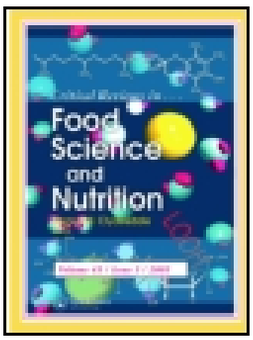

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Natural Colorants: Pigment Stability and Extraction Yield Enhancement via Utilization of Appropriate Pretreatment and Extraction methods

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Abstract

Natural colorants from plant-based materials have gained increasing popularity due to health consciousness of consumers. Among the many steps involved in the production of natural colorants, pigment extraction is one of the most important. Soxhlet extraction, maceration and hydrodistillation are conventional methods that have widely been used in industry and laboratory for such a purpose. Recently, various non-conventional methods such as supercritical fluid extraction, pressurized liquid extraction, microwave-assisted extraction, ultrasound-assisted extraction, pulsed-electric field extraction and enzyme-assisted extraction have emerged as alternatives to the conventional methods due to the advantages of the former in such terms as smaller solvent consumption, shorter extraction time and more environmental friendliness. Prior to the extraction step, pretreatment of plant materials to enhance the stability of natural pigments is another important step that must be carefully taken care of. In this article, a comprehensive review of appropriate pretreatment and extraction methods for chlorophylls, carotenoids, betalains and anthocyanins, which are major classes of plant pigments, is provided by using the pigment stability and extraction yield as the assessment criteria.

Keywords: Anthocyanins; Betalains; Carotenoids; Chlorophylls; Natural products; Non-conventional extraction.

1. Introduction

Among many characteristics of food, color is one of the most important as it can lead to a good first impression of a product. Unfortunately, most food processing operations, especially those involving the use of heat, generally causes alteration, degradation or even loss of food color. For this reason, a wide array of food colorants (i.e., pigments and dyes) has been added into food to enhance or intensify its original color. Colorants can also be added to ensure color uniformity or to obtain the best food appearance or even to provide color to otherwise uncolored food (Delgado-Vargas and Paredes-López, 2003).

Most food colorants that are in use today are derived from minerals or synthetic processes. Seven types of synthetic pigments, which are Brilliant Blue FCF (FD&C Blue No. 1), Indigotine (FD&C Blue No. 2), Fast Green (FD&C Green No. 3), Erythrosine (FD&C Red No. 3), Allura Red AC (FD&C Red No. 40), Tartrazine (FD&C Yellow No. 5) and Sunset Yellow FCF (FD&C Yellow No. 6), are permitted in foods under the Food and Drug Administration (FDA) regulations (Harp and Barrows, 2015). Although synthetic colorants possess higher stability, more diverse hue and vibrant color, their consumption has been reported to be related to many adverse health effects such as attention problems, hyperactivity, irritability, sleep disorders and aggressiveness in children (Masone and Chanforan, 2015). Due to health consciousness of modern consumers, colorants produced from natural sources (natural colorants) are an attractive alternative and have gained increasing popularity. Chlorophylls, carotenoids, betalains and anthocyanins are four important classes of natural pigments that are abundant in nature and their extracts have been approved as food additives by FDA and Codex (Francis, 1996). Nevertheless,

less stability and limited diversity in terms of color shade are still problems of natural colorants (Delgado-Vergas and Paredes-Lopez, 2003).

In general, to produce a natural colorant, extraction process is firstly required to extract crude pigment from a starting material, which is most of the time a plant material. Extraction of a plant material can be done by various extraction techniques. Normally, conventional methods such as Soxhlet extraction are used. More recently, however, many non-conventional methods such as supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), pulsed-electric field (PEF) extraction and enzyme-assisted extraction (EAE) have been proposed due to their enhanced extraction efficiency and environmental friendliness (Azmir et al., 2013). A suitable extraction technique helps increase the extraction yield and prevent the degradation of extracted pigments, leading to an ability to produce natural colorants of higher quality.

A number of researchers have also proposed the use of various pretreatment methods prior to extraction to increase the extraction yield and/or to enhance the stability of pigments. Thermal pretreatments (e.g., steam and hot-water blanching) and chemical soaking are among the most popular methods that can be used to pretreat a plant material. Thermal treatments are mainly used to inactivate deleterious enzymes, which cause pigment degradation; these treatments also help induce changes in the physical structure of a plant material, resulting in an enhanced extraction yield (Heaton and Marangoni, 1996; Stamatopoulos et al., 2012). Enhancement of pigment stability by using chemical solutions (e.g., organic acid solutions, alkaline solutions, ionic solutions) is also well-recognized (Maharaj and Sankat, 1996; Reynoso et al., 1997; Bąkowska et al., 2003; Hiranvarachat et al., 2011). However, selecting a proper

pretreatment method for each pigment group is of paramount importance and must always be done with great care.

This article aims to provide a comprehensive review of appropriate pretreatment and extraction methods for each group of the four important pigments, namely, chlorophylls, carotenoids, betalains and anthocyanins, by using the stability and extraction yield of the pigments as the assessment criteria.

2. Important classes of natural pigments and their stability

Two systems have been used to classify natural pigments. The first one is based on the structural affinities; natural pigments are divided into six major structural classes, which are tetrapyroles, tetra-terpenoids, quinones, *N*-heterocyclic, *O*-heterocyclic and metallo-proteins (Hendry, 1996). This system has an advantage of brevity, but biosynthetic compounds, which are quite unrelated, are grouped together. The other classification system is based on natural occurrence of the pigments (Hendry, 1996), and is the system adopted in the present article. According to this system of classification, pigments in plant materials are classified into chlorophylls, carotenoids, betalains and anthocyanins.

2.1 Chlorophylls

Chlorophylls are oil-soluble and the most widely distributed pigments responsible for the characteristic green color of plants. The structure of chlorophylls is porphyrins or closed ring

tetrapyroles chelated with a centrally located magnesium atom (Marquez and Sinnecker, 2008) as shown in Figure 1. There are five classes of chlorophylls that have been found in plants and photosynthetic organisms. However, the major chlorophylls in foods are chlorophyll *a* and chlorophyll *b*. These chlorophylls are different at position 7, where chlorophyll *a* is composed of $–CH_3$ and chlorophyll *b* is composed of –CHO. This difference leads to the difference in color; chlorophyll *a* appears blue-green, while chlorophyll *b* possesses yellow-green color (Steet and Tong, 1996).

Chlorophylls are highly sensitive to heat, light, oxygen, acids and enzymes, leading to their easy degradation and color change (Özkan and Bilek, 2015). Acids and Mg-dechelatase, which is an enzyme found in algae and plants, are the main causes of conversion of the chlorophylls color from green to olive brown (Marquez and Sinnecker, 2008). These changes occur due to the loss of central magnesium atom in the chlorophylls structure, with the replacement by hydrogen ions, leading to the transformation of the chlorophylls structure from native chlorophylls to pheophytin, which exhibits olive brown color (Delgado-Vargas and Paredes-López, 2003). Other enzymes such as chlorophyllase and oxidative enzymes (e.g., lipoxygenase, chlorophyll oxidase and peroxidase) are also related to chlorophylls degradation and cause color changes (Marquez and Sinnecker, 2008). Thus, inhibition of enzyme activity and avoidance of acidic condition must be exercised to prevent changes in the chlorophylls color.

Heat is another important factor that indirectly causes the conversion of the chlorophylls structure, which subsequently leads to color changes. Many researchers have investigated the effect of thermal processes on the change in the color of chlorophylls. For example, Lau et al. (2000) studied the color change of green asparagus during thermal treatments and found that the

color of the sample changed to olive color due to chlorophylls degradation by heat. In fact, heat simply induces breaking up or disruption of plant cell walls; intracellular acids thus release out of the cells, resulting in the change of pH to an acidic condition (Fellows, 2000; Marquez and Sinnecker, 2008). For these reasons, the structure of native chlorophylls changes to pheophytin and hence the color change from green to olive brown as mentioned earlier.

2.2 Carotenoids

Carotenoids are lipid-soluble pigments responsible for many of the brilliant red, orange and yellow colors of fruits, vegetables and flowers (Ötles and Çagindi, 2008). Based on their structure, carotenoids can be divided into two groups, which are carotenes and xanthophylls (Figure 2). Carotenes are constituted by carbon and hydrogen, while xanthophylls are constituted by carbon, hydrogen and oxygen (Britton, 1996). Carotenoids can be divided based on their functionality into primary and secondary carotenoids. Primary carotenoids (e.g., *β*-carotene, lutein, zeaxanthin, antheraxanthin) are pigments required for the photosynthetic process, while secondary carotenoids (e.g., α-carotene, lycopene, astaxanthin, canthaxanthin) are pigments that are not directly related with plant survival (Delgado-Vargas et al., 2000).

Degradation of carotenoids is mainly caused by reactions of oxidation and isomerization, which lead to a decrease in the redness and yellowness of a plant material (Provesi and Amante, 2015). There are several factors affecting the occurrence of oxidation and isomerization of carotenoids. Oxygen naturally causes oxidation of carotenoids; the reaction can also be stimulated by light, heat, peroxide, metal ions and enzymes (Boon et al., 2010). Most carotenoids in plants are *trans*-isomer, while isomerization of the *trans*-isomer to *cis*-isomers occurs during

food processing (Khoo et al., 2011). Heat and light as well as acids are main factors that can promote isomerization of carotenoids from *trans*-isomer to *cis*-isomers (Provesi and Amante, 2015). However, isomerization of carotenoids leads only to slightly reduced biological activities and color saturation, while oxidation leads to complete losses of the activities and color of carotenoids (Takyi, 2001). Several precautions (e.g., processing under low pressure, storage in dark under low-emperature and low-oxygen condition) have therefore been proposed to prevent oxidation reaction (Ötles and Çagindi, 2008; Khoo et al., 2011).

2.3 Betalains

Betalains are *N*-heterocyclic water-soluble pigments, which exhibit red-purple or yellow color depending on the pigment structure (Delgado-Vargas and Paredes-López, 2003). These pigments are found in many fruits, vegetables and flowers (e.g., prickly pear, red beet root, aizoaceae). Betalains can be classified based on their structure into two groups, which are betaxanthins (yellow pigment) and betacyanins (red-purple pigment) as shown in Figure 3 (Chandrasekhar et al., 2015).

Instability of betalains to light, heat, alkaline, oxygen and metal ions restricts them from extensive applications in food (Khan and Giridhar, 2014). Among various factors, heat is the most critical factor affecting the degradation of betalains (Reshmi et al., 2012), while other factors affect heat sensitivity of betalains. Heat mostly induces oxidation (i.e., dehydrogenation in the presence of oxygen), aldimine bond hydrolysis and decarboxylation of batalains, which results in color change to orange-yellow (Gonçalves et al., 2013). Since betalains are susceptible

to heat, it is most important to immediately cool down a treated plant material after thermal treatment (Henry, 1996).

Besides heat, stability of betalains is also related to pH. These pigments are most stable in a pH range of 3 to 7. Betacyanins are more resistant to acidic condition, while betaxanthins are most stable at neutral pH (Stintzing and Carle, 2008). Within the pH range of 3 to 7, the color of betalains does not change. When the pH is below 3, the structure of betalains is converted from red anionic to violet cationic, resulting in the color change from red to blue-violet shade (Henry, 1996). On the other hand, alkaline condition (pH higher than 7) brings about aldimine bond hydrolysis, which results in rapid degradation of betalains into betalamic acid (BA) and cyclodopa-5-*O*-glucoside (CDG) and the color change to yellow-brown (Henry, 1996).

Metal ions (e.g., Sn^{2+}, Al^{3+}, Ni^{2+}, Cr^{2+}, Fe^{2+}, Fe^{3+} and Cu^{2+}), which may contaminate a plant (from soil accompanying the plant) can accelerate oxidation of betalains, resulting in color losses (Stintzing and Carle, 2008). However, contamination of metal ions can be minimized by washing. Light does not significantly cause degradation of this group of pigments (Ötles and Çagindi, 2008).

2.4 Anthocyanins

Anthocyanins are water-soluble pigments found in many flowers, fruits and leaves (e.g., butterfly pea, red onion, grape, purple corn) with various shades (orange, red or blue) (Delgado-Vargas and Paredes-López, 2003). The basic structure of anthocyanins is glycosides of anthocyanidins, which is known as flavylium cation (Mercadante and Bobbio, 2008). The term anthocyanidins is referred to anthocyanins without sugar molecules. The most abundant

anthocyanins in nature are based on six anthocyanidins (Wu and Prior, 2005), which are pelargonidin (plg), cyanidin (cyd), delphindin (dpd), penodin (pnd), petunidin (ptd) and malvidin (mvd), as shown in Figure 4. The differences in the number of hydroxyl and methoxyl groups in the structure affect the color variation of the pigments. Pigments that have a larger number of hydroxyl groups exhibit more bluish shade, while those having a larger number of methoxyl groups exhibit more redness (Delgado-Vargas and Paredes-López, 2003).

The color stability of anthocyanins is influenced not only by the structural features, but also by the pH, temperature, light, presence of co-pigments, enzymes, oxygen and sugars (Rodríguez-Saona et al., 1999). Change in pH causes reversible transformation of anthocyanins structure and significantly affects the shift in color of anthocyanins. In an aqueous solution, the molecular species of anthocyanins, which are flavylium cation, carbinol pseudobase, quinoidal base and chalcone, are in equilibrium. Flavylium cation, which exhibits red color, dominates at pH 1, but when the pH increases to 4 or 5, the concentration of carbinol pseudobase, which is colorless, increases at an expense of the flavylium cation. At pH 6, hydroxyl groups present in the structure and the concentration of quinoidal base increases, yielding unstable blue or violet color. Quinoidal base continuously increases until the pH rises to 8; quinoidal base would then transform into chalcone, which exhibits yellow color (Delgado-Vargas and Paredes-López, 2003; Ananga et al., 2013). Structural transformation and color change of anthocyanins with pH are shown in Figure 5.

Heat is well known as an important factor affecting the color and stability of natural pigments. However, anthocyanins are more stable to heat than the aforementioned pigments. Degradation of anthocyanins occurs when temperature approaches 100 °C or even higher

(Jackman and Smith, 1996). The first step of thermal degradation involves the formation of colorless carbinol pseudobase and subsequent opening of the pyrylium ring to form chalcone, before transforming into a brown degradation product (Perera and Baldwin, 2001).

The color stability of each pigment is briefly summarized in Table 1.

3. Stability enhancement via pretreatments

Due to the instability of natural pigments, various pretreatment methods have been suggested to enhance the stability and also to modify the structure of plant materials. Pretreatment methods may be divided into three groups, which are physical, chemical and biological pretreatments. Chemical pretreatments (e.g., acid and alkaline treatments) are proposed to enhance the stability of pigments. On the other hand, the main purpose of physical pretreatments (e.g., microwave heating, pulsed-electric field and ultrasonic treatments) and biological pretreatments (e.g., enzymatic treatment) are plant structural modification to enhance subsequent extraction; the use of physical and biological pretreatments in combination with extraction will be discussed in the later section. Since the stability depends on the type of pigments, a suitable method of pretreatment that should be used to increase the stability also depends on the type of the pigments.

3.1 Stability enhancement of chlorophylls

Enzymes and acids are the major factors affecting the degradation of chlorophylls as mentioned in Section 2.1. Thus, enhancing the stability of chlorophylls pigments may be done by inactivating the enzymes and/or preventing an acidic condition. Many pretreatment methods

have been proposed for such purposes. Blanching is a short-time thermal treatment, which is usually performed by subjecting a sample to hot water or steam, with the aim to inactivate enzymes involving in the change of color of plant materials (Bahçeci et al., 2005). Most enzymes normally suffer decreased activity when being heated at above 80 °C (von Elbe and Schwartz, 1996). Since peroxidase is one of the highly thermal resistant enzymes and its activity is easy to measure, it is generally used as an indicator for adequate blanching (Bahçeci et al., 2005; Viña et al., 2007). Olivera et al. (2008), for example, reported that blanched Brussels sprouts exhibited more greenness than the un-blanched sample after storage at -18 °C for 8 months. However, after 8 months of frozen storage, the greenness values of both blanched and un-blanched samples decreased. Blanching nevertheless leads to changes in the plant cellular structure, resulting in sometimes undesirable softening of plant tissues (Fellows, 2000). In addition, blanching cannot solve the problem of acidic condition that may occur in further processing steps.

Alkaline treatment is proposed to prevent an acidic condition. Alkalinizing agents (e.g., calcium hydroxide, magnesium hydroxide, potassium hydroxide, sodium hydroxide and potassium bicarbonate) in combination with blanching are usually used to enhance the stability of green color. Alkalinizing agents raise the pH of a blanched sample, thus enabling the retention of green color of chlorophylls (Negi and Roy, 2001; Ahmed et al., 2013). Maharaj and Sankat (1996) reported that blanching dasheen leaves in alkaline solution (0.06% magnesium carbonate solution) at 100 °C for 10 s prior to drying at 60 °C resulted in better retention of green color of the dried product when compared with the products obtained from unblanched and even steam-blanched samples. Unfortunately, commercial application of alkaline treatment is still not too effective as alkalizing agents do not have the ability to neutralize interior tissue acids over a long

period of time, leading, for instance, to the change in the color of canned green peas after less than 2 months of storage at room temperature (von Elbe and Schwartz, 1996).

Complexation of chlorophylls into metal complexes of chlorophyll derivatives (e.g., copper pyropheophytin and zinc pyropheophytin) has been proposed to alleviate the aforementioned problems. Chlorophylls can be transformed into metallo-chlorophyll derivatives, which exhibit green color like native chlorophylls but are more stable to acids and heat (Marquez and Sinnecker, 2008). Although both copper and zinc ions can be utilized for metallo-chlorophyll derivatives formation, zinc ions are of greater interest due to the toxic nature of copper ions (Delgado-Vargas and Paredes-López, 2003).

To create metallo-chlorophyll derivatives, chlorophylls are first acidified to change the structure of chlorophylls to pheophytin prior to blanching in a medium containing metal ions such as zinc or copper ions to form metallo-chlorophyll derivatives (Tonucci and von Elbe, 1992) as illustrated in Figure 6. Many researchers have indeed investigated the thermal stability of these metallo-chlorophyll derivatives and reported improved results. For example, Ngo and Zhao (2005) reported that blanching green pear chunks at 94 °C in 2600-ppm Zn^{2+} solution for 13 min could preserve the green color of the samples during sterilization process (94 °C, 20 min). The color of the sample did not change for 19 weeks at 38 °C under intensive illumination or for at least 35 weeks at 10 °C.

3.2 Stability enhancement of carotenoids

Degradation of carotenoids, with their highly conjugated double-bond structure, is mainly due to the oxidation reaction (von Elbe and Schwartz, 1996). Inactivation of oxidative enzymes

is among the possible means of solving the problem of carotenoids oxidation. Hot-water or steam blanching is a simple pretreatment method that can be used to inactivate enzymes such as lipoxygenase, which catalyzes oxidative decomposition of carotenoids (von Elbe and Schwartz, 1996). However, although blanching has a benefit of enzyme inactivation, many investigators have reported that blanching in some cases led to degradation of the carotenoid pigments (Hiranvarachat et al., 2011). Marx et al. (2003) and Lavelli et al. (2007) who studied the effect of blanching on the carotenoids degradation of carrot juice and dehydrated carrot, respectively, reported that carrot juice and freeze-dried carrot produced from blanched carrot suffered more extensive degradation of carotenoids than those produced from un-blanched carrot; the degradation led to the fading color of the products. This observation might be caused by the change in the carotenoids structure from *trans*- to *cis*-isomers due to heat upon blanching.

Chemical pretreatments are another alternative method to prevent the oxidation of carotenoids. Antioxidant agents (e.g., butylated hydroxyanisol, pyrogallol and citric acid) are proposed to retard pigment degradation by oxidation (Ötles and Çagindi, 2008). Hackett et al. (2004) reported that addition of antioxidants (α-tocopherol or butylated hydroxytoluene) helped decrease the degradation rate of lycopene in tomato heated at 50 °C. Hiranvarachat et al. (2011) showed that β-carotene content of carrot soaked in citric acid (pH 4.0-5.0) tended to be unchanged during drying (hot air drying), while β-carotene content of untreated carrot decreased continuously. However, adding acid-based antioxidants leads to a decrease in the pH of a sample; when the pH of a sample becomes less than 3.0, carotenoids would start to degrade. Note that carotenoids would significant degrade at pH below 3.0, while become more stable at pH between 4.0 and 6.0. Under acidic condition (pH < 3.0), carotenoids are protonated, resulting

in isomerization of their structure from *trans*-isomer to *cis*-isomers (Qian et al., 2012). Thus, the pH of a sample should be carefully controlled when acid-based antioxidants such as organic acids are used.

3.3 Stability enhancement of betalains

To improve the stability of betalains, blanching is usually required to inactivate the betacyanin decoloring enzyme, which leads to color fading (Delgado-Vargas and Paredes-López, 2003). Nevertheless, heat is the most crucial factor affecting the stability of betalains. As mentioned in Section 2.3, the color loss due to the degradation of betalains is mainly caused by heat and alkalinity, which induce aldimine bond hydrolysis, leading to the separation of betalains structure into BA and CDG (Henry, 1996).

Betalains can be regenerated after thermal processing via the use of acids (Herbach et al., 2006b). Various organic acids (e.g., ascorbic acid, isoascorbic acid, citric acid and gluconic acid) have been proposed to aid the regeneration as well as to retain the stability during processing and storage of betalains. Bilyk et al. (1981) noted that addition of isoascorbic acid into a betacyanins solution either before or after heating (at 100 °C) helped regenerate betacyanins. The regenerated betacyanins was restored almost completely after 24 h (storage in dark at 25 °C). On the other hand, the degraded betaxanthins could not be regenerated by the use of isoascorbic acid due to its instability in acidic condition (Stintzing and Carle, 2008). Then, organic acid is a suitable pretreatment for enhancing the stability of red colorant from betacyanins but not for yellow colorant from betaxanthins.

The quantity of regenerated betacyanins is affected by pH, storage temperature, type of additives and the presence or absence of oxygen (Bilyk and Howard, 1982). Han et al. (1988) studied the regeneration of degraded red beet betacyanins in the presence of different types of acid-based additives, which were antioxidants (ascorbic acid, isoascorbic acid and gluconic acid), organic acids (acetic, citric, lactic, gallic and gluconic acids) and inorganic acids (metaphosphoric acid, phosphoric acid and Na_2EDTA). Acid-based additives helped regenerate the red beet pigments that were degraded by heating at 100 °C for 5 min. The degraded pigments were restored under 10 °C for 10 min. The effect of pH on pigment regeneration was also evaluated and it was found that the addition of ascorbic acid and isoascorbic acid resulted in the highest retention of the pigments (98.3%) for the sample controlled at pH 3.0. Addition of gluconic acid and metaphosphoric acid also resulted in high retention of the pigments, with the retention of 81.7% and 75.4%, respectively, for the sample controlled at pH 6.8.

Besides the ability to regenerate the pigment after heating, ascorbic acid and isoascorbic acid could also help stabilize betacyanins during food processing and storage. Reynoso et al. (1997) reported that garambullo juice that was added with 0.1% ascorbic acid exhibited higher redness than the untreated sample after sterilization at 121 °C for 15 min. The presence of the acid in red beet juice and garambullo juice (adjusted pH to 5.5) also resulted in an increase in the stability of betalains during storage at 25 °C for 5 days. Moreover, ascorbic acid could improve the stability of betalains when metal ions (chromium and iron) are present since the acid could act as a chelating agent (Pokorny, 2007).

3.4 Stability enhancement of anthocyanins

Anthocyanins are highly sensitive to the change in pH, which results in the shift of color. Using anthocyanins as a natural colorant is suggested at low pH (pH < 4.0). At pH below 4, anthocyanins are primarily in the form of flavylium cation, which is more stable than the other structures (Tan et al., 2014). Cevallos-Casals and Cisneros-Zevallos (2004), for instance, reported that at pH in the range of 0.9 to 4.0, the colorant samples from red sweet potato and purple carrot showed high stability during storage at 20 °C for 134 days; hue angle of the red sweet potato colorant at pH 4.0 still exhibited red-violet color after the storage. In contrast, the color of the samples that were controlled at pH above 4.0 shifted from purple-blue to brown and yellow and after 134 days the color of all the samples was predominated by the yellow-colored chalcone.

Besides a proper pH adjustment, the source of anthocyanins must be considered to obtain the most stable colorant. Different sources of plant materials contain different anthocyanins structure, which affect their stability. Anthocyanins from red cabbage, black carrot, red radish and red sweet potato are reported to be more stable to heat and pH change than anthocyanins from other sources due to their acylation of the structure (Bąkowska-Barczak, 2005). Acylation of the anthocyanin molecules can enhance the stability through intramolecular copigmentation (Rodríguez-Saona et al., 1999). Higher stability of acylated anthocyanins is attributed to the stacking of acyl group with the pyrylium ring of the flavalium cation, thus preventing the nucleophile attack of water and subsequent formation of chalcone (Bąkowska-Barczak, 2005). Many researchers have indeed demonstrated the stability of acylated anthocyanins in comparison with that of non-acylated anthocyanins. For example, Cevallos-Casals and Cisneros-Zevallos (2004) who studied the stability of anthocyanins from red sweet potato, purple carrot, purple

corn and red grape reported that anthocyanins from red sweet potato and purple carrot, which mainly consist of acylated anthocyanins, were more stable than those from purple corn and red grape, which mainly consist of non-acylated anthocyanins. Anthocyanins extracted from red sweet potato exhibited longer half-life than those from purple carrot, purple corn and red grape when heated at 98 °C and controlled at pH 3.0.

Intercellular copigmentation has been suggested to improve the stability of anthocyanins. Intercellular copigmentation is defined as interactions between anthocyanins molecules and other molecules e.g., flavonoids, alkaloids, amino acids, organic acids, metals and other anthocyanins (Castañeda-Ovando et al., 2009). The basic role of intercellular copigmentation is the same as that of intracellular copigmentation, which is to protect the flavalium cation from the nucleophile attack of the water molecules (Mazza and Brouillard, 1990; Castañeda-Ovando et al., 2009). Gauche et al. (2010) reported that addition of organic acids (caffeic, ferulic, gallic and tannic acids) into an anthocyanins solution could retard the color change when the pH of the solution increased to 4.0-6.0, whereas the solution without organic acids became colorless. Moreover, the half-life of the anthocyanins solutions with added organic acids was longer than that of the untreated sample during storage at 28 °C; addition of tannic acid resulted in the longest half-life of the sample maintained at pH 1.0.

Although intercellular copigmentation can help increase the stability of anthocyanins, this reaction induces an increase in the absorbance (hyperchromic effect: ΔA) and wavelength of the maximum absorbance (bathochromic shift: $\Delta\lambda$) of the pigment. Bathochromic shift results in the color change from red to red-orange or blue (Bąkowska et al., 2003). Addition of tannic acid (1:1 w/v) to an anthocyanins solution from Isabel grapes (*Vitis labrusca* L.) controlled at pH 3.0 and

4.0, for example, led to an increase in the absorbance and wavelength of the solution, resulting in bluer and brighter color of the treated sample (Bordignon-Luiz et al., 2007). Gauche et al. (2010) also illustrated that copigmentation of anthocyanins with tannic, gallic, caffeic and ferulic acids resulted in hyperchromic effect and bathochromic shift at every pH value studied (1.0, 2.0, 3.0, 3.3, 3.5, 3.7, 4.0, 4.5). Exception was noted in the cases of caffeic and ferulic acids at pH 1.0 and 2.0, where only the bathochromic shift was observed. At pH 1.0 and 2.0 these hydroxycinnamic acids induced the formation of pyranoanthocyanins, which is anthocyanins-derived pigments, hiding the hyperchromic effect of copigmentation (Gómez-Miguez et al., 2006).

Since only a few natural blue-tone colorants are commercially available, production of blue natural colorants from anthocyanins sources is of interest. Blue hue colorants can be produced by complexation of metal-anthocyanins. However, metal-anthocyanins complexes (blue colorant) are reported to be stable only in the vacuolar matrix (Buchweitz et al., 2013) and would rapidly precipitate in an aqueous solution (Buchweitz et al., 2012). Addition of some polysaccharides or gelatin to form a gel structure is proposed to enhance the stability of metal-anthocyanins complexes by preventing the complexes precipitation. Buchweitz et al. (2013) noted that gelatin and blend agar-agar with amidated pectin could improve the stability of blue color of ferric anthocyanins chelates during storage at both 20 °C in dark and at 25 °C under illumination. The most stable sample was obtained by using gelatin as the gel matrix; storage should be done at 20 °C in the dark.

Although the stability of anthocyanins can be enhanced by both intra- and intercellular copigmentation as well as preparation of the gel matrix as mentioned earlier, controlling the pH

of a food product should be done to prevent the shift of color. Storage should be done in dark at cool condition (Bordignon-Luiz et al., 2007).

Examples of stability enhancement of chlorophylls, carotenoids, betalains and anthocyanins via pretreatments are given in Table 2.

4. Effects of extraction methods on pigment extractability

Extraction of pigments from plant materials can be performed by various techniques. Conventional methods are often used to extract crude pigments and other compounds. Recently, however, non-conventional extraction methods, which have been regarded as green extraction techniques, are alternatives to conventional extraction since they require less amount of solvent as well as shorter extraction time and are more environmentally friendly (Cheok et al., 2014). Selecting an appropriate extraction technique for each type of natural pigments must be done to improve the efficiency and productivity of natural colorant production. The effects of selected extraction methods on the stability and extraction yield of pigments are discussed here to serve as a guideline for a proper selection of the extraction technique.

4.1 Conventional extraction methods

Conventional methods such as Soxhlet extraction, maceration and hydrodistillation are simple, inexpensive and easy to handle (Veggi et al., 2013). These methods have therefore widely been used to extract essential oils, bioactive compounds as well as natural pigments from

a wide array of plant materials. Soxhlet extraction was initially designed for lipid extraction but is nowadays not only used for such a purpose. In fact, Soxhlet extraction is now often used as a reference method for comparing the yield of an advanced extraction technique (Azmir et al., 2013). During the extraction, a plant sample contained in a thimble is repeatedly percolated with condensed vapor of solvent until the extraction is completed (or the solvent is no longer able to solubilize interested compounds in the sample), which is noted when the solvent has become colorless (Veggi et al., 2013). Although Soxhlet extraction is simple and easy to handle, this method requires large amount of solvent, long extraction time and leads to pigment degradation due to heat.

Maceration is a conventional extraction method that can be performed at room temperature. This method can thus be used for extracting heat-sensitive pigments. Cha et al. (2010a), for instance, noted that the pheophytin content, which is an indirect indicator of chlorophylls degradation, of a crude extract of green microalga (*Chlorella vulgaris*) obtained from maceration at 25 °C for 6 h was lower than that from Soxhlet extraction at 100 °C for 2 h. To extract crude pigments, a plant sample is ground and mixed with an extraction solvent; the mixture is left in an extraction vessel with occasional shaking or stirring. After the process is finished, the liquid is strained off and the residue is pressed by a mechanical press or centrifuged to repeat the extraction with a fresh solvent until the solvent exhibits no color (Azmir et al., 2013). Since the extraction is generally performed at room temperature, the time required for the extraction is long; large amount of solvent is also needed to repeat the extraction.

Hydrodistillation is a traditional method that is usually used for extracting essential oils from a plant material. The extraction medium, which is hot water and/or steam, is directly in

contact with a sample to extract interested compounds (Azmir et al., 2013). Since hydrodistillation involves the use of water for the extraction, an obtained extract is not contaminated with any organic solvent, which can be toxic. However, since hydrodistillation is normally operated at a rather high temperature (about 100 °C), significant pigment degradation is expected.

The efficiency of conventional extraction methods directly depends on the solubility of a solute from a plant material into an extraction solvent (Cowan, 1999); extraction temperature also plays an important role on the extraction efficiency. Examples of solvents used for extracting chlorophylls, carotenoids, betalains and anthocyanins are listed in Table 3. Although conventional extraction methods have many advantages, these methods require large amount of solvent, long extraction time) and may lead to significant pigment degradation (Cheok et al., 2014. Non-conventional methods or green extraction methods have therefore been proposed to alleviate such limitations.

4.2 Non-conventional extraction methods

4.2.1 Supercritical fluid extraction (SFE)

SFE utilizes the advantages of supercritical fluids, which exhibit gas and liquid-like properties, to enhance extraction. A supercritical fluid can be produced by subjecting a solvent to a temperature and pressure beyond its critical point. In supercritical state, fluid possesses high diffusivity and low viscosity like gas but exhibits high solvation power like liquid (Macías-Sánchez et al., 2005). For these reasons, supercritical fluids can better penetrate into a sample matrix; this subsequently leads to a more efficient extraction.

Carbon dioxide is considered an ideal solvent for SFE since its critical temperature (T_c) and critical pressure (P_c) are not too high, 31 °C and 74 bars, respectively (Greibrokk, 1991). SFE is generally operated within a pressure range of 8 and 40 MPa and a temperature range of 30 and 60 °C. For this reason, SFE can be applied to extract heat-sensitive pigments. Vági et al. (2002) reported that the pheophytin contents of crude extracts of *Origanum majorana* L. obtained from SFE at 40, 50 and 60 °C were lower than those obtained from Soxhlet extraction at 70 and 80 °C.

Since most extraction solvents for SFE are non-polar, this method is suitable for the extraction of low-polar pigments such as carotenoids and chlorophylls. On the other hand, SFE is not suitable for extraction of betalains and anthocyanins, which are high-polar pigments. Macías-Sánchez et al. (2005) reported that SFE was faster and more selective than ultrasound-assisted maceration for extracting carotenoids and chlorophylls from *Nannochloropsis gaditana*. The highest extraction yields of both carotenoids and chlorophylls *a* via SFE were achieved when using an extraction temperature of 60 °C and a pressure of 400 bar. Nevertheless, SFE with supercritical carbon dioxide as a solvent resulted in lower carotenoids and chlorophylls *a* yields than ultrasound-assisted maceration employing methanol as a solvent.

To improve the extraction yields of carotenoids and chlorophylls, combination of carbon dioxide and organic solvent (e.g., methanol, ethanol) has been proposed. Addition of 5% (v/v) ethanol to supercritical carbon dioxide was noted to increase the extraction yield of *β*-carotene from carrot by about 7% (Baysal et al., 2000). Using 7% (v/v) ethanol as a co-solvent with supercritical carbon dioxide could also increase the extraction yields of chlorophylls *a* and *b* from 0.027 and 0.023 to 0.848 and 0.356 mg/$g_{\text{dry sample}}$, respectively (Guedes et al., 2013).

Although SFE is quite efficient and involves small solvent consumption, no or less use of toxic solvent, can extract heat-sensitive pigments and can be automated, this extraction method requires high capital and operating costs because of the high pressure required for the operation (Veggi et al., 2013).

4.2.2 Pressurized liquid extraction (PLE)

PLE utilizes a liquid solvent at elevated pressure (10.3-13.8 MPa) and temperature (40-200 °C) for the extraction (Antunes et al., 2008). High temperature results in better diffusion of solvent into the sample matrix and also helps disrupt plant cells, resulting in a more effective release of pigments from the cells and hence more effective extraction. High pressure, on the other hand, forces the solvent into the matrix pores and hence allows better contact between the solvent and compounds to be extracted (Cha et al., 2010b; Mustafa and Turner, 2011). Thus, PLE requires shorter time and involves the use of less solvent for extraction (Antunes et al., 2008).

PLE can extract both water- and oil-based pigments, depending on the selection of an extraction solvent. However, PLE cannot effectively extract heat-sensitive pigments since the method involves the use of high temperature. PLE is therefore normally used to extract less heat-sensitive pigments (i.e., anthocyanins, carotenoids and chlorophylls). Machado et al. (2014) studied the use of PLE in comparison with conventional methods (Soxhlet extraction and maceration) to extract monomeric anthocyanins from blackberry. PLE at 100 °C exhibited a higher extraction rate than Soxhlet extraction at 80 °C and maceration at 25 °C. Time required to extract monomeric anthocyanins in the cases of PLE, Soxhlet extraction and maceration was 30,

300 and 1440 min, respectively. In terms of carotenoids and chlorophylls, Cha et al. (2010a) reported that PLE at 160 °C for 120 min yielded higher contents of carotenoids, chlorophylls *a* and *b* than maceration extraction at 25 °C for 360 min and Soxhlet extraction at 100 °C for 120 min. Although PLE gave the highest yields of carotenoids, chlorophylls *a* and *b*, the method resulted in the highest pheophytin content due to the use of a higher temperature for the extraction.

To increase the stability of pigments during PLE, some pretreatment methods, which are mentioned in Section 3, can be used. Adjusting the pH of an extraction solvent to acidic values can help retard the degradation of anthocyanins during PLE because acidic condition could lead to the formation of flavylium cation, which is the most stable form of anthocyanins (Tan et al., 2014). Sharif et al. (2010) reported that the most redness (or highest *a** value) and highest yields of anthocyanins (cyanidin 3-glucoside, cyaniding 3-rutinoside, cyandin chloride) extracted from onion (*Allim cepa*) skin could be achieved by adding 0.1% (v/v) hydrochloric acid into an extraction solvent (methanol) and performing PLE at 80 °C, 689.48 bar. Besides adjusting the pH, intra- and intermolecular copigmentation may be used to increase the stability of anthocyanins during PLE. For chlorophylls, the conversion of native chlorophylls to metallo-chlorophyll derivatives prior to pigment extraction may be employed to retain the green color of chlorophylls extracts obtained by PLE. Nevertheless, this extraction method again requires high capital and operating costs due to the use of a higher pressure for extraction.

4.2.3 Microwave-assisted extraction (MAE)

MAE is gaining interest as an alternative for extracting both water- and oil-based plant pigments due to its speed and small solvent consumption when compared with conventional extraction methods (Dahmoune et al., 2014). Rapid heating by microwave radiation results in an expansion of plant cell structure with subsequent rupture of plant cell walls. Thus, compounds, including pigments can easily migrate out of cells, resulting in an enhanced extraction rate (Zou et al., 2013). Dabiri et al. (2005), for example, illustrated that MAE could reduce the time and solvent volume requirement when extracting pigments (alizarin and purpurin) from Rubiaceae plants. MAE required the time and solvent volume of only 20 min and 20 mL, while Soxhlet extraction required the time and solvent volume of up to 360 min and 100 mL, respectively. Moreover, MAE at an optimized condition yielded higher alizarin and purpurin recovery than Soxhlet extraction operated also at its optimum condition.

Combination of stability enhancement and pigment extraction has been proposed for MAE. Cardoso-Ugarte et al. (2014) proposed a two-stage MAE with the addition of L-ascorbic acid into 50% (v/v) ethanol for the extraction of betanines and betaxanthins from red beet. Both extraction steps were performed at a power level of 400 W and 100% duty cycles. A cooling step was added between the first and second stages to delay the degradation of the pigments already extracted. The addition of ascorbic acid could retard the degradation of betanines. However, the stability of betaxanthins could not be retained by the addition of ascorbic acid since betaxanthins are not stable in acidic condition (Stintzing and Carle, 2008).

Combination of MAE with vacuum, resulting in the so-called vacuum microwave-assisted extraction (VMAE), has recently been proposed to extract heat-sensitive bioactive compounds and pigments (Hiranvarachat et al., 2015). VMAE can reduce pigment degradation

by oxidation since less oxygen is available in the extraction process. Xiao et al. (2012) noted that VMAE could prevent the degradation of vitamin C extracted from peppers and guava, β-carotene extracted from carrot and aloin A extracted from aloe vera. The extraction yields of vitamin C, β-carotene and aloin A extracted by VMAE were also noted to be higher than those obtained via MAE.

4.2.4 Ultrasound-assisted extraction (UAE)

UAE utilizes cavitation bubbles created by ultrasound waves to enhance the extraction efficiency (Rastogi, 2011). Cavitation bubbles are formed when ultrasound waves pass through a medium, creating alternative compression and decompression cycles, which in turn result in compression and expansion of the bubbles. When bubbles grow too large to be contained by the surface tension force, the bubbles collapse, resulting in shearing forces to break up or disrupt cell walls of a contacted plant material (Pitt et al., 2004). As a result, release of intracellular compounds is enhanced. UAE has received considerable attention because of its benefits, which include the ability to perform extraction at lower temperatures due to the absence of external input heat and at low solvent consumption (Tao and San, 2013).

Both water- and oil-based pigments can be extracted by UAE. Since UAE is a non-thermal process, it can be used to extract heat-sensitive pigments. Tao et al. (2014), for example, investigated the use of UAE to extract anthocyanins from wine lees in comparison with the use of maceration and found that at the same extraction time and temperature (36 min and 60 °C) the yield of anthocyanins extracted by ultrasound at 40 kHz was higher than that by maceration. UAE has also been employed as an efficient technique for the extraction of betalains and

chlorophylls. Kong et al. (2012), for example, reported that UAE could increase the extraction rate by up to 88.9% in comparison with that of maceration when extracting chlorophylls from *Chlorella vulgaris*.

UAE is known for its lower extraction effectiveness when compared with some other non-conventional extraction processes (i.e., SFE, PLE, MAE and PEF extraction). Cha et al. (2010a), for instance, compared the extraction yields of carotenoids and chlorophylls from *Chlorella vulgaris* by PLE and UAE and noted that PLE at 160 °C for 30 min gave higher yields of both carotenoids and chlorophylls than UAE at 25 °C for 360 min. Nevertheless, due to its higher-temperature operation, PLE caused more chlorophylls degradation, which resulted in higher pheophytin content in the extract. To increase the extraction yield of UAE, repeated extraction is required; larger volume of solvent would be needed to repeat the extraction, however.

Recently, combined use of UAE and MAE is proposed to increase the extraction yield of UAE and, at the same time, reduce the time a material needs to undergo MAE. UAE and MAE in combination leads to more extensive damage of plant structure than employing UAE or MAE alone (Pongmalai et al., 2015). Lianfu and Zelong (2008) indeed reported that UAE+MAE at 98 W of microwave power with 40 kHz of ultrasound gave higher yield of extracted lycopene from tomato than UAE alone. The percentage of lycopene yield was 97.4% and 89.4% for UAE+MAE and UAE, respectively. Moreover, UAE+MAE required only 6 min to obtain such a yield, while UAE needed as long as 29 min to obtain a similar yield.

4.2.5 *Pulsed-electric field (PEF) extraction*

Pulsed electric field (PEF) has noted to be useful for enhancing the many processes of food production, including the extraction process. Short and high-voltage electric field is used to induce pore formation in the cell walls of a plant material, which leads subsequently to better release of cellular constituents and hence enhanced extraction (Azmir et al., 2013; Donsì et al., 2010).

Most solvents used to extract betalains and anthocyanins are polar solvents, which possess electrical conductivity and can let electricity to pass thorough to sample cells. On the other hand, electric field cannot pass thorough a non-polar solvent since it is an electrical resistance possessing low or negligible conductivity (Yuhas Jr., 1995). For this reason, PEF is more suitable for the extraction of betalains and anthocyanins than for chlorophylls and carotenoids.

A number of works exist on the use of PEF to extract (or to assist the extraction of) plant pigments. Puértolas et al. (2013), for instance, applied PEF at 3.4 kV/cm and 105 µs (35 pulses of 3 µs) to extract anthocyanins from purple-fleshed potato. The anthocyanins yield was noted, as expected, to be higher than those from the non-PEF treated sample and also from the macerated sample. pH also exerts a significant effect on the PEF extraction yield. López et al. (2009), for example, studied the effect of pH of the solvent on the PEF extraction yield of betanines from red beetroot and found that McIlvaine buffer at pH 3.5 led to the highest yield of betanines. This is probably because an acidic solvent could prevent the degradation of betanines during the extraction (Bilyk and Howard, 1982).

Despite its potential, PEF-based extractor, especially at an industrial scale, suffers from the need of a high-power supply equipment and treatment chamber (Nip, 2007), which are nowadays still rather expensive.

4.2.6 Enzyme-assisted extraction (EAE)

EAE involves the use of enzymes to enhance extraction of bioactive compounds, including pigments, from a plant material. Various enzymes such as pectinase, cellulase and hemicellulase are used to hydrolyze plant cellulosic cell walls (Socaciu, 2008), resulting in easier release of cellular constituents and hence facilitated extraction. EAE is of interest due to its non-thermal nature as well as lower toxicity and solvent consumption (Socaciu, 2008). EAE can be divided into two common approaches, which are enzyme-assisted aqueous extraction (EAAE) and enzyme-assisted cold pressing (EACP) (Latif and Anwar, 2009). In EAAE, enzymes are used to help destroy cell walls and hence rupturing polysaccharide-protein colloids. On the other hand, enzymes are used only to hydrolyze cell walls in the case of EACP (Hassan and Gökçe, 2014).

Many researchers investigated the effect of enzyme treatment on the extraction yields of chlorophylls, carotenoids, betalains and anthocyanins. Barzana et al. (2002), for example, noted that the extraction yield of carotenoids from marigold flower treated by enzymes (viscozyme, proteolytic and pectinolytic enzymes) was higher than that from the untreated sample. Kammerer et al. (2005) proposed a two-stage process for the extraction of anthocyanins from grape pomace via EAE. The process involved a pre-extraction step, which was then followed by EAE. Release of phenolic acids during the pre-extraction step nevertheless caused a decrease in the pH, leading

to an unfavorable condition for enzyme activity. For this reason, before EAE, the pH of the residue obtained from the pre-extraction step was adjusted to an optimum range for enzyme hydrolysis, which resulted in enhanced extraction.

Enzyme treatment can also be applied to assist other green extraction methods. Lenucci et al. (2015), for instance, proposed an enzyme-aided supercritical carbon dioxide extraction and found that enzymatic pretreatment could help increase the extractable lycopene concentration from the matrix of tomato puree due to the degradation of tomato cell walls by the enzymes. However, the denseness of the enzyme-treated sample hindered the diffusion of supercritical carbon dioxide through the sample matrix. Addition of a co-matrix (hazelnut seeds) into the base matrix was therefore proposed to facilitate the penetration of supercritical carbon dioxide through the sample matrix and hence increased lycopene extractability. Although EAE can improve the extraction yield and can be applied to extract heat-sensitive pigments, EAE requires a very long extraction time; enzymes are also normally quite expensive (Hardouin et al., 2014).

Examples of pigment extraction via different methods as well as pros and cons of each extraction method are listed in Table 4 and Table 5, respectively.

Conclusions

Selected methods that can be used to enhance the stability of natural pigments during their processing and storage are first outlined. Selection of an appropriate pretreatment method depends on the type of the pigments. Selected non-conventional extraction methods are then reviewed as a means to enhance the yield and maintain the stability of the pigments without sacrificing the friendliness to the environment. Suitability of the extraction methods is discussed

based on the thermal sensitivity and polarity of the pigments. MAE seems to be a proper method for the extraction of carotenoids, chlorophylls and anthocyanins but not heat-sensitive pigments like betalains. UAE, EAE and PEF, which are classified as non-thermal extraction processes, can be used to extract betalains.

Although only guidelines for the selection of appropriate pretreatment and extraction methods for each type of pigment are given, other factors such as investment cost, operating cost as well as applicability of the selected techniques at an industrial scale must also be considered besides the extraction yield and stability of the pigments. Combination of different extraction and stability enhancement methods can be performed to increase both the pigment stability and extraction yield.

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Table 1. Color stability of chlorophylls, carotenoids, betalains and anthocyanins to heat, light, oxygen and pH

Pigment	Typical source	Stability			
		Heat	**Light**	**Oxygen**	**pH change**
Chlorophylls	Leaves	Moderate	High	High	Low
Carotenoids	Leaves	Moderate	Low	Low	High
Betalains	Beetroot	Moderate to low	High	High	Moderate
Anthocyanins	Fruits	High to moderate	High	High	Low

Table 2. Pigment stability enhancement via pretreatments

Pigment	Source	Pretreatment method	Result	Reference
Chlorophylls	Rocket (*Eruca sativa*)	Combination of blanching and acid/alkaline treatment	• Sample blanched in 0.1% NaOH exhibited maximum greenness, while blanching sample in HCl yielded a drop in green color	Ahmed et al. (2013)
	Stevia rebaudiana leaves	Copper treatment	• Cu-chlorophylls suffered less color losses than Mg-chlorophylls after storage at 60 °C for 20 days • Color losses were 16% and 36% for Cu-chlorophyll and Mg-chlorophyll complexes,	Bobbio and Guedes (1990)

			respectively	
	Green pear	Blanching and zinc treatment	• Green pigments of control sample and sample blanched in water were destroyed after heating at 94 °C for 12 min • Blanching and zinc treatment helped retain green pigment of sample after thermal processing	Ngo and Zhao (2005)
Carotenoids	Pumpkin (*Cucurbita moschata*, Duchesne ex Poiret)	Adjusting pH by addition of ascorbic acid and potassium sorbate	• Addition of ascorbic acid and potassium sorbate to pH 4.0 minimized losses of redness and yellowness of sample after storage at 25 °C	Gliemmo et al. (2009)

			for 6 weeks	
	Carrot	Blanching and acid treatment	• β-carotene content of sample treated by blanching in citric acid (targeted pH 4–5) tended to be unchanged during thermal processing, whereas β-carotene content of untreated sample decreased continuously	Hiranvarachat et al. (2011)
Betalains	*Rivina humilis* L. berry juice	Addition of ascorbic acid	• Addition of ascorbic acid (0.25 g/100 mL) helped retain betacyanins at 93% and 78% after heating at 90 °C for 3 min every 24 h for 6 consecutive days and after heating at 90 °C for 24 min,	Khan and Giridhar (2014)

			respectively	
Anthocyanins	Blood orange juice	Addition of ascorbic acid	• Total carotenoids content of sample added with ascorbic acid (30 mg/100 mL) decreased only 2.8%, while that of untreated sample decreased up to 6.6% after storage at 4.5 °C for 7 weeks	Choi et al. (2002)
	Honeysuckle (*Lonicera kamtschatica*)	Copigmentation by using QSA, NaMSA, rutin, quercetin, chlorogenic acid, tannic acid, and flavones	• Color of copigment anthocyanin complexes slowly changed during heating at 180 °C for 1 h at pH 2.5, 3.5 and 4.5	Bąkowska et al. (2003)
	Cabernet Sauvignon grape	Adjusting pH by 0.1-M citric acid–sodium citrate and	• Copigmentation by adjusting to pH 4 yielded retention of color by 96% after	Gris et al. (2007)

		copigmentation by using caffeic acid	storage at 4 °C for 72 h, while control sample had only 90% retention of color • Sample extracts added with caffeic acid and adjusted to pH 4 had half-life of anthocyanins up to 291 days when storage at 4 °C in dark, while control sample had half-life of only 96 days	

Table 3. Examples of solvents used for extracting different groups of pigments (Cowan, 1999; Kujala et al., 2001; Hosikian et al., 2010; Machmudah and Goto, 2013)

Chlorophylls	Carotenoids	Betalains	Anthocyanins
Methanol	Methanol	Methanol	Methanol
Ethanol	Ethanol	Water	Water
Acetone	Acetone		
	n-hexane		
	Pentane		
	Chloroform		

Table 4. Natural pigments extracted via different methods

Extraction technique	Plant material	Operating condition	Extracted pigment and yield (in bracket) (μg/mg sample)	Reference
SFE	Carrot (*Daucus carota* L.)	Temperature: 40 °C Pressure: 505 bar Solvent: CO_2 Flow rate: 600–750 mL/min Time: 60 min	*α*-carotene (0.12) *β*-carotene (0.15)	Chandra and Nair (1997)
SFE	*Dunaliella salina*	Temperature: 60 °C Pressure: 300 bar Solvent: CO_2 Flow rate: 4.5 mmol/min Time: 180 min	Carotenoids (14.92) Chlorophylls (0.27)	Macias-Sanchez et al. (2009)
SFE	*Scenedesmus obliquus*	Temperature: 40 °C Pressure: 250 bar Solvent: CO_2 with	Carotenoids (0.30) Chlorophyll *a*	Guedes et al. (2013)

		7.7% (v/v) ethanol Flow rate: 2 g/min Time: 240 min	(0.85) Chlorophyll *b* (0.36) Chlorophyll *c* (0.02)	
PLE	*Chlorella vulgaris*	Temperature: 160 °C Pressure: 103.42 bar Solvent: 90% ethanol Time: 30 min	*β*-carotene (0.50) Chlorophyll *a* (9.63) Chlorophyll *b* (5.77) Pheophytin *a* (5.64)	Cha et al. (2010a)
UAE	*Chlorella vulgaris*	Temperature: Room temp. Solvent: 90% ethanol Solvent volume: 50 mL Time: 120 min	*β*-carotene (0.10) Chlorophyll *a* (5.12) Chlorophyll *b* (3.17) Pheophytin *a* (2.64)	
Soxhlet extraction	*Chlorella vulgaris*	Temperature: 100 °C Solvent: 90% ethanol	*β*-carotene (0.26) Chlorophyll *a* (3.32)	

		Solvent volume: 100 mL Time: 120 min	Chlorophyll *b* (3.45) Pheophytin *a* (3.90)	
Maceration	*Chlorella vulgaris*	Temperature: Room temp. Solvent: 90% ethanol Solvent volume: 50 mL Time: 360 min	*β*-carotene (0.08) Chlorophyll *a* (4.26) Chlorophyll *b* (2.58) Pheophytin *a* (2.31)	
PLE	Blackberry (*Rubus fruticosus* L.)	Temperature: 100 °C Pressure: 75 bar Solvent: Water with 50% (v/v) ethanol Flow rate: 3.35 mL/min Time: 30 min	Anthocyanins (1.02)	Machado et al. (2014)
Soxhlet extraction	Blackberry (*Rubus fruticosus* L.)	Temperature: 80 °C Solvent: Methanol Solvent volume: 200 mL	Anthocyanin (1.33)	

		Time: 300 min		
Soxhlet extraction	Blackberry (*Rubus fruticosus* L.)	Temperature: 80 °C Solvent: Ethanol Solvent volume: 200 mL Time: 300 min	Anthocyanin (1.68)	
Maceration	Blackberry (*Rubus fruticosus* L.)	Temperature: Room temp. Solvent: Methanol with 0.01% (v/v) HCl Solvent volume: 200 mL Time: 1440 min	Anthocyanin (1.21)	
MAE	Purple corn (*Zea mays* L.)	Solvent: 95% ethanol with 1.5-M HCl Solvent to sample ratio (mL/g): 20/1	Anthocyanins (0.18)	Yang and Zhai (2010)

		Microwave power: 555 W Time: 19 min		
Maceration	Purple corn (*Zea mays* L.)	Solvent: 95% ethanol with 1.5-M HCl Solvent to sample ratio (mL/g): 30/1 Temperature: 55 °C Time: 60 min	Anthocyanins (0.16)	
MAE	Red beet (*Beta vulgaris*)	Solvent: 50% (v/v) ethanol with 0.04-M ascorbic acid Solvent to sample ratio (mL/g): 250/1 Microwave power: 400 W Time: 3.50 min	Betanines (0.19)	Cardoso-Ugarte et al. (2014)
Maceration	Red beet (*Beta vulgaris*)	Solvent: 50% (v/v) ethanol Solvent to sample ratio (mL/g): 250/1	Betanines (0.15)	

		Temperature: 80 °C Time: 60 min		
MAE	Carrot	Temperature: 58 °C Solvent: Mixed solvent (50% v/v hexane, 25% v/v acetone, 25% v/v ethanol) Solvent volume: 75 mL Microwave power: 180 W Time: 3 min	Total carotenoids (0.52) β-carotene (0.23)	Hiranvarachat et al. (2013)
Soxhlet extraction	Carrot	Temperature: 58 °C Solvent: Mixed solvent (50% v/v hexane, 25% v/v acetone, 25% v/v ethanol) Solvent volume: 75 mL	Total carotenoids (0.61) β-carotene (0.29)	

		Time: 360 min		
UAE (Ultrasonic probe)	*Bougainvillea glabra* flower	Temperature: 55 °C Solvent: 50% methanol Solvent to sample ratio (mL/g): 17/1 Power and frequency: 88 W, 20 kHz Time: 37 min	Betacyanin (1.72) Betaxanthin (5.78)	Maran et al. (2015)
UAE (Ultrasonic bath)	*Chlorella vulgaris*	Temperature: 50 °C Solvent: 80% ethanol Solvent to sample ratio (mL/g): 200/1 Power and frequency: 200 W, 40 kHz Time: 30 min	Total chlorophylls (13.2)	Kong et al. (2012)

Maceration	*Chlorella vulgaris*	Temperature: 50 °C Solvent: 80% ethanol Solvent to sample ratio (mL/g): 200/1 Time: 30 min	Total chlorophylls (8.32)	
UAE (Ultrasonic bath)	Light wine lees (60% Cabernet Sauvignon, 30% Merlot, 10% Cabernet Franc)	Temperature: 60 °C Solvent: 50% ethanol Solvent to sample ratio (mL/g): 60/1 Acoustic energy density: 48 W/L Frequency: 40 kHz Time: 36 min	Total anthocyanins (6.69)	Tao et al. (2014)
Maceration	Light wine lees (60% Cabernet Sauvignon, 30% Merlot, 10% Cabernet Franc)	Temperature: 60 °C Solvent: 50% ethanol Solvent to sample ratio (mL/g): 60/1 Time: 36 min	Total anthocyanins (5.55)	

PEF-assisted extraction	Grape by-product	PEF treatment Temperature: Room temp. Number of pulses: 30 Pulse frequency: 2 Hz Electric field strength: 9 kV/cm Extraction (maceration) Temperature: 70 °C Solvent: 50% (v/v) ethanol Solvent to sample ratio (mL/g): 4.5/1 Time: 60 min	Anthocyanins (14.05)	Corrales et al. (2008)
UAE (Ultrasonic bath)	Grape by-product	Temperature: 70 °C Solvent: 50% (v/v) ethanol Solvent to sample ratio (mL/g): 4.5/1	Anthocyanins (7.76)	

		Frequency: 35 kHz Time: 60 min		
Maceration	Grape by-product	Temperature: 70 °C Solvent: 50% (v/v) ethanol Solvent to sample ratio (mL/g): 20/1 Time: 180 min	Anthocyanins (7.93)	Corrales et al. (2008)
PEF-assisted extraction	Red beetroot	PEF treatment Temperature: 30 °C Number of pulses: 5 Pulse frequency: 1 Hz Electric field strength: 7 kV/cm Extraction (maceration) Temperature: 30 °C Solvent: McIlvaine buffer at pH 3.5	Betanines (90% of total)	López et al. (2009)

		Solvent volume: 400 mL Time: 300 min		
Maceration	Red beetroot	Temperature: 50 °C Solvent: McIlvaine buffer at pH 3.5 Solvent volume: 400 mL Time: 400 min	Betanines (66% of total)	
EAE	Marigold Flower (*Tagetes erecta*)	Enzyme treatment Enzyme to sample ratio (mL/g): 0.1/1 Time: 60 min Extraction (maceration) Temperature: 25 °C Solvent: Hexane Solvent to sample ratio (mL/g): 4/1 Time: 1440 min	Carotenoids (0.5)	Barzana et al. (2002)

Table 5. Advantages and drawbacks of different extraction methods

Extraction method	Solvent	Advantage	Drawback
Soxhlet extraction	• Organic solvents (both polar and non-polar)	• Easy to handle • Low investment • Automated system	• Not suitable for heat-sensitive pigments • Long extraction time • Large solvent consumption
Maceration	• Organic solvents (both polar and non-polar)	• Easy to handle • Low investment • Applicable for heat-sensitive pigments	• Very long extraction time • Large solvent consumption • Filtration step required • Non-automated system • Repeated extraction may be required
Hydrodistillation	• Water	• Easy to handle • Low investment • Automated system	• Not suitable for heat-sensitive pigments • Long extraction time
SFE	• CO_2 (non-polar) • CO_2 with polar solvent	• Moderate extraction time • Applicable for heat-	• Required sophisticated safety controls • High pressure leading

		sensitive pigments • No use of toxic solvents • Automated system	to high capital and operating costs
PLE	• Organic solvents (both polar and non-polar)	• Fast extraction • Small solvent consumption • Automated system	• Not suitable for heat-sensitive pigments • High investment
MAE	• Organic solvents (both polar and non-polar)	• Fast extraction • Easy to handle • Moderate solvent consumption • Moderate investment	• Not suitable for heat-sensitive pigments • Filtration step required • Non-automated system
PEF extraction	• Organic solvents (both polar and non-polar)	• Fast extraction • Applicable for heat-sensitive pigments • Moderate investment	• Large solvent consumption • Filtration step required • Non-automated system
UAE	• Organic solvents (both polar and non-polar)	• Fast extraction • Applicable for heat-sensitive pigments • Simple • Low investment	• Large solvent consumption • Filtration required • Non-automated system • Repeated extraction may be required

EAE	• Organic solvents (both polar and non-polar)	• Applicable for heat-sensitive pigments • Easy to handle • Moderate solvent consumption	• Long extraction time • Filtration required • Non-automated system

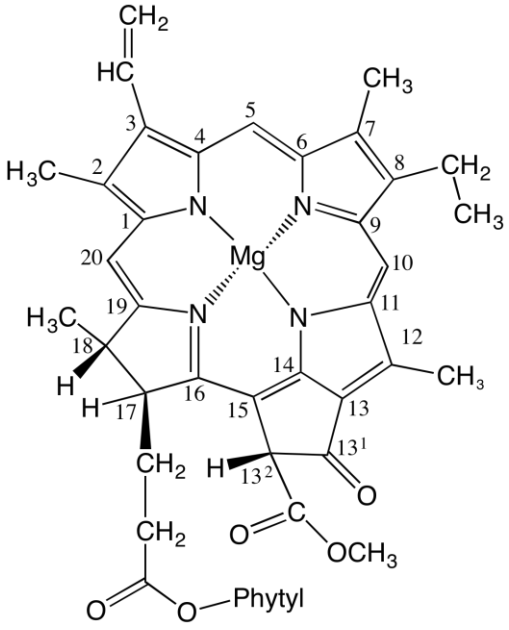


Figure 1. Basic structure of chlorophylls pigment.

(Marquez and Sinnecker, 2008)

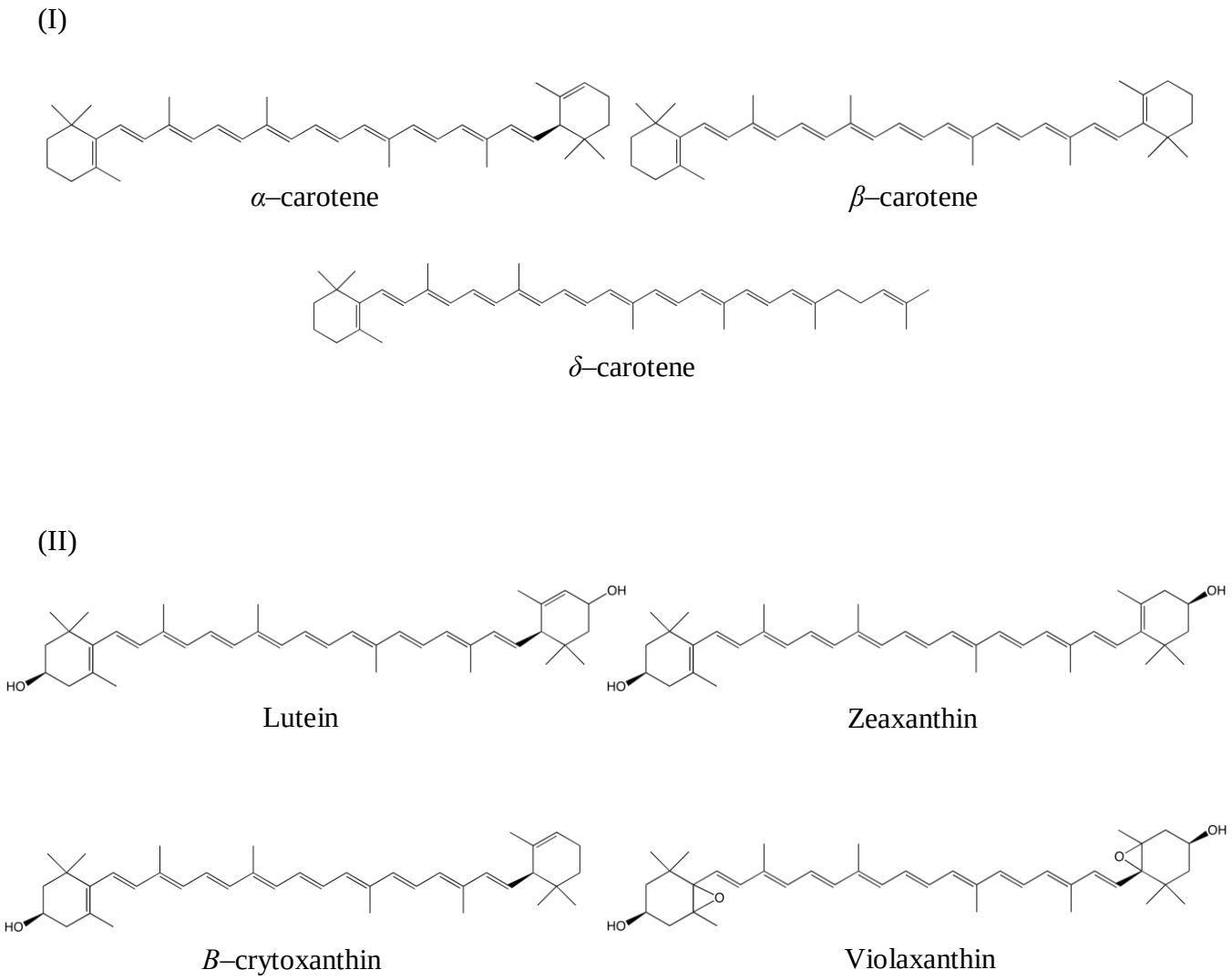
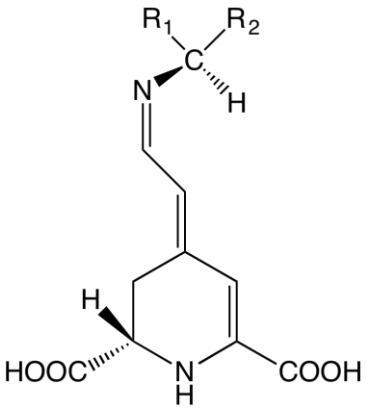


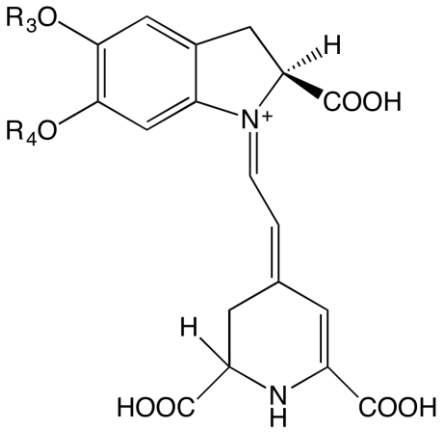
Figure 2. Structures of major carotenes (I) and xanthophylls (II).

(Ötles and Çagindi, 2008)

(I)



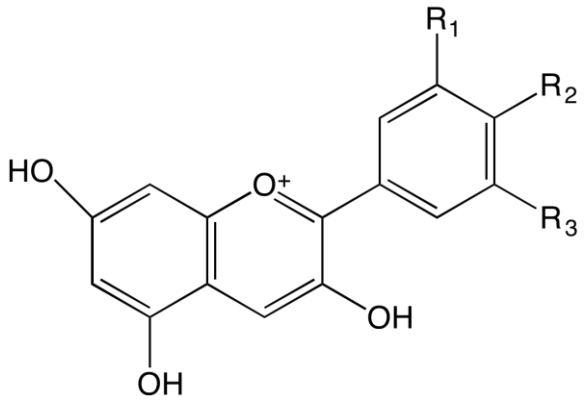
(II)



Substitution pattern				Trivial name
R_1	R_2	R_3	R_4	
H	Glutamic acid	-	-	Vulgaxanthin I
Proline	Proline	-	-	Indicaxanthin
-	-	*β*-glucose	H	Betanine
-	-	6´-O-(malonyl)-*β*-glucose	H	Phyllocactin
-	-	6´-O-(3´´-hydroxy-3´´-methylglutaryl)-*β*-glucose	H	Hylocerenin
-	-	H	*β*-glucose	Gomphrenin I

Figure 3. Structures of betaxanthins (I) and betacyanins (II).

(Adapted from Herbach et al., 2006a)



Anthocyanidin	Substitution pattern			Color
	R_1	R_2	R_3	
Pelargonidin (plg)	H	OH	H	Orange
Cyanidin (cyd)	OH	OH	H	Magenta, Crimson
Delphinidin (dpd)	OH	OH	OH	Purple, mauve, blue
Peonidin (pnd)	OCH_3	OH	H	Magenta
Petunidin (ptd)	OCH_3	OH	OH	Purple
Malvidin (mvd)	OCH_3	OH	OCH_3	Purple

Figure 4. Structures of six important anthocyanidins in nature.

(Adapted from Delgado-Vargas and Paredes-López, 2003; Mercadante and Bobbio, 2008)

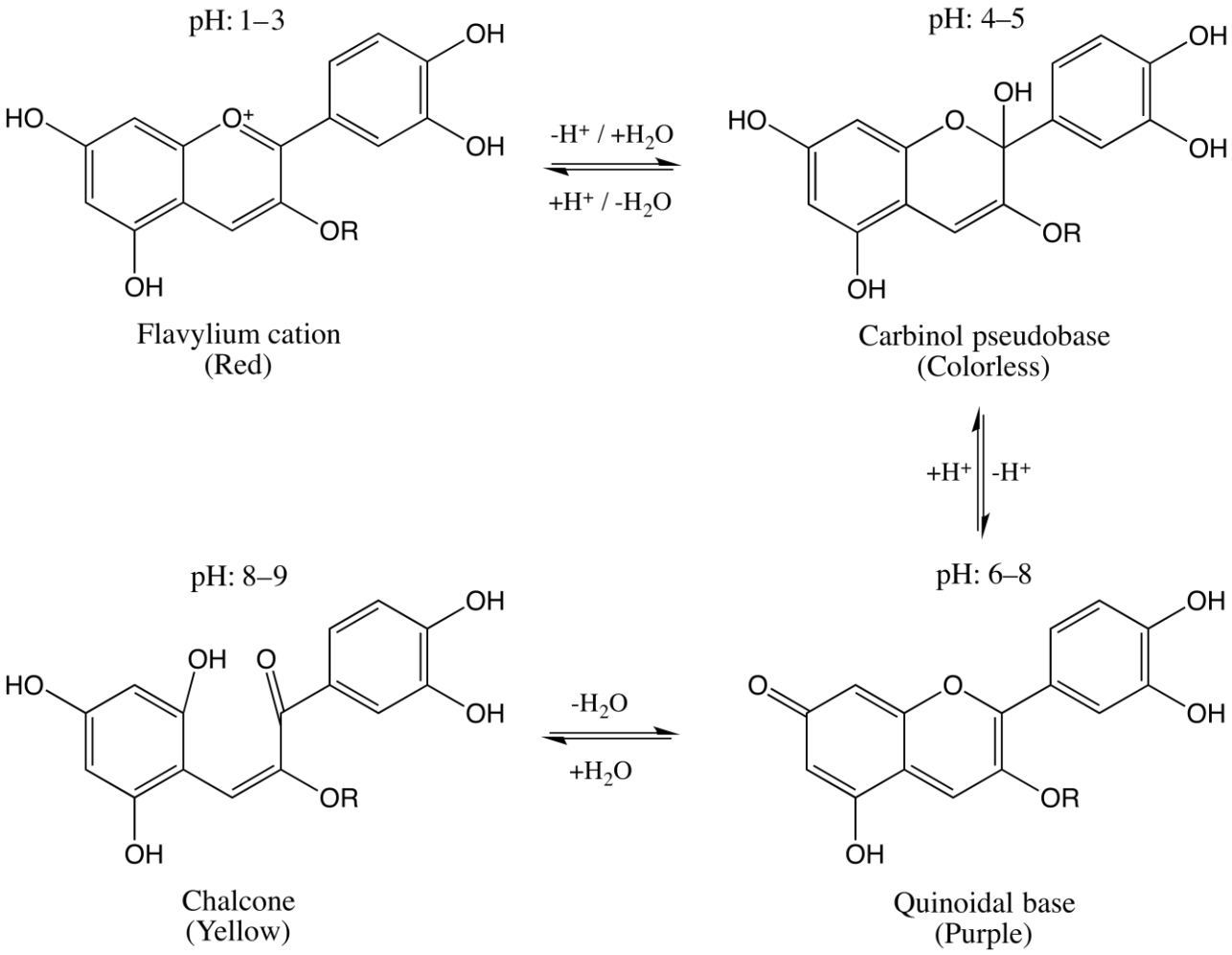


Figure 5. Changes in structure and color of anthocyanins at various pH.

(Adapted from Ananga et al., 2013)

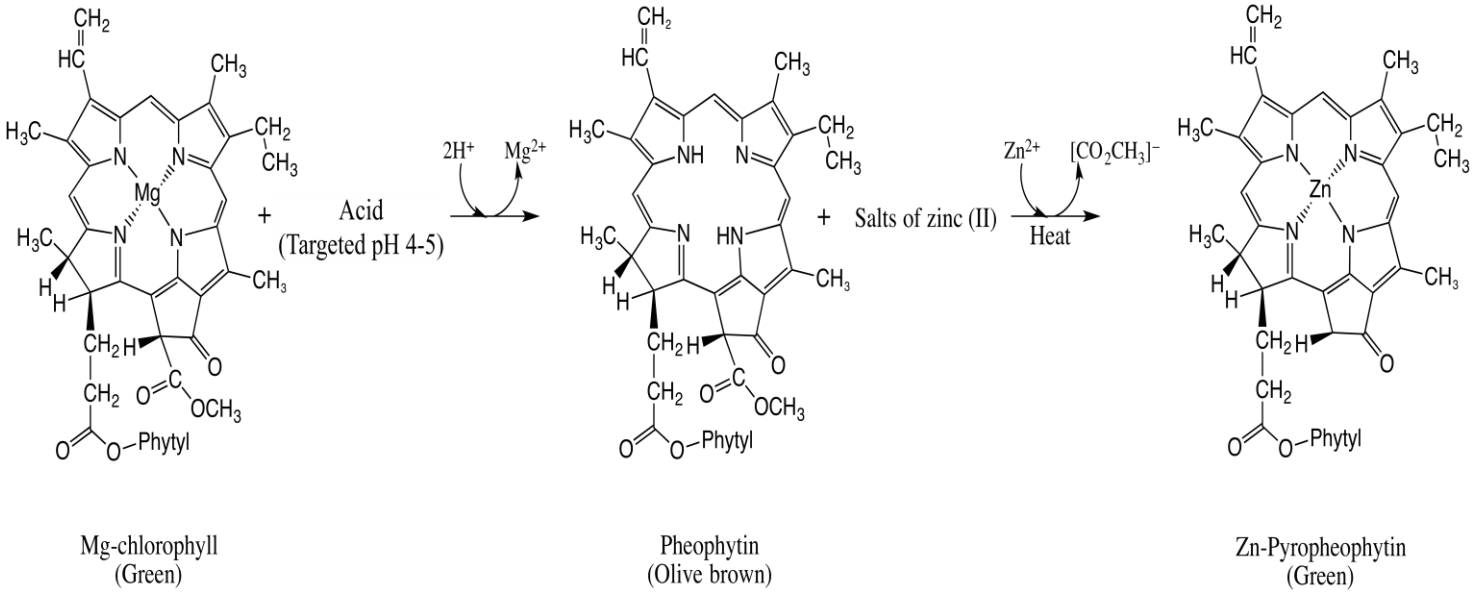


Figure 6. Chemical reactions for creating zinc-chlorophylls derivative.

(Adapted from Socaciu, 2008)