Flower Color and Means to Determine Causal Anthocyanins And Their Concentrations[1]

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Abstract

This paper looks at the issue of the coloration of plants and the ability to estimate the concentrations of certain colorants such as anthocyanins based upon commonly available spectrometer methods. The approach is to begin with classic color theory which has been employed extensively elsewhere and then to develop a model for reflectance using the Beer's model and in turn provides a set of methodologies to estimate the concentrations of all colorants in a cell. This approach can then be employed in several areas; first in the determination of the genetic networks generating the colorants, the gene expression identification problem, and secondly the issue of flower color patterning, namely tessellation.

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1 INTRODUCTION

Flower color is a direct result of light absorption in the cells of the petals and sepals. The process of absorption may be complex and many but the result is that the reflected light spectra will absorb certain parts of the visible spectrum and allow other parts to be reflected back out by the cell walls. To some degree there are many complex and yet to be understood or characterized processes at play. However, if one is seeking to estimate the concentrations of the chemical elements which lead to the coloration, then it is possible to do so using the means and methods proposed herein.

Once the concentrations of such elements such as the anthocyanins has been determined then it is possible to use that data and work backwards to assess and determine the nature and workings of the genetic pathways which have given rise to these colorants (see McGarty, 2007). In this paper we develop a method to determine the concentrations of colorants resulting from secondary pathways in flowers. The method employs the use of standard spectroscopic techniques and using the basic principles of color absorption provides a detailed set of methodologies to estimate separate concentrations. It is assumed that for each secondary pathway colorant its individual profile of extinction or absorption is known.

In this paper we address several issues. The objective of addressing these issues will be twofold. First we will need the understanding to proceed to the issues of understanding genetic pathways and to understand and explain the complex issue of flower color. The second use will be the establishment of a base to perform measurements and analyze the resulting data to validate the theories. Thus the issues we must join are:

1. **Color and Human Factors:** In previous work we used the Tristimulus model to analyze the results obtained from the measurements in the genetic pathway efforts. We argue here that this is a limited approach which on the one hand must be understood and integrated in what we are doing but on the other hand must be gone beyond if we hope to obtain the resolution required.

2. **Color in Plants:** This discussion is a complex set of issue regarding plant color. On the one hand we review and position the anthocyanins and other colorants and on the other hand develop constructs for explaining the passage, absorption and reflectance of light as color in plants.

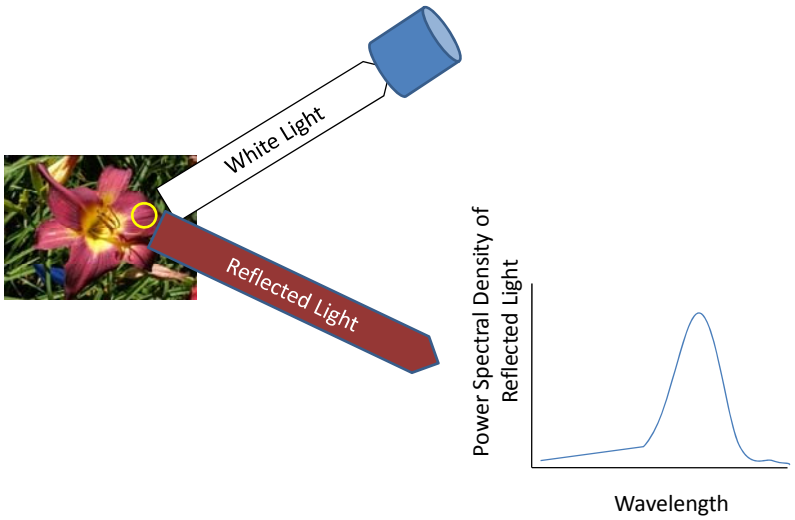
3. **Measurements and Methods:** Spectrometry has been a mainstay of assessing molecular structure especially of complex organic molecules. We review the physics of the underlying phenomena and then review the experimental techniques employed. We argue that the use of Fourier Transform Spectroscopy is best suited in this environment.

4. **Data Analysis and Concentration Estimation:** Once the data has been captured, we then seek methods using the inverse of Beer's law to ascertain the concentrations of and types of anthocyanins and other colorants in the cells. Multiple methods are presented, developed and compared.

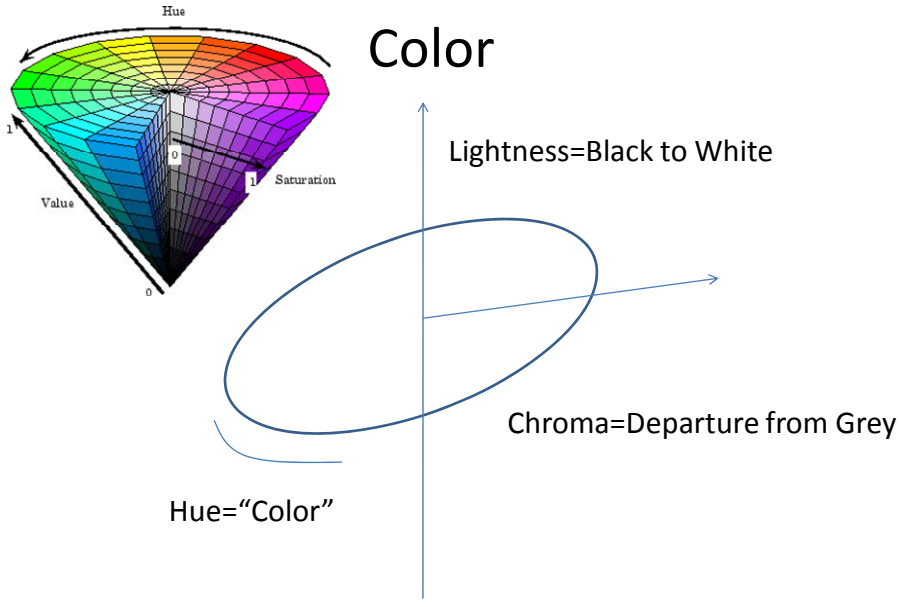
2 CLASSIC COLOR THEORY

Color can be viewed from several perspectives and the two focused on herein are the human eye and the measure power spectrum. The human eye views color in a complex manner since the eye receives color stimuli via sets of sensors which are tuned to three possible visible frequencies, the classic red, green and blue.

The Figure below depicts the basic concept. We see a flower as a certain color. There is "white" light shown upon the flower and the light is reflected from the petals and sepals and what we perceive is a red flower. This perception is a combination of two things; what part of the incident white light is reflected, and how our eyes process that reflected light. Thus color has two meanings for us. First color is nothing more than a reflected spectrum of electromagnetic waves in the optical frequency band. Second it is what we perceive as a human observer and in turn name as a color. The latter approach can and often is quite subjective. This latter approach is the basis of print color, paints, dyes, pigments and the like where the end point is the presentation of some artifact of a desired color. It is the former or first approach we seek to use, namely what is there independent of the observer, specifically the spectrum.

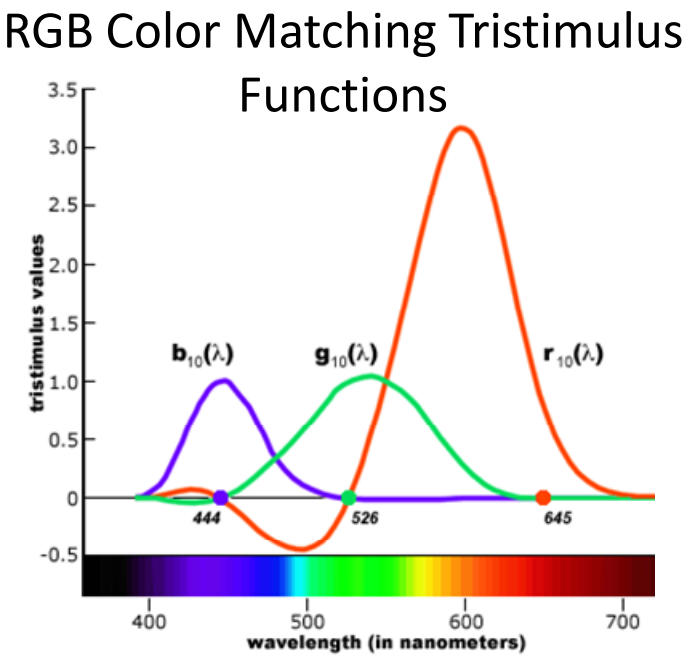


In the context of color as perception there is a collection of terms which should be understood. Hue is a synonym for a color. Red is thus a hue as is orange. The hues cover the visible spectrum. Then there is the lightness, ranging from whit to black. The third element is the chroma which is the departure of the hue from gray. We show these elements below.



The color school then takes three more steps. These are the CIE models for color. The first step is the Tristimulus models. The Tristimulus function is shown below. This is NOT a spectrum. In addition negative values mean more

positive stimulus. These are also the result of extensive experimental modeling. The red, green and blue Tristimulus model as shown below characterizes three stimuli which affect the three receptors of color in the eye.

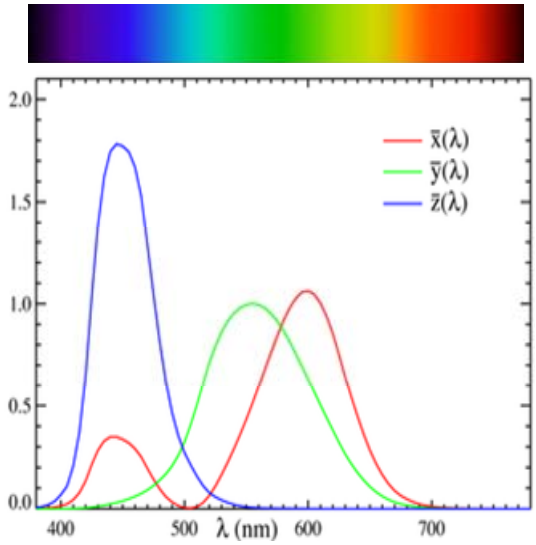


The above Tristimulus curve, called the color matching function, were experimentally determined through an experiment where a person looked at a test color centered at a wavelength as shown on the horizontal axis above and tried to match it by adjusting a red, blue and green lamp in the reference field. This could be accomplished for all regions except between 444 and 526 nm. In that region a red light had to be added to the test field to adjust the color to match. In effect the test color was changed by adding red. This adding of red is accounted for by the negative portion of the above curve[2].

Following this above model based upon experiment is the spectral approach called the standard observer consisting of the X Y Z model as shown below. They do effectively represent quasi spectral responses since they are all positive. It is possible to transform between the RGB and the XYZ formats.

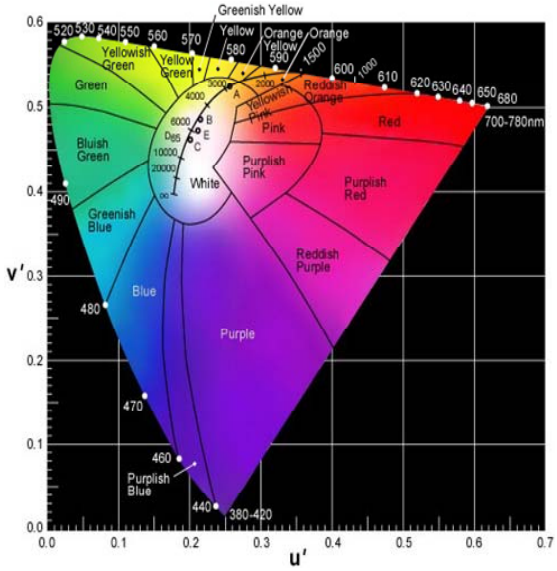
[2] See Berns p. 49.

CIE Standard Observer Curves



The CIE Chart is a manifestation of how the three stimuli above can be added together to create a broad set of colors, hues. It is possible to go from red, thru green and then to blue. One need only mix the three stimuli in the proper ratio. Then other hues can also be generated.

CIE Chart



To understand this a bit better we analyze the RGB system first. We start with a source specified by intensity I dependent on wavelength. This allows us to define the following:

$I(\lambda) =$ *the spectrum of a specific sample*

Define

$$R = \int I(\lambda) r(\lambda) d\lambda$$
$$G = \int I(\lambda) \bar{g}(\lambda) d\lambda$$
$$B = \int I(\lambda) b(\lambda) d\lambda$$

Note, as we had stated, the color matching primaries show negative values because the negative was the way the CIE arbitrarily represented an excess positive contribution required to be added to a primary to achieve the desired spectrum response while keeping the elements normalized. Specifically:

$$\int \bar{r}(\lambda) d\lambda = \int \bar{g}(\lambda) d\lambda = \int \bar{b}(\lambda) d\lambda$$

Now this also implies the following are to be true:

$$r = \frac{R}{R+G+B}$$
$$g = \frac{G}{R+G+B}$$
$$b = \frac{B}{R+G+B}$$

and

$$r + g + b = 1$$

In a similar manner we can do the same for the XYZ system. This is done as follows:

$I(\lambda) =$ *the spectrum of a specific sample*

Define

$$X = \int I(\lambda) \bar{x}(\lambda) d\lambda$$
$$Y = \int I(\lambda) \bar{y}(\lambda) d\lambda$$
$$Z = \int I(\lambda) \bar{z}(\lambda) d\lambda$$

And as was the case for RGB we also have the normalizing factor. Not that it is this normalizing factor which assures our ability to deal with the triangular plot of color.

$$x = \frac{X}{X+Y+Z}$$
$$y = \frac{Y}{X+Y+Z}$$
$$z = \frac{Z}{X+Y+Z}$$
and
$$x + y + z = 1$$

Finally there exists a set of transforms which allows one to convert from one to the other. This is shown below:

$$\begin{bmatrix} r \\ g \\ b \end{bmatrix} = A \begin{bmatrix} x \\ y \\ z \end{bmatrix}$$
A such that sums of r,g,b and x,y,z are unitary

Therefore for any color C we can write it in one of the following two manners:

$$C = rR + bB + gG$$

$$C = xX + yY + zZ$$

Thus an x,y plane can be constructed such that any color can be characterized as a pair of coordinates (x,y). This is the CIE Chart which we have shown above. It must be noted that all of this analysis is predicated on how "we" see color and not in any context of how it is created or the underlying physics of color.

Now there are two other brief examples worth noting. First is the concept of additive colors, such as those we see when we add lights. This was the basis of what Newton did in his early experiments. By adding lights we can ultimately create white. We show that below.

Additive Primary Colors

Additive Colors combine to form white. Traditionally adding lights is additive whereas adding colorants, pigments or dyes, is subtractive.

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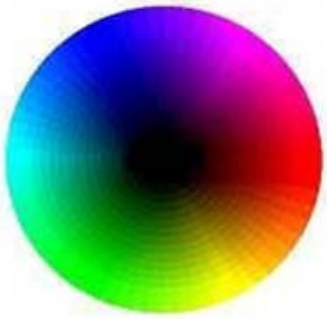
The opposite is the subtraction of light, and this is the result of adding pigments of different colors together in an oil painting. If we were to add all the colors together then we obtain black and not white. This is subtractive, for we are in reality removing colors by the use of those pigments. In many ways this is the difference between water colors and oil paint. We show this subtractive result below:

Subtractive Primary Colors



Subtractive Colors form black. Subtractive mixing involves the removal, subtraction, of light from the mix. Removing all light ultimately results in black. Absorption only is called simple subtractive mixing whereas combining this with scattering is complex subtractive mixing.

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Notwithstanding the above detail and its use in many industrial processes, these methods used in classic colorimetry are methods that rely upon the human by necessity being part of the process. We when looking at plants, shall disregard the human.

3 SPECTRA AND MEASUREMENTS

The measurement of absorption spectra can be accomplished by a variety of means. We present here two methods; classic spectrometry and Fourier Transform Spectrometry (FTS) also called Fourier Transform Infrared Spectrometry, FTIS. However FTS can be applied to the optical bands as well.

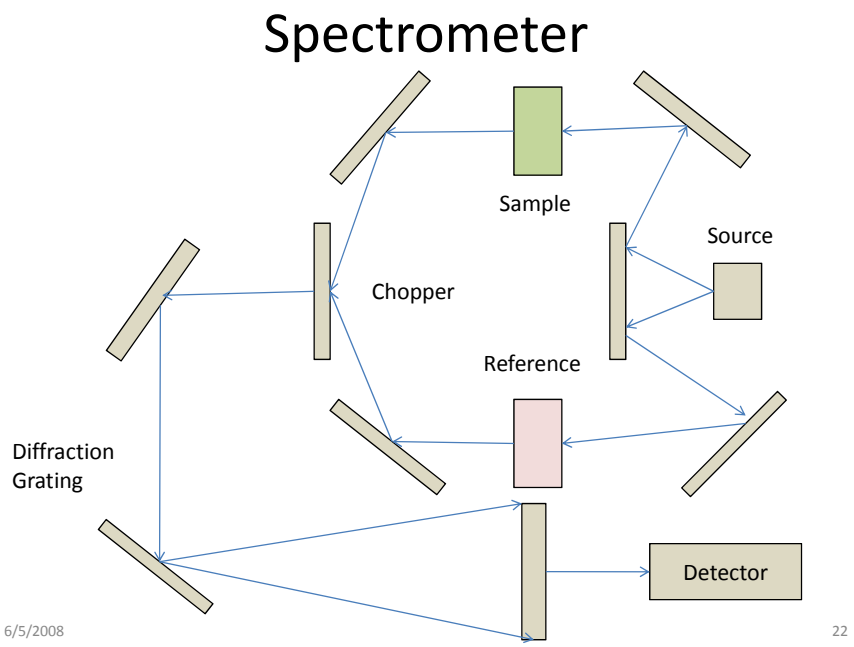
The goals using these methods are as follows. First to determine the absorbance and extinction coefficients of the secondary products that are colorants. This means that solutions of purified anthocyanins, Peonidin for example, would be used and their absorbance and extinction coefficients determines for all wavelengths over the optical band[3]. This is accomplished for all targeted absorbents. Second, perform the same on all known colorants found in a target plant cell. This could include any secondary product or even proteins which have absorbent properties. Generally the other chemical elements react in an absorbent manner out of the optical band. Third, perform the analysis on target cells. Our approach is to perform this on a cell by cell basis thus requiring focused optical positioning.

3.1 Classic Spectrometer

We first look at the classic spectrometer. It uses two paths for transmission, one through a cell with the target secondary and another cell without any secondaries or colorants. The second cell is a reference cell. The reason for this approach is to calculate the difference in absorption. The spectrometer is shown below. It functions as follows:

- Select a Reference and a Sample. Source Spectrum is to be determined using reference.
- Send light from source through both sample and reference. The source must be broadband wavelength. There will be no need to regulate amplitude across the band since a difference signal is obtained and the result will be expressed as a ratio.
- Chop the signals using electronic chopper so that half interval it is sample and other half it is reference. This can be accomplished with a time controlled electronic device or even a mechanical rotating wheel which can be synchronized to the measurement elements.
- Send to Diffraction Grating to spread out signals over visible spectrum.
- Sample from one end of spectrum to the other by mechanically sampling the diffraction grating spread out. Remember that the diffraction grating act as a prism and spreads out the signal spatially over the optical band.
- Use the reference as the baseline and then measure the ratio or the difference of sample to reference and plot. This generally requires just a difference amplifier at the measuring point and synchronizing it with the chopping signal.

[3] See Cantor and Schimmel, Part II, pp 380-388.



The spectrometer, as shown above, functions well for the determination of relative absorption. It is a long and sometimes cumbersome process because the screen in front of the detector is scanned slowly and this provides the signal used to ascertain the difference measurement. There is an issue of accuracy and precision in the collection of data and there is also the issue regarding the amount of light intensity requires. One should remember that as we spread the light out through the grating we see the spectrum now spatially but in so doing reduce the signal strength of each segment. The spectrometer has advantage and disadvantage in this configuration.

3.2 Fourier Transform Spectrometer

The FTS is a more recent embodiment of a spectrometer and it eliminates many of the accuracy and precision issue of the classic spectrometer. In many ways it may be viewed as a mini-CAT scanner in that it collects data which is the Fourier Transform of the desire waveform, namely the absorption spectrum.

The FTS works as follows:

1. A target sample is placed in front of a detector. The detector is a broadband detector and it provides at its output the integral of all the power entering across the optical spectrum. The optical spectrum will be the target spectrum of interest so we delimit the detector to that. We also assume we know the detector response and that this can be adjusted for by means of signal pre-emphasis. This means that the detector works as follows:

$$P = \int \tilde{S}(f)df$$

Here P is the total power and S(f) is the power spectral density of the combined signal. We will look at that in some detail in a moment. Now we assume that the detector may itself have a spectral sensitivity given by H(f). Thus what we really receive if we do not pre-process is:

$$P = \int \tilde{S}(f)H(f)df$$

Which may bias out answer? The way to avoid this is to do some pre-emphasis on the front end by using filters which do the following:

$$P = \int H(f)G(f)P(f)df$$

$$= \int P(f)df$$

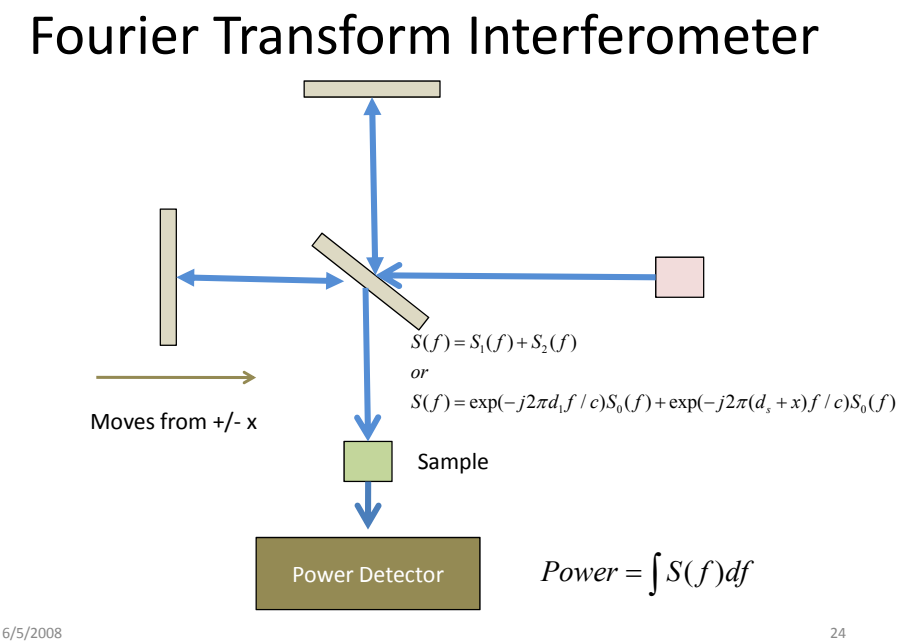
$$if$$

$$H(f)G(f) = 1$$

This is optical pre-emphasis filtering as one does with FM radio. This is a standard approach.

2. Now let us go back to the input. We assume we have a flat frequency broadband source of radiation emitted from the source. If now we can also pre-emphasize that as well. Then this source follows two paths. Path 1 is a fixed path up and down and through the sample. Path 2 is one that goes to a reflector whose portion is changing uniformly in time and is accurately measures. This second path then send the same signal with the sole exception that it is phase offset from the main path. At times it may be totally in phase and at time totally out of phase. For every position x of the reflecting mirror we measure the combined power spectrum received, the integral of both signals, measure as their amplitude.

3. It can be shown, we do so below, that if one collects the P values and notes them as P(x) then if we sample x properly we obtain samples of the Fourier Transform of the S(f) function. Thus collecting P(x) for the correct values of x and doing so with enough samples we can then perform an inverse Fourier Transform to readily obtain S(f). This is FTS.



The details can be displayed simply as follows. The signal received is the direct and the reflected and they are combined in complex space to account for the phase difference as shown below:

$$S(f) = \exp(-j2\pi d_1 f / c)S_0(f) + \exp(-j2\pi(d_s + x)f / c)S_0(f)$$

$$\approx S_0(f)\cos(2\pi fx / c),\ \textit{so that the received power is:}$$

$$P(x) = \int S_0(f)\cos(\frac{2\pi}{c} fx)df$$

The result is that P(f) is the real FT of S(f).

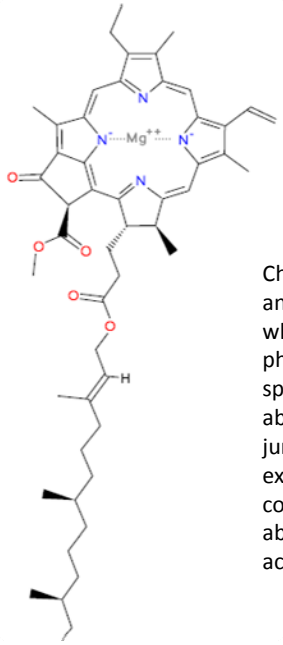
FTS shows that we can obtain P(x) and it is the Fourier Transform of S(f) the absorption spectrum of the sample. We take P(x) for many values of x and then inverse FT.

4 PLANTS, COLOR AND CHEMISTRY

We can now consider plants and their colors. We have discussed this before and it is covered generally in the literature. However we want to focus on specific colorants.

4.1 Molecular Issues

We begin with the most common colorant, chlorophyll, the element which makes leaves green



Chlorophyll

Chlorophyll contains many bonds and many outer shell electrons which in turn can absorb many photons across the incoming spectrum of visible light. They are absorbed by causing electrons to jump to more distant orbits and by exciting bonds between the constituent atoms. The result is the absorption of photons selectively across the optical spectrum.

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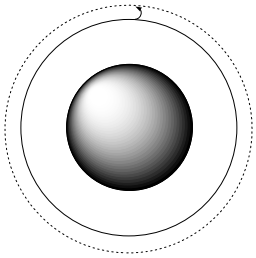
What causes absorption? The answer is a complex one but it may be simplifies into two parts/ first is the excitation of outer layer electrons, using the exact energy of the incoming photon. The second is the resonance excitation of the bonds in the carbon elements especially. There is a complex set of issues here. The correct manner to approach this is via the quantum mechanical methods. They are extremely complex due to the complex structure of the compounds. However phenomenologically it is easy to measure.

We show these phenomena in the Figure below.

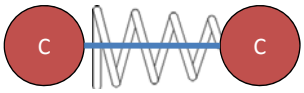
Absorption

- Molecular: Electrons and bonds
  - Electrons in outer shells

$$\varepsilon = h\nu = \frac{hc}{\lambda} = energy\ of\ photon\ absorbed$$



  - Bond vibrations



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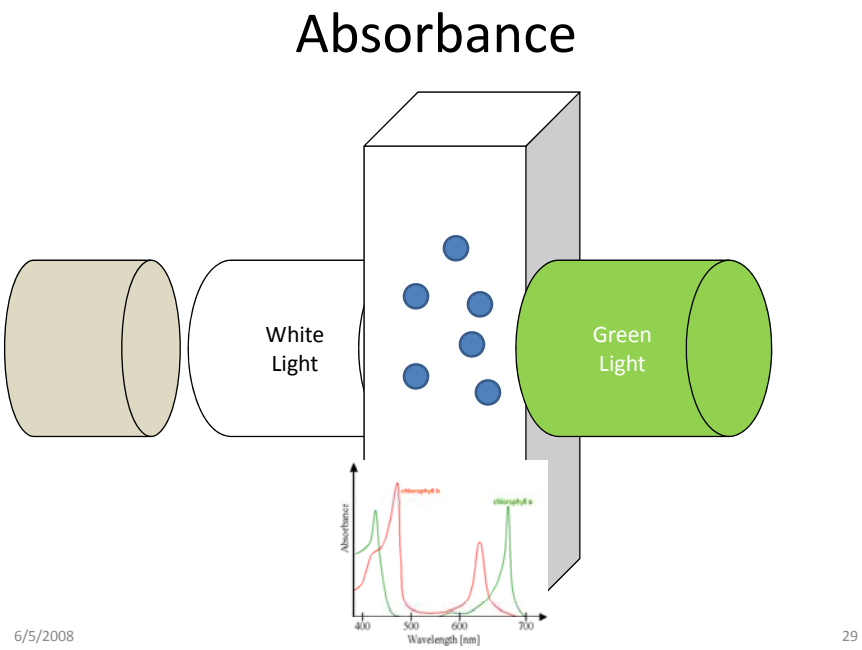
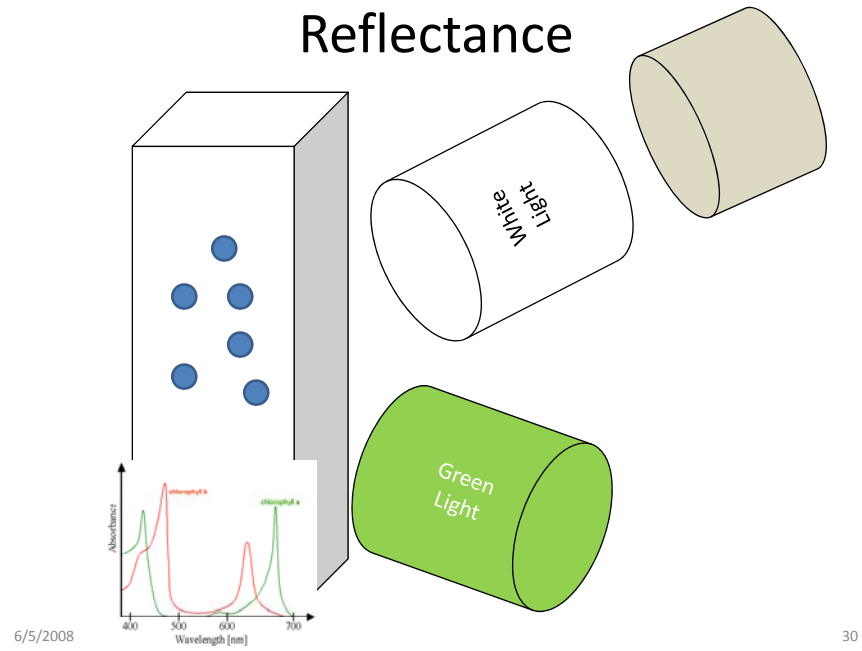
4.2 Absorbance and Reflectance

The next question is what are leaves green and flowers red? The answer is simple obtained by understanding absorbance and reflectance. In the figure below we show white light passing through a cell or sell filled with chlorophyll. Chlorophyll absorbs red and blue light and lets the green part of the spectrum pass unabsorbed. The absorption results from the very atomic interactions of the photons on the structure of the chlorophyll molecule we have shown previously.

In the Figure below we show a white light as we may see in the sun and we shine it upon a cell or set of cells and we assume that it is absorbed but manages to pass through. The light emitted is green.

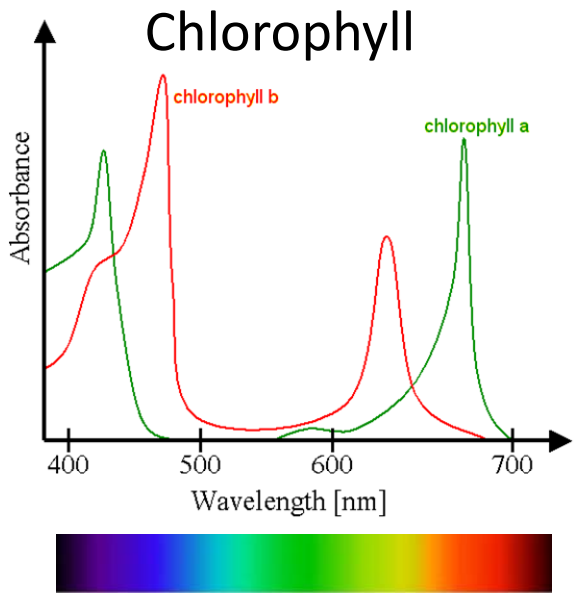


In contrast to absorbance we also have reflectance. Reflectance results when we shine white light upon a cell or set of cells and the light is refracted and in turn reflected back out into the general direction of the incident light. In the Figure below we show white light impinging on a cell and the cell is filled with chlorophyll. The chlorophyll absorbs the red and blue so the reflected light is green. This has the same characteristics as Transmittance.



The absorbance of chlorophyll is shown in the following Figure. We shall define absorbance latter. However what we see is that chlorophyll absorbs the low and high frequencies, the long and short wavelengths, and leaves the middle wave lengths relatively un-absorbed. This means that what is reflected back from a plant cell composed of chlorophyll is primarily green. Thus the green leaves of plants. Of course we will not see the same in flowers. In

addition, in the fall when the chlorophyll degrades as the plant goes dormant, what is left in the leaf are the anthocyanins which we shall show have a reddish orange tint.



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The above Figure also depicts the two types of chlorophyll, but for our purposes we need not be concerned with them.

4.3 Plant Cell Reflection

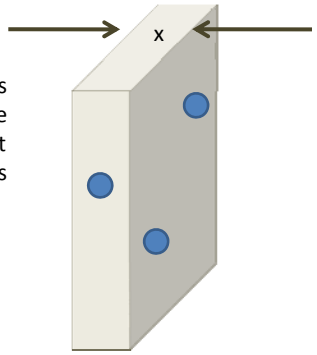
The next issue is to understand how reflection occurs in plants. This is somewhat of a complex issue but has a simple explanation.

A plant cell on the surface of a plant has a cell coating composed of cellulose, hemicelluloses, pectin and proteins which are all relatively transparent to light in the visible spectrum[4]. Cellulose is a long chain of glucose residues which form a ribbon. Visible light penetrates this wall easily and then is passed through the cell. The light then is reflected back out, most of it, and some continues to flow to lower layer cells.

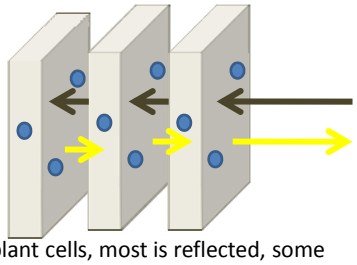
[4] See Taiz p. 22.

Plant Cell Reflectance

Back of the plant cell acts as a reflective surface. Most of radiation is reflected outward and very little passes through.



Within the cell there is a concentration of [C] of a substance which absorbs. The absorption depends on the wavelength, the concentration and the effective thickness.



Several layers of plant cells, most is reflected, some penetrates to lower layer and most again is reflected.

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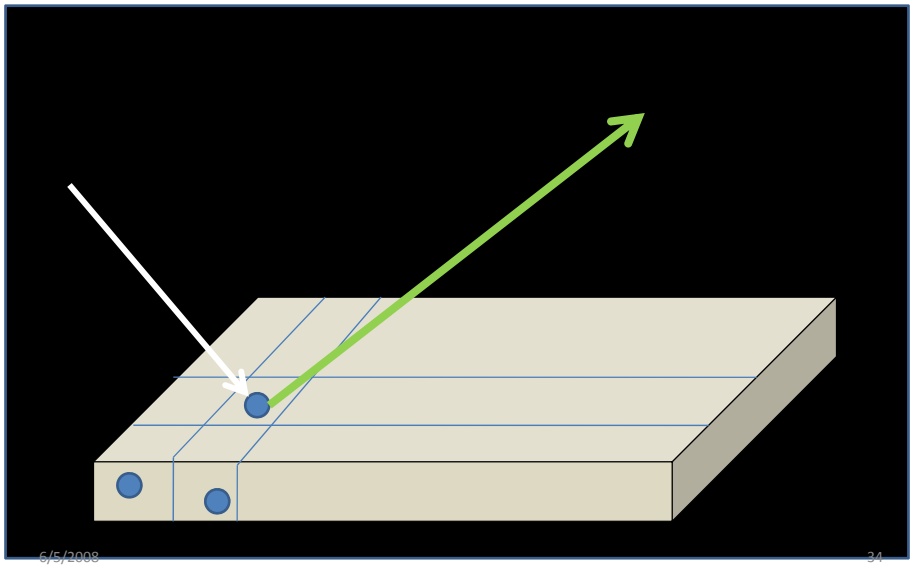
Reflection occurs in a plant cell as a result of the standard process of light being reflected at the boundary where we have an abrupt change in the index of refraction. This index changes as we go from air into the cell, and indeed even in the cell itself. The cell has water, proteins, colorants, a nucleus and many other constituents. The refraction at the interface changes the direction of propagation and the change may reflect the light out or further into the cell. The issue is dependent upon the incident angle and the index of refraction. We show a prototypical example below.

Cells



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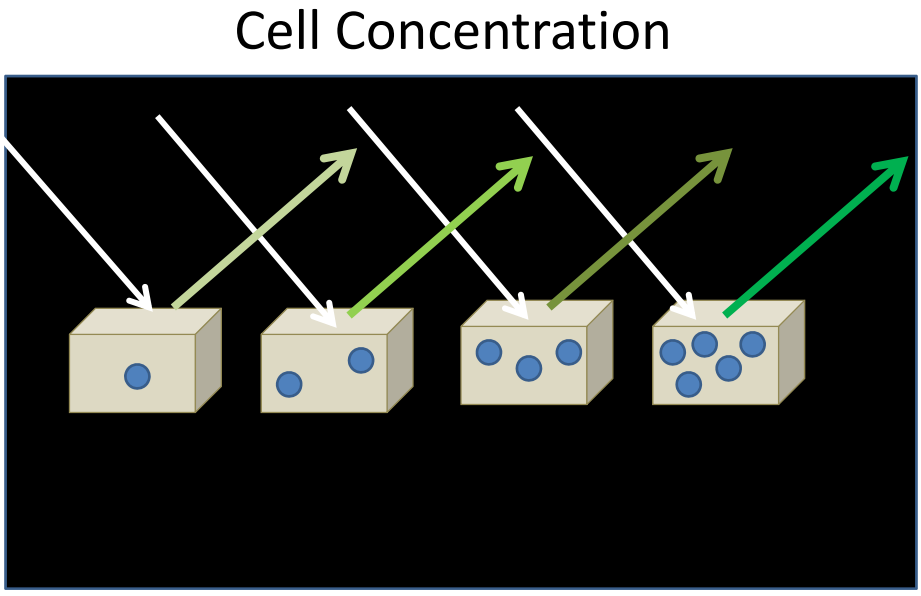
The Figure below shows another phenomenon. As the cell concentration increase more absorption occur and the color may actually change. Thus cell concentration is a major factor. In addition the thickness of the cells and the number of total cells will also be a significant factor and we shall discuss that next.



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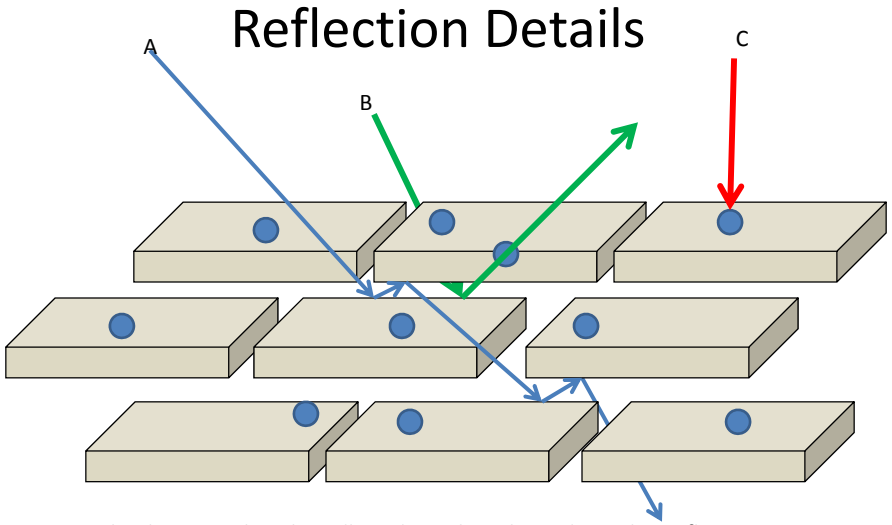
The process of intracell reflection is somewhat complex to say the least. It has been studied for over a century and there are still many theories to explain various cell structures. However for the case of a flower as Hemerocallis the presentation can be simplified. The Figure below, adapted from Lee, shows three paths through several layers of cells. One path bounces about and finally is reflected albeit attenuated by the colorant molecules. A second path manages to go through the cells and out the other side,. and a third path gets fully absorbed.



Path A bounces thru the cells and out the other side. Path B reflects from a second layer cell and passes through an anthocyanin. Path C gets fully absorbed by an anthocyanin patch.

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The details of the above are generally difficult to analyze due to the random nature of the cells and the colorants. McGarty (1971)[5] provides a summary of the approaches. However there are certain metrics which can be useful. The plant cell has a cellulose wall which is rigid. The dominant substance inside the cell, especially in a flower, is the vacuoles filled with water. The colorants are mixed in the cell. A plant cell is about 100 μm in width and about 20-50 μm in depth. The wavelength of light in the visible region is 500 nm which is 20 time less. Thus scatter is not a major factor. However refraction and reflection are. The index of refraction of the cell is about 1.2-1.4 (see Lee)[6] and from Born and Wolfe (p. 41) we know that for an interface of this type the reflection and refracted components can be calculated. If we define A as the incident amplitude, with two components, parallel and perpendicular, then we can calculate the two components of the reflected component R and the refracted or transmitted component T. This is shown below:

$$T_0 = \frac{2}{n+1} A_0$$

$$T_1 = \frac{2}{n+1} A_1$$

$$R_0 = \frac{n-1}{n+1} A_0$$

$$R_1 = -\frac{n-1}{n+1} A_1$$

$$0 = parallel$$

$$1 = perpendicular$$

Thus when we enter the index of refraction we see that transmission is greater than reflection for perpendicularly incident light. This analysis provides some insight into the nature of the cell. Also one must remember that the reflected light is reflected off the outer layer of the cell. This process then continues again as the light leaves the cell and enters ones below.

We now move to a deeper analysis of the specific colorants and their impacts on the light entering the cells.

5 COLORANTS FROM SECONDARY PATHWAYS

The anthocyanin molecules is shown below. Note on the B ring we have six sites to which we can attach differing molecular chains. This will be an important element when we see the different configurations and their implications.

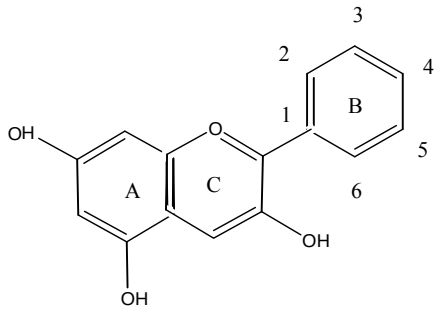
5.1 Anthocyanins

The anthocyanin or anthocyanidin molecule comes from two different secondary pathways in the plant cell. One is from the shikimic pathway and the other from the malonate pathway. This means that we have to understand both pathways to understand the ultimate abundance of the product. Anthocyanins are not the only elements which are secondary products which produce color. There are three classes of chemicals which give rise to color; anthocyanins, flavones or flavonols, and carotenoids. The basic structure of the anthocyanin is shown below.

[5] See McGarty, 1971. This Thesis details the complex issues of multiple scattering in complex media such as a cell matrix. The Thesis also summarizes the experimental and theoretical work to the date of publication. Some extensions have been made since that time but the solution to the problem is still somewhat intractable except in a statistical sense. We use Beer's Law in the next section as a means to handle the complex nature of the optical problem.

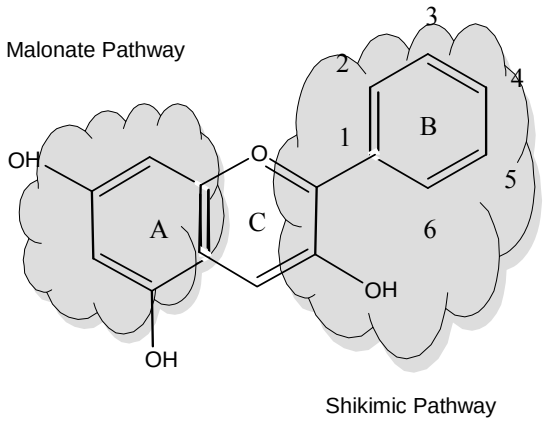
[6] See Lee p. 84. The author states that the index of refraction of water in a cell is 1.3, with the molecules in solution in vacuoles is 1.34 and that cellulose of the wall has an index of refraction of 1.4.

Anthocyanidin



The anthocyanin or Anthocyanidin molecules comes from two different pathways. In the figure below we have taken the basic resulting molecule and have shown that there are two elements; one is from the shikimic pathway and the other from the malonate pathway. This means that we have to understand both pathways to understand the ultimate abundance of the product.

Anthocyanidin



Before continuing we want to look at what the results would look like if we have different substitutes on the B ring. In the Table below we show that the terminations on the 3, 4 or 5 elements yield different results. The results give pelargonadin, cyanidin, delphinidin, peonidin, and petunidin. Each obviously named after their related flower and each resulting in an anthocyanin of a different color.

Anthocyanin Colors

Anthocyanidin	*Substituents*	*Color*
Pelargonidin	4'-OH	orange-red
Cyanidin	3'-OH, 4'-OH	purplish red
Delphinidin	3'-OH, 4'-OH, 5'-OH	bluish purple
Peonidin	3'-OCH_3, 4'-OH	rosy red
Petunidin	3'-OCH_3, 4'-OH, 5'-OCH_3	purple

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In the Table below we have shown the colors of each of these as well as the weighting of a red, green and blue combination which best matches the color. Thus one can in an 8 bit color schemes, as one would find in any PC color scheme, get the resulting anthocyanin colors by blending the R, B, G elements to yield what we are seeking. This relating the colors back to RBG is critical since it get reflected in the ultimate flower color.

Colors (R, G, B)

Pelargonadin (255, 102, 0)	
Cyanidin (255, 0, 255)	
Delphinidin (153, 102, 255)	
Peonidin (255, 153, 204)	
Petunidin (153, 0, 153)	

Now if we assume we have only anthocyanins for color, and that we have the above combinations available, we ask; how do we combine these colors in a weighted manner to obtain the desired color? This approach is critical to the overall understanding. First we show by a weighted RBG we get the color we seek or the color which is presented. Then we assume that if we can then do the same for each anthocyanin, then we can create any desired color from a weighted collection of anthocyanins. This means that we can then determine what the relative percents of expression of any anthocyanin is and this lets us then go back to how strongly the gene for that anthocyanin is expressed. The model we presented earlier will be a key element in this overall process.

No let us start with a simple expression. For any color we have by definition:

$$\text{Color} = \alpha\ \text{Red} + \beta\ \text{Blue} + \delta\ \text{Green}$$

For example, we may have a (0,0,255), or a (128, 128, 128). Or any other set of combinations.

Likewise we could state this by means of some combination of anthocyanins. Namely:

$$\text{Color} = \text{a}\,[\text{Pelargonidin}] + \text{b}\,[\text{Cyanidin}] + \text{c}\,[\text{Delphinidin}]$$

But we can relate the anthocyanins to the basic colors or red, blue and green as:

$$\left[Pel\,\text{arg}\,onidin\right] = \alpha_P\,\text{Re}\,d + \beta_P Blue + \delta_P Green$$

$$\left[Cyanidin\right] = \alpha_C\,\text{Re}\,d + \beta_C Blue + \delta_C Green$$

$$\left[Delphinidin\right] = \alpha_D Red + \beta_D Blue + \delta_D Green$$

If we define a color vector of Red, Blue and Green as:

$$C = \begin{bmatrix} Red \\ Blue \\ Green \end{bmatrix}$$

Thus if we define the mix vector as **m** then we have:

Color = $\mathbf{m}^{\,T}\,\mathbf{C}$

Or:

$$m = \begin{bmatrix} \alpha \\ \beta \\ \delta \end{bmatrix}$$

But we have the following matrix:

$$A = \begin{bmatrix} \alpha_P & \beta_P & \delta_P \\ \alpha_C & \beta_C & \delta_C \\ \alpha_D & \beta_D & \delta_D \end{bmatrix}$$

These yields:

$$\text{Color} = \mathbf{m}^{\,T}\,\mathbf{A}\,\mathbf{C}$$

The above analysis shows us that we can analytically determine the expression of the anthocyanins from the color of the cell by means of the above formulas. These are relative expressions but by benchmarking any one element we can make them all absolute in the cell as well.

5.2 Other Color Elements

Anthocyanins are not the only elements which are secondary products which produce color. There are three classes of chemicals which give rise to color; anthocyanins, flavones or flavonols, and carotenoids. The Table below depicts the different elements and their colors. The approach we took above for the anthocyanins can be taken for the

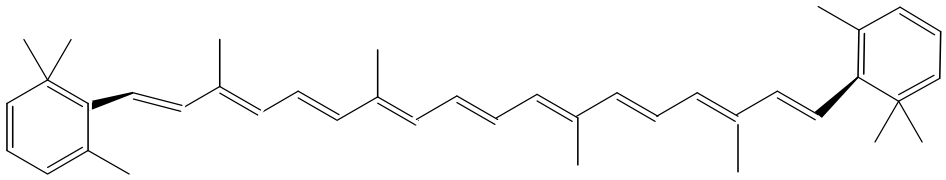
flavones and carotenoids as well. It should be noted that there may not be a unique solution here but there are several possible but they can be narrowed down by actual determination of one to three elements as baseline.

Class	*Agent*	*Color*[7]
Anthocyanidin		
	Pelargonidin	orange-red
	Cyanidin	purplish-red
	Delphinidin	bluish-purple
	Peonidin	rosy red
	Petunidin	purple
	Malvinidin	
Flavonol		
	Kaempferol	ivory cream
	Quercetin	cream
	Myricetin	cream
	Isorhamnetin	
	Larycitrin	
	Syringetin	
	Luteolin	yellowish
	Agipenin	Cream
Carotenoids		
	Carotene	orange
	Lycopene	Orange-red

We now summarize the other element classes.

5.2.1 Carotenoids

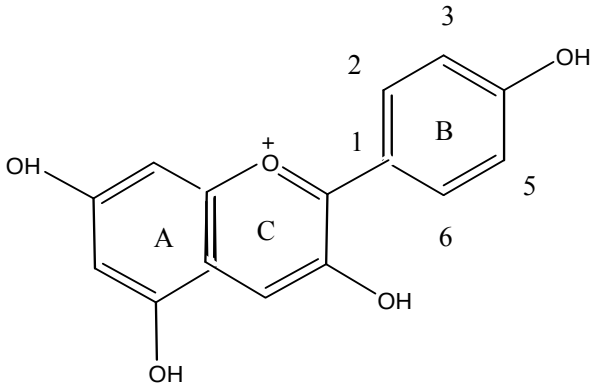
Carotenoids are what is quite common in the carrot, the orange hew we see in that root. Its molecular structure is shown below, this is beta carotene.



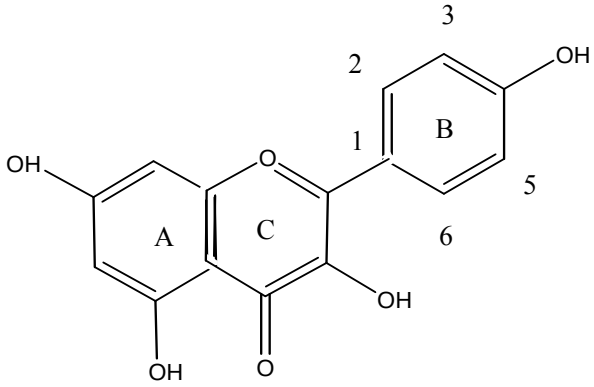
[7] See Taiz p. 334 for the Anthocyanidin color and Bernhardt for the Flavonol and carotene.

5.2.2 Flavones

The flavonols, or flavones are quite similar to anthocyanin. Their structure is shown below. Note that we have compared it to that of anthocyanin.



Anthocyanidin



Flavonol

We can also show how closely they relate in substitutions and colors. This is shown in the Table below.

Flavonol	Anthocyanidin	Substitution	
		3’	5’
Kaempferol	Pelargonidin	H	H
Quercetin	Cyanidin	OH	H
Myricetin	Delphinidin	OH	OH
Isorhamnetin	Peonidin	OCH_3	H
Larycitrin	Petunidin	OCH_3	OH
Syringetin	Malvinidin	OCH_3	OCH_3

5.3 Anthocyanin Absorbance and Reflectance

There have been many studies on the absorbance and reflectance of the various anthocyanins. The Figure below shows the results from a 1957 paper by Harborne. Harborne used the following procedure:

"*Spectrophotometry. All measurements were made with a Unicam SP. 500 spectrophotometer. The pure dry pigments were dissolved in methanol containing 0-01 % of conc. HCI and the solutions diluted to give an optical density reading in the range 0-800-1-300 at the visible maxima. For measurements in the ultraviolet region, the solutions of those anthocyanins obtained from eluting chromatograms were measured against a solution obtained from an appropriate blank area of the chromatogram, prepared at the same time as the corresponding pigment solution.*

For the purpose of measuring spectral shifts in the presence of aluminum chloride, three drops of a solution of the anhydrous salt in ethanol (5%, w/v) were added to the cell solution. Measurements of the shift were made as quickly as possible, since in some cases the color of the resulting solution faded on standing.

For measuring the spectra of mixtures of Pelargonidin 3:5-diglucoside and p-coumaric, caffeic and ferulic acids, 1 m-mole of each compound in pure, anhydrous form was dissolved in 50 ml. of methanol containing 0-01% of conc. HCI. Portions (1 ml.) of these stock solutions were mixed together in varying proportions and the solution was made up to 10 ml. The compounds were previously purified by recrystallization and then dried in vacuo at 1000 over phosphorus pentoxide."

He continues:

"In searching for new methods of characterizing anthocyanins, it should be remembered that they are difficult compounds to deal with by the usual techniques of organic chemistry. In solution, they are unstable to light and pH changes. They are difficult to isolate in a pure state as a general rule. Many of them do not have sharp melting points and do not give meaningful results on elementary analysis. No suitable derivatives are known for characterizing them. The procedure of methylation and hydrolysis, commonly used with flavones for determining the position of sugar residues, is of limited value in the anthocyanin series…"

The Harborne result for Pelargonidin is shown below.

Pelargonidin

Spectral Methods of Characterizing Anthocyanins

By J. B. HARBORNE

John Innes Horticultural Institution, Bayfordbury, Hertford, Herts

(Received 1

Note that the anthocyanidins block out the green colors found in chlorophyll. The absorb the green and reflect back the reds and purples.

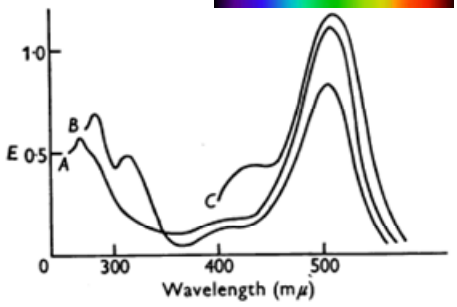


Fig. 2. Absorption spectra: curve *A*, pelargonidin 3:5-diglucoside; curve *B*, pelargonidin 3:5 diglucoside acylated with *p*-coumaric acid (monardein); curve *C*, pelargonidin 3-monoglucoside. The concentrations of the pigments were about 50 μM, in methanolic 0·01% (w/v) HCl.

http://www.biochemj.org/bj/070/0022/0700022.pdf

The following Table details all of the anthocyanins and their peak spectral line as well as the relative peaks compared to 440 nm[8].

[8] From Harborne, 1958.

Anthocyanidin Class	Anthocyanidin Specific	Wavelength nm	E440/Emax
Pelargonidin and derivatives	Pelargonidin	520	39
	Pelargonidin 3-monoglucoside	506	38
	Pelargonidin 3-rhamnoglucoside	508	40
	Pelargonidin 3-gentiobioside	506	36
	Pelargonidin 3-diglucosido-7 (or 4'.)-glucoside	498	42
Cyanidin and derivatives	Cyanidin	535	19
	Cyanidin 3-monoglucoside	525	22
	Cyanidin 3-rhamnoglucoside	523	23
	Cyanidin 3-gentiobioside	523	25
	Cyanidin 3-xyloglucoside	523	22
	Peonidin	532	25
	Peonidin 3-monoglucoside	523	26
Delphinidin and derivatives	Delphinidin	544	16
	Delphinidin 3-monoglucoside	535	18
	Delphinidin 3-rhamnoglucoside	537	17
	Petunidin	543	17
	Petunidin 3-monoglucoside	535	18
	Malvidin With 5-hydroxyl group free	542	19
	Malvidin 3-monoglucoside With 5-O-substituent	535	18
Pelargonidin and derivatives	Pelargonidin 5-glucoside	513	15
	Pelargonidin 3:5-diglucoside	504	21
	Pelargonidin 3-rhamnoglucosido-5.glucoside	505	19
	Pelargonidin 3-diglucosido-5-glucoside	503	21
	Monardein	505	21
	Salvianin	505	20
Cyanidin and derivatives	Cyanidin 3:5-diglucoside	522	13
	Peonidin 3:5-diglucoside	523	13
	Peonidin 3-rhamnoglucosido-5-glucoside	523	12
	Peonidin 5-glucoside	528	12
	Peonidin 5-benzoate	528	11
Delphinidin and derivatives	Delphinidin 3:5-diglucoside	534	11
	Petunidin 3:5-diglucoside	533	10
	Petunidin 3-rhamnoglucosido-5-glucoside	535	10
	Malvidin 3:5-diglucoside	533	12
	Malvidin 3-rhamnoglucosido-5-glucoside	534	9
	Negretein	536	9

6 ESTIMATING ANTHOCYANIN CONCENTRATIONS

This section addresses the ability to determine the detailed concentrations of each of the colorants in a cell if one knows the cell effective optical length and the extinction coefficients for each of the constituents. The models for performing these tasks also show what the maximum resolution that can be achieved as well and the maximum number of constituents. The results in the maximum bounding resemble the same results that are found in such areas as ascertaining the accuracy in ambiguity functions for phased arrays. The latter problem was solved by the author in the mid 1970s.

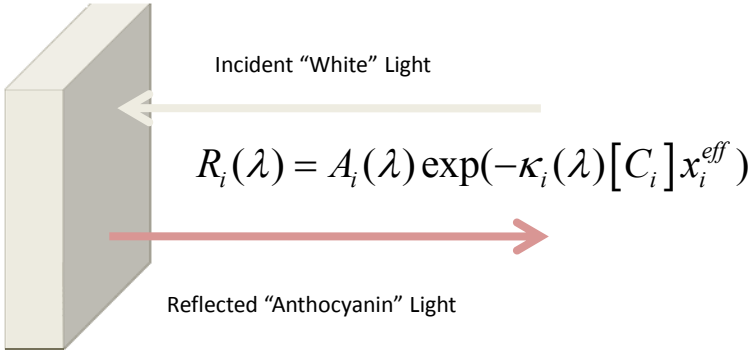
6.1 The Model

Let us begin with a simple model of reflectance. We look at the Figure below and see a white light impinging on a cell and the light reflected back is seeing at one specific wavelength, frequency, as an attenuated version of what was transmitted at the wavelength. A is the amplitude of the transmission and the exponentially reduced A value is what is reflected. Thus if absorption is in the red and blue as we saw with chlorophyll then we reflect green and that is what we see. This is an application of Beer's Law[9]. Beer's law is a statistical approach to absorption. It reflects

[9] See Cantor and Schimmel, pp. 60-68.

what experimentally is obtained and does not provide a detailed analysis as we had been developing in prior sections.

Example



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We define the reflect light at a specific frequency, wavelength, as follows:

$$R_i(\lambda) = A_i(\lambda)\exp(-\kappa_i(\lambda)[C_i]x_i^{eff})$$

where [C] is a concentration and x is the effective thickness of the cell.

Here R is the reflected light we see at the wavelength specified and at the ith anthocyanin.
A is the incident light amplitude at the wavelength specified. The exponent is Beer's law where C is the concentration of anthocyanin I and x the effective depth of that anthocyanin.

Now we can write Beer's law for one or two or even more absorbents. We show the case for one and two absorbents as follows:

$$\frac{dR}{R} = -C_k\kappa_k dx$$

or

$$\frac{dR}{R} = -C_k\kappa_k dx - C_{k+1}\kappa_{k+1}dx$$

Note that the reduction in reflected light or in transmitted light is reduced by a result of the additive reduction of separate collisions with separate molecules.

$$R_{Total}(\lambda) = A(\lambda)\exp(-\sum_{i=1}^{N}\kappa_i(\lambda)[C_i]x_i(\lambda))$$

The log of the ratio of intensities is the sum of the weighted concentrations. We assume we know the κ values for each absorbing element at each wavelength. Then we can use the above to estimate the separate concentrations

$$I(\lambda) = \ln \frac{R_{Total}(\lambda)}{A(\lambda)} = -\sum_{i=1}^{N} \kappa_i(\lambda)[C_i]x_i(\lambda)$$

The problem is simply stated. We measure the intensity at say M values of wavelength and this gives us M samples. We then must find values of the [C] which give the best fit to the measurements obtained using the model assumed. That is for every wavelength, we define an error as the difference between the measurement and what the measurement would have been using the estimates of the [C] values and the best [C] values are those which minimize the sum of the squares of these errors. There are M measurements and N concentrations and M is much larger than N. That is:

Choose $[C_n]$ *such that they minimize*

$$\min\left(\sum_{m=1}^{M}\left(I(m) - \hat{I}(m)\right)^2\right.$$

where

$I(m)$ *is the mth measurement*

and

$$\hat{I}(m) = \sum_{i=1}^{N} \kappa_i(\lambda)[\hat{C}_i]x_i(\lambda)$$

This is an optimization problem which can be solved in many ways. We address some of them in the next section.

6.2 The Approaches

Some Examples, this is an example of the Inverse Problem already solved by McGarty:

1. CIE approach: This assumes that one can unravel the exponents of the x,y,z model. The problem is that we will not have an adequate number of degrees of freedom.
2. Splines: This assumes we can generate curves and then separate them and then focus on their coefficients[10].
3. Steepest Descent: This is the incremental approach of best fit. It assumes we are trying to solve an optimization problem.
4. Least Squares Fit: A statistical best fit method.
5. Kalman Filter: This is the statistical solution using steepest descent but with correlation matrices.
6. Matched Filter: This approach assumes we know the waveforms of each absorption curve for each colorant and that we receive a resulting absorption curve which is the sum of all of them, and that we then try to estimate the "amplitudes" of each curve, in effect the concentrations.

We may define the problem as follows:

[10] See Hildebrand pp. 478-494. The use of splines is an approach which tries to match coefficients of polynomials.

Let $R(\lambda)$ be determinable for a given set of $[C_i]$ and let
$\widetilde{R}(\lambda)$ be the measured received spectrum power and
$I(\lambda)$ be the log of the received to incident power
at the wavelength
Find the set of $[\widehat{C}_i]$, i=1...N, such that
$\left(\widetilde{R}(\lambda)-\widehat{R}(\lambda)\right)^2$ is minimized where $\widehat{R}(\lambda)$is the
estimated received spectral element

We may also characterize the variables as follows:

Let

$$x(k)=\begin{bmatrix} C_1 \\ . \\ . \\ C_n \end{bmatrix}=x(k+1)$$

and

$$z(k)=c^T(k)x(k)+n(k)$$

where

$$c(k)=\begin{bmatrix} -\kappa_1(k)x_1 \\ . \\ . \\ -\kappa_n(k)x_n \end{bmatrix}$$

and for this case k and λ are identical increments

We now consider three possible approaches.

6.2.1 Newton Steepest Descent

The Newton Steepest descent approach is one where we define an optimization and this optimization results in solving a polynomial equation. We then employ an iterative method to solve that equation. We now seek the following:

Find the a such that:

$$\hat{a} = \begin{bmatrix} \hat{a}_1 \\ .. \\ \hat{a}_n \end{bmatrix} = \begin{bmatrix} [C_1] \\ . \\ . \\ [C_N] \end{bmatrix}$$

such that

$$\min\left[\sum_{i=1}^{M}(I_i - \hat{I}_i)^2\right]$$

Let us recall the simple optimization result:

$$h(a) = \left[\sum_{i=1}^{M}(I_i - \hat{I}_i)^2\right]$$

and

$$\frac{\partial h(a)}{\partial a_n} = g_n(a) = 0$$

is the optimal point, so we seek to solve the vector equation:

g(a)=0

We can now state the general solution in terms of Newton's Method[11]:

$$g(a) = 0$$

is the desired result. Define:

$$A(a) = -\left[\frac{\partial g(a)}{\partial a}\right]^{-1}$$

where we define:

$$\left[\frac{\partial g(a)}{\partial a}\right] = \begin{bmatrix} \frac{\partial g_1}{\partial a_1} & \cdots & \frac{\partial g_1}{\partial a_n} \\ \frac{\partial g_n}{\partial a_1} & \cdots & \frac{\partial g_n}{\partial a_n} \end{bmatrix}$$

and the estimate at sample k+1 is:

$$\hat{a}(k+1) = \hat{a}(k) + A(\hat{a}(k))g(\hat{a}(k))$$

Note that we use this iterative scheme as one of several means to achieve the result. For each tuple of data we do the following:

[11] See Athans et al, Systems, Networks and Computation, Multivariable Methods, McGraw Hill (New York) 1974, pp-115-122.

$\hat{a}(0) = a^0$, an n x 1 vector guess. Then we use the first data tuple:

$\hat{a}(1) = \hat{a}(0) + A(\hat{a}(0))g((\hat{a}(0))$

where we use the difference:

$a_{k,measured}(0) - \hat{a}(0)$

as the data entry element for each of the elements of a.

The Newton algorithm is but one of many possible algorithms. We know the conditions for Newton convergence. We can also estimate the accuracy of this algorithm as well.

6.2.2 Kalman Filter

The method of estimating the structural elements of the gene expression can be structured using a standard set of methodologies. In particular we use the two approaches. The approach was applied to estimating the constituent chemical concentrations of the upper atmosphere, namely the inversion problem, using transmitted light as the probe mechanism. In this case we seek to estimate the gene expression matrix using the concentrations of secondary chemicals as expressed in color concentrations. This is in many ways a similar problem.

6.2.2.1 The Model

Let us consider a six gene model, two color modifying genes and four control genes, two each. The model is as follows. First is a general linear model for the gene production:

$$\frac{dx(t)}{dt} = Ax(t) + u(t) + n(t)$$

Then the entries are as follows:

$$A = \begin{bmatrix} a_{11}..a_{12}..a_{13}..0..0..0 \\ 0...a_{22}..0...0...0...0 \\ 0...0...a_{33}..0...0...0 \\ 0...0...0...a_{44}..a_{45}..a_{46} \\ 0...0...0...0...a_{55}..0 \\ 0...0...0...0...0...a_{66} \end{bmatrix}$$

and

$$u(t) = \begin{bmatrix} u_1 \\ ... \\ u_6 \end{bmatrix}$$

And we assume a system noise which is white with the following characteristic:

$$E[n(t)] = 0$$

and

$$E[n(t)n(s)] = N_0 I\delta(t-s)$$

Now we can define:

$$A = \begin{bmatrix} A_1 ... 0 \\ 0 ... A_2 \end{bmatrix}$$

Where we have partitioned the matrix into four submatrices. This shows that each gene and its controller are separate. Now we can determine the concentrations of each protein in steady state as follows, neglecting the Gaussian noise element for the time being:

$$\begin{bmatrix} x_1 \\ x_2 \\ x_3 \end{bmatrix} = -A_1^{-1} \begin{bmatrix} u_1 \\ u_2 \\ u_3 \end{bmatrix}$$

and

$$\begin{bmatrix} x_4 \\ x_5 \\ x_6 \end{bmatrix} = -A_2^{-1} \begin{bmatrix} u_4 \\ u_5 \\ u_6 \end{bmatrix}$$

We argue that finding either the matrix elements or their inverse relatives is identical. Thus we focus on the inverse elements. Now the concentrations of the anthocyanins are given by the 2 x 2 vector as follows:

$$\begin{bmatrix} z_1 \\ z_2 \end{bmatrix} = \begin{bmatrix} c_{11} ... 0 ... 0 ... 0 ... 0 ... 0 \\ 0 ... 0 ... 0 ... c_{24} ... 0 ... 0 \end{bmatrix} \begin{bmatrix} x_1 \\ x_2 \\ x_3 \\ x_4 \\ x_5 \\ x_6 \end{bmatrix} = Cx$$

The color model remains the same.

6.2.2.2 The Estimator Model

The system model is as follows. Let us begin with a model for the vector a that we seek:

$$\frac{da(t)}{dt} = 0 : where$$

$$a(t) = \begin{bmatrix} a_1 \\ ... \\ a_5 \end{bmatrix}$$

In this case we have assumed a is a 5 x 1 vector but it can be any vector. The measurement system equation is given by:

$$z(t) = g(a,t) + w(t)$$

Where z is an m x 1 vector. In this case however we have for the measurement the following:

$$z(t) = \begin{bmatrix} m_1 \\ m_2 \\ m_3 \\ x_1 \\ \dots \\ x_6 \end{bmatrix} = g(a,t) + w(t)$$

We now expand in a Taylor series the above g function:

$$g(a,t) = g(a_0,t) + C(a_0,t)\left[a(t) - a_0(t)\right] +$$
$$\frac{1}{2}\sum_{i=1}^{N} \gamma_i \left[a - a_0\right]^T F_i \left[a - a_0\right] + \dots$$

Where we have:

$$C = \begin{bmatrix} \frac{\partial g_1}{\partial a_1} & \dots & \frac{\partial g_1}{\partial a_n} \\ .. & .. & .. \\ \frac{\partial g_m}{\partial a_1} & \dots & \frac{\partial g_m}{\partial a_n} \end{bmatrix}$$

Thus we have for the measurement:

$$z(t) = C(t)a(t) + \left[g(a_0) - C(a_0)a_0(t)\right]$$

We now use standard Kalman theory to determine the mean square estimate;

$$\frac{d\hat{a}(t)}{dt} = P(t)C^T(t)K^{-1}(z - C(t)\hat{a}(t))$$
where
$$\frac{dP(t)}{dt} = -P(t)C^T(t)K^{-1}C(t)P(t) +$$
$$\sum_{i=1}^{N} PF_iP\gamma_i{}^T K^{-1}(z - g(a_0)$$
where
$$K\gamma(t-s) = E\left[w(t)w^T(s)\right]$$

In discrete time we have the equation:

$$\hat{a}(k+1) = \hat{a}(k) + PCK^{-1}\left[z(k) - \hat{z}(k)\right]$$

This is identical to the equation we derived from the Newton method.

6.2.3 The Matched Filter Approach

This is a different approach and it is an application of signal detection taken from classic communication theory. It assumes we have N signals and each signal shape is known but the amplitude of the individual signals is not known. Then we ask how we can estimate the amplitude of each signal if what we have is a received signal which is the sum of the N plus noise. We begin this approach as follows:

Let us assume there are two waveforms bounded on an interval [0, T]

Let

$$s_1(t) = s_1^{orthog}(t) + s_1^{remain}(t)$$

$$s_2(t) = s_2^{orthog}(t) + s_2^{remain}(t)$$

such that

$$\int_0^T s_1^{orthog}(t) s_2^{orthog}(t) dt = 0$$

Now there are three questions which we may pose:

1. Does such a decomposition exist, if so under what terms?

2. What is a constructive way to perform the decomposition?

3. Is there an optimum decomposition such that the "distance between the two orthogonal signals is maximized"?

Namely:

$\exists$ *a set* $\{s_1, s_2\}$

such that

$$\int_0^T s^2{}_1(t) dt = E_1$$

$$\int_0^T s^2{}_2(t) dt = E_2$$

and

$\exists \max$

$$\int_0^T s^{2,orth}{}_1(t) dt = \widetilde{E}_1$$

$$\int_0^T s^{2,orth}{}_2(t) dt = \widetilde{E}_2$$

Let us approach the solution using the theory of orthogonal functions[12]. Now we can specifically use a Fourier series approach. We do the following:

Let

$$s_1(t) = \sum_{n=1}^{\infty} s_1^n \cos(\frac{2\pi}{T} nt) + r_1(t)$$

where

$$r_1(t) = s_1(t) - FS\cos$$

and

$$FS\cos = \sum_{n=1}^{\infty} s_1^n \cos(\frac{2\pi}{T} nt)$$

Likewise

Let

$$s_2(t) = \sum_{n=1}^{\infty} s_2^n \sin(\frac{2\pi}{T} nt) + r_2(t)$$

where

$$r_2(t) = s_2(t) - FS\sin$$

and

$$FS\sin = \sum_{n=1}^{\infty} s_2^n \sin(\frac{2\pi}{T} nt)$$

Clearly FScos and FSsin are orthogonal. The residual functions r are the sin and cos elements respectively of the expansions. We could have just as easily transposed the sin and cos allocations between the two s functions. As to answering the third question we are effectively asking if the r residual functions can be minimized. The answer is not with a Fourier Transform. Then the question would be; is there another set of orthogonal functions which would minimize the residuals, namely:

$$\int_0^T r_1^2(t)dt = R_1$$

and

$$\int_0^T r_2^2(t)dt = R_2$$

are to be minimized. For a Fourier Transform as the orthogonal base we are left with residuals, R, at whatever they may be. However using the Fourier Transform approach we can extract the two signals as follows:

$$P(\lambda) = [C_1]\tilde{s}_1(\lambda) + [C_2]\tilde{s}_2(\lambda) + r_{TOT}(\lambda)$$

[12] See Sansone, Orthogonal Functions.

Note we can interchange t and λ since they represent the same variable. We now have a "signal" with amplitudes to be determined and a bias which is known. Using standard "signal detection theory" we can readily solve this problem as well. This becomes the "matched filter problem"[13].

7 CONCLUSIONS

What we have sought to accomplish in this paper is to describe color and it generation in plants and to present a set of methods and means to determine the constituents which give rise to those colors. In effect we have created a world view of color, apart from the classic colorimetry approach, and used this and the physical measurements related thereto to affect a method and means to determine concentrations of colorants in flowers.

The simple application of Beer's law and the use of the known spectra of anthocyanins and other colorants allow us to use data from FTS to determine the concentrations of each colorant on literally a cell by cell basis. Beer's law is a simplistic but fairly accurate and consistent method. It would be interesting to explore the details of the transmission of light to a deeper level but the complexity of the cell structure prohibits that at this time.

Having a methodology of the type developed herein we can now more readily examine the genetic pathways and expression systems in the genus Hemerocallis. This paper details multiple ways to ascertain concentrations on a cell by cell basis.

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[13] See VanTrees, Detection, Estimation and Modulation Theory. He presents details on this solution.

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