A review on analytical methods for the determination of natural colorants (green) in food commodities and beverages

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ABSTRACT
A wide variety of natural colorants are generally used for coloring food stuffs to make them more attractive, informative and reestablish the original color lost during the production process. However, in many countries the usage of natural colorants is limited due to their low stability. Green is one of the most important secondary colors as far as consumer goods are concerned due to looking fresh and natural. Chlorophyll and its metal derivatives like copper or zinc chlorophylls have been used as green colorant in food and pharmaceuticals. The regulatory authorities and food analysts have adopted precise analytical methods in assessing quality and safety of food products. These techniques mainly deal with determination of quality, quantity, colorant stability, safety and utility limits of colorants in food stuffs. These aspects are sensitively regulated by regulatory authorities as well as domestic and food suppliers. In this review, we discuss various extraction methods that are executed for extracting the chlorophyll and metallochlorophyllins from complex food commodities and beverages. In addition, different extraction methods used for food commodities have been presented. Exclusively the present manuscript reviews various analytical techniques used for the analysis of Na-Cu-Chlorophyllin (Na-Cu-Chl) employed in food products as food colorant.

Keywords: Chlorophyll; metallochlorophyllins; analytical techniques; colorant extraction; commodities; beverages; safety.

1. INTRODUCTION
Over the past few decades apart from the natural pigments, the production of synthetic food colorants has gained more importance in food processing industries. In our day to day life usage of artificial food colorants have been increased in all kinds of foods and beverages [1]. The growing trend in substitution of synthetic colorants to natural derivatives has facing the challenge of stability [2]. Apparently, green is one of the most complicated colors for both natural set-up and restrain. Colorants are appended to food and beverages mainly: (i) to compensate color lost during processing, (ii) to improve the color already existing, (iii) to avoid batch to batch disparity, and (iv) to add colorant to colorless foods. Nowadays the marketing methodology mainly has been motivated based upon the addition of food colorants to the food products. Food colorants which are added to the foods, may work like codes to differentiate the food products on sight. In ancient periods the color additives were used in food, cosmetics, drinks and drugs and those colorants were extracted from naturally available pigments [3]. Although the utility of natural pigment is good for health they too possess disadvantages such as low stability at moderate temperature, pH and light sensitivity. Therefore long term storage is not possible [4]. In recent years the consumer expectations are specific, as the food products must have pleasing color and also the added food colorant must be natural. The methods of food processing and long term storage conditions are two crucial steps in food industries. Hence the natural pigments are replaced by synthetic pigments [5].

As consumer expectation is increased on natural colorants the requirement for the natural pigments is frequently raised by the food industry. This demand for natural colorants could be resolved by researchers by increasing the stability of chlorophyll and chlorophyll derivatives which offers a more healthy way of coloring food commodities and beverages. Hence, studies of plant pigments should be undertaken in finding new sources of pigments. It does not only give a way of finding common alternatives for synthetic colorants, but also encourages discovering new methodologies for the natural colorant production. The range of tropical and subtropical vegetation offers the unknown plant compounds may prove appropriate for human needs. Chlorophylls, betalains, carotenoids and anthocyanins are the most common plant pigments. Generally, the most research has been focused on betalains and carotenoids but in recent day’s metallochlorophyllins have gained an interest in food science [6]. The extract which contains anthocyanin is usually used as food color till now in food industries. Compare to all other natural colorants the commercially available sodium, copper chlorophyllin (Na-Cu-Chl) is an ideal semi-natural green colorant for coloring food stuffs [7].

1.1. Distribution of Natural Colorants.
Food colorants can be classified as (1) natural colors, (2) nature-identical colors, (3) synthetic colors and (4) inorganic colors. Natural colorants are made by living organisms. Usually, colorants which are prepared by modifying components obtained from living organisms like metallochlorophyllins, caramel, and vegetable carbon, are also considered natural colorants although they are not found in nature. The man-made colorants, which also exist in nature, are carotene, canthaxanthin and riboflavin. Even though natural colorants are structurally diversified and obtained from various sources, it can be grouped into a few classes like tetrapyrrolic, flavonoids and tetratetrapenoic. The chlorophyll is an important member of the tetrapyrrolic, which is abundantly available in most of the higher plants. Tetratetrapenoic are carotenoids similar to chlorophyll ubiquitously available in most
of the plants [8]. Carotenoids and chlorophylls are the most abundant pigments in nature. The main sources of the organic colorants are photosynthetic bacteria, protozoa and plants which are essential for the development of other living organisms like vertebrate and invertebrate. Carotenoids are found in animals (e.g., skin of fish, bird plumage) while chlorophyll is not found [9]. Generally animal carotenoids are taken from the common diet and some other pigments such as riboflavin and heme proteins are also observed in animals with very essential functions but the function of few colorants such as melanins and flavonoids are not yet completely found out. Some other organisms have interesting colorants which have been utilized or have potential application. Lichens can generate depsides, the most significantly used colorant as textile dyeing agents [10]. Also, it acts as a chemical indicator i.e. inhibitor of prostaglandin synthesis, sunlight filters, and cytological stains. Some colorants such as parietin and orcein are derived from lichen substances. Nearly 1000 colorants have been found in fungi, whereas Fungi do not contain chlorophyll. Riboflavin as yellow color supplement is used as natural colorant in food production. In addition, natural colorants act as multifunctional agents such as chlorophyll as essential vitamin source in food, betalains as an essential source of amino acid and anthocyanins for quality marker of food stuffs, and flavonoids as pharmaceutical drugs. It is suggested that carotenoid does not exist in its original form due to influence of radicals and microenvironments during intake [11]. Hence the selected method development, use of advanced technologies for during production and optimization of process parameters are the very essential area of research handling natural food grade colors. Hence, a thorough review of analytical techniques applied for the determination of natural food colorant is necessary.

1.2. Food Grade Natural Biocolorants.

Natural biocolorants like canthaxanthin, carotene, annatto extract, tagetes extract, dehydrated red beet, dactylopus coccus extract, grape skin extract, cotton seed meal, fruit and vegetable juices, saffron, carrot and corn endosperm oil, paprika and riboflavin, paprika oleoresin, turmeric and turmeric oleoresin and xanthophylls are in exempt category of FDA and EU certification for use in food stuffs [12]. The bixin, carotenoids and norbixin are responsible for the yellow to orange color in plants. Annatto (Yellow to orange color) comes from the layer of seeds of Bixa orellana tree and its color hue depends on its solubility and pH; higher oil solubility creates brighter color hue. Normally annatto are available in three forms i.e. oil soluble, water soluble and oil/water dispersible forms. Hence it is available in emulsion to avoid precipitation at low pH and used for over two centuries in cheese and various other food stuffs as a food color [13]. Beta vulgaris (Red beet) extract demonstrates a variety of colors, it is mainly based on their content of yellow compound. Also a bluish-red color produced through betain is very stable at higher pH range than red cabbage extract. It has wide range of function in various food stuffs from candy to beverages [14]. The algae Haematococcus lacustris can produce carotenoids, whereas it is the natural colorant of orange yellow chanterelle mushroom. Canthaxanthin can be converted into vitamin A under stress. It is used in poultry due to its color shade of the yolk, in foods, especially in dairy products, fish and meat products, confectionary (soft and hard candy), snacks, beverages, fruit products, beer and wine. Also, it has more stability during photo degradation compared to carotene [15]. Interestingly, EU regulation does not approve canthaxanthin as food additive. Carnimic acid obtained from the extract of Opuntia ficusindica is used as magenta red while its water insoluble form carmine acts as a range of color from pink to purple. Except low pH it is very stable than synthetic food colorants towards chemical oxidation, light and heat. The water soluble form i.e. calcium carmine is used in alcoholic drinks but water insoluble form is used in beverages, meat, processed poultry products, sausages, bakery, alcoholic drinks, dairy products, sweets and desserts [16]. A fat soluble gossypol obtained from cotton seed is a yellow pigment [17]. Also, anthocyanin like cherry, cyaninid in apple and anthocyanins in grape provide reddish purple color to beverages. In addition, grape juice is used to impart variety of colors like yellow, purple and red (shades of raspberry, cherry) to a number of non-beverage food products like fruit fillings, gelatin desserts, and some confectionaries. Sometimes banana bract and Oxalis triangularis are potential source of anthocyanins which acts as food colorants [18].

Acylated anthocyanins from various edible sources (black carrot) are used as food colorants in food industry. Lycopen (E 160d) obtained from tomato which is approved by US and EU regulation is used as conventional food colorant due to its high stability under moderate temperature and pH. Riboflavin or vitamin B2 is present in several food products as yellow food colorant. It is permitted worldwide in different applications such as dressings, beverages, ice creams, tablets and other products but its naturally bitter taste and slight odor limit its uses. Green resembles natural sensation and is an additive of many consumer goods to show freshness [19]. Hence in this review paper we have focused on chlorophyll and metallochlorophyllins as green food colorants in food products and beverages.

2. CHLOROPHYLL AND CHLOROPHYLLINS

2.1. Chlorophyll in food products and beverages.

Chlorophyll being green photosynthetic pigment is found in green plants, algae, and cyanobacteria. Based on the chemical changes during food processing and storage, it can cause apparent discoloration of green vegetable to olive brown. Fig. 1 shows different forms of chlorophylls in food processing, cooking or storage with detailed structures and origin of them. During processing or storage, chlorophyll transforms into four major compounds. Pheophytinization is the most common product where two hydrogen atoms replace the central magnesium atom of the tetrapyrole in acidic condition under mild heat treatment [20]. Consequently green color changes to a brownish color. Chlorophylls can form pheophytins under acid and heat treatment, but can produce chlorophyllides under alkaline condition enzymatically followed by pheophorbide formation. Pheophytins formation is observed during steam-blanching of chlorophyll [21], while the de-esterification of the phyto chain is observed as second reaction leading to more polar compounds under enzymatic or alkaline conditions. Chlorophyllase being one of the chlorophyll catalytic enzymes exist in photosynthetic organisms and becomes active when the cell integrity is lost. Chlorophyll yields chlorophyllides on dephytlation followed by pheophorbide
on heating, but pheophytin produces pheophorbide enzymatically under alkaline conditions. At very high temperatures chlorophylls is converted to pyroderivatives due to the loss of the carboxymethoxy group. Normally pyrophophorbides and pyropheophytins are very common in cooked foods and hence pyrochlorophylls are the most common form of chlorophyll in cooked foods. Mild heat may generate the epimers from the native chlorophyll structure and oxidations may generate chlorophyll derivatives as follows: (i) the substitution at C13\(^2\) by a hydroxyl group by hydrogen atom, (ii) The rearrangement of isocyclic ring, setting up a lactone group, led to the formation of 15-hydroxy-lactone derivatives (Fig. 1). The oxidized chlorophyll derivatives produced in dehydrated food products or the stored items could be promoted by either chemically or enzymatically [22, 23]. Hence different enzymes such as lipoxygenase, peroxidase, and oxidase induce the chlorophyll oxidization in plant tissues [24-27]. Sometimes free radicals can oxidize chlorophylls at room temperature in model solutions [28, 29]. In Mg-Chlorophyllins [30], the reaction pathway might associate with polyphenols and hydrogen peroxide, lead to presence of 13\(^\circ\)-hydroxy- and 15-hydroxy-lactone chlorophylls in mature fruits and vegetables (Fig. 1a). Among the various food processing methods, dehydration promotes minimizing water related problems of the product and freeze-drying among sun-drying, oven-drying and freeze-drying are used preferentially to preserve green color [31]. Hence, the freeze dried system is less drastic than hot-air-dried one, because the higher amounts of oxidized derivatives pyroderivatives, pheophytinization, and epimerization take place in hot-air-dried food materials.

During fermentation of raw materials [32, 33] chlorophyllase enzyme is active which yields chlorophyllides at the beginning and later pheophytins and pheophorbides are produced by pheophytinization process. Actually chlorophyll degrades to pheophytin and pyropheophytin as a result of both heat and time conditions [34]. It happens also at high temperatures greatly manufacturing processes like pasteurization and sterilization. Sometimes heat resistant microorganisms like microbial spores are deactivated by pressure-assisted thermal processing (PATP) or high-pressure high-temperature processing (HPHT) in industries, but a thorough investigation is needed on the effects of these novel methodologies on food quality and safety features. Moreover studies have proved the pheophytinization of chlorophylls under high temperature heat treatment, but insignificant changes on chlorophyll molecule occur without high temperatures and pressure treatment [35]. Before the consumer ingestion, the commercial food products undergo various kinds of storage conditions and deteriorate to some extent, because external factors like changes in temperature, increased levels of light, oxygen, ethylene, water stress, and internal influence like enzymes and acids are the major causes for chlorophyll degradation.

Pheophytinization of chlorophyll in olive oil is observed even in dark storage conditions forming pyroderivatives and oxidative chlorophylls via chemical reactions. In the case of fruits and vegetables, the degraded enzymes can impact greatly on chlorophyll during different processing, storage, packaging methodologies as well as changes in the chemical composition of foods [36, 37]. In addition to storage conditions, appropriate cooking methodologies can retain the antioxidant property or maintain their nutrition level of fruits and vegetables [38, 39]. It is seen that heating increased the level of pheophytins in seaweeds [40], whereas different heating methods of steaming, boiling and microwaving pheophytinization percentage has decreased in green beans, leek, squash, peas, spinach and broccoli [41].

2.2. Stability of chlorophyllins in foods.

Different process and storage parameters are responsible for stability of phytochemicals and additive added to different food stuffs and beverages. Judicial management and controlling of these parameters are necessary for stability of these phytoconstituents and added chemicals in the food matrices.

*pH Value or Acidity*

The color shade of some natural colors is affected by the pH of food system. Anthocyanin (E163), common natural color additive is commercially available as purple/black colour which is prepared from carrot and grape skin extract and requires a low pH 3.5 in order to promote a red color, but above pH (7.5 to 8) the red color will change through purple to blue. Also, the high pH decreases anthocyanin’s stability. Similarly, although different studies have provided ways to achieve a stable natural green color, the stability of the green chlorophyllins is low in aqueous food system, but metallochlorophyllins can be used to provide a green color in low water activity applications. Generally, food and beverages are stored in warehouse under ambient conditions with a 9 to 12 month shelf life, which warrants the stability limits of natural colors. Hence innovated storage conditions and modified colorants are urgently required to follow the regulatory and design requirement of the food product in keeping a color system within the specification limits or criteria. It has been seen that many factors such as highly stable synthetic colorants and modifying process conditions like heating/cooling cycle during processing must be considered to increase the sustainability of color in foods and beverages. In addition sufficient care is undertaken to avoid instability of the color during food processing for longer product shelf life [42]. Although chlorophylls are highly stable in their biological environment, food processing and cooking cause instability as well as isolated forms from their biological environment are highly sensitive to chemical and physical changes.

2.3. Biological Actions.

The different physicochemical properties of chlorophyll and metallochlorophyllins support favorable biological effects on human health suppressing the harmful effect of potential chemical carcinogens. The sensitizing activity of chlorophyll to singlet oxygen (\(^1\)O\(_2\)) encourages its extended evaluation of the antioxidant activity of this group of colorants [43] and results suggest that the unbroken chlorin structure is responsible for its antioxidant property via a hydrogen transfer mechanism. Normally chlorophylls differ in *in vitro* antioxidant activity compared to their metal derivatives. Cu-chlorin e\(_6\), one of the main constituent of commercial Na-Cu-Chl is a more effective radical quencher of DPPH (2, 2-diphenyl-1-picrylhydrazyl), and Cu-pheophorbide a shows the strongest scavenging activity in ABTS\(^+\) (2,2'-azinobis(3-ethylbenzothiazoline-6- sulphonic acid) than chlorophyll [44]. Animal studies on chlorophyllins indicate that it is an antioxidant, can reduce oxidative damage caused by radiation or chemical carcinogens [45]. The potential antiradical property of
these chlorophyllin colorants develops due to chelated metal ion and the π-cation radical in the porphyrin structure. Interestingly the chelated metal ions supply electron to break the chain reaction and π-cation radical reinforces electron density surrounding central bounded metal and away from porphyrin backbone.

It is interesting that the bioavailability of some chemical carcinogens is reduced due to their molecular complexes with chlorophyll and the chemical carcinogens are absorbed less and low concentration available in inner tissues after co-ingested with chlorophyll. Also, it has been observed in the case of heterocyclic amine [46, 47]. This effect proves that chlorophyllins can be used as a potential anticarcinogen. Although the mechanism of anticarcinogenic activity exerted by chlorophyll is not clear, it may inhibit the activity of cytochrome P450 enzymes or the activity of phase II detoxification enzymes enhanced by chlorophyll and their derivatives by enhancing excretion of carcinoogens and preventing procarcino gens conversion to their active form, respectively. Based on animal studies and cytotoxicity studies in hepG2 human hepatoma cell lines Cu-chlorin e4 ethyl ester could be effective promotor of phase II detoxification enzymes [48].

The similarity in structures between the iron porphyrin of hemoglobin and porphyrin ring of chlorophyll and the myoglobin is the basis for reinforce chemical interactions between these compounds. Chlorophyllins prevent the formation of cytotoxic factors arising from metabolism of heme in the gut and red meat consumption which decreases colon cancer as well as colonic mucosa. This effect is observed in rats fed with purified chlorophyll assimilated into heme-rich diet [49]. Balder et al. (2006) [50] and Fonseca-Nunes et al. (2013) [51] proposed after conducting population-based cohort investigation that colon cancer risk is positively related to a diet of low intake of chlorophyll and high intake of heme iron. In addition, chlorophylls show anticlastogenic activity against inorganic metal salts mediated clastogenic activity like cobalt chloride and cesium chloride. Na-Cu- chlorophyllin is used regularly and demonstrated to be effective as a natural chlorophyll supplement. It is observed that chlorophyll colorants suppress the genotoxicity of orally delivered chemotherapeutic agent 4-nitroquinoline 1-oxide in Drosophila with less DNA damage [52]. The use of chlorophyllin colorants for the dietary and medical applications is also proposed like wound healing, control of calcium oxalate formation and anti-inflammatory properties and relieving fecal odors for elderly patients [53]. The derivatives chlorophylls like pheophorbide (dephytylated chlorophyll) are applied in the development of photodynamic therapy. Hence they are potential photosensitizers which can catalyze the production of ROS in cancer cells during irradiation at a selected wavelength [54].

The results regarding digestive nature of chlorophyllin derivatives are less due to common assumption that chlorophylls cannot be absorbed by humans, but some study has shown that chlorophyllin derivatives are absorbed in human serum in the level of ∼2.0 μg/mL as Cu- chlorin e4 and its ethyl ester being the main constituents after ingestion of 300 mg chlorophyllin per day in a controlled clinical trial [55]. Moreover, the stability of chlorophyllin derivatives against several pH changes and enzyme reactions, suggest partial oxidation and pheophytinization of original ingested chlorophyllin structure with modified property [56], On the other hand metallochlorophyllins such as copper chlorin e4 and zinc-pheophytin are relatively stable in the gastrointestinal phases [57].

Naturally abundant chlorophyll pigments in habitual dietary food sources with low absorption could be considered physiologically relevant. Furthermore, different chlorophyllin derivatives with wide range of polarity show different absorption rates in humans and release efficiency from the food matrix. Moreover micellization extent of chlorophyllin derivatives varies with their hydrophobicity and food matrixes. Normally dephytylated chlorophylls have a significant micellization rate (70%–95%) than their phytlylated analogs (10%–55%), while about 3%–10% of the micellar chlorophylls can be internalized through intestinal cells. The both in vivo and in vitro studies using dried spinach leaves show about 3.5% of phytlylated chlorophylls absorption [58]. This rate of absorption is quite similar to that of carotenoids which commonly co-exist with chlorophyll in food materials, while the commercial food colorant Na-Cu-chlorophyllin shows a higher efficient absorption rate of 45%–60% [46].

![Figure 1. Structures of chlorophyll (Chl) molecule and its derivatives during food processing or cooking and storage.](image-url)
3. EXTRACTION OF CHLOROPHYLLINS FROM DIFFERENT FOOD MATRIXES

The extraction of chlorophyllins was done by selective extraction techniques based on nature of food matrices. In prior to extraction of chlorophyllins from food products about 1 to 5 g of food sample was taken in a centrifuge tube and 200 mL of internal standard Green 3 (50 mg/kg), 10 mL. ethyl acetate (EA): acetone (5: 1 v/v) and 15 mL of citrate/phosphate buffer (pH 2.6) were added. Samples like boiled sweets, jelly, and jelly sweets were dissolved in warm water. For samples like flour confectionery and dried soup mixes were homogenized in the buffer solution by micro dispersion tool followed by solvent extraction of 2-6 h using orbital shaker. For the extraction chlorophyllins from biscuit samples it was defatted using homogenization with 10 mL hexane and centrifuged to remove hexane. Either using vortex mixer or micro dispersion tool for 1 min the contents were homogenized.

The sample mixture was centrifuged for 5 min at 1600 x g in a clean centrifuge tube and the upper layer was removed. If upper layer was not separated well or gel-like or emulsifiers, about 0.5 mL ethanol was slowly added without shaking, centrifuged and then the upper solvent layer was removed as mentioned above. If a colored emulsion layer was there, the lower aquatic layer was carefully removed using a pipette. Then 3 mL of acetone was added and vortex mixed for 5 sec, centrifuged and with the pooled ethyl acetate: acetone extract the supernatant were mixed. If necessary these steps were repeated. Further 5 mL of 5: 1 ethyl acetate: acetone with buffer mixture was vortexed for 1 min, centrifuged and the upper layer was separately taken into the first extraction. This process must be repeated until no more color was perceived in the upper layer. Through gentle blow drying under nitrogen at less than 40°C the solvent was evaporated. The extracted sample was dissolved in 2 mL acetone: methanol solution (2: 1 v/v). The solids adhering to the sides of the container wall was dispersed using sonication if necessary. Then the extracted sample was filtered using 0.2 mm nylon filter and stored for analysis. Generally this procedure could be efficient to remove small amounts of precipitate, but the sample extracts like ice cream, the additional clean-up steps were required for the presence of co-extracted emulsifiers. If a significant precipitate was noticed, then the extract was centrifuged at 1600 x g for 5 min and carefully the supernatant was transferred to a clean glass vial. The colored residue was extracted twice using 2 mL acetone: methanol mixture (2: 1 v/v) if the residue was green or yellowish green, and mixed with the original supernatant [59, 60].

The solvent was dried by gentle blow-drying of N₂ gas and the residue was dissolved in 2.0 mL acetone:methanol mixture (2:1 v/v) by vortex mixing for 1 min. Also, dispersed solids cohere on container wall was collected by sonication if required. Finally the extract was diluted to a known volume and filtered using 0.2 µm filters. The extracted sample was stored at -18°C for longer term. To study the original chlorophyll molecule in food products, it is very important to isolate the green colorants using suitable analytical methodologies that can give minimal changes in their original state. Due to sensitive nature of chlorophylls the extent of some conditions such as alkaline, acidic or heating extraction must be avoided or at least minimized and the use of dark or green-light condition is necessary. Till now there is no single proper and selective analytical methodology for extraction of chlorophyll, as the process should be suitable to the property of the food matrix and the interfering compounds. Practically green leaves are used as the common plants tissue for chlorophylls extraction and study.

Homogenization of green leaves in appropriate solvent is necessary and chlorophylls are isolated from the substrate by multiple back to back extractions with solvents like ethanol, acetone and methanol mixed with water. Generally the extracting solvent is saturated with MgCO₃ to avoid Pheophytinization. In addition, it is necessary to neutralize the acid released during homogenization. For quick removal of residual plant debris and to avoid phytol cleavage induced by chlorophyllase activity the extraction procedure was performed under low temperature [61]. Consequently the extract was stored at low temperatures and all subsequent analyses were done immediately. Sometimes changes in analytical procedure are important for the extraction of chlorophylls from oily materials due to the high content of triglyceride which delays the extraction. Generally the saponification step is not recommended, because this may completely alter the chlorophyll structure. It is observed that hexane and dimethylformamide (DMF) mixture is used to extract chlorophyll and xanthophylls (DMF) from olive oil, whereas hexane is used for extraction of carotene and triglycerides. Normally, cellular structure in aged botanical materials inhibits pigment extraction, because aged plant tissues hold many of secondary metabolic products like polysaccharides [62]. In such this case, for complicated materials the combination of solvents is used [63].

To inhibit the degradation of chlorophyll during its size reduction by different methodologies such as cell rupture technologies [64], ultrasound and freeze-drying [65], the use of liquid nitrogen, solid-phase extraction to concentrate chlorophylls from oily materials by diol-phase cartridges and supercritical fluid extraction are used.

4. CHLOROPHYLLINS (E141) AS FOOD ADDITIVE

Nowadays the commercial availability of chlorophyll and its derivatives (sodium copper chlorophyllin, copper-pheophytin chlorophyll α, pheophytin α) is increasing. At the same time, isolation and preparation techniques are essential for the rest of other chlorophyll derivatives. Thin-layer chromatography is an easy and quick method to isolate chlorophylls. Preparative high performance liquid chromatography (HPLC) technique allows the separation of chlorophylls from their allomerized and epimers counterparts. Metal free chlorophyll derivatives (pheophytin) can be easily procured by acidification with a few drops of 5 N HCl, which results pheophorbide from chlorophyllide and pheophytin from chlorophyll. By mixing 20 mL ZnCl₂ (1 M) in acetone the desired chlorophyll metal-pheophorbide or metal-pheophytin can be obtained [66, 67]. Na-Cu-Chl is obtained by saponification of chlorophyll extract in methanolic potassium hydroxide followed by neutralization with hydrochloric acid and finally chelation with copper (II). Generally, synthesis of dephtyalted chlorophyll like chlorophyllide or pheophorbide is obtained by the enzymatic conversions using partially purified chlorophyllase enzyme extract.
at pH 8.5. As the crude enzyme extracts are often utilized, several side reactions i.e. oxidation reactions are induced by related plant enzymes like peroxidase and lipoxidase. Similarly, chlorophyllide is modified by adding some hydroxyl groups of tetrapyrrol ring side chain, whereas heating is used to get the desired products like lactone- or hydroxyl (pyroderivatives). It is proved that for the formation of pyropheophytin the energy requirement is higher to procure the oxidized phophydrin derivatives or 13'-hydroxy and 15-hydroxy-lactone [68].

Chen et al. (2015) described a complete methodology to procure chlorophyll derivatives [69]. Initially, hydroxy chlorophyll containing 0.2-0.8 mg pigment and 7 mg/mL SeO$_2$ in pyridine solution was heated at 70°C for 3 h, but formation of 15'-hydroxy-lactone derivatives was noticed after heating for prolong the time of 7 h. Pyrochlorophyll derivatives were prepared by heating parent chlorophyll in pyridine at 80°C for 4 h. Chlorophyll is unstable to high heat treatment and hence decarbomethoxylation (at C13$^3$) was induced by heating phophytin or pheophorbide (magnesium-free derivatives) at higher reaction temperatures (>100°C).

Natural green and other colours are biocompatible and benign to human health. Hence European Union (European legislation regulation, EC No 1333/2008 and amendments) allowed in 1962 the utilization of natural green colorants Eu140 and E141, which are structurally similar to chlorophyll and was published with the Council Directive No of 62/2645/EEC. E140 comprises direct chlorophyll derivative extraction using organic solvent from natural sources such as alfalfa, grass, spinach, nettles and from edible plant components. In addition, the final product contains other pigments, lipids and waxes and the final product of chlorophyllin colorant is waxy. Generally chlorophyll or E140 derivative is lipid-soluble, while water-soluble foods used an alternative marketed powder Na-Cu-Chlorophyllins (E141 (i) and (ii)).

It is prepared by saponification of the extracted product from edible plant material. The ester-phytol bond is broken during saponification improving the polarity of the chlorophyll derivatives. These metal derivatives of chlorophylls show good stability which favors the food industries to use the metallochlorophyllins as green colorant prepared from copper salts with lipid or water-soluble chlorophyll solutions. The insertion of metal ion into the chlorophyll structure stabilizes the molecule, and the green color remains the same which is called regreening effect and provides high stability to the colorants that can be stable during processing and the storage of the colored food [70, 71]. These metallochlorophyllins’ permitted level is summarized in different food stuffs recommended by FSSAI (Table 1).

| Food category (E 141 (i) and (ii)) | Permitted maximum level (mg/Kg) |
|-----------------------------------|---------------------------------|
| Unripened cheese                  | 50                             |
| Ripened cheese includes rind      | 15                             |
| Cheese powder (for reconstitution; e.g., for cheese sauces) | 100                           |
| Flavored processed cheese, including containing fruit, vegetables, meat, etc. | 50                             |
| Fruit in vinegar, oil             | 100                            |
| Canned or bottled (pasteurized) fruit | 100                         |
| Fruit preparations, including Fruit pulp, purees, fruit toppings and coconut milk | 100                        |
| Fermented fruit products          | 100                            |
| particulate drinks, includes carbonated fruit beverages, carbonated beverages with fruit | 300                          |
| Snacks and savouries –potato, cereal, flour or starch based | 350                          |
| Candied / glazed / crystallised fruit including murrabba | 250                          |

5. COMPOSITION OF SODIUM COPPER CHLOROPHYLLIN

The composition of Na-Cu-Chl has been examined thoroughly by many scientists, and the present studies are in agreement with some of these reports [72]. More than fifty years ago only Cu- isoclorin e4 was found as the major analogs of Na-Cu-Chl [73]. Later another study found Cu-pyropeophorbide a, Cu-chlorin e6, Cu-rhodochlorin, Cu-purpurin 18 and Cu-isoclorin e4. In addition, the 3'-hydroxy derivatives were found due to interaction among water and vinyl group of all the compounds mentioned above except Cu-chlorin e6. These reports match the present findings, but Cu-purpurin 18 was not established. Cu-purpurin 18 is an anhydride of chlorin p6, and probably the purpurin 18 was generated from chlorin p6 during analysis. Strell et al. (1956) decoppeered the Na-Cu-Chl and extracted derivatives of chlorophyllin with hydrochloric acid from an ether solution [74]. It was observed that at pH 6 the purpurin 18 was stable but at higher pH it hydrolysed to chlorin p6 [75]. Therefore, Na-Cu-Chl was prepared from chlorophyll under alkaline conditions. As Cu chlorin p6 can dominate over Cu purpurin 18 under alkaline condition, Strell and co-workers prepared the purpurin 18 under the acidic conditions. Also, Cu-isoclorin e4 and Cu-chlorin e6 were the derivatives of Na-Cu-Chl [76]. Some other studies reported the same main derivatives i.e Cu rhodin g7, Cu pheophorbide a, Cu chlorin e6, and Cu chlorin e4) of Na-Cu-Chl [77]. These reports concluded that the Cu-chlorin e4 might be the main analog constituting 35–60% range of four samples [78] and 81% of them observed Cu-chlorin e6, Cu-isoclorin e4 and Cu-pheophorbide derivatives as the main analog of Na-Cu-Chl [79]. Egner et al. (2001) and others reported Cu-chlorin e4 (33%) and Cu-chlorin e6 (31%) are the major components of Na-Cu-Chl during storage [46, 80]. Scott et al. (2011) reported that Cu-chlorin e4 showed absorption maxima at 410 nm and 634 nm, while Cu-isoclorin e4 also had absorption maxima at 406 nm and 627 nm, respectively [100]. Hymnen et al. (2006) [81] reported expected rhodin g7 and chlorin e6 as the major products of chlorophylls after saponification. However Mortensen and Geppel, (2007) [82] found that Cu-chlorin e6, Cu-chlorin p6 and Cu-isoclorin e4 were the major components of Na-Cu-Chl during storage and suggested that the formation of...
chlorophyll p6 from chlorophyll was due to oxidation at C-13, but the occurrence of Cu-isochlorin e4 as a major analog in Na-Cu-Chl was somewhat surprising, because decarboxylation of chlorin e6 on heating may lead to the formation of chlorin e4. Streel et al. (1956) [74] found Cu-isochlorin e4 in Na-Cu-Chl sample, but they did not find any isochlorin e4 (uncoppered) and chlorin e4 chlorophyllins. Therefore it seems that the formation of Cu-isochlorin e4 is directly related to Cu²⁺ involvement, but the actual formation mechanism is not known yet. Fischer and Stern, (1940) [83] reported the formation of porphyrins through the coppering of chlorin p6. It appears that Cu²⁺ does not oxidise chlorins to porphyrins, but Cu-chlorins is oxidised to porphyrins when unbound Cu²⁺ acts as a catalyst. In plants the chlorophyll-a and chlorophyll-b ratio is generally 3:1 and hence a notable composition of rhodins could be expected in Na-Cu-Chl. However, rhodin g7 was found as a major component of Na-Cu-Chl with a trace amount of Cu-rhodin g7 [44]. One possible justification might be that Cu²⁺ oxidizes the formyl group of rhodin g7 to a carboxylic acid and carboxylic acid compounds could still illustrate a rhodin type of spectrum. This type of sequence reactions would transform rhodin g7 to 7-demethylchlorin e6 with a similar spectrum of chlorin (mass as 643 amu with copper). Also, Cu-chlorin p6 was not detected by mass spectroscopy and the fate of chlorophyll-b during manufacturing of Na-Cu-Chl was not established yet [80].

6. INTAKE OF COLORS

The intake of food colors is determined by the Fractional Flow Reserve (FFR) method using a food frequency Questionnaire (FFQ) which consists of respondent’s name, gender, age and food habit. Furthermore the queries on the intake of specified colored food commodities and frequency of consumption are collected with information on product type and an identical amount of colors added in foods. Normally the consumption information of corresponding colored foods has been utilized for calculating the intake of food colors which are then correlated with different acceptable daily intake (ADI) based on maximum allowable limits of each food colors. In addition, the quantity of colours is considered on the basis of their permissible limits in various aged peoples. The approach of ADI offered an expression of safety to use and also implement administrative authorities to take sufficient legislative measures for their control. The measure of ADI is followed by the approval of an additive in different food products and their probable intake. Although this type of survey is essential for exposure and safety study of very toxic substances via foods in most of the countries, developing countries do very less national level studies on this issue. Moreover actual quantity and the type of colors are engaged in food products and the consumption data of corresponding colored products are used to calculate the consumption of food color for ADI calculation. The corresponding ADI based maximum permissible limits of each color are compared with the concentration of permissible limits in various aged people.

For each individual the mean color absorption per day is calculated by the analytically identified concentrations of colors which contain in the ingested foods. To assess the potential issue correlated with the consumption of synthetic colors by the population inspection, a comparison was done between the ADIs recommended by JECFA and through the survey observed mean color intake. By the concentration of colors in foods the mean color intake per day by each individual was calculated to identify the ADI of the colors ingested in the food [84]. The ADI can be calculated by this formula: Color ingested per day/body weight of an individual. The ADI value indicates the volume of a substance that could be intake everyday all over the lifetime of an individual with no health effect and has been used to assess the issue associated with intake of synthetic food colors using a selected population. Children weights were not included in the survey time, but standard National Centre for Health Statistics (NCHS) weight for height/age table was used to consider the weights of the children [85].

\[
\text{Color (mg kg}^{-1}\text{bwt)} = \frac{\text{Amount of colored food consumed (g or mL)} \times \text{concentration of color intake present in food (mg kg}^{-1}\text{)})}{\text{body weight (kg)}}
\]

Plenty of analytical methods for food colors identification have been suggested like thin layer chromatography (TLC), polarography, spectrometry, adsorptive voltammetry, ion chromatography, capillary electrophoresis (CE), differential pulse polarography, spectrophotometry, immunoassay, derivative spectrometry and other spectrophotometric methods which related to chemometrics. Although all these methods are rapid and simple, they are unable to identify different food colors in the complex food matrixes. Capillary electrophoresis provides good resolution in short analysis time, although in the electropherograms tailing or fronting peaks of food colors are determined. Generally reverse phase-HPLC (RP-HPLC) and ion-pair liquid chromatography (IP-IC) is used for fast identification of food colors in drinks as well as all other foods like candy, sweets, mixtures etc. Specifically, when extensive treatment is not possible, there is a possibility to do selective detection using spectrometry in the visible region [86].

7. SAFETY AND QUALITY EVALUATION OF NATURAL COLORANTS

Colorants, especially food colorants are evaluated sensitively in general by national and international regulators to judge, whether i) the colorants are technically suitable for future usage of the products, ii) are batch-to-batch reproducible, (iii) are compatible to various safety and purity conditions are given in national and international regulations, (iv) are noninteractive to food molecules and beverages, and (v) finally are nontoxic and biocompatible to humans and benign to environment. A range of
analytical methods is employed to evaluate the safety and quality attributes (aspects) of food colorants. The choice of method depends on the nature of colorants (artificial or natural) as well as its method of manufacturing and its physicochemical properties. Also, these information on colorants is highly useful to select the most suitable analytical methodology for quantitation [87]. In order to meet application requirements of different food colorants in a number of formulations, stringent quality measures are required for different categories of colorants which vary product to product.

Hence, the analytical requirements for safety and quality evaluation vary different for different categories of food products and beverages. In addition, once the color is added to food, the quality and safety criteria could vary due to complex food matrices. Therefore, the food manufacturers are highly selective to choose analytical methods in analyzing accurately its quantity in color formulation of food products. Noticeably (i) the product should have the desired shade, hue, strength, and stability, (ii) low product cost, (iii) obeying regulations with its chemical identity, and (iv) correct concentration of colorant and water or solvent solubility. For natural colorants manufacturers look into acid/alkali stability, heat and light effects, additives or processing methodology as well as human psychological aspects in dealing visual findings of food, control and measurement of color by the standardized International Commission on Illumination (CIE) system [88].

In EU all the permitted food color additives were evaluated thoroughly for safety on regular basis, including natural colorants which are obtained from well known food sources like spinach. In addition, other information such as purity criteria i.e. total percentage of colorant, the main color components including all isomers and derivatives as well as known processing artefacts and impurities were monitored. Also, evaluation process considered extracting solvents, other co-extracted materials including resins, volatiles, and oils during monitoring. As per EU directions when new research will be available, or when there will be an considerable variations to manufacturing conditions or new application, the food additives safety should be reevaluated. For instance, European Food Safety Authority (EFSA) announced opinions on food additives safety of use on regular basis. The scope of these evaluation includes attributes of chemical stability, characterization, and reaction in food products in addition to the biochemical (or toxicological) safety aspects. The latter includes risk assessment, exposure estimates, mode of action, including animal/human studies, and acceptable daily intakes (ADIls), which make available to scientific recommendations for risk managers [89].

8. METHODS FOR DETERMINATION OF NATURAL COLORS IN DIFFERENT FOOD MATRIXES

Normally natural food colorants provide a more different and complex chemical classes compare to synthetic colorants which led to an analytical challenge in identifying and quantifying using different analytical methodologies. Therefore, the development of a range of proficient extraction and analysis methods is essential, especially most of the natural coloring components are unstable during extraction at ambient conditions. Clean up techniques are used to separate the colors from the sample mixture and moreover the co-extracts present in overwhelming excess after extraction made more difficult due to the extensive usage in raw materials and foodstuffs. Alternately if a compound contains mixture of colorants after extraction or degradation products of analyte and the interfering compounds in a range of polarity, then the development of chromatographic separation is usually necessary. Identification and quantification of isolated coloring components are generally achieved by spectrophotometric technique such as UV-vis absorbance, but inorganic colors like titanium dioxide (E171), vegetable carbon (E153), calcium carbonate (E170), iron oxides and hydroxides (E172) require moderate analytical approaches. These methods are different from the extraction and chromatographic methodologies utilized for other synthetic and natural colors. However, there are very few standardized or validated methods are available for the determination of natural colors in food stuffs. Till now there is no legal definition for natural colors available as well as several disparities in their classification [10, 25]. The spectroscopic conditions used for the determination, characterization, and assay of all natural coloring components permitted in the EU by JECFA [90] are summarized as follows (Table 2).

8.1. Riboflavin (E101).

Since there is no method of assay is suggested for riboflavin in EU purity standard, the JECFA recommended spectrophotometry based identification methods at 267, 375, and 444 nm with specific rotation α267-D and color response which is similar to that of recommended riboflavin-5'-phosphate with an absorption band at 444 nm. In almost all biological system riboflavin is discovered in low levels, but is highly abundant in meat at ca. 2 mg/kg, in liver at ca. 30 mg/kg, in cheese at ca. 5 mg/kg and milk at ca. 1.5 mg/kg respectively. Furthermore it is also used to reinforce certified foods for nutritional purposes as food colorant. Microbiological assay routinely used in olden days to determine the levels of riboflavin in foods assay is time consuming. Generally chemical assay process involves dil. HCl at moderate temperature to remove the protein bound riboflavin followed by extraction, isolation and measurement through fluorimetry [91].

Usually acid hydrolysis (typically 0.1 M HCl or H2SO4 at 100-120 °C) step is used to extract analyte from protein bound moiety and to promote the conversion of polysaccharides like starch to sugars, but hydrolysis step is time consuming and results different efficacy due to involvement of enzymes and varying conditions. As riboflavin shows fluorescence HPLC equipped with fluorimetric detector (FLD) was considered accurate, specific and sensitive method for quantitation at satisfactory level of detection sensitivity and selectivity in food samples. Arella et al. (1996) established a HPLC method to identify and quantify the riboflavin in chocolate powder, yeast, powdered milk, baby food, fruits, food complements, and tube feeding solutions [92]. For fluorimetric analysis different food samples such as mackerel, pork, yeast, powdered milk, veal, wheat flour, porridge oats, carrots, rice, peas, orange juice and milk powder were hydrolyzed in dil. HCl at 100 °C for 30 min and then hydrolyzed by enzyme [93]. Isocratic RP-HPLC equipped with fluorimetric detector was monitored at 422 nm and 522 nm using 0.05 M sodium acetate and methanol mobile
of curcuminoids in solvent (ACN) extracts of turmeric products [99]. Scotter (2011) described the extraction and analysis conditions for a choice of existing methods for curcumin [100]. In addition, RP-HPLC with MS detector could be employed to characterize curcuminoids and other components in turmeric through acetonitrile and ammonium acetate buffer as mobile phase. Moreover, thermospray and particle beam interfaces are used for the identification and quantitation of curcuminoids in methanolic extract against an external standard (technical grade curcumin, 80%) [101].

8.3. Chlorophylls (E140i) and chlorophyllins (E140ii).

There are six types of chlorophylls in nature. The two main types in plants are chlorophyll-a (Chl-a) and chlorophyll-b (Chl-b). Chl-a absorbs violet and orange light (at 430 and 660 nm), while Chl-b absorbs mostly blue and yellow light (at 450 and 640 nm). Chlorophylls have diverse spectroscopic properties due to their characteristic sites and the absorption maxima such as S and Q bands denote Chl-b and Chl-a (Fig. 2). These absorption and fluorescence methods were used for the identification and quantitation of chlorophyll [102]. The chlorophyll can be identified using absorbance spectrum and can be quantified through its molar extinction coefficient however the occurrence of one or more other chlorophyll derivatives causes difficulty of quantification. If carotenoids are present in chlorophyll sample, they are usually determined using wavelengths at 430 nm and 660 nm. The method of assay using spectrophotometric was suggested in EU purity measure for chlorophylls is mainly based on $E_{1\text{cm}, 1\%}$  value of 700 and an absorbance at 409 nm in diethyl ether, whereas the assay for chlorophyllins was performed at two wavelengths 405 nm ($E_{1\text{cm}, 1\%}$ 700) and 653 nm ($E_{1\text{cm}, 1\%}$ 140). The identification tests suggested by JECFA are TLC and solubility. The JECFA assay for chlorophylls depends on measurement of absorbance in diethyl ether at six specific wavelengths demonstrating the absorption maxima of Chl-a & Chl-b and phaeophytins a and b (PP-a and PP-b), respectively, the each of derivative content is measured from the changes in absorption size after and before the treatment of oxalic acid. For the determination of chlorophylls several methods have focused on natural derivatives which are present in fresh and processed vegetables and fruit [103 and different foodstuffs] [104-108].

The most important methodological approaches are solvent extraction, liquid-liquid partition and evaluation using reverse-phase HPLC coupled UV-Vis spectroscopy. Because chlorophylls are labile colorants which are sensitive in the air leading to oxidation and degradation through enzymes like chlorophyllase and the loss of chelated metals (i.e. Mg$^{2+}$) by acid. Consequently appropriate care is needed; extraction and assessment must be performed rapidly (promptly) in hazy light and at moderately low temperatures [107, 108]. Generally, chlorophyll extractions are performed by homogenization of the sample with methanol and acetone particularly for samples of high water content, which promotes break down of pigment:protein complex. To isolate and purify chlorophyll derivatives from crude extracts of leaf solvent partitioning methods are also used but the rate of recovery is poor. RP-SPE is used to separate phytlylated and dephtylylated colorants from acetone extracts of plants and furthermore it is used in isolation and clean-up of PPa and PPh in aloe, chlorella, kale, spirulina, mallow, Jews and green tea leaves prior to HPLC extraction, followed by filtration. The limit of quantitation (LOQ) measured through raw data is ranged between 0.1 and 0.4 mg/kg. Furthermore, the isomers of thermal degradation products of annatto displayed similar chromatographic performance to the curcuminoids and therefore it should be clearly distinguished. RP-HPLC with both isocratic and gradient elution with THF: water as mobile phase has been employed for the separation of annatto and curcuminoids. Annatto and curcuminoids are simultaneously extracted from fish through crushing with Celite and HCl in the presence of ascorbyl palmitate and RP-HPLC equipped with PDA detection at 422 nm where the curcuminoids peaks are clearly noticeable from norbixin isomers and noise peaks [96].

High performance thin layer chromatography (HP-TLC) silica gel plates with methanol: chloroform mobile phase could be used for the evaluation of curcuminoids in benzene extracts of *Curcuma longa* germplasm and quantitation through densitometry at 425 nm. Phattanawasin et al. (2009) reported methanol extraction prior to TLC with detection through imaging analysis [97]. RP-TLC using THF: acetonitrile: oxalic acid for quantitation by scanning densitometry can be examined turbidic oleoresin and RP-HPLC equipped with PDA detection at 422 nm where the curcuminoids peaks are clearly noticeable from norbixin isomers and noise peaks [96].
analysis [109]. Counter-current chromatography technique also
be used to isolate Chl-a and Chl-b from spinach [110].

Although quantification of chlorophyll in fermented olives is
done using rapid spectrophotometry followed by TLC [111], the
better specificity and sensitivity is achieved by fluorescence
spectroscopy at 409-468 nm and the emission wavelengths of 650-
670 nm [112]. The solvent nature also has significant differences
in emission spectra which could be noticed between polar and
non-polar solvents, ligation to protein which intensively influences
the position and shape of UV/Visible spectra [108]. For isolation
of chlorophyll derivatives from crude extract, one or more
chromatographic techniques are employed, while low cost easy
TLC separation is relatively used for qualitative analysis of
colorants. The drawback of these methods includes choice of
stationary phase and the separation of isomers [108]. Chlorophylls
and carotenoids separation will be achieved by conventional column
chromatography in different phases such as DEAE-Sepharose,
powdered sucrose, cellulose/ MgO/Hyfloupercel [108]. HPLC
coupled with photodiode array (PDA) can separate chlorophyll
derivatives in food stuffs, and fluorescence detector facilitates the
determination of chlorophyll derivatives. NP- HPLC also could be
used to separate carotenoids and chlorophyll derivatives but RP-
HPLC is a widely used method for regular analysis due to the
wide range of column availability [100, 101, 107, 108]. Retention
is mostly affected by modern stationary phases, whereas
combinations of water, methanol and ethyl acetate as mobile phase
are usually used as mobile phases based on the polarity of
compounds [113]. Generally the retention time in RP-HPLC varies
as chlorophyllid-b < chlorophyllid-a < phaeophorbide
<chlorophyll-b < chlorophyll-a < phaeophytin-b < phaeophytin-a
[112]. Moreover, the commercially available chlorophyllin purity
can be assessed using RP-HPLC [104, 105]. RP-HPLC method
has been used for the identification and quantification of
chlorophyll derivatives in a limited range of food products using
UV-Vis, fluorescence, and mass spectrometer. This method has
been used for analyzing green tea leaves [103], vegetables like
celery leaves and beans [114], green peas, kiwi fruit, stored fruit,
health foods [100] and in rapeseed [115].

![Figure 2. UV-Visible spectrum of chlorophyll showing the location of the Soret (S) and Q bands.](image)

In addition to PDA detector, fluorescence and mass
spectrometric detectors are developed for chlorophyll and its
derivatives. Post column addition of formic acid is reported to
improve sensitivity of chlorophyll in spinach extract by LC–MS
[116]. Gauthier-Jaques et al. (2001) reported the analysis of
canned beans and rehydrated spinach powder and found the major
fragment at [M + H278] in all chlorophyll analogs owning the
phytly chain at [M + H 338] which related to the elimination of
CH2COOC2H5 [117]. Other characteristic fragments can be
assigned for large number of degradation products which occur
during food process and extraction, transformation products, and
chemical moieties. Although the clear structural information of
chlorophyll analogues can be obtained using NMR and other
identification modes like infrared and circular dichroism [107],
chromatographic method show better and easy determination of
natural colorants. Moreover a review of various analytical
techniques used for the analysis of Na-Cu-Chlorophyllin (Na-Cu-
Chl) employed in food stuffs is summarized in Table 3.

### 8.4. Cu-Chlorophylls (E141(i) and Cu-Chlorophyllins (E141(ii))

The Cu-Chlorophyllins of E141(i) and E141(ii) are highly
stable in mineral acids, light and moderate heat exposure when
compared to native chlorophyll derivatives. Basically Cu-
Chlorophylls (E141(i) is water-soluble and Cu-Chlorophyllins
(E141(ii)) is oil-soluble. The spectroscopic determination was used
to characterize chlorophyllin and its derivatives. Generally
derifferent copper derivatives are present due to chemical
conversion during purification of non-coppered chlorophylls,
rhodochlorins, phaeophytins, phaeophorbid as well as the
coopered chlorophyllins by using different extraction and
purification methods to get purity standards [59, 91, 100]. The
spectrophotometric method of assay suggested in the EU purity
standards for copper chlorophylls is mainly based on absorbance
at 422 nm in chloroform and an E1cm,1% value of 540 [70] which
is similar to that suggested by JECFA in the EU test method. Also,
silica-based TLC for separation from carotenoids is also proposed.
Furthermore the JECFA assay measured absorbance of
chlorophyllin like chlorophyllin in diethyl ether at six specific
wavelengths without oxalic acid treatment. The EU assay for Cu-
Chlorophyllins was performed at two different wavelengths 405
nm (E1cm,1% 565) and 630 nm (E1cm,1% 145) in pH 7.5 buffer
[70]. The corresponding JECFA standards for Cu complexes of
chlorophyllin derivatives particularizes determination by
spectrophotometry through similar conditions except for the
extinction coefficient, a color response analysis using ammonia,
and precipitation analysis with diethyl dithiocarbamate and a
purity check through the presence of basic dyes are also
recommended. The JECFA spectrophotometric assay for Cu-
chlorophyllins was performed at two different wavelengths in
buffer pH 7.5 using the same EU E1cm,1% value of 565 with an
experimentally resulted maximum absorbance between 403 and
406 nm. The RP-HPLC equipped with UV-Vis detection at 407
nm using methanol: water (97:3 v/v) mobile phase with 1% acetic
acid was used for the separation of copper chlorin e6 (CuCe6),
copper rhodin g7 (CuRg7), copper pheophorbide a (CuPpa) and
copper chlorin e4 [118].

The RP-HPLC coupled with PDA detection using mobile
phase methanol: ammonium acetate: acetone (gradient elution) is
also available [78]. Almela et al., (2000) performed analysis with
similar chromatographic conditions to monitor the chlorophyll
derivatives with a wide range of polarities yielded during the
ripening of fruit through PDA detection at 660 nm and FLD
detection (lex/440 nm, lem/660 nm) [119]. Furthermore HPLC-
PDA was used to separate and determine the copper (II) chlorophyll derivatives and iron (III) derivatives of chlorophyllin using ion-pair RP-HPLC [120]. A few analytical methods are available for determination of E141(i) and E141(ii) in foodstuffs. Generally TLC technique can be utilized to separate metal derivatives (M = Cu, Zn, Fe) of chlorophylls and chlorophyllins extracted from vegetable and fruits using ethanol and segregated against n-butyl acetate from chewing gum using ethanol, n-butyl acetate, and in addition hot water prior to filtration. A similar procedure has been used to the study of candies, chewing gum, processed seaweeds, chocolate and processed edible wild plants, and in boiled bracken, gar, and, chewing gum [121]. Scott et al. (2005) determined Na-Cu-Chl (E141(i) and E141(ii)) from dried soup mixes, jellies, boiled sweets, biscuits and flour confectionery against the artificial dye Solvent Green 3 as internal standard using mobile phase phosphate buffer (pH 2.6) and ethyl acetate: acetone can be used as mobile phase (under gradient elution) using RP-HPLC equipped with both DAD and FLD detection [59]. The peak spectra library compared with reference standards in the range of 300–700 nm were applied for a qualitative analysis of Na-Cu-Chl derivatives (Chla Ce6, Ce4, PPa, rhodin g7 and Chlb) in foodstuffs, with commercially available Na-Cu-Chl as reference material. Mortensen and Geppel, (2007) performed separation of a larger number of compounds including commercially available Na-Cu-Chl using HPLC with MS and PDA technique [82]. C30 column was used in order to separate chlorophyllin derivatives and the mass spectra of the peaks were studied through a characteristic splitting due to copper and carbon isotopes. Furthermore, the spectral data for chlorophyllin derivatives were obtained from PDA detector shown in Table 4.

Table 2. EU spectrophotometric purity specifications for natural colorants.

| Compound                      | E No. | E_{urm} (×1%),% | λ_{max} (nm) | Solvent            | Minimum purity (%) |
|-------------------------------|-------|-----------------|--------------|--------------------|--------------------|
| Curcumin                      | 100   | 1607            | 426          | Ethanol            | 96                 |
| Riboflavin                    | 100 (i)| 328             | 444          | water              | 98                 |
| Riboflavin-5c-phosphate       | 100 (ii)| 250             | 375          | water              | 95                 |
| Chlorophylls                  | E 140 (i) | 700            | 409          | chloroform         | 10                 |
| Copper chlorophylls (i)       | E 140 (ii) | 700          | 405          | water pH 9         | 90                 |
| Copper chlorophylls (ii)      | E 141 (ii) | 540 and 300  | 422 and 652 | chloroform         | 10                 |
| Lutein                        | 160a(ii)| 2500           | 440-457      | Cyclohexane        | 20                 |
| b-carotene                    | 160a(iii)1 | 2500       | 440-457      | Cyclohexane        | 96                 |
| b-carotene from Blakesea fritspora | 160a(iii)2 | 2500  | 440-457      | Cyclohexane        | 96                 |
| Solvent extracted annatto; bixin norbixin | 160b (i) | 2870             | 502 and 482  | Chloroform and Aqueous KOH | 75 |
| Lycopene                      | 160D   | 3450            | 472          | Hexane             | 5                  |
| Lutein                        | 161 b  | 2550            | 445          | mixed              | 4                  |
| Beet red                      | 162    | 1120            | 535          | Water pH 5         | 0.4                |

Table 3. HPLC procedures for the determination of Chlorophyll derivatives in food samples.

| Sample Type                        | Analyzed Chlorophylls | Stationary Phase | Mobile Phase | Detection | Ref. |
|------------------------------------|-----------------------|------------------|--------------|-----------|------|
| Plant extracts, vegetable powders and canned vegetables | Chlorophylls, hydroxochlorophylls, Methoxochlorophylls, Methoxalactochromechlorophylls, Pheophytins, Hydroxyphophytins, Porphobides, Chlorophyllides, Pyrochlorophyllides | Lichrospher 100-RP-18C18, 5 μm, 250 × 4.6 mm | A: 1 M aq. ammonium acetate: methanol (1:4, v/v) B: acetone:methanol (1:4 v/v) | UV/Vis: 430, 650, 670 nm; triple-quadrupole mass spectrometer | 11 |
|                                   |                       |                  |              |           | 7    |
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| Sample Type                              | Analyzed Chlorophylls                                                                 | Stationary Phase                        | Mobile Phase                                                                                          | Detection                        | Ref. |
|------------------------------------------|---------------------------------------------------------------------------------------|-----------------------------------------|--------------------------------------------------------------------------------------------------------|-----------------------------------|------|
| Food additives                           | Fe (III) complexes of chlorophyll derivatives                                          | Inertsil ODS C18-5 μm, 250 × 4.6 mm     | Acetonitrile: phosphate buffer pH 2 (6:4 v/v) containing 0.01 M tetramethylammonium chloride           | UV/Vis and FAB mass spectrometry | 124  |
| Barley leaves                            | Biosynthetic intermediates of chlorophyll and pheophytin                              | Senshupak silica 2151-N, 150 × 6 mm     | Hexane:toluene: methanol (100:40:0.8 v/v)                                                            | UV/Vis                           | 125  |
| Allomerization reaction of chlorophyll a standard | Allomers and epimers of chlorophyll a                                                 | Spherisorb S5W, 250 × 4.6 mm             | A: Hexane containing 1.5% 2-propanol:- methanol (1:1, v/v) B: Hexane containing 10% 2-propanol:- methanol (1:1, v/v) | UV/Vis and APCI-ion trap mass spectrometer | 29, 126 |
| Plant extracts                           | Chlorophylls, pheophytins, chlorophyllides                                            | Zorbax ODS C18, 5 μm, 250 × 4.6 mm      | Methanol                                                                                              | Fluorimetric at Ex 428 nm; Em 672 nm | 127  |
| Food additives with Cu(II) complexes of chlorophyll derivatives | Copper(II) pheophorbide a, copper(II) chlorin e6, copper(II) rhodin g7, copper(II) chlorin e4 | Inertsil ODS C18, 5 μm, 250 × 4.6 mm     | Methanol:water (97.3, v/v) containing 1% acetic acid                                                  | UV/Vis: 407 nm; FAB mass spectrometer | 77   |
| Food additives with Cu(II) complexes of chlorophyll derivatives | Copper (II) pheophorbide a, copper(II) chlorin e6, copper(II) rhodin g7, copper(II) chlorin e4 | Vydac 201TP54 C18, 5 μm, 250 × 4.6 mm | A: 1 M Aqueous ammonium acetate: Methanol (1:4, v/v) B: methanol:acetone (3:2 v/v) | UV/Vis: 410 nm; Fluorimetric at Ex 400 nm; Em 640 nm | 59   |
| Commercial Na-Cu-Chlorophyllin (E141)    | Chlorin e6, Cu purpurin 7, Cu rhodin g7, Uncoppered rhodin, Cu rhodin, Cu chlorin e6, Cu chlorin p6, Cu rhodin, Cu isochlorin e4, Cu porphyrin of chlorin e6, Cu porphyrin of chlorin p6, Cu rhodochlorin, Cu pyropheophorbide a, Cu porphyrin of isochlorin e4, Cu porphyrin, | YMC (C30, 3 μ, 250 × 4.6 mm)             | A:methanol:water: acetic acid (90:10:0.5) and B: tert-butyl methyl ether:methanol:acetic acid (100:10:0.5) | LC-MS                            | 81   |
| Blood serum                              | CuCl24 Ethyl Ester                                                                     | C18 Prodigy column (250 mm 4.6 mm, 5 μm) (Phenomenex, Torrance, CA) | solvent A, methanol:water (80:20, v/v) containing 1% (v/v) acetic acid (J9); solvent B, methanol | LC-MS                            | 55   |
| Food and beverages                       | Na-Cu-Chlorophyllin                                                                    | Luna C18 column (250 *4.6nm id, 5 μm particle size, 100 Å pore size) | Mobile phase A comprised of methanol:acetonitrile (1:1 v/v) and mobile phase B consisted of 40M ammonium acetate aqueous solution | HPLC-UV/Vis                      | 128  |
| Candy                                    | Na-Fe-Chlorophyllin, Na-Cu-Chlorophyllin                                               | Inertsil ODS-2 columns (5 μm, 4.6×250 mm) | The mobile phase consisting of (A) MeOH–water (97.3, v/v) containing 1% acetic acid and (B)          | HPLC and UPLC equipped with Mass spectrometry | 129  |
Table 4. Spectral data for Chlorophyll derivatives by HPLC analysis.

| Compound              | Wavelength Maxima (nm) |
|-----------------------|------------------------|
|                       | Soret band | Q band |
| Chlorophyll a         | 432        | 664    |
| Chlorophyll b         | 466        | 650    |
| Pheophytin a          | 410        | 666    |
| Pheophytin b          | 436        | 654    |
| Pheophorbide          | 410        | 666    |
| Pyropheophytin        | 435        | 654    |
| Cu-pyropheophytin     | 443        | 633    |
| Cu-13'-OH-pheophytin  | 408        | 635    |
| Cu-chlorin e0         | 406        | 627    |
| Cu-isochlorin e4      | 427        | 657    |
| Cu-purpurin 7         | 410        | 628    |
| Cu-chlorin e4         | 430        | 614    |
| Cu-rhodin g7          | 434        | 624    |
| Cu-rhodin             | 406        | 636    |
| Cu-pyropheophorbide a | 404        | 653    |

9. FUTURE TRENDS IN NATURAL COLORING OF FOODS

Chlorophylls are constantly playing an important role in our lifecycle. Green is the color of nature which brings to us numerous positive views (e.g., presence of water, balance, freshness) when available in our environment. A lot of research endeavours have been made in different fields of science and technology to search biological actions, biosynthesis, chemistry, function, catabolism, and industrial applications of chlorophylls and its derivatives. Still, now screening of chlorophylls and its derivatives is not yet completely characterized in new foods and plants sources. Determination also applies to products formed from catabolism in senescent tissues. These structures should be characterized further by scientists, mainly because the biochemical instruction of catabolic methods and the involved enzymes are still unfamiliar.

Several aspects of food safety organizations, presence of regulations in developing countries and the consumers’ claim for “natural” foodstuffs warrant separation of chlorophylls from diverse biological samples or preparations is urgently needed. The mushrooming of natural alternatives for color preparations with complete description of the colorant profile in the preparation demands complete specifications of resources used. In addition, consumers and health organizations request a complete and possible evidence possible about contaminants, concerning source, intended uses and restrictions applied in some food stuffs, and specifications for the colorants separations. In recent years, significant developments have been made on chlorophylls digestion and metabolism in human. Information about their biological actions conventionally related to soluble chlorophyll derivatives bioavailable in humans is now broadening to those chlorophyllin structures or metabolites resulting from digestion of them. However the constant efforts should be made to check their beneficial effect on human health after injection through food stuffs and beverages. Also, a proper analytical method is necessary for screening them in an easy and quick way with reliable and reproducible output.

10. CONCLUSIONS

The instability of natural colorants used for coloring food products has become a critical issue to analytical chemists as well as to monitoring agencies.

The chlorophylls and metallochlorophyllins are complex mixture of analogs and the composition varies from suppliers to suppliers or product to product. Hence the susceptible stability of these natural colorants stresses the development of highly sensitive and selective analytical methods to determine in various food matrices.

The sample treatment must be undertaken to avoid matrix effect during analysis which would be compatible with the type of food analyzed.

Hence, development of simple, selective and environmentally friendly methods consisting of chromatographic and spectrometric techniques would be urgently needed for their determination in food products.
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