CHAPTER I
INTRODUCTION

1.1 Background

Spectrophotometry is one of the quantitative measurement methods in analytical chemistry on the reflection or light transmission properties of a solution to determine the concentration of a particular substance. Spectrophotometry can be used to analyze the concentration of a particular substance in solution based on the absorbance or transmittance of the color of the solution at a certain wavelength. Absorbance is the ratio of the intensity of the absorbed light to the intensity of the incident light. Then for transmittance itself is the fraction of incident light at a certain wavelength that passes through the sample. The absorbed light is measured as absorbance and the light released is measured as transmittance so that it can be concluded that the relationship between absorbance and transmittance is inversely proportional. The spectrophotometric method requires a standard solution of known concentration. This method is a very simple method for analyzing very small amounts (concentrations) of samples.

Examples of the use of spectrophotometry in the food and pharmaceutical fields in this case include spectrophotometry useful for determining anthocyanin levels in food ingredients and can also determine the concentration of β-estradiol. Anthocyanins are secondary metabolites of flavonoids which are often found in fruits and vegetables in the form of water-soluble pigments which usually consist of blue, red, magenta, orange, purple, and violet colors. Anthocyanins can be found at wavelengths between 490-545 nm from these various colors. Anthocyanins also have benefits as natural colorants for food and can be used as an alternative to synthetic dyes that are safer for health.

1.2 Practical Purpose

1. Determining the optimum wavelength of anthocyanins by spectrophotometer spectrophotometric method.
2. Determine the standard curve of the relationship of anthocyanin concentration vs. absorbance at the optimum wavelength by spectrophotometer spectrophotometric method.
3. Determine the concentration of anthocyanins in the sample by spectrophotometer spectrophotometric method.

1.3 Practical Benefits

1. Students are able to perform accurate quantitative analysis of a chemical substance using a spectrophotometer instrument.
2. Students are able to understand the step process on the instrument used until the desired results are obtained.

CHAPTER II
LITERATURE REVIEW

2.1 Definition of Spectrophotometry

Spectrophotometry is a way of quantitative analysis based on the transmittance or absorbance of a solution to light at certain wavelengths using a spectrophotometer instrument. When a light containing the entire spectrum of wavelengths passes through a medium, such as colored glass or a solution that transmits light with certain wavelengths and absorbs other light, the medium seems to be colored. The color absorbed by the spectrophotometer is usually called the adsorbed color and the color adsorbed by the spectrophotometer is a complementary type of color. Complementary colors are visible light or light seen in everyday life, for example, a substance will be orange if it absorbs the blue color of the visible light spectrum with the wavelength of the visible light spectrum.

The wavelength used is the optimum wavelength, which is the wavelength that is absorbed most by the compound / mixture being analyzed. At the optimum wavelength the sensitivity will be maximum because the change in absorbance for each unit of concentration is the greatest. Around the optimum wavelength, the shape of the absorbance curve is flat and in these conditions the Lambert-Beer law will be fulfilled. If repeated measurementsare made, the error caused by resetting the wavelength will be very small.

Table 2.1 Relationship between absorbed energy and molecular motion

Molecular Movement	Absorbed Light	Energy
Rotation	Microwave,	Low
Vibration	Infrared	Medium
Electron transition	Visible, Ultraviolet	High

The electromagnetic spectrum range such as infrared, visible light, ultraviolet or X-ray can be used to interact with substances. The device used in this practicum can also be called a colorimeter, because it can measure the absorption of light in the visible light spectrum. A schematic of the light

absorption process by a sample solution can be seen in Figure 2.1.

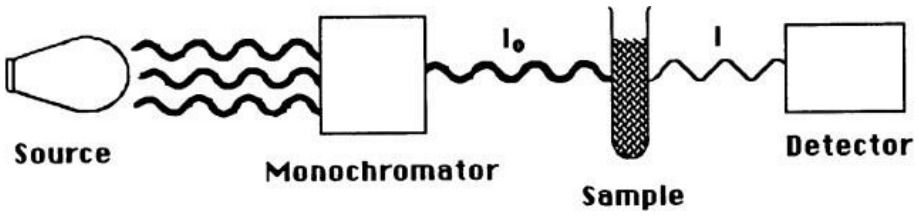

Figure 2.1 Absorption of light by the sample solution

2.2 Equipment for Spectrophotometry

A very important component of a spectrophotometer, which is schematically shown in the figure below:A very important component of a spectrophotometer, which is schematically shown in the figure below:

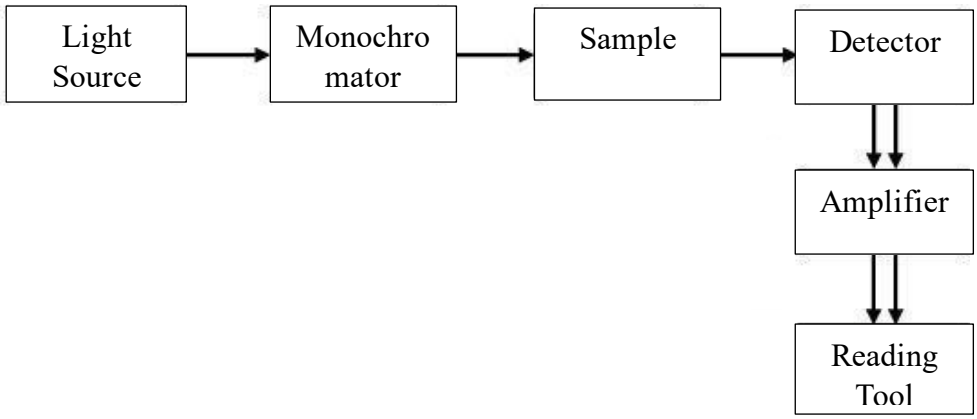

Figure 2.2 Principle of spectrophotometer

1. A continuous source of light energy covering the region of the spectrum in which the instrument is designed to operate. The light source used is usually a lamp. The lamp used is of course different based on the type of spectrophotometry used.
2. A monochromator, which is a device for isolating a narrow band of wavelengths from the wide spectrum emitted by the light source (100% monochromaticity is not achieved, of course). A monochromator consists of:
    - The entrance slit for determining the narrow beam of radiation from the source.
    - A collimation lens for collecting light.

- Prisms are for scattering light into specific wavelengths.A focusing lens to capture the dispersed light and sharpen the light to enter the cuvet through the exit slit.
- Exit slit to let the corrected wavelength of light into the sample cuvet

3. A container for the sample. The container used for the sample is a cuvet. The cuvet has two sides, the transparent side and the opaque side.
4. A detector, which is a transducer that converts light energy into an electrical signal. The detector should be sensitive and have a fast response over a large enough wavelength range. In addition, the electrical signal generated by the detector should be directly proportional to the emitted intensity.
5. An amplifier and associated circuitry that makes the electrical signal adequate for reading.
6. A reading system in which the magnitude of electrical signals is demonstrated.

Table 2.2 Light and dye uptake

λ (nm)	Forwarded Color	Absorbed Color/ Complementary Color
400 – 435	Violet	Yellow Green
435 – 480	Blue	Yellow
480 – 490	Aquamarine	Orange
490 – 500	Blue Green	Red
500 – 560	Green	Purple
560 – 580	Yellow Green	Violet
580 – 595	Yellow	Blue
595 – 610	Orange	Aquamarine
610 - 750	Red	Blue Green

The amount of light that is absorbed depends on the type of solution, the length of the cell/cuvet, the concentration of the solution. These parameters can be expressed mathematically with Beer's law:

$$A = \log (Io/It) = a.b.c \quad \text{..... (3)}$$

with:

Io = Intensity of incident light

It = Intensity of transmitted light

A = Absorbance

a = Absorbivity

b = Thickness of cuvet (cm)

c = Concentration (mg/L)

Molecules do not absorb all wavelengths equally. This depends on the color of the object. A flower will appear to have a red color, if it absorbs all colors except red or if it absorbs light from the complementary color of red, which is blue-green. Table 2.2 shows the colors and complementaries of various colors.

In this practicum, the sample compound and cuvet used are the same so that the absorbivity value and cuvet thickness are the same. Because the absorbivity and cuvet thickness are the same, the ab value from equation 3 is considered as a new constant (k), so that equation (3) becomes

$A = k.c$ .....(4)

Equation (4) can be expressed as a straight line equation. From Beer's law it can also be stated that the relationship between absorbance vs concentration will give a straight line (Underwood, 1999).

Beer's law is the basis for the quantitative aspect of spectrophotometry where concentration can be calculated based on equation (4). Absorptivity (a) is a constant that is independent of concentration, cuvet thickness and intensity of radiation hitting the sample solution. Absorptivity depends on temperature, solvent, molecular structure, and wavelength of radiation.

2.3 Types of Spectrophotometry and Mechanism of Action

2.3.1 Visible Spectrophotometry

In this spectrophotometry, what is used as energy is visible light with λ 380-750 nm. The way this spectrophotometry works is that the sample to be analyzed must have color. Therefore, colorless samples must first be colored with specific reagents that will give color to the compound.

2.3.2 UV Spectrophotometry

UV spectrophotometry is based on the interaction of the sample with UV light which has a λ of 190-380 nm. UV light arena cannot be detected by our eyes, so compounds that can absorb this light are sometimes compounds that have no color, clear, and transparent. Therefore, colorless samples do not need to be made colored by adding certain reagents. But keep in mind that cloudy samples must first be made clear by filtration or centrifugation.

2.3.3 UV-Vis Spectrophotometry

It is a combination of visual and UV spectrophotometry because it uses two different light sources. So it can be used for both colored and colorless samples. UV-Vis spectrophotometry can identify molecules in solid or liquid samples, determine the concentration of certain molecules in solution, identify absorbance or transmittance through liquids or solids of various wavelengths, identify the reflectance properties of a surface or measure the color of a material, and study chemical reactions or biological processes.

2.3.4 IR (Infrared) Spectrophotometry

This spectrophotometry is based on the absorption of λ infrared. Infrared light is divided into near, mid, and far infrared. Infrared in spectrophotometry is far infrared and mid-infrared which has a wavelength of approximately 2.5-1000 μm. Generally, IR spectrophotometry is used in qualitative analysis, usually used to identify functional groups in a compound, especially organic compounds. The results of the analysis are usually in the form of a

signal chromatogram of the relationship between IR intensity and wavelength.

2.4 Benefits of Spectrophotometry in Industry

1. Measurement of protein content in milk
2. Analyzing the levels of inorganic compounds in wastewater effluent
3. Quality level testing on vegetable oil
4. Analyze the levels of chemical compounds in the pharmaceutical industry

2.5 Lambert-Beer Law

Lambert formulated the relationship between absorbance and the thickness of the medium layer traveled by light in solution.

$log\ Po = k.\ b$ ……(1)

Where $log\ \frac{Po}{P}$ = absorbance

P = radiation power that comes out of the medium

Po = radiation power entering the medium

b = medium layer thickness

According to Beer, absorbance is affected by concentration so that

$log\ Po = k.\ c$ ……(2)

If k_1' = f (c) and k_2' = f (b) then the substitutions of equations (1) and (2) are:

$$\frac{f(c)}{c} = \frac{f(b)}{b} = k$$

$$f(c) = k.c\ dan\ f(b) = k.b$$

Substitute into the initial equation

$$log\frac{Po}{P} = f(c).b$$

$$log\frac{Po}{P} = f(b).c$$

$$log\frac{Po}{P} = k.c.b$$

$$log\frac{Po}{P} = k.b.c$$

If the concentration of solution in

- mol/liter then k should be written as ε, where ε = molar abortivity

$$log\frac{Po}{P} = \varepsilon.\,b.\,c$$

- grams/liter then k should be written as a where a = absorptivity

$$log\frac{Po}{P} = a.\,b.\,c$$

$$A = a.\,b.\,c$$

If absorbance (A) = $log\frac{Po}{P}$

$$\%T = \frac{Po}{P}.100\%$$

$$A = log\frac{Po}{P} = log\frac{1}{T} = -\log T = 2 - \log \%T$$

2.6 The Least Square Method

The Least Square method was chosen for the spectrophotometer approach according to Beer's Law which is the basis of light absorption.

A = a.b.c, where:

a = absorbtivity

b = cuvet thickness

c = concentration of the absorbing substance

When A is flowed for c against a sample that is b cm thick, it will produce a region where Beer's law applies to a straight line with slope ab.

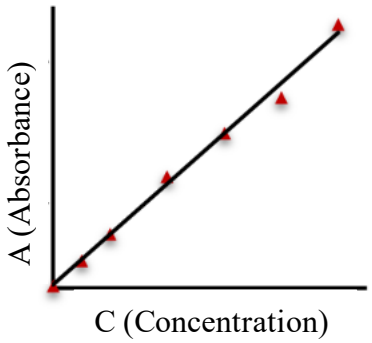

Figure 2.3 Standard curve of absorbance vs concentration relationship

The linear relationship between absorbance and concentration shows that absorbance depends on concentration. The greater the concentration of a substance, the greater the absorbance, and vice versa. Beer's Law, A = a.b.c, with variable A (Absorbance) as the dependent variable and a,b are constants in the form of absorptivity and cuvet thickness (always fixed value using the

same type of cuvet) and c is the concentration. From the equation it can be seen that A is equal to y and a,b is equal to m and c is equal to the slope (x) so that the equation y = mx is obtained.

CHAPTER III
RESEARCH METHODOLOGY

2.1 Materials and Tools

3.1.1 Materials

1. Adequate aquadest
2. KCl 0,025 M as much as 20 ml
3. Natrium Asetat 0,4 M sebanyak 20 ml
4. Liquid sample as much as 100 ml

3.1.2 Tools

1. Optima Sp-300 Spectrophotometer
2. Cuvet
3. Beaker glass 250 ml
4. Test tubes and test tube racksPipet ukur 10 cc
5. pH meter
6. Beaker glass 50 cc

3.1.3 Main Tool Figure

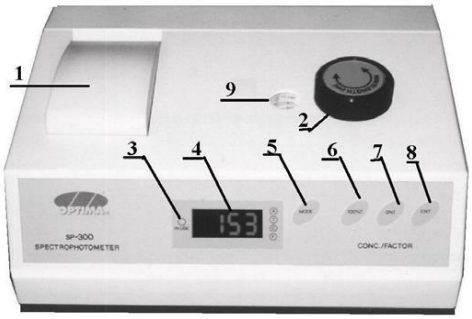

Figure 3.1 Optima Sp-300 Spectrophotometer

Explanation :

1. Sample place
2. Wavelength controllerIndikator power ON/OFF
3. Digital LCD ReadoutTombol pengganti mode
4. 100% control button T
5. 0% control button T
6. Print button
7. Wavelength reading window

Figure 3.2 Cuvet

3.3 Procedure

3.3.1 Determine the optimum wavelength for anthocyanins.

1. Calibrate the instrument first before conducting the optimum wavelength determination test on the sample.
2. Put the sample with a certain composition on the cuvet until ¾ of the cuvet and wait until the transmittance value can be read on the screen.
3. Record the transmittance value read on the screen and calculate the absorbance value using the formula A = 2-log%T.
4. Every time you change wavelengths, you must calibrate the spectrophotometer and repeat the calibration steps.

3.3.2 How to calibrate spectrophotometry equipment

1. The sample holder (1) on the spectrophotometer is emptied.
2. The cuvet (a simple box-shaped but super-quality glass material, quite expensive, so care is needed) is taken and cleaned and then filled with distilled water up to ¾ (called the blank). The outside of the cuvet is cleaned with a cotton swab carefully (do not scratch it).
3. The sample cap on the spectrophotometer (1) is opened, and the cuvet holder is taken out.
4. The cuvet is inserted in the cuvet holder with the clear side facing outward and closed again (the solution height is adjusted according to the markings).
5. Set at a specific wavelength using button (2).
6. The transmittance reading is set to 100% (A=0) for the blank solution using button (6).

7. The cuvet is removed from the sample holder and closed. The transmittance scale reading can be seen on the display (4). At this stage, the transmittance reading must be 100% until the word blank appears. If not, repeat from step 3 until a consistent transmittance reading is obtained.
8. When a consistent 100% transmittance reading is obtained, the cuvet is kept with the blank solution until the lab is completed.
9. The spectrophotometer is ready to be used for optimum wavelength measurements and calibration curves on samples.

3.3.3 Make a calibration curve between absorbance versus anthocyanin concentration.

1. Samples that are colored solutions are made at various concentrations [samples 1 (original), 2, 3, 4, 5, and 6], making samples can be done by dilution using distilled water in a measuring flask.
2. The spectrophotometric apparatus is calibrated (steps 1 to 6 in 3.3.2.).
3. The wavelength was set according to the results obtained in the optimum wavelength test using the optimum wavelength (having the highest absorbance value).
4. Another cuvet was filled with sample solutions having various concentrations, the cuvet was cleaned on the outside, inserted into the sample holder, closed again, read the transmittance scale and calculated the absorbance, A = 2-log (%T). Then repeated for samples 3, 4, 5, and 6.

3.3.1 Determine the total anthocyanin content in solution.

1. KCl 0.025 M solution was used as a pH 1 buffer solution. Then the pH was measured and adjusted to have a pH of 1 using HCl solution.
2. Sodium Acetate ($CH_3CO_2Na.3H_2O$) 0.4 M solution was made as a pH 4.5 buffer solution. Then measured the pH and adjusted the pH so that the solution has a pH of 4.5 using HCl solution.

3. a 50 ml beaker glass was filled with one sample of the composition divided by 5 ml with a measuring pipette. The pH of the solution was made the same as sample 1 by adding KCl buffer solution with a measuring pipette, count and record how much volume has been added to the buffer solution so that pH = 1. The same thing was done for sample no. 3,4,5, and 6.
4. Add (CH3CO2Na.3H2O) using a measuring pipette to the various samples as in step number 3, count and record the final volume that has been added to the buffer solution so that pH = 4.5.
5. The wavelength was set at 520 nm, then calibrated the device as steps 1-6 in 3.3.2 (done for each wavelength change). After that, sample no.1 with pH = 1 was inserted into the cuvet until ¾ part. The % transmittance was recorded and the absorbance was calculated, as well as for samples no. 3,4,5, and 6.
6. The wavelength is set at 700 nm. Then calibrate the tool steps 1-6 (done for each wavelength change). After that, put sample no.1 pH = 1 into the cuvet until ¾ part. The % transmittance was recorded and the absorbance was calculated, as well as for samples no. 3,4,5, and 6.
7. Steps 1-5 are repeated, but the pH of the solution is made equal to 4.5 by adding Na Acetate buffer solution.
8. Anthocyanin concentration was calculated according to the formula:

$$C = \frac{A \times MW \times DF \times 1000}{E \times b} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (5)$$

Dengan : C = Konsentrasi antosianin (mg/l)
A = (A520-A700)pH1 - (A520-A700)pH4,5
MW = Bobot molekul = 449,2 gram/mol
DF = Faktor Pengenceran
E = 26900 L/mol cm
1000 = konversi dari gram ke mgr
b = Tebal sel atau cuvet (cm)

9. 9. Beer's equation for anthocyanin concentration, made by equation (4):

A = k.c

with :

A = absorbance

k = slope

c = concentration

C (Concentration)

REFERENCES

Groggins, P. H. (1950). *Unit Procesess in Organic Synthesis (5th ed.)*. New York: McGraw-Hill Book Company Inc.

Kerr, R. W. (1950). *Chemistry and Industry of Starch (2nd ed.)*. New York: Academic Press Inc. Publishers.

Lee, J., Durst, R. W., & Wrolstad, R. E. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the ph differential method: Collaboration study. *Journal of AOAC International*, *88*(5), 1269- 1278.

Mukramin, B. (2012). *Spektrofotometri-Absorbansi dan Konsentrasi (Hukum Lambert-Beer)*. Diakses pada tanggal 27 April 2013.

Perkins-Veazie, P., Maness, N., & Roduner, R. (2002). Composition of orange, yellow, and red-fleshed watermelons. *Cucurbitaceae*, 436-440.

Underwood, A. I., & Day, R. A. (1983). *Analisa kimia kuantitatif (5th ed.)*. Diterjemahkan oleh R. Soendoro. Jakarta : Erlangga.

Vertzoni, M., Kartezini, T., Reppas, C., Archontaki, H., & Valsami, G. (2006). Solubilization and quantification of lycopene in aqueous media in the form of cyclodextrin binary systems. *International Journal of Pharmaceutics*, *309*(1-2), 115-122.

Vogel. (1989). *The Textbook of Quantitatif Chemical Analysis*. New York: Longman Scientific and Technical.

Woodman, A. (1941). *Food analysis (4 ed.)*. NewYork: Mc-Graw Hill Book Company Inc.