# SEPARATION OF ALGAL PIGMENTS BY THIN LAYER CHROMATOGRAPHY (TLC) AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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## ABSTRACT

The pigment content in microalgae is one of the important features of each species. Four green algae such as G11, G12, G13 and G14 were isolated from different freshwater habitat in Madurai. The isolated microalgae G11, G12, G13 and G14 were closely related to *Chloromonas pichinchae* (G11), *Grasiella vacuolata* (G12), *Watanabea reniformis* (G13) and *Scenedesmus deserticola* (G14). Thin layer chromatography revealed the presence of xanthophylls, chlorophyll and β-carotene in the isolated green algal strains. In this study, using an optimized method of HPLC, trace pigments were also identified. Chlorophyll a, chlorophyll $c_2$, chlorophyll $c_3$ and β-carotene were identified as the major pigments present in all the four green algae. The retention time decreased in the following order (Phaeophytin a > Phaeophytin b > chlorophyll b > chlorophyll a > chlorophyllide a) and predominantly was dependent on the polarity of the mobile phase. Algal products have widening scope for industries in the form of dyes and bio-plastics which can increase profitability and reduce the biomass waste.

**KEYWORDS:** Green algae, Pigments, TLC, HPLC.

## INTRODUCTION

Algae contain a wide variety of pigments that absorb light for photosynthesis. Aquatic light environments can be extremely variable. The variety of pigments in algae has contributed to their successful acclimation to light environments of different quality and intensity. The major photosynthetic pigments are chlorophylls, carotenoids and phycobiliproteins. Chlorophyll a is common to alloxygenic photosynthetic organisms including land plants

which, like green algae, also contain chlorophyll b. Inchromophytes, chlorophyll c and the carotenoid fucox-anthin are also important light-harvesting pigments. Photosynthetic microorganisms have evolved a wide range of photoprotective and photosynthetic pigments capable of collectively harvesting most of the wavelengths of visible light available in underwater marine habitats (Jeffrey *et al*., 2011). In recent decades, HPLC has emerged as the gold standard analytical tool for qualitative and quantitative analysis of phytoplankton pigments in seawater and culture samples because of its ease of use, rapidity, sensitivity, resolution, and potential for development on research vessels (Zapata *et al*., 2000, Claustre *et al*., 2004). HPLC is the technique of choice for the standardized quantification of chlorophyll *a* and identification and quantification of minor pigments. Methodological optimization of HPLC performance demonstrated that, in addition to the major pigments easily identified by their absorbance spectrum, band ratio and polarity, several minor unidentified chlorophyll and carotenoids derivatives are usually present in extracts from environmental samples or cultivated species (Serive *et al*., 2017). In this study, using an optimized method of HPLC, trace pigments were identified from isolated green algae.

## MATERIALS AND METHODS

### Isolation of green algae

Microalgae isolated from the water bodies of Madurai, Tamilnadu, was maintained in Chu10 medium with a photoperiod of 12 hours light/ 12 hours dark, light intensity of 2000 lux at a temperature of 25°C. The cultures were identified by their morphological features. All the isolated green algae were maintained as pure culture in Chu10 medium at pH 6.5 (Safferman, 1964). The isolated microalgae were identified by 18srRNA method (Yang *et al*., 2012).

### Extraction of pigments

A known volume of green algal suspension was centrifuged at 5000 rpm for 10 minutes. The pellet was washed twice in distilled water. The pellet was suspended in 4ml of 80% acetone and vortexed. To prevent solvent evaporation, the tube was covered with an aluminium foil. It was incubated in a boiling water bath at 60°C for 1 hour in dark. The tube was cooled and centrifuged at 5000 rpm for 5 minutes. The supernatant was transferred to another tube and extracted with 2 ml of the solvent. The supernatants were pooled and made upto 10 ml. The extractions were carried out in dim light (Hansmann, 1973). The extracted pigments were identified by TLC and HPLC.

### Thin Layer Chromatography

20g of silica gel G was added to 40ml of distilled water and mixed well. This slurry was poured on the TLC plates. Plates were activated in an oven at 100°C for 2 hours. Concentrated pigment extract was spotted on the silica gel plates using a capillary tube. Plates were developed using petroleum ether: acetone (7:3). The pigments were detected by the presence of green or yellow spots. $R_f$ values were calculated using the following formula.

$$R_f \text{ value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

### High Performance Liquid Chromatography (HPLC)

Algal cells were harvested before the end of the log phase and 50ml of culture aliquots were taken for pigment analysis. These were filtered through cellulose nitrate filter paper (0.45µm) under a low vacuum. The filters were then immediately folded and packed by an aluminium foil and then frozen at -80°C until analysis. Pigment extraction was done under dim light to prevent photooxidation. The filters containing the algal sample were cut into small pieces and then homogenized in 100% acetone. The extract plus filters were kept for 24hours in the dark at -20°C. During the dark period, the extracts were stirred for two to three times. The extracts were then filtered immediately before HPLC analysis (Hou *et al*., 2011).

### HPLC system

HPLC measurements were performed on an Agilent G1311C HPLC system (Agilent G1311C, Agilent Co., USA), which consists of a binary bump, an auto sampler, a diode array detector (DAD) and a fluorescence detector (FLD). The separating column Eclipse plus C18 (dimendion: 4.6×250 nm) was used for the separation of pigments. A gradient mixture of two solvents was used in the mobile phase: Solvent A – 80:20 methanol: 0.5 M ammonimum acetate (v/v); Solvent B – 60:40 methanol: acetone (v/v). The 10 minutes linear gradient (A-B) was followed by 25 minutes isocratic hold (100%B) with 1ml flow rate. Pigments were detected at 430,460,610 and 660 nm and in parallel by FLD (230 nm excitation and 460 nm emission). All reagents were of HPLC grade and solvents were prepared in Milli-Q water. Identification and calibration of the HPLC peaks were done with standard pigments such as chlorophyll a and β-carotene obtained from Sigma Aldrich. Other pigments were identified by their relative retention times of well characterized pigments.

### RESULTS AND DISCUSSION

Four green algae such as G11, G12, G13 and G14 were isolated from different freshwater habitat in Madurai. Microscopic observation of selected indigenous algal isolates revealed its colony existence and purity (Fig.1). The four isolated green algae G11, G12, G13 and G14 were identified by morphological examination under microscope based on their cell shapes. Morphological data are frequently unreliable when used to identify green microalgae to species level. For identification of the microalgae at the species level, DNA was extracted. All the 4 green algae were subjected to preliminary PCR amplification for primer pair evaluation and selection. All green algae showed positive bands with the primer pair of 18S rRNA (Moon-van der Staay *et al*., 2000, Zhu *et al*., 2005) (Fig.2). The isolated microalgae G11, G12, G13 and G14 were closely related to *Chloromonas pichinchae* (G11), *Grasiella vacuolata* (G12), Watanabea reniformis (G13) and Scenedesmus deserticola (G14) based on 99%, 99%, 94% and 99% sequence similarities respectively. The DNA sequences were published in the NCBI database. (GenBank Accession numbers are provided in the Table 1).
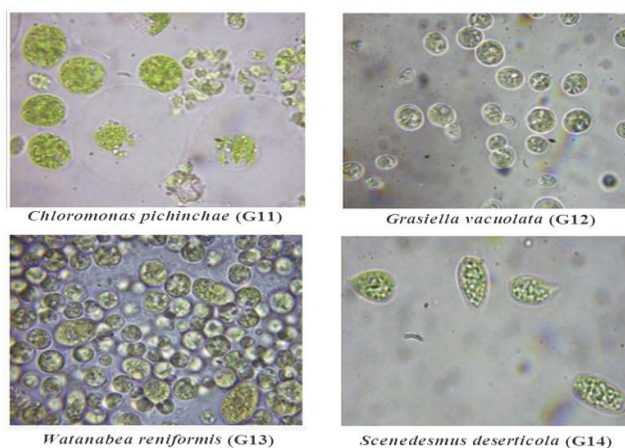


*Chloromonas pichinchae* (G11)     *Grasiella vacuolata* (G12)

*Watanabea reniformis* (G13)     *Scenedesmus deserticola* (G14)

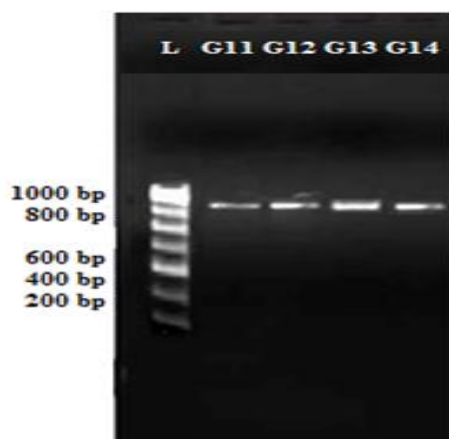**Fig. 1: Light microscopic observation of isolated of Green algae.**



**Fig. 2: Molecular Identification of Green algae.**

**Table. 1: Molecular identification of green algae, based on BLAST similarity search.**

| Green algae | GenBank Accession Numbers | Closest Relative | % Similarity |
|---|---|---|---|
| *Chloromonas pichinchae* (G11) | KF209345 | *Chloromonas pichinchae* | 99 |
| *Graesiella vacuolata* (G12) | KF209346 | *Graesiella vacuolata* | 99 |
| *Watanabea reniformis* (G13) | KF209347 | *Watanabea reniformis* | 94 |
| *Scenedesmus deserticola* (G14) | KC701525 | *Scenedesmus deserticola* | 99 |

**TLC separation of pigments in green algae**

Thin layer chromatography revealed the presence of 3-4 pigment fractions extracted from the isolated green algal strains (**Fig. 3**). Chlorophyll a and β-carotene are the dominant pigments present in the green algal strains. Xanthophylls were clearly observed in *Watanebea reniformis* (G13). The identified pigments were xanthophylls, cholorophyll b, chlorophyll a and β-carotene and their Rf values 0.42, 0.54, 0.68, and 0.94 respectively. $R_f$ values were compared with the literature data (Jeffrey, 1974). Photosynthetic pigments such as chlorophylls, xanthophylls and ß-carotene are soluble in organic solvents (Quach wt al., 2004). The pigments extracted into the particular organic solvent can then be separated and presumptively identified with a thin layer of silica (Sherma *et al*., 2003). Chlorophyll a and b spots were separated into two zones during development in the chloroform-light petroleum solvent. This was mainly due to the pigment extracted from green algal cells in late exponential growth (Jeffrey, 1961). Chlorophyll a, carotenes, chlorophyll b and lutein are the definitive pigments present in all the green algae (Jeffrey, 1981).
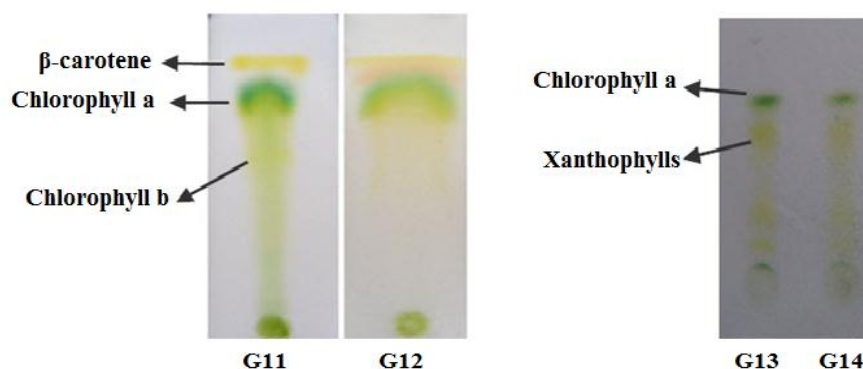


**Fig. 3: TLC separation of pigments in green algae.**

**HPLC analysis – Pigment Identification:** Pigments were identified from the absorbance spectra and retention time. Area percentage was calculated from the signals in the photodiode

array detector. Calibration of the HPLC peaks was performed using commercial standards, namely, chlorophyll a and β- carotene obtained from Sigma Aldrich. The absorbance spectra of other algal pigments and their elution order were compared with the literature data (Wright *et al*., 1991). Chlorophyll a, chlorophyll $c_2$, chlorophyll $c_3$ and β-carotene were identified as the major pigments present in all the four green algae (**Fig.4**). Chlorophyll breakdown products are particularly abundant in the isolated algae. The other carotenoids detected from chlorophyceae are zeaxanthin, lutein and violaxanthin. Name of the identified pigment fraction and their retention time are presented in **Table 2**. Separate peaks for chlorophyll $c_3$ and chlorophyll $c_2$ were obtained. At the polar end of the chromatogram, the phytol free chlorophyll derivatives such as chlorophyllide *a* and chlorophyll $c_3$ were almost completely resolved from chlorophyll $c_1+c_2$ which coeluted as a single peak in *W.reniformis* (G13). At the non-polar end of the chromatogram, the phytolated chlorophylls *a* and *b*, Phaeophytins and the less polar carotenoids were resolved. The native chlorophylls predominated with a very small proportion of chlorophyll a epimer in *S.deserticola* (G14). The resolution of polar chlorophylls and carotenoids has been enhanced, while retaining the resolution of later eluting pigments (Wright *et al*., 1997). Chlorophyll $c_3$ was clearly resolved from other chlorophyll *c* pigments such as chlorophyll $c_1$, and $c_2$. The importance of optimized extraction and automation on the reproducibility and sensitivity of the HPLC method, particularly when less microalgal biomass is present (Leeuwe *et al*., 2006). Chlorophyllide *a* is the result of the loss of the phytol chain and is a common degraded form of chlorophyll a whose occurrence has been associated with cell senescence due to the enzymatic activity of chlorophyllases (Louda *et al*., 1998, Louda *et al*., 2002). The retention time decreased in the following order (Phaeophytin a > Phaeophytin b > chlorophyll b > chlorophyll a > chlorophyllide a) and predominantly was dependent on the polarity of the mobile phase. Similar results were obtained using non polar C18 column (Gauthier, 2001). Changes in pigment stoichiometry are a key element in the acclimatization of plants to fluctuating environmental conditions. The exact evaluation of the ecophysiological meaning of pigment composition requires the separation and quantification of all carotenoids and chlorophylls (Esteban *et al*., 2010).
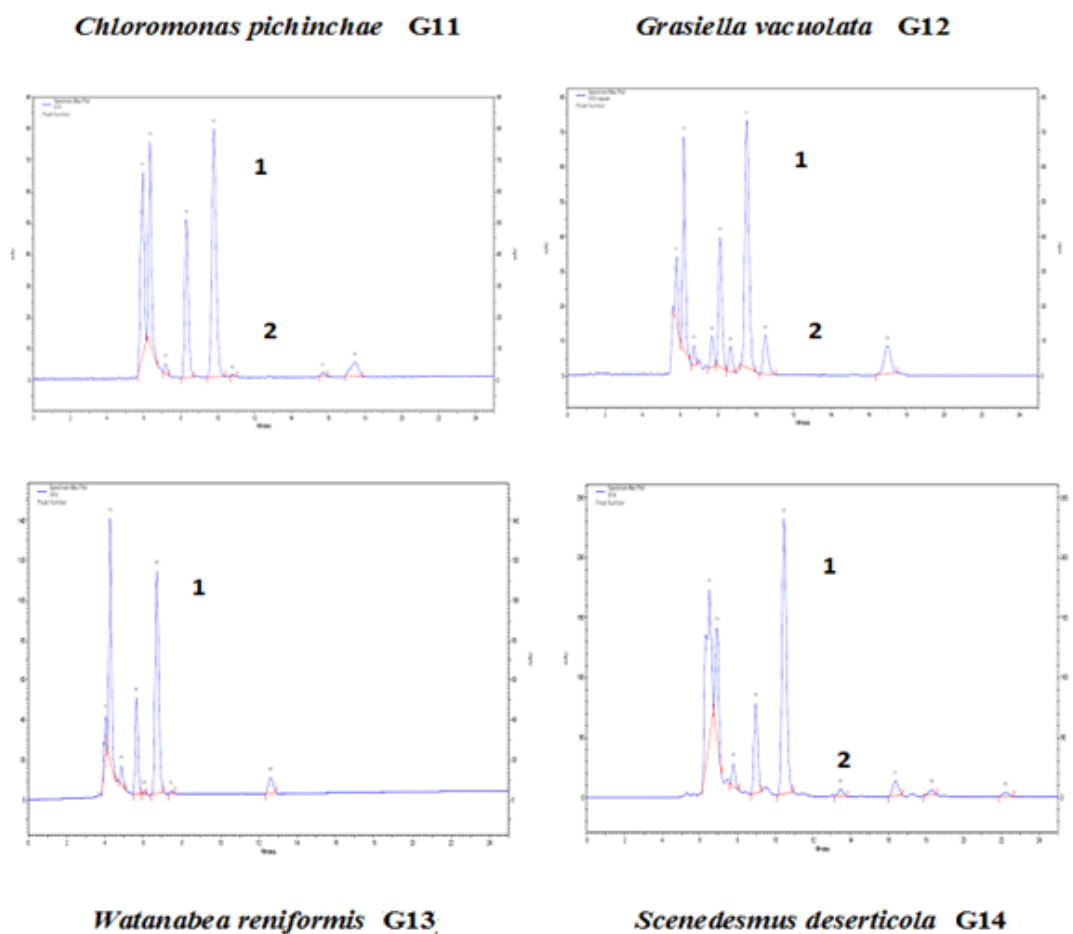
*Chloromonas pichinchae*   G11          *Grasiella vacuolata*   G12



*Watanabea reniformis*   G13          *Scenedesmus deserticola*   G14

**Fig.4 - HPLC chromatograms of pigments in green algae**

Peak number 1, 2 indicate the presence of chlorophyll a and β-carotene in all isolated green algae Chlorophyll a, chlorophyll b appeared in a shorter retention time in non polar C18 column. Chlorophyll epimers have identical absorption spectra in diode array detector (DAD). Chlorophyll a was eluted before chlorophyll a epimer, because the –CHOOCH$_3$ substituent at the C$_{13}$ position in the chlorophyll a molecule was not on the same plane of the C$_{17}$ phytol group and was therefore less hindered and more polar than chlorophyll a . Epimers of chlorophylls are present in the chlorophyll and its derivatives. They are naturally present in small amount in photosynthetic organisms (Lim, 2009). As the analysis of chlorophyll and its derivatives predominantly uses C18 column, the retention time always decreased in the same order (Phaeophytin a > chlorophyll a > Phaeophytin b > chlorophyll b > chlorophyllide a > chlorophyllide b) and predominantly depended on the polarity of the mobile phase (Milenkovic *et al*., 2012).

**Table. 2: HPLC analysis of pigments in green algae.**

| S. No | Pigments | Retention Time | G11 | G12 | G13 | G14 |
|-------|----------|----------------|-----|-----|-----|-----|
| 1 | Chlorophyllide a | 5.01 | - | + | + | - |
| 2 | Chlorophyll $c_3$ | 5.38 | + | + | + | + |
| 3 | Chlorophyll $c_2$ | 6.40 | + | + | + | + |
| 4 | Peridinin | 7.42 | + | + | + | + |
| 5 | Siphonaxanthin | 8.11 | + | + | + | - |
| 6 | Chlorophyll a | 8.40 | + | + | + | + |
| 7 | Chlorophyll b | 8.69 | + | + | + | + |
| 8 | Chlorophyll a epimer | 8.90 | - | - | - | + |
| 9 | cis-fucoxanthin | 9.68 | - | + | - | - |
| 10 | cis-19-hexanoyloxyfucoxanthin | 9.97 | + | - | - | - |
| 11 | Violaxanthin | 10.59 | - | + | - | + |
| 12 | β-carotene | 10.76 | + | + | - | + |
| 13 | Lycopene | 17.59 | + | + | - | - |

The absorbance spectra of other algal pigments and their elution order were compared with the literature data (Wright *et al*., 1991). Note: + Indicate the presence of pigments; - Indicate the absence of pigments. G11 – *C.pichinchae*, G12 – *G.vaculata*, G13 – *W.reniformis*, G14 – *S.deserticola.*

**CONCLUSION**

In this paper, an optimized method of TLC and HPLC were developed for the separation of pigments in green algae. Major pigments such as xanthophylls, chlorophyll and carotenoids which can exhibit colours ranging from orange, green and yellow in TLC. In this study, using an optimized method of HPLC, trace pigments were also identified. This green algal pigment analysis can be useful as a prioritization tool for aquaculture, drug discovery and other applications involving pigment chemodiversity. Algal products have widening scope for industries in the form of dyes and bio-plastics which can increase profitability and reduce the biomass waste.

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