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DETERMINATION OF CHLOROPHYLL IN MARINE WATERS: INTERCOMPARISON OF A RAPID HPLC METHOD WITH FULL HPLC, SPECTROPHOTOMETRIC AND FLUOROMETRIC METHODS

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ABSTRACT

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A statistical comparison was made between chlorophyll measurements made on a suite of marine samples by four methods. The methods were one complete and one rapid high-performance liquid chromatographic (HPLC) method, a fluorometric method and the traditional spectrophotometric method. Generally good agreement was found, provided that consideration was given to the presence of chlorophyllide *a* and allomers of chlorophyll *a* as determined by the HPLC methods. The rapid HPLC method (10 min per sample) is recommended as a convenient and accurate alternative to traditional methods for chlorophylls.

INTRODUCTION

Determination of particulate chlorophyll, as an indicator of phytoplankton biomass, is one of the most important measurements in oceanography and freshwater science. Measurement of chlorophyll concentration helps us to understand natural systems and is important in studies aimed at assessing the impact of pollution or other disturbances.

The standard analytical methods for chlorophylls are based on spectrophotometry or fluorometry (Strickland and Parsons, 1972). Despite continual refinement, these methods do not always cope well with the complex range of pigments and pigment degradation products found in natural samples (Holm-Hansen et al., 1965; Moed and Hallegraeff, 1978; Gibbs, 1979).

In recent years, high-performance liquid chromatographic (HPLC) methods have received much attention (Abaychi and Riley, 1979; Mantoura and Llewellyn, 1983; Wright and Shearer, 1984). The use of HPLC in plant pigment analysis has been reviewed by Schwartz and von Elbe (1982). HPLC methods provide a physical separation of the pigments and hence solve

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many interference and calibration problems. Lorenzen and Jeffrey (1980) considered a chromatographic method to be essential whenever precise knowledge of pigment composition is required and recommended the wider use of chromatography in pigment determination. However, environmental studies often require large numbers of analyses for chlorophylls alone and in these circumstances spectrophotometric and/or fluorometric methods may be preferable in view of their greater simplicity, speed and the convenience of a simple result. It must also be remembered that, where biomass estimation is the ultimate aim, analytical error may be far outweighed by other uncertainties (Lehman, 1981).

In our laboratories we routinely use the traditional chlorophyll methods or one of two HPLC procedures as circumstances demand. The first HPLC method is similar to that described by Wright and Shearer (1984): the method operates in the reversed-phase mode, uses absorbance detection (and auxiliary fluorescence detection) for the determination of the full range of pigments and takes about 35 min per sample (Murray et al., 1985). The second HPLC method is used whenever the accuracy provided by chromatographic resolution of the chlorophylls is necessary, but time or sample size is limited. This is an isocratic method with fluorescence detection which yields values for chlorophylls *a* and *b* and the corresponding phaeophytins in about 10 min. The rapid method does not normally give values for the non-phytolated pigments (chlorophyll *c*, chlorophyllides, phaeophorbides) because these co-elute at the beginning of the chromatogram. However, as will be shown, chlorophyll *c* can be determined if the fluorescence excitation and emission wavelengths are set appropriately.

The use of multiple chlorophyll methods leads to concern about the comparability of results. Only very limited intercalibration between methods has been reported in the literature. Mantoura and Llewellyn (1983) tabulated results for a single sample, comparing their HPLC method with traditional spectrophotometric methods. They found discrepancies between 3.5- and 12.6-fold which were ascribed to inadequacies in the spectrophotometric methods. Jacobsen (1978) compared his rapid HPLC procedure with both spectrophotometric and fluorescence methods and also found very poor agreement. Gieskes and Kraay (1983) found that their normal-phase HPLC procedure gave lower results than spectrophotometric and fluorometric methods and attributed the differences to the presence of unknown chlorophyll derivatives. Recently, a comparison has been made between HPLC and various spectrophotometric methods using freshwater algae (Sartory, 1985). The spectrophotometric procedures were found to overestimate chlorophyll *a* and underestimate chlorophyll *b*.

We report here a comparison between the results of chlorophyll determinations made on a typical suite of marine and estuarine samples by four methods: traditional spectrophotometric, fluorometric, rapid HPLC (with fluorescence detection) and full HPLC (with absorbance detection). Most attention has been directed towards comparison of estimates for chlorophyll *a*, but chlorophyll *b*:*a* ratios were also compared. No attempt was

made to compare estimates for chlorophyll *c* since this is only measured routinely by the full HPLC and spectrophotometric methods and, in the latter case at least, the values given are considered unreliable (Strickland and Parsons, 1972).

The ability of the chromatographic methods to resolve chlorophyllides and chlorophyll *a* allomers allowed us to observe and quantify the effect of these compounds on the spectrophotometric and fluorometric determination of chlorophyll *a*.

METHODS

Sample sources

Natural phytoplankton samples were obtained from various locations in Eastern Bass Strait (sample numbers 1—19), Western Port Bay (sample numbers 20—22) and the Gippsland Lakes (sample numbers 23—27). The lakes provided samples from a range of salinities between 10 and 35‰. All but one of the samples represented surface waters, the exception being a sample taken at a depth of 2500 m on the continental slope in Eastern Bass Strait. The range of chlorophyll *a* concentrations in these field samples was approximately 0.02 $\mu g l^{-1}$ (the deep water sample) to 11 $\mu g l^{-1}$, with most samples being less than 1 $\mu g l^{-1}$. To represent higher chlorophyll concentrations (ca. 30 $\mu g l^{-1}$), two further samples (numbers 28 and 29) were taken from algal culture tanks at our laboratories. In total, twenty-nine field samples were analysed by the spectrophotometric, fluorometric and full HPLC methods. A subset of these comprising the Bass Strait and Gippsland lakes samples (24 samples) was analysed by the rapid HPLC procedure.

In addition to the natural phytoplankton samples, a pigment mixture distributed by the United States Environmental Protection Agency (USEPA) was also analysed by the four methods. This sample, which consisted of a mixture of chlorophylls, carotenoids and degradation products in 90% acetone, was distributed as a check sample for the spectrophotometric method. For our purposes it was diluted tenfold for the full HPLC and spectrophotometric analyses, 100-fold for the rapid HPLC and fluorometric analyses and was analysed in duplicate. In other respects it was treated exactly as the natural samples.

Samples for traditional methods and full HPLC were obtained by filtering up to 13 l of water through 49 mm diameter GF/C filters under slight positive pressure of nitrogen. Samples for the rapid HPLC procedure were obtained by filtering 250 ml water through 22 mm GF/C filters using a syringe. Filters were stored on dry ice, then at − 20°C until analysis.

Standards

Commercial chlorophyll *a* and *b* preparations were obtained from Sigma Pty. Ltd. (Cat. Nos. C-5753 and C-5878). Analytical HPLC showed the

absence of significant impurities in these materials though small amounts of allomers were present. A stock solution of 50 mg l^{-1} chlorophyll *a* and *b* in anhydrous acetone was prepared and working standards were made from this stock immediately before use.

Spectrophotometric and fluorescence analysis

Filters were disrupted in 25 ml of 90% aqueous acetone using a Labsonic 2000 ultrasonicator (45 s, 90 W) with a needle probe. Magnesium carbonate was not added (Moed and Hallegraeff, 1978). Spectrophotometric and fluorometric measurements were made according to Strickland and Parsons (1972) with the substitution, in the spectrophotometric method, of the equations of Jeffrey and Humphrey (1975). Absorbance was measured on a Uvidec spectrophotometer in 10-cm cells while the fluorescence was read at excitation 431 nm, emission 667 nm on a Hitachi 650-40 spectrofluorometer operated in the ratio mode and with slits set to 5 nm for both monochromators. It should be noted that the spectral bandpass of the spectrofluorometer was much less than that of the filter instruments more commonly used for fluorometric analysis of chlorophylls. A total of 15 ml of the extract was used for the absorbance and fluorescence measurements, the remaining 10 ml being used for the full HPLC method.

HPLC analysis

For the full HPLC method the pigments in the sample extract obtained above were immediately transferred into diethyl ether according to the method of Jeffrey (1968). Just before analysis the ether was removed in a stream of nitrogen and the pigments were redissolved in 2 ml of 90% aqueous methanol. For the rapid HPLC method the smaller filters were sonicated in 10 ml of 100% acetone which was then centrifuged and filtered (0.45 μm). A 3-ml aliquot was then diluted with 1 ml of water and subjected to HPLC analysis within 5 min of the cell disruption.

In both methods 200 μl of the respective extracts were injected on-column via a Rheodyne loop injector using a Waters instrument consisting of dual pumps, a radially compressed 10 x 0.8 cm (5 μm) C-18 column and a Waters model 440 absorbance detector with a 436 nm filter. The spectrofluorometer described above was connected in series with the absorbance detector using an 18-μl flowcell and a valve system which allowed pigments to be trapped in the cell for recording of their excitation and emission spectra. The fluorometer slits were set to their maximum width of 20 nm during its use as an HPLC detector.

The solvent program for the full HPLC method consisted of 10 min elution with 9:1 methanol:water followed by the addition of ethyl acetate at 3% min^{-1} for 20 min. Before the next injection the column was re-equilibrated with the initial solvent for 5 min.

For the rapid method, an isocratic elution with a solution of 45% ethyl acetate, 49.5% methanol and 5.5% water was used.

Total flow rate was 2 ml min^{-1} for both methods and peak areas were measured manually from the chart recorder traces, with the aid of a magnifying graticule.

Calibration

In principle the spectrophotometric method does not require external calibration for each set of analyses. However, calibration standards in the range 50—1000 $\mu g\, l^{-1}$ chlorophyll *a* in 90% acetone were prepared for the calibration of the full HPLC method and these were also analysed by the spectrophotometric method to which they showed the expected response with no deviations from linearity. A similar set of standards was used to calibrate the fluorometer, but in keeping with our normal practice, the fluorescence of each pure chlorophyll *a* standard was related to its corresponding absorbance on the spectrophotometer, rather than to the nominal concentration.

To calibrate the full HPLC method, the standards were subjected to the same ether partitioning and concentration as described for samples. Thus, sample results were automatically corrected for losses caused by the treatment and for the concentration factor. Previous experience with the ether partitioning has indicated that the actual recovery of total chlorophyll *a* is about 90%.

Calibration of the rapid HPLC method employed standards in a much lower concentration range owing to the high sensitivity of the fluorescence detector. The range was 0.5—4 $\mu g\, l^{-1}$ and again, standards were treated identically to the samples. Calibration curves for chlorophylls *a* and *b* were found to be linear over this range.

Calibration of both HPLC methods for phaeophytins *a* and *b* employed standards made by acidification of the parent chlorophylls and was carried out in the same manner as for the chlorophylls.

As well as the calibrations described above (which were repeated at the end of the sample set) at least one standard, containing chlorophylls *a* and *b*, was run through all methods on each day that analyses were performed.

Comparisons made between the optical and chromatographic methods were based on the three separate measures of chlorophyll *a* which can be derived from the full HPLC chromatograms:

(1) Chlorophyll *a*, i.e. the major chlorophyll *a* peak only.

(2) Chlorophyll *a* + allomers.

(3) Total chlorophyll *a*, i.e. chlorophyll *a* + allomers + chlorophyllide *a*.

The term 'allomers' is used here to describe the group of pigments with retention times and optical properties similar to chlorophyll *a*. They are thought to arise from keto-enol tautomerism at C-9 and epimerisation at C-10 (Hynninen and Assandri, 1973). Allomers and chlorophyllides were

calculated as chlorophyll *a* equivalents, i.e. the mass response factors (peak area/nanogram injected) were assumed to be the same as for chlorophyll *a*. The rapid HPLC method could only measure (1) and (2) above since the relatively polar chlorophyllides co-eluted with chlorophyll *c* and other pigments at the start of the chromatogram.

Statistical treatment

The results given by each method for chlorophyll *a* were compared using weighted linear regressions (WLR) in the manner described by Thompson (1982). With this procedure, the values given by one method are plotted against those given by another and a bias between the methods appears as a significant deviation of the slope of the regression line from unity (rotational bias) or of the intercept from zero (translational bias). A significant deviation is defined as more than $\pm 2s$, where s represents the standard error of the slope or intercept, respectively.

Use of the WLR model requires an estimate of the variance in the y-axis variable at every point. To obtain these variance estimates, the precision of each method was first assessed by repetitive application to typical samples. This precision was then assumed to be constant (i.e. variance changing in proportion to the value) across the concentration range, with the exception of the lowest concentration sample. The concentration of chlorophylls in this latter sample was near to the detection limit for all of the methods and it was arbitrarily assigned a precision of 50% regardless of the measurement method. In all cases, the regression analysis was carried out using the method with the better precision as the independent variable. This is a practical constraint imposed by the use of a weighted rather than a maximum likelihood regression (Thompson, 1982).

Since the use of a regression method assumes that the random errors in each method are distributed normally, a non-parametric test was also used to compare the methods: the Wilcoxon signed rank test was applied to the differences between paired results. In all cases, however, the regression and non-parametric tests were in agreement.

RESULTS AND DISCUSSION

Table I gives the full set of results for chlorophylls *a* and *b* while Fig. 1 shows typical chromatograms resulting from the two HPLC procedures, applied to the same sample. Figure 2 shows the full HPLC chromatogram of the deep water sample, with the fluorometer set to be sensitive to phaeopigments. Peak assignments shown in Figs. 1 and 2 are the result of retention time matching, absorbance and fluorescence scanning of similar peaks from phytoplankton cultures (Murray et al., 1985) and comparison with the literature. For example the pair of peaks in the centre of Fig. 2 is probably attributable to phaeophorbides (Wright and Shearer, 1984).

Repetitive application of each of the analysis methods gave the following

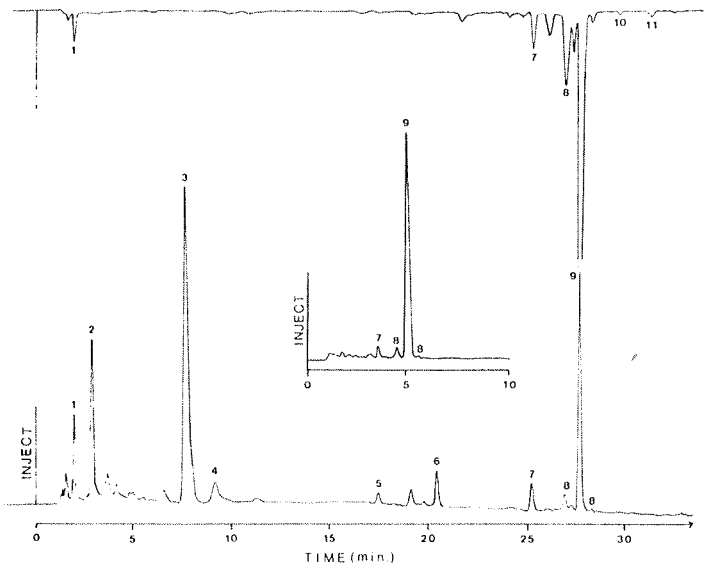

Fig. 1. Full HPLC chromatogram of sample 11, Table I. Lower trace: absorbance at 436 nm, 0.05 AUFS. Upper trace: fluorescence at excitation 431 nm, emission 667 nm. INSET: rapid HPLC chromatogram (fluorescence) of the same sample. (1) Chlorophyllide *a*, (2) chlorophyll *c* (c_1 and c_2), (3) fucoxanthin, (4) neoxanthin, (5) diadinoxanthin, (6) lutein/zeaxanthin, (7) chlorophyll *b*, (8) chlorophyll *a* allomers, (9) chlorophyll *a*, (10) phaeophytin *b*, (11) phaeophytin *a*.

coefficients of variation for measurement of chlorophyll *a* ($1\,\mu g\,l^{-1}$ concentration, $n = 10$): spectrophotometric method (uncorrected for phaeophytin), 4.3%; fluorometric method (uncorrected for phaeophytin), 5.2%, full HPLC, 8.3%; rapid HPLC, 6.4%. The precisions obtained for the spectrophotometric and fluorometric methods are consistent with those previously reported (Strickland and Parsons, 1972). The slightly worse precision found for the full HPLC method is to be expected in view of the greater sample handling required.

During sampling for the rapid HPLC method, it was suspected that the combination of syringe and the small filters was occasionally resulting in rupture of the filters. This was confirmed in the case of five samples which were discarded, reducing the data set for evaluation of the rapid HPLC method to nineteen samples. A further problem experienced with the rapid method was that the very low concentration standards and samples were subject to a quite rapid proportional loss of chlorophyll *a*. This difficulty was circumvented by minimising the time between disruption of the filter and injection of the sample into the HPLC.

Chlorophyll a

The full HPLC analyses indicated that the field samples contained, in addition to the normal chlorophylls *a*, *b* and *c*, varying amounts of

TABLE 1

Results for chlorophyll *a* ($\mu g l^{-1}$) and chlorophyll *b* : *a* ratio obtained by different methods

	Spectrophotometric			Fluorometric		Full HPLC				Rapid HPLC		
Sample No.	Uncorr. chl.*a*	Corr. chl.*a*	*b* : *a* ratio	Uncorr. chl.*a*	Corr. chl.*a*	Total chl.*a*[a]	*a*+ alls.	Chl.*a* only	*b* : *a* ratio	*a*+ alls.	Chl.*a* only	*b* : *a* ratio
1	0.72	0.63	0.32	0.56	0.46	0.58	0.47	0.38	0.23	0.50	0.48	0.31
2	0.33	0.32	0.30	0.27	0.22	0.32	0.19	0.11	0.16	0.25	0.23	0.32
3	0.37	0.30	0.22	0.29	0.25	0.39	0.27	0.21	0.14	0.36	0.34	0.24
4	0.46	0.40	0.28	0.39	0.33	0.48	0.36	0.28	0.18	0.55	0.53	0.29
5	0.98	0.95	0.09	0.76	0.69	0.98	0.72	0.57	0.06	0.97	0.90	0.12
6	1.09	1.03	0.10	0.82	0.73	1.06	0.92	0.73	0.07	1.03	0.95	0.12
7	0.68	0.70	0.13	0.58	0.52	0.73	0.55	0.33	0.08	0.72	0.68	0.16
8	0.65	0.58	0.18	0.51	0.45	0.81	0.57	0.45	0.09	0.47	0.44	0.24
9	0.49	0.45	0.14	0.42	0.37	0.52	0.34	0.27	0.08	0.69	0.63	0.21
10	0.29	0.26	0.24	0.26	0.22	0.23	0.19	0.15	0.13			
11	0.28	0.23	0.25	0.23	0.20	0.32	0.25	0.22	0.12	0.27	0.25	0.24
12	0.20	0.17	0.25	0.18	0.15	0.15	0.12	0.10	0.18			
13	0.25	0.24	0.28	0.22	0.18	0.21	0.16	0.14	0.13			
14	0.45	0.41	0.18	0.37	0.33	0.39	0.28	0.16	0.11	0.49	0.46	0.19
15	0.30	0.29	0.20	0.24	0.21	0.28	0.17	0.09	0.20	0.14	0.13	0.25
16	0.28	0.25	0.18	0.24	0.21	0.27	0.20	0.15	0.05			
17	0.31	0.29	0.26	0.28	0.24	0.31	0.23	0.10	0.18	0.22	0.20	0.21
18	0.87	0.75	0.14	0.66	0.57	0.87	0.54	0.36	0.07	0.86	0.79	0.06
19	0.02	0.02	0.50	0.01	0.01	0.02	0.02	0.01	<0.5	<0.01	<0.01	<1

20	0.51	0.44	0.14	0.37	0.31	0.47	0.35	0.25	0.07			
21	1.05	0.91	0.11	0.87	0.77	1.25	0.74	0.59	0.07			
22	0.60	0.46	0.12	0.48	0.41	0.63	0.44	0.37	0.08			
23	5.35	5.49	<0.02	5.31	5.05	7.57	6.90	5.46	0.03			
24	7.07	7.23	0.07	6.33	5.86	7.37	6.84	4.83	0.07	7.72	7.02	<0.02
25	5.36	5.39	0.05	5.35	4.96	5.56	5.05	3.39	0.06	4.32	4.00	0.08
26	8.25	8.48	0.02	8.91	8.42	11.59	10.92	8.07	0.06	6.97	6.48	0.08
27	2.81	2.41	0.08	2.47	2.22	2.60	2.45	1.45	0.06	2.86	2.08	0.08
28	26.95	28.73	0.12	19.00	17.74	27.95	25.50	22.12	0.16			
29	25.54	26.66	0.13	19.27	17.37	29.35	23.85	20.62	0.17			

[a]'Total chlorophll *a*' for the full HPLC method means chlorophyll *a* + allomers + chlorophyllides.
Note: samples 1—19 were from Bass St., 20—22 from Western Port Bay, 23—27 from the Gippsland Lakes and 28 and 29 from algal culture tanks.

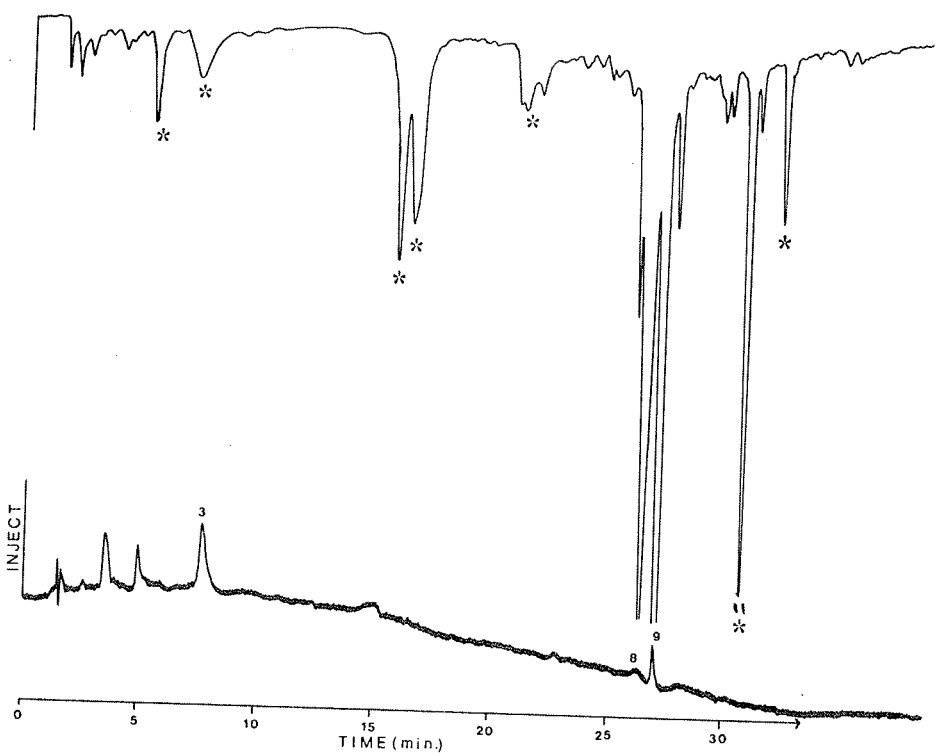

Fig. 2. Full HPLC chromatogram of a deep water sample (sample 19, Table I). Lower trace: absorbance at 436 nm, 0.01 AUFS. Upper trace: fluorescence at excitation 410 nm, emission 674 nm. Peaks numbered as for Fig. 1. Peaks marked with an asterisk were trapped in a separate run and found to have excitation maxima between 408 and 411 nm.

chlorophyllide *a*, chlorophyll *a* allomers and phaeophytin *a*. The amount of phaeophytin in these samples was, however, quite low, averaging 9% of the total chlorophyll *a* (when both pigments were measured by full HPLC). Pure chlorophyll *a* standards passed through the ether partitioning were found to have been partly converted to allomers. The extent of this conversion varied from 5 to 16% of the original chlorophyll *a*. No significant conversion of chlorophyll to chlorophyllides or phaeophytin occurred. Wright and Shearer (1984) found that ether partitioning caused an 8% loss of chlorophyll *a* and a 46% increase in the amount of allomers.

Although some fraction of the allomers was produced by the sample handling, they are a common feature of natural samples (Jensen and Sakshaug 1973; Jeffrey and Hallegraeff, 1980; Gieskes and Kraay, 1983). The effect of both allomers and chlorophyllides on the spectrophotometric and fluorometric methods must be considered when carrying out a method comparison. While the results of these methods can be corrected for the phaeopigments, neither can distinguish between the parent chlorophyll *a*

and its allomers and chlorophyllides. One would thus expect agreement between 'corrected' spectrophotometric or fluorometric results and the sum of chlorophyll *a*, allomers and chlorophyllides as determined by HPLC.

Table II shows the results of the comparison of the four methods, taking the modified forms of chlorophyll *a* into account. The figures represent the slopes of weighted linear regressions of each method against the other. In all cases comparing HPLC with spectrophotometric or fluorescence results, the intercept deviated slightly from zero with a small positive reading for fluorescence or absorbance. This corresponded to approximately 30 $ng\,l^{-1}$ chlorophyll *a* and probably arose from minute quantities of other fluorescent and absorbing material in the samples. In all combinations listed in Table II correlation coefficients (r^2) were 0.90 or greater.

It may be seen from Table II that the full HPLC results for chlorophyll *a* plus allomers plus chlorophyllide agreed most closely with those of the spectrophotometric method, uncorrected for phaeophytin, rather than with the corrected values as expected. This set of samples being rather low in phaeophytin (as shown chromatographically) suggests that the inherent imperfections in the phaeophytin correction artificially reduced the value for corrected chlorophyll *a*.

The discrepancy between the spectrophotometric and fluorescence methods for natural samples (following intercalibration with pure chlorophyll *a*) was surprising. It had been assumed that chlorophyllides would behave as chlorophyll *a* in both methods. However it is noteworthy that the fluorescence results agreed most closely with the full HPLC method for chlorophyll *a* plus allomers (i.e. excluding chlorophyllides). If chlorophyllides respond less in the fluorometric method than in the spectrophotometric method, both of these results would be understandable.

Table II also shows that the major chlorophyll *a* peak alone (i.e. excluding both allomers and chlorophyllides) from full HPLC did not account fully for the response of the two optical methods. In fact only 57% of the spectrophotometric response and 72% of the fluorescence response could be attributed to intact chlorophyll *a* as measured by full HPLC.

Examination of the rapid HPLC results, however, shows a very different picture. Table II shows that there was no significant difference between the full HPLC results for chlorophyll *a* + allomers + chlorophyllides and the combination of chlorophyll *a* + allomers given by the rapid HPLC method. In fact, the chlorophyll *a* peak in the rapid HPLC chromatograms accounted for almost all of the total chlorophyll *a* found by the full method. Conversely, the chlorophyll *a* peak in the full HPLC chromatograms represented on average only 54% of the chlorophyll *a* found by the rapid method. The higher values given by the rapid method for unchanged chlorophyll *a* were parallelled by much smaller values for the allomers. The rapid HPLC method found that the samples contained an average of 8% of the chlorophyll *a* as allomers compared with the greater than 20% values indicated by the full method (Table I). It is clear that some chlorophyll *a* was converted to

TABLE II

Slopes of regression lines comparing different methods

x-axis[a]	y-axis						
	Rapid HPLC		Full HPLC			Fluorometric	
	Chlor. *a* + alls.	Chlor. *a* only	Chlor. *a* + alls. + c'phyllide	Chlor. *a* + alls.	Chlor. *a* only	Corr.	Uncorr.
Spectrophotometric							
corrected	0.90	0.83	1.10	0.86	0.57	0.77	0.87
uncorrected	0.85	0.78	1.04[b]	0.80	0.54	0.76	0.83
Fluorometric							
corrected	1.12	1.01[b]	1.39	1.10	0.72		
uncorrected	0.99[b]	0.90	1.23	0.97[b]	0.63		
Full HPLC							
chlorophyll *a* + alls. + c'phyllide	1.05[b]	0.94					
chlorophyll *a* + alls.	1.39	1.28					
chlorophyll *a* only	2.04	1.87					

[a] Appearance of a variable on the x-axis does not imply that it was used as the independent variable.
[b] These slopes were not statistically different from one.

TABLE III

Results for the USEPA sample ($mg\ l^{-1}$)

	Stated	Spectro-photometric	Fluoro-metric	Full HPLC	Rapid HPLC
Uncorr. chlorophyll *a*	7.76	7.51	6.30		
Corr. chlorophyll *a*	6.29	6.20	5.35		
Chl.*a* + alls. + c'phyllide				6.34	
Chl.*a* + alls.				5.67	3.97
Chlorophyll *a* only				4.44	3.87
Phaeophytin *a*	2.11	2.35		1.81	2.73
Chlorophyll *b*	0.60	0.94		1.37	1.78

Note: phaeophytin was not determined by fluorescence due to its low response with slits set at 5 nm.

allomers during the preparative step of the full HPLC method. These data provide an indication that sample handling for the full method may have also produced chlorophyllide-like material. That is, the slope of the regression line for rapid HPLC chlorophyll *a* + allomers against full HPLC chlorophyll *a* + allomers + chlorophyllides is close to one. This is not the case when the chlorophyllide contribution is excluded from the full HPLC results. Although we found no conversion of pure chlorophyll *a* standards to chlorophyllides, this does not preclude such a conversion having occurred in the samples (Barrett and Jeffrey, 1971; Owens and Falkowski, 1982).

The USEPA sample differed from the field samples in having a larger proportion of phaeophytin. The results of the different methods applied to this sample are shown in Table III. In this case the full HPLC result for chlorophyll *a* + allomers + chlorophyllides agreed more closely with the corrected spectrophotometric figure than it did with the uncorrected one. This is of course to be expected. For this sample the rapid HPLC method gave results for chlorophyll *a* approximately 35% lower than the other methods.

Thus in general for chlorophyll *a* there is no evidence for a systematic difference between the HPLC and optical methods, provided allowance is made for allomers and chlorophyllides. Fairly large differences can occur for individual samples however, though we have not observed discrepancies of the scale reported by others (Jacobsen, 1978; Mantoura and Llewellyn, 1983). We believe the unreliable nature of the phaeophytin correction in the spectrophotometric and fluorometric method to be responsible for some of the differences we have observed. In particular this explains the better agreement of uncorrected spectrophotometric results with HPLC in the case of samples low in phaeophytin.

Chlorophyll b

Chlorophyll *b* and other pigments could not be measured by the HPLC methods with the same precision as chlorophyll *a*. This was because peak areas were measured manually and it was necessary to scale chromatograms for optimum measurement of the chlorophyll *a* peak. Hence, no statistical comparison of chlorophyll *b* results was made. However, Table I includes values for the chlorophyll *b*:*a* ratio. The ratios are included instead of absolute concentrations in order to avoid reiteration of arguments relating to chloropyll *a* and because they can be used, for example, as an index of the relative abundance of green algae. Very good agreement was found between the ratios determined by the spectrophotometric and rapid HPLC methods, especially considering the HPLC results often represent very small chlorophyll *b* peaks. In contrast, chlorophyll *b*:*a* ratios given by full HPLC analysis were generally lower than those given by the other methods. These lower values for chlorophyll *b* may have been due to the exclusion of allomers and chlorophyllides from the results. Given the apparent conversion of chlorophyll *a* to allomers and chlorophyllide derivatives, some conversion of chlorophyll *b* might have been expected and the full HPLC chromatograms occasionally showed a very small peak eluting just before the main chlorophyll *b* peak. This peak was, however, too small to be reliably identified or measured. No allomerisation of chlorophyll *b* standards was observed.

Chlorophyll *b* values found for the USEPA sample are given in Table III. Both chromatographic methods indicated higher concentrations for this pigment than the spectrophotometric procedure. The reason for this discrepancy, which was greatest in the case of the rapid method, is unknown, but the spectrophotometric method has been shown to give low values for chlorophyll *b* when phaeopigments are present (Lorenzen and Jeffrey, 1980; Sartory, 1985).

The sensitivity of the rapid HPLC method for chlorophyll *b* was quite low, due to the spectral selectivity of the fluorometric detector. It is worth noting, however, that this selectivity can be used to advantage if a more sensitive analysis for chlorophyll *b* is required. Figure 3 shows how chromatographic and spectral selectivity can be combined to provide good peaks for chlorophylls *b* and *c* in a rapid HPLC run. The wavelengths can be chosen to be quite sensitive to chlorophylls *b* and *c* and very insensitive to forms of chlorophyll *a* and phaeopigments. This means that it may be possible to determine chlorophyll *c* from a rapid HPLC chromatogram even though it may not be resolved chromatographically from various chlorophyll *a* degradation products.

CONCLUSIONS

The data presented show little evidence for a fundamental difference between the results of chromatographic and spectrophotometric or fluorometric methods for chlorophyll *a*. Further work will have to be done,

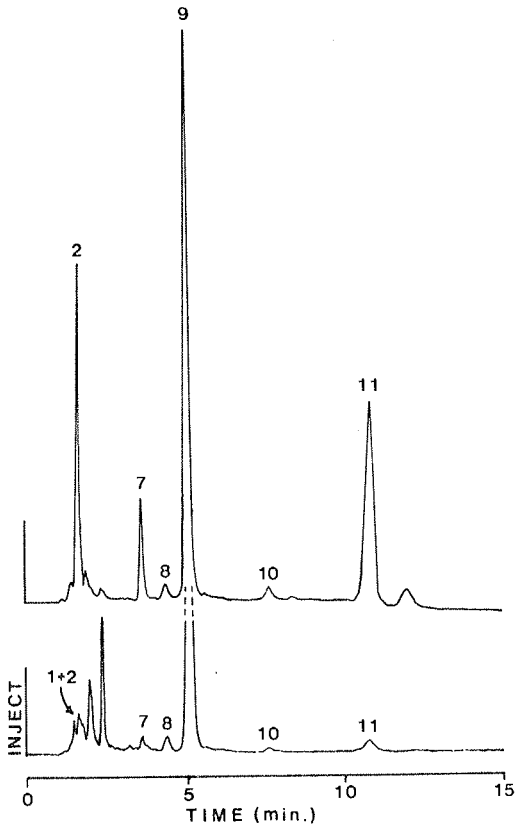

Fig. 3. Rapid HPLC chromatograms of pigments collected from a Bass Strait sample. Lower trace: excitation 431 nm, emission 667 nm as normal. Upper trace: the same sample with adjustments made to the excitation/emission wavelengths and sensitivity during the run: 0—4 min 460/650, sens. × 4; 4—7 min 431/667 sens. × 1; 7—9 min 431/667 sens. × 4; 9—15 min 410/674 sens. × 4. Peak assignments as for Fig. 1.

however, to determine why rapid HPLC gave such a low result for the USEPA sample.

Though both chromatographic methods gave acceptable results for total chlorophyll *a*, it is clear that the rapid method caused much less conversion of chlorophyll to degradation products. This appears to arise from the concentration step included in the full HPLC method and it might be argued that such a step is unnecessary for the determination of chlorophylls since more sensitive fluorescence detection could have been used. Nevertheless, the usual application of a full HPLC procedure includes the determination of the non-fluorescent carotenoid pigments and this necessitates a concentration step in many cases.

With the advent of HPLC methods, the pigment analyst can now select,

from a range of techniques, one which gives a balance of convenience, accuracy and information appropriate to the task in hand. The data reported herein should engender confidence that results given by different methods will be comparable. Care must be taken, however, to ensure that reported values refer to the same combination of the many possible forms of chlorophyll *a*.

The rapid HPLC method, as used for this work, seems to provide a good compromise between chromatographic simplicity, speed and accuracy of results. Furthermore, its high sensitivity means that small sample volumes can be used. This is an important advantage for much oceanographic work. We have recently carried out a depth profiling of chlorophyll in Eastern Bass Strait (Australia) using the rapid HPLC procedure (Gibbs et al., in preparation). Even in this oligotrophic water body, a sample size of 10 ml would have been theoretically adequate. The rapid method also causes minimal chlorophyll degradation during preparation, whilst overcoming much of the uncertainty inherent in traditional spectrophotometric and fluorometric methods.

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REFERENCES

Abaychi, J.K. and Riley, J.P., 1979. The determination of phytoplankton pigments by high performance liquid chromatography. Anal. Chim. Acta, 107: 1—11.

Barrett, J. and Jeffrey, S.W., 1971. A note on the occurrence of chlorophyllase in marine algae. J. Exp. Mar. Biol. Ecol., 7: 255—262.

Gieskes, W.W. and Kraay, G.W., 1983. Unknown chlorophyll *a* derivatives in the North Sea and tropical Atlantic Ocean revealed by HPLC analysis. Limnol. Oceanogr., 28: 757—765.

Gibbs, C.F., 1979. Chlorophyll *b* interference in the fluorometric determination of chlorophyll *a* and 'phaeo-pigments'. Aust. J. Mar. Freshwater Res., 30: 597—606.

Holm-Hansen, O., Lorenzen, C.J., Holmes, R.W. and Strickland, J.D.H., 1965. Fluorometric determination of chlorophyll. J. Cons. Perm. Int. Explor. Mer, 30: 3—15.

Hynninen, P.H. and Assandri, S., 1973. Chlorophylls II. Allomerisation of chlorophylls *a* and *b*. Acta Chem. Scand., 27: 1478—1486.

Jacobsen, T.R., 1978. A quantitative method for the separation of chlorophylls *a* and *b* from phytoplankton pigments by high pressure liquid chromatography. Mar. Sci. Commun., 4: 33—47.

Jeffrey, S.W., 1968. Quantitative thin-layer chromatography of chlorophylls and carotenoids from marine algae. Biochim. Biophys. Acta, 162: 271—285.

Jeffrey, S.W. and Hallegraeff, G.M., 1980. Studies of phytoplankton species and photosynthetic pigments in a warm core eddy of the East Australian current. II. A note on pigment methodology. Mar. Ecol. Prog. Ser., 3: 295—301.

Jeffrey, S.W. and Humphrey, G.F., 1975. New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1 and c_2 in higher plants, algae and natural phytoplankton. Biochem. Physiol. Pflanz., 167: 191—198.

Jensen, A. and Sakshaug, E., 1973. Studies on the phytoplankton ecology of the Trondheimsfjord. II. Chloroplast pigments in relation to abundance and physiological state of the phytoplankton. J. Exp. Mar. Biol. Ecol., 11: 137—145.

Lehman, P.W., 1981. Comparison of chlorophyll *a* and carotenoid pigments as predictors of phytoplankton biomass. Mar. Biol., 65: 237—244.

Lorenzen, C.J. and Jeffrey, S.W., 1980. Determination of chlorophyll in seawater — report of intercalibration tests. UNESCO Tech. Pap. in Marine Science No. 35, 20 pp.

Mantoura, R.F.C., and Llewellyn, C.A., 1983. The rapid determination of algal chlorophyll and carotenoid pigments and their breakdown products in natural waters by reverse-phase high performance liquid chromatography. Anal. Chim. Acta, 151: 297—314.

Moed, J.R. and Hallegraeff, G.M., 1978. Some problems in the estimation of chlorophyll *a* and phaeopigments from pre- and post-acidification spectrophotometric measurements. Int. Rev. Ges. Hydrobiol., 63: 787—800.

Murray, A.P., McShane, P.E., Errey, B. and Terhell, D., 1985. Analysis of phytoplankton pigments and their degradation products by high pressure liquid chromatography. Mar. Sci. Lab. Tech. Rep. 38, Ministry for Conservation, Forests and Lands, Victoria, Australia, 17 pp.

Owens, T.G. and Falkowski, P.G., 1982. Enzymatic degradation of chlorophyll *a* by marine phytoplankton in vitro. Phytochem., 21: 979—984.

Sartory, D.P., 1985. The determination of algal chlorophyllous pigments by high performance liquid chromatography and spectrophotometry. Water Res., 19: 605—610.

Schwartz, S.J. and von Elbe, J.H., 1982. High performance liquid chromatography of plant pigments — a review. J. Liquid Chromatogr., 5 (Suppl 1): 43—73.

Strickland, J.D.H. and Parsons, T.R., 1972. A Practical Handbook of Seawater Analysis. Fisheries Res. Board of Canada, Ottawa, 310 pp.

Thompson, M., 1982. Regression methods in the comparison of accuracy. Analyst (London), 107: 1169—1180.

Wright, S.W. and Shearer, J.D., 1984. Rapid extraction and high performance liquid chromatography of chlorophylls and carotenoids from marine phytoplankton. J. Chromatogr., 294: 281—295.