carotenoids (lycopene and ϐ-carotene) in sufficient percentages (96%). For lycopene isolation a mixture of hexane with acetone and ethanol or methanol is often used because other solvents, such as diethyl ether and THF, may contain peroxides that react with carotenoids (Xianquan et al., 2005). The stability of lycopene extracts obtained with hexane/acetone or hexane/ethanol is

shown to be higher than that of extracts obtained with other organic solvents, such as chloroform, methanol or dichloromethane (Kaur et al., 2008). Olives Barba et al. (2006) reported that the hexane/acetone/ethanol mixture (50:25:25 v/v/v) showed the best results in comparative studies with three solvents (methanol, ethyl ether, THF) and two other solvent mixtures, i.e. methanol/THF (92:8 v/v) and methanol/THF (50:50 v/v).

Comparison of efficiencies of different solvents for carotenoid extraction from various plant materials is presented in the literature, but few of them concern tomato waste, with limited data on the optimisation of extraction conditions (Kaur et al., 2008; Periago et al., 2004). In such an investigation (Strati and Oreopoulou, 2011b) of lycopene extraction from tomato residue, a mixture of ethyl acetate and hexane was reported to give the highest carotenoid extraction yield among the other examined solvents (ethanol, hexane, ethyl acetate, acetone and their mixtures). In that study (Strati and Oreopoulou, 2011b) the extraction conditions, such as percentage of hexane in the mixture with ethyl acetate, ratio of solvent to residue and particle size were optimised using a statistically designed experiment. The optimised conditions for maximum carotenoid yield (37.5 mg/kg dry waste) were 45% hexane in solvent mixture, solvent mixture to waste ratio of 9.1:1 (v/w) and particle size 0.56 mm (Strati and Oreopoulou, 2011b).

Recently, ethyl lactate has been also proposed as extraction solvent with promising results (Ishida and Chapman, 2009). Because of its low toxicity, it is commonly used in pharmaceutical preparations, food additives and fragrances. It has been approved for use in food products by the US FDA. According to Strati and Oreopoulou (2011a), ethyl lactate is more advantageous than ethyl acetate which is currently used; these authors found that 30 min was adequate for each extraction step, with a recovery of 55ï 74% of total carotenoids achieved in the

first extraction step. In that study it was also shown that a temperature increase up to 70°C increased significantly the carotenoid yield. The maximum amount of total carotenoids extracted from tomato waste was obtained using ethyl lactate for three successive extractions of 30 min, at 70°C (243 mg/kg dry tomato waste). However, even at 25°C the total yield obtained by ethyl lactate amounted to 202.73 mg/kg, indicating that this solvent extracted more carotenoids at ambient temperature than other solvents used at higher temperatures; consequently, the energy-related process cost would be reduced compared to other solvents (Strati and Oreopoulou, 2011a).

If red tomato powder is extracted at 45 °C for 1 h with ethyl lactate, then the amount extracted is almost equal (541 mg/g of dry weight) to that obtained using dichloromethane/ methanol/H_2O (DMH) (40:40:20) for 2 h (Ishida and Chapman, 2009). Or, red tomato powder can be extracted at 45 °C for 2 h using 60% ethanol in ethyl lactate, and the extracted amount goes up to 724 mg/g of dry weight, which is much greater than that extracted using DMH (558 mg/g of dry weight). If red tomato powder is processed in ethyl lactate for 1 h at 60 °C, the total lycopene extracted increases to 586 mg/g of dry weight, compared to 558 and 489 mg/g of dry weight in DMH and absolute ethanol (Ishida and Chapman, 2009).

In case of watermelon, a mixture of hexane/methanol/acetone (1:1.7:3.3 v/v) for 4 h at 5ŭC in darkness was used by Tarazona-Díaz et al. (2011) for the lycopene extraction but there was no attempt to optimize extraction conditions. The extraction processing of lycopene from watermelon was investigated by Shi et al. (2012a) using response surface methodology (RSM) with key parameters the number of extraction stages, the temperature and time in order to increase extraction yield. They found that the optimum extraction conditions were obtained with

hexane (containing 2% dichloromethane) as extraction solvent, ratio of solvent to raw material of 3:1 (mL/g), two extraction stages, extraction time 1.9 h and temperature of 29.8°C. Under these conditions the lycopene content was 14.71 mg/kg, which is in agreement with a value predicted by their model.

There are only few reports dealing with lycopene extraction from microorganisms *after its biotechnological production*. Lycopene extraction from *Rhodopseudomonas palustris* with various solvents has been recently reported by Bao et al. (2010). Nine polar and six nonpolar solvents were used in this study to test the extraction efficiency of lycopene from *R. palustris* cells. In order to effectively extract the intracellular hydrophobic lycopene from bacterial cells, an important function of the selected solvent must both improve the solubility of lycopene and the permeability through cell membrane. Dichloromethane and benzene were the best solvent for the lycopene recovery, with a content of 0.85 mg lycopene/g dry cells and 0.68 mg/g dry cells, respectively. Polar dichloromethane and nonpolar benzene have a relatively high solubility of lycopene, which may be responsible for their high extraction efficiencies. These solvents with relatively high extraction efficiency of lycopene were selected and used for further investigations (Bao et al., 2010).

However, methanol was not an efficient extraction solvent, despite its simplest molecular structure and smallest molecular weight, which enables an easy permeation of the bacterial cell wall and membrane; this might be due to its low solubility of lycopene (Bao et al., 2010). Nonpolar petroleum ether is widely used in the extraction of lycopene from tomato puree; however, its extracting efficiency of lycopene from *R. palustris* was low, which might be caused by its low permeability through cell wall and membrane. Some studies have focused on the

extraction of lycopene from tomatoes using the common polar solvent ethyl acetate. However, the polar solvent dichloromethane was found to be a better solvent to extract lycopene from *R. palustris*, which suggested that the solvent polarity was not a very important factor for extracting lycopene from *R. palustris*. Periago et al. (2004) reported that although the extraction efficiency of lycopene with ethanol was low, it would raise the recovery of lycopene from tomatoes when ethanol was combined with n-hexane, probably due to a synergistic secondary effect; this effect between the combined reagents was suggested to be a key factor in the efficient extraction of lycopene.

A mixture of the polar reagents dichloromethane or THF with the nonpolar reagents benzene, n-hexane, or cyclohexane at ratios of 1:1 v/v was used to extract lycopene from bacterial cells. Applying the immiscible two-phase systems of n-hexane/ethanol, cyclohexane/ethanol, n-hexane/methanol, and cyclohexane/ methanol (1:1 v/v,), higher recoveries were obtained using the combination of immiscible n-hexane/ methanol, that might also result from a synergistic secondary effect. The amount of extracted lycopene was 1.5 mg/g dry cells, which was much higher than that extracted by any single polar or nonpolar reagent. The immiscible two-phase systems of n-hexane/methanol at volume ratios of 1:2, 1:1, 2:1, and 4:1 v/v were further investigated. The maximum extraction efficiency was obtained when the ratio between n-hexane and methanol was set at 1:1 v/v. The maximum extraction efficiency achieved with n-hexane and methanol (1:1 v/v), was approximately double compared to that obtained with a single solvent. Methanol could easily enter the bacterial cells through wall and membrane to dissolve intracellular lycopene and then transfer the extracted lycopene to the phase of n-hexane, which was responsible for the highest extraction efficiency of lycopene from the

cells of *R. palustris*. Most lycopene (83 %) could be found in the top phase of n-hexane and a small amount (17%) was detected in the low phase of methanol, indicating that almost all extracted lycopene from *R. palustris* cells was dissolved in the n-hexane phase. This partitioning behavior of lycopene is helpful in concentration and purification processes of lycopene in industrial production. The effective extraction of lycopene from microorganisms forms the basis of further purification of lycopene. The extraction efficiency of lycopene with combined solvents was apparently lower than that with dichloromethane.

Further studies indicated that the extraction of lycopene was much improved after an alkaline wash of *R. palustris* cells with 2 mol $L^{ï 1}$ NaOH where a lycopene content of 3.29mg/g dry cells was obtained. The saponification reaction between sodium hydroxide and fatty acids on the cell membrane of *R. palustris* was apprently responsible for the improved extraction of lycopene, which is expected to facilitate the potential application in the recovery of lycopene (Bao et al., 2010).

In another biotechnological production case, the effect of *Blakeslea trispora* biomass pretreatment and solvent extraction on carotenoid recovery is reported by Papaioannou et al. (2008). The biomass was used after its extensive washing without further treatment (wet biomass **a**), disrupted by grinding after freezing with liquid N_2 (wet biomass **b**), lyophilized (dry biomass **c**) or dried for 40 h at 50^{o}C (dry biomass **d**). Eight food-compatible solvents of class II and III of the International Conference of Harmonization were used (in combination with the differently pretreated biomass **a-d**), namely: ethanol, methanol, acetone, 2-propanol, pentane, hexane, ethyl acetate, and ethyl ether. Furthermore, HPLC analysis was used for the evaluation of their selectivity towards the three main carotenoids, ɓ-carotene, ɔ-carotene and lycopene. The highest

carotenoid extraction yield was obtained in the case of wet *B. trispora* biomass (**a** and **b**), where 44ï 56% of total carotenes were recovered with one solvent and three extractions, whereas the remaining percentage was recovered only after subsequent treatment with acetone; thus, four extraction stages of 2.5 h total duration each were needed. On the contrary, two extraction stages of 54 min each were enough to recover carotenoids from dehydrated *B. trispora* biomass (**c** and **d**), with the disadvantage of a high degree of carotenes degradation (about 76-86%). The degradation of carotenoids during lyophilization is attributed to the fact that this process renders the samples more porous, thus increasing their exposure to oxygen. For maximum carotenoid recovery, ethyl ether, 2-propanol, and ethanol could be successfully used with biomass without prior treatment (**a**), whereas fractions enriched in ϐ-carotene or lycopene could be obtained by extraction with the proper solvent, thus avoiding degradation due to time consuming processes. HPLC analysis showed that the final acetone extract from wet and dry biomass was rich in lycopene. Interestingly, the acetone extract of biomass **a** after it was extracted with another solvent (three times) gave a fraction of over 85% in lycopene. Ethyl acetate and hexane recover lycopene as effectively as acetone from the initial stages, leading to a lycopene rich extract, around 45%, compared to the three main carotenoids. In the case of biomass **b** with disrupted cells, it seems that ethyl acetate is still capable of recovering lycopene preferentially, while hexane is not. This is considered to be the main difference emerging between wet biomass **a** and **b**. In that study, the percentage of the three major carotenoids recovered from wet biomass **a** is higher than in the other three cases of biomass investigated, suggesting that wet biomass offers advantages compared to dehydrated biomass, where a high degree of degradation is observed. The data from a kinetic analysis indicate that the solvents exhibit better characteristics regarding

maximum total carotenoid recovery and required extraction time in the following order: ethyl ether, 2-propanol, and ethanol. In addition, selecting the proper solvent from the first isolation step, enriched fractions in ϐ-carotene or lycopene can be obtained, rendering further purification of these two carotenoids relatively easy (Papaioannou et al., 2008).

The *B. trispora* cells in another study (Wang et al., 2011) was dried in vacuum freeze dryer at -75^{o}C under 45 Pa for 48 h, and extracted with acetone using an ultrasonic cell disruption apparatus for 180 s for lycopene quantification and there is no further evidence about extraction process. For lycopene isolation from the metabolically engineered *E. coli* cells, acetone was used at 55°C for 15 min with intermittent stirring, only in order to analyze its content; however, no further data concerning its extraction was given (Kim et al., 2011).

In conclusion, the mixtures of hexane with methanol, ethanol, acetone, and ethyl acetate are generally proven to be more efficient for lycopene extraction; moreover, they are meeting the health safety and environmental protection standards. The proper percentage of each solvent in the mixture seems to vary from case to case, as well as their efficiency which depends mainly on the nature of treated material (wet or dry, particle size etc). However, the use of solvent mixtures in practice appears to be problematic; this is due to alteration in mixture solvent proportion during lycopene extraction from raw wet materials of different initial water content. This solvent mixture alteration after lycopene recovery should be taken into account from batch to batch in industrial scale, something quite tedious in such scales.

6.3 Super-Critical Fluid Extraction (SCFE)

The available separation techniques for food products and medicines are limited by the requirement of low temperatures, since the product usually has poor stability at high temperatures. SCFE is an extraction method which operates above the solvent critical pressure and temperature, enhancing its solvating power (Machmudah et al., 2012). Supercritical fluids combine the physical properties of fluids and gases in an advantageous way, so that they exhibit higher solvent power and higher density than the gases, together with fast diffusion, low surface tension and low viscosity in comparison to liquids. The combination of high solubility and low capillary action is especially attractive for extracting compounds from solid raw materials when the target substances are well embedded in the solid matrix. Since physical properties can be manipulated over a wide range by pressure and temperature, the selectivity can be controlled.

Carbon dioxide (CO_2) is a solvent frequently used in the SCFE method due to its low critical temperature, no toxicity or flammability, and its availability at low cost and high purity (Machmudah et al., 2012). In SC CO_2, substances such as alcohols, aldehydes, ketones, esters and some alkaloids are readily soluble, whereas others like amino acids, glycosides, proteins and carbohydrates are less soluble (Bart and Pilz, 2011). Although CO_2 is the most frequently used supercritical solvent today, due to its aforementioned distinct attributes, other solvents like ethane or propane have also been successfully employed in such processes (Nobre et al., 2012). The solubility of substances in SC CO_2 can be manipulated by additives, usually ethanol and water; furthermore, processes using a mixture of gases are possible; e.g. a mixture of CO_2 and propane combines the low capillary resistance with high solubility of these solvents, respectively (Bart and Pilz, 2011).

Several papers have reported on the use of SC CO_2 extraction to obtain lycopene and other carotenoids from tomato by-products at low temperature and pressure as well as on the use of organic solvents as a modifier (Vagi et al., 2007; Huang et al., 2008). Improving the extraction efficiency of carotenoids with SC CO_2 is essential for developing processes at an industrial scale. Through response surface regression, Huang et al. (2008) predicted a 93% lycopene recovery using supercritical CO_2 and 16% ethanol as a co-solvent. Topal et al. (2006) reported on the effects of temperature, pressure, and CO_2 flow rate on extraction of lycopene from tomato peel by-product without the addition of any modifier; they did, however, use the toxic organic solvent THF for rinsing the remaining lycopene in the system. It was shown that temperature and pressure had an effect on lycopene extraction, and that the highest yield of lycopene, 85%, was obtained at 40 MPa, 100^{o}C, and 2.5 mL/min of CO_2 with the THF rinsing method. Rozzi et al. (2002) examined the effects of temperature, pressure, flow rate, and CO_2 volume on extraction of lycopene from tomato peel and seed. A lycopene recovery of 61.0% was obtained at 86^{o}C and 34.47 MPa with 500 mL of CO_2 at a flow rate of 2.5 mL/min. The use of vegetable oil as a co-matrix with the tomato matrix for supercritical CO_2 extraction of lycopene has also been reported, with mixed olive oil and ethanol (Shi et al., 2009) and hazelnuts oil (Lenucci et al., 2010; Vasapollo et al., 2004). Vasapollo et al. (2004) showed that the presence of hazelnut oil helped both to improve up to three times the yield of extracted lycopene (over 8 h) and to prevent its degradation during storage. The maximum amount of extractable lycopene from dried tomato obtained at 45 MPa and 66^{o}C in the presence of hazelnut oil (10%) at a flow rate of 20 kg CO_2/h for extraction time more than eight hours was 60%. Lenucci et al. (2010) reported that the addition of an oleaginous co-matrix consisting of roughly crushed hazelnuts made it possible to

recover approximately 80% of the lycopene in the oleoresin. Machmudah et al. (2012) recently studied the SC CO_2 lycopene extraction in the temperatures range 70-90^oC, pressure 20-40 MPa, particle size 1.05 mm and flow rates 2ï 4 mL/min of CO_2 for 180 min extraction time. They found that the optimum operating condition to extract lycopene (achieving 56% lycopene extraction) was 90^oC, 40 MPa, and a ratio of tomato peel to seed of 37/63. The presence of tomato seed oil appeared to improve the recovery of lycopene from 18% to 56%.

The extraction yield increased with temperature and pressure using pure SC CO_2 to extract lycopene from tomato peels (Topal et al., 2006) and from tomato peels and pulp (Gomez-Prieto et al., 2003). However, Vagi et al. (2007) indicated that only temperature had a significant effect on the yield. Different lycopene recoveries were reported, such as 61% at 86^oC and 345 bar (Rozzi et al., 2002), and 94% at 100^oC and 400 bar (Topal et al., 2006). Furthermore, Gomez-Prieto et al. (2002) observed that the amount of the trans form of lycopene extracted increased and the cis form decreased at higher extraction pressures. In a study on the stability of lycopene at very high hydrostatic pressures of 1000ï 6000 bar, Qui et al. (2006) concluded that lycopene was stable up to 4000 bar.

De la Fuente et al. (2006) reported lycopene solubility in SC CO_2 ranging from 0.3 to 1.5x 10^{-6} mol solute/ mol mixture using purified lycopene as the feed material. Gomez-Prieto et al. (2002) reported lower lycopene solubility in SC CO_2 (ranging from 4.1 to 11 x 10^{-8} mol solute/ mol mixture using tomato peel waste as their starting material and only 30 min of dynamic extraction at a flow rate of 4 mL/min. Shi et al. (2009) also performed a dynamic extraction for 90 min using tomato peel to measure the solubility in SC CO_2 of lycopene (0.7ï 1.9 x 10^{-6}). Both Gomez-Prieto *et al.* (2002) and Shi et al. (2009) used tomato matrix rather than

pure lycopene in their solubility measurements. Moreover, in both studies, a dynamic solubility measurement method was employed, but it was based on a single point measurement; considering that relatively high flow rates prevail, no other evidence or comments were offered to demonstrate that equilibrium was achieved. More studies are, therefore, needed to determine the solubility of lycopene and ß-carotene in SC CO_2 and to understand the effects of the matrix and the presence of co-solvents on the solubility.

The SC CO_2 extraction of lycopene and b-carotene from tomato peel and pulp was studied by Saldaña et al. (2010) at 40^{o}C, 400 bar, and 0.5 L/min of CO_2 flow rate; using three different supercritical solvents, they found the following order of process efficiency: SC CO_2 + 5% canola oil > SC CO_2 + 5% ethanol > SC CO_2. Lycopene apparent solubility values for the multicomponent complex system (lycopene extracted from tomatoes with SC CO_2 and SC CO_2 and co-solvent), under the same processing conditions, were in the range of 0.4×10^{7} to 2.2×10^{-7} mole fraction. The findings show the difference between the true solubility based on binary system measurements reported in the literature and the apparent solubility based on multicomponent system measurements. This difference can be attributed to the impact of tomato cell matrix on the free availability of lycopene for solubilization into the supercritical fluid as well as on the interactions between different components, which can change over time throughout the dynamic extraction period. The use of oil as a continuous co-solvent is promising for industrial recovery of carotenoids from tomato matrix. The carotenoid-saturated vegetable oil product can be used as is in a variety of nutraceutical and functional food applications (Saldaña et al., 2010).

Lycopene extraction from *other sources such as watermelon* was also reported using SC CO_2 with ethanol as a co-solvent, reaching a maximum yield at 70^{o}C, 21 MPa and ethanol at 15% by volume (Vaughn Katherine et al., 2008). The SC CO_2 extraction of lycopene from watermelon was studied by Vaughn Katherine et al. (2008) in respect to extraction temperature (70-90^{o}C), pressure (20.7-41.4 MPa) and co-solvent addition (10-15% ethanol). A lycopene yield of 38 ε g per gram of wet weight was reported for the freeze dried sample when extracted by SC CO_2 at 70^{o}C, 20.7 MPa, and 15% by volume ethanol. The extraction of fresh (non-freeze-dried) watermelon yielded 103 ε g lycopene per gram fresh fruit weight. Of the parameters tested, temperature had the greatest effect on lycopene yield. Thus, in another set of experiments, the temperature was varied from 60-75^{o}C at an extraction pressure of 20.7 MPa in the presence of 15% ethanol. This study also showed that freeze-dried watermelon flesh loses lycopene in storage (Vaughn Katherine et al., 2008).

In order to improve the efficiency of processes using SC CO_2, Varona et al. (2013) examined the solubility of lycopene in mixtures of ethyl acetate (EA) and CO_2 at elevated pressures. The solubility of lycopene was determined for different temperatures (313ï 333 K), pressures (12ï 16 MPa) and CO_2 molar fractions (0.58ï 1). Their data showed that CO_2 acted as an antisolvent in the system lycopene/EA/CO_2 of CO_2 molar fractions analysed (0.58ï 1) and that the solubility increased with temperature and pressure. Since lycopene has a relative low solubility in CO_2, this solubility can be increased by adding an organic solvent like ethyl acetate. The solubility of lycopene is rather small with lycopene molar fractions ranging from $0.1\times10^{ï 6}$ to $46\times10^{ï 6}$ (Varona et al., 2013).

Nobre et al. (2012) have recently reported on the supercritical extraction of lycopene from industrial tomato wastes in a semi-continuous flow apparatus using *ethane* as solvent. Ethane as a supercritical solvent, although more expensive than CO_2, may offer several advantages, since it has a rather low critical temperature (near that of CO_2) and a low critical pressure (305.4 K and 48.2 atm) which would allow energy cost reduction. Moreover, ethane is an apolar solvent, with a higher polarizability than CO_2, which apparently renders it more appropriate for the type of carotenoids found in tomato. The effects of pressure, temperature, feed particle size, solvent superficial velocity and matrix initial composition were evaluated. The effect of the temperature was somewhat complicated; i.e., with temperature increasing from 40 to 60°C lycopene recovery increased from 80 to 90%, but for a further increase to 80 °C the recovery remained almost the same. The latter indicates that some isomerization could have occurred as well as some lycopene degradation. The final recovery of all-trans-lycopene was found to be practically independent of the tomato-waste particle sizes. Comparison of extraction yield with that obtained with other supercritical solvents (supercritical CO_2 and a near critical mixture of ethane and propane) showed that the mixture of ethane/propane led to the highest recovery (about 100%) of all-trans-lycopene, followed by ethane (90%) and CO_2 (86%). Also the extraction was reported to be faster when ethane and the mixture ethane/propane were used; this behavior could be explained in terms of the compound solubility in these supercritical solvents which is greater than in CO_2.

The supercritical fluid extraction is an attractive process alternative, often applied with success; however, the capital expenses necessary for the extraction setup are relatively high and this tends to inhibit application of this technique. In fact, from an industrial point of view, the

separation of compounds from solid materials by means of SCFE (using a solvent under high pressure) is generally troublesome and requires significant capital cost for high-pressure extraction equipment (Montesano et al., 2008). However, for relatively high-value products (e.g. caffeine removal from the coffe) the application of SC CO_2 at production scale is advantageous (Telo et al., 2011) mainly because CO_2 can be easily separated from the extracts, thus leaving no traces of solvent in the final product. Such benefits regarding product quality have to be weighted against energy and capital expenses resulting from the use of high pressure technology (Montesano et al., 2008; Naviglio et al., 2008). Nevertheless, supercritical fluids are suitable for the extraction of compounds that can be easily degraded by light, oxygen and high temperatures like carotenoids, even though the solubility of these substances in SCF is still relatively low compared to their solubility in organic solvents, and high pressures must be used to obtain reasonable extraction yields. Therefore, from an industrial point of view, solvent extraction is still in general the first option because of its simplicity and relatively low total processing cost, especially in relatively small capacity plants.

7 Alternative extraction methods

7.1 Ultrasound/microwave assisted extraction

Microwave-assisted extraction (MAE) has drawn significant research attention in various fields, particularly in medicinal plant research, due to its special heating mechanism, moderate capital cost and its good performance under atmospheric conditions. Microwaves are electromagnetic waves in the frequency range 0.3 to 300 GHz, which can penetrate into certain materials and interact with the polar components to generate heat. Microwave energy acts

directly on the molecules by ionic conduction and dipole rotation, and thus only selected and targeted materials can be heated based on their dielectric properties. Some drawbacks associated with MAE are the requirement of additional clean up step to remove solvent from sample matrices and the restriction to only polar solvent application in the system.

Ultrasound can be used as an alternative to MAE in natural compounds extraction; thus in such a case the process is referred to as *Ultrasound Assisted Extraction (UAE)*. The mechanical effect of ultrasound provides a greater penetration of solvent into the cellular materials and result in the disruption of biological cell walls to facilitate the release of its contents (Dolatowski et al., 2007). The advantages of sonochemistry application in food processing include increase in both the extraction yield and extraction rate, resulting in reduced extraction time and a higher throughput (Rastogi, 2011; Chemat et al., 2011; Kumcuoglu et al., 2013). Recent reports (Eh and Teoh, 2012) suggest that sonication can enhance the efficiency of relative lycopene recovery yield (enhancement of 26% extraction yield of lycopene at 40.0 min, 40.0^{o}C and 70.0% v/w in the presence of ultrasound), lower the extraction temperature, shorten the total extraction time and at the same time, minimise the degradation and isomerisation of lycopene. Kumcuoglu et al. (2013) have reported very recently that UAE of lycopene requires less time, lower temperature and smaller amount of solvent than conventional organic solvent extraction (COSE) processes. The effects of various factors, including temperature, solvent to solid ratio and ultrasonic power on lycopene yield were investigated. A mixture of hexane:acetone:ethanol (2:1:1) was used as solvent and three different solvent to solid ratios; 50:1, 35:1 and 20:1, (v/w) were tested in both COSE and UAE. COSE experiments were performed at 20 °C, 40 °C and 60 °C for 10, 20, 30 and 40 min, whereas, 50, 65 and 90 W of

ultrasonic power were applied for 1, 2, 5, 10, 15, 20 and 30 min in UAE case. The results of this study indicate that the most efficient extraction process in case of COSE was with 50:1 solvent to solid ratio at 60°C for a 40 min run, whereas for UAE, the 35:1 (v/w) solvent to solid ratio, 90 W ultrasonic power for 30 min extraction was enough (Kumcuoglu et al., 2013).

The simultaneous application of two of the green chemistry tools, namely *sonication and biocatalysis,* is also reported to improve extraction of lycopene from tomato peel (Konwarh et al., 2012). In this case an significant increment in the enzyme (cellulase ‘Onozuka R-10’)-mediated extraction of lycopene was recorded. Furthermore, use of sonication resulted in reduction of the required optimal enzyme concentration and incubation period. The coupled system showed improved extraction by 662%, 225% and 150% over the reference, only cellulase ‘Onozuka R-10’treated and only sonication treated samples, respectively (Konwarh et al., 2012).

On the contrary, a recent study by Anese et al. (2013) claims that ultrasound processing of tomato pulp may induce changes in the viscoelastic properties of tomato pulp causing partial de-esterification of tomato pectins, which increase with increasing processing time. It is inferred that the partially de-esterified pectin molecules gave rise to a new molecular organization leading to the formation of a stronger network with increased gel-like properties. Therefore, ultrasound processing is proposed that causes a marked decrease of in vitro bioaccessibility of lycopene in the tomato pulp. Such an effect seems to be related to the viscosity changes induced by ultrasound processing. In other words, the formation of a stronger network by the application of ultrasound may entrap lycopene in the matrix, rendering it less accessible for digestion. More in-depth research on the mechanisms governing these changes is obviously needed in order to exploit ultrasound technology as a means of influencing structure and functionality of tomato

derivatives. Moreover, the results on the in vitro bioaccessibility of lycopene need to be compared with the results obtained from human studies (Anese et al., 2013).

Industrial processing of tomatoes into different end-products includes mechanical treatments, several thermal treatment steps, and the addition of ingredients which might induce changes in lycopene bioaccessibility. Colle et al. (2013) have studied the influence of high pressure homogenisation (HPH) (100 bar) and microwave heating (20 min at 70, 90, and 120 °C) of tomato pulp; in the absence and in the presence of three different oils (5%: coconut oil, olive oil, and fish oil) with distinct fatty acid composition. In that study lycopene bioaccessibility in the processed samples was examined by determining the fraction of lycopene transferred from the food matrix to the aqueous micellar phase during in vitro digestion. Adding lipids prior to processing clearly enhanced the lycopene bioaccessibility. However, the type of lipid added was of minor importance compared to the process conditions applied. HPH or microwave heating of tomato pulp in the presence of lipids during 20 min at 70 and 90 °C did not improve the lycopene bioaccessibility significantly. When HPH was applied prior to the heat treatment, microwave heating could improve the lycopene bioaccessibility. It is, therefore, hypothesised that HPH damages the cellular barriers for lycopene bioaccessibility, which can be further disrupted by thermal processing improving lycopene release during digestion. Finally, it was proved that applying firstly high pressure homogenization at 100 bar and afterwards 20 min microvave heating at 120 °C facilitated the lycopene bioaccessibility remarkably (Colle et al., 2013).

Zhang and Liu (2008) developed a more efficient method for the extraction of lycopene from tomatoes by ultrasound/microwave assisted extraction (UMAE). Enhancement of mass transfer mechanism in extraction can be achieved by UMAE that combines microwave and

ultrasonic waves providing high momentum and energy to rupture the cell and elute the active compounds to the extraction solvent (Chan et al., 2011). As a result, extraction proceeds over shorter extraction time and with smaller solvent consumption. UMAE has been used to extract a variety of active compounds such as lycopene from tomatoes (Lianfu and Zelong, 2008), vegetable oil (Cravotto et al., 2008) and polysaccharides (Chen et al., 2010) from various plants. In the extraction of lycopene from tomatoes, the extraction time of UMAE was 6 min with 97.4% yield, compared to 29 min and 89.4% yield by using ultrasonic-assisted extraction (UAE). Some of the commercial MAE equipment is tailored to cater to the needs of specific extraction requirements for instance the Ultrasonic/Microwave extractor system (CW-2000 model) was developed by the Xin Tuo Company (China) for the extraction of lycopene from tomatoes (Lianfu and Zelong, 2008). The main drawbacks of UMAE overall appear to be safety limitations and relatively high equipment cost (Liu et al., 2010).

7.2 High pressure extraction

According to mass transfer theory, pressurized cells show increased permeability. The higher the pressure, the more solvent can enter into the cell and the greater the variety of compounds that can permeate through the cell membrane. Thus, an increase in pressure can lead to an increase in the extraction yield of natural products. On one hand, the weaker bonds such as the hydrogen bond, and those resulting from electrostatic, Van der Waals and hydrophobic interactions, can be broken by high pressure, whereas the covalent bonds cannot be broken due to energy level limitations; this means that small molecules will not change under high pressure processing (HPP). Furthermore, based on the phase behavior theory, the solubility is greater as the pressure increases. Experiments (Xi, 2006) showed that the extraction yield of lycopene

obtained by HPP was higher than that obtained with solvent extraction for 30 min. The extraction by HPP performed with 75% ethanol concentration in water, over a 5 min period, 1:5 (g/mL) solid/liquid ratio, at room temperature, shows that when the pressure increased from 100ï 500 MPa, the extraction yield of lycopene increased from 182 to 417 mg/kg. It is obvious that pressure offers the means for improving the extraction yield of lycopene. However, when the pressure level was greater than 500 Mpa, the extraction yield did not change significantly with further increase of the pressure level. Therefore, the optimum pressure level for extraction of lycopene from tomato paste waste was reported (Xi, 2006) as 500 Mpa; moreover, the lycopene molecular structure did not change during HPP treatment at room temperature (Xi, 2006).

The extraction of pure lycopene from tomato waste that uses the Naviglio® extractor (a pressure prosess) and water as extracting phase has been also reported. This extractor was developed by Naviglio et al. (2008) to recover a solid lycopene fraction in a paracrystalline form from tomato waste in tap water. The Naviglio extractor can operate at room or lower temperatures; it works by applying a pressure increase on the surface of the liquid phase containing the solid material (matrix) to be extracted. The use of low or room temperature greatly reduces the thermal stress for any heat-susceptible substances present in the matrix, e.g. lycopene. The optimum extraction time in this case was found to be 4 h, since continuing the extraction beyond this time period did not increase recoveries; the recovery in tap water was between 1.5 and 3.9 mg/kg, corresponding to a percentage recovery of 7.5% and 19.5% (w/w), respectively, compared with solvent extraction. A higher-grade of pure lycopene (>98%) is achieved using the Naviglio extractor compared with the solvent extraction procedure. It is worth

noting that the extraction efficiency and lycopene recovery could be further improved by applying higher pressure in the extraction process (Naviglio et al., 2008).

7.3 Enzyme assisted extraction

Streamlining research in various domains, in accordance with the dictates of green chemistry and green engineering, has gained tremendous impetus in the last decade. Biocatalytic lysis of the cell wall is an effective and green route to release intracellular contents for the extraction of myriad of substances (Konwarh et al., 2012). The enzymatic pretreatment of agro-wastes is an already established approach, with many applications for recovering compounds of biological significance and other high added value products from their compact highly structured plant tissue. The use of cell-wall degrading enzymes, i.e., enzymes that are capable of hydrolyzing the main polysaccharide components of the plant structures, where the natural target compound accumulates, is a mild and efficient means to facilitate its recovery. In particular, enzymes have been used for plant material treatment prior to conventional extraction methods, thus facilitating the extraction. The useful application of enzymes enhances the effectiveness of solvent treatment, either reducing the amount of solvent needed for extraction or increasing the yield of extractable compounds (Puri et al., 2012). Since the plant cell wall is comprised of cellulose, hemicellulose and pectins, cellulases, hemicellulases and pectinases have been used to disrupt the structural integrity of the plant cell wall, thereby enhancing the extraction of bioactive compounds from plants. Such enzymes are widely used in juice processing and beer clarification, to degrade cell walls and improve juice extractability. The disruption of the cell wall matrix also releases components such as phenolic compounds into the juice, thus improving product quality. These enzymes hydrolyze cell wall components thereby increasing cell wall permeability, which

results in higher extraction yields of bioactive compounds (Puri et al., 2012). These enzymes have successfully been used to facilitate the release from vegetable tissues of phenolic compounds (Landbo and Meyer, 2001), sweeteners (Puri et al., 2012), non-volatile grape aroma precursors (Bautista-Ortín et al., 2005), and carotenoids (Choudhariand Ananthanarayan, 2007; Papaioannou and Karabelas, 2012; Zuorro and Lavecchia, 2010) from a variety of plant materials. Recent studies on enzyme assisted extraction have shown faster extraction, higher recovery, reduced solvent usage and lower energy consumption when compared to non-enzymatic methods.

Enzymes can be derived from bacteria, fungi, animal organs or vegetable/fruit extracts. To use enzymes most effectively for extraction applications, it is important to understand their catalytic property and mode of action, optimal operating conditions as well as the enzyme or enzyme combination appropriate for the plant material selected (Puri et al., 2012). With a view to industrial applications, a method is preferable which utilizes commercial enzyme preparation and organic solvents, such as ethyl acetate that are approved for food applications in most countries (Zuorro and Lavecchia, 2010).

Enzyme-aided extraction of lycopene from tomato tissues using cellulases and pectinases under optimized conditions resulted in a significant increase (206%) in lycopene yield with reference to control experiments (Choudhariand Ananthanarayan, 2007). Similarly, lycopene-assisted pancreatin digestion of tomato skin provided a 2.5-fold increase in yield. A digestion step prior to extraction by solvents was necessary to efficiently extract lycopene from the raw material (Dehghan-Shoar et al., 2011). Zuorro et al. (2011) have shown that the recovery of lycopene with hexane from the peel fraction of tomato processing waste can be greatly enhanced

by the use of mixed enzyme preparations. These enzymes have cellulolytic and pectinolytic activities, resulting in an 8- to 18-fold increase in extraction yields. The factors investigated included extraction temperature (10-50ŭC), pretreatment time (0.5ï 6.5 h), extraction time (0.5ï 4.5 h), enzyme solution-to-solid ratio (10ï 50 dm^3/kg) and enzyme load (0ï 0.2 kg/kg). A response surface data analysis pointed to the following optimal extraction conditions: temperature 30ŭC, extraction time 3.18 h and enzyme load 0.16 kg/kg (Zuorro et al., 2011). In the very recent study of Ranveer et al. (2013), cellulase (1.5%) and pectinase (2%) of *Aspergillus niger* at 4 h of incubation period, followed by solvent extraction, proved to be advantageous. Hexane: acetone:ethanol (50:25:25) was found to be the best solvent with lycopene recovery (176.22 ε g/g), by comparison with tests carried out individually with hexane (120.55 ε g/g), ethyl acetate (122.03 ε g/g) and petroleum ether (75.63 ε g/g). It was shown that the treated sample with cellulose and pectinase gave better extraction yields of lycopene (Ranveer et al., 2013). The enzyme-assisted extraction of lycopene from tomato peels, using a multi-enzyme preparation from a selected strain of *Aspergillus niger,* was optimized in a recent study (Zuorro et al., 2012); it was found that the optimum lycopene extraction was achieved under the following conditions: temperature 25°C, pretreatment time 4 h, extraction time 3 h, liquid-to-solid ratio 20 mL/g and enzyme load 0.25 g/g. The isolated lycopene in this case was successfully incorporated into tomato seed oil and a new process for the integrated utilization of these two by-products was proposed (Zuorro et al., 2012).

The use of commercial pectinolytic enzyme preparations and the subsequent extraction with ethyl acetate was recently evaluated by Papaioannou and Karabelas (2012), for lycopene recovery from tomato peels; it has been shown that this could lead to an increased lycopene

recovery ~9.5-11% from the first extraction stage, in respect to the reference untreated peels. The reported lycopene recovery yields are expressed as the percentage of the lycopene amount extracted in a single extraction step over the total amount of lycopene in the peels that could be extracted after three repeated extraction cycles (2h each). The lycopene extracted from untreated tomato peels after just 1h stirring in distilled water was also measured as reference, giving 6.08 %. The optimum incubation time was found to be 1h for the enzymatic pre-treatment of peels, for both enzyme preparations examined, under the same enzymatic activity of 250 Units/mL. Afterwards, the influence of the solution with different enzyme activity in the pre-treatment of peels has been further investigated, showing that a solution with enzyme activity 250 Units/mL and 1 h was effective in extracting the lycopene from the peels (Papaioannou and Karabelas, 2012).

Enzyme-assisted extraction of bioactive compounds from plants has potential commercial and technical limitations: (i) enzymes are relatively expensive for processing large volumes of raw material; (ii) currently available enzyme preparations cannot completely hydrolyze plant cell walls, limiting extraction yields of compounds; (iii) enzyme-assisted extraction can be difficult to scale up to industrial scale because enzymes behave differently at different scales. However, if the above limitations can be overcome, then enzyme-based extraction could provide an opportunity to not only increase extraction yields, but also to enhance product quality by enabling the use of milder processing conditions such as lower extraction temperatures (Puri et al., 2012).

7.4 Surfactant assisted extraction

Lycopene as mentioned above is highly hydrophobic and insoluble in water, so that an organic solvent is usually involved in the extraction process. However, most organic solvents are flammable and may cause environmental pollution. Because of this limitation and the enhanced solubility of lycopene in an aqueous emulsion (Spernath et al., 2002), surfactant assisted extraction represents an organic solvent-free extraction method, which is environmentally friendly. This method involves a solvent-free emulsion in place of an organic solvent; it has proven to be a better method than the traditional one, requiring less extraction time and removing the lycopene from tomato paste more efficiently (Zhu et al., 2010). Systems based on surfactants are well known in the area of biotechnology, e.g. for the extraction of protein from cells.

Surfactants are amphiphilic compounds consisting of a hydrophilic and a lipophilic part. The hydrophilic part has a polar group with an affinity for polar solvents (particularly water), whereas the lipophilic part has a nonpolar group with an affinity for nonpolar substances like organic solvents, carotenoids etc. Due to their amphiphilic structure, surfactants are capable of reducing the surface tension of a liquid, allowing easier spreading, and reduce the interfacial tension between two liquids. Therefore, they are soluble in both organic solvents and water. The spherical micelles formed above critical micelle concentration (CMC) are capable of containing hydrophobic substances which are removed from the biomass. In a second step, the system is brought into the two ï phase region above the cloud point. This leads to an increase in concentration and purity for the extracted hydrophobic substances. Depending on its nature, the target substance is either in the surfactant ï rich phase or in the water phase. These systems are not only used in the downstream processing of crude extracts but also in solid - liquid extraction.

Because the surfactant ï rich mesophase has a volume of 10 to 50% of the total volume a concentration of the target compound by a factor 2 to 10 is feasible. Mainly the lamellar phase is used to absorb hydrophobic and amphiphilic substances (Bart and Pilz, 2011). Several papers and patents deal with applications of the micellar and the cloud point extraction. On the contrary, surfactant molecules have a very low volatility which renders the solvent recovery and product removal from the extract more complicated than for typically used organic solvents. However, for the extraction of natural compounds from plant, economically feasible methods still have to be developed (Bart and Pilz, 2011).

Another case using surfactants for lycopene extraction from tomato paste is the *foam fractionation method* (Wang et al., 2010), with about three hours duration. The foam fractionation is reported to have advantages such as milder operating conditions and simpler experimental setup. Foam fractionation is a process for separating and concentrating chemicals that utilizes differences in their surface activities. It involves the production of stable foam by forcing gas into the liquid, which causes formation of bubbles in the solution. Hydrophobic materials are attached by adsorption to the bubbles, which then rise to the surface of the liquid. At the liquid surface, the bubbles often create a foam phase, which tends to collapse into a concentrated liquid product. It has been reported that this method is economically viable and environmentally friendly (Wang et al., 2010).

A surfactant comprised of co-polymer of n-butyl acrylate, styrene, and acrylic acid, (defined as CPS) was used by Zhu et al. (2010) for lycopene extraction by the emulsion method and compared to that obtained using an organic solvent. The emulsion lycopene samples were dissolved in acetone before analysis. This type of emulsion extraction was suggested as a viable,

environmentally friendly method for the extraction of lycopene from tomatoes. Compared with the traditional methods, emulsion extraction appears to be more efficient and needs no organic solvents, while it exhibits several typical attributes of ñgreenò chemistry; in summary

ÅNo waste generation; there is no organic solvent used during extraction.

ÅUse of renewable feedstock; the emulsifier can be easily reused.

ÅUse of safer solvents; water is the only solvent used in the extraction process (Zhu et al., 2010).

The lycopene solubilization by a variety of amphiphiles from watermelon tissue was examined by Fish (2006). Detergents were evaluated either at or just below their reported CMC in water and at a concentration sufficiently greater than the CMC to ensure a significant concentration of detergent micelles. Of the detergents that were tested, only the n-alkyl sulfate detergents, n-octyl sulfate, and n-dodecylsulfate (SDS) appeared to solubilize appreciable quantities of lycopene. The carotenoids solubilized in the n-alkyl sulfates were subsequently extracted with hexane, and the total amount of extracted lycopene was compared to that directly extracted from identical tissue by hexane. The results of this report (Fish, 2006) indicated that the n-alkyl sulfate solutions extracted >95% of the lycopene from the watermelon tissue, whereas using 0.3% SDS, the carotenoids could be extracted from watermelon, pink grapefruit, guava, cantaloupe, fresh red tomatoes, and papaya. For acidic fruit such as grapefruit, it was necessary to adjust the pH of the puree to near neutrality before the SDS would completely extract the carotenoids. Conversely, only limited quantities of carotenoids could be extracted from processed tomato products, even after pH adjustment. From the processed tomato sources, only small amounts of lycopene were extracted, along with larger quantities of Ŭ- and ɓ-carotenes. Attempts to improve lycopene extraction from tomato sauce by heating at 60^{o}C for up to 30 min

or at 100^{o}C up to 15 min did not appreciably increase the amounts of carotenoids solubilized. No significant difference in the amount of lycopene extracted from watermelon tissues (or loss after solubilization) could be detected for extra treatment times with 0.2% SDS at room temperature from 1 h up to 24 h or at 60^{o}C from 10 min up to 30 min. Samples treated at 100^{o}C for longer than 5 min began to show a decline in lycopene levels. It was also observed that only part of the lycopene from noticeably over-ripe watermelons could be extracted into 0.2% SDS. Centrifugation after SDS treatment of over ripe watermelon yielded a band of intense red material pelleted on top of the colorless cell debris. According to this method, 120 SDS molecules were bound to the chromoplast membranes and corresponded to one molecule of lycopene inside the chromoplast membrane. HPLC analysis indicated that no changes in carotenoid composition or isomer distribution had occurred during the 10 days of dialysis at room temperature that it took to reach system equilibrium (Fish, 2006).

Eight commonly used surfactants with quite broad range of Hydrophilic Lipophilic Balance (HLB) were examined recently by Papaioannou and Karabelas (2012) and found that surfactants with a HLB value in the range 4 to 7 (i.e. Span 20, Span 40 and Span 60) exhibited the best performance; Span 20 was the most effective, resulting in lycopene recovery ~23-25%. Furthermore, the influence of ñSpan 20 over lycopeneò molar ratio on extraction was investigated and was found that lycopene recovery was strongly dependent on Span 20 concentration; i.e. the recovery increased with Span 20 concentration, up to a specific ratio. The optimum range of ñSpan 20 over lycopeneò molar ratio was 5 to 7 and led to a lycopene recovery ~20-25%. It should be mentioned here in respect of an industrial application that a low ratio

indicates an economically more effective process (less surfactant used) and an easier separation of the entrapped (into the emulsion) lycopene from the surfactant, if it is necessary.

The results of sequential enzyme tomato peel pre- treatment and surfactant assisted extraction were studied as well by Papaioannou and Karabelas (2012), for six surfactant (Span 20) over lycopene molar ratios (i.e. 1, 3, 4, 5, 8, 11); an improvement was obtained in lycopene recovery, with a somewhat lower molar ratio of surfactant over lycopene molecule, i.e. 4 to 5. Combined treatment led to lycopene recovery almost four times greater compared to simple 1 hr enzymatic pretreatment, and approx. ten times greater compared to recovery from untreated peels.

The results of this study (Papaioannou and Karabelas, 2012) show that the recovery of lycopene from the peel fraction of tomato processing waste can be greatly enhanced by the combined use of commercial enzyme preparation and surfactant assisted extraction. In particular, initial pretreatment of tomato peels by Citrozym® CEO followed by surfactant assisted extraction, for 30 min in each step, resulted in extraction yields four and ten times greater compared to enzymatically pretreated and untreated peels, respectively. This fact, together with the comparatively low cost of commercial food-grade enzyme preparations and surfactants used, lend strong support to the possible implementation of the process at industrial scale. Furthermore, the recovered lycopene is in the form of an aqueous emulsion, readily usable for applications in the food and cosmetics industries; this is of paramount importance for such a lipophilic compound recovery, avoiding the use of organic solvents and thus being more environmentally friendly (Papaioannou and Karabelas, 2012).

8 Conclusions

Lycopene extraction using organic solvents remains the most common, simplest and easiest to perform method for laboratory and small scale extraction applications from various natural sources. However, special care should always be taken for avoiding toxic solvents, e.g. benzene, chlorinated hydrocarbons etc. Ethyl acetate, acetone, ethanol, hexane and mixtures thereof are the most frequently employed solvents, with fair results (that depend on the natural source), fulfilling both satisfactory yield and safety requirements. On the negative side, solvent extraction is time-consuming, prone to product degradation because of lycopene lability; the latter is the main reason for the relatively high lycopene purification cost. Furthermore, the solvents mixture is rather problematic for industrial use, due to composition alterations resulting from raw material water removal.

Supercritical solvent extraction is a promising technique with significant benefits, especially in respect of product purity, although it is not easily applicable at small scale and for analysis purposes; moreover, it requires relatively high capital expenses.

Other proposed alternative extraction techniques, including microwave, ultrasound assisted extraction etc, are sophisticated and still in the development stage; i.e. they are applicable only in laboratory scale at present.

The use of cell wall degrading enzymes and surfactant-assisted extraction, on the other hand, are processes constantly gaining attention for practical applications. These techiques are rather easily implemented and are in accord with green chemistry principles; i.e., they can be easily applied at any scale with ordinary equipment, without any special precautions and high cost. Furthermore, the recovered lycopene through such process steps is in the form of an

aqueous emulsion, readily usable for applications in the food and cosmetics industries. Therefore, the lycopene aqueous emulsion form is of particular importance for the recovery of such a lipophilic compound, as it leads to either minimization (under these extraction conditions) of the amount of necessary solvent, or to its total elimination if there is no need for lycopene separation from the emulsifier before commercial exploitation; the resulting economic and environmental benefits are obvious.

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Table 1. Lycopene content of the fruits and vegetables with high concentrations (Rao et al., 2006).

Plant source	*Lycopene (µg/g wet weight basis)*	*Reference*
Gac fruit	348-6,200	(Rao et al., 2006; Collins et al., 2006; Vuong et al., 2006 ; Nhung et al., 2010; Kubola and Siriamornpun 2011)
Tomatoes	8.8-200	(Toprak Aktas and Yildiz, 2011; Jaswir et al., 2011; Ishida and Bartley, 2005; Bao et al., 2010; Rao et al., 2006; Saldaña et al., 2010; Machmudah et al. 2012)
Watermelon	11.6-112	(Jaswir et al., 2011; Rao et al., 2006; Tarazona-Díaz et al., 2011; Vaughn Katherine et al., 2008; Perkins-Veazie et al., 2001; Perkins-Veazie et al., 2006 ; Shi et al., 2012a)
Guava (pink)	52.3-55	(Rao et al., 2006)
Grapefruit (pink)	3.5-33.6	(Rao et al., 2006)
Papaya	1.1-53	(Jaswir et al., 2011;, Rao et al., 2006)
Rosehip puree	6.8-7.1	(Rao et al., 2006)
Carrot	0.02-7.8	(Rao et al., 2006)
Pumpkin	3.8-4.6	(Rao et al., 2006)
Sweet patato	0.2-1.1	(Rao et al., 2006)

Apricot	0.1-0.5	(Rao et al., 2006)

Table 2. Lycopene content of common tomato based products (Jaswir et al., 2011; Rao et al., 2006; Collins et al., 2006; Shi and Le Maguer, 2000)

Tomato product	*Lycopene (µg/g wet weight basis)*
Cooked tomatoes	37
Tomato sauce	62-194.5
Tomato paste	54-1500
Tomato ketchup	99-414
Tomato juice	50-616
Tomato puree	89.3-193.7
Tomato powder	1126.3-1264.9

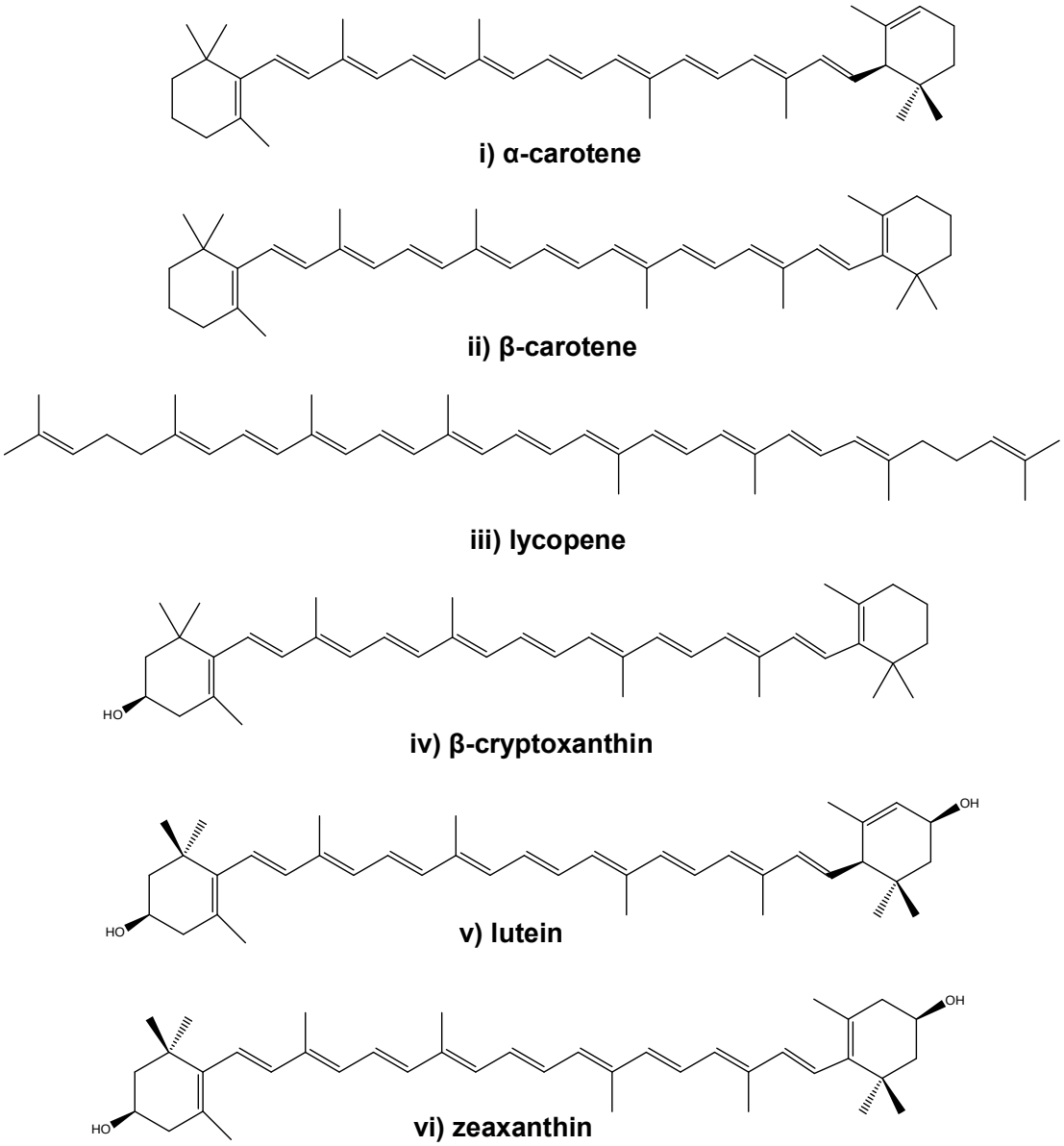

Figure 1. The six main carotenoids found in human blood.

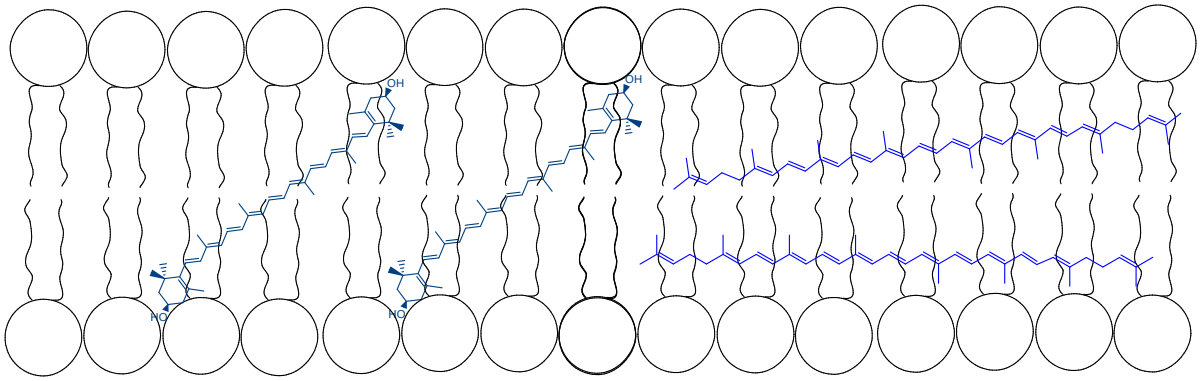

Figure 2. Carotenoids conformation in bilayer lipid membranes according to their polarity; lycopene in blue and zeaxanthine in deep blue.

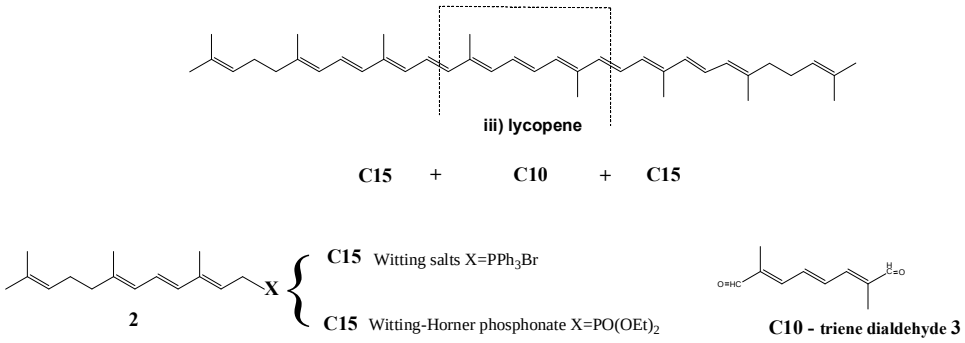

Figure 3. Chemical synthesis of lycopene iii via Wittig or Wittig-Horner reaction (C15+C10+C15 condensation, Shen et al., 2011).

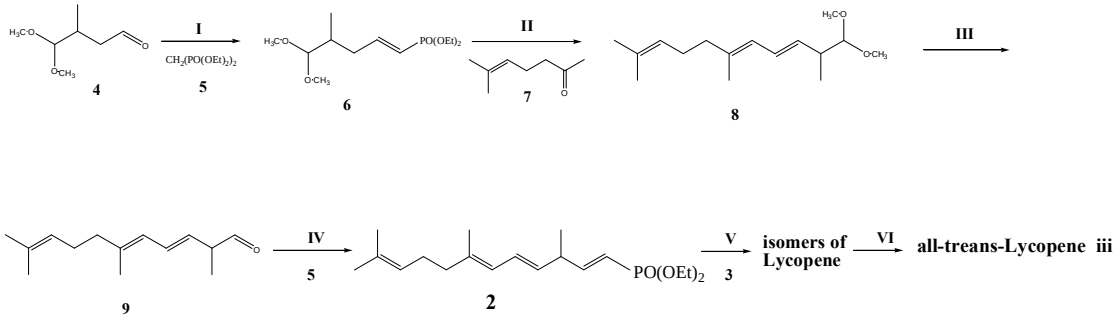

Figure 4. Total chemical synthesis of lycopene iii based on: (I) a condensation between 4,4-dimethoxy-3-methylbutanal **4** and methylenebisphosphonic acid tetraethyl ester **5**, leading to the C6-phosphonate **6**, followed by (II) a modified WittigeHorner reaction between **6** and 6-methyl-5-hepten-2-one **7** producing dimethoxy-3,5,9-triene **8**, and (III) another modified Wittige-Horner reaction between C15-phosphonate **2** and C10-triene dialdehyde **3** producing all-E-lycopene (Shen et al., 2011).