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HPLC analysis of algal pigments: a comparison exercise among laboratories and recommendations for improved analytical performance

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Abstract

Pure individual and mixed pigment standards were distributed among Joint Global Ocean Flux Study (JGOFS) pigment analysts to estimate the variability of their spectrophotometric and chromatographic systems. To monitor the integrity of the pigments during the comparison exercise, chlorophyll and carotenoid standards were archived and periodically analyzed by high-performance liquid chromatography (HPLC). Pigment standards stored in the dark under nitrogen at −20°C were found to be stable for periods of at least one year. Results from three separate intercalibration exercises document a better agreement for spectrophotometric analyses than for HPLC. For the spectrophotometric comparisons, 90% of the pigments analyzed by participant laboratories were within ±6% of the mean "consensus" values. By contrast, 65 and 85% of the laboratories agreed to within ±10 and ±20%, respectively, when chromatographic analyses were compared. Chlorophyll absorption measurements obtained with a diode array-type spectrophotometer were 6–9% lower than those obtained with monochromator-type spectrophotometers. These underestimates probably result from chlorophyll fluorescence contamination associated with the optical configuration of the diode array spectrophotometer. It was also determined that HPLC methods which are not capable of separating monovinyl chlorophyll *a* from divinyl chlorophyll *a* can produce 15–25% overestimates of total chlorophyll *a* concentration in *Prochlorococcus*-dominated oceanic waters. A simple dichromatic approach is described for eliminating this variable source of error caused by co-elution of these structurally-related pigments. The use of internal standards and periodic calibration checks with external standards is highly recommended for improving analytical performance.

1. Introduction

The analysis of photosynthetic pigments by aquatic biologists is widely used to estimate phytoplankton biomass and to identify the presence of different algal groups. Characterization of the complex pigment mixtures found in natural waters has been greatly improved by the development of high-performance liquid chromatographic (HPLC) techniques (Roy, 1987; Millie et al., 1993). Unfortunately, there is a limited availability of authentic pigment standards for instrument calibration. Pigments which are commercially available include chlorophyll *a*, chlorophyll *b*, α-carotene, β-carotene, lutein, zeaxanthin, and canthaxanthin. As a consequence, most pigment laboratories resort to

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purification of their own standards. Once purified, pigments are transferred to appropriate solvents and concentrations are determined spectrophotometrically based on published extinction coefficients. Variations in the purity of isolated pigments and in the choice of the extinction coefficients make it difficult to compare pigment data originating from different laboratories. To estimate analytical variability among analytical laboratories, an intercalibration exercise was organized as part of the US Joint Global Flux Study (US JGOFS) Program.

The goals of the study were to (1) provide laboratories participating in JGOFS-related research with highly pure pigment standards, (2) provide mixed standards for interlaboratory comparisons, (3) monitor the stability of standards during the course of the intercalibration exercise, and (4) provide recommendations for improving the precision and accuracy of HPLC-determined pigment analyses.

2. Materials and methods

2.1. Preparation of pigment standards

Individual pigment standards were obtained from various sources, dissolved in solvent and sealed in 5 ml amber ampules under a stream of purified nitrogen. A total of twelve different external standards were supplied as part of the intercalibration exercise (Table 1). 19′-hexanoyloxyfucoxanthin, 19′-butanoyloxyfucoxanthin, prasinoxanthin and alloxanthin were purified from *Emiliania huxleyi* (clone CCMP373), *Pelagomonas calceolata* (clone CCMP1214), *Pycnococcus provasolii* (clone CCMP1203), and *Chroomonas salina* (clone CCMP1319), respectively, following the methods described by Bidigare et al. (1991). A chlorophyll c_{1+2} standard was also provided but not included as part of the HPLC comparisons since it was not possible to produce a stable preparation of this pigment. Chlorophyll c_{1+2} stored in acetone produced a series of polar chlorophyll *c*-related compounds, possibly including chlorophyll *c* dimers (cf. Jeffrey, 1972). Three potential internal standards were also sent for evaluation, including canthaxanthin (Roth Chemical Co.), methyl pyrophaeophorbide *a* (Spirutec, Inc.), and etioporphyrin I (Aldrich Chemical Co.).

Concentrations of the standards were determined spectrophotometrically (prior to ampule sealing) using the extinction coefficients given in Table 1. The standards were individually labeled (pigment identity, solvent description, batch number, and pigment

Table 1
Pigment standards documentation sheet

Standard	Abbreviation	Source	E (L g^{-1} cm^{-1})	λ_{max} (solvent)
Chlorophyll *a*	CHL A	Sigma	88.15 [a]	662 nm (acetone)
Fucoxanthin	FUCO	Repeta	150.7 [b]	446 nm (acetone)
Diadinoxanthin	DIADIN	Repeta	262.0 [c]	448 nm (ethanol)
β-carotene	B-CAR	Sigma	262.0 [d]	452 nm (ethanol)
Canthaxanthin	CANTH	Repeta	222.9 [b]	474 nm (acetone)
Etioporphyrin I	ETIOPOR	Aldrich	(231.9) [e]	394 nm (acetone)
Chlorophyll *b*	CHL B	Sigma	51.36 [a]	646 nm (90% acetone)
Peridinin	PERIDININ	Repeta	134.0 [f]	468 nm (acetone)
Zeaxanthin	ZEAX	Roth	254.0 [d]	452 nm (ethanol)
Lutein	LUT	Roth	255.0 [d]	446 nm (ethanol)
19′ but-fucoxanthin	19′BUT	Isolated	160.0 [g]	446 nm (ethanol)
19′hex-fucoxanthin	19′HEX	Isolated	160.0 [g]	446 nm (ethanol)
Alloxanthin	ALLOX	Isolated	262.0 [h]	454 nm (ethanol)
Prasinoxanthin	PRASINOX	Isolated	160.0 [i]	454 nm (ethanol)
Methyl pyrophaeophorbide *a*	MPP-A	Spirutec	(75.53) [e]	666 nm (Acetone)

[a] Jeffrey and Humphrey (1975); [b] Repeta (pers. commun., 1988); [c] Goodwin (1955); [d] Davies (1976); [e] measured in the study; [f] Jeffrey and Haxo (1968); [g] value for fucoxanthin (cf. Vesk and Jeffrey, 1987); [h] value for diadinoxanthin (cf. Gieskes and Kraay, 1984); [i] value for fucoxanthin.

concentration) and stored in the dark at −20°C prior to distribution. An absorption spectrum and HPLC chromatogram were supplied with each standard. Three replicates of each standard and five unknown mixed standards (see below) were provided to each laboratory. A strict protocol was provided to each laboratory for sample storage upon arrival.

The ampules were shipped on dry ice to participating laboratories by various express mail services. Shipment times were on the order of two to three days and all shipments arrived with sufficient dry ice to ensure pigment stability.

To validate the experimental results, the standards were monitored for stability during the intercalibration exercises. Every two months for a period of approximately one year, ampules from each batch were randomly selected, opened and analyzed in duplicate by HPLC (Bidigare et al., 1989) at Texas A&M University.

2.2. Interlaboratory comparisons

Eight laboratories, randomly designated as laboratories *A*, *B*, *C*, *D*, *E*, *F*, *G*, and *H*, participated in the intercalibration exercise (under conditions of anonymity) and included JGOFS pigment analysts from four countries (United States, Germany, The Netherlands and Canada).

The interlaboratory comparison consisted of three separate exercises, each designed to evaluate spectrophotometer and HPLC analytical performances. To avoid sources of error other than instrumental, a thorough protocol was provided to each laboratory for preparation and analysis of the pigment standards and mixtures.

The ampules containing the pigment standards were opened by participating laboratories just prior to HPLC analysis and transferred to a 1 cm pathlength cuvette to determine absorbances at the wavelengths (i.e. λ_{max}) specified in Table 1. A reading at 750 nm was subtracted from the absorbance measured at λ_{max} to correct for scattering. The average absorbance reading of the three replicate ampules was reported.

Immediately following spectrophotometric analysis, the pigment standards were injected onto a chromatographic system to calculate individual pigment response factors (i.e. ng of pigment injected divided by the resulting peak area) (Table 2). Peak areas were calculated as the area of the parent pigment plus the areas of any structurally-related derivatives or isomers (e.g. chlorophyll *a'*, cis-fucoxanthin, etc.; cf. Bidigare, 1991). The response factors were calculated using the concentrations provided with the standards, thus, the chromatographic and spectrophotometric errors could be quantified separately. These

Table 2
Specifications of equipment used by participant laboratories during the HPLC intercomparison exercises

Lab ID	Flow rate (ml/min)	Loop size (μl)	Volume injected (μl)	Column length (mm)	Column width (mm)	Column part. size (μm)	Absorbance detector (nm)	Fluorescence detector (yes/no)
*A*1–3	6.0	500	Full	100	8.0	5	436	yes
*B*1	1.0	100	Full	150	4.6	3	440	no
*B*2,*B*3	1.0	200	Full	160	4.6	3	440	no
*C*1,*C*2	1.0	20	Full	250	4.0	5	436	yes
*C*3	1.0	20	Full	300	3.9	5	436	yes
D	2.5	400	50–100	250	4.0	5	440	yes
E	3.0	100	Full	250	4.0	5	436	yes
*F*1	1.1	190	Full	250	4.6	5	436	yes
*F*2	1.1	100	Full	250	4.6	5	436	yes
*F*3	1.0	100	Full	250	4.0	5	436	no
*G*1–3	1.5	100,1000	Full	150	3.5	3	440	no
*H*1–3	1.0	500	250	75	4.6	3	430	yes

All laboratories used C_{18} columns and solvent gradients for separation. All laboratories used absorption detection for quantification. When used, fluorescence was an aid for pigment identification. Letters in the first column refer to laboratory designation and numbers refer to a specific intercomparison exercise.

response factors were used to determine the pigment composition of the unknown mixtures of algal pigments.

After instrument calibration, ampule 1 of the three replicates of the unknown mixed standard was injected three times, and ampules 2 and 3 were each injected once. Three injections from ampule 1 were used to estimate chromatographic precision. The mean of the three injections, was used together with single injections from the other two replicate ampules to examine interampule variability.

This same protocol was repeated during each of the three intercalibration exercises, and a total of five mixed standards were analyzed by participant laboratories. Intercalibration I (Mixed Standards I, II and III) was carried out in winter 1989, Intercalibration II (Mixed Standard IV) in summer 1989, and Intercalibration III (Mixed Standard V) in summer 1990. Mixed Standard I (EPA Spectrophotometric Standard, Concentration No. 2) contained chlorophyll *a*, fucoxanthin, and β-carotene; Mixed Standards II and III contained chlorophyll *a*, fucoxanthin, diadinoxanthin, and β-carotene; Mixed Standard IV contained chlorophyll *a*, chlorophyll *b*, peridinin, lutein, and zeaxanthin; and Mixed Standard V contained chlorophyll *a*, 19′-hexanoyloxyfucoxanthin, alloxanthin, 19′-butanoyloxyfucoxanthin, and prasinoxanthin.

3. Results and discussion

3.1. Pigment stability study

Based on HPLC analysis, parent pigment concentrations in ampules were stable for at least one year under the storage conditions used to maintain the standards (−20°C, dark, N_2 atmosphere). The results shown in Fig. 1 are expressed as a percent of the parent pigment peak area measured during the course of the stability study relative to those measured at t_0 (i.e. the initial parent pigment peak areas). Most of the points fell within ±5% of the initial values and trends suggesting degradation were not observed. Most of these deviations probably result from time-dependent variations in HPLC detector response and not from changes in parent pigment concentration. Periodic detector calibration during the stability study may have reduced this variability.

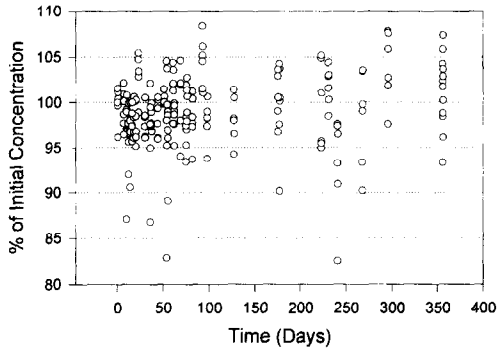

Fig. 1. Stability of external and internal standards measured over the course of approximately one year.

3.2. Comparison of spectrophotometric determinations

The accuracy of spectrophotometric measurements could not be evaluated directly since certified pigment standards were not available for use in this study. As such, the deviations of individual laboratories from "mean" consensus values were examined. The variability of spectrophotometric measurements between laboratories was relatively low, as 90% of the pigments analyzed were within ±6% of the mean (consensus) values. Only three data points reported (for chlorophylls *a* and *b*, and prasinoxanthin) out of 98 could be considered outliers (Sprent, 1993). As expected, the highest variability was found in standards with the lowest pigment concentrations. The chlorophyll *a* and chlorophyll *b* standards measured during the second exercise exhibited the highest degree of variability. Chlorophyll *a* was analyzed during each of the three exercises and its variability was always high relative to the carotenoid standards (data not shown). A potential source of this interlaboratory variability may be related to spectrophotometer optical configuration and is discussed in more detail below.

3.3. Comparison of HPLC pigment determinations

The interampule (i.e. intralaboratory) coefficient of variation (C.V.) was calculated using the mean of

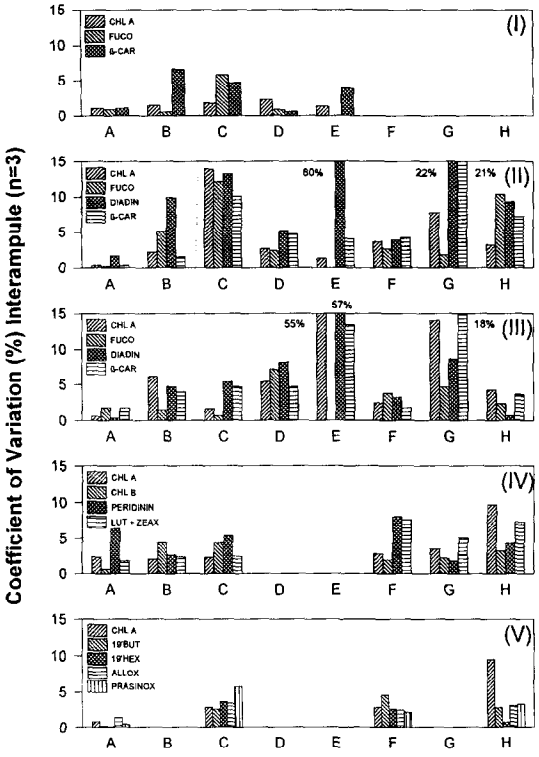

Fig. 2. Chromatographic interampule variability for the different pigments from Mixed Standards I, II, III, IV and V. Missing points correspond to no reported data. *A*, *B*, *C*, *D*, *E*, *F*, *G* and *H* are the assigned codes for each laboratory.

the three injections from ampule 1 as a single replicate and the two single injections from ampules 2 and 3 (Fig. 2). Most laboratories had a C.V. of 3–4% for most pigments. However, some of the laboratories did not show a consistent pattern throughout the study. Laboratories *A*, *B* and *F* were the most precise with an average C.V. of $< 4\%$. In general, C.V. values for laboratories *D* and *H* were $< 5\%$. With the exception of Mixed Standard II (C.V. $> 10\%$), laboratory *C* also exhibited a good agreement among replicates. Laboratories *E* and *G* showed poor reproducibility for Mixed Standards II and III, and good reproducibility for Mixed Standards I, IV and V. The largest variability was found for diadinoxanthin (Mixed Standard II), chlorophyll *a* (Mixed Standard III), and diadinoxanthin (Mixed Standard III) reported by laboratory *E* (C.V. = 60%, 55% and 57%, respectively).

When examining interlaboratory variability, outliers were detected (Sprent, 1993) in all of the Mixed Standards. The mean of the three ampules was used for this comparison. To avoid the bias produced by these outliers and find a "consensus value", pigment concentration estimates were compared against the median of the pooled results from the different laboratories, not the mean. The results are shown in Fig. 3 after normalization by dividing the difference between the estimate and the median, by the median.

The estimation of individual pigment concentrations within exercises were statistically different ($p < 0.05$) using parametric (ANOVA) or non-parametric (Kruskal-Wallis) tests. Pairwise comparisons between laboratories (Tukey HSD test, SYSTAT software package) showed that some of these differences were due to differences of a single laboratory against the rest. On some occasions, the unique measurement coincided with a detected outlier, but not in all cases. For example, for Mixed Standard I, the esti-

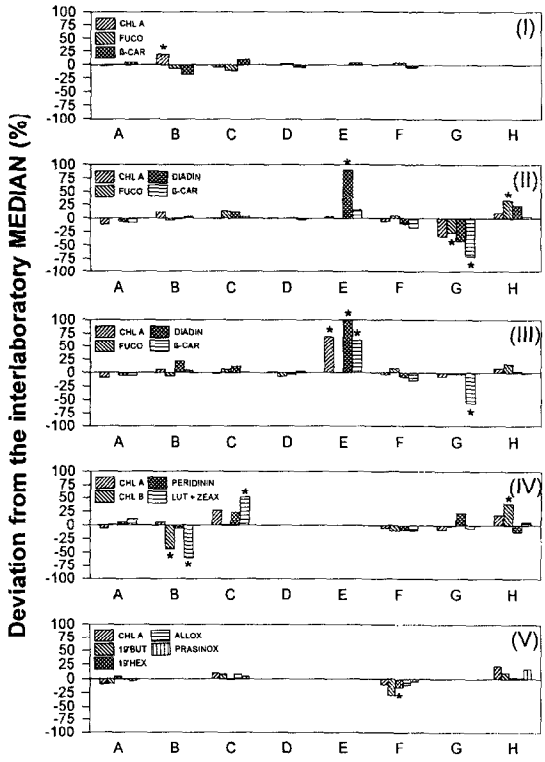

Fig. 3. Chromatographic variability observed among laboratories during the intercalibration exercises. Missing points are no reports. Asterisks indicate outlier values. Labels as in Fig. 2.

mates of chlorophyll *a* and β-carotene by laboratory B were statistically different from those of the other laboratories, but only the result for chlorophyll *a* could be considered as an outlier (cf. Sprent, 1993). Furthermore, it is surprising that the estimates of diadinoxanthin concentration for Mixed Standards II and III by laboratory E were not different from all the other laboratories (Fig. 3). The explanation for this result is the high interampule variability in the estimation of this pigment by laboratory E (Fig. 2). Clearly, interampule variability could distort the interpretation of results if conclusions are based on statistics alone. For example, while a 6% difference between the estimates of 19-hexanoyloxyfucoxanthin concentration by laboratories *A* and *C* (Mixed Standard V) was statistically significant ($p < 0.05$), a difference of 37% between diadinoxanthin estimates by laboratories *F* and *H* (Mixed Standard II) was not.

Examination of individual pigment concentrations, however, does not provide a general picture of the interlaboratory comparison because on many occasions paired comparison between laboratories yielded significant differences for only some of the pigments analyzed. A Friedman test (using the approach of Iman and Davenport, 1980) was applied designating the different pigments as random blocks. This test looks for biases among laboratories within exercises taking into account all the pigments. Mixed Standard I was the only case where laboratories did not have a significant effect on pigment estimates. This result indicates that, when considering all pigments together, the laboratories showed consistent trends in their estimates. Two laboratories, *F* and *H*, showed tendencies to underestimate and overestimate, respectively. The average underestimation by *F* was ~ 10%, and in most cases *H* overestimated by < 20%. It is very likely that these trends are the reason for the significant differences in the overall estimation of pigment concentrations among laboratories.

The differences among exercises shown by some laboratories and the differences in the concentration estimate (relative to the consensus value) for the different pigments from the same sample suggest that the largest variability (> 20% of the median) was due to random factors. There are several factors that could account for the more systematic intermediate deviations exhibited by laboratories *F* and *H*. Peak integration parameters and sample handling could be important sources of error. However, repetitive injections from the same ampule and replicates from different ampules gave similar variability (a result not mentioned until now), indicating that sample handling did not increase chromatographic variability. Manipulation of chromatograms by computer software can considerably improve the accuracy of peak integration. Another source of error could be the use of inadequate HPLC equipment for pigment analysis, but there was not evidence of a connection between erroneous results and equipment specifications (Table 2).

3.4. Variability between spectrophotometer types

As discussed above, the variability associated with the spectrophotometric measurements of chlorophylls *a* and *b* was always high. One possible explanation could be the use of different measurement systems as both diode array and monochromator spectrophotometers were used in this study. Because of its optical configuration, the diode array spectrophotometer is potentially susceptible to fluorescence contamination artifacts because a "white" light source is used for making absorption measurements. Since the red fluorescence emitted by chlorophylls *a* and *b* overlaps with the wavelengths used for determining their concentrations (662 and 646 nm, respectively; Table 1), an underestimation of absorption could result. This was investigated by comparing the absorbance readings obtained for chlorophyll *a*, chlorophyll *b* and β-carotene using two monochromator-based instruments (Perkin Elmer Lambda 3B and Beckman DU 640) and a diode array-based instrument (Hewlett Packard 8452A). Absorbance readings obtained for chlorophylls *a* and *b* with the two monochromator spectrophotometers were not statistically different ($p > 0.05$; Table 3). However, chlorophyll *a* and chlorophyll *b* absorbances measured using the diode spectrophotometer were significantly lower ($p < 0.05$) and resulted in 6 and 9% underestimations, respectively. By contrast, absorbance readings obtained for the non-fluorescent β-carotene standard were not statistically different among instruments ($p > 0.05$). To minimize this source of error, it is strongly recommended

that concentrations of pigment standards only be determined with monochromator-based spectrophotometers.

3.5. Errors associated with co-eluting pigments

Another source of error in chromatographic measurements is the co-elution of more than one compound in a single peak. For example, many of the participant laboratories were unable to separate lutein from zeaxanthin in Mixed Standard IV. Thus, the results presented in Figs. 2 and 3 are expressed in terms of "lutein plus zeaxanthin". This co-elution problem may in part explain the ±60% deviation exhibited by laboratories *B* and *C* for this pigment pair. Currently, there are no published HPLC methods capable of separating all algal pigments of interest in a single chromatographic analysis. While the method recommended by SCOR Working Group 78 (Wright et al., 1991) is capable of separating lutein from zeaxanthin, it lacks the ability to separate monovinyl chlorophyll *a* from divinyl chlorophyll *a* (among other pairs). This co-elution can lead to a significant overestimation of total chlorophyll *a* concentration (Goericke and Repeta, 1993; Letelier et al., 1993) and, thus, has serious implications for the accurate "ground-truthing" of ocean color satellite sensors (e.g. SeaWiFS).

This source of analytical error could be minimized by taking advantage of the different spectral signatures displayed by these pigments in the blue region of the visible spectrum. Response factors for these chlorophyll *a*-related pigments were determined at two wavelengths (436 and 450 nm) by monitoring the absorption signal at each wavelength during separate injection of pure standards of these co-eluting pigments. These response factors were then used in conjunction with dichromatic equations (Kolthoff et al., 1969) to spectrally resolve mixtures of these co-eluting pigments:

$$A_1 = \varepsilon_{x1} C_x + \varepsilon_{y1} C_y \quad (1)$$

$$A_2 = \varepsilon_{x2} C_x + \varepsilon_{y2} C_y \quad (2)$$

where ϵ_{x1}, ..., ϵ_{y2} are the reciprocal of the response factors calculated for pigments X and Y at λ_1 and λ_2 and, A_1 and A_2 are the respective peak areas at those wavelengths. Solving these two simultaneous equations allows the calculation of individual concentrations of the co-eluting pigments, C_x and C_y, from a single chromatographic analysis as follows:

$$C_x = (\varepsilon_{y2} A_1 - \varepsilon_{y1} A_2)(\varepsilon_{y2}\varepsilon_{x1} - \varepsilon_{y1}\varepsilon_{x2})^{-1} \quad (3)$$

$$C_y = (\varepsilon_{x1} A_2 - \varepsilon_{x2} A_1)(\varepsilon_{x1}\varepsilon_{y2} - \varepsilon_{x2}\varepsilon_{y1})^{-1} \quad (4)$$

This approach was evaluated with the SCOR-recommended Wright et al. (1991) HPLC method and was found to provide excellent results for several mixtures of monovinyl and divinyl chlorophyll *a*. The comparison of this spectral approach and the physical separation of both pigments using a recently published reverse-phase C_8 HPLC technique (Goericke and Repeta, 1993) is shown in Fig. 4. Recent studies document that, in natural phytoplankton populations, divinyl chlorophyll *a* can represent 20–40% of the total chlorophyll *a* (Goericke and Repeta, 1993; Letelier et al., 1993; Bidigare and Ondrusek, 1996). The use of a single response factor (i.e. that determined for monovinyl chlorophyll *a*) would have resulted in a 15–25% overestimation of total chlorophyll *a* concentration (Fig. 5). The co-elution of two or more pigments in a single peak could potentially represent an unavoidable source of a variable error. However, for the monovinyl–di-

Table 3
Comparison of spectrophotometer types

Pigment (λmax, solvent)	PE Lambda 3B Mean ± SD (*n*)	BM DU 640 Mean ± SD (*n*)	HP 8452A Diode Array Mean ± SD (*n*)	% Difference (DA − MC)/MC
Chlorophyll *a* (662 nm, acetone)	0.1827 ± 0.0006 (3)	0.1873 ± 0.0017 (3)	0.1740 ± 0.0006 (3)	−5.95 [a]
Chlorophyll *b* (646 nm, 90% acetone)	0.1753 ± 0.0006 (3)	0.1754 ± 0.0003 (3)	0.1594 ± 0.0004 (3)	−9.10 [a]
β-carotene (452 nm, ethanol)	0.1703 ± 0.0006 (3)	0.1710 ± 0.0002 (3)	0.1689 ± 0.0007 (3)	−1.03

PE = Perkin-Elmer; BM = Beckman; HP = Hewlett-Packard; DA = diode array type; MC = monochromator type.
[a] These significant underestimates probably result from chlorophyll fluorescence contamination.

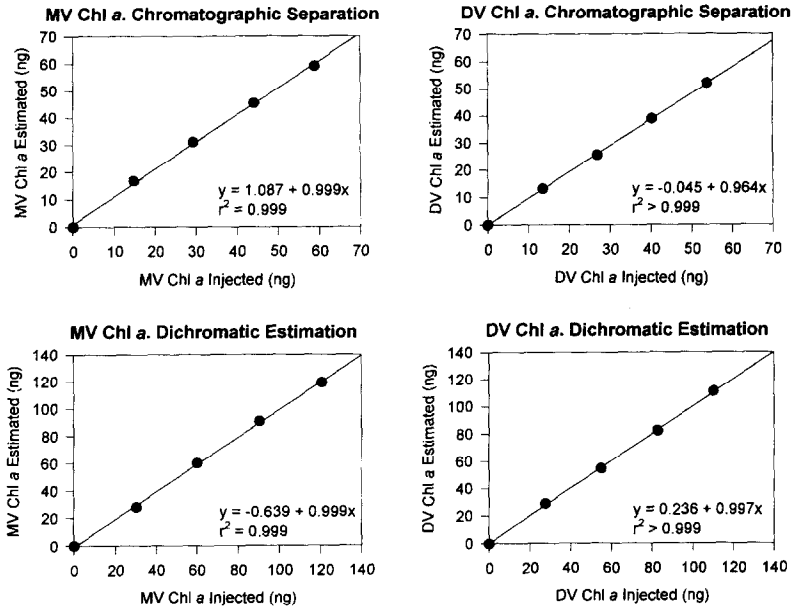

Fig. 4. Accuracy of the chromatographic and the dichromatic methods for the estimation of monovinyl chlorophyll *a* (MVChl *a*) and divinyl chlorophyll *a* (DVChl *a*) concentrations. The concentration of the divinyl chlorophyll *a* standard was calculated using the same extinction coefficient as for monovinyl chlorophyll *a*.

vinyl chlorophyll *a* pair (and probably for the monovinyl–divinyl chlorophyll *b* pair) a simple and reliable corrective procedure is recommended. It should be noted that the magnitude of the analytical error shown in Fig. 5 depends on the differences in the response factors for each pigment at the monitored wavelengths and, thus, it is different for each HPLC system configuration.

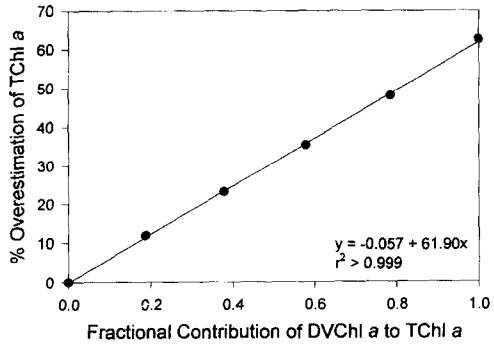

Fig. 5. Overestimation of total chlorophyll *a* (i.e. TChl *a* = MVChl *a* + DVChl *a*) concentration in the presence of DVChl *a* when using the MVChl *a* response factor for the co-eluting chromatographic peak. The % overestimation is calculated as: (Chl *a* − TChl *a*)(TChl *a*)$^{-1}$ × 100%. Chl *a* is the concentration of total chlorophyll *a* calculated using the MVChl *a* response factor.

4. Conclusions

In this comparison exercise, the minimum interlaboratory variability is shown. Usually, laboratories prepare their own standards and choose among several extinction coefficients available in the literature for standard calibration. In the current study, however, standards of known concentration were provided to calibrate HPLC detectors. The comparison among spectrophotometric determinations gave good agreement, as 90% of the pigments analyzed were within ±6% of the "mean" consensus values (Fig. 6A). Deviations from the median consensus values for HPLC-determined chlorophyll *a*, carotenoid, and pooled pigment concentrations are shown in Fig. 6B, C and D, respectively. Seventy and 90% of the chlorophyll *a* measurements agreed to within ±10 and ±20% of the median consensus values, respectively. For the carotenoids and pooled pigments, the results were quite similar as 65 and 85% of the

measurements agreed to within ±10 and ±20% of the median consensus values, respectively. When comparing both the spectrophotometric and the chromatographic sources of error, the error associated with the spectrophotometric analyses was small. However, it is an additional source of variability which should be considered in the analysis of algal pigments. As mentioned above, there are several steps in the chromatographic protocol that give rise to variability. The results of the present intercalibration suggest that systematic error for any given laboratory is usually < 20%. Larger differences seemed to be a consequence of random errors and some of them could be detected by the large variability between replicate injections. Besides operator error, two possible sources of random error are (1) the precipitation of non-polar pigments (e.g. β-carotene) resulting from the addition of water prior to injection (this step is required in some protocols for HPLC pigment analysis), and (2) in the case of manual injections, the incomplete filling of the sample loop (an injection of 2–3 times the loop volume is required for > 95% filling of the sample loop and 4–5 times for > 99%). It is recommended that an

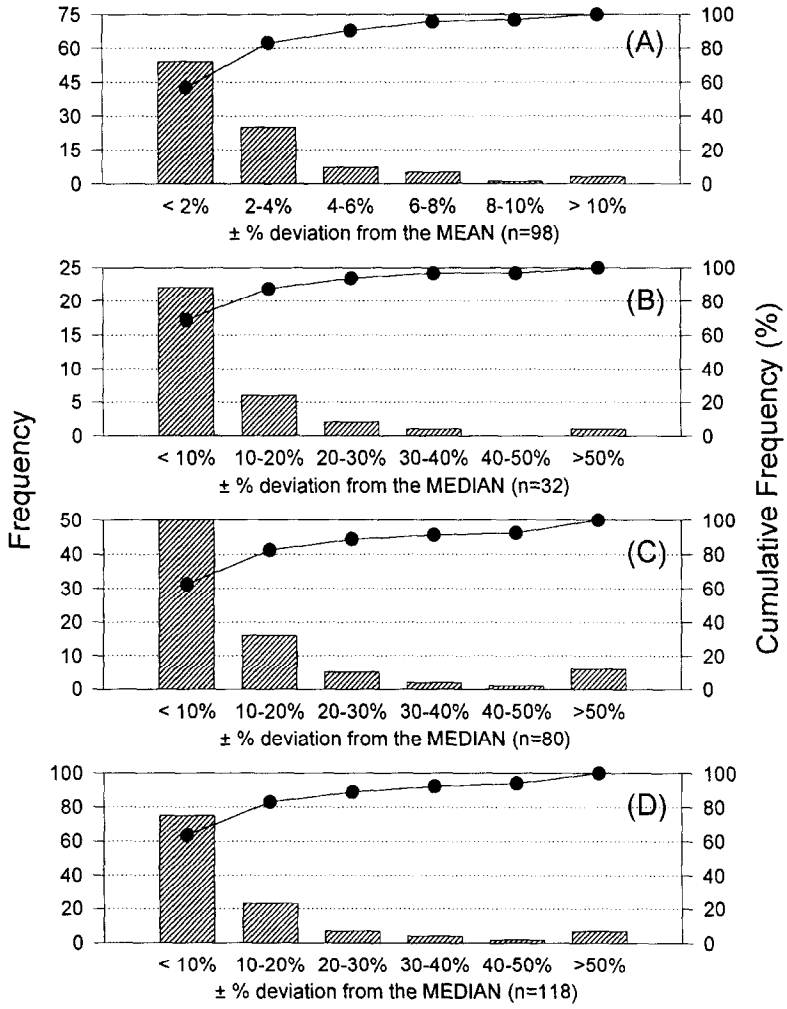

Fig. 6. Summary of results from the intercalibration exercises. Bars represent frequency. Lines indicate the cumulative frequency. (A) shows the variability among spectrophotometer readings; (B), (C), and (D) show the chromatographic variability for chlorophyll *a*, carotenoids, and all pigments (including chlorophyll *b*), respectively.

internal standard (e.g. canthaxanthin) be used to correct for errors associated with partial HPLC injections. Periodic injections of stable external standards can also reduce errors associated with variations in detector response.

In summary, good agreements among spectrophotometric and chromatographic measurements were achieved by most of the participant laboratories. However, outliers were not rare for chromatographic analyses. Since the samples were processed with replication, problems with single injections are not a source of error. The substantial variability found in this exercise where standards were provided, strongly suggests the necessity of periodic calibration checks using external standards and/or standard reference materials to ensure reliable results for laboratory and field pigment studies.

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