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# *A New Method for the Microdetermination of Chlorophyll* c *in Sea Water*

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## ABSTRACT

A method developed for the microdetermination of chlorophyll *c* by conversion of chlorophyll *c* to pheophytin *c* is described. This method is found to be more sensitive and specific than previously reported methods for the determination of this pigment and is suitable for open-ocean samples of only a few liters in volume.

*Introduction.* Parsons and Strickland (1963) have demonstrated, in this issue, the difficulty of determining small amounts of chlorophyll *c* by trichromatic absorption readings in the 630-m$\mu$ region. The following procedure, offered as an alternative method, is of greater accuracy and specificity than other procedures for the determination of chlorophyll *c*, especially in oceanic waters of relatively low pigment content.

The method described here depends on the removal of other chlorophylls and carotenoids by partition of the pigments between an aqueous acetone extract and hexane. The chlorophyll *c* that remains in the aqueous acetone phase is determined by measuring the optical density at 450 m$\mu$ before and after a small amount of hydrochloric acid is added. The decrease in optical density due to the conversion of chlorophyll *c* to pheophytin *c* has been standardized under the procedural conditions to give a measure of the amount of chlorophyll *c* in the original extract.

*Preparation of the Pigment.* Spectrophotometrically pure chlorophyll *c* was prepared from the seaweed *Sargassum muticum* after the method described by Smith and Benitez (1955: 143–196). This material was employed to standardize the method described here. The quantities of chlorophyll *c* shown in Fig. 1 and Table 1 were determined from the specific absorption coefficient of 15.8 l/g cm at 630 m$\mu$ in 100% acetone (Jeffrey, 1962).

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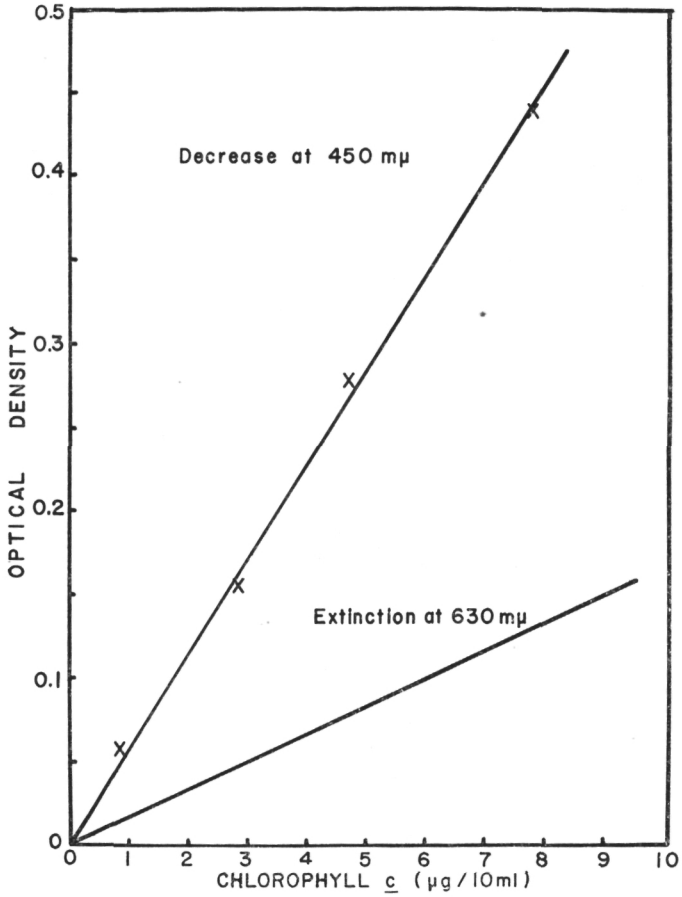


Figure 1. Standard curve for chlorophyll *c*.

*Procedure.* The following reagents were used: (1) 90% v/v acetone; (2) hexane (redistilled); (3) 0.05% sodium chloride in water; (4) acetone (redistilled from anhydrous $Na_2CO_3$-$Na_2SO_3$).

Transfer the 10 ml of 90% acetone extract containing plant pigments (obtained as described by Strickland and Parsons, 1960) into a 50-ml separatory funnel. Drain but do not rinse the tube. Add 3.5 ml of 0.05% NaCl solution and 13.5 ml of hexane. Shake the funnel to partition the pigments and precipitate the Millipore filter. (The addition of 0.05% saline instead of distilled water improves the precipitation of the Millipore-filter material and its subsequent coagulation at the interface of the hexane and aqueous acetone.)

Run off 8.5 ml of the lower aqueous-acetone phase into a 15-ml graduated centrifuge tube, and add 100% acetone to bring the volume up to exactly

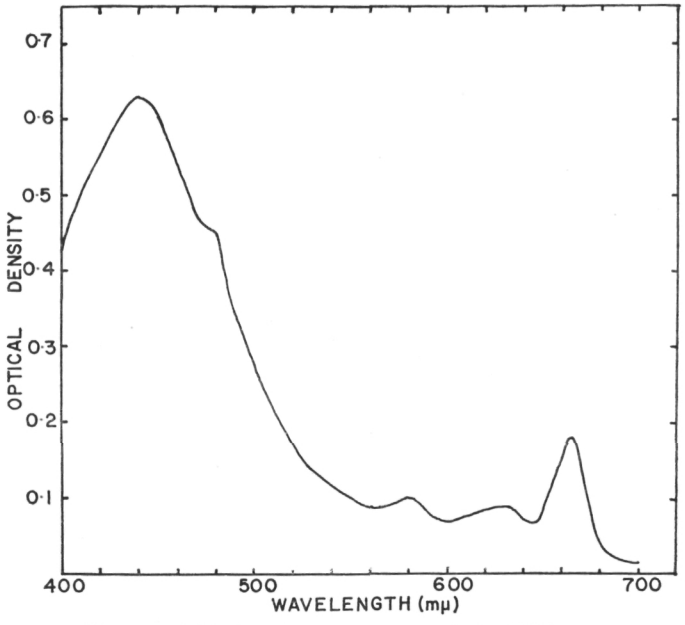


Figure 2. Original 90% acetone extract of *Amphidinium carteri*.

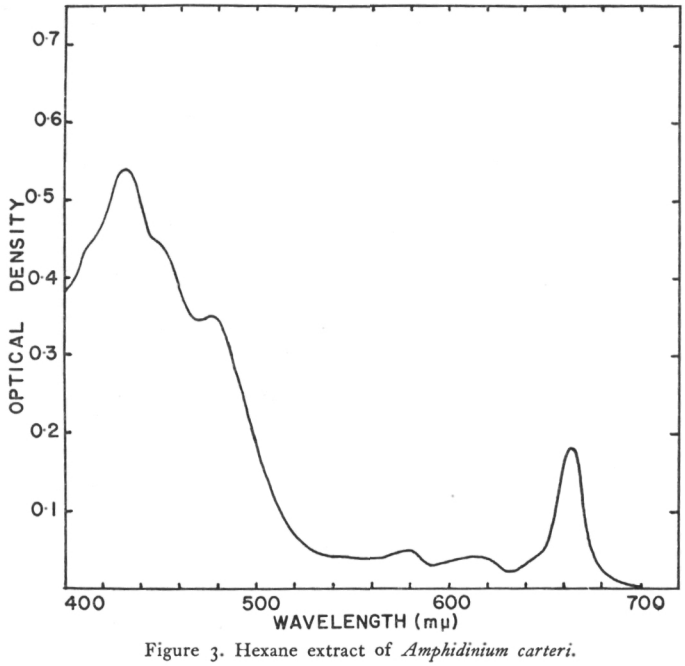


Figure 3. Hexane extract of *Amphidinium carteri*.

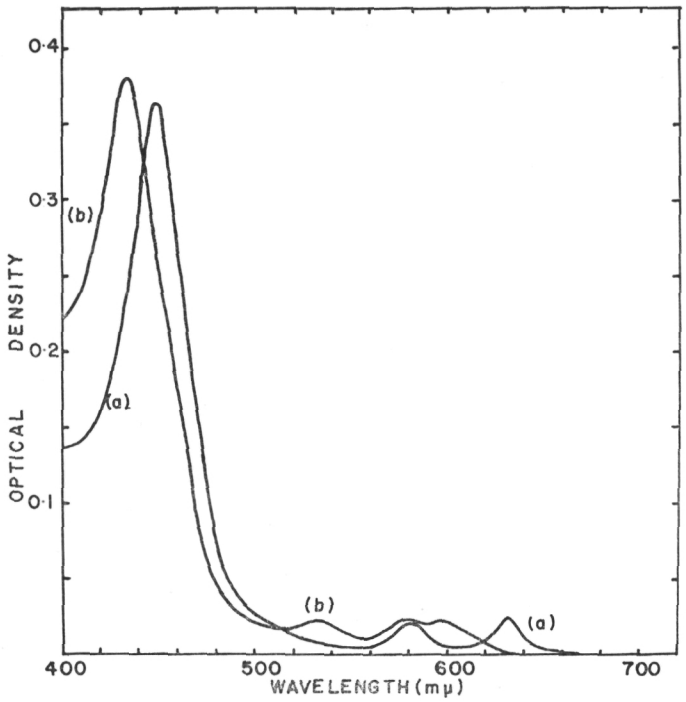


Figure 4. Aqueous acetone extract of *Amphidinium carteri* after hexane treatment. Curve (a) aqueous acetone extract. Curve (b) aqueous acetone extract after hydrochloric acid treatment.

TABLE I. DETERMINATION OF CHLOROPHYLL *c* IN THE PRESENCE OF ACETONE EXTRACTS OF *Dunaliella salina*.

| Material | Chl. *a* ($\mu$g) | Total Carot. ($\mu$ SPU) | Optical Density at 450 m$\mu$ | Optical Density at 450 m$\mu$ with HCl | Decrease at 450 m$\mu$ |
|---|---|---|---|---|---|
| 1. Extract alone .... | 5.8 | 2.8 | 0.128 | 0.129 | +0.001 |
| 2. Extract alone .... | 11.6 | 5.6 | 0.170 | 0.170 | 0.000 |
| 3. Extract (1) + 2.85 $\mu$g chl. *c* ... | 5.8 | 2.8 | 0.492 | 0.341 | −0.151 |
| 4. Extract (2) + 2.85 $\mu$g chl. *c* ... | 11.6 | 5.6 | 0.506 | 0.354 | −0.152 |
| 5. 2.85 $\mu$g chl. *c* ... | None | None | 0.374 | 0.220 | −0.154 |

10 ml. Mix and transfer the solution to a cuvette of 10-cm lightpath, which holds slightly less than 10 ml of fluid. Measure the optical density at 450 m$\mu$ and then add one *small* drop (ca. 0.02 ml) of concentrated HCl to the cuvette. Stopper the cuvette and invert it several times to mix the acid and aqueous acetone. Within one minute, reread the optical density at 450 m$\mu$. The differ-

ence in optical density between the first and second readings multiplied by 17.5 (a factor obtained from Fig. 1) gives the amount of chlorophyll *c* in the original 10 ml of 90% acetone extract. Thus:

$$\text{difference in optical density at } 450\ \text{m}\mu \times 17.5 \equiv \mu\text{g chlorophyll } c$$

This value divided by the volume of sea water originally filtered (in liters) gives, in $mg/m^3$, the amount of chlorophyll *c* in the seawater sample.

It is important, when a number of chlorophyll *c* determinations are being performed, to remove all acid from the cuvette between each determination. Cuvettes should be rinsed with acetone, then water, and finally with acetone again in order to remove all the acid.

An example of the extraction procedure is shown in Figs. 2, 3, and 4. Fig. 2 is the spectrum of a 90% acetone extract of a culture of *Amphidinium carteri*. Fig. 3 shows the same extract transferred to the hexane layer. The principal difference between the spectrum in Figs. 2 and 3 is the absence of an amount of material absorbing in the 450-m$\mu$ region. Thus in Fig. 3 the 430-m$\mu$ chlorophyll *a* peak and the 480-m$\mu$ carotenoid peak are more pronounced than in Fig. 2. In Fig. 4, curve (a), the 450-m$\mu$ absorbing material is clearly identified as chlorophyll *c* by its two additional peaks at 580 and 630 m$\mu$; it is also apparent that the partition procedure effects transfer of virtually all of the strongly hypophasic carotenoids to the hexane layer. Fig. 4, curve (b), which shows the effect of HCl on curve (a), is similar to the pheophytin *c* curve shown by Smith and Benitez. The maximum difference between points on curves (a) and (b) in Fig. 4 occurs as an increase in optical density at 430 m$\mu$. This value, compared with the decrease at 450 m$\mu$, is less reliable, however, due to possible interference from other compounds. Compounds that may interfere with the estimation of chlorophyll *c* are discussed later.

*Standardization.* Standard amounts of chlorophyll *c* in 90% acetone were pipetted into 15-ml graduated centrifuge tubes. One Millipore AA filter, treated with magnesium carbonate as described for the determination of total pigments (Strickland and Parsons, 1960), was added to each tube, and the volume was made up to 10 ml with 90% acetone. The solutions were taken through the procedure for the estimation of chlorophyll *c* described in the previous section; the results have been plotted in Fig. 1. From this figure the factor 17.5 was determined. The increased sensitivity of this method over that used by Richards with Thompson (1952) and Parsons and Strickland (1963) is also illustrated in Fig. 1, where the optical density of the chlorophyll *c* at 630 m$\mu$ in the original 90% acetone extract is shown.

*Sensitivity, Specificity, and Precision.* Ten replicate samples of a culture of *A. carteri* were analyzed for chlorophyll *c* as described above. The calculated

standard deviation from the mean (6 $\mu$g chlorophyll *c*/10 ml of 90% acetone extract) is expressed below as the range in which the mean of n determinations would lie with a probability of 95%. The correct value lies in the range:

$$\text{mean of n determination} \pm \frac{1.64}{n^{\frac{1}{2}}} \mu\text{g Chl. } c\text{/10 ml 90\% acetone extract.}$$

*Interference.* In order to determine the interference from other compounds, standard amounts of chlorophyll *c* were added to an extract of a culture of *Dunaliella salina*; this organism contains chlorophylls *a* and *b* and the HCl-labile carotenoids violaxanthin and neoxanthin, but it contains no chlorophyll *c* (Parsons, 1961). The results of these experiments are shown in Table 1. The recovery of the added chlorophyll *c* from the 90% acetone extracts of *Dunaliella salina* was within the experimental error of the determination as calculated in the previous section. Further, the extracts of *Dunaliella salina* alone gave no indication of containing chlorophyll *c*.

The only compounds not yet tested that could cause interference with this method for chlorophyll *c* are the chlorophyllides of chlorophyll *a* and *b*. These, which would tend to remain in the acetone layer after partition of the pigments, would form pheophorbides upon addition of HCl, with an accompanying shift in the wavelength of maximum absorption. Judging by our own experiments, these compounds are seldom present in oceanic samples. Two precautions should be taken, however, to detect and eliminate possible interference from chlorophyllides, especially in coastal waters. The first is the present use of the decrease in optical density at 450 m$\mu$, rather than the increase in optical density at 430 m$\mu$, which greatly reduces the possibility of optical density changes due to chlorophyllides. A further positive detection of chlorophyllides can be made by measuring the extinction at 665 m$\mu$ of the original 90% acetone solution and of the hexane extract. To measure the latter, the hexane layer must be freed of water by adding a small amount of anhydrous sodium sulfate. The value obtained at 665 m$\mu$ in hexane, corrected for the larger volume of the hexane layer and multiplied by the factor 0.85 (to allow for different absorption coefficients in the two solvents), should closely approximate the reading obtained at 665 m$\mu$ on the original 10 ml of extract. In the presence of chlorophyllides, the reading at 665 m$\mu$ in the two solvents will be appreciably different, being lower in hexane.

If organisms are encountered that show a chlorophyllide *a* peak at 665 m$\mu$ in the spectrum shown in Fig. 4 (a), or if chlorophyllide *a* is shown to be present by the above procedure, which is essentially for persons not having a self-recording spectrophotometer, then the following correction should be applied to the determination of chlorophyll *c*:

A measurement of the optical density at 665 m$\mu$ is made of the acetone extract at the same time as the first measurement at 450 m$\mu$. This value

TABLE II. DETERMINATION OF CHLOROPHYLL *c* BY DIFFERENT METHODS ($\mu$g OR $\mu$SPU/10 ml 90% ACETONE EXTRACT): RICHARDS WITH THOMPSON, 1952 (R. WITH T.), PARSONS AND STRICKLAND, 1963 (P. AND S.), AND THE PRESENT METHOD (PRES. METH.).

| | R. with T. | P. & S. | Pres. Meth. |
|---|---|---|---|
| *Amphidinium carteri* culture | 7.27 | 3.64 | 2.40 |
| | 9.85 | 5.10 | 5.35 |
| Sea water (50°N, 145°W) | | | |
| 0 m | 2.71 | 1.32 | 0.97 |
| 12 m | 2.35 | 1.18 | 0.82 |
| 50 m | 1.99 | 1.00 | 0.24 |

multiplied by 0.1 closely approximates the decrease in optical density that can be expected from chlorophyllide *a* after the acid is added. The 665 m$\mu$ optical density $\times$ 0.1 should be subtracted from the decrease in optical density at 450 m$\mu$ to obtain the true decrease resulting from the conversion of chlorophyll *c* to pheophytin *c*. If the chlorophyllide correction exceeds about half of the chlorophyll *c*, then the method should be abandoned as unsuitable, but we believe this is unlikely to occur often with natural samples.

*Comparison of Different Methods.* In Table II the determination of chlorophyll *c* using the equations reported by Richards with Thompson and by Parsons and Strickland are compared with the determination of chlorophyll *c* by the method presented here. It is seen that the values obtained by this method are appreciably lower than those calculated from equations. This is particularly true in the determination of small amounts of pigments, as in oceanic samples and in a culture containing the least amount of pigment. The ratios of chlorophyll *c* to chlorophyll *a* obtained for oceanic samples when chlorophyll *c* is determined by this new method are similar to those found in phytoplanktonic cultures by Jeffrey (1961) and Parsons (1961). Neither author found the excessively large chlorophyll *c*-to-*a* ratios that are apparent from the use of equations in Table II and which have been reported for some oceanic areas (see Currie 1958, Humphrey 1960, McAllister *et al.* 1960, *in* Parsons and Strickland, 1963: 163). Thus, with the specificity of the chlorophyll *c* method already outlined and the observations from laboratory cultures, it is very likely that the values presented in Table II for the determination of chlorophyll *c* by the equation methods are in error, and high chlorophyll *c* to chlorophyll *a* ratios (exceeding unity) are suspect.

*Summary.* A new method for chlorophyll *c* determination suggests several advantages over methods involving trichromatic readings in the 630-m$\mu$ region. The advantages are as follows:

1. The sensitivity of the determination has been increased by a factor of approximately four.

2. Under the conditions of the determination the measurement is highly specific for chlorophyll *c*.
3. The correction of optical density measurements for turbidity blanks is unnecessary.
4. The precision on individual estimations is much greater than that with methods involving trichromatic readings, especially when concentrations of chlorophyll *c* are relatively low.

The major disadvantages of the proposed method are the additional time required for a determination over that required for other pigments, and possibly, but unlikely, interference from chlorophyllides. The whole procedure can be carried out in approximately five minutes, however, which is not excessive when a precise estimate of chlorophyll *c* is required in waters of low pigment content. The method can be carried out on the same pigment extract that is used for the determination of chlorophylls *a* and *b* by trichromatic readings.

## REFERENCES

JEFFREY, S. W.
1961. Paper-chromatographic separation of chlorophylls and carotenoids from marine algae. Biochem. J., *80*: 336–342.
1962. Purification of chlorophyll *c* from *Sargassum flavicans*. Nature, London, *194*: 600.

PARSONS, T. R.
1961. On the pigment composition of eleven species of marine phytoplankters. J. Fish. Res. Bd. Canada, *18*: 1017–1025.

PARSONS, T. R., AND J. D. H. STRICKLAND
1963. Discussion of spectrophotometric determination of marine-plant pigments, with revised equations for ascertaining chlorophylls and carotenoids. J. Mar. Res., *21*: (3): 155–163.

RICHARDS, F. A., WITH T. G. THOMPSON
1952. The estimation and characterization of plankton populations by pigment analyses. II. A spectrophotometric method for the estimation of plankton pigments. J. Mar. Res., *11*: 156–172.

SMITH, J. H. C., AND A. BENITEZ
1955. Chlorophylls: Analysis in plant materials, Vol. 4, *in* Modern methods of plant analysis. Springer-Verlag, Berlin.

STRICKLAND, J. D. H., AND T. R. PARSONS
1960. A manual of sea water analysis. Bull. Fish. Res. Bd. Canada, 125; 185 pp.

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