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Studies on the Isolation of Plant Pigments from Spinach Leaves

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ABSTRACT: *The leaves of plants contain a number of colored pigments generally falling into two categories, chlorophylls and carotenoids. The green chlorophylls a and b, which are highly conjugated compounds capture the light energy used in photosynthesis. Carotenoids are part of a larger collection of plant-derived compounds called terpenes. These naturally occurring compounds contain 10, 15, 20, 25, 30 and 40 carbon atoms, which suggest that there is a compound with five carbon atoms that serves as their building block. Their structures are consistent with the assumption that they were made by joining together isoprene units, usually in a "head to tail" fashion. Isoprene is the common name for 2-methyl-1,3-butadiene. The branched end is the "head" and the unbranched is the "tail". That isoprene units are linked in a head to tail fashion to form terpenes is known as the isoprene rule. Carotenoids are tetraterpenes. Lycopene, the compound responsible for the red coloring of tomatoes and watermelon, and β-carotene, the compound that causes carrots and apricots to be orange, are examples of carotenoids. β-Carotene which cleaves to form two molecules of vitamin A when it is ingested, is the major dietary source of this vitamin.*

*Spinach leaves, which you will use in this assignment, contain chlorophyll **a** and **b** and β- carotene as major pigments as well as smaller amounts of other pigments such as xanthophylls. The xanthophylls, which are oxidized versions of carotenes and pheophytins, look like chlorophyll except that the, Mg+2 is replaced by two hydrogen ions. In this assignment you will isolate and separate the plant pigments using differences in polarity to effect the separation. Since the different components are colored differently, the separation is easily followed visually.*

Keywords: Isolation, Extraction, Column Chromatography, Thin-layer chromatography

INTRODUCTION

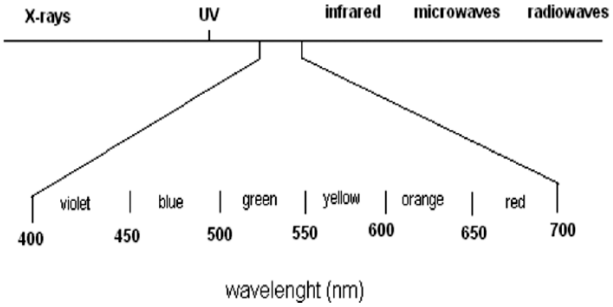
Plants owe their appearance in part to colorful substances consisting of biochromes, which either absorb or reflect light of varying wavelengths. The absorbed light is dissipated in the pigment, and the reflected light is visible as color. The colors are also the result of a mix of residual wavelengths that are reflected. The human eye is capable of seeing light within the range of 400–700 nm (Fig. 1), which corresponds to the colors of the rainbow identified by Newton: violet, indigo, blue, green, yellow, orange and red (Heldt, 2005).

The secrets of plant pigmentation are among the oldest interests of botanists. The first publications on the subject of carotenoids came in the beginning of the nineteenth century, and the term "chlorophyll" was first used in 1818 (Davies, 2004). The variety of colors and easy identification of mutations resulted in flowers becoming one of the most popular genetic research subjects, starting with Mendel's pioneering experiments on pigment inheritance in pea and leading to the identification of mobile colored elements called transposons in corn, for which Barbara McClintock received the 1983 Nobel Prize for Physiology and Medicine (McClintock, 1983). Research into pigments has led to many breakthrough discoveries in the field of molecular biology; for example, the first transcription factor was identified when anthocyanins were studied, and the first isolation of cDNA in plants came from anthocyanin research (Holton et al., 1993).

Pigments responsible for the appearance of colors in higher plants are classified in several groups: chlorophylls, carotenoids (carotenes, xanthophylls), flavonoids (chalcones, anthocyanins, flavones, flavonols) and betalains (betaxanthin, betacyanin) (Tab. 1).

In prokaryotic (Cyanobacteria) and eukaryotic cells (cyanelles, red algae) there are water-soluble photosynthetic pigments called phycobilins, which are found in the cytoplasm or in the stroma of the chloroplast (Marsac, 2003). There are two classes of phycobilins – phycocyanin and phycoerythrin (Tab. 1).The bluish pigment phycocyanin is found in cyanobacteria and gives them the common name "blue-green algae", and the reddish phycoerythrin is found only in red algae, *Rhodophyta* (Beale, 2003).

These pigments are covalently bound to the proteins forming phycobiliproteins and are organized into



a supramolecular complex called the phycobilisome (Beale, 2003). Two major phycobilins are phycocyanobilin and phycoerythrobilin, which absorb light at 620 nm and 560 nm, respectively (Mimuro, 2002). Numerous data on known pigments have shown the richness and variety of plant colors. So far approximately 600 carotenoids, 7,000 flavonoids and more than 500 anthocyanins have been identified (Davies, 2004).

The main pigment groups are located at various sites in plant organs. Flavonoids appear in almost all tissues; carotenoids, for example, are present in leaves, roots, seeds, fruits and flowers. Some pigments such as anthocyanins or chlorophylls have a specific cellular or subcellular location. Anthocyanins are usually found in epidermal cells of flower petals, whereas chlorophylls and carotenoids are in plastids in subepidermal photosynthetic cells of leaves. Like anthocyanins, betalains are water-soluble and appear in vacuoles (Davies, 2004).

Anthropocentrism leads us to believe that flowers were created for our pleasure, to delight our eyes with colorful tones, and for artists as an inspiration for creation in the different arts. However, neither the brilliant colors of plants nor their intoxicating aromas were designed for people. All the characteristics that form the beauty of plants are actually information addressed to animals, insects, birds and bats, which visit plants in search of food. In these almost discourteous visits, they bring grains of dust effecting the pollination and thus the continuation of the species. For this reason, plants must be visible from a distance for airborne pollinators, and must stand out from their surroundings. Plants therefore developed, through evolution, a set of features (pigmentation, fragrance) that can be compared to aggressive advertising campaigns effectively targeting pollinators. The first plants, which appeared in the Mesozoic era, were probably creamcolored and only with time developed sharper colors, increasing the concentration of various pigments (Raven, 2005).

TABLE 1. Major pigments of plants and other organisms

Pigment groups	Type	Localization	Color
Phycobillins	Phycocyanin Phycoerythrin	Cyanobacteria Phodophyta	Bluish Red
Chlorophylls	Chlorophyll *a, b*	All photosynthetic plants	Green
Carotenoids	Carotenes Xanthophyll s	Plants, bacteria & some crustaceans	Yellow, orange, red
Flavonoids	Chalcones Flavonols Anthocyanins	Gymnosperms & Angiosperms	Cream, pale yellow, pink, red, blue, balck
Betalains	Betaxanthins Betacyanins	Caryophyllales, cactus, & some fungi	Yellow, red

GREEN – CHLOROPHYLLS

The most eye-catching and widespread plant pigment is chlorophyll, which appears in leaves and other green parts of plants exposed to light. Chlorophyll is located, together with carotenoids, in chloroplasts, and there its physiological function is to absorb light energy and use it in photosynthesis. There are two kinds of chlorophyll in higher plants: green-blue chlorophyll *a*, and green-yellow chlorophyll *b*. Their amounts depend on the species of plant, light conditions, and the availability of minerals such as magnesium (Mg). Normal chlorophyll *a* content is 2–4 times that of chlorophyll *b* although spectrometry of acetone extracts of fresh mint, melissa and nettle leaves showed chlorophyll *a/b* ratios varying from 3:1 in melissa and nettles to 1:1 in mint (Dżugan, 2006). Plants growing in shade contain less chlorophyll *a* and more chlorophyll *b*. Replacing Mg with Fe ions gives a grey-brown chlorophyll product and the presence of Zn and Cu ions increases the stability of the natural green color (Dżugan, 2006).

The chlorophyll molecule is a porphyrin derivative whose main skeleton is an arrangement of four pyrrole rings containing an Mg ion in the center. The presence of Mg in the center of a chlorophyll molecule plays an important role in absorption of light energy, and the pyrrole ring creates a structure with single and double bonds, which facilitates the

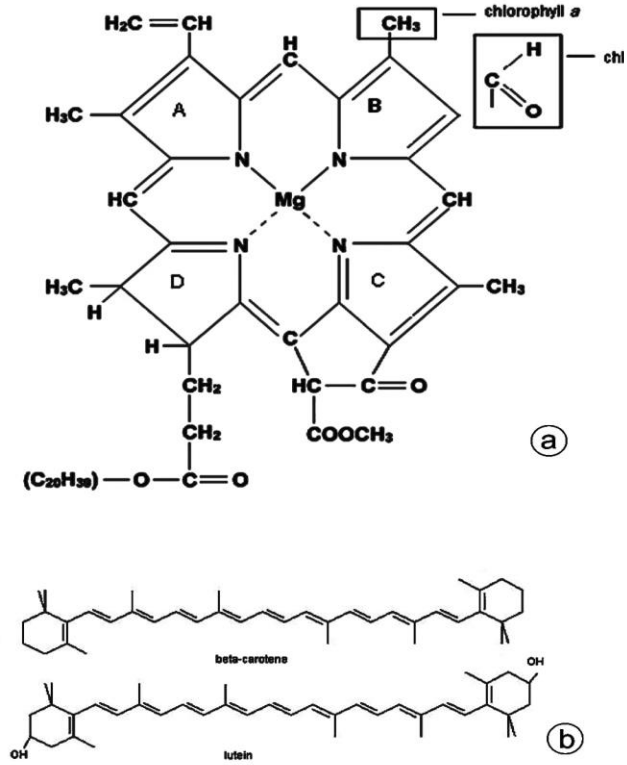


Fig. 2. The chemical structure of plant pigments. (a) Structure of the chlorophyll molecule. All plants, algae and cyanobacteria that photosynthesize contain chlorophyll *a*. A second kind of chlorophyll is chlorophyll *b*, which occurs only in green algae and in plants (Taiz and Zeiger 2006, modified), (b) Chemical structure of carotenoids, (c) Structure of the basic flavonoid skeleton with 15 carbons in two aromatic rings (A and B) connected by a three-carbon bridge, (d) Betalains: betacyanin and betaxanthin with nitrogen atom in the chromophore.

absorption of light photons. A phytol tail consisting of 20 carbon atoms is attached to this tetrapyrrole part of chlorophyll. The differences in structure between chlorophyll *a* and *b* are minimal and involve the various groups attached to the third carbon atom in the II pyrrole ring. Chlorophyll *a* attaches the CH3 group, hereas chlorophyll *b* binds with CHO (Fig. 2a) (Heldt, 2005). Reverse conversion from chlorophyll *a* to chlorophyll *b* may occur in chloroplasts as a result of the activity of CAO oxygenases encoded by a class of genes identified in *Arabidopsis thaliana* and *Oryza sativa* (Espineda et al., 1999).

Removal of Mg from the chlorophyll molecule results in its conversion to pheophytin, grey-brown in color. Pheophytin accumulates in leaves during senescence of plants or as a result of damage caused by environmental pollution, such as acid rain (Dżugan, 2006).

The green pigment of plant leaves is due to the ability of chlorophyll to absorb light in the visible wavelength, primarily in the blue (420–460 nm) and red (650–700 nm) range, and reflection of green light (~550 nm). However, the physiological cause of plants' greenness is still not completely known. It is thought that green leaves adapted exceptionally well to varying light conditions, which prevail in many habitats. The success of green pigment results not only from chlorophyll's ability to absorb light under changing irradiance and maximal utilization of light energy in photosynthetic electron transport, but also from its ability to protect chloroplasts from light excess by dissipating it in the form of heat or fluorescence radiation (Nishio, 2000). Thus the greenness of plants is a result of complex reactions of chlorophyll biosynthesis. The final light-dependent reaction is catalyzed by NADPH-protochlorophyllide oxidoreductase (POR). In *Arabidopsis thaliana*, three POR isoforms occur: POR A, POR B and POR C (Beale, 2005). Angiosperms growing in darkness accumulate only small amounts of protochlorophyll-lide, which is associated with NADPH and POR in a ternary complex, so these plants are not capable of chlorophyll synthesis. In these etiolated seedlings a large amount of oxidoreductase POR A proteins and protochlorophyllide, as well as POR A mRNA, are accumulated. Following exposure to light, the level of POR A falls rapidly as a result of proteolytic degradation. Rapid proteolysis of this isoform is the reason

that plants growing in darkness do not contain chlorophyll. Yellow pigmentation is produced by carotenoids, which mask the green color of protochlorophyllide. The POR B isoform, responsible for chlorophyll synthesis, has been found in barley and *Arabidopsis thaliana* growing in light. The enzyme has been shown to be active only in light, probably as a result of its insensitivity to proteolytic hydrolysis (Lebedev et al., 1995; Tanaka and Tanaka, 2006).

In *A. thaliana*, 30 genes encoding enzymes involved in chlorophyll synthesis have been identified. Mutations in those genes are usually lethal to plants (Beale, 2005). Chlorophyll synthesis mutants are often pale green, sensitive to light, and characterized by overproduction of protochlorophyllide (von Wettstein, 2000). These mutants are particularly helpful in understanding the regulation of chlorophyll synthesis and its integration with plant growth and development. Of particular interest is a corn mutant with mutations in the gene encoding uroporphyrinogen III decarboxylase. This mutation has characteristic necrotic patches on the leaves due to the accumulation of phototoxic intermediates. This is the only case of natural porphyria in plants (Hu et al., 1998).

YELLOW, ORANGE AND RED – CAROTENOIDS

Carotenoids are a large family of terpenoid pigments (more than 600 compounds) found in photosynthetic bacteria, algae, fungi, and cells of higher plants and animals. These pigments can be divided into two groups: orange-red carotenes, which are unsaturated hydrocarbons; and yellow-orange xanthophylls, which contain additional oxygen within their molecules (Fig. 2b).

In higher plants they are involved in light harvesting in photosynthesis and protection against excessive light, participating in energy and electron transfer. In light-harvesting reactions, carotenoids act as accessory pigments and transfer the excitation energy to chlorophyll *b*. However, the important function of these pigments is photoprotection of the reaction center (RC) of photo system II from photooxidative damage by either quenching triplet chlorophyll or quenching singlet oxygen (Young et al., 1997). The role of these pigments in photoprotection is based on fluorescence-quenching mechanisms. The excess energy is dissipated as heat; it is a feature of the interconversion between two carotenoids, zeaxanthin and violaxanthin, in the xanthophyll cycle. Under excess light, the violaxanthin is biochemically transformed (de-epoxidated) into zeaxanthin via intermediate antheraxanthin. In darkness or under low photon flux density, the process is reversed and violaxanthin content increases in plant leaves. The quenching mechanism is complex and not entirely understood in molecular detail (Horton and Ruban, 2005). Carotenoids are also present in chromoplasts, giving them a characteristic yellow-red-orange color. This pigmentation determines the color of some plants (roses and marigolds), fruits (tomatoes), roots (carrot) and seeds (red peppers). The unusual blue color of some carotenoids is the result of interaction between certain carotenoids with apoproteins such as astaxanthin with crustacyanin, which give the blue pigmentation of lobsters (Grotewold, 2006). β-carotene and lycopene are among the most common carotenoids to carrots and tomatoes; the most frequently represented xanthophylls are zeaxanthin, violaxanthin and lutein. Besides antenna functions, some xanthophylls (violaxanthin, lutein) are responsible for the yellow color of autumn leaves (xanthos = yellow, phyll = leaf) (Davies, 2004).

Carotenoids absorb light of 400–500 nm wavelength, which accounts for their orange coloring. In addition to their role in energy transfer to chlorophylls, carotenoids also have a key function in chloroplast protection under excess light conditions. By dissipating the excess energy they prevent reactive oxygen species production, photo-oxidation and damage to the photosynthetic system (Britton, 1995; Niyogi, 2000).

An important application of biotechnology is improvement of the dietary value of crops to supplement the local diet with nutrients that do not occur naturally in it. Carotenoids are an important part of the diet, comprising a source of vitamin A and antioxidants. One of the earliest genetic modifications was the introduction of β-carotene, a vitamin A precursor, into rice, which does not contain vitamin A or any of its biochemical precursors. Rice lines genetically modified with genes encoding phytoene synthase and lycopene β-cyclase originating from *Narcissus pseudonarcissus* and *Erwinia uredovora* were produced. The transformed lines produced β-carotene manifested phenotypically as orange pigmentation of rice grains, giving rise to the name "Golden Rice" (Giuliano et al., 2000; Al-Babili and Beyer, 2005).

Carotenoids present in leaves, together with anthocyanins, are responsible for the beautiful colors of autumn. One hypothesis (Hamilton and Brown, 2001) suggests that the colors of leaves have the same significance as colored lines painted on the face of a native American: strength and readiness to fight. Trees that quickly change their foliage color to yellow, purple and gold are demonstrating their vitality and strong metabolic processes. This is a

signal for insects that the tree is strong and that in case of attack it may attack the parasite with a harmful substance produced as a result of the metabolic process (Lee and Gould, 2002). Another suggestion, the resorption protection hypothesis, is that anthocyanins produced during senescence help to photoprotect plants from excess light and can affect a plant's ability to resorb important foliar nutrients such as nitrogen and phosphorus. In this case, anthocyanins serve as a barrier cream in leaves by shielding the photosynthetic apparatus from potentially harmful light levels (Hoch et al., 2003).

RED, BLUE AND PURPLE – ANTHOCYANINS AND BETALAINS

Flavonoids

Flavonoids are chemical substances of the polyphenol group whose primary skeleton comprises 15 carbon atoms arranged in two phenyl rings, A and B, joined by a three-carbon bridge (Fig. 2c).

So far more than 7,000 derivatives containing this structure have been found. In higher plants these compounds are divided into several classes differing in he number and location of hydroxyl groups on rings A and B, the degree of oxidation of ring C, and the presence of dimeric structures, that is, repetition of the C6-C3-C6 structure. The flavonoids include anthocyanins, chalcones, aurones, flavones and flavonols. They all are water soluble, located in vacuoles often as glycoconjugates, and absorb visible light in the 280–315 nm range. Flavonoids are biochemically active substances with varied functions: they are responsible for the color of flower petals and fruits, they create a UV protective filter, they serve as antioxidants, and they have antibacterial and antiviral activity. They also control gene transcription for proteins involved in auxin transport and give important visual signals to insect and animal pollinators (Davies, 2004; Winkel-Shirley, 2002). The names of many known anthocyanins reflect their history and originate from the names of the families in which they were first discovered (e.g., *Centaurea, Delphinium, Pelargonium*). In 1913, Willstätter and Everest identified the first anthocyanin in the blue cornflower, and since then the structures of over 600 different anthocyanins have been identified (Winkel-Shirley, 2006). As early as 1664 the violet pigment in the pansy (*Viola* sp.), belonging to the anthocyanins, was discovered, and was used as the first natural pH indicator.

The rich variety of flavonoids and their role in providing color to plants are one of the oldest botanical research areas. Anthocyanins are the most common pigments of the flavonoid group, which often give a bright red, red, blue or violet color to plant petals,

TABLE2. The most common anthocyanidins and their Colors

Anthocyanidyn	**Type of ring substitute (R1-R7)**	**Color**
Pelargoniodin	R2,4,5,7-OH; R1, 3, 6-H	Orange Red
Cyaniding	R1,2,4,5,7-OH; R 3,6-H	Purplish red
Delphinidin	R1,2,3,4,5,7-OH; R6-H	Blue
Mavidin	R2,4,5,7-OH; R1,3-OCH_3	Purple Violet
Peonidin	R2,4,5,7-OH; R1,OCH_3	Rose
Petunidin	R1,2,4,5,7-OH; R3-OCH_3	Purple Violet

fruits and stems. The key to this variety of color is the degree of oxidation of the central chromophore group, anthocyanidin, as well as the number and type of attachments to the main skeleton. Anthocyanins are glycosides that have sugars attached to the anthocyanidin. In the structure of anthocyanins, 17 anthocyanidins have been distinguished, the most common of which are cyanidin, delphinidin, pelargonidin, peonidin, malvidin and petunidin. Anthocyanin color is influenced by many factors. One of them is the number of hydroxyl and methoxyl groups. If many OH groups are present,
the color is blue. The presence of many OCH_3 groups shifts the color towards red (Tab. 2) (Winkel-Shirley, 2002; Grotewold, 2006). A characteristic property of these pigments is the change of tone with environmental factors (Davies, 2004). In this respect, anthocyanins are true chameleons in the pigment world. In acidic pH, anthocyanins take a red color, and in alkali pH, blue. Anthocyan color also depends on the presence of metal ions such as aluminum (Al), iron (Fe) and magnesium (Mg); the intense blue color of the dayflower (*Commelina communis*) is brought about by the anthocyan complex with two Mg atoms. The co-occurrence of anthocyanins in association with other pigments such as
carotenoids and flavonoids also has a significant effect on petal pigmentation (Griesbach, 2005; Grotewold, 2006). Flavones usually have a yellowish or cream color, and also absorb ultraviolet, so they can provide protection against UV radiation. In such

complex structures, anthocyanins take on more intense and stable colors.

The application of electron microscopy in research on plant petals revealed the presence of spherical bodies known as anthocyanic vacuolar inclusions (AVIs) in some taxa, for example *Lisianthius, Dianthus caryophyllus, Salvia, Antirrhinum, Eustoma* and *Delphinium*. These structures may be formed by anthocyanins attached by H-bonding to the protein matrix, and their concentration intensifies petal color, especially in areas rich in AVIs (Markham et al., 2000).

Recent electron microscopy studies of the AVIs of lisianthus (*Eustoma grandifolium*) and carnation (*Dianthus carryophyllus*) report that AVIs are compact, irregular bodies formed from ER-derived vesicles in epidermal cells in different regions of the petal, and are
not surrounded by membranes. Based on the subcellular evidence, it is suggested that anthocyanins may be first packed into prevacuolar compartments (PVCs) localized in the vicinity of their biosynthesis sites. Subsequently these PVCs develop and merge with the central vacuole, where anthocyanins are finally sequestered (Zhang et al., 2006).

Many genes associated with the biosynthesis of various anthocyanins have been identified. The first plants serving as color models in genetic experiments were corn, snapdragon and petunia, and work on these subjects led to the isolation of many structural and regulatory genes coding key proteins involved in the biosynthesis of anthocyanins and their sub-cellular organisation. Recognition of the complete *Arabidopsis thaliana* genome enriched our knowledge of the mechanisms regulating the expression of genes typically active in petal epidermal cells and which may react to environmental factors such as wavelength and quality of light (Winkel- Shirley, 2001; Grotewold, 2006). Synthesis of anthocyanins may also be induced by many otherstress factors such as cold, high temperatures, salinity and deficiency of minerals, particularly nitrogen and phosphorus, which is usually manifested by purple pigmentation of stems and leaves. This shows that flavonoid synthesis is one of the general plant reactions to stresses (Winkel-Shirley, 2002). The most common environmental factors influencing pigment color is soil pH. It can affect pH within the cell compartments and in this way can change the subcellular accumulation of flavonoids or other cellular components that interact with flavonoid pigments (Verweij et al., 2008). Mutations in glucosyltransferase genes (*GTs*) changed the location of flavonoids from vacuole to cytoplasm, resulting in flower yellowing. Some of the genes regulating vacuole pH also controlled anthocyan biosynthesis in the petunia (Davies, 2004; Verweij et al., 2008).

Research on anthocyanins in dicotyledons provides evidence that plant coloration involves two levels of genetic regulation of flavonoid synthesis. Thefirst comprises genes encoding the enzymes involved in early steps of flavonoid synthesis: chalcone synthase (*CHS*) and chalcone isomerase (*CHI*). The second involves regulation of the expression of genes involved in the late stages of anthocyan production, such as *DFR* (dihydroflavonol reductase) or *LDOX/ANS* (leucoanthocyanidyn dioxygenase/anthocyanidins synthase) (Winkel-Shirley, 2002; Davies, 2004). Mutation of *CHS* in both corn and petunia produced white instead of yellow pollen in wild plants (Winkel-Shirley, 2002). In the case of *DFR*, for example, genetic modifications using antisense gene structures for nucleotide sequencing of later stages in flavonoid synthesis changed the flower pigmentation from white to pink in petunia and from red to pink in gerbera (Davies, 2004). Genetic engineering has created the possibility to use these genes
to transform plant species and strains to attain the required color.

Blue Growers of decorative plants have been trying for centuries to obtain a blue rose. In this genus blue flower color is not found in nature, as its petal cells do not accumulate delphinidins or any delphinidin derivatives, due to the lack of a key enzyme involved in their synthesis: flavonoid 3',5'- hydroxylase. Roses do accumulate large amounts of pelargonidins, cyanidins and carotenoids. Apart from this, the pH in the vacuoles of rose petal epidermal cells ranges between 3.69 and 5.78. At such a low pH, anthocyanins have a red or pink tone. One strain of rose, "Rhapsody in Blue," contains a small amount of the blue pigment rosacyanin in AVIs. Color changes in this rose from purple to blue progressing with age are caused by increasing accumulation of anthocyanins in vesicle-like AVI structures. However, the molecular mechanism of this phenomenon is not yet clears (Katsumoto et al., 2007).

Scientists from Florigene in Australia transformed plants with the gene coding flavonoid hydroxylase enzyme, vital for the synthesis of blue derivative delphinidins, to grow blue roses and carnations. The first transgenic rose with the flavonoid hydroxylase gene originating from the pansy, the petals of which contain delphinidin, was created in 1992 (http://www.florigene.com). The next achievement of the scientists at Florigene was successful transformation of selected rose strains with a

construct of two genes: the gene encoding F3'5' H from the pansy and the *DFR* gene from the iris. This transformation caused greater delphinidin accumulationin genetically modified roses. The ability to pass on the blue color became a fully inherent trait, which became apparent in successive generations.

Overexpression of the *F3'5'H* gene alone was not enough to create the blue pigmentation of carnations. However, transformation of the white carnation with both *F3'5'H* from the pansy and *DRF* from the petunia resulted in the production of a blue pigment named "Moondust" (Katsumoto et al., 2007; http://www.florigene.com/research).

The first anthocyan isolated from plants was cyanidin, originating from the cornflower petal. The same pigment was later identified in the rose; the difference in pigmentation between the flowers of those two species was explained by the difference in their petal pH. The crystallographic structure of pigment isolated from the cornflower shows it to be a large complex composed of six cyanidin molecules chelated with Fe3+ and Mg2+ and six flavones associated with Ca2+. This new supermolecule, named protocyanin, may be the challenge awaiting breeders looking for a source of blue pigment for many decorative plant strains (Shiono et al., 2005).

Betalains

Betalains substitute anthocyanins in most species of Caryophyllales. They have also been found in certain fungi (*Amanita*, *Hygrocybe*) and cactii. Since betalains derive from tyrosine, an amino acid common to all organisms, the presence of nitrogen in their molecule is characteristic. Betalains differ structurally in the sidechain groups, which may be amino acids or amines (Fig. 2d), and are divided into two groups: betaxanthins (yellow; e.g., indicaxanthin in cactii) and betacyanins (red to violet; e.g., amaranthin in *Amaranthus caudatus*). Sometimes they form complexes with tyrosine or glycine (e.g., portulacaxanthin II and III identified in *Portulaca grandifolia*). The most well known betalains, betanin and vulgaxanthin, were isolated from beetroot. Betalains are present also in fungi in the fruiting body (musca-aurin). The toadstool contains, instead of betacyanin, betalamic acid-derived compounds: musca-purpurin and musca-flavin, which give its cap its characteristic red color (Davies, 2004).

Spanish researchers made an interesting observation in a very original flower known as Mirabilis (*Mirabilis jalapa*), whose petals display fluorescence. The petals of this plant open in the late evening or overcast days in order to be more noticeable to insects through their fluorescence. Thisplant's petals contain two types of pigment: one of them emits fluorescence and the other is an antifluorescent pigment. Some petals, yellow in color, contain betaxanthin and emit green fluorescent light, and other parts of flowers contain both yellow betaxanthin and violet betacyanin pigments. The violet pigment absorbs green light emitted during fluorescence. The combination of these two pigments can create green patterns which have been suggested to act as a guide for insects and bats sensitive to thiscolor (Gandía-Herrero et al., 2005).

Research on flower pigments sometimes yields unexpected discoveries for different fields in biology. Gene silencing and siRNA (small interfering RNA) have become subjects of interest for many researchers, opening new doors in molecular biology for research into plant color properties.

MATERIAL AND METHODS

The spinach leaves used in this study were procured from farm land near college, Parvathapur.

Isolation of pigment from leaves: Weigh about 0.5 – 1.0 g of fresh spinach leaves (avoid using stems or thick veins). Cut or tear the spinach leaves into small pieces and place them in a mortar along with 2.0 mL of acetone. Grind with a pestle until the spinach leaves have been broken into particles too small to be seen clearly. If too much acetone has evaporated, you may need to add an additional portion of acetone (0.5-1.0 ml). Using a Pasteur pipette or spatula, transfer the mixture to a centrifuge tube. Rinse the mortar and pestle with 2.0 mL of cold acetone, and transfer the remaining mixture to the centrifuge tube. Cap tightly. Centrifuge the mixture being sure to balance the centrifuge first.

Add ~2.0 mL of hexane to the centrifuge tube, cap the tube, and shake the mixture thoroughly. Then add 2.0 mL of water and shake thoroughly with occasional venting. Centrifuge the mixture to break the emulsion, which usually appears as a cloudy green layer in the middle of the mixture. The pigment layer is the top hexane layer, which should be dark green. Most of the acetone will dissolve in the water.

Using a dry Pasteur pipette, carefully, transfer the top organic layer (dark green hexane solution of spinach pigments) into a clean test tube. Add another 1.0 mL of hexane to the centrifuge tube that contains the aqueous layer, cap the tube and centrifuge the mixture to break the emulsion. Again withdraw the top organic layer and add it to the test tube.

The dark green hexane solution of spinach pigments in the test tube may contain traces of water that must be removed before separating the components through chromatography. To dry the solution, add 0.5g of anhydrous sodium sulfate, Na SO to the hexane solution. Cap and gently swirl to allow the sodium sulfate to contact all parts of the hexane. After standing for 5 minutes, use a clean, dry Pasteur pipette to transfer the liquid into another clean test tube. Label this test tube with an E for extract so that you don't confuse it with the test tubes you will be working with later in this experiment.

Add about 0.5 mL hexane to rinse the hydrated sodium sulfate and transfer this liquid to the same test tube, E.

Column Chromatography of Spinach Pigments: Clamp a clean, dry Pasteur pipette
vertically and push a very small plug of cotton to the bottom of the pipette. Weigh out about 1.25g of alumina. Pack the column with alumina and gently add a little less than one half cm of sea sand to the top of the column. Tap gently to achieve a flat surface.

Once the procedure is started, it should not be stopped: The alumina must be kept wet with solvent all the time. Since the column does not have a stopcock to stop the flow of solvent during the procedure, all the solvents must be at your desk before starting the process. In separate labeled containers obtain 15 mL hexane, 15 mL 70% hexane-30% acetone solution, 15 mL acetone and 15 mL 80% acetone-20% methanol solution. Also, label six test tubes 1 through 6 and a beaker as "waste solvent."

First, transfer about 0.5mL of your spinach extract (from test tube, E) to a small vial for use later in the thin layer chromatography analysis.

Then, when you are ready to begin the column separation, place the waste solvent beaker under the column and add about 3.0 mL of hexane to the top of the column. As the alumina is wetted, the hexane will flow into the beaker. When the solvent level drains to the top of the sand add your spinach extract to the top of the column. As the extract drains onto the alumina, the pigments should begin to separate into a yellow carotene band and a green chlorophyll band. If a separation is seen, add an additional 4 mL of hexane and continue collecting solvent in your waste solvent beaker until the yellow band reaches the bottom of the column and the solvent draining out turns yellow. Replace the waste beaker with test tube #1. Continue to add hexane until the yellow band passes through the column switching to test tubes #2, #3, etc as necessary until the yellow eluant becomes clear. Then replace the test tube with the waste beaker.

If the yellow band has not begun to separate from the green band after the initial 4mL of hexane has drained through the column, add 4mL of the next more polar solvent (70% hexane-30% acetone). When changing solvents, do not add the new solvent until the level of the last solvent is almost at the top of the alumina. When the appropriate solvent is found add this solvent until the yellow band is completely removed from the column.

Once the yellow band has eluted from the column, add several mL of the next more polar solvent when the level of the last solvent is almost at the top of the see sand. If the green band moves down the column, continue to add this solvent until the green band is eluted completely from the column. If the green band does not move, change to the next polar solvent. Collect the green band in a clean test tube. Cover and save these fractions for the next lab period when you will study the visible spectrum and TLC of the green and yellow fractions.

Before you can carry out the thin layer chromatography (TLC) on your fractions and original extract, you must save about 3-mL of the chlorophyll and ß-carotene fractions for UV-visible spectra. For the TLC you must concentrate the separated fractions. Pass a gentle stream of air over the solutions in a hood to evaporate the solution to solvent about 1 mL of yellow and 1 mL of green solution. If you have several test tubes of the same pigment, combine the contents as you evaporate so that you end up with just one vial of yellow carotenes and one vial of green chlorophylls.

If necessary due to time constraints, the vial can be labeled with your name, capped, and stored until the next laboratory period.

PERIOD 2
CHARACTERIZATION
Thin Layer Chromatography: Gently draw a horizontal pencil line about 1 cm from
the bottom of a thin layer chromatography (10 cm x 4 cm). As shown in the figure below, spot your three solutions (E, yellow and green) on this line. Use a separate microcapillary tube for each solution. Fill each capillary by dipping it in the solution and gently touch it to the plate to empty it.

Use several short touches to empty each capillary so that the spots will be small. If the spots look light in color, re-spot on top of the original spots until the spots are fairly dark. Allow the spots to dry. Obtain a

chromatography jar, and to the jar add about 10 mL of 5 developing solvent (70% hexane-30% acetone) then place the TLC plate in the developing jar with the spotted end at the bottom of the jar and allow the solvent to rise up the plate undisturbed until about 80% of the plate is wet. Remove the plate and quickly draw a pencil line across the plate to mark the farthest reach of the solvent. This is called the solvent front. Allow the wet plate to dry in the hood. Circle the visible spots in pencil since the colors may fade over time.

Retention factor (R): Different compounds should move different distances on your
TLC plates; however the exact distance a particular compound moves depends on how far the solvent is allowed to rise up the plate. The further the solvent moves, the further the spots travel. To take this variation into account, the ratio of the two distances is calculated and reported. This ratio, called the retention factor R allows comparison with reported values and other people's work. Specifically, the retention fraction is defined as the fractional distance the spot spot moves compared to the distance travelled by the solvent front. The R value for a compound is dependent on the developing solvent and the composition of the adsorbent on the chromatography plate

OBSERVATIONS:
Isolation:
Keep a detailed record of all your observations during the extraction of the pigment and the chromatographic separation. In tabular format record the number of the fraction being collected, the time over which the fraction is collected, the concentration of the solvent on the column, the appearance of colors on the column, and the color of the solution in the fraction being collected.

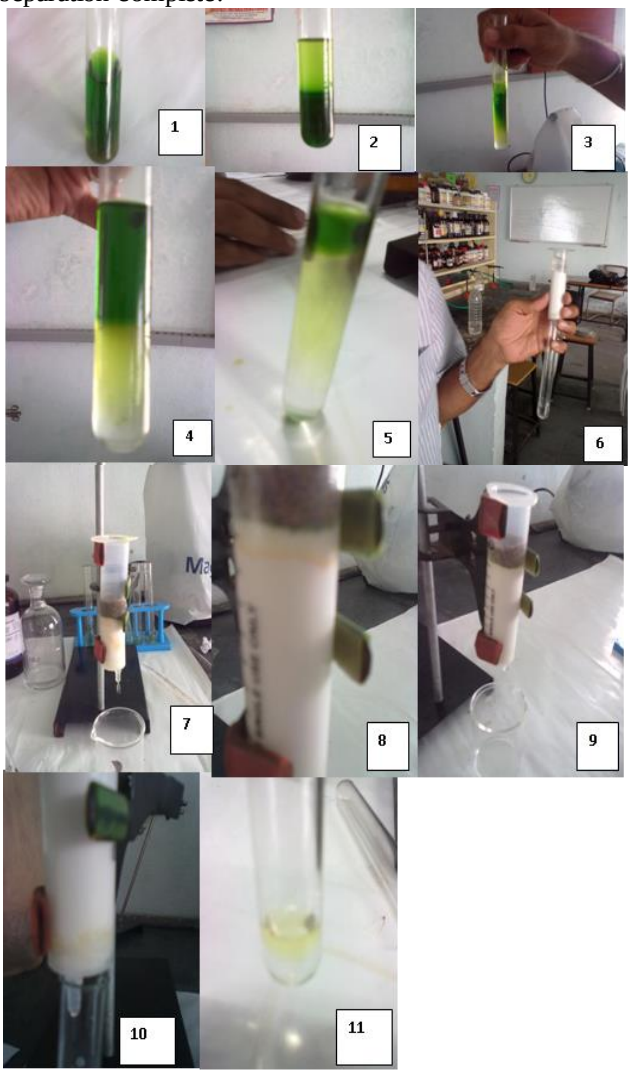
Characterization:
Prepare a detailed sketch of your developed TLC plate in your notebook. In tabular format record the "lane" the spot is in, the color of the spot, and its R value.

DATA ANALYSIS:
Identify the absorption(s) in the UV spectra that give rise to the colors of the chlorophyll and beta-carotene solutions.

Using the guide below, identify as many spots in the TLC as you can. Determine which pigments were present in the yellow band and which were present in the green band from your column.
Pigments in spinach in order of decreasing R values:

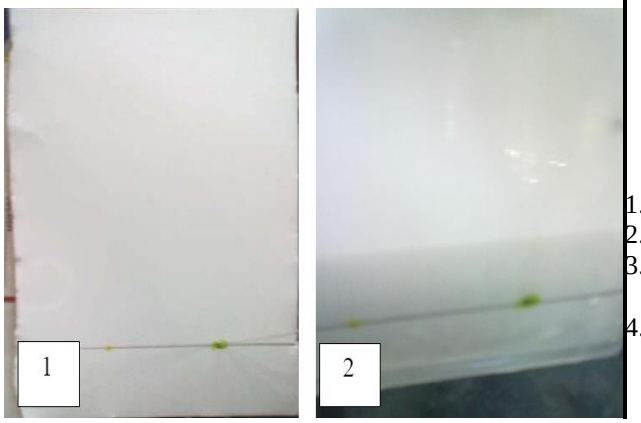
Carotenes (1 spot) (yellow-orange)
Pheophytin a (gray, may be nearly as intense as chlorophyll b)
Pheophytin b (gray, may not be visible)
Chlorophyll a (blue-green, more intense than chlorophyll b)
Chlorophyll b (green)
Xanthophylls (possibly 3 spots: yellow)

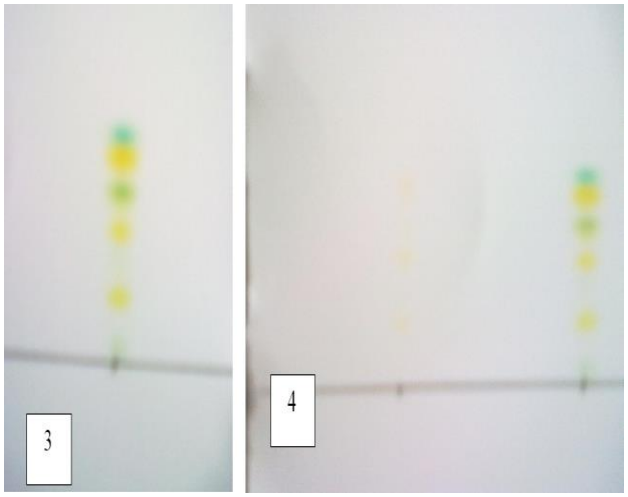
CONCLUSION:
In your conclusion address how well the column separated the various pigments. Compare the separation with the TLC plate. Could TLC be used for quantitative separations? Explain your answer. Based on your UV spectra, was the column separation complete?



1. Spinach leaves into small pieces and place them in a mortar along with 2.0 mL of acetone.
2. Add~2.0 mL of hexane to the centrifuge tube.
3. Add 2.0 mL of water and shake thoroughly with occasional venting.
4. Pigments in hexane layer and acetone completely dissolved in water phase.

5. Separate the Pigment-Hexane layer from water-acetone mixture.
6. Packing the column with Alumina (Al_2O_3) and washing the column with hexane.
7. Washing the column with hexane.
8. Sample loading on to the column and separation of the xanthophylls
9. Running of the column.
10. Separation of the xanthophylls.
11. Collection of the xanthophylls.





1. Load the samples on TLC sheet.
2. Run the samples by using developing solvent.
3. separation of the all pigments like chlorophyll, xanthophylls & caretonoids.
4. Seperation of the xanthophylls.

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