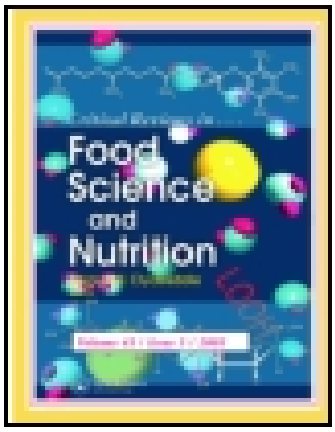

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Antioxidant Compounds from Vegetable Matrices: Biosynthesis, Occurrence, and Extraction Systems

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Antioxidant compounds from vegetable matrices: biosynthesis, occurrence, and extraction systems

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Natural antioxidants such as vitamin C, tocopherols and tocotrienols, carotenoids, and phenolic compounds are largely distributed in plant products. Most of them are not synthesized by human and need to be introduced with diet according to the Recommended Daily Intake (RDI). This work was aimed to give a comprehensive overview on the occurrence of these antioxidants in plants, in particular in plant foods, on the mechanisms of biosynthesis, and on conventional (liquid-liquid or solid-liquid extraction, Soxhlet) and innovative (enzymatic-assisted, pressurized fluid, supercritical fluid, ultrasound-assisted, microwave-assisted, pulsed electric field) extraction systems.

Keywords Ascorbic acid, bioactive compounds, carotenoids, phenolics, solid-waste, tocopherols

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INTRODUCTION

Biological processes in the human body may produce oxygen-centered free radicals and other reactive oxygen species as byproducts (Cai et al., 2004). The term ‘‘antioxidants’’ is referred to compounds that can delay or inhibit the oxidation of varies molecules by inhibiting the initiation or propagation of oxidative chain reaction (Velioglu et al., 1998) and acting as reducing agents, free radical scavengers, potential complexers of prooxidant metals, and quenchers of singlet oxygen (Brewer, 2011). Several researches have demonstrated the occurrence of antioxidants in all higher plants, and in all parts of the plant (wood, bark, stems, pods, leaves, fruit, roots, flowers, pollen, and seeds) (Chanwitheesuk et al., 2005).

Natural antioxidants include compounds such as vitamins, carotenoids, and phenolic compounds. The most abundant types of antioxidants contained in fruit and vegetable include vitamin C, carotenoids, and phenolics whereas tocopherols and tocotrienols are present in relatively low levels in fruit and vegetable as compared with nuts and grains (Kalt, 2005). According to Rice-Evans and Miller (1996), vitamin C acts as a chain-breaking scavenger for peroxy radicals and, furthermore, donates a hydrogen atom to the vitamin E-derived phenolate radical, thus regenerating its activity. Vitamin E and carotenoids are quenchers for singlet oxygen. The most effective antioxidants are those that interrupt the free radical chain reaction. This type of antioxidants, which contains aromatic or phenolic rings, donate a hydrogen atom to the free radicals formed during oxidation and become radical themselves. These radical intermediates are stabilized by the resonance delocalization of the electron within the aromatic ring and formation of quinone structures (Nawar 1996). In addition, many of the phenolics lack positions suitable for molecular oxygen attack. In a special ranking, the antioxidant activity is

most often due to phenolic acids (gallic, protocatechuic, caffeic, and rosmarinic acids), phenolic diterpenes (carnosol, carnosic acid, rosmanol, and rosmadial), flavonoids (quercetin, catechin, naringenin, and kaempferol), and volatile oils (eugenol, carvacrol, thymol, and menthol) (Brewer, 2011). Furthermore, plant pigments such as anthocyanins and anthocyanidins can chelate metals and donate H to oxygen radicals thus slowing oxidation via 2 mechanisms.

Concerning phenolic compounds, epidemiological data have indicated beneficial effects in the prevention of several disease states, including cancer, cardiovascular disease, and neurodegenerative disorders (Hsu and Yen, 2008; Sung Kang et al., 2012) although other researches highlighted a dual function of dietary phenolics with possible health hazard and beneficial effects (Stich, 1991). Recent investigations on phenolic compounds suggested that cellular effects of flavonoids may be mediated by their interactions with specific proteins of intracellular signaling cascades (Williams et al., 2004).

Most of the antioxidant compounds cannot be synthetized by the human body and must be introduced them through a diet rich in fruit and vegetable. According to Kyoung Chun et al. (2005), the American daily intake of phenolics, flavonoids and antioxidants from fruit and vegetable was 450 mg gallic acid equivalents, 103 mg catechin equivalents and 591mg vitamin C equivalents, respectively. Generally, the levels of total phenolics, total flavonoids and antioxidant capacity of fruit were higher than those of vegetables. In fact, among 14 fruit and 20 vegetables, orange contributed about 26 and 25% of total phenolics and antioxidant, followed by apples. Even though potatoes had lower levels of phenolics and antioxidant capacity, they were third due to the fact that their consumption is the highest. The average adult South African total dietary antioxidant capacity (TDAC) was estimated at 11433 μmoles Trolox

Equivalent/person/day and comprised contributions from fruit, vegetables, grains (which include bread, cereal, rice and pasta), legumes and nuts, beverages (tea and coffee) (Louwrens et al., 2009). The beverage group supplied the largest contribution (38.5%, tea in particular), followed by grains (25.6%), vegetable and fruit (11.0% and 19.5%, respectively), legumes and nuts (5.5%). The total dietary antioxidant capacity of the Spanish Mediterranean diet was estimated at 6014 and 3549 μmoles Trolox equivalents by FRAP (ferric reducing antioxidant power) and ABTS (free radical-scavenging capacity) procedures, respectively (Saura-Calixto and Goñi, 2006). About 68% of TDAC came from beverages, 20% from fruit and vegetable, nearly 8% from nuts and legumes, the remaining part from cereals from cereals. Foods such olive oil and wine are known to have a high antioxidant capacity but their contribution to the TDAC depends upon their intake. According to the study of Saura-Calixto and Goñi (2006), the contribution of olive oil account for just 0.6% while wine consumption represents about 14% of the TDAC in the Spanish diet. Coffee was the single greatest contributor (44.5%). The total phenolics intake was estimated as 1171 mg gallic acid/person/day by the Folin–Ciocalteau method. According to Joudalová and Réblová (2012), the daily intake of extractable antioxidants in the Czech Republic amounted to 8300 μmol Trolox equivalents for men and 7500 for women. The largest sources of antioxidants were coffee (43.1% of overall intake for men and 54.6% for women) and beer (15% for men vs. 1.8% for women). Other significant sources of antioxidants were tea, vegetables and vegetable products, fruit and fruit products, cereal products, wine. Within the fruit and fruit products category, apples were the most significant source of extractable antioxidants, whereas peppers were the largest source of antioxidants in the vegetable and vegetable products category. The estimated dietary intake of total antioxidants in Japan,

calculated as the average H-ORAC value for "typical vegetables" was 594.3 μmol Trolox equivalents/100 g (Takebayashi et al., 2010). In a Norwegian diet, fruit, berries, cereals, vegetables, roots, dried fruits, and pulses contributed 43.6%, 27.1%, 11.7%, 8.9%, 7%, 1.5%, and 0.2%, respectively (Halvorsen et al., 2002). It is difficult to compare these data since they were calculated and expressed in different ways, nevertheless they highlight noticeable differences due to the different diet followed. The Institute of Medicine (2000) - an independent, nonprofit organization based in Washington (U.S.A.), which works outside of government to provide unbiased and authoritative advice to decision makers and the public - has established the recommended dietary allowance (RDA) for dietary antioxidants: vitamin C, 90 mg/day for men and 75 mg/day for women (tolerable upper intake level 2000 mg/day); vitamin E, 15 mg/day for men and women; selenium, 55 micrograms/day for men and women; no RDA was established for beta-carotene.

Living plant tissues are under constant oxidative stress and, for that reason, many of these tissues have developed antioxidant systems to control free radicals, lipid oxidation catalysts, oxidation intermediates, and secondary breakdown products (Brown and Kelly 2007; Iacopini et al., 2008). The plants that contain most antioxidants include members of several families, such as Rosaceae (dog rose, sour cherry, blackberry, strawberry, raspberry), Empetraceae (crowberry), Ericaceae (blueberry), Grossulariaceae (black currant), Juglandaceae (walnut), Asteraceae (sunflower seed), Punicaceae (pomegranate), and Zingiberaceae (ginger) (Halvorsen et al., 2002). However, content, activity and bioavailability of bioactive compounds in fruit and vegetable depend on a number of intrinsic (genus, species, cultivars) and extrinsic (agronomic, environmental, handling, storage, and processing) factors (Nicoli et al., 1999; Rapisarda et al.,

1999; Tomas-Barberan and Espin 2001) although the conclusions reached by different authors are not always consistent with each other. For example, Asami et al. (2003) reported that organically grown strawberries had higher phenolics content than conventionally grown crops, though another study could not establish such a correlation (Hakkinen et al., 2000). Bartoli et al. (1999) investigated the drought and watering-dependent oxidative stress in *Triticum aestivum* L. leaves. They found that the contents of α-tocopherol and β-carotene in wheat leaves were increased by 2.4-fold and 2.6-fold, respectively, after drought while drought decreased by 28.5% the content of reduced ascorbic acid. Scaglionet al. (2012) observed increases of antioxidant concentration of both Montepulciano grapes and wine as a consequence of canopy management such as early defoliation, cluster thinning and cluster cutting. In a study performed by Dabbou et al. (2010) on the effect of pedoclimatic conditions on the chemical composition of the Sigoise olive cultivar, the content of total phenols and lipoxygenase (LOX) oxidation products was higher for olives grown at the higher altitude, whereas that of α-tocopherol, carotenes, and chlorophylls was higher for olives grown at the lower altitude. Nadgórska-Socha et al. (2013) studied the accumulation of heavy metals and antioxidant responses in *Vicia faba* plants grown on monometallic contaminated soil. The antioxidant responses appeared to be metal specific. The elevation of guaiacol peroxidase activity in leaves and stems as well as the proline in leaves was the only more general reaction to metal exposure. Nitrogen limitation produces higher leaf content of phenolic compounds in pepper plants while a mild water stress slightly increased it (Estiarte et al., 1994). According to Ruhland et al. (2007), the solar ultraviolet-B radiation increases phenolic content in *Avena sativa*. Domestic and commercial food processing typically has drastic effects on the structural integrity of fruit and vegetable (Kalt, 2005). Compared with

other antioxidants, ascorbic acid is more susceptible to significant loss during postharvest handling, storage, and processing. When different methods of packaging were investigated, there was essentially no loss of vitamin C in fruit and vegetable stored in a modified atmosphere package, whereas unpackaged products or products packed in perforated film, lost great amounts of their vitamin C during storage. Large losses of vitamin C occur in processes that use water due to its water solubility (Gil et al., 1999). Depending on storage conditions, the content of certain phenolic compounds can increase or decrease. For example, accumulation of anthocyanins was observed in strawberries and raspberries when ripe fruit stored for various periods at 20 °C but total phenolic content and antioxidant capacity did not change (Kalt et al., 1999). Like vitamin C, phenolic antioxidants are water-soluble and can be leached from fruit and vegetable tissues by processing in water. Furthermore, the retention of carotenoids during storage is also affected by packaging. Modified atmosphere packaging prevented any loss in carotenoids whereas unpackaged products or products packed in perforated film lost about half of their carotenoid content under the same storage conditions (Barth and Zhuang, 1996). Therefore, it is advisable to increase the daily intake of fruit and vegetable obtained through minimally processing technologies designed to limit the impact of processing and packaging on nutritional and sensory quality of foods. Furthermore, it is getting interesting the introduction in the usual diet of the so-called “functional” foods, defined as “natural or processed food that contains known biologically-active compounds which, when in defined quantitative and qualitative amounts, provides a clinically proven and documented health benefit, and thus, an important source in the prevention, management and treatment of chronic diseases of the modern age” (Martirosyan, 2011). Antioxidants naturally present in fruit and vegetable can be extracted from their original

matrix and become useful candidates for functional ingredients in fortified foods. A noticeable source of antioxidants is constituted by solid wastes resulting from processing of minimally processed fruit and vegetable where peels, skins, seeds, stems and other lignocellulosic fractions that are usually discarded (Laroze et al., 2008).

This paper was intended to provide an overview of natural antioxidants, their biosynthesis and occurrence in edible plants, and, where available, their extraction systems.

VITAMIN C

The name vitamin C is referred to the L-enantiomer of ascorbic acid and its oxidized forms. The D-enantiomer is called D-ascorbate and while having equal antioxidant power, it is not found in nature, and has no physiological significance. (Aboul-Enein, 1999). L-ascorbic acid is the trivial name for the six-carbon sugar derivative L-threo-hex-2-enono-1,4-lactone.

The richest natural sources are fruit and vegetable although the amount depends on variety of the plant, soil condition, climate, length of time since it was picked, and storage conditions. Vitamin C is present in all living and actively metabolising parts of plants. It is not present in dormant seeds, but is made in large amounts when seeds germinate. In plants, vitamin C reaches concentrations over 20 mM in chloroplasts (where apparently plays a role in ameliorating the oxidative stress of photosynthesis) and occurs in all cell compartments, including the cell wall.

The vitamin C content of fruit is generally higher when they are immature, but for a few, such as the jujube fruit, the vitamin C content rises with increased ripeness. In foods of plant origin, the method of preparation must be included among the factors affecting vitamin C

content. According to the USDA (2010), the richest raw plant sources are represented by fruit unknown to most such as kakadu plum (1000-5300 mg/100g), camu camu – a cherry-like fruit (2800 mg/100g), and acerola (1677 mg/100g). The vitamin C content of the most consumed fruit and vegetable are relatively low: green chili pepper (190 mg/100g); kiwifruit and broccoli1 (90 mg/100g); strawberry (95 mg/cup); orange (50 mg/100g); lemon, melon, cauliflower (40 mg/100g); grapefruit, mandarin orange, spinach (30 mg/100g); potato, melon (20 mg/100g); tomato, blueberry, pineapple, grape, plum, watermelon (10 mg/100g); banana, carrot (8 mg/100g); cherry, peach (7 mg/100g); apple, asparagus (6 mg/100g); lettuce, pear (4 mg/100g); eggplant, fig (2 mg/100g). Most species of animals (but not humans or guinea pigs) make their own vitamin C. Therefore, some animal products can be considered as sources of dietary vitamin C: pork, beef, calf, chicken, lamb liver (23, 31, 36, 13, and 12 mg/100g, respectively), goat and cow milk (2 mg/100g, respectively). Although humans are not able to synthetized it, the human milk contains about 4 mg/100g vitamin C. Another important information is represented by the ratio between vitamin C and sugar content in fruit and vegetable. Among vegetables, the highest values of that ratio, expressed as mg/g, are 32.1 for green bell pepper and 29.8 mg/g for red bell pepper. Among fruits, the most interesting values are the following: strawberry 11.8, papaya 10.2, peeled orange 5.7, pineapple 5.4, and cantaloupe 4.7.

It is a metabolite with strong antioxidant activity and also it represents a cofactor for enzymes catalyzing numerous biochemical reactions, including those neutralizing the effects of reactive oxygen species. It is a necessary nutrient for only a limited number of animals, including humans, that are incapable of its synthesis and that must introduce vitamin C by means of dietary uptake (Giovannoni, 2007). Ascorbic acid can also act as a pro-oxidant (McGregor and

Biesalski, 2006). In fact, ascorbic acid reduces transition metals, such as cupric ions (Cu^{2+}), to cuprous (Cu^{1+}), and ferric ions (Fe^{3+}) to ferrous (Fe^{2+}) during conversion from ascorbate to dehydroascorbate in vitro (Satoh and Sakagami, 1997), but this reaction can generate superoxide and other reactive oxygen species (ROS). Vitamin C is found in high concentrations in immune cells, and is consumed quickly during infections. It has been hypothesized that vitamin C interacts with the immune system by modulating the activities of phagocytes, the production of cytokines and lymphocytes, and the number of cell adhesion molecules in monocytes (Preedy et al., 2010).

Most of animals and the plants are able to synthesize vitamin C, through a sequence of enzyme-driven steps, which convert monosaccharides to vitamin C. In plants, a biosynthetic pathway without inversion of the carbon skeleton and with the occurrence of L-galactono-1,4-lactone as the immediate precursor of L-ascorbic acid has been proposed (Wheeler et al., 1998). This pathway involves the conversion of GDP-D-mannose to GDP-L-galactose catalysed by a GDP-mannose-3',5'-epimerase. L-galactose released from the nucleotide is the immediate precursor of L-galactono-1,4-lactone, which is converted to L-ascorbic acid by action of a dehydrogenase. In some plant tissues, another pathway called uronic acid pathway has been proposed. It highlighted the role played by methyl ester of D-galacturonic acid as precursor and the occurrence of an inversion pathway (Smirnoff et al., 2001). In this pathway, pectin-derived D-galacturonic acid is reduced to L-galactonic acid, which in turn is spontaneously converted to L-galactono-1,4 lactone. This compound is the substrate of the L-galactono-1,4-lactone dehydrogenase enzyme. Alternative pathways have been discovered. GDP-L-gulose and myo-inositol are proposed as new intermediates in L-ascorbic acid biosynthesis, so that part of the

animal pathway might also be operating in plants. Furthermore, the GDP-mannose-3',5'-epimerase and L-galactono-1,4-lactone dehydrogenase are important regulatory steps for L-ascorbic acid biosynthesis. In some animals, the ascorbate synthesis is a glycogenolysis-dependent process since the glucose needed to produce ascorbate derived from glycogen. The process can occur in the liver (in mammals and perching birds) or in the kidneys (in reptiles and birds) (Bánhegyi and Mándl, 2001).

TOCOPHEROLS AND TOCOTRIENOLS

Vitamin E exists in eight different forms, four tocopherols and four tocotrienols. Tocopherols are a series of benzopyranols (or methyl tocols) whose C16 side chain is saturated while the C16 side chain of tocotrienols contains three trans double bonds at the 3', 7' and 11' positions, attached to the benzene ring. The unsaturation of the tails gives tocotrienols only a single stereoisomeric carbon (and thus two possible isomers per structural formula, one of which occurs naturally), whereas tocopherols have eight possible stereoisomers per structural formula, again, only one of which occurs naturally. The unnatural l-isomers of tocotrienols and half of the possible 8 isomers of the tocopherols lack vitamin activity. Among the stereoisomers that retain activity, increasing methylation (especially the full methylation related to the α-form) increases vitamin activity. The individual tocopherols are called 'vitamers' Together, tocopherols and tocotrienols are termed as tocochromanols. All feature a chromanol ring, with a hydroxyl group that can donate a hydrogen atom to reduce free radicals and a hydrophobic side chain that allows for penetration into biological membranes. The four main constituents of each of the two classes are termed: α (5,7,8-trimethyl), β (5,8-dimethyl), γ (7,8-dimethyl) and δ (8-methyl).

Tocochromanols are only synthesized by plants and other oxygenic, photosynthetic organisms, thus their intake must be guaranteed by the diet of animals. α-Tocopherol is the main source of vitamin E in the European diet due to the high intake of olive and sunflower oils while γ-tocopherol is the most common form in the American diet due to a higher intake of soybean and corn oils (Wagner et al., 2004).

The richest sources of vitamin E are vegetable oils, nuts, and seeds (including whole grains). Tocochromanols are found almost exclusively in the chloroplasts, where they limiting the damage from photosynthesis-derived reactive oxygen species during conditions of oxidative stress, including high-intensity light stress. According to USDA (2010), the vitamin E content, expressed in mg per 100g, of some food sources are the following: wheat germ oil (215.4); sunflower oil (55.8); almond oil (39.2); almond (26.2); hazelnut (26.0); peanut oil (17.2); olive oil (12.0); poppyseed oil (11.4); peanut (9.0). Several marine fish species contain, together with α-tocopherol, an unusual tocopherol called marine-derived α-tocomonoenol that is a more efficient radical scavenger at low temperatures. A related isomer with a Δ11 double bond has been found in palm oil and kiwi fruit whereas α- and γ-tocomonoenols have been detected in pumpkin seeds.

The biosynthesis of tocopherols, whose site is chloroplast, starts from tyrosine, which is oxidized to p-hydroxypyruvic acid. The enzyme p-hydroxyphenylpyruvate dioxygenase catalyze its convertion to homogentisic acid, which is condensed with phytyl diphosphate (geranylgeranyl diphosphate if the final products are represented by tocotrienols) by a prenyl transferase to give the 2-methyl-6-phytyl-plastoquinol. The latter is first methylated to form 2,3-dimethyl-5-phytyl-1,4-benzoquinol and then converted by the enzyme tocopherol cyclase to γ-tocopherol. A further

methylation reaction produces α-tocopherol, while changes of the pathway produce β- and δ-tocopherols.

CAROTENOIDS

Carotenoids are an important group of yellow, orange, and red pigments that are found in the chloroplasts and chromoplasts of plants and some other photosynthetic organisms like algae, and some bacteria and fungi. The colour of many flowers and fruit is due to carotenoid-containing chromoplasts, which are devoid of chlorophyll. In leaves, the carotenoids are generally masked by the chlorophylls but, in the autumn, the quantity of chlorophyll declines, thus making visibile the carotenoids and producing the yellow and red colours of the autumn foliage. For the same reason, carotenoid colours often predominate in ripe fruit such as oranges, tomatoes, bananas, after the disappearance of chlorophylls.

In plants, carotenoids work as accessory light-harvesting pigments since they cover regions of the visible spectrum not utilized by chlorophylls. Carotenoids absorb light most strongly in the blue portion of the spectrum (lutein has its maximum absorption at 450 nm, cryptoxanthin at 453 nm, zeaxanthin at 454 nm), thus enabling the chloroplast to trap a larger fraction of the radiant energy. Carotenoids don't transfer sunlight energy directly to the photosynthetic pathway, but pass it to chlorophylls. Furthermore, they protect against excessive light by quenching both singlet and triplet states of chlorophylls (Armstrong and Hearst, 1996).

Carotenoids are tetraterpenoids. This means that since they contain 40 carbon atoms, being built from four terpene units, each containing 10 carbon atoms. They are classified into two groups: those containing oxygen, which are named xanthophylls (for example, lutein and

zeaxanthin); and pure hydrocarbons, which don't contain oxygen and are named carotenes (α-carotene, β-carotene, and lycopene are some example). The known carotenoids are over 600. Carotenoids naturally occurred in form of: hydrocarbons; alcohols; alcohol esters; glycosides; ethers; epoxides; aldehydes; acid; acid esters; ketones; apo-, nor-, and seco-carotenoids; retro- and retro-apo-carotenoids; higher carotenoids. A list of high β-carotene foods, with concentration expressed as μg/100g, is the following: sweet potatoes 9444, raw kale 9226, raw carrots 8285, cooked kale 8173, raw turnip greens 6952, mustard greens 6300, dried basil 5584, dried parsley 5380, cooked collards 4814, marjoram 4806, cooked turnip greens 4575, red-leaf lettuce 4495, green-leaf 4443, raw butternut squash 4226, dried oregano 4112, cooked mustard greens 3794, ground sage 3485μg, dried coriander 3407, fresh thyme 2851, and iceberg 299 (http://www.healthaliciousness.com/articles/natural-food-sources-of-beta-carotene.php).

Carotenoids cannot be synthesized by humans and animals, thus they need to guarantee their intake through the diet. The exception is the red pea aphid, which has the genes necessary for synthesizing carotenoids, probably acquired from fungi via horizontal gene transfer. The most common carotenoids in North American diets are α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene (Institute of Medicine, 2000). Carotenoids in foods mainly exist in the all-trans form, although cooking may result in the formation of other isomers. The relatively low bioavailability of carotenoids from most foods is partly due to their association with proteins in the plant matrix (Yeum and Russell, 2002). Another reason of the different bioavailability is their antagonistic effect. The results of a study of van den Berg (1998) stated an inhibitory effect of lutein on β-carotene absorption, but apparently not on β-carotene cleavage. In a comparative study with two β-carotene/lutein ratios (2:1 and 1:2, respectively), this

inhibitory effect of lutein was found to be most marked when lutein was the predominant carotenoid. In studies on plasma (serum) response also an inhibitory effect of β-carotene on lutein response was observed, in agreement with the observations of Kostic et al. (1995). The study of Albanese et al. (1997). indicated that the β-carotene supplemented subjects had significantly higher serum concentrations of β-carotene (1483%), α-carotene (145%), β-cryptoxanthin (67%) and retinol (6%) while lutein was 11% lower in the supplemented group. Instead, serum lycopene, zeaxanthin, and α-tocopherol did not differ according to β-carotene-supplementation status. Bioavailability of carotenoids is also affects by the food matrix and the form in which carotenoids exist. The relative bioavailability of β-carotene from vegetables compared with purified β-carotene ranges between 3 and 6% for green leafy vegetables, 19 and 34% for carrots and 22 and 24% for broccoli (van het Hof et al., 2000). Based on their study on β-carotene and total carotenoids blood concentration from different food sources, de Pee et al. (1998) established that the equivalent of 1 retinol equivalent (RE) would be 12 μg β-carotene for fruit and 26 μg β-carotene for leafy vegetables and carrots. These differences may result from differences in intracellular location of carotenoids. In leaves, they are present in chloroplasts, whereas in fruits, and other parts of the plant, carotenoids are located in chromoplasts, where β-carotene is dissolved in oil droplets (de Pee et al., 1998). This has led to the speculation that chloroplasts may be less efficiently disrupted in the intestinal tract than chromoplasts. In carrots, β-carotene is present as crystals, which dissolve very slowly (de Pee et al., 1998). The relative bioavailability of lutein from a diet supplemented with a variety of vegetables is much greater than that of β-carotene (i.e., 67 and 14%, respectively) (van het Hof et al., 1999). The release of lutein into an aqueous environment is probably higher than that of β-carotene because of its

lower lipophilicity compared with β-carotene. The presence of dietary fibre in fruit and vegetable may explain in part the lower bioavailability of carotenoids from plant foods. It has been suggested that fibre interferes with micelle formation by partitioning bile salts and fat in the gel phase of dietary fibre but the results of the researchers are often contraddictory. Disruption of the food matrix and release of carotenoids constitute the first step in carotenoid absorption (van het Hof et al., 2000). In fact, operations such as chopping, homogenizing, and cooking disrupt the plant matrix, increasing their bioavailability (van Het Hof et al., 2000). The bioavailability of tomato lycopene is substantially improved by heating in oil (Gartner et al., 1997; Stahl and Sies, 1992). The Recommended Dietary Allowances for vitamin A are given as mcg of retinol activity equivalents (RAE) to account for the different bioactivities of retinol and provitamin A carotenoids. Since the body converts all dietary sources of vitamin A into retinol, 1 mcg of physiologically available retinol is equivalent to: 1 mcg of retinol, 12 mcg of β-carotene, and 24 mcg of α-carotene or β-cryptoxanthin from dietary sources. On food and supplement labels, vitamin A is listed in International Unit (IU). The conversion rates between mcg RAE and IU are the following (Otten et al., 2006): 1 IU retinol = 0.3 mcg RAE; 1 IU β-carotene from food = 0.05 mcg RAE; 1 IU α-carotene or β-cryptoxanthin = 0.025 mcg RAE. The RDA for adolescent and adult men is of 900 mcg RAE (Institute of Medicine, 2001). The tolerable Upper Intake Level for preformed vitamin A in adults is 3000 mcg RAE or 10000 IU (Institute of Medicine, 2001).

α-Carotene is mainly present in pumpkins and carrots whereas β-carotene is mainly contained in carrots, pumpkins, spinach, sweet potatoes, collards, kales, and turnip greens. Pumpkin and papayas are the foods richest in β-cryptoxanthin. Lycopene is abundant in tomatoes whereas lutein and zeaxanthin are present, in a decreasing order, in: spinaches, kales, turnip

greens, collards, dandelion greens, and mustard greens (USDA, 2008). de Pee et al. (1998) quantified the effectiveness of dietary retinol sources such as orange fruit and dark-green leafy vegetables in improving vitamin A status, and tested whether orange fruit is a better source of vitamin A and carotenoids than are leafy vegetables. They found that the apparent mean vitamin A activity of carotenoids in fruit and in leafy vegetables and carrots was 50% and 23% of that assumed, respectively. This has important implications for choosing strategies for controlling vitamin A deficiency.

α-Carotene, β-carotene, and β-cryptoxanthin are provitamin A carotenoids (i.e. they can be converted by the body to retinol - vitamin A). Vitamin A is essential for normal growth and development, immune system function, and vision. Lutein, zeaxanthin, and lycopene have no vitamin A activity. Currently, it would seem that the only essential function of carotenoids recognized in humans is that of provitamin A (Institute of Medicine, 2000). Carotenoids are also antioxidants and can inhibit the lipid peroxidation, but their actions in humans appear to be more complex (Young and Lowe, 2001) and it is unclear whether the biological effects of carotenoids in humans are a result of their antioxidant activity or of non-antioxidant mechanisms.

Carotenoids originate in the plastid-localized 2-C-ethyl-D-erythriol 4-phosphate (MEP) pathway that starts with the reaction between pyruvate and glyceraldehydes -3-phosphate. The first steps are regulated by the enzymes 1-deoxy-D-xylulose-5-phosphatate synthase and 1-deoxy-D-xylulose-5-phosphatate reductoisoerase. The second step is catalyzed by 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase and lead to the production of isopentenyl diphosphate and dimethylallyl diphosphate. The geranyl-geranyl diphosphate synthase catalyze the conensation of three molecules of isopentenyl diphosphate and one molecules of

dimethylallyl diphosphate to produce geranyl-geranyl diphosphate, a 20-carbon molecule. Two molecules of geranyl-geranyl diphosphate are condensed by phytoene synthase to form phytoene. Phytoene undergoes four sequential reactions to give lycopene. The introduction, catalyzed by lycopene β-cyclase, of a β-ring at both ends of lycopene gives origin to the β-carotene. Lycopene β-cyclase and lycopene ε-cyclase catalyze the introduction of a β-ring and a ε-ring to the ends of lycopene to form α-carotene. α-Carotene is acted upon by a β-ring hydroxylase to form zeinoxanthin, which is hydroxylated by a ε-ring hydroxylaseto produce lutein. β-carotene can be hydroxylated to give zeaxanthin, with β-cryptoxathin as an intermediate product (Farré et al., 2010; Walter and Strack, 2011).

PHENOLIC COMPOUNDS

Phenolics are ubiquitous and occur naturally in humans, plants, and animals where are involved in many processes (Harbone and Williams, 2000; Rispail et al., 2005). The terms “phenolic” and "polyphenol" includes more than 8,000 compounds with great structural diversity. As a general rule, they refer to the most abundant secondary natural metabolites arising biogenetically from the shikimatephenylpropanoids-flavonoids pathways, producing monomeric and polymeric phenols and polyphenols (Oksana et al., 2012). Phenolics are aromatic compounds containing a benzene ring with one or more hydroxyl groups attached directly to the benzene ring (Taylor and Chom, 2006).

Phenolic compounds have been categorized on the basis of their skeleton: C6 (simple phenol, benzoquinones); C6-C1 (phenolic acid); C6-C2 (acetophenone, phenylacetic acid); C6-C3 (hydroxycinnamic acids, coumarins, phenylpropanes, chromones); C6-C4 (naphthoquinones);

C6-C1-C6 (xanthones); C6-C2-C6 (stilbenes, anthraquinones); C6-C3-C6 (flavonoids, isoflavonoids); (C6-C3)2 (lignans, neolignans); (C6-C3-C6)2 (bioflavonoids); (C6-C3)n (lignins); (C6)n (catechol melanins); (C6-C3-C6)n (condensed tannins) (Lattanzio and Ruggiero, 2003).

The most abundant phenolic compounds in the diet are phenolic acids (hydroxybenzoic and hydroxycinnamic acids.), and flavonoids (30 and 60% of the total, respectively) (Escarpa and Gonzalez, 2008). Phenolic acids occur in different forms in plants, including aglycones (free phenolic acids), esters, glycosides, and/or bound complexes. Hydroxycinnamic acid compounds occur most frequently as simple esters with hydroxy carboxylic acids or glucose while hydroxybenzoic acid compounds are present mainly in the form of glucosides. Flavonoids are the largest group of plant phenols and the most studied and include more than 5000 compounds (Yao et al., 2004). They can be subdivided in 13 classes: chalcones, dihydrochalcone, auron, flavones, flavonols, dihydroflavonol, flavanones, flavanols (catechins), flavandioles or leucoanthocyanidins, anthocyanidins (its glycoside is called anthocyanin), isoflavononas, flavonoids, and condensed tannins or proanthocyanidins. Furthermore, they can be found as aglycones, although they are usually found as glycosides contributing to the colour (blue, scarlet, orange) of leaves, flowers, and fruit.

Phenolic substances are mainly deposited in leaves or bark (in case of trees or bushes), together with other waste products (Yanishlieva, 2001). The best phenolic acid sources are: rowanberry (103 mg/100 g), coffee (97 mg/100 g); chokeberry (96 mg/100 g), blueberry (85 mg/100 g), sweet rowanberry (75 mg/100 g), saskatoon berry (59 mg/100 g), green and black teas (30-36 mg/100 g); dark plum, cherry, and one apple variety (Valkea Kuulas) (28 mg/100 g).

Anthocyanins are present in fruit such as: bilberry (300-648 mg/100g); black berry (82-325 mg/100g); blueberry (25-495 mg/100g); sweet cherries (350-450 mg/100g); cranberry (50- 80 mg/100g). The richest sources of phenolic compounds, in terms of mg gallic acid equivalent/g of dry weight, are: strawberries (14.8-23.7); bilberry (29.7); hop (23.1); black currant (20.3); red currant (12.6); apple (11.9-12.1). The acai (*Euterpe oleracea* Mart.) and pomegranate (*Punica granatum* L.) fruits deserve a separate discussion. Acai is an amazonian palmberry, which has a dense pigmentation and a high antioxidant power due to its anthocyanin content (319 mg/100g) (Schauss et al., 2006a). Cyanidin-3-glucoside and cyaniding-3-rutinoside were the major anthocyanins detected by Lichtenthäler et al., 2005). Flavonoids are the major polyphenols in acai pulp including including homoorientin, orientin, isovitexin and scoparin (Schauss et al., 2006). Vitexin and quercetin were identified for the first time in acai pulp by Kanga et al. (2010). Proanthocyanidins amounted to 1.3 mg/100g of the freeze dried pulp skin berry with a profile similar to that of blueberry. Resveratrol was also found although at low levels (1.1 μ/g). According to the ORAC (Oxygen Radical Absorbance Capacity) assay, the antioxidant effect against peroxyl radical of a freeze-dried Acai powder was about 1027 micromol Trolox Equivalent/g (Schauss et al., 2006b). Pomegranate is a Mediterranean fruit, which is receiving an increasing attention due to its antioxidant content and activity. It a so-called "super food" that has been cultivated since ancient times. It is native to northern India but are grown everywhere from China and the Middle East to Arizona and California. Pomegranates have a yellow, red or pink tough, leathery rind. Transparent sacs filled with tart, juicy pulp and seeds called arils are found on the inside. Pomegranates is found in the raw form, in juice and in a hundreds of products from candy to breakfast bars. One cup of pomegranate juice contains 5923 μmol Trolox

Equivalent/100g (http://www.livestrong.com/article/324436-antioxidant-level-in-pomegranates/). According to Gil et al. (2000), commercial pomegranate juices showed an antioxidant activity (18-20 TEAC) three times higher than those of red wine and green tea (6-8 TEAC). HPLC-DAD and HPLC-MS analyses revealed that commercial juices contained the pomegranate tannin punicalagin (1500-1900 mg/L) thus indicating that industrial processing extracts some of the hydrolyzable tannins present in the fruit rind. This could account for the higher antioxidant activity of commercial juices together with anthocyanins, ellagic acid derivatives, and other hydrolyzable tannins.

Phenolics are formed by three different biosynthetic pathways: the shikimate/chorizmate or succinylbenzoate pathway, which produces the phenyl propanoid derivatives (C6–C3); the acetate/malonate or polyketide pathway, which produces the side-chain-elongated phenyl propanoids, including the large group of flavonoids (C6–C3–C6) and some quinones; the acetate/mevalonate pathway, which produces the aromatic terpenoids, mostly monoterpenes, by dehydrogenation reactions (Bhattacharya et al., 2010; Knaggs, 2001). The aromatic amino acid phenylalanine, synthesized in the shikimic-acid pathway, is the common precursor of phenol containing amino acids and phenolic compounds.

EXTRACTION SYSTEMS

Plant products and solid waste produced during food plant processing contain bioactive compounds with potential application in foods, cosmetic and pharmacologic formulations. Peels, skins, seeds, stems and other lignocellulosic fractions, usually discarded, are attractive sources of antioxidants (Laroze et al., 2008). Nevertheless, some of these compounds must be extracted

from the vegetable matrix. The extraction stage is extremely important, as its outcome will determine the release of analytes from the vegetable matrix into the medium (Escarpa and Gonzalez, 2008). Many extraction systems have been developed by researchers and their description is reported in the following paragraphs, together with an overview of the most representative applications, where available, for the different class of antioxidants.

Liquid-liquid extraction and solid-liquid extraction

They are the traditional extraction methods consisting in separation processes where the distribution of the analyte between two immiscible phases or between the solid and the liquid phase is made in order to arrive at the appropriate distribution coefficient (Dobiáš et al., 2010). The extraction procedure is performed using aqueous organic solvents, alone or mixed together, to extract antioxidants from plant tissues. Solvents are chosen in order to eliminate or reduce potential matrix interferences (Luthria, 2008). The optimisation of the extraction conditions (temperature, time, pH, solid-to-liquid liquid-to-liquid ratio, stirring, solvent polarity) is essential for a quantitative extraction of antioxidants from different food matrices.

The limit of these methods are the requirement of expensive and hazardous organic solvents (which are undesirable for health reasons) and of long time *per* analysis, giving rise to possible degradations (Proestos and Komaitis, 2008). For these reasons, these traditional extraction sample methods have been replaced by other systems, which are more sensitive, selective, fast, and environmentally friendly (Mahugo et al., 2009).

An alternative to the traditional solvent extraction is represented by the Soxhlet extraction. It was created over a century ago. It consists of placing the solid matrix particles into

a cartridge inside the extraction chamber, and the extraction solvent into a flask connected to the extractor chamber. The solvent is heated and condenses inside the cartridge thereby contacting the matrix particles and extracting the compounds of interest. The main advantages to this technique are the repeated washing of the matrix particles with fresh extracting solvent, the better solubilization because of the use of hot solvent, the versatility since it can be applied to nearly any food matrix. The main disadvantages are the long extraction time, the large amount of organic solvent used, the possibility of degradation for temperature sensitive compounds (Luque de Castro and Priego-Capot, 2010). Ahmad et al. (2010) employed the Soxhlet extraction technique for the extraction and separation of chemical constituents in the medicinal plant, Herba Leonuri. The study showed that methanol extracted almost double yield than n-hexane and that, for methanol extraction, the mass yield percent extracted increased with increasing length of extraction period whereas, for n-hexane extraction, the mass yield percent extracted was not consistent with increasing length of extraction period.

Extraction of tocopherols and tocotrienols

Solvent extraction commonly used hexane as a solvent to extract carotenoids and vitamin E from crude palm oil (Chiu et al., 2009). However, hexane possesses potential fire, health and environmental hazards. Thus, short-chain alcohols, especially ethanol and isopropanol, have been proposed as alternative extraction solvents due to their greater safety and reduced probability of regulation (Ping and Gwendoline, 2006). Rice bran can be submitted to solvent extraction in order to extract vitamin E and oryzanols. According to Hu et al. (1996), isopropanol could be considered a promising alternative solvent to hexane since preheated isopropanol (3:1

solvent/bran ratio and 60°C, 15 min.) extracted less crude oil but more vitamin E and similar amounts of oryzanol relative to preheated hexane.

Extraction of carotenoids

Carotenoids can be conveniently extracted from tomato waste with hexane/ethyl acetate (45:55 v/v), a solvent to waste ratio of 9.1:1 (v/w), and particle size 0.56 mm. In those conditions, the yield amounted to 37.5 mg kg^{-1} dry waste. The use of double extraction, each with 35 ml of hetanol/hezane (4:3 v/v) resulted in good recoveries of carotenoids (lycopene 96%, α-carotene 102%, and β-carotene 93-100%) from canned tomato juice (Taungbodhitham et al., 1998). According to Sarkar et al. (2012), grinding followed by homogenization and an acetone:methanol/ethanol (7:3) solvent system may be used for better extraction of carotenoids from algae.

Extraction of phenolic compounds

Solubility of phenolic compounds is governed by their chemical nature in the plant, which may vary from simple to very highly polymerized. Furthermore, they may interact with other plant components such as carbohydrates and proteins that may lead to the formation of complexes that may be quite insoluble. Additional steps may be required to remove the unwanted phenolics and non-phenolic substances such as waxes, terpenes, fats, and chlorophylls, which are soluble in the same solvents of the desired phenolics (Gómez et al., 2005; Naczk and Shahidi, 2006). Solvents such as methanol, ethanol, propanol, acetone, ethyl acetate, and their combinations have been used for the extraction of phenolics, often with different proportions of

water. For example, phenolic compounds can be efficiently extracted from beans and eggplants using an methanol/water (80:20 v:v) system (Espinosa-Alonso et al., 2006; Singh et al., 2009). Anthocyanins are usually extracted with acidified organic solvents, most commonly methanol. This solvent system destroys the cell membranes, dissolves the anthocyanins, and stabilizes them. However, the acid may bring about changes in the native form of anthocyanins by breaking down their complexes with metals and co-pigments (Naczk and Shahidi, 2009).

Enzyme-assisted extraction

Enhanced release of bioactive compounds from plant cells by cell disruption and extraction through the cell wall can be optimized using enzyme preparations either alone or in mixtures (Puri et al., 2012). The enzyme-assisted extraction methods are gaining more attention due to the need for eco-friendly extraction technologies. Enzymes are able to degrade or disrupt cell walls and membranes thus enabling better release and extraction of bioactives and enhancing product yield by minimizing the application of severe processing conditions. Application of enzymes is not more exploited within the food industry. The main limitation for the application of enzymes in industrial extraction processes is their high cost, although it is already possible to obtain enzymatic formulations with lower costs and better quality (Bhat, 2000; Zúñiga et al. 2003).

Extraction of carotenoids

Bunea et al. (2010) used cellulases, hemicellulases and pectinases from the beginning of the extraction to extract carotenoids from the marigold flowers. HPLC analysis of the extract

indicates that the original carotenoid profile was not altered. Çinar (2005) enzymically extracted carotenoid pigments from orange peel, sweet potato, and carrot using different concentrations of cellulase and pectinase combinations. It was observed that the same colour yield can be reached at lower concentrations of enzymes by extending the extraction time, thus resulting in lower costs of enzymes. A study of Arnous and Meyer (2010) examined the release of phenols during pectinolytic and cellulolytic degradation of the cell wall polysaccharides in skins of Merlot and Cabernet Sauvignon wine grapes (*Vitis vinifera* L.). Anthocyanins were released from skins during the early phases of the enzymatic treatments, but were then degraded during further enzymatic treatment. Flavonols underwent transformation from glycosylated (rutin) to deglycosylated (quercetin) during the enzymatic treatment. Phenolic acids were released as a function of monosaccharides liberation, i.e. as a function of the enzyme catalyzed cell wall degradation of the skins, and with some of the phenolic acids perhaps released from the lignin. Cuccolini et al. (2013) investigated the possibility of extracting lycopene from tomato waste peels through an enzymatic-assisted extraction. Cells were lysed thanks to a combination of pH changes and hydrolytic enzyme treatments. The lycopene-containing chromoplasts were then precipitated and centrifuged. At this stage the lycopene content of the isolated chromoplasts showed a 10-fold increase with respect to untreated tomato peels. A further improvement in lycopene concentration is obtained by a second enzymatic treatment using a protease cocktail. The final product showed a 30-fold increase with respect to the lycopene concentration of the untreated peels.

Extraction of phenolic compounds

Bioprocesses such as enzyme technology represent an alternative for production of bioactive compounds from agro-industrial byproducts. Gomez-García et al. (2012) used different types of commercial enzymes such as Celluclast® 1.5 L, Pectinex® Ultra, and Novoferm® were used to release phenolic compounds from grape wastes. Novoferm® had the strongest effect on phenolic release from grape waste, followed by Pectinex® Ultra and Celluclast® 1.5 L. The increment of antioxidant activity is associated with the release of *o*-coumaric acid. Li et al. (2006) determined the total phenolic contents of five citrus peels (Yen Ben lemon, Meyer lemon, grapefruit, mandarin and orange) extracted by enzyme-assisted aqueous extraction. The highest recovery using Celluzyme MX in the enzyme-assisted extraction process was up to 65.5% (about 87.9% of the solvent extraction).

Pressurized Fluid Extraction

Pressurized liquid extraction uses organic solvents at high pressures and temperatures above their normal boiling point. In general, a solid sample is packed into a stainless steel extraction cell and extracted with a suitable solvent under high temperatures (40–200 ºC) and pressure (500-3000 p.s.i.) for short periods of time (5–15 min). The extract is purged into a collection vial with the aid of a compressed gas (Garcia-Salas et al., 2010).

Extraction of tocopherols and tocotrienols

Milagros Delgado-Zamarreño et al. (2009) applied the pressurized fluid extraction for the simultaneous extraction of tocotrienols and tocopherols from cereals. The extraction recoveries were in the 80-114% range.

Extraction of carotenoids

Mustafa et al. (2012) applied the pressurized hot ethanol to the extraction of carotenoids from carrot by-products. The extraction procedure was optimized by varying the extraction time (2–10 min) and the temperature (60–180 °C). Time and temperature of the extraction had significant effect in the yield of carotenoids. Optimized conditions for extraction were found to be 60 °C, 50 bars, 5 min pre-heating plus 10 min extraction (5 × 2 min). Rodríguez-Meizoso et al. (2008) used three solvents of different polarities (i.e., hexane, ethanol, and water) to obtain pressurized liquid extracts with different compositions to extract bioactive compounds from unknown species of microalgae. Moreover, extractions were performed at four different extraction temperatures (50, 100, 150, and 200 °C) with 20 min as extraction time. In general, hexane and ethanol extracts showed higher antioxidant capacity that was mainly attributed to carotenoid compounds. The high antioxidant activity of the 200 °C water extracts was related to the presence of Maillard reaction compounds produced by thermal degradation of the sample. β-Carotene, lutein, violaxanthin, and neoxanthin were identified in 150 °C ethanol extracts.

Extraction of phenolic compounds

Luthria (2008) applied pressurized fluid extraction to phenolic compounds from parsley (*Petroselinum crispum*) flakes. He analyzed the effects of six pressurized liquid extraction parameters (temperature, pressure, particle size, flush volume, static time, and solid-to-solvent ratio). All extractions were carried out with either one or two solvent mixtures, ethanol-water (50:50, v/v) and/or acetone-water (50:50, v/v). The extraction yield of the phenolic compounds

was influenced by temperature, particle size, and solid-to-solvent ratio. Temperature had a major impact on the phenolic profile, as at higher extraction temperatures, malonyl-apiin was partially degraded to acetylapiin and apiin. Higher extraction yields of phenolic compounds were obtained with the smallest particle size fraction. The extraction efficiency of phenolic compounds per unit mass of sample matrix decreased with the increase of the solid-to-liquid ratio. Extraction of phenolic compounds was carried out at three pressure settings (1000, 1250, and 1500 psi) that are commonly applied to evaluate whether pressure influences the extractability of phenolics by increasing diffusivity of extraction solvent within the sample matrix (Carabias-Martínez et al., 2005). The results showed that pressure did not influence the extraction efficiency. Herrero et al. (2009) compared the performance of pressurized liquid extraction (using water and ethanol as solvents), supercritical fluid extraction (using neat CO_2 and supercritical CO_2 modified with ethanol), and a procedure called 'water extraction and precipitation on-line' for the extraction of antioxidants from rosemary (*Rosmarinus officinalis*). The results obtained in this study show that pressurized liquid extraction using ethanol at high temperatures (200 ºC) and 1500 psi was able to produce extracts with high antioxidant activity and high yield.

Supercritical Fluid Extraction

It is a relatively recent technique that presents various advantages over traditional methods, such as the use of low temperatures, reduced energy consumption, and high product quality due to the absence of solvents in the solute phase. Nevertheless the application of this technique is limited to compounds of low or medium polarity (Garcia-Salas et al., 2010). Supercritical fluid extraction is the process of separating one component (the extractant) from

another (the matrix, which is usually solid, but can also be liquid) using supercritical fluids as the extracting solvent. Carbon dioxide is the most used supercritical fluid, sometimes modified by co-solvents such as ethanol or methanol, due to its particular characteristics, such as moderate critical conditions (31.1 ºC and 73.8 MPa) and ready availability. It is also nontoxic, inflammable and chemically stable. The extraction selectivity may be increased by varying the pressure and temperature.

The solvent extraction is a diffusion-based process, the solvent must diffuse into the matrix, and the extracted material to diffuse out of the matrix into the solvent. In supercritical fluid, diffusivities are much faster than in liquids, and therefore extraction can occur faster. Furthermore, there is no surface tension, viscosity is lower than in liquids, so the solvent can penetrate into small pores inaccessible to liquids. An extraction using an organic liquid may take several hours, whereas supercritical fluid extraction requires 10 to 60 minutes (Skoog, 2007).

The system must contain a pump for the carbon dioxide, a pressure cell to contain the sample, a means of maintaining pressure in the system and a collecting vessel. The liquid is pumped to a heating zone, where it is heated to supercritical conditions, then passes into the extraction vessel, where it rapidly diffuses into the solid matrix and dissolves the material to be extracted. The dissolved material is sent into a separator at lower pressure, and the extracted material settles out. The resulted carbon dioxide can then be cooled, re-compressed and recycled, or discharged to atmosphere.

Extraction of tocopherols and tocotrienols

Kwon et al. (2010) observed that the best supercritical extraction conditions for the extraction of α- and β-tocopherols were 60 ºC and pressure of 30 MPa with a carbon dioxide (CO_2) flow rate of 26.81 g/min. According to Leo et al. (2005), the maximum recovery was obtained in the first 2–3 h of extraction at a pressure of 420 bar, a temperature of 50 °C, and a flow rate of 30 kg h^{-1} CO_2. According to Mariod et al. (2011), extraction of tocopherols using high pressure at 600 bar/40 °C or 600 bar/80 °C) gave higher total tocopherols when compared with hexane extraction from kenaf seeds. Olive tree leaves were treated with supercritical carbon dioxide to obtain tocopherol concentrates. The conditions applied were pressure in the range 25-45 MPa, particle size of 0.25-1.5 mm, solvent flow 0.5-1.5 SL/min and temperature of 313-333 K (De Lucas et al., 2002).

Extraction of carotenoids

Concerning carotenoids, numerous chemical modifiers (water, ethanol, methylene chloride, and hexane) have been tested to enhance the extraction process. Modifiers are similar to co-solvents (since they aid extraction), but they are added directly to the sample prior to extraction, instead of with the solvent. Talisic et al. (2012) investigate the response of the extractability of β-carotene from D. Salina algae using supercritical ethane and ethylene and making used of supercritical carbon dioxide as basis. The solubility of the non-polar β-carotene in supercritical ethane and ethylene are higher than those in carbon dioxide at the same reduced temperature and reduced density. Ethane and ethylene were better supercritical solvents than carbon dioxide since their greater polarizability, which resulted in an induced dipole. The induced dipole served as an attractive force between the solute and solvent molecules and

allowed the solvent to solubilize the solute molecules. According to Wei et al. (2005), the extraction of carotenoids from crude palm oil (which have a very low solubility in supercritical CO_2 was governed by its solubility in the supercritical CO_2 and can be enhanced by increasing pressure at a constant temperature or decreasing temperature at a constant pressure. Increasing the flow rate and decreasing the sample size can reduce the extraction time but do not enhance the solubility. Rozzi et al. (2002) investigated the extraction of caotenoids from tomato by-products. The results indicated that the percentage of lycopene extracted increased with elevated temperature and pressure until a maximum recovery of 38.8% at 86 °C and 34.47 MPa, after which the amount of lycopene extracted decreased.

Extraction of phenolic compounds

Carbon dioxide as extracting solvent is of no use for phenolic compounds because of its low polarity in comparison to most phenols (Castro-Vargas et al., 2010; Liazid et al., 2007). In fact, Castro-Vargas et al. (2010) compared different extraction systems for guava seed samples and found that the yield of the supercritical carbon dioxide extraction process in terms of phenolic fraction is lower than the value achieved by Soxhlet extraction with ethanol. The yield increase directly with solvent polarity and the use of EtOH as a co-solvent is useful to enhance the phenolic fraction yield. At constant temperature, the rise in pressure increases the yield due to density enhancement. At constant pressure, the phenolic yield decrease with rising temperatures due to the solvent density reduction (Garcia-Salas et al., 2010). Literature is rich of references concerning the application of supercritical fluids to the extraction of phenolics. Scalia et al. (1999) extracted flavonoids from chamomile owners at 40-45 °C and 203 bars, for 30 min

using 5% methanol as co-solvent. Antioxidants were extracted from cocoa hulls at 50°C and 100-200 bars, using methanol and acetone as co-solvent (Arlorio et al., 2005). Arlorio et al. (2005) extracted anthocyanins from edelberries and grape marcs at 30-40 °C and 250, 300 bars. Grape seeds were submitted to supercritical fluids in order to extract complex phenols and tannins (Murga et al., 2000) at 40 °C and 200-300 bars, for 180 min., using methanol and ethanol as-cosolvents. Tena et al. (1998) used supercritical fluids to extract *t*-resveratrol and other phenolics from an inert support. They found that only some of the phenolics studied (e.g. *p*-coumaric acid, *t*-resveratrol and salicylic acid) could be successfully extracted and the use of methanol as modifier was essential in order to extract them. Nevertheless, neither methanol percentages higher than 5%, flow rates above 2 mL/min nor extraction times longer than 15 min are recommended since they produced analyte losses. Water negatively affected the recovery of phenolics, as a result of undesirable partitioning.

Ultrasound-assisted extraction

This method uses ultrasounds, i.e. a cyclic sound pressure with a frequency greater than 20 kHz. In this method, the crushed sample is mixed with the suitable solvent and placed into the ultrasonic bath, where temperature and extraction time are set (Klejdusa et al., 2009). Ultrasounds induce cavitation that forms small bubbles in liquids and the mechanical disruption of cell walls, facilitating the release of contents. This causes a local increase in temperature and pressure, which increases solubility, diffusivity, penetration and transport of solvent and template molecules (Cintas et al., 1999; Luque-Garcia et al., 2003). The main targets have been polyphenols and carotenoids and in both aqueous and solvent extraction systems. The ultrasound

extraction trials have demonstrated improvements in extraction yield ranging from 6 to 35% (Vilkhu et al., 2008).

Extraction of carotenoids

Almahy et al. (2013) applied the ultrasound-assisted extraction to carotenoids to be used as natural dyes from carrots. Authors compared the % yield for water extraction using ultrasound and magnetic stirrer compared to conventional techniques. The results indicate that there was a significant improvement in the % yield of colouring matter extract obtained due to the use of ultrasound.

Extraction of phenolic compounds

Han et al. (2011) checked the application of an ionic liquid-based ultrasonic-assisted extraction method to the extraction of phenolic compounds from *Laminaria japonica* Aresch. Three kinds of 1-alkyl-3-methylimidazolium with different cations and anions were evaluated for extraction efficiency in comparison with the conventionally used water and methanol. Compared with the conventional solvents, they provided higher extraction efficiency. Ghafoor and Choi (2009) optimized the ultrasound-assisted extraction of phenolic compounds from grape peels. The optimal conditions included; 53.14% ethanol, 46.03 °C, and 24.03 min for the maximum total phenolic compounds; 53.06% ethanol, 50.65 °C, and 25.58 min for the maximum antioxidant activity.

Microwave-assisted extraction

Microwaves are non-ionizing electromagnetic waves of frequency between 300 MHz to 300 GHz and positioned between the X- ray and infrared rays in the electromagnetic spectrum. Microwave technology is commonly known for its use as heat treatment. Recently, microwave-assisted extraction has been applied in the development of extraction methods for organic compounds from soil, sediment, seed, and food matrices. Studies showed that the extraction is more effective when microwave energy is used (Mandal et al., 2007). A microwave device comprises four main components: a microwave generator – magnetron - which generates microwave energy; a wave guide, which is used to propagate the microwave from the source to the microwave cavity; the applicator, which receives the sample; the circulator, which allows the microwave to move only in the forward direction (Mandal et al., 2007). Microwaves are made up of two oscillating perpendicular fields, the electric field and the magnetic field. The former is responsible for heating. In microwave-assisted extraction, the principle of heating is based upon its direct impact with polar materials/solvents and is governed by two phenomena: the ionic conduction and the dipole rotation (Letellier and Budzinski, 1999). Ionic conduction refers to the electrophoretic migration of ions under the influence of the changing electric field. The resistance offered by the solution to the migration of ions generates friction, which determines the solution heating. Dipole rotation refers to the realignment of the dipoles of the molecule with the rapidly changing electric field. Obviously, only dielectric material or solvents with permanent dipoles do get heated up under microwave. The principle on the basis of microwave-assisted extraction is that the moisture inside the plant cells when heated, evaporates and generates high pressures on the cell. The pressure pushes the cell walls from inside and causes their rupture. In this way, the active constituents leaching out from the cells to the surrounding

solvent (Wang and Weller, 2006). The main advantage of microwave-assisted extraction is that similar recoveries to those of supercritical fluid extraction were achieved. However, care must be taken when working with flammable solvents or in the case of samples that contain constituents which couple strongly with microwave radiation to cause a rapid rise in temperature and thereby lead to potentially hazardous situations (Jáuregui and Galceran, 2001). The choice of solvent to be used in microwave-assisted extraction depends on the solubility of the target analyte, the interaction between solvent and plant matrix, and the microwave absorbing properties of the solvent. The latter increases as the so-called 'dissipation factor' (tan δ) increases. For example, hexane is transparent to microwave (it doesn't heat up under microwave), whereas water, methanol, ethanol has good microwave absorbing capacity, as indicate by the high tan δ value, and hence heats up faster and can enhance the extraction process. Small amount of water in the extracting solvent can penetrate easily into the cells of the plant matrix, thus facilitating heating and increasing the mass transfer of the active constituents into the extracting solvent. Sometimes mixtures of high and low microwave absorbing solvent composition were found to produce optimum results since a good microwave absorber could be a bad extraction solvent and *viceversa*. Zhou and Liu (2006) applied the microwave-assisted extraction to solanesol from tobacco. Ethanol was a relatively good absorber of microwave energy but not a good extraction solvent for solanesol. Hexane, instead, was found to be a good extraction solvent but not a good absorber of microwave energy. Therefore, the two solvents were mixed and the ratio able to give the best extraction yield was hexane:ethanol (1:3). Other important factors are solvent-to-solid ratio, microwave power and irradiation time, which influences each other.

Extraction of tocopherols and tocotrienols

Zigoneanu et al. (2008) found that isopropanol was the best solvent for the extraction of rice bran oil rich in γ-tocopherol and γ-tocotrienol as compared with hexane for both microwave-assisted and conventional solvent extraction. They also observed that no differences in oil yield, total vitamin E, and antioxidant activity of oil was noticed between microwave-assisted and solvent extractions at 40 °C.

Extraction of carotenoids

Vasu et al. (2010) applied microwave-assisted extraction to obtain bixin, a carotenoid widely used in the food industry as colourant, from *Bixa orellana* seeds. Theu treated fresh, whole seeds with ethyl acetate and distilled water using microwave power at 210W intensity for 18 min. In comparison with traditional heating, microwave assisted extraction was found as the most effective extraction procedure for isolation of bixin.

Extraction of phenolic compounds

According to Chen et al. (2004), 80% (v/v) aqueous methanol was found to be the optimum extracting solvent mixture for the extraction of chlorogenic and geniposidic acid in microwave-assisted extraction of *Eucommia ulmodie*. Japon-Lujan et al. (2006) applied the microwave-assisted extraction to oleuropein and related phenolics from leaves of *Olea europeaea*, used a mixture of ethanol:water (80:20) as solvent whereas Stebova et al. (2004) extracted phenolic compounds from *Hypericum perforatum* and *Thymus vulgaris* using aqueous

HCl as solvent. Rafiee et al. (2011) studied the extraction of phenolic compounds from olive leaves of Koroneiki, Roghani and Mission

varieties by maceration and microwave-assisted extraction with different solvents. They found that microwave-assisted extraction had a higher extraction yield compared with the maceration and that the highest phenolic concentration was achieved at 24 h in maceration method and at the 15 min of exposure to microwaves with ethanol. Singh et al. (2011) applied a response surface methodology to optimize the microwave-assisted extraction parameters (extraction time, methanol concentration, and microwave power level) for extraction of phenolics from potato peels. The conditions to achieve the maximum yields of total phenolics or of specific phenolic compounds were different. The maximum total phenolic content was obtained at methanol concentration of 67.33%, and treated the samples for 15 min at a power level of 14.67%. To obtain the highest level of ascorbic, caffeic, and ferulic acids, the optimum values were methanol 100%, 15 min, and 10% power level while the max chlorogenic acid content was obtained with methanol 100%, 5 min, and 10% power level. Alupului et al. (2012) applied microwave-assisted extraction to flavonoids and phenolic acids from *Cynara scolymus* leaves. The solvent was 50% solution water/ethanol while the solid-to-liquid ratio was 1/8 (w/v). The system was irradiated for 1, 2, 3 and 5 minutes at 70, 90, and 100 °C, with stirring. According to the results, 70 °C was the maximum allowable extraction temperature in that experiment and was considered as the optimum for achieving a high recovery yield of active constituents. At 70 °C, when the extraction time was longer than 3 min, the yield decreased because of the thermal oxidation caused by the excessive exposure.

Pulsed electric field extraction

Pulsed electric field processing is a technique requiring placement of food between two electrodes in a batch or continuous treatment chamber and its exposure to a pulsed voltage (typically, 0.1–5 kV/cm with pulses of 10–1000 μs for electroporation (electropermeabilization) of plant cells and non thermal extraction from solid foods). The duration and number of pulses should be limited to reduce the temperature increase, which is generally of no more than 3-5 °C (Vorobiev and Lebovka, 2010).

Extraction of phenolic compounds

The application of pulsed electric field combined to a control extraction at 70 °C for 1 h of anthocyanins from grape by-products enhanced the antioxidant capacity of the extracts up to four-fold higher than the control extraction. The solvent used was a mixture of ethanol and water (50:50, v/v), the solid-to-liquid ratio was 1:4.5. Liu et al. (2008) applied unconventional extraction methods including ultrasonication, high voltage electrical discharge, and pulsed electric field to extract polyphenols from grape seeds. The treatment were conducted with grape seeds and distilled water (liquid-to-solid ratio 5:1, w/w) at T= 50°C. At the same energy consumption, high voltage electrical discharge permitted higher polyphenol extraction than the other two methods and the results obtained through the application of pulsed electric field were the worst.

CONCLUSIONS

Naturally antioxidants are largely distributed in the plant kingdom, where they occur thanks to complex biosynthetic pathways including many enzymatic reactions. Since there are hierarchies in antioxidant concentration among different plant and plant products, the knowledge of the richest fruit and vegetable helps to focus on them the extraction procedures.

The application of advanced technologies such as enzymatic-assisted, pressurized fluid, supercritical fluid, ultrasound-assisted, microwave-assisted, and pulsed electric field extractions has demonstrated to offer an extraordinary potential in terms of enhancing of the antioxidant extraction yields. Furthermore, they represent feasible green alternative methods, which often utilizes cheap and non-toxic solvents. Under optimized conditions, they can be thought as suitable techniques for scale up to handle larger sample sizes for industrial applications.

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