Exercise 1. Determination of the Anthocyanin Concentration in Table Wines and Fruit Juices Using Visible Light Spectrophotometry

A. Introduction

Anthocyanins are natural plant pigments that give various fruits, vegetables and flowers red, blue and purple color. Blueberries, blackberries, raspberries and grapes, and wines and fruit juices made of these fruits are relatively rich in anthocyanins. Unlike chlorophylls and carotenoids, anthocyanins are water soluble; they are considered one of the largest and most important group of water-soluble pigments in the plant kingdom.

YakimaBlueberries.com

Anthocyanins are relatively strong natural antioxidants. They have recently caught the attention of scientists and the public because of their possible use in fighting the effects of aging and reducing the risk of cancer and cardiovascular disease through their antioxidant power. In recent years, research into the cellular effects of anthocyanins has intensified, and many research groups are working to determine the concentration of anthocyanins in fruits, vegetables, fruit juices, and wines.

The specific objective of the experiment to be performed in this exercise is to determine the concentration of anthocyanin pigment in various wines and fruit juices. To accomplish this objective, students will use a variety of pipets to prepare various standards and samples. Once these samples are prepared, students will employ visible-light spectrophotometry to measure the amount of anthocyanin pigment in samples. In the process, students will gain a working understanding of pipeting and spectrophotometry, two tools commonly used in cell biology, biochemistry and molecular biology laboratories.

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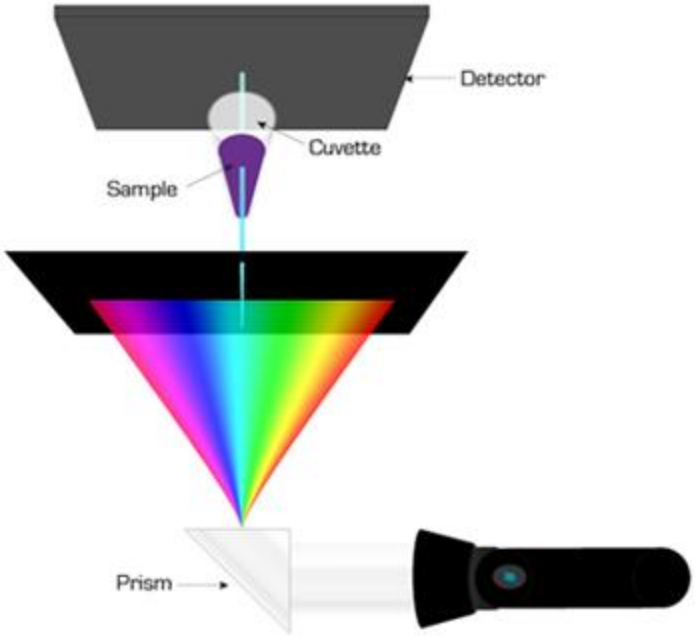
B. Spectrophotometry

Introduction

Spectrophotometry is the science of measuring the light-absorbing and light-transmitting characteristics of a substance.

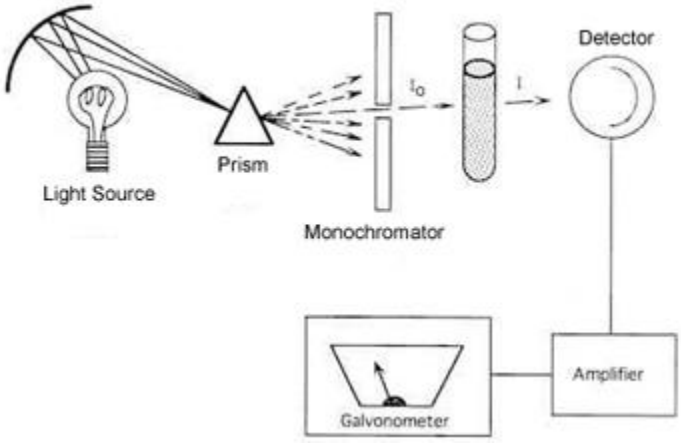
Spectrophotometry is the science of measuring the light-absorbing and light-transmitting characteristics of a substance. Many substances absorb light and transmit light of specific wavelengths within the ultraviolet (200 - 400 nm), visible (400 - 700 nm) and near-infrared (700 - 1000 nm) regions of the electromagnetic spectrum. These light-absorbing / light-transmitting characteristics of a substance are useful in determining the presence and concentration of that substance in a sample.



ADInstruments.com

The Spectrophotometer

The instrument used to measure the amount of light of a specific wavelength absorbed or transmitted by a substance is called a **spectrophotometer**. In a spectrophotometer, a sample of the substance is placed across the path of a light beam of a chosen specific wavelength. The spectrophotometer determines the intensity of the light entering the sample and the intensity of the light leaving the sample, then calculates the amount of light transmitted and absorbed by the substance.



http://faculty.uca.edu/march/bio1/scimethod/spectro.htm

A diagram of the light path through a visible-light spectrophotometer is shown to the right. A beam of light emerges from its source and passes through a prism, which dissects the light into a continuous spectrum of wavelengths. The user can select which single specific wavelength of

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light passes to the sample with the monochromator. Light of a single wavelength is called **monochromatic light**. The monochromatic light that passes to the sample is the known as **incident light**, its intensity is represented the value I_o. As the incident light passes through the sample, a certain amount of the light will be absorbed by the sample. The monochromatic light that is not absorbed emerges from the sample; it is called **transmitted light**, and its intensity is represented by the value I (or I_1). The intensity of the transmitted might is detected by a photodetector.

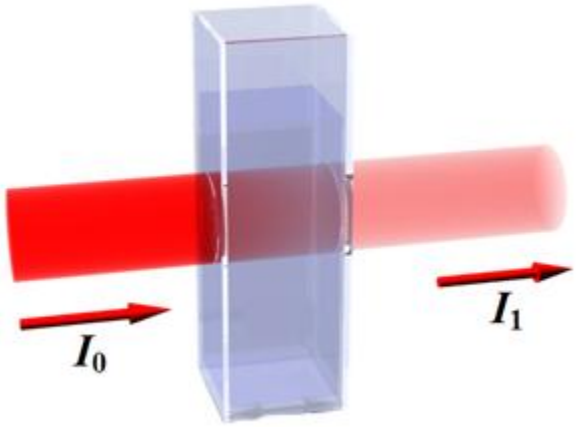
Once the transmitted light is detected, the instrument calculates the fraction of the incident light transmitted by the sample, a value known as **transmittance (T), $T = I_1 / I_0$**. From the transmittance value, the instrument will calculate the amount of the monochromatic light absorbed by the sample, a value known as **absorbance (A)**, using the formula **$A = - \log_{10} T$**. Both the transmittance and absorbance are displayed on the display screen of the instrument.

Transmittance & Absorbance

The amount of monochromatic light absorbed by a sample is determined by comparing the intensities of the incident light (I_0) and transmitted light (I_1). The ratio of the intensity of the transmitted light (I_1) to the intensity if the incident light (I_0) is called ***transmittance (T)*** .

$$T = I_1 / I_0$$

Because the intensity of the transmitted light (I_1) is never greater than the intensity of the incident light (I_0), transmittance (T) is always less than 1.



http://en.wikipedia.org/wiki/File:Beer_lambert.png

In practice, one usually multiplies T by 100 to obtain the ***percent transmittance (%T)***, which ranges from 0 to 100%.

$$\%T = T * 100$$

If the T of a sample is 0.40, the %T of the sample is 40%. This means that 40% of the photons in the incident light emerge from the sample as transmitted light and reach the photodetector. If 40% of the photons are transmitted, 60% of the photons were absorbed by the sample.

From the transmittance or % transmittance, one can calculate the quantity known as ***absorbance (A)***. Absorbance is the amount of light absorbed by a sample. It is calculated from T or %T using the following equations:

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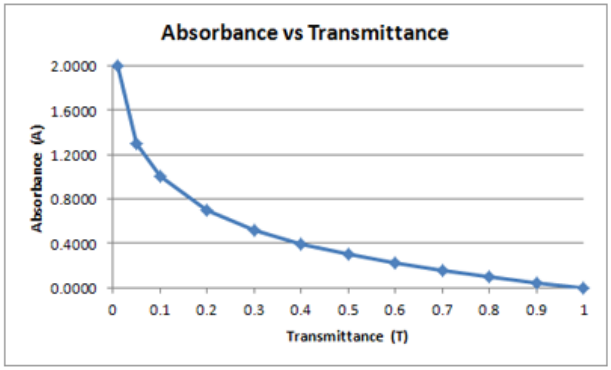
$$A = -\log_{10} T \text{ or } A = \log_{10} (1/T)$$

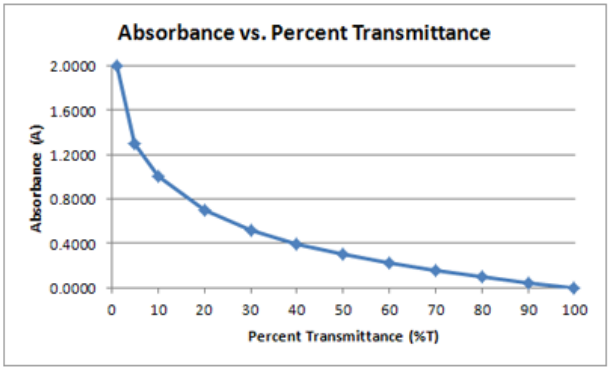
$$A = 2 - \log_{10} \%T$$

These equations reveal that transmittance and absorbance are inversely related. That is, the more a particular wavelength of light is absorbed by a substance, the less it is transmitted. Moreover, the inverse relationship between A and T is not linear, it is ***logarithmic***. Therefore, if 50% of the photons of monochromatic light are transmitted by a sample, and 50% of the photons are absorbed, T = 0.5, but A is not 0.5, A is 0.3, due to the inverse logarithmic relationship beween T and A. If 10% of the photons of monochromatic light are transmitted by a sample, and 90% of the photons are absorbed, T = 0.1, but A is not 0.9, A = 1.0. When A is 2.0, 99% of the photons of monochromatic light are absorbed, and when A is 3.0, 99.9% of the photons of monochromatic light are absorbed.

The inverse logarithmic relationship between absorbance and transmittance and between absorbance and %T are clearly shown in the graphs below. In these graphs, as transmittance (top graph) and %T (bottom graph) increase from 0 to 1.0 and 0% to 99%, respectively, absorbance decreases logarithmically from 2.0 to 0.



a) Inverse logarithmic relationship between transmittance and absorbance.



b) Inverse logarithmic relationship between percent transmittance and absorbance.

Stephen Gallik, Ph. D.

Sample Calculations

Three Sample Calculations of Absorbance from T and %T

Calculation #1:

if $T = I_1 / I_0 = 0.999$

then $\%T = T * 100 = 99.9$

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and $A = 2 - \log_{10} \%T = 2 - \log_{10} 99.9 = 2 - 1.9995 = 0.0005$

Calculation #2:

if $T = I_1 / I_0 = 0.50$

then $\%T = T * 100 = 50$

and $A = 2 - \log_{10} \%T = 2 - \log_{10} 50 = 2 - 1.69897 = 0.301$

Calculation #3:

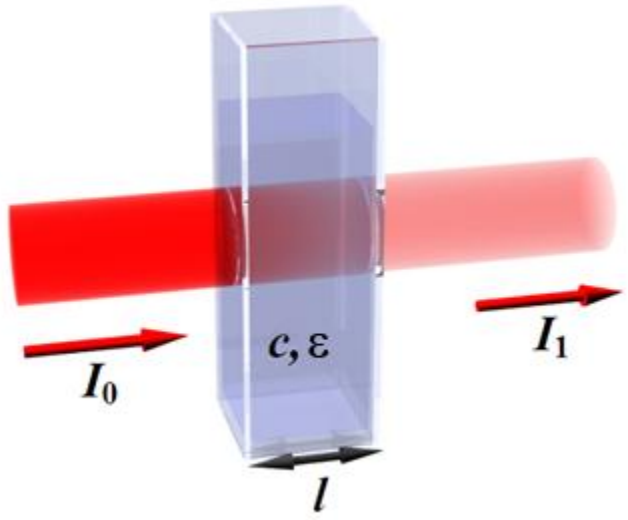
if $T = I_1 / I_0 = 0.20$

then $\%T = T * 100 = 20$

and $A = 2 - \log_{10} \%T = 2 - \log_{10} 20 = 2 - 1.301 = 0.699$

Beer's Law

The amount of light absorbed by a sample is dependent on the concentration of the pigment in the sample (c), path length (l), and the extinction coefficient of the pigment (E).



http://en.wikipedia.org/wiki/File:Beer_lambert.png

Determining the amount of monochromtic light absorbed by a substance is most-commonly used to determine the concentration of that substance in a sample. The concentration (c) of a substance in a sample is one of three factors that affect the amount of light absorbed by a sample. The other two are path length (l), that is the distance the light travels through the sample, and the extinction coefficient of the absorbing substance (E). The extinction coefficient is simply a measure of how strongly a substance absorbs light of a given wavelength. The relationship between transmittance or absorbance and these three factors is expressed by ***Beer's Law***, one of the fundamental laws of spectrophotometry. **Beer's Law states that the intensity of transmitted light decreases exponentially as each of these three factors increases.** That is,

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$$T = I_1/I_0 = 10^{-Elc}$$

Putting this in terms of absorbance, the absorbance of a given wavelength of absorbed light increases linearly as each of these three factors increases. That is,

$$A = \log_{10} (1/T) = Elc$$

Since spectrophotometry is most-commonly used to determine the presence and concentration of a particular substance in a sample, we are most interested in understanding the relationship between absorbance or transmittance and concentration. The two graphs to the right show the relationship between %T and C (right graph) and the relationship between A and C (left graph). The graphs show that %T decreases exponentially as concentration rises while A increases linearly as concentration rises. Understanding these relationships, we can easily use %T and/or A to determine the concentration of light-absorbing substance in a sample. Since the relationship between A and C is a relatively simple linear one, scientists usually use absorbance measurements when determining the concentration of a particular substance rather than %T.

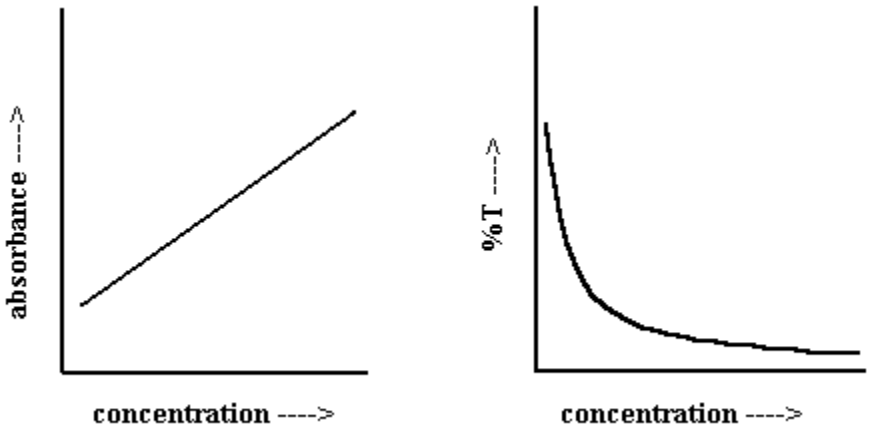

Image Source: Unknown

Graphs show the relationship between %T and C (right graph) and the relationship between A and C (left graph).

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C. The Light-Absorbing Characteristics of Biological Pigments

Biological Pigments

Biological pigments are chemical compounds produced by living organisms that selectively absorb certain wavelengths of light and selectively transmit certain wavelengths of light, giving them color. Biological pigments are found in both plants and animals. Their distribution in animals is more limited than in plants. Hemoglobin, myoglobin and melanin are three good examples of animal pigments.

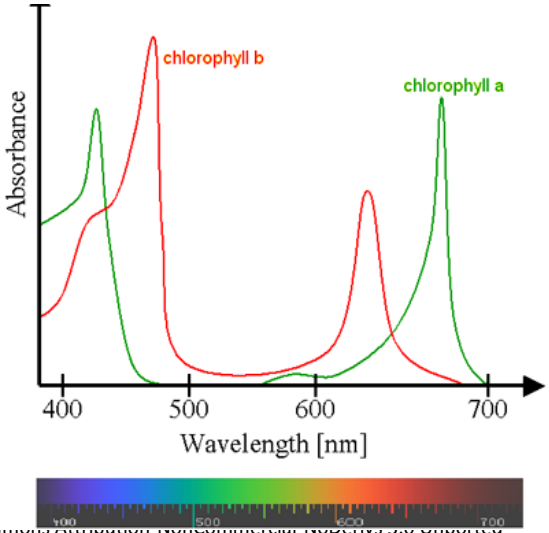
http://www.ehow.com/facts_6750947_new-guinea-impatiens-information.html

Plant pigments, on the other hand, are more widely distributed. They fall into five classes: **chlorophylls (porphyrins)**, **carotenoids**, **phycobilins**, **anthocyanins** and **betalains**. The chorophylls, carotenoids and phycobilins are used primarily for photosynthesis in leaves. While sometimes found in the leaves of a limited number of plants, anthocyanins and belalains are found primarily in flowers and fruits, where they are used to attract pollinators and for seed dispersal.

Light Absorption and Absorption Spectra

http://en.wikipedia.org/wiki/File:Chlorophyll_ab_spectra2.PNG

By their very nature, pigments do not absorb all wavelengths of light equally. Usually, a particular pigment will strongly absorb light of within a relatively narrow range of wavelengths. The wavelengths that are most strongly absorbed by a pigment are called **absorption maxima (Amax)** of the pigment. The wavelengths that are least absorbed are called **absorption minima (Amin)**. A graph showing the absorbance of light at various wavelengths, i.e., absorbance vs. wavelength, is called an **absorption spectrum** of that substance.



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To the right is the absorption spectrum for chlorophyll a an chlorophyll b. It shows that both types of chlorophyll strongly absorb visible-light in two regions of the visible-light spectrum, between 425 nm and 475 nm (violet - to blue range) and between 625 nm and 680 nm (red light). Thus each of the chlorophylls has two absorption maxima. Each type of chorophyll absorbs little light between 500 nm and 600 nm, thus transmitting green light to the eye.

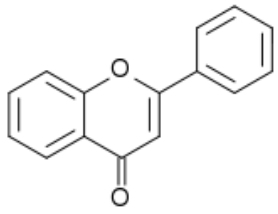
Because the absorption maximum is the wavelength most strongly absorbed by a pigment, it is the wavelength one uses in spectrophotometry when determining the presence or concentration of a particular pigment in a sample.

D. The Anthocyanins

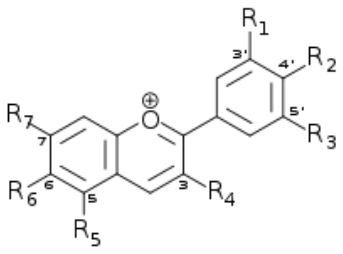
The Biochemistry of Anthocyanins

As stated in the introduction, anthocyanins are natural plant pigments that give various fruits, like blueberries, blackberries, raspberries and grapes, vegetables and flowers red, blue and purple color. In flowers and fruits, anthocyanins are not photosynthetic pigments, but are rather used to attract pollinators and for seed dispersal. Unlike chlorophylls and carotenoids, anthocyanins are water soluble; they are considered one of the largest and most important group of water-soluble pigments in the plant kingdom.

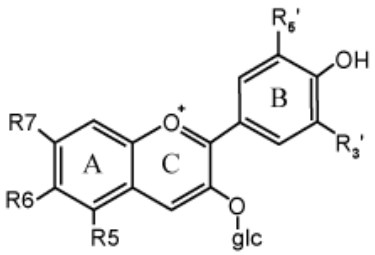
Anthocyanins belong to a class of organic molecules called ***flavonoids***, a group of polyphenol compounds found in plants. All flavonoids have a three-phenol backbone (see figure a below). Flavonoids that are hydroxylated (-OH) or methoxylated (-OCH^3) to various extents at seven specific positions (R_1 - R_7) around the flavonoid backbone belong to a class of flavonoids called ***anthocyanidins*** (see figure b below). ***Anthocyanins are glucosides of anthocyanidins.*** That is, they have a sugar molecule, often glucose, covalently attached to the flavonoid backbone, usually at the #3 position on ring C. In figure c below, the sugar molecule is represented by the ***0-glc*** group covalently linked to the #3 carbon. The sugar molecule increases the anthocyanin's solubility in water.



(a) Flavonoid Backbone



(b) Anthocyanidins have hydroxyl or methoxyl groups at positions R1 - R7



(c) Anthocyanin

http://www.micro-ox.com/chem_antho.htm

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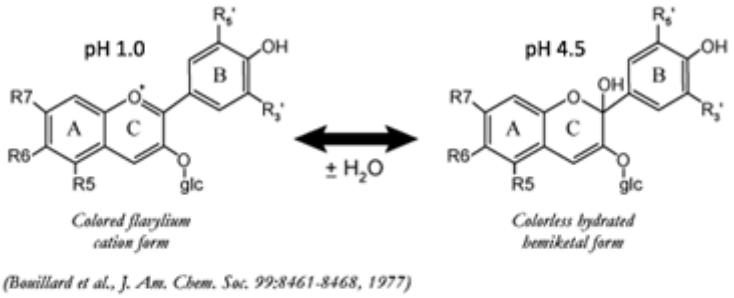
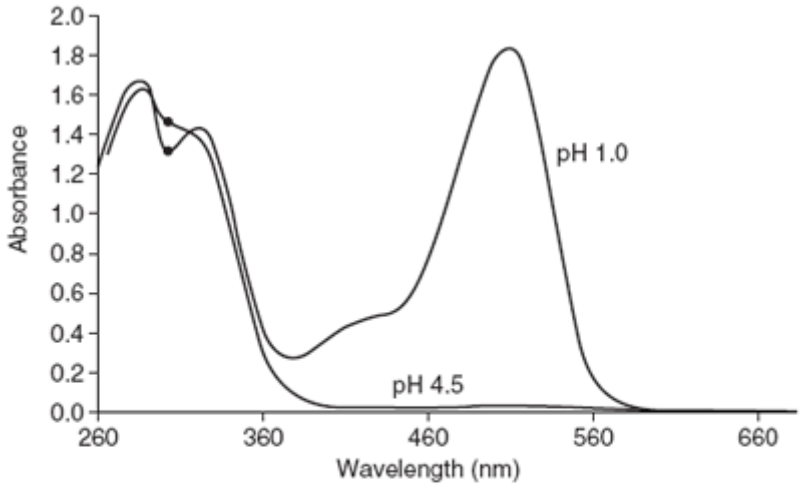
Anthocyanins are Pigments

Anthocyanins absorb light of selected wavelengths and thus transmit light of selected wavelengths. Thus, they have color. Anthocyanin pigmentation is largely due to the positive charge on the C ring of the molecule. This charge, however, is pH dependent. At a pH of 1.0, ring C carries the positive charge and the molecule is pigmented. At a pH of 4.5 and higher, however, the the C-ring is hydrated, the positive charge is neutralized, and the anthocyanins lose their pigmentation and become colorless.



(Bouillard et al., J. Am. Chem. Soc. 99:8461-8468, 1977)

a. pH dependency of ring C



b. Absorption spectrum of anthocyanins

a, Bouillard et al. J. Am. Chem. Soc.99:8461-8468, 1977. , b, M. Mónica Giusti and Ronald E. Wrolstad, Current Protocols in Food Analytical Chemistry (2001) F1.2.1-F1.2.13 Copyright © 2001 by John Wiley & Sons, Inc., Permission pending.

The pH-dependent chemical reaction of anthocyanins and the anthocyanin absorption spectrum is shown in the figure to the right. Clearly, the spectrum shows its pH dependency. At a pH of 1.0, due to the positive charge on the C ring, anthocyanins strongly absorb light between 460 and 550 nm (long blue, cyan and green light) and **have an absorption maximum of about 520nm**. Thus, at a pH of 1.0, anthocynanins are colored; they transmit violet, short blue and red light to the eye. However, at a pH of 4.5, due to the loss of the positive charge in the C ring, anthocyanins absorb no light in the visible range. At this higher pH they transmit all wavelengths of visible light equally to the eye, and thus are colorless.

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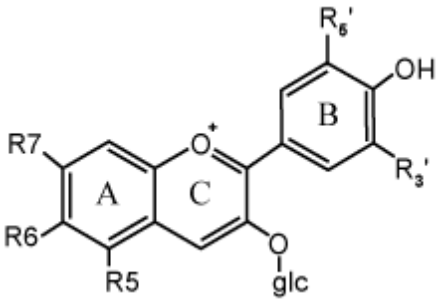
Anthocyanins are Antioxidants

Anthocyanins are not only pigments, they are also antioxidants. That is, they can inhibit the oxidation of other molecules. Anthocyanins can do this because they are **reducing agents** and tend to become oxidized themselves, thus inhibiting the oxidation of other molecules. According to Cooke et al (2005), the antioxidant capacity of anthocyanins is associated with the hydroxyl groups of the anthocyanin B-ring.



http://www.micro-ox.com/chem_antho.htm

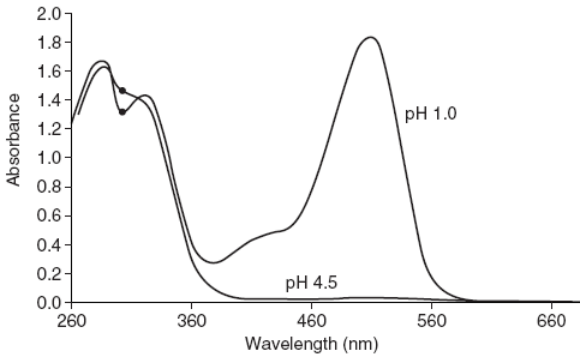
Oxidation reactions can be dangerous to cells. Oxidation reactions produce free radicals, highly reactive molecules with unpaired electrons. In the cell, free radicals can start chain reactions that can damage or even kill the cell. Antioxidants are valuable to cells because by inhibiting the oxidation of other molecules, they prevent free radical formation.

Some studies suggest, that the antioxidant capacity of anthocyanins is **likely lost once anthocyanins are consumed**. Studies have shown that anthocyanins are poorly conserved in the body, and **most of what is absorbed is chemically modified and rapidly excreted**. Nonethless, research into the cellular effects of anthocyanins continues at a brisk pace, and many research groups are working to determine the concentration of anthocyanins in fruits, vegetables, fruit juices, and wines.

E. Determining the Concentration of a Pigment in a Sample Using Spectrophotometry

Step 1. Determine the Wavelength of Light to Measure.

When analyzing a substance spectrophotometrically, the specific wavelength of light chosen to measure is critical. The wavelength of light chosen must be one that is strongly absorbed by the substance of interest, and preferably the wavelength that is most strongly absorbed, that is, the substance's **absorption maximum (A_{max})**. Therefore, before you can begin your work, you must identify the Amax of the substance you are interested in.



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The easiest way to determine the A_{max} of a substance is to simply perform a literature search to see if the absorption spectrum and A_{max} of the substance has already been determined. If the Amax of the substance has not preveiously been determined, one would need to empirically determine the absorption spectrum of the substance, then identify the absorption maxima from that spectrum.

Fortunately for us, the absorption spectrum for anthocyanins has been also been determined. It is shown on the right. As discussed previously. Anthocyanins have only one absorption maximum in the visible light range (400 nm - 700 nm), and that is at 520nm. So, 520 nm is the wavelength of light we need to measure when spectrophotometrically determining the concentration of anthocyanins in any sample.

However, there is a problem. When determining the concentration of anthocyanins in complex mixtures like wines and fruit juices, we need to consider the fact that wines and fruit juices often contain both degraded pigments and additives that also have Amaxs of 520nm. The problem: How can we selectively determine the concentration of anthocyanins and eliminate the other interfering substances from the measurement?

The solution to the problem is found in the unique pH-dependency of anthocyanin light absorption. Unlike anthocyanins, the aborption maximum for these additional substances is NOT pH-dependent. That is, they strongly absorb 520nm light at a pH of 1.0 AND a pH of 4.5. Thus, we can exploit the unique pH-dependency of the anthocyanin absorption spectrum to selectively determine anthocyanin concentration spectrophotometrically and eliminate the other interfering substances from the measurement. The method used to do this is called the ***pH differential method***. It is a simple method of measuring the absorption of 520 nm light (the Amax wavelength) by anthocyanin standards and wine and fruit juice samples at two pHs, 1.0 and 4.5. The absorbance values obtained at a pH of 1.0 are due to ALL substances in the sample that absorb 520 nm light, including anthocyanins. However, the absorbance values obtained at pH 4.5 are only due to the other substances, and not anthocyanins. To determine the absorbance by anthocyanins, one simply subtracts the absorbance values at 4.5 from absorbance values at 1.0.

Step 2. Plotting the Standard Curve.

One needs a standard curve if one is to spectrophotometrically determine the concentration of a substance in a sample. A standard curve establishes the quantitative relationship between the absorbance (or transmittance) of Amax light by the substance of interest and the concentration of that substance. If that sounds familiar, that's because this is Beer's Law.

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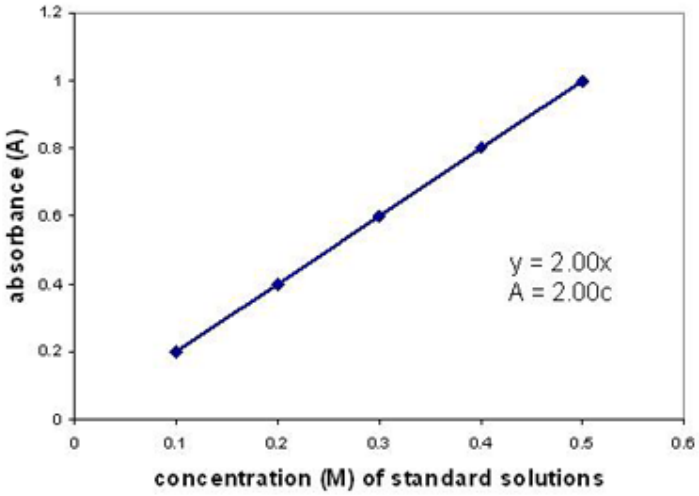
You need to create a new standard curve every time you run this type of experiment. To create your standard curve, you need to measure the absorbance (or transmittance) of the Amax light by several samples of the substance of interest at known concentrations. Samples of a specific substance of known concentration are called standards. So, if you want to measure the concentration of chorophyll a in a sample of leaf extract, for example, you first need to create a set of chorophyll a standards, measure the absorbance of Amax light by these standards, then plot your data as shown in the figure. The line created by the plotted data is your standard curve, and the equation for the straight line (of the form y = ax + b) will establish the quantitative relationship between absorbance of Amax light (y) and concentration (x).



http://www.wellesley.edu/Chemistry/Chem105manual/Lab04/lab04.html

Step 3. Collecting the Unknown Data.

http://www.lpdlabservices.co.uk/analytical_techniques/chemical_analysis/uv_spectrophotometer.php

The final step in determining the concentration of a substance in a sample spectrophotometrically is to measure the absorbance (or transmittance) of Amax light in your unknown samples. We call these samples *unknowns* because while we know the identity of the samples, we do NOT know the concentration of the substance of interest in these samples.

Once you collect the absorbance data from the unknowns, you can use the standard curve, or better stated the equation for the straight line of the standard curve, to determine the concentration of the substance in your unknown. With the standard curve straight line of the form

$$y = ax + b$$

with **y** being absorbance, **a** being the slope of the line and **b** being the y-intercept of the line, all of which are known values, one simply solves for concentration (**x**).

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F. This Week's Experiment

Introduction, The Question Being Asked and The Hypothesis

Introduction: Anthocyanins are relatively strong natural antioxidants, and they have recently caught the attention of scientists and the public because of their possible use in fighting the effects of aging and reducing the risk of cancer and cardiovascular disease through their antioxidant power. In recent years, research into the cellular effects of anthocyanins has intensified, and many research groups are working to determine the concentration of anthocyanins in fruits, vegetables, fruit juices, and wines.

Question: The question being asked here is *"What is the anthocyanin concentration of commonly-available table wines and fruit juices?*

Hypothesis: The anthocyanin concentration of the table wines and fruit juices used in this study are typical of those reported by other scientists.

Specific Objective of the Experiment and Predictions

Specific Objective: The **specific objective** of this study is to determine the concentration of anthocyanin pigment in various table wines and fruit juices using visible light spectrophotometry. To accomplish this objective, students will employ a special application of visible-light spectrophotometry called **the pH differential method** to determine the concentration of anthocyanin pigment in two unknowns, one table wine and one fruit juice.

Predictions: The anthocyanin concentration of the table wines and fruit juices used in this study will fall within the range of values reported by other scientists.

Experimental Design

This is an **observational study**. As such, there are no treatment or control groups. This observational study is divided into two basic parts: **Part 1: Constructing the Standard Curve**, **Part 2: Collecting Raw Data from the Unknowns**.

Part 1: Constructing Your Standard Curve.

In part 1 of this experiment, you will collect raw absorbance data from 4 anthocyanin standards and construct a standard curve, establishing the quantitative relationship between absorbance

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of Amax light and anthocyanin concentration. To do this, you will use the **pH Differential Method**, measuring the absorbance of 520 nm light (the Amax for anthocyanins) in 3 replicate samples of 4 anthocyanin standards at a pH of 1.0 and at a pH of 4.5. There will be a total of 24 measurements taken.

You will record your raw data in a spreadsheet, then calculate the mean absorbance taken at pH 1.0 and the mean absorbance taken at pH 4.5 for each standard along with the standard deviation (SD) and 2 X the standard error of the mean (SEM). You will then subtract the mean absorbance taken at pH 4.5 from the mean absorbance taken at pH 1.0 to get the difference for each standard. You will then plot the differences against the known concentrations of the 4 standards.

Once the differences are plotted, the spreadsheet will calculate and display the best straight line that fits your data. This straight line, or more accuraetly the equation for the straight line, will be your standard curve.

Part 2: Collecting the Raw Data from the Unknowns.

In part 2 you will use the pH Differential Method to measure the absorbance of 520 nm light in 3 replicate samples of 2 unknowns, 1 red wine and 1 fruit juice, for a total of 12 measurements. You will record your raw data in a spreadsheet, then calculate the mean absorbance taken at pH 1.0 and the mean absorbance taken at pH 4.5 for each unknown along with the standard deviation (SD) and 2 X the standard error of the mean (SEM). You will then subtract the mean absorbance taken at pH 4.5 from the mean absorbance taken at pH 1.0 to get the difference for each unknown.

Once the pH differences are determined, you will use the standard curve equation to determine the concentration of anthocyanins in each of your unknowns.

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Equipment & Materials

Buffers (to be used for blanks):

- One small brown bottle containing pH 1.0 buffer.
- One small brown bottle containing pH 4.5 buffer.

Anthocyanin Standards:

- Four small brown bottles, each containing a kerocyanin chloride standard prepared in pH 1.0 buffer.
    - Standard #1 (pH 1.0 - 1), 0.33 mg kerocyanin chloride / 100 ml
    - Standard #2 (pH 1.0 - 2), 0.69 mg kerocyanin chloride / 100 ml
    - Standard #3 (pH 1.0 - 3), 1.33 mg kerocyanin chloride / 100 ml
    - Standard #4 (pH 1.0 - 4), 2.66 mg kerocyanin chloride / 100 ml
- Four small brown bottles, each containing an anthocyanin standard prepared in pH 4.5 buffer.
    - Standard #1 (pH 4.5 - 1), 0.33 mg kerocyanin chloride / 100 ml
    - Standard #2 (pH 4.5 - 2), 0.69 mg kerocyanin chloride / 100 ml
    - Standard #3 (pH 4.5 - 3), 1.33 mg kerocyanin chloride / 100 ml
    - Standard #4 (pH 4.5 - 4), 2.66 mg kerocyanin chloride / 100 ml

Unknowns:

- One small brown bottle containing your wine unknown diluted in pH 1.0 buffer.
- One small brown bottle containing your wine unknown diluted in pH 4.5 buffer.
- One small brown bottle containing your fruit juice unknown diluted in pH 1.0 buffer.
- One small brown bottle containing your fruit juice unknown diluted in pH 4.5 buffer.

Miscellaneous:

- A supply of graduated transfer pipets
- A pipetting device to be used with the graduated transfer pipets
- Automatic pipettors and pipet tips
- A supply of cuvettes and required cuvette racks
- A Genesys 20D visible light spectrophotometer

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Preparation of the Spectrophotometer

You are using a Thermo Scientific Genesys 20D Spectrophotonmeter.

1. Turn on the spectrophotometer and allow it to warm up.

Before the start of the experiment, turn on the spectrophotometer and allow the spectrophotometer to warm-up for at least 30 minutes before using it. You will find the power switch along the back left corner of the instrument.

Note: A spectrophotometer should always be turned on 30 minutes before its use. The lamp that serves as the source of incident light must warm up to produce stable incident light.

2. Familiarize yourself with the instrument.

If you are unfamiliar with your spectrophotometer, familiarize yourself with the rest of the spectrophotometer while the instrument is warming up. You will find a guide to your spectrophotometer in the appendix of this manual.

3. Set your spectrophotometer to the Amax wavelength

Set your spectrophotometer to 520 nm, which is the Amax wavelength for anthocyanins.

4. Set the measurement mode to absorbance.

Set the measurement mode of the instrument to absorbance. (You will be making all measurements in absorbance mode.)

Preparation of the Blanks

You need to prepare the two blanks you will use to calibrate your spectrophotometer.

1. Gather 1 cuvette and label it "B1.0". Pipette 1000 ul (1 ml) of pH 1.0 buffer into the cuvette. This is your pH 1.0 blank. You will use this blank to calibrate your spectrohotometer for all the measurements taken at pH 1.0.

2. Gather another cuvette and label it "B4.5". Pipette 1000 ul (1 ml) of pH 4.5 buffer into the cuvette. This is your pH 4.5 blank. You will use this blank to calibrate your spectrohotometer for all the measurements taken at pH 4.5.

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Experimental Protocol

Part 1: Collecting Your Standard Curve Data

A. The Spreadsheet

A Microsoft Excel© worksheet for today's experiment has been prepared for you. The worksheet contains all the tables and graphs programmed to perform the necessary calculations needed to construct and display your standard curve and calculate the anthocyanin concentration of your unknowns. Go to the on-line lab manual to download the spreadsheet to your computer. Once you've downloaded the spreadsheet, open and inspect it. Wait for your instructor to discuss it with you before you begin collecting data.

B. Protocol: Collect Raw Absorbance Data from your Anthocyanin pH 1.0 Standards.

1. Perform the following procedure to each of 3 replicates of your anthocyanin pH 1.0 standard #1.

a. Calibrate the spectrophotometer with the pH 1.0 blank (B1.0) by inserting the blank into the cuvette holder, closing the lid, then pressing the 100%T/0A button. Wait for a stable reading of 0 (or -0). Repeat if necessary. When you are satisfied the absorbance reading is stable, remove the blank from the spectrophotometer.

b. Pipette 1000 ul (1 ml) of anthocyanin pH 1.0 standard #1 into a fresh cuvette. Insert the cuvette into the cuvette holder of the spectrophotmeter, close the lid and measure the absorbance (A) of the standard. Record the data in the appropriate cell in Table 1a of the Microsoft Excel© spreadsheet below (Buffer 1.0, Standard 1, Replicate 1).

c. Repeat this procedure on two more replicates of anthocyanin pH 1.0 standard #1. Make sure you re-calibarate the spectrophotometer with the pH 1.0 blank before every new cuvette.

2. When you have measured the absorbance of 3 replicates of anthocyanin pH 1.0 standard #1, repeat steps a, b and c on 3 replicates of pH 1.0 standard #2, #3 and #4.

C. Protocol: Collect Raw Absorbance Data from your Anthocyanin pH 4.5 Standards.

1. Perform the following procedure to each of 3 replicates of your anthocyanin pH 4.5 standard #1.

a. Calibrate the spectrophotometer with the pH 4.5 blank (B4.5) by inserting the blank into the cuvette holder, closing the lid, then pressing the 100%T/0A button. Wait for a stable reading of 0 (or -0). Repeat if necessary. When you are satisfied the absorbance reading is stable, remove the blank from the spectrophotometer.

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b. Pipette 1000 ul (1 ml) of anthocyanin pH 4.5 standard #1 into a fresh cuvette. Insert the cuvette into the cuvette holder of the spectrophotmeter, close the lid and measure the absorbance (A) of the standard. Record the data in the appropriate cell in Table 1a of the Microsoft Excel© spreadsheet below (Buffer 4.5, Standard 1, Replicate 1).

c. Repeat this procedure on two more replicates of anthocyanin pH 4.5 standard #1. Make sure you re-calibarate the spectrophotometer with the pH 4.5 blank before every new cuvette.

3. When you have measured the absorbance of 3 replicates of anthocyanin pH 4,5 standard #1, repeat steps a, b and c on 3 replicates of pH 4.5 standard #2, #3 and #4.

D. Save your Microsoft Excel© Worksheet

When all of the data have been collected & recorded, save the Microsoft Excel© worksheet to your computer. Make sure you change the name of the file to reflect that it contains your data. For example, **YourName_CellBiologyOLM_Lab01_2012.xlsx**

E. Examine your Standard Curve

Once you have saved your Excel spreadsheet, examine your standard curve. As your instructor explained, your standard curve was automatically constructed and displayed as you entered your raw data. Examine your standard curve line and find the equation that defines the line in the upper right corner of the graph. Make sure you understand the equation. You should recognize that Y is the absorbance (of 520 nm light) and X is anthocyanin concentration. Do you know which value is the slope of the line? Which value is the Y-intecept? Write down the equation. You will need this equation for the next part of the experiment.

Part 2: Collecting Raw Absorbance Data from your Unknowns

A. The Spreadsheet

Before you begin, click on the Part II. Unknowns tab on your Excel worksheet, read the instructions, then enter the following information in the appropriate cells in table 2.

1. Type your name in the appropriate gray box

2. Enter the slope of your standard curve in the appropriate gray cell.

3. Enter the y-intercept of your standard curve in the appropriate gray cell.

4. Enter the name of your unknown #1 in the appropriate gray cell.

5. Enter the name of your unknown #2 in the appropriate gray cell.

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B. Protocol: Collect Raw Absorbance Data from your Unknown #1.

1. Perform the following procedure to each of 3 replicates of unknown #1.

a. Calibrate the spectrophotometer with the pH 1.0 blank (B1.0) by inserting the blank into the cuvette holder, closing the lid, then pressing the 100%T/0A button. Wait for a stable reading of 0 (or -0). Repeat if necessary. When you are satisfied the absorbance reading is stable, remove the blank from the spectrophotometer.

b. Pipette 1000 ul (1 ml) of unknown #1 pH 1.0 into a fresh cuvette. Insert the cuvette into the cuvette holder of the spectrophotmeter, close the lid and measure the absorbance (A) of the unknown. Record the data in the appropriate cell of the Microsoft Excel© spreadsheet table below (Unknown 1, Buffer 1.0, Replicate 1).

c. Repeat this procedure on two more replicates of unknown #1 pH 1.0. Make sure you re-calibarate the spectrophotometer with the pH 1.0 blank before every new cuvette.

2. When you have measured the absorbance of 3 replicates of unknown #1 pH 1.0, repeat steps a. and b. on 3 replicates of unknown #1 pH 4.5. Make sure you use the pH 4.5 blank when calibrating the machine.

C. Protocol: Collect Raw Absorbance Data from your Unknown #2.

1. Perform the following procedure to each of 3 replicates of unknown #2.

a. Calibrate the spectrophotometer with the pH 1.0 blank (B1.0) by inserting the blank into the cuvette holder, closing the lid, then pressing the 100%T/0A button. Wait for a stable reading of 0 (or -0). Repeat if necessary. When you are satisfied the absorbance reading is stable, remove the blank from the spectrophotometer.

b. Pipette 1000 ul (1 ml) of unknown #2 pH 1.0 into a fresh cuvette. Insert the cuvette into the cuvette holder of the spectrophotmeter, close the lid and measure the absorbance (A) of the unknown. Record the data in the appropriate cell of the Microsoft Excel© spreadsheet table below (Unknown 2, Buffer 1.0, Replicate 1).

c. Repeat this procedure on two more replicates of unknown #2 pH 1.0. Make sure you re-calibarate the spectrophotometer with the pH 1.0 blank before every new cuvette.

2. When you have measured the absorbance of 3 replicates of unknown #2 pH 1.0, repeat steps a. and b. on 3 replicates of unknown #2 pH 4.5. Make sure you use the pH 4.5 blank when calibrating the machine.

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D. Save your Microsoft Excel© Worksheet

When all of the data have been collected & recorded, save the Microsoft Excel© worksheet to your computer.

E. Examine Your Results

Now that you have collected your raw data and saved your Excel tables, examine the results. The anthocyanin concentration of each of your two unknowns in both their diluted and undiluted forms have been calculated.

F. Homework Assignment

Your instructor will likely follow today's work with a homework assignment. Make sure you understand the assignment before you leave lab today.

G. Clean Up

Once you saved the Excel table to you computer, today's experiment is complete. Before you shut down, you should make sure the two Excel files are saved to your computer. You can logout of the lab manual. Before you leave you must clean up your place so it looks the way it did when you walked into lab today.

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