Asian Journal of Chemistry; Vol. 23, No. 11 (2011), 5001-5003

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Determination of Chlorophyll-a, Pheophytin-a and β-Carotene Contents of Isolated Photosynthetic Reaction Centre Complexes

E.D. DIKIO[1,*] and D.A. ISABIRYE[2]

[1]Department of Chemistry, Vaal University of Technology, Private Bag X021, Vanderbijlpark 1900, South Africa
[2]Department of Chemistry, North West University, P.O. Box X2046, Mafikeng 2735, South Africa

*Corresponding author: Fax: +27 16 9509794; Tel: +27 16 9509606; E-mail: ezekield@vut.ac.za

(*Received*: 5 January 2011; *Accepted*: 22 July 2011) AJC-10198

Stoichiometric quantities of chlorophyll-a, pheophytin-a and β-carotene extracted from spinach leaves have been determined by analysis of the UV-VIS spectrum of extracted pigments. Two methods of using extinction coefficients of purified pigments at different wavelengths are investigated in this paper. The results indicate that these methods are suitable for the routine determination of pigment stoichiometry.

Key Words: Chlorophyll, Pheophytin, Carotene, Photosynthesis, Pigment.

INTRODUCTION

Quantitative analysis of chlorophyll, pheophytin and their degradation products have been the focus of research in many fields of science[1-4]. Biochemists and biophysicist show great interest in the mechanism of photosynthesis that is related to chlorophyll[5-7].

Environmental scientists use photosynthetic degradation products to monitor the fate of algae in sea water and medical scientists investigate chlorophyll, porphyrin and their degradation products as potential photosensitizers in photodynamic therapy (PDT) for the treatment of cancer and hyper-proliferative diseases[8].

Chlorophyll and their degradation products are used as markers of food processing because chlorophyll is sensitive to light, pH and temperature.

Chlorophylls and carotenoids are components of antenna complexes in the photosynthetic apparatus of higher plants where they fulfill the function of light absorption and excitation energy transfer. Carotenoids on the other hand, are involved in photoprotection[9]. The spectroscopic properties of these pigments are strongly influenced by the surrounding environment: the polarizability of the medium has been shown to induce bathochromic shifts of chlorophylls and carotenoids in organic solvents[10].

Carotenoids are essential constituents of chlorophyll binding protein in all higher plants and perform several functions in photosynthetic membranes. The most important being the preventing of the formation of singlet oxygen and protecting chlorophylls by quenching their triplet states *via* thermal dissipation of energy. Also, carotenoids play a central structural role for chlorophyll binding proteins of both the antenna system and the reaction centre[11].

Chlorophyll is easily extracted from plant sources with acetone solution but the amount of chlorophyll extracted is not easily determined and the amounts extracted vary widely depending on the procedure[12-15]. This is because of the sensitivity of chlorophyll to light. Many researchers make great effort to quantify the amount of chlorophyll extracted from plant source[16-19]. Although chlorophyll could be determined by a titration method[12], through the analysis of the central magnesium ion in the porphyrin ligand, this method does not differentiate between chlorophyll and its degradation products[12].

Spectrometry is therefore the most widespread and commonly used method for chlorophyll quantification[1,20,21]. The limitations of this method include spectral interferences by chlorophyll degradation products and sample handling techniques which reduce the accuracy and precision of the analysis. Despite intensive research, determination of the amount of chlorophyll extracted from plant sources has presented a formidable obstacle to researchers.

In this paper, we present a simple spectroscopic method well suited for routine analysis to determine chlorophyll, pheophytin and β-carotene extracted in 80 % acetone. The results obtained are compared with usual methods of chlorophyll quantification.

EXPERIMENTAL

All the reagents and solvents were obtain from commercial suppliers and used without further purification (petroleum ether (60-80 ºC), diethyl ether, glacial acetic acid, acetone and methanol). Spinach leaves were frozen for 24 h before analysis.

A Finnigan LCQ-MS spectrometer with an ESI source was employed for mass spectrum determination. The determination conditions were as follows: sheath gas flow rate 0.55 L min^{-1}; auxillary gas flow rate, 0.05 L mi; ion spray voltage, 3.5 kV; capillary temperature, 200 ºC, capillary voltage 27 V; tube lens offset, 55 V. Sample was injected by a Finnigan syringe pump connected to the ESI source by a 100 μm id fused-silica capillary, flow rate was 3.5 μL/min. IR spectra were recorded on a Nicolet impact 410 Fourier transform infrared spectrometer in the 4000-400 cm^{-1} spectral region with potassium bromide cells. A pye unicam 8700 and a Cary 50 (Varian Mulgrave USA) spectrophotometers were used to determine the UV-VIS spectrum and for measurement of absorbance in quartz cuvets in 1 cm path length.

Pigment extraction: Fresh frozen spinach leaves (100 g) were crushed and extracted as described previously[15]. The chlorophyll-a obtained was analyzed by ultraviolet and visible spectroscopy. The chlorophyll-a extract is then acidified with glacial acetic acid, to obtain pheophytin-a. Each extraction product is analyzed by ultraviolet-visible spectroscopy and by electrospray ion mass spectroscopy (EPI-MS). The yield from 100 g of freeze-dried spinach leaves was 23-24 mg of chlorophyll-a (obtained in triplicate).

RESULTS AND DISCUSSION

The absorption spectra of chlorophyll-a and pheophytin-α show strong bands in the red region of the spectrum and a more intense band in the blue region. Four distinct absorption bands in chlorophyll labelled B_y, B_x, Q_x and Q_y bands in order of decreasing transition energy are observed[15,22,23]. The absorption of spectra pheophytin-a, on the other hand, show six absorption bands. These bands are observed at 429 nm (B_y) and 530 nm (B_x) and at 618 nm (Q_x) and 661 nm (Q_y)[24].

To determine the concentration *c*, of chlorophyll-a, pheophytin-a and β-carotene in 80 % acetone extract, the spectra of whole photosynthetic pigment extracted were normalized to published molar extinction coefficients ε, in 80 % acetone[1,10,12,25] (86.3 m/M cm at 663 nm for chlorophyll-a, 140 m/M cm at 454 nm for β-carotene and 51.9 m/M cm at 665 nm for pheophytin-a. Normalization of our measurement to published molar extinction coefficient of chlorophyll-a, pheophytin-a and β-carotene enable us to determine the concentration of these pigments in our extract as shown in Table-1. At each wavelength λ, the absorption A_λ of the extract is given by eqn. 1:

$$A_\lambda = (C_{chl} \times \varepsilon_{chl,\lambda}) + (C_{phe} \times \varepsilon_{phe,\lambda}) + (C_{car} \times \varepsilon_{car,\lambda}) \quad (1)$$

The concentration of total chlorophyll obtained, Table-2, from this normalization of molar extinction coefficient method is compared to the concentration of total chlorophyll obtained from the method of Vernon[12], eqns. 2 and 3, for the determination of total chlorophyll in 80 % acetone as follows:

$$\text{Total chlorophyll (mg/L)} = 20.2\ D_{645} + 8.02\ D_{663} \quad (2)$$

$$\text{Chlorophyll-a (mg/L)} = 12.7\ D_{663} - 2.69\ D_{645} \quad (3)$$

where, D_{645} and D_{663} are the absorbances of the pigments at the specified wavelengths.

After purification of the extract by column chromatography, electronic absorption spectra of the pigments in diethyl ether were taken and the total amount of chlorophyll extracted determined using first the method of normalized molar extinction coefficient in diethyl ether followed by the formula of Vernon[12] for the determination of total chlorophyll in diethyl ether.

The concentration of chlorophyll and pheophytin obtained after normalization of molar extinction coefficient is found to be 23.85 M and 36.89 M respectively (Table-3). While the concentration of chlorophyll and pheophytin obtained after

TABLE-1
CONCENTRATION OF DIFFERENT PIGMENT EXTRACTS IN 80 % ACETONE SOLUTION

Pigment	Wavelength (λ, nm)	Absorbance	Molar extinction coefficient (ε) (m/M cm)	Concentration (m/M)	Concentration (M)
Chlorophyll-a	663	0.813	86.3	0.00942	9.42
Pheophytin-a	665	0.390	51.9	0.00751	7.51
β-Carotene	454	0.700	140	0.0050	5.0

TABLE-2
TOTAL CHLOROPHYLL EXTRACTED IN 80 % ACETONE CALCULATION USING VERNON'S METHOD[12]

	Formula	Calculation	Concentration (mg/L)
Total chlorophyll (mg/L)	$20.2\ D_{645} + 8.02\ D_{663}$	$20.2 \times 0.273 + 8.02 \times 0.609$	= 10.399
Chlorophyll-a (mg/L)	$12.7\ D_{663} - 2.69\ D_{645}$	$12.7 \times 0.813 - 2.69 \times 0.273$	= 9.591

TABLE-3
CONCENTRATION OF DIFFERENT PIGMENT EXTRACTS IN DIETHYL ETHER FROM A CHLOROPHYLL EXTRACT

Pigment	Wavelength (λ, nm)	Absorbance	Molar extinction coefficient (ε) (m/M cm)	Concentration (m/M)	Concentration (M)
Chlorophyll-a	430	2.97	131.5	0.02259	22.59
	662	2.406	1009	0.02385	23.85
Pheophytin-a	409	2.12	132.0	0.01606	16.06
	667	2.35	63.7	0.03689	36.89

TABLE-4
CONCENTRATION OF DIFFERENT PIGMENT EXTRACTS IN DIETHYL ETHER FROM PHEOPHYTIN EXTRACT

Pigment	Wavelength (λ, nm)	Absorbance	Molar extinction coefficient (ε) (m/M cm)	Concentration (m/M)	Concentration (M)
Chlorophyll-a	430	0.64	131.5	0.00488	4.88
	662	0.639	100.9	0.00633	6.33
Pheophytin-a	409	1.459	132.0	0.01105	11.05
	667	0.659	63.7	0.01035	10.35

acidification of the chlorophyll solution with glacial acetic acid, 6.33 M and 10.35 M also give an accurate amount of the conversion of chlorophyll to pheophytin (Table- 4).

Conclusion

In this paper, normalized published molar extinction coefficients and published equations for the determination of chlorophyll-a, pheophytin-a and β-carotene in diethyl ether have been used to determine the concentration of chlorophyll-a, pheophytin-a and β-carotene in purified extracts of photosynthetic pigment in 80 % acetone.

The results obtained from the methods used in the determination of chlorophyll-a, pheophytin-a and β-carotene show that it is possible to estimate the concentrations of chlorophyll, pheophytin and β-carotene and other pigments in 80 % acetone extract.

This method of estimation and calculation which involves spectrophotometric analysis has the advantage of being very quick. An extract could easily be analyzed and estimated. It is also possible to directly compare the pigment content of an extraction.

ACKNOWLEDGEMENTS

This work was supported by a research grant from the Faculty of Applied and Computer Science Research and Publications Committee of Vaal University of Technology, Vanderbijlpark and the Faculty of Science Research and Publications Committee of North West University (Mafikeng Campus).

REFERENCES

1. T. Bohn and T. Walczyk, *J. Chromatogr. A*, **1024**, 123 (2004).
2. C. Eijckelhoff and J.P. Dekker, *Photosyn. Res.*, **52**, 69 (1997).
3. P. Folly and N. Engels, *J. Biol. Chem.*, **274**, 21811 (1999).
4. Y. Tan, J.-H. Jiang, H.-L. Wu, H. Cui and R.-Q. Yu, *Anal. Chim. Acta*, **412**, 195 (2000).
5. J.W. Kimball, Biology, Addison-Wesley, Reading MA, edn. 4 (1979).
6. R. Eder, in ed.: Leo M.L. Nollet, Pigments in Handbook of Food Analysis 1 (Physical Characterization and Nutrient Analysis), Marcel Dekker Inc. New York (1996).
7. J.B. Harbone, Phytochemical Methods, Chapman and Hall, London, edn. 2 (1988).
8. J. Helaja, Ph.D. Dissertation, University of Helsinki (2000).
9. R. Croce, G. Cinque, A.R. Holzwarth and R. Bassi, *Photosyn. Res.*, **64**, 221 (2000).
10. H.A. Frank and R.J. Cogdell, *Photochem. Photobiol.*, **63**, 257 (1996).
11. C.-W. Yang, C.-L. Peng, J. Duan and Y.-Z. Chen, *Bot. Bull. Acad. Sin.*, **43**, 181 (2002).
12. L.P. Vernon, *Anal. Chem.*, **39**, 1144 (1960).
13. R. Livingston and E. Fujimori, *J. Am. Chem. Soc.*, **80**, 5610 (1958).
14. A.F.H. Anderson and M. Calvin, *Nature*, **194**, 285 (1962).
15. E.D. Dikio and D.A. Isabirye, *Bull. Chem. Soc. Ethiop.*, **22**, 301 (2008).
16. Q. Wang, Y. Lai, L. Yang and B. Huang, *Anal. Sci.*, **17**, 789 (2001).
17. K.M. Smith, Synthesis and Preparation of Porphyrin Compounds in Kelvin. M. Smith (editor), Porphyrins and Metalloporphyrins, Elsevier Scientific Publishing Company, Amsterdam (1975).
18. M. Holden, in ed.: T.W. Goodwin, Chlorophylls, In Chemistry and Biochemistry of Plants Pigments, Academic Press, London (1976).
19. A.H. Jackson, Structure, Properties and Distribution of Chlorophylls in Goodwin, T.W. (editor), Chemistry and Biochemistry of Plant Pigments edn. 2, Vol. 1, Academic Press, London (1976).
20. P.H. Hynninen and K. Hyvärinen, *J. Org. Chem.*, **67**, 4055 (2002).
21. P.H. Hynninen and S. Lötjönen, *Synthesis*, 705 (1983).
22. G. Hartwich, C. Geskes, H. Scheer, J. Heinze and W. Mantele, *J. Am. Chem. Soc.*, **117**, 7784 (1995).
23. C. Geskes, G. Hartwich, H. Scheer, W. Mantele and J. Heinze, *J. Am. Chem. Soc.*, **117**, 7776 (1995).
24. G. Hartwich, L. Fiedor, I. Simonin, E. Cmiel, W. Schäfer, D. Noy, A. Scherz and H. Scheer, *J. Am. Chem. Soc.*, **120**, 3675 (1998).
25. K. Kalyanasundaram, Photochemistry of Polypyridine and Porphyrin Complexes, Academic Press, London (1992).