Bulletin of Pure and Applied Sciences.
Botany, Vol.41 B, No.2.
July-December 2022: P.79-86
Original Research Article

Print version ISSN 0970 4612
Online version ISSN 2320 3196
DOI: 10.5958/2320-3196.2022.00009.X
Available online at www.bpasjournals.com

Comparative Study of Estimation Methods for Efficient Extraction of Chlorophyll *a* and Carotenoids Using Different Solvents

[1]Amrish Saini, [2]Jyoti Singh, [3]Rama Kant*

Author's Affiliation
[1,2,3]Department of Botany, Chaudhary Charan Singh University Meerut, Meerut, Uttar Pradesh 250001, India.

***Corresponding Author:**
Rama Kant
Department of Botany, Chaudhary Charan Singh University, Meerut, Uttar Pradesh 250001, India.
E-mail:
ramakant.algae@gmail.com

Received on 30.01.2022
Revised on 11.05.2022
Accepted on 23.05.2022
Published on 15.12.2022

Keywords:
Algae, Spectroscopy, Solvents, Chlorophyll and carotenoids content.

Abstract
Photosynthetic pigments chlorophyll and carotenoids are primary pigments in green plants and algae. These pigments have the potential to turn light energy into chemical energy. In this study, different solvents acetone, ethanol and methanol were used to estimate chlorophyll and carotenoids through UV-VIS spectroscopy from different algal species viz. Anabaena sp., Nostoc sp., Merismopedia sp. and Microcystis sp., Methanol solvent contain the eminent potential for extraction of plant pigments. UV-VIS spectrum (300-800nm) gives the best detection spectrum to evaluate the efficiency of pigments with different solvents. Three methods were used to estimate the best efficiency for the extraction of chlorophyll and carotenoid pigments from algae cells. Results were recorded that Methanol > Ethanol >Acetone in the manner of extraction of plant pigments.

How to cite this article: Saini A., Singh J. and Kant R. (2022). Comparative Study of Estimation Methods for Efficient Extraction of Chlorophyll *a* and Carotenoids Using Different Solvents.*Bulletin of Pure and Applied Sciences-Botany,* 41B(2), 79-86.

INTRODUCTION

Photosynthetic pigments have the potential to turn light energy into chemical energy in all photosynthetic organisms, phenomena were first time determined by Stokes (1864). Plants contain photosynthetic pigments Chl and CARs, sterols, prenylquinones and phenols. Algae are classified on the bases of aquatic habitats, type of colors or pigments. Chl-*a* pigment study is helpful in monitoring algae growth. Therefore, the estimation of Chl content in plant physiology and ecology is an important measurement. Chl *a* is the primary photosynthetic pigment and other Chls viz. b, c1, c2, c3 and d are present or absent in plants with their taxonomic importance (Ritchie, 2006). Chl *a* and Chl *b* both have different ranges in light intensity and spectral quality of light (Atwell *et al*. 1999). LED fluorescence lidar system is useful in estimating Chl *a* concentration in plant tissue (Cadondon, 2022). A lot of research has been done to estimate the efficient extraction of Chl pigments from plant tissue. Aqueous acetone (polar aprotic solvents) is widely used for the efficient extraction of Chl from plant tissues, leathery leaves and algae. Acetone solvent is a good choice and versatile properties carrying solvent, which gives good Chl absorption peaks in Chl assays spectra

(Arnon 1949; Porra *et al*. 1989; Jeffrey *et al.* 1997; Wright *et al*. 1997, Porra, 2002), while many studies suggested that acetone is a poor solvent for extracting of Chl from many vascular plants, green algae (*Scenedesmus, Chlorella, Chlorococcum* and *Nannochloris* (Sartory and Grobbelaar 1984; Porra *et al*. 1989; Wright *et al*. 1997; Porra 2002). Methanol and Ethanol are polar protic solvents, which is a good choice for efficient extraction of Chls from plants and green algae (Lichtenthaler and Wellburn 1983; Sartory and Grobbelaar 1984; Wright *et al*. 1997; Lichtenthal 1987). With the help of methanol and ethanol solvents, Chls viz. Chl c_1 + c_2 or c_2 can't determine (Ritchie, 2006), while acetone is a good source to extract Chl *a, b* and c_1 + c_2 (Jeffrey and Humphrey 1975). Several studies were carried out on Mackinney's coefficients that pure dried solid samples of Chl *a* and Chl *b*, specific extinction coefficients of Chl *a* and Chl *b* in aqueous 80% acetone were totally imprecise (Lichtenthaler 1987; Wellburn, 1994, Porra 2002). 90% acetone solvent gives good extraction of lipophilic compounds based on the cell wall and pigment composition (Shankar, 2022).

MATERIALS AND METHODS

Culture algal species

Purified algae species cultures were used in the present investigation. For better and maximum quantitative observation, algae were cultivated in 150 ml sterilized flask containing 100 ml BG - 11 medium (Stanier *et al.* 1971), maintained light 4000 Lux to 14:10hrs (light: Dark) cycle condition and 27±1 ^{o}C temperature were used for growth enhancement of all algal species. Exponential growth cultures were used of *Merismopedia* sp., *Nostoc* sp., *Anabaena* sp. and *Microcystis* sp. for this study.

Experimental design

Several equations or methods are available to determine of total Chl pigments present in plant tissues. The exponential growth of four algae was used for the purpose of determining Chl *a* and CARs value with the help of different solvents (acetone, methanol, ethanol) by using different methods (viz: Arnon, 1949; Lichtenthaler and Wellburn, 1983; Lichtenthaler and Buschmann, 2001). Action spectrum line graphs were obtained between solvents (acetone, methanol, ethanol) and four algae (*Merismopedia* sp., *Nostoc* sp., *Anabaena* sp., *Microcystis* sp.) with the help of UV-2600, UV-VIS Spectrophotometer Shimadzu. The amount of Chl and CARs was in µg/ml.

(1) Chlorophyll estimation:

5ml volume of homogenized culture was centrifuged (5000 rpm) for 10 min and the pellet was treated with different solvents and concentrations. After that, it was properly shaken. It was kept at room temperature in dark for 1hrs and then UV-VIS spectrophotometer-118 (Systronic) was used absorbance measure. These methods have been used especially keeping in mind for extraction of Chl *a* and CARs from algae cells.

(**i**)Using wavelength (λ) total Chl was estimated by Arnon (1949):

(a) Acetone 80% (v/v)

Chl *a* (µg/ml) = 12.7 ($A_{\lambda= 663}$) - 2.69 ($A_{\lambda= 645}$)

Chl *b* (µg/ml) = 22.9 ($A_{\lambda= 645}$) - 4.68 ($A_{\lambda= 663}$)

Total Chl (µg/ml) = Chl a + Chl b.

The OD of the supernatant was measured at λ= 645 nm and λ= 663 nm using 80% acetone as blank. The whole experiment was done under dark conditions to avoid photoreaction and loss of pigments.

(**ii**)Using wavelength (λ) total Chl was estimated by Lichtenthaler and Wellburn (1983):

(a) Acetone 80% (v/v)

Chl *a* (µg/ml) = 12.21($A_{\lambda= 663}$) - 2.81($A_{\lambda= 646}$)

Chl *b* (µg/ml) = 20.13($A_{\lambda= 646}$) - 5.03($A_{\lambda= 663}$)

(b) Ethanol 96% (v/v)

Chl *a* (µg/ml) = 13.95($A_{\lambda= 665}$) - 6.88($A_{\lambda= 649}$)

Chl *b* (µg/ml) = 24.96($A_{\lambda= 649}$) - 7.32($A_{\lambda= 665}$)

(c) Methanol 100% (v/v)

Chl *a* (µg/ml) = 15.65($A_{\lambda= 666}$) - 7.34($A_{\lambda= 653}$)

Chl *b* (µg/ml) = 27.05($A_{\lambda= 653}$) - 11.21($A_{\lambda= 666}$)

(**iii**) Using wavelength (λ) total Chl was estimated by Lichtenthaler and Buschmann (2001):

(a) Acetone 80% (v/v)

Chl *a* (µg/ml) = 12.25 ($A_{\lambda= 663.2}$) - 2.79 ($A_{\lambda= 646.8}$)

Chl *b* (µg/ml) = 21.50 ($A_{\lambda= 646.8}$) - 5.10 ($A_{\lambda= 663.2}$)

(b) Ethanol 95% (v/v)
Chl *a* (µg/ml) = 13.36 ($A_{\lambda= 664.2}$) - 5.19 ($A_{\lambda= 648.6}$)
Chl *b* (µg/ml) = 27.43 ($A_{\lambda= 648.6}$) - 8.12 ($A_{\lambda= 664.2}$)

(c) Methanol 100% (v/v)
Chl *a* (µg/ml) = 16.72 ($A_{\lambda= 665.2}$) - 9.16 ($A_{\lambda= 652.4}$)
Chl *b* (µg/ml) = 34.09 ($A_{\lambda= 652.4}$) - 15.28 ($A_{\lambda= 665.2}$)

(2) Estimation of Carotenoids:
A complete homogenized 5ml sample of algal culture was taken and it was centrifugation at 5000 rpm for 10 minutes. With obtained pallet, 5ml solvent is added and shaken well. It was kept at room temperature in dark for 1hr and then absorbance was measured in the spectrophotometer.

(i) Wavelength (λ) used for estimated total CARs by Lichtenthaler and Wellburn (1983)
(a) 80 % acetone (v/v)
Total CARs $_{(x + c)}$ (µg/ml) = ($1000A_{\lambda 470}$ -3.27C_a - 104C_b)/229

(b) Ethanol 96% (v/v)
Total CARs $_{(x + c)}$ (µg/ml) = ($1000A_{\lambda 470}$ -2.05C_a - 114.8C_b)/245

(c) Methanol 100% (v/v)
Total CARs $_{(x + c)}$ (µg/ml) = ($1000A_{\lambda 470}$ -2.86C_a - 129.2C_b)/245

(ii) Wavelength (λ) used for estimated total CARs by Lichtenthaler and Buschmann (2001)
(a) Acetone 80% (v/v)
Total CARs $_{(x + c)}$ (µg/ml) = ($1000A_{\lambda 470}$ -1.82C_a– 85.02C_b)/198

(b) Ethanol 95% (v/v)
Total CARs $_{(x + c)}$ (µg/ml) = ($1000A_{\lambda 470}$ -2.13C_a - 97.64C_b)/209

(c) Methanol 100% (v/v)
Total CARs $_{(x + c)}$ (µg/ml) = ($1000A_{\lambda 470}$ -1.63C_a - 104.96C_b)/221

Note: $A_{\lambda=}$ absorbance at the respective wavelength
Total CARs $_{(x + c)}$ = xanthophylls and carotenes

RESULT
According to Lambert-Beer law, spectroscopic quantification of pigments has specific light absorption characteristics with the respective solution and dissolved compounds in the used solution (Lichtenthaler and Buschmann 2001).

(1) Estimation of Chlorophyll:
Estimation of efficient Chl from algae *Nostoc* sp., *Anabaena* sp., *Merismopedia* sp. and *Microcystis* sp. show a direct relative response against different organic solvents. Variance in efficient extraction of Chl pigments from algae was recorded highest in methanol. Chl *a* content present in four algae species were determined with the help of three different methods, against their weight, *Anabaena* species contain high Chl amount, although the rest three species contain Chl *a* amount was very close to each other against their weight. For evaluation of the extraction efficiency of Chl, three methods are used Arnon, 1949; Lichtenthaler and Wellburn, 1983 and Lichtenthaler and Buschmann, 2001 (Table 1, Fig.1).

Table 1: Chlorophyll pigment extraction efficiency of different solvents with different methods

Species ⇓	**Estimation methods** ⟹	**Arnon (1949)**	**Lichtenthaler and Wellburn (1983) Chl amount= µg/ml**			**Lichtenthaler and Buschmann (2001) Chl amount= µg/ml**		
	Solvents ⟹	**Acetone 80%**	**Acetone 80%**	**Ethanol 96%**	**Methanol 100%**	**Acetone 80%**	**Ethanol 95%**	**Methanol 100%**
Nostoc sp.		1.116	1.030	1.077	1.089	1.074	1.524	1.689
Anabaena sp.		1.968	1.880	1.937	2.078	2.095	2.743	3.213
Merismopedia sp.		0.329	0.309	0.471	0.479	0.319	0.380	0.683
Microcystis sp.		0.451	0.428	0.458	0.650	0.447	0.499	0.595

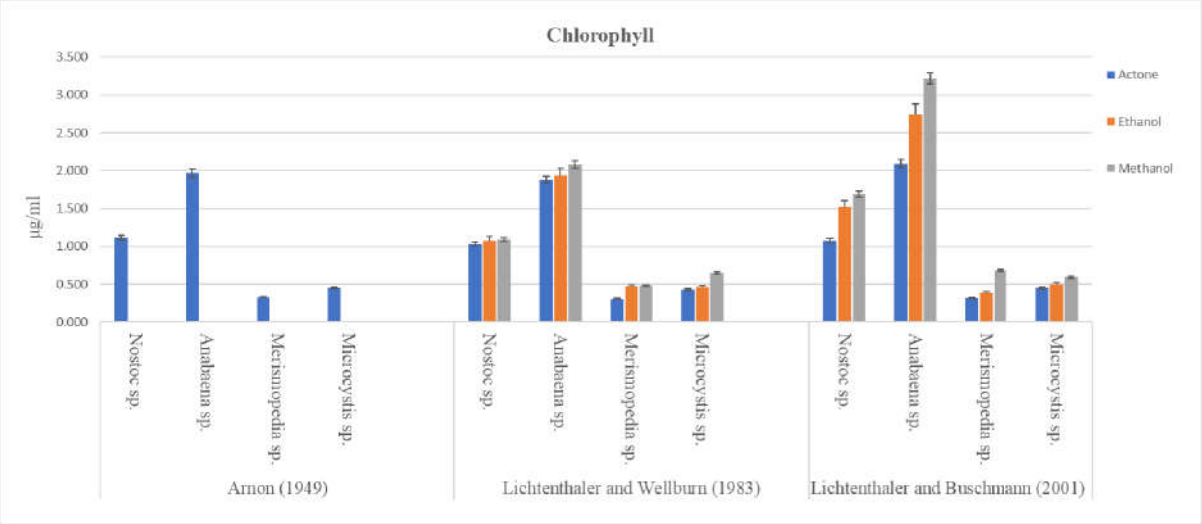


Figure 1: Chlorophyll pigment µg/ml with different solvents.

(2) Estimation of Carotenoids:
Estimation of total CARs from cyanophycean algae *Nostoc* sp., *Anabaena* sp., *Merismopedia* sp. and *Microcystis* sp. show a direct relative response against different organic solvents. Completely extraction of total CARs content from algae cells determined that methanol solvent was more successful in the effective extraction of total CARs. For the evaluation of the extraction efficiency of total CARs, two methods were used Lichtenthaler and Wellburn, 1983 and Lichtenthaler and Buschmann, 2001 (Table 2 and Fig. 2).

Table 2: CARs pigment extraction efficiency of different solvents with different protocols

Species ⇓	**Estimation methods** ⟹	**Lichtenthaler and Wellburn (1983) CARs amount = µg/ml**			**Lichtenthaler and Buschmann (2001) CARs amount = µg/ml**		
	Solvents ⟹	**Acetone 80%**	**Ethanol 96%**	**Methanol 100%**	**Acetone 80%**	**Ethanol 95%**	**Methanol 100%**
Nostoc sp.		0.114	0.073	0.111	0.150	0.108	0.384
Anabaena sp.		0.510	0.540	0.569	0.686	1.008	1.296
Merismopedia sp.		0.097	0.179	0.201	0.132	0.128	0.318
Microcystis sp.		0.197	0.260	0.316	0.214	0.328	0.366

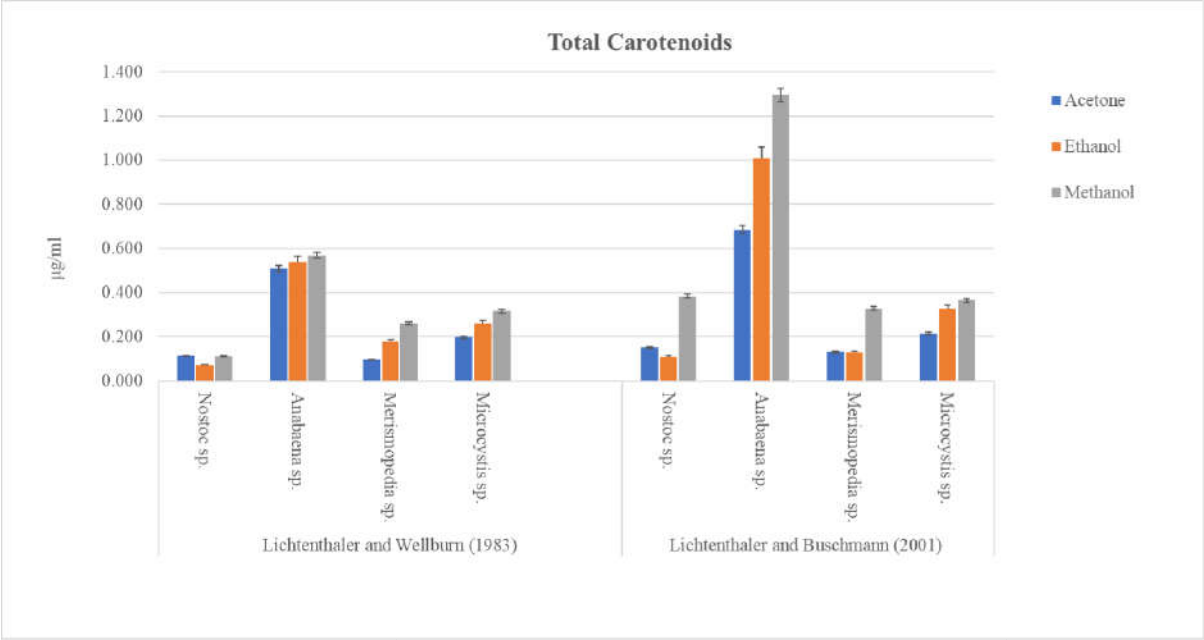
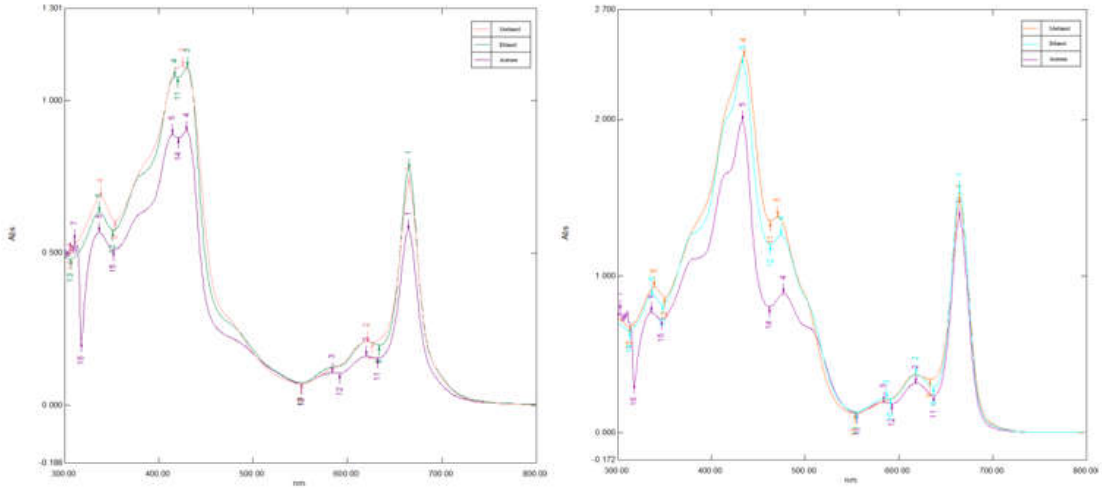


Figure 2: Total Carotenoids pigment µg/ml with different solvents.

(3) Comparison of the efficient extraction with solvents:

Different solvents have their own properties for extracting efficient pigments on the basis of structural composition, acetone shows weak acidic properties, Ethanol is poor acidic (sometimes basic properties) and methanol is slightly acidic properties shown in the comparison of water. Acetone is a dimethyl ketone and consists of 'oxo' group, which gives reaction with the cell wall composition and helps in releasing pigments. Methanol consists mainly 'carbon bond methyl' group and ethanol consist 'hydroxy group', which gives maximum releasing efficiency of pigments from plant cells and algae cells. Due to these properties, these solvents provide different action spectrum lines with the same algae on the basis of extracted pigments in solvents (Fig. 3).



Nostoc sp. *Anabaena* sp.

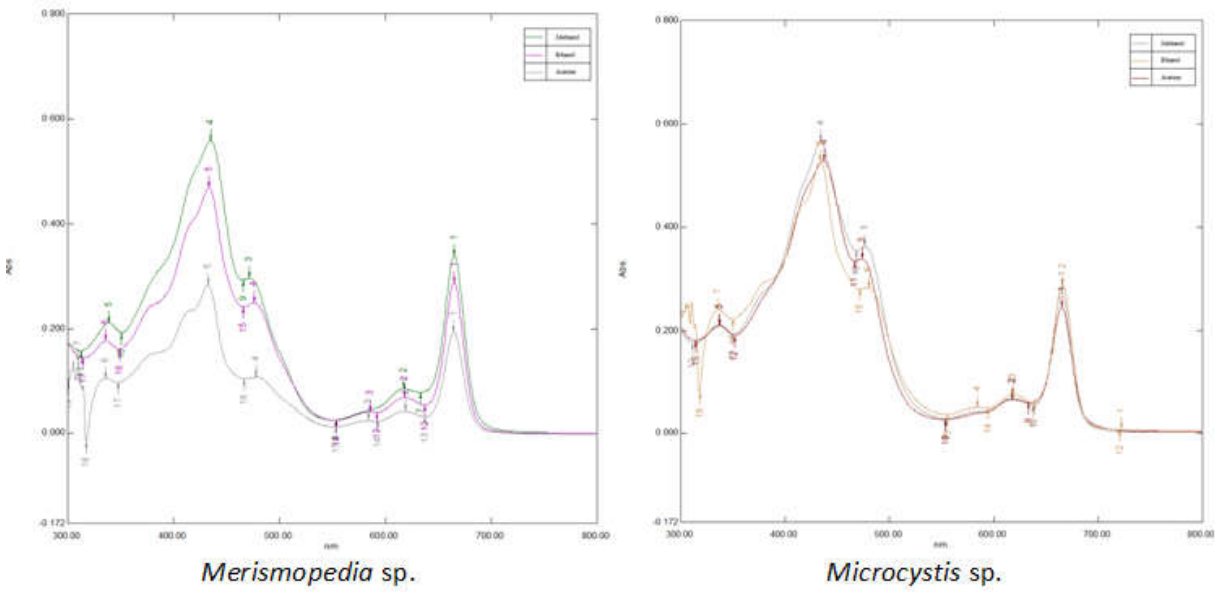


Fig. 3: Action spectrum line between solvents and algae.

DISCUSSION AND CONCLUSION

Chlorophylls and CARs are usually extracted with many organic solvents like acetone, methanol, ethanol, diethyl ether and hexane solvent. Standard-grade organic solvents significant variation was recorded with absorption coefficients of pigments with wavelength maxima. The efficiency to react with the cell wall and help in releasing pigments is mainly based on intact plant cell material and the water content of the cell. Chls and CARs are unstable with water containing organic solvents. Chl *a* and CARs are easily soluble in acetone, ethanol and methanol. Due to its polar nature and acidic properties, the magnesium holding group is easily affected by acids. So, methanol solvent contains high efficacy to extract plant pigments. The effect of methanol, ethanol and acetone was recorded with *Scenedesmus quadricauda* and *Selenastrum capricornutum* and ethanol gives the best efficiency (Sartory and Grobbelaar 1984). On the behalf of difference in cell wall composition, methanol gives better extraction of pigments (Dere *et. al.*, 1998). For estimation of Chls and CARs from *Synechococcus*, *Phaeodactylum*, *Spinacia* and *Rhodomonas* algae, 90% acetone, 100% methanol, and 100% ethanol solvents efficiency were recorded as good (Ritchie 2006). This study concluded that with selected algae *Merismopedia* sp., *Nostoc* sp., *Anabaena* sp. and *Microcystis* sp., methanol has eminent efficiency for better extraction of chlorophyll. After that ethanol gives good extraction efficacy and followed by acetone solvent.

Acknowledgment:
The authors are thankful to the Head, Department of Botany, Chaudhary Charan Singh University, Prof. Y. Vimala and Dr. Ishwar Singh for providing instrument facility UV-2600, UV-VIS Spectrophotometer Shimadzu in his laboratory. One of us (Amrish Saini) is thankful to Prof. Raj Singh, Department of Biotechnology, MMEC, Maharishi Markandeshwar, Mullana-Ambala, Haryana for the help of mensuration written and also thankful to C. C. S. University, Meerut for financial support as University Fellowship for one year.

Abbreviations:
Chl =Chlorophyll,
CARs =carotenoids,
hrs= hours,
sp. = species.

REFERENCES

1. Arnon, D.I. (1949). Copper enzymes in isolated chloroplasts: polyphenol oxidase in Beta vulgaris. *Plant Physiol* 24, 1–15.
2. Atwell, B. J., Kriedemann, P. E., and Turnbull, C. G. (Eds.). (1999). *Plants in action: adaptation in nature, performance in cultivation*. Macmillan Education AU. Search Google Scholar
3. Cadondon, J. G., Ong, P. M. B., Vallar, E. A., Shiina, T., and Galvez, M. C. D. (2022). Chlorophyll-a Pigment Measurement of Spirulina in Algal Growth Monitoring Using Portable Pulsed LED Fluorescence Lidar System. *Sensors* 22(8), 2940.
4. Dere, S., GUNES, T., and Sivaci, R. (1998). Spectrophotometric Determination of Chlorophyll-A, B and Total carotenoid Contents of Some Algae Species Using Different Solvents. *Turkish Journal of Botany* 22(1), 13-18.
5. Jeffrey, S. W., Mantoura R. F. C., Bjornl and T. (1997). Data for the identification of 47 key phytoplankton pigments, *Phytoplankton pigments in oceanography*: *guidelines to modern methods*. UNESCO Publishing, Paris, 10, 449–559
6. Jeffrey, S.W., Humphrey, G.F. (1975). New spectrophotometric equations for determining Chlorophyll *a, b, c*1 and *c*2 in higher plants, algae and natural phytoplankton. – *Biochem. Physiol, Pflanz*.167, 191-194.
7. Lichtenthaler, H. K. (1987).Chlorophyll and carotenoids: pigments of photosynthetic biomembranes, In: PACKER, L. and DOUCE, R. eds. *Methods in Enzymology*. Washington, Academic Press, 148, 350-382.
8. Lichtenthaler, H. K., and Buschmann, C. (2001). Chlorophyll and carotenoids: Measurement and characterization by UV-VIS spectroscopy. *Current protocols in food analytical chemistry*, 1(1), 4-3. https://doi.org/10.1002/0471142913.faf0403s01 F4.3.1-F4.3.8.
9. Lichtenthaler, H. K., and Wellburn, A. R. (1983). Determinations of total carotenoids and Chlorophyll *a* and *b* of leaf extracts in different solvents. *Biochem. Soc. Trans.,* 11, 591–592.
10. Porra, R.J. (2002) The chequered history of the development and use of simultaneous equations for the accurate determination of Chlorophyll a and b. Photosynth Res. 73, 149–156.
11. Porra, R. J., Thompson W. A., Kreidemann P. E. (1989). Determination of accurate extinction coefficients and simultaneous equations for assaying Chlorophyll a and b extracted with four different solvents: verification of the concentration of Chlorophyll standards by atomic absorption spectrometry. *Biochim Biophys Acta.* 975, 384–394.
12. Ritchie, R. J. (2006). Consistent sets of spectrophotometric Chlorophyll equations for acetone, methanol and ethanol solvents. *Photosynthesis research*, 89(1), 27-41.
13. Sartory, D. P., Grobbelaar J. U. (1984). Extraction of Chlorophyll *a* from freshwater phytoplankton for spectrophotometric analysis. *Hydrobiologia,* 114,177–187
14. Shankar, U., Lenka, S. K., Ackland, M. L., and Callahan, D. L. (2022). Comparison of different solvent extraction compositions for the analysis of phytoplankton pigments and lipids using a rapid and sensitive LC-MS method. https://doi.org/10.21203/rs.3.rs-1706520/v1
15. Stanier, R. Y., Kunisawa, R., Mandel, M. C. B. G., and Cohen-Bazire, G. (1971). Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriological reviews,* 35(2), 171-205.
16. Stokes, G. G. (1864). II. On the supposed identity of biliverd in with chlorophyll, with remarks on the constitution of chlorophyll. *Proceedings of the Royal Society of London,* 13, 144-145.
17. Wellburn, A.R. (1994). The Spectral Determination of Chlorophyll a and b, as well as Total Carotenoids, Using Various Solvents with Spectrophotometers of

Different Resolution, *J. Plant Phys.* 144, 307-313.

18. Wright, S. W, Jeffrey, S. W., Mantoura F. R. C. (1997) Evaluation of methods and solvents for pigment analysis. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton pigments in oceanography: guidelines to modern methods. *UNESCO Publ., Paris* pp 261–282.

****