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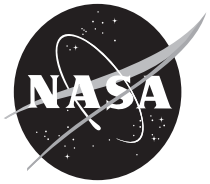

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December 2009

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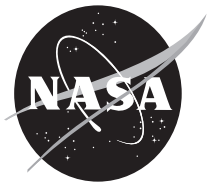

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Abstract

Seven international laboratories specializing in the determination of marine pigment concentrations using high performance liquid chromatography (HPLC) were intercompared using *in situ* samples and a mixed pigment sample. The field samples were collected primarily from oligotrophic waters, although mesotrophic and eutrophic waters were also sampled to create a dynamic range in chlorophyll concentration spanning approximately two orders of magnitude (0.020–1.366 $\mathrm{mg\,m^{-3}}$). The intercomparisons were used to establish the following: a) the uncertainties in quantitating individual pigments and higher-order variables (sums, ratios, and indices); b) the reduction in uncertainties as a result of applying quality assurance (QA) procedures; c) the importance of establishing a properly defined referencing system in the computation of uncertainties; d) the analytical benefits of performance metrics, and e) the utility of a laboratory mix in understanding method performance. In addition, the remote sensing requirements for the *in situ* determination of total chlorophyll *a* were investigated to determine whether or not the average uncertainty for this measurement is being satisfied.

PROLOGUE

The first Sea-viewing Wide Field-of-view Sensor (SeaWiFS) High Performance Liquid Chromatography (HPLC) Round-Robin Experiment (SeaHARRE-1) took place in 1999 (Hooker et al. 2000). It emphasized oligotrophic and mesotrophic regimes (northwest African upwelling and the Mediterranean Sea), involved four laboratories using four different methods (three C_8 and one C_{18}), and was based on 11 duplicates and triplicates (12 triplicates were planned). SeaHARRE-2 took place in 2002 (Hooker et al. 2005) and emphasized mesotrophic and eutrophic regimes (Benguela Current), involved eight laboratories using five different methods (four C_8 and four C_{18}), and was based on 12 duplicates (12 triplicates were planned).

The planning for SeaHARRE-3 coincided with an anticipated field deployment across the central part of the South Pacific gyre as part of the Biogeochemistry and Optics South Pacific Experiment (BIOSOPE) cruise. The field campaign began in Papeete (Tahiti), included a stop at Rappa Nui (Easter Island), and ended in Tulcahuano (Chile) after sampling in the Chilean upwelling. The opportunity to sample oligotrophic, mesotrophic, and eutrophic waters in one cruise ensured the field samples would come from a significant dynamic range in chlorophyll *a* concentration. Because part of the data set would include the unusual opportunity of including hyper-oligotrophic samples with many more pigments at detection limits than usual, 24 triplicates were planned to ensure statistical reliability.

Seven international laboratories agreed to participate in SeaHARRE-3 with so-called *validated* HPLC methods:

1. The Australian Commonwealth Scientific and Industrial Research Organisation (CSIRO),
2. The Danish DHI Institute for Water and Environment (DHI),
3. The American Horn Point Laboratory (HPL) University of Maryland Center for Environmental Science (UMCES),
4. The European Joint Research Centre (JRC),
5. The French *Laboratoire d'Océanographie de Villefranche* (LOV),
6. The South African Marine and Coastal Management (MCM†), and
7. The American Center for Hydro-Optics and Remote Sensing (CHORS) at the San Diego State University (SDSU).

The scientists involved in SeaHARRE-3 are given in Appendix A. The other laboratories that have participated in SeaHARRE intercomparisons are the Canadian Bedford Institute of Oceanography (BIO), and the British Plymouth Marine Laboratory (PML). As shown in Table 1, of all the laboratories involved with SeaHARRE activities, only HPL, LOV, and MCM have participated in every one, although CSIRO, DHI, and SDSU have now participated in two each.

Table 1. The laboratories, with their corresponding countries and codes, that have participated in the three SeaHARRE activities.

Laboratory and Country		*Code*	*SeaHARRE*		
BIO	Canada	*B*		2	
CSIRO	Australia	*C*		2	3
DHI	Denmark	*D*		2	3
HPL	United States	*H*	1	2	3
JRC	Italy	*J*	1		3
LOV	France	*L*	1	2	3
MCM	South Africa	*M*	1	2	3
PML	United Kingdom	*P*		2	
SDSU	United States	*S*		2	3

The aforementioned concept of a validated method requires some additional explanation, because there is no

† MCM is the marine branch of the South African Department of Environmental Affairs and Tourism.

Table 2. The methods used by all the laboratories in the three HPLC round-robin intercomparisons. The laboratory codes indicate the method used by a particular laboratory as a function of the three round robins.

Intercomparison Activity	*Gieskes and Kraay (1989)*[1]	*Wright et al. (1991)*[1]	*Vidussi et al. (1996)*[2]	*Barlow et al. (1997)*[2]	*Van Heukelem and Thomas (2001)*[2]
SeaHARRE-1		*J*	*L*	*M*	*H*
SeaHARRE-2	*B*	*C D S*	*L*	*M P*	*H*
SeaHARRE-3		*J* S^3		*M*	*C D H L* S^3

[1] A C_{18} column method.
[2] A C_8 column method.
[3] SDSU switched from the Wright et al. (1991) method to the Van Heukelem and Thomas (2001) method, but executed both, so they could be compared. The C_8 method is denoted S_8 and the C_{18} method is denoted S_{18}.

external process or independent agency that certifies an HPLC method is validated. The validation process is currently conceived and executed by the individual laboratory based on the sampling requirements and research objectives associated with the method. Consequently, validation occurs largely in isolation and relies heavily on a temporal evaluation of the calibration procedures, although some laboratories use more sophisticated evaluation criteria. As first demonstrated during SeaHARRE-1 (Hooker et al. 2000), intercomparing methods is a more robust mechanism for demonstrating the degree of validation for a particular method, and this is a permanent objective of the SeaHARRE activity.

Method validation procedures are important because they describe the level of measurement uncertainty associated with reported pigment concentrations. In the absence of the aforementioned external process or independent agency, however, validation activities of individual laboratories have emerged with varying emphases, often tailored to the specific research conducted by the individual laboratory. The products of validation, therefore, may not always yield the kind of information useful to intercomparing a diverse set of laboratory results over time or between laboratories. Consequently, for a more thorough understanding of measurement uncertainty and its relationship to accuracy in the analysis of field samples, intercalibration exercises are necessary, but the methods of the participating laboratories are best evaluated according to a common set of procedures and products.

The culmination of this philosophy of quantitative assessment was the drafting of a set of *performance metrics* during SeaHARRE-2 (Hooker et al. 2005). The evaluation of the performance metrics—in particular the corresponding accuracy and precision parameters—resulted in some participants abandoning the methods they were using for a single (more modern) method with superior performance parameters: the Van Heukelem and Thomas (2001) method. Consequently, the maximum diversity in methods was achieved during SeaHARRE-2, and the least during SeaHARRE-3 (Table 2).

The reduction in method diversity was not expected. There was a strong feeling that the approach used in the joint Global Ocean Flux Study (JGOFS)—selecting one method, in this case the Wright et al. (1991) method, and making it the protocol—should not be repeated, even unintentionally, because it stifles creativity. The practical benefit of adopting a proven method instead of investing an unknown amount of time and resource in trying to improve a method, however, was simply too alluring. The potential pitfall of this approach—which is quantified here in many aspects—is underestimating the difficulty of implementing a new method with all its attendant detail.

The overall results of SeaHARRE-3 are presented in Chapter 1 and the individual methods of the eight laboratories are presented in Chapters 2–8, respectively. A summary of the material presented in each chapter is given below.

1. SeaHARRE-3 Methods, Data, and Analysis

The focus of this study was the estimation of uncertainties in quantifying a diverse set of chlorophyll and carotenoid pigment concentrations for a variety of HPLC methods and related procedures used in the analysis of predominantly oligotrophic waters. The results suggest uncertainties are at a minimum in mesotrophic samples and increase as concentrations become larger and smaller, with the largest increase associated with eutrophic samples (confirmed by prior SeaHARRE activities). The chlorophyll *a* accuracy requirements for ocean color validation activities can be reliably satisfied in predominantly oligotrophic conditions by a quality-assured method. The performance metrics proposed during SeaHARRE-2 were shown to be well conceived, and properly distinguished the methods from one another at a level in keeping with the expected accomplishments of quantitative analysis. Based on the robustness of the performance metrics, it seems warranted to establish performance metrics for the higher-order data products, and proposed thresholds are presented. The higher-order associations in pigments (i.e., sums and ratios) repeatedly confirmed the basic conclusions regarding method performance first established with the individual pigments, and showed that the uncertainties of the individual pigments strongly influence the uncertainty budget for the higher-order variables. There is a significant statistical difference between the results obtained with the C_8 and

C_{18} methods with the former outperforming the latter, but the degraded performance of the latter was likely caused by the procedures used by the laboratories involved rather than intrinsic deficiencies with the C_{18} method being used. The laboratory mix proposed during SeaHARRE-2, and now marketed by DHI, can provide considerable insight into many of the performance aspects of the HPLC method being used.

2. The CSIRO Method

The CSIRO method is a modified version of the Van Heukelem and Thomas (2001) method. This method has the capability to resolve approximately 35 different pigments with baseline resolution of divinyl and monovinyl chlorophyll *a*, zeaxanthin and lutein, and partial separation of divinyl and monovinyl chlorophyll *b*. Samples are extracted over 15–18 h in an acetone solution before analysis by HPLC using a C_8 column and binary gradient system, with an elevated column temperature. Pigments are identified by retention time and absorption spectra from a photo-diode array (PDA) detector. The method is regularly validated with the use of both internal and external standards and individual pigment calibration. The detection limit of most pigments is within the range of from 0.001–0.005 mg m^{-3}. The method has been used at CSIRO since August 2004, and has proven to offer a good balance between accuracy of pigment composition and concentration, and the number of samples analyzed. The separation of the divinyl and monovinyl forms of chlorophylls *a* and *b* has allowed a complete analysis of samples from the oligotrophic regions of the world ocean.

3. The DHI Method

The HPLC method used at DHI is a somewhat modified version of the HPL method (Van Heukelem and Thomas 2001). In comparison with the DHI method used during SeaHARRE-2 (Wright et al. 1991), the HPL method separates divinyl from monovinyl chlorophyll *a*, and chlorophyll c_1 from chlorophyll c_2. Furthermore, the HPL method provided state-of-the-art results during SeaHARRE-2, so the capabilities of the method were as highly rated as possible. When adapted to the DHI HPLC system, the HPL method, however, did not initially provide the same excellent results for the analysis of the SeaHARRE-3 samples, in terms of the precision of the results. Subsequent troubleshooting identified three problems: a) a fault in the autoinjector, b) an inappropriate tetrabutyl ammonium acetate (TbAA) buffer, and c) the HPLC vials were not completely airtight (so there was some evaporation of the extract while it resided in the autosampler compartment). The poor precision was identified prior to submitting the SeaHARRE-3 results by checking the reproducibility of standardized mixed pigments (from DHI Mix-101), which were distributed to all of the participants as part of the intercalibration exercise. This emphasizes the usefulness of such pigment mixtures and the necessity of quality assurance for detecting method problems, which have an impact on the results.

4. The HPL Method

The HPL method was developed for use with a variety of water types. Many pigments important to freshwater, estuarine, and oceanic systems are baseline resolved and quantitatively reported, including divinyl and monovinyl chlorophyll *a*. The method is based on a C_8 HPLC column, a methanol-based reversed-phase gradient solvent system, a simple linear gradient, and an elevated column temperature (60°C). The method can provide quantitative results for up to 25 pigments with qualitative information for additional pigments. Quality assurance measurements are made during sample analysis to confirm that the method performance is within expectations. Investigations into the uncertainties in the method show the 95% confidence limits were estimated as a) 0.5–3.8% for precision of replicate injections within and across sequences, b) 3.2% for chlorophyll *a* calibration reproducibility, and c) 5.1% for chlorophyll *a* method precision, including filter extraction and analysis.

5. The JRC Method

The HPLC method used at the JRC follows the JGOFS protocols (JGOFS 1994) and is a modified version of the method presented in Wright et al. (1991). It does not allow the separation of divinyl chlorophyll *a* and *b* from their respective monovinyl forms. Filters are sonically disrupted, and the pigments are extracted within a 100% acetone solution including an internal standard (*trans*-β-apo-8′-carotenal). The HPLC system used includes a quaternary pump, a three-solvent gradient method, a reversed-phase C_{18} column with an autosampler (both with thermostats), a diode array detector, and a fluorescence detector. The effective limit of detection (computed for the SeaHARRE-3 samples) for the chlorophylls and carotenoids is about 0.0008 mg m^{-3} (for the typical filtration volumes used). In terms of routine sample analysis, this method has been applied almost exclusively to coastal water samples.

6. The LOV Method

The LOV method is derived from the Van Heukelem and Thomas (2001) technique, and applies a sensitive, reversed-phase HPLC procedure for the determination of chloropigments and carotenoids within 28 min. The different pigments, extracted in 100% methanol, are detected using a diode array detector, which permits automatic pigment identification based on absorption spectra. Optical densities are monitored at 450 nm (chloropigments and carotenoids), 667 nm (chlorophyll *a* and derived pigments) and 770 nm (bacteriochlorophyll *a*). The method provides good resolution between most pigments, but uncertainties may arise because of the partial separation of chlorophyll *b* and divinyl chlorophyll *b*, and for the resolution of chlorophyll *c* pigments. It has proven to be efficient over a wide range of trophic conditions, from eutrophic upwelling waters, to the hyper-oligotrophic South Pacific subtropical

gyre. Short- and long-term quality control is monitored regularly to ensure *state-of-the-art* analyses. The injection precision of the method is estimated at 0.4%, and the effective limits of quantitation for most pigments are low ($0.0004\,\mathrm{mg\,m^{-3}}$ for chlorophyll *a* and $0.0007\,\mathrm{mg\,m^{-3}}$ for carotenoids, for the typical filtration volumes used).

7. The MCM Method

The MCM method is a reversed-phase HPLC technique using a binary solvent system following a step linear gradient on a C_8 chromatography column. Baseline separation of monovinyl and divinyl chlorophyll *a* and of lutein and zeaxanthin, partial separation of monovinyl and divinyl chlorophyll *b*, and resolution of other key chlorophylls and carotenoids are achieved in an analysis time of approximately 30 min. The use of *trans*-β-apo-8′-carotenal as an internal standard improves the accuracy of pigment determinations. Providing a pragmatic balance between good analyte resolution and acceptable sample throughput, the method is suitable for the analysis of a wide range of oceanographic seawater samples.

8. The SDSU (CHORS) Method

The CHORS method was developed to provide HPLC phytoplankton pigment analysis support for the NASA Moderate Resolution Imaging Spectroradiometer (MODIS) program. The method is a modified version of Van Heukelem and Thomas (2001) and uses a reversed-phase C_8 column, with a binary solvent gradient. A temperature-controlled autosampler provides continuous sample injection to maintain the quota of 4,000 samples per year run by CHORS. System calibration is monitored and recorded to ensure repeatability and consistency of data products.

Chapter 1

SeaHARRE-3 Methods, Data, and Analysis

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Abstract

The focus of this study was the estimation of uncertainties in quantifying a diverse set of chlorophyll and carotenoid pigment concentrations for a variety of HPLC methods and related procedures used in the analysis of predominantly oligotrophic waters. The results suggest uncertainties are at a minimum in mesotrophic samples and increase as concentrations become larger and smaller, with the largest increase associated with eutrophic samples (confirmed by prior SeaHARRE activities). The chlorophyll *a* accuracy requirements for ocean color validation activities can be reliably satisfied in predominantly oligotrophic conditions by a quality-assured method. The performance metrics proposed during SeaHARRE-2 were shown to be well conceived, and properly distinguished the methods from one another at a level in keeping with the expected accomplishments of quantitative analysis. Based on the robustness of the performance metrics, it seems warranted to establish performance metrics for the higher-order data products, and proposed thresholds are presented. The higher-order associations in pigments (i.e., sums and ratios) repeatedly confirmed the basic conclusions regarding method performance first established with the individual pigments, and showed that the uncertainties of the individual pigments strongly influence the uncertainty budget for the higher-order variables. There is a significant statistical difference between the results obtained with the C_8 and C_{18} methods with the former outperforming the latter, but the degraded performance of the latter was likely caused by the procedures used by the laboratories involved rather than intrinsic deficiencies with the C_{18} method being used. The laboratory mix proposed during SeaHARRE-2, and now marketed by DHI, can provide considerable insight into many of the performance aspects of the HPLC method being used.

1.1 INTRODUCTION

The results obtained in the first two SeaHARRE activities established a strong interest in understanding the uncertainties associated with the principal terms in the equation governing the calculation of the concentration (C) of an individual pigment (P_i) from a field sample. Ignoring the specific details of the basic HPLC processes, because they are presented in detail by Jeffrey et al. (1997a) and Bidigare et al. (2003), the formulation for determining pigment concentration begins with the terms describing the calibration of the HPLC system:

$$\tilde{C}_{P_i} \;=\; \hat{A}_{P_i}\, R_{P_i}, \qquad (1)$$

where $\tilde{C}_{P_i}$ is the amount of pigment injected (usually in units of nanograms), $\hat{A}_{P_i}$ is the area of the parent peak and associated isomers for pigment P_i (usually in milli-absorbance units† or microvolts as a function of time), and R_{P_i} is the response factor. The latter is the calibration coefficient for the HPLC system, and it takes on a separate value for each pigment being quantitated. For the general problem, the response factor is denoted R, but for the specific problem of a particular pigment, it is denoted R_{P_i}. R values are usually expressed as the amount of pigment divided by the peak area.

† A milli-absorbance unit is denoted mAU.

The formulation given in (1) is based on a single-point calibration wherein one or more injections of a calibration standard at a known concentration is injected onto the HPLC column. An alternative approach is to create a dilution series of the pigment standard, inject these one at a time, and then fit the response of the HPLC system to a linear function ($y = mx + b$) using least-squares analysis (this is also referred to as a *multipoint* calibration). In this case, pigment concentration is computed as

$$\tilde{C}_{P_i} = \frac{\hat{A}_{P_i} - b_i}{m_i}, \tag{2}$$

where m_i is the slope (equating change in peak area with change in amount) and b_i is the y-intercept.

The formulation presented in (2) can be expressed to follow (1) as follows:

$$\tilde{C}_{P_i} = \hat{A}_{P_i} \left[\frac{1 - (b_i / \hat{A}_{P_i})}{m_i} \right], \tag{3}$$

where the equivalent R_{P_i} for (1) is given by the terms in brackets. If the linear regression is forced through zero, $b_i = 0$, and (3) becomes

$$\tilde{C}_{P_i} = \frac{\hat{A}_{P_i}}{m_i}, \tag{4}$$

and $R_{P_i} = 1/m_i$ (note that the inverse slope is change in amount divided by change in peak area, which matches the definition for R). In this context, it is convenient to reconsider the definition of R_{P_i}, which some authors have done (Bidigare et al. 2003), as the inverse of the original definition, that is, $F_{P_i} = 1/R_{P_i}$ and (1) becomes

$$\tilde{C}_{P_i} = \frac{\hat{A}_{P_i}}{F_{P_i}}. \tag{5}$$

The advantage of this approach is F_{P_i} follows directly from the slope of the linear calibration curve and, for the common case of forcing the slope through zero, $F_{P_i} = m_i$. For the purposes of this study, the majority of the methods used the original definition of R, so it is retained hereafter.

The governing equation for the determination of pigment concentration can be expressed as

$$C_{P_i} = \frac{V_x}{V_f} \frac{\tilde{C}_{P_i}}{V_c}, \tag{6}$$

where V_x is the extraction volume, V_c is the volume of sample extract injected onto the HPLC column (measured in the same units as V_x), and V_f is the volume of water filtered in the field to create the sample (usually through a 0.7 µm pore size glass-fiber filter and measured in liters).

Inquiries into HPLC uncertainties are guided by the terms and underlying processes associated with quantifying the parameters given in (6). The factors influencing proper pigment identification and quantification of peak areas, are an explicit part of understanding HPLC uncertainties. More subtle aspects are discerned from the procedures associated with the individual methods or parameters. For the work presented here, the assumption is all laboratories have properly validated the methods being used, so the uncertainties in (6) have been properly minimized and estimated, or at least the laboratories involved have enough information available to estimate the uncertainties.

The consideration of these types of refinements resulted in the following goals for SeaHARRE-3:

1. Estimate the uncertainties in quantifying a diverse set of chlorophyll and carotenoid pigment concentrations for a variety of HPLC methods and related procedures used in the analysis of predominantly oligotrophic waters.
2. Confirm whether or not the chlorophyll *a* accuracy requirements for ocean color validation activities (approximately 25%, although 15% would allow for algorithm refinement) can be met in predominantly oligotrophic conditions.
3. Evaluate the efficacy of the performance metrics for evaluating methods, which were proposed during SeaHARRE-2 (Hooker et al. 2005), determine their utility in establishing the reference system for computing uncertainties, and compare the uncertainties from the various methods to the so-called semi-quantitative performance thresholds, i.e., an average accuracy (and precision) of 25% (8%) for the primary pigments (Sect. 1.2.3) and 15% (5%) for total chlorophyll *a* (i.e., TChl *a*).
4. Investigate how higher-order associations in individual pigments (i.e., sums and ratios) influence the uncertainty budget, while also determining how this information can be used to minimize the variance within larger pigment databases.
5. Determine if there is a statistical difference between the results obtained with C_8 versus C_{18} methods.
6. Establish whether or not the laboratory mix proposed during SeaHARRE-2, and now marketed by DHI, is a useful and suitable substitute for a mixed standard.

The second objective requires additional explanation, because in the time period since the first round robin, the accuracy requirements for marine pigments were still only associated with the remote sensing requirements for chlorophyll *a*. In the absence of having any other accuracy criteria—which are needed if the evaluations for all the other pigments are to be placed in a useful context—during SeaHARRE-2 the accuracy thresholds for all the pigments were arbitrarily set to be the same as those initially established for chlorophyll *a*: 25% for compliance, and 15% for improvement.

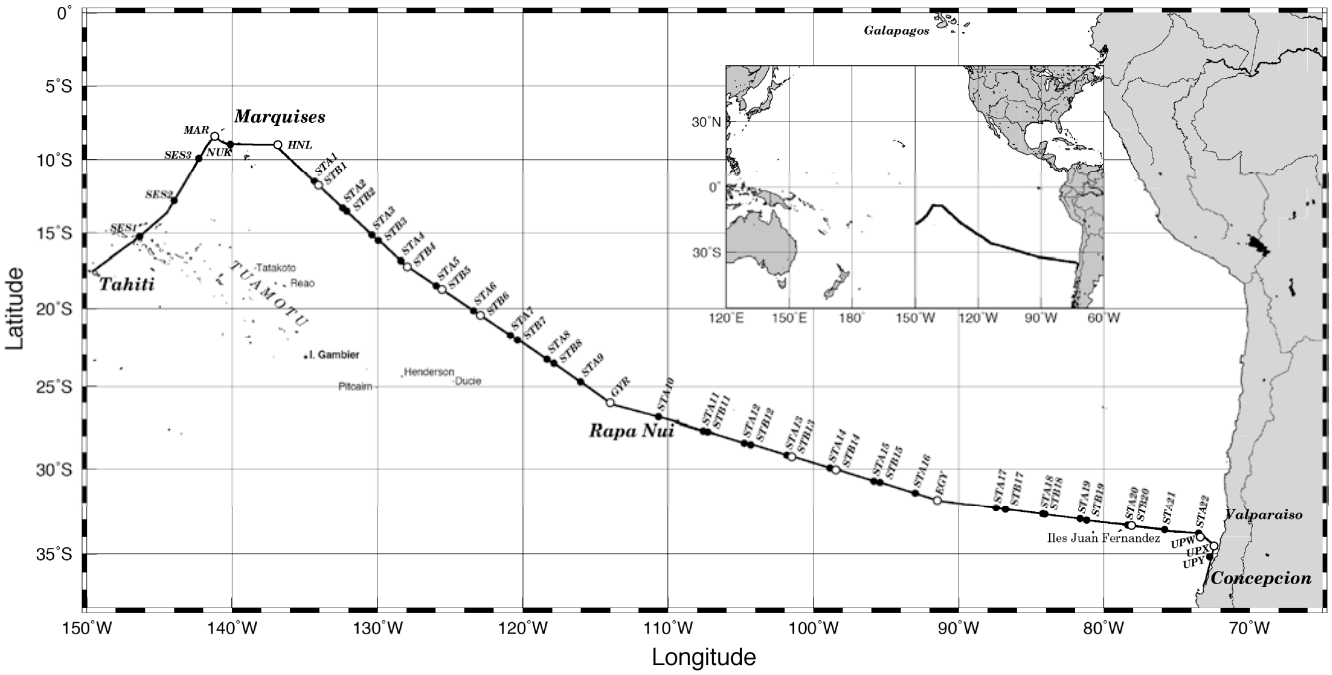

Fig. 1. The overall BIOSOPE cruise track (inset panel), which began in Papeete (Tahiti). The cruise was split into two legs. The first leg included sampling around the Marqueses Islands and ended in Rappa Nui (Easter Island). The second leg continued from Rappa Nui through the Chilean upwelling and ended in Tulcahuano (Chile) outside Conception. The oligotrophic, mesotrophic, and eutrophic regimes are associated with the Marqueses Islands, the South Pacific central gyre waters around Easter Island, and the Chilean upwelling, respectively. Generalized station locations are shown as solid circles, and the SeaHARRE-3 sample stations (Table 3) are shown as slightly larger open circles.

Setting arbitrary accuracy thresholds, because none were available, was noted during SeaHARRE-2 as being an unsatisfactory state of affairs. Consequently, a significant part of the SeaHARRE-2 activity was to discuss and establish performance-based metrics independent of any special emphasis from a particular subset of the community (like remote sensing). As noted above, part of the objectives of the SeaHARRE-3 activity was to evaluate the performance metrics.

1.2 THE DATA SET

The SeaHARRE-3 analyses were derived from field samples and a laboratory mixture of natural pigments. The activity involved seven laboratories (Table 1) using three different methods (Table 2), with one laboratory (S) performing two methods. Six C_8 and two C_{18} methods were executed.

In some parts of this document, abbreviations from the Scientific Committee on Oceanic Research (SCOR) Working Group (WG) are used for pigment presentations (Appendix B), but the majority of the analysis results are presented using a more compact lexicon. This lexicon was developed to satisfy the diversity of presentation requirements spanning text, tables, and formulas. The latter is particularly important to summarizing the statistical description of the results.

1.2.1 The Field Samples

The SeaHARRE-3 sampling plan emphasized oligotrophic waters. The samples were collected as part of the BIOSOPE field campaign and the locations of the sampling stations are shown in Fig. 1. Details about which BIOSOPE stations were used for SeaHARRE-3 samples are presented in Table 3. All samples were collected on 25 mm GF/F filters and stored in liquid nitrogen as soon as filtration was completed. The filters within each batch were randomly selected for each laboratory and distributed using (liquid nitrogen) dry shippers, and there were no anomalies in the shipment of the samples—all filters were received properly frozen by each laboratory.

1.2.2 The Laboratory Mix

One of the recommendations from the SeaHARRE-2 activity was to find a commercial source for an algal mixture of the primary pigments. After SeaHARRE-2, DHI successfully produced a laboratory mix of pigments and made it commercially available. The mixed pigments are made from cultures of different phytoplankton species, and each lot contains approximately 20 different pigments, which all can be present in oligotrophic oceans: Chl c_3, Chl c_2, Peri, But, Fuco, Neo, Pras, Viola, Hex, Diad, Allo, Diato, Zea, Lut, Chl b, Chl a, $\beta\beta$-Car, and $\beta\varepsilon$-Car. The

Table 3. The sampling log for the 24 batches of field samples, which were all collected in triplicate. Enough samples were collected for eight laboratories, which means each batch involves 24 filters. The individual filters were identified alphabetically from `a` to `x`. Only seven batches were used during the initial analyses, because only seven laboratories participated in SeaHARRE-3 (Table 2). Although laboratory *S* executed two methods, only one set of samples were used, because the extract for each individual filter was split between the two methods. The water samples were collected either from Niskin bottles during conductivity, temperature, and depth (CTD) profiles or from an over-the-side pump. The sampling depth is shown with the volume of water filtered, V_f, for all filters within a batch.

Batch Number	*Station Code*	*Sample Type and Seqeunce*		*Depth* [m]	V_f [L]	*Batch Number*	*Station Code*	*Sample Type and Sequence*		*Depth* [m]	V_f [L]
1	MAR1	CTD	8	5	2.8	13	STB13	CTD	130	5	2.8
2	MAR2	CTD	16	90	2.8	14	STB14	CTD	134	5	2.8
3	HNL1	CTD	39	5	2.8	15	EGY1	CTD	141	80	2.8
4	HNL2	CTD	47	80	2.8	16	EGY4	CTD	165	20	2.8†
5	STB1	Pump	9	80	3.4	17	EGY5	CTD	173	70	2.8
6	STB4	CTD	66S	5	2.8	18	STB20	CTD	191	5	1.5
7	STB5	CTD	70S	100	2.8	19	UPW1	CTD	201	5	1.0
8	STB6	CTD	73S	200	2.8	20	UPW2	CTD	207	5	1.0
9	GYR2	CTD	91	170	2.8	21	UPW3	CTD	211	40	1.0
10	GYR4	CTD	107	170	2.8	22	UPX1	CTD	215	20	1.0
11	GYR5	CTD	115	20	2.8	23	UPX1	CTD	216	10	1.0
12	GYR5	Pump	16	180	7.5	24	UPX2	CTD	221	5	1.0

† Samples `u` and `v` were filtered using only 2.1 L of seawater.

content varies slightly in different lots, and may also contain Phide *a*, Phytin *a*, Chlide *a*, MgDVP, and Chl c_1 and other Chl *c*-type pigments.

The mixed pigments allow an HPLC analyst to check that all pigments are detected and separated. They can also be used for quality assurance, for example, monitoring retention time and response factor stability, verifying the correct elution and identity of peaks, and for documenting the precision of the HPLC. The mix is not really a proxy for a natural field sample, because they contain less of some pigments and more of some others, so the relative abundance is not in keeping with a natural sample.

The mix analyzed during SeaHARRE-3 is denoted DHI Mix-101. Each laboratory received several ampules of the mix and was requested to make at least three analyses of the mix, and to use the mix to whatever quality assurance (QA) advantage was deemed appropriate. For example, some analysts make an analysis of the mix at the start of the field sample analyses and after every 20 samples (with at least one mix sample analyzed in each *sample set* of field samples, e.g., for each complete analysis of an autosampler compartment). In most cases, more than three analyses were performed.

1.2.3 The Pigments

Each participating laboratory established and validated an HPLC method based on the pigment content of the samples they typically analyze. This is an important point, because some laboratories were exposed to atypical pigment types or concentrations. The variety of methods means some pigments were analyzed by only a few methods, whereas others were analyzed by all methods. The latter constitute a group of pigments that are routinely useful to many aspects of marine studies and, following the nomenclature of Claustre et al. (2004), are referred to here as the *primary pigments* (PPig).

The utility of the pigments for biogeochemical inquiries along with the number of methods that actually quantitated a particular pigment were used to separate the pigments into four groups:

- The *primary* pigments are the total chlorophylls and the carotenoids most commonly used in chemotaxonomic or photophysiological studies in the open ocean or in coastal waters (Gieskes et al. 1988, Barlow et al. 1993, Claustre et al. 1994, and Bidigare and Ondrusek 1996);
- The *secondary* pigments are the individual pigments used to create a primary pigment composed of separate contributions (e.g., the total chlorophylls);
- The *tertiary* pigments are those pigments not included in the composition of the primary and secondary pigments for which three or more laboratories provided quantitations; and
- The *ancillary* pigments are those remaining pigments only analyzed by one or two laboratories.

Although this nomenclature implies some precedence or ranking of the pigments, this is only true from the current perspective of the SeaHARRE activity and marine phytoplankton pigment research for which certain pigments are

routinely used more often than others (e.g., chlorophyll *a*). The primary reason for establishing a unique vocabulary is to provide an appropriate categorization scheme for grouping the analytical results. A listing of the secondary, tertiary, and ancillary pigments are given in Table 4. Table 4 also provides the methods used for the quantitation of each pigment, the names and abbreviations of each pigment, and the corresponding variable forms, which are used to indicate the concentration of each pigment.

All laboratories quantitated the individual primary pigments, which are used to create the higher-order pigment associations: sums, ratios, and indices (Table 5). The grouping of pigments to form sums permits the formulation of variables useful to different perspectives. For example, the pool of photosynthetic and photoprotective carotenoids (PSC and PPC, respectively) are useful to photophysiological studies (Bidigare et al. 1987) and the total amount of accessory (non-chlorophyll *a*) pigments (TAcc) are useful in remote sensing investigations (Trees et al. 2000). The ratios derived from these pooled variables, e.g., $\left[\text{PSC}\right]/\left[\text{TChl } a\right]$, are dimensionless, and have the advantage of automatically *scaling* the comparison of results from different areas and pigment concentrations.

An important pigment sum is the total diagnostic pigments (DP), which was introduced by Claustre (1994) to estimate a pigment-derived analog to the f-ratio (the ratio of new-to-total production) developed by Eppley and Peterson (1979). The use of DP was extended by Vidussi et al. (2001) and Uitz et al. (2006) to derive size-equivalent pigment indices that roughly correspond to the biomass proportions of pico-, nano-, and microphytoplankton, which are denoted $\left[\text{pPF}\right]$, $\left[\text{nPF}\right]$, and $\left[\text{mPF}\right]$, respectively, and are also referred to as *macrovariables*. They are composed of pigment sums and are ratios, so they should be particularly useful in reconciling inquiries applied to databases from different oceanic regimes.

Together with the individual primary pigments, the pigment sums, ratios, and indices are presented in Table 5. Note that $\left[\text{TChl } a\right]$, $\left[\text{TChl } b\right]$, and $\left[\text{TChl } c\right]$ do not represent individual pigment concentrations—each represents a group of pigments roughly characterized by the same absorption spectra (including some degradation products). These chlorophyll sums allow the comparison of results originating from HPLC methods that differ in the way the pigments within the same family are quantitated (e.g., chlorophyll *c* types) or whose extraction procedures might or might not generate degradation forms (e.g., chlorophyllide *a*). Perhaps most importantly, these sums permit the comparison of methods that differ in their capability of differentiating monovinyl from divinyl forms.

The symbols used to indicate the concentration of the so-called primary pigments, which were reported by all of the laboratories, are as follows:

C_{Ta} Total chlorophyll *a*,
C_{T_b} Total chlorophyll *b*,
C_{T_c} Total chlorophyll *c*,
C_C Carotenes,
C_A Alloxanthin,
C_B 19′-Butanoyloxyfucoxanthin,
C_{Dd} Diadinoxanthin,
C_{Dt} Diatoxanthin,
C_F Fucoxanthin,
C_H 19′-Hexanoyloxyfucoxanthin,
C_P Peridinin, and
C_Z Zeaxanthin.

These are the same 12 pigments given in the topmost portion of Table 5. The first three are the (total) pigment associations for the chlorophylls and the other nine are all carotenoids, of which only Caro is a sum.

The secondary and tertiary pigments that are also of interest to this study, in terms of the presentation of some of the statistical analysis of the results and a historical perspective for the SeaHARRE activity, are as follows:

C_a Chlorophyll *a*,
C_{D_a} Divinyl chlorophyll *a*,
C_{Ca} Chlorophyllide *a*,
C_L Lutein,
C_N Neoxanthin,
C_{N+V} Neoxanthin plus violaxanthin,
C_{Pb_a} Phaeophorbide *a*,
C_{Pt_a} Phaeophytin *a*,
C_{Pr} Prasinoxanthin,
C_V Violaxanthin, and
C_{Z+L} Zeaxanthin plus lutein.

These 11 pigments are a mixture of chlorophylls and carotenoids. They are not the full subset of secondary and tertiary pigment analyzed by three or more laboratories, but are representative of the most important secondary pigments for marine studies (the chlorophyll *a* family of pigments) plus some of the usually minor pigments that many methods quantitate (the tertiary carotenoids).

The symbology presented here is used primarily to represent the final pigment concentrations for each field sample, because this is the way most laboratories report their results, and replicate sampling is not a normal procedure in field campaigns. The symbology does not represent the concentrations associated with the individual samples that were used to determine the final sample value.

1.3 LABORATORY METHODS

SeaHARRE is based on a global perspective, so it is likely that some laboratories would receive samples that were atypical of those for which their HPLC method was originally intended. For example, it would not necessarily be true that a method developed for oligotrophic samples would perform optimally with eutrophic samples. The

Table 4. The secondary (top portion) and tertiary (middle portion) pigments shown with their variable forms, names, and calculation formulas (if applicable). The absence of allomers and epimers for pigments other than Chl *a* is not indicative of a lack of understanding that they might be present—it is simply a reflection that the SeaHARRE participants have agreed to quantitate and include the allomers and epimers in the definition of [Chl *a*]. The methods used to quantitate the various pigments are indicated by their one-letter codes. The variable forms, which are used to indicate the concentration of the pigment, are patterned after the nomenclature established by the SCOR WG 78 (Jeffrey et al. 1997b). Abbreviated pigment forms are shown in parentheses. The ancillary pigments (bottom portion) were quantitated by the indicated methods, but are not used in this study, because less than three laboratories analyzed them; they are included to fully summarize the complete capabilities of each method. Only a subset of the tertiary pigments are presented and discussed. The ones selected for inclusion were deemed representative of the types of tertiary pigments of general interest to the marine phytoplankton community. In addition, the presence of [Neo+Vio] and [Zea+Lut] is to maintain continuity with prior SeaHARRE activities for which these sums were important, because of the methods involved.

Variable	*Method*	*Secondary Pigment*	*Calculation*
[Chl *a*]	*C D H J L M* S_8 S_{18}	Chlorophyll *a* (Chl *a*)	Including allomers and epimers
[DVChl *a*]	*C D H* § *L M* S_8 §	Divinyl chlorophyll *a* (DVChl *a*)	
[Chlide *a*]	*C D H J L M* S_8 S_{18}	Chlorophyllide *a* (Chlide *a*)	
[Chl *b*]	*C L*	Chlorophyll *b* (Chl *b*)	
[Chl c_{1+2}]	*C D H J L M*	Chlorophyll c_{1+2} (Chl c_{1+2})	[Chl c_1] + [Chl c_2]
[Chl c_3]	*C D H J L M* S_8 S_{18}	Chlorophyll c_3 (Chl c_3)	
[$\beta\varepsilon$-Car]	*L* S_{18}	$\beta\varepsilon$-Carotene† ($\beta\varepsilon$-Car)	
[$\beta\beta$-Car]	*L* S_{18}	$\beta\beta$-Carotene‡ ($\beta\beta$-Car)	
Variable	*Method*	*Tertiary Pigment*	*Calculation*
[Chl c_1]	*C H L*	Chlorophyll c_1 (Chl c_1)	
[Chl c_2]	*C D H L* S_8 S_{18}	Chlorophyll c_2 (Chl c_2)	
[Lut]	*C D H J L M* S_8 S_{18}	Lutein (Lut)	
[Neo]	*C D H J L* S_8	Neoxanthin (Neo)	
[Neo+Vio]	*C D H J L* S_8	Neoxanthin and Violaxanthin (Neo+Viola)	[Neo] + [Viola]
[Phide *a*]	*C D H L* S_8 S_{18}	Phaeophorbide *a* (Phide *a*)	
[Phytin *a*]	*D H J L*	Phaeophytin *a* (Phytin *a*)	
[Pras]	*C D H J L M* S_8 S_{18}	Prasinoxanthin (Pras)	
[Viola]	*C D H J L M* S_8 S_{18}	Violaxanthin (Viola)	
[Zea+Lut]	*C D H J L M* S_8 S_{18}	Zeaxanthin and Lutein (Zea+Lut)	[Zea] + [Lut]
Variable	*Method*	*Ancillary Pigment*	*Calculation*
[DVChl *b*]	*C L*	Divinyl chlorophyll *b* (DVChl *b*)	
[Myxo]	*D*	Myxoxanthophyll (Myxo)	
[MgDVP]	*L*	Mg 2,4-divinyl phaeoporphyrin a_5 monomethyl ester¶ (MgDVP)	
[BChl *a*]	*L*	Bacterial Chlorophyll *a* (BChl *a*)	
[Pyro *a*]	*C D*	Pyro-phaeophytin *a* (Pyro *a*)	

§ Methods *J* and S_{18} used the Latasa et al. (1996) simultaneous equations to estimate [DVChl *a*].

† Also referred to as α-Carotene.

‡ Also referred to as β-Carotene.

¶ MgDVP is frequently present in Chl *c* pigment products even if it is not explicitly represented in the quantitation.

Table 5. The (individual) primary pigments, pigment sums, pigment ratios, and pigment indices shown with their variable forms, names, and calculation formulas (if applicable). All methods produced all of these variables. The variable forms, which are used to indicate the concentration of the pigment or pigment association, are patterned after the nomenclature established by the SCOR Working Group 78 (Jeffrey et al. 1997b). Abbreviated forms for the pigments are shown in parentheses.

Variable	*Primary Pigment (PPig)*	*Calculation*
$[\text{TChl } a]$	Total chlorophyll a† (TChl a)	$[\text{Chlide } a] + [\text{DVChl } a] + [\text{Chl } a]$
$[\text{TChl } b]$	Total chlorophyll b† (TChl b)	$[\text{DVChl } b] + [\text{Chl } b]$
$[\text{TChl } c]$	Total chlorophyll c† (TChl c)	$[\text{Chl } c_1] + [\text{Chl } c_2] + [\text{Chl } c_3]$
[Caro]	Carotenes† (Caro)	$[\beta\beta\text{-Car}] + [\beta\varepsilon\text{-Car}]$
[Allo]	Alloxanthin (Allo)	
[But]	19′-Butanoyloxyfucoxanthin (But-fuco)	
[Diad]	Diadinoxanthin (Diadino)	
[Diato]	Diatoxanthin (Diato)	
[Fuco]	Fucoxanthin (Fuco)	
[Hex]	19′-Hexanoyloxyfucoxanthin (Hex-fuco)	
[Peri]	Peridinin (Perid)	
[Zea]	Zeaxanthin (Zea)	
Variable	*Pigment Sum*	*Calculation*
[TChl]	Total Chlorophyll (TChl)	$[\text{TChl } a] + [\text{TChl } b] + [\text{TChl } c]$
[PPC]	Photoprotective Carotenoids (PPC)	[Allo] + [Diad] + [Diato] + [Zea] + [Caro]
[PSC]	Photosynthetic Carotenoids (PSC)	[But] + [Fuco] + [Hex] + [Peri]
[PSP]	Photosynthetic Pigments (PSP)	[PSC] + [TChl]
[TAcc]	Total Accessory Pigments (TAcc)	$[\text{PPC}] + [\text{PSC}] + [\text{TChl } b] + [\text{TChl } c]$
[TPig]	Total Pigments (TPig)	$[\text{TAcc}] + [\text{TChl } a]$
[DP]	Total Diagnostic Pigments (DP)	$[\text{PSC}] + [\text{Allo}] + [\text{Zea}] + [\text{TChl } b]$
Variable	*Pigment Ratio*	*Calculation*
$[\text{TAcc}]/[\text{TChl } a]$	The [TAcc] to $[\text{TChl } a]$ ratio	$[\text{TAcc}]/[\text{TChl } a]$
$[\text{TChl } a]/[\text{TPig}]$	The $[\text{TChl } a]$ to [TPig] ratio	$[\text{TChl } a]/[\text{TPig}]$
[PPC]/[TPig]	The [PPC] to [TPig] ratio	[PPC]/[TPig]
[PSC]/[TPig]	The [PSC] to [TPig] ratio	[PSC]/[TPig]
[PSP]/[TPig]	The [PSP] to [TPig] ratio	[PSP]/[TPig]
Variable	*Pigment Index*	*Calculation*
[mPF]	Microplankton Proportion Factor‡ (MPF)	$\dfrac{[\text{Fuco}] + [\text{Peri}]}{[\text{DP}]}$
[nPF]	Nanoplankton Proportion Factor‡ (NPF)	$\dfrac{[\text{Hex}] + [\text{But}] + [\text{Allo}]}{[\text{DP}]}$
[pPF]	Picoplankton Proportion Factor‡ (PPF)	$\dfrac{[\text{Zea}] + [\text{TChl } b]}{[\text{DP}]}$

† Considered as individual pigments, although computed or equivalently represented as sums by some methods.
‡ As a group, also considered as *macrovariables*.

Table 6. A summary of the extraction specifications for each of the methods. The volume of solvent added is given in milliliters. Each filter was disrupted for the indicated amount of time, allowed to soak for the specified number of hours, and then clarified.

Meth. Code	*Volume Added*	*Extraction Solvent*	*Internal Standard*	*Mode and Time of Disruption*	*Soak Time* [h]	*Clarification*
C	4.2	97.5% Acetone	Vitamin E acetate	Sonicating bath 15 min	15–18	Centrifuge and 0.2 µm Teflon syringe filter
D	3.0	95% Acetone	Vitamin E acetate	Sonicating bath ∼10 min	24	0.45 µm Teflon syringe filter
H	1.832	95% Acetone	Vitamin E acetate	Sonic probe ∼15 s	4	0.45 µm Teflon syringe filter
J	1.5	100% Acetone	*trans*-β-apo-8′-carotenal	Grinder 30 s	24	0.45 µm Teflon syringe filter
L	3.0	100% Methanol	Vitamin E acetate	Sonic probe ≤10 s	1†	1.3 µm GF/C filter
M	2–6	100% Acetone	*trans*-β-apo-8′-carotenal	Sonic probe 30 s	0.5	Centrifuge 10 min (3,500 rpm)
S_8	4.0	100% Acetone	*trans*-β-apo-8′-carotenal	Sonic probe <15 s	≥ 24	Centrifuge 4 min (5,100 rpm)‡
S_{18}	4.0	100% Acetone	*trans*-β-apo-8′-carotenal	Sonic probe <15 s	≥ 24	Centrifuge 4 min (5,100 rpm)‡

† The sum of soaking for 0.5 h, sonicating, and then soaking for another 0.5 h.

‡ Plus a 0.2 µm Teflon membrane filter.

HPLC methods presented here (Chapts. 2–8) are based on diverse objectives, but are most commonly used with samples from a variety of environmental regimes:

C Based on the Van Heukelem and Thomas (2001) method and used predominantly with temperate water samples;

D Based on the Van Heukelem and Thomas (2001) method and used mostly with samples from freshwater estuaries and coastal areas;

H Based on the Van Heukelem and Thomas (2001) method and used with a wide variety of water samples from freshwater lakes, estuarine ecosystems, and the oligotrophic ocean;

J Based on the Wright et al. (1991) method and used predominantly for the analysis of coastal samples;

L Based on the Van Heukelem and Thomas (2001) method and used initially with Case-1 (open ocean) samples, but also successfully with Case-2 (coastal) waters;

M Based on the Barlow et al. (1997) method and used with a wide range of oceanic samples;

S Based on the Van Heukelem and Thomas (2001) method and used with a wide range of pigment concentrations from water types throughout the world ocean, but the original Wright et al. (1991) method was also executed (the two methods are denoted S_8 and S_{18}, respectively).

Note there are seven laboratories and eight methods, but only three methods (*J*, *M*, and S_{18}) are completely different. It is important to remember, however, that the implementation of a common method always results in differences that will distinguish the seemingly identical methods from one another over time.

A summary of the filter extraction procedures is presented in Table 6. All of the methods used acetone as an extraction solvent, except *L* used methanol. Sonic disruption predominated, although one method relied on a mechanical grinder (*J*). The soak time for the extract ranged from 0.5 h to more than 24 h, and clarification was an almost equal combination of centrifugation and filtration. The internal standard used by *C*, *D*, *H*, and *L* was vitamin E acetate, whereas the *M*, S_8, and S_{18} methods all used *trans*-β-apo-8′-carotenal.

A summary of the HPLC column separation procedures and solvent systems used by the SeaHARRE-3 participants are given in Tables 7 and 8, respectively. The type of stationary phase divides the eight methods into two groups, C_8 and C_{18}, with the former predominating. Additional distinction can be seen with a) column temperature (*J* and S_{18} do not control column temperature while *C*, *D*, *H*, *L*, and S_8 used high temperature control), and b) multiple- versus single-wavelength monitoring systems (*C* only used 436 nm). Note also the diversity in equipment manufacturers. All methods used an injection buffer, and the majority of the methods are two-solvent systems, with S_{18} using a three-solvent system. The flow rates of the methods are very similar except for laboratory *L*, which had a flow rate

Table 7. A summary of the HPLC separation procedures used by the SeaHARRE-3 methods. Column particle size (P_s) is in units of micrometers, and column length (L_c) and diameter (D_c) are given in millimeters. Column temperature is denoted by T_c, and wavelength is denoted as λ.

Meth. Code	*Stationary Phase*	*Column*				*Detector and Monitoring Wavelength*	
		P_s	L_c	D_c	T_c	*Manufacturer and Model*	λ [nm]
C	C_8	3.5	150	4.6	55°C	Waters PDA 996	436
D	C_8	3.5	150	4.6	60°C	Shimadzu SPD-M10A VP-DAD	222†, 450, and 665
H	C_8	3.5	150	4.6	60°C	Agilent 1100	222†, 450, and 665
J	C_{18}	5.0	250	4.6	Room	Agilent 1100	436 and 405
L	C_8	3.5	150	3.0	60°C	Agilent 1100	440 and 667
M	C_8	3.0	100	4.6	25°C	ThermoQuest UV6000	440 and 665
S_8	C_8	3.5	150	4.6	60°C	ThermoQuest UV6000	436 and 450
S_{18}	C_{18}	5.0	250	4.6	Room‡	ThermoQuest UV6000	436 and 450

† Used for monitoring vitamin E (the internal standard).
‡ Maintained at 18°C by a specialized air conditioner.

Table 8. A summary of the HPLC solvent systems used with the SeaHARRE-3 methods: MeCN is acetonitrile, NH_4Ac is ammonium acetate, EtOAc is ethyl acetate, MeOH is methanol, and TbAA is tetrabutyl ammonium acetate. The flow rate is in units of milliliters per minute.

Meth. Code	*Injection Buffer*	*Flow Rate*	*Mobile Phase Solvent*			*Initial Conditions*
			A	B	C	
C	TbAA:MeOH†	1.1	70:30 28 mM TbAA:MeOH	MeOH		95% A:5% B
D	TbAA:MeOH†	1.1	70:30 28 mM TbAA:MeOH	MeOH		95% A:5% B
H	TbAA:MeOH†	1.1	70:30 28 mM TbAA:MeOH	MeOH		95% A:5% B
J	1.0 M NH_4Ac	1.0	80:20 MeOH:0.5 M NH_4Ac	90:10 MeCN:Water	EtOAc	100% A
L	TbAA:MeOH†	0.55	70:30 28 mM TbAA:MeOH	MeOH		95% A:5% B
M	1.0 M NH_4Ac	1.0	70:30 MeOH:1.0 M NH_4Ac	MeOH		75% A:25% B
S_8	TbAA:MeOH†	1.1	70:30 28 mM TbAA:MeOH	MeOH		95% A:5% B
S_{18}	Water	1.0	80:20 MeOH:0.5 M NH_4Ac	90:10 MeCN:Water	EtOAc	100% A

† 28 mM TbAA:MeOH in a 90:10 (v:v) mixture, and the TbAA has a 6.5 pH.

that is half as much as the others (a lower flow rate is required with the narrower internal diameter column used by *L*).

1.4 DATA ANALYSIS METHODS

This study uses a laboratory mix and field samples, both with unknown concentrations. Each laboratory participated as if the analyses were performed as a result of normal operations; that is, a single concentration value was reported by each laboratory for each pigment in each filter. For subsequent data analyses, solitary pigment concentrations per batch were used and were determined as the average concentration of a pigment across a set of triplicates, also referred to as a "sample." To ensure a consistency in reporting, all values were converted to concentrations of milligrams per cubic meter.

In the analytical approach adopted here for field samples, no one laboratory (or result) is presumed more correct than another—all the methods were considered properly validated by the individual analysts. Furthermore, there is no absolute truth for field samples, so an unbiased approach is needed to intercompare the methods. The first step in developing an unbiased analysis is to calculate the average concentration, $\bar{C}$, for each pigment from each sample as a function of the eight contributing laboratories:

$$\bar{C}_{P_i}^{L_j}(S_k) = \frac{1}{N_R}\sum_{l=1}^{N_R} C_{P_i}^{L_j}(S_{k,l}), \tag{7}$$

where P_i identifies the pigment or pigment association (following the symbology established in Sect. 1.2.3); L_j is the laboratory (or method) code; $S_{k,l}$ sets the batch (or station) number and replicate number, using the k index for the former (following Table 3) and l for the latter ($l = 1$, 2, or 3); and N_R is the total number of replicates (3).

In (7), the i index represents an arbitrary ordering of the pigments, and the j index is used for summing over the eight laboratory (or method) codes. Although any ordering for the pigments and methods is permissible, the former are ordered following their presentation in Table 6; for the latter, $j = 1, 2, \ldots, 8$ corresponds to the *C*, *D*, *H*, *L*, *M*, *P*, S_8, and S_{18} methods, respectively (which is based on a simple alphabetic ordering of the one-letter codes).

Only one value for each pigment is computed for each station, and this is generically referred to as a "sample,"

so the number of samples equals the number of stations. Averages of a sample across the methods reporting a particular pigment in a sample are used to estimate the true value of the pigment for each sample (or station):

$$\bar{C}^{A}_{P_i}(S_k) \;=\; \frac{1}{N_L}\sum_{j=1}^{N_L} \bar{C}^{L_j}_{P_i}(S_k), \tag{8}$$

where the superscript A denotes an average across all (applicable) methods, and N_L is the number of laboratories quantitating a pigment. For the primary pigments, $N_L = 8$, but for the secondary and tertiary pigments N_L is frequently less than 8 (Table 4).

The unbiased percent difference (UPD), ψ, for each pigment of the individual laboratories with respect to the average values are then calculated for each sample as

$$\psi^{L_j}_{P_i}(S_k) \;=\; 100\,\frac{C^{L_j}_{P_i}(S_k) \;-\; \bar{C}^{A}_{P_i}(S_k)}{\bar{C}^{A}_{P_i}(S_k)}. \tag{9}$$

Note that the formulation in (9) provides a relative percent difference (RPD), because it is signed: a positive ψ value indicates the pigment concentration for a particular laboratory was greater than the average for that pigment (a negative value indicates the laboratory pigment concentration was less than the average). Although $\bar{C}^{A}_{P_i}$ is not considered truth, it is the reference value or proxy for truth by which the performance of the methods with respect to one another are quantified.

When RPD values for methods that do not present any trend relative to the average consensus are summed, however, there is the risk of destroying some or all of the variance in the data. To preserve an appropriate measurement of the variance in the data, absolute UPD values, $|\psi|$, are averaged over the number of samples (N_S) to give the average absolute percent difference (APD) of each laboratory for each pigment across all the samples:

$$|\bar{\psi}|^{L_j}_{P_i} \;=\; \frac{1}{N_S}\sum_{k=1}^{N_S} \left|\psi^{L_j}_{P_i}(S_k)\right|, \tag{10}$$

where S_k is the k^{th} batch (or station) number (Table 3) associated with pigment P_i. For the analysis of field samples, $N_S = 24$, but when the analysis is extended to mixed laboratory samples, N_S is less and depends on the number of times a laboratory analyzed and reported results for the mixed laboratory sample (e.g., the DHI Mix).

Absolute values are used in the overall averages, so positive and negative ψ values do not cancel out and artificially lower the average difference. The latter is particularly important for pigments with low concentrations, but also in terms of a general philosophy: the primary measure of dispersion between the methods are the ψ and $|\bar{\psi}|$ values, so it is important to ensure they are not underestimated.

Another useful parameter is the average of the APD, $|\bar{\psi}|$, values for a particular pigment across the number of laboratories (N_L) reporting the pigment involved:

$$|\bar{\psi}|^{A}_{P_i} \;=\; \frac{1}{N_L}\sum_{j=1}^{N_L} |\bar{\psi}|^{L_j}_{P_i}, \tag{11}$$

where the A code indicates all the laboratories were averaged (and $\bar{\psi}^{A}_{P_i}$ values are formed in a similar fashion from the UPD values). In general, (11) is only computed for the primary pigments, so $N_L = 8$.

To examine the replicate data for each method more closely, the coefficient of variation (ξ) is used, which is expressed as the percent ratio of the standard deviation in the replicate (σ) with respect to the average concentration ($\bar{C}$):

$$\xi^{L_j}_{P_i}(S_k) \;=\; 100\,\frac{\sigma^{L_j}_{P_i}(S_k)}{\bar{C}^{L_j}_{P_i}(S_k)}, \tag{12}$$

where S_k is the k^{th} sample number, and the number of replicates is three for all methods. Individual ξ values are computed for each pigment, for each sample, and for each method; and then all the ξ values for a particular method are averaged to yield an average precision ($\bar{\xi}$) for the method and pigment:

$$\bar{\xi}^{L_j}_{P_i} \;=\; \frac{1}{N}\sum_{k=1}^{N_S} \xi^{L_j}_{P_i}(S_k). \tag{13}$$

The formulations presented in (7)–(13) are for the field samples, but they are applicable to the laboratory standards by redefining the indexing limits and setting S_k to match the laboratory samples.

1.5 RESULTS

Before presenting any results, it is useful to clarify the definitions of certain key terms required for arriving at any statistical description of the various methods. Although not all of these terms are used in this study explicitly, they are all defined to provide complete clarification:

- *Accuracy* is the estimation of how close the result of the experiment is to the *true* value.
- *Precision* is the estimation of how exactly the result is determined independently of any *true* value.
- *Repeatability*, also called *within-run* precision, is obtained from a single operator, using the same instrument, and analyzing the same samples from the same batch.
- *Reproducibility*, also called *between-run* precision, is obtained from different operators, using different instruments and analyzing separate samples from the same batch.

Table 9. The performance metrics for the four categories established during SeaHARRE-2 for validating the determination of marine pigments using an HPLC method (left to right): concentration (average precision, $\bar{\xi}$, and accuracy, $|\bar{\psi}|$, for TChl *a* and PPig); separation (minimum resolution, $\check{R}_s$, and average retention time precision, $\bar{\xi}_{t_R}$); injection precision, $\bar{\xi}_{\text{inj}}$ (the average of an early- and late-eluting pigment standard, e.g., Perid and Chl *a*); and calibration (average residual, $|\bar{\psi}|_{\text{res}}$, for Chl *a* and the precision of the dilution devices, $\bar{\xi}_{\text{cal}}$). The PPig and TChl *a* performance metrics are based on using the analysis of a laboratory mixture of pigments and replicate field samples with approximately equal weights applied to each (remembering that uncertainties are assumed to combine in quadrature and that the latter presupposes the inclusion of replicate filter collection during field sampling). The corresponding values for method H are given as an example. The overall performance of H is considered "state-of-the-art," because the average score of the weights is 3.7, (4+4+4+3+3+4+4+4+3+4)/10.

Performance Weight, Category, and Score	*TChl* a		*PPig*		*Separation*†		*Injection*‡ ($\bar{\xi}_{\text{inj}}$)		*Calibration*§	
	$\bar{\xi}$	$\lvert\bar{\psi}\rvert$	$\bar{\xi}$	$\lvert\bar{\psi}\rvert$	$\check{R}_s$	$\bar{\xi}_{t_R}$	*Perid*	*Chl* a	$\lvert\bar{\psi}\rvert_{\text{res}}$	$\bar{\xi}_{\text{cal}}$
1. Routine 0.5	8%	25%	13%	40%	0.8	0.18%	10%	6%	5%	2.5%
2. Semiquantitative 1.5	5	15	8	25	1.0	0.11	6	4	3	1.5
3. Quantitative 2.5	3	10	5	15	1.2	0.07	4	2	2	0.9
4. State-of-the-Art 3.5	≤2	≤5	≤3	≤10	≥1.5	≤0.04	≤2	≤1	≤1	≤0.5
Method H	*1*	*5*	*2*	*12*	*1.2*	*0.02*	*<1*	*<1*	*1.1*	*0.4*

† The $\check{R}_s$ parameter is the minimum resolution determined from a critical pair for which one of the pigments is a primary pigment. The retention time precision, $\bar{\xi}_{t_R}$, values are based on sequential replicate injections of pigments identified in a laboratory mix. In the absence of a diverse set of early- through late-eluting pigments, a practical alternative is to compute $\bar{\xi}_{t_R}$ based on three sequential injections of Perid, Fuco, Diad, Chl *a*, and $\beta\beta$-Car.

‡ The $\bar{\xi}_{\text{inj}}$ terms are calculated from the average of replicate injections of an early- and late-eluting pigment in the same run. (Perid is chosen here to incorporate the possible effects of peak asymmetry, which is not presented as a separate parameter.)

§ The $|\bar{\psi}|_{\text{res}}$ values presented here are based on calibration points within the range of concentrations typical of the SeaHARRE-2 field samples. To determine this metric for an arbitrary sample set, $|\bar{\psi}|_{\text{res}}$ is computed using those calibration points within the range of concentrations expected in the field samples to be analyzed.

Note that alternative definitions and quantifications are possible, and the ones advocated above are simply the ones deemed suitable for this study.

Two of the most important variables in the results presented here are accuracy and precision, and a simpler definition for these parameters is:

> *Accuracy is telling a story truthfully*, and *precision is how similarly the story is repeated over and over again.*

For the analytical approach adopted here, the average accuracy is represented by the average APD values across the 24 samples, $|\bar{\psi}|$, and the average precision is given the average coefficient of variation (CV) across the 24 samples, $\bar{\xi}$. These are the principal parameters for determining method performance and the uncertainties in the methods.

The first step in the analysis of the SeaHARRE data is to establish the QA subset using the performance metrics (Table 9) established during SeaHARRE-2 (Hooker et al. 2005). This is initially based on the precision obtained with the field samples. A method with an average precision not satisfying the semiquantitative performance metric—more than 8% plus 2% for field sample variability (or approximately 10%)—is excluded from the QA subset (S_{18}). In addition, a laboratory with three or more primary pigments with a precision exceeding routine capabilities (13%) is considered for exclusion (none for this round robin).

The second step is to consider a permutative analysis of the results versus different selections of which laboratories constitute the reference set for computing uncertainties. For this inquiry, laboratories with an established QA capability are intercompared for consistency (for SeaHARRE activities, the QA subset satisfies the quantitative analysis performance metrics in Table 9). The results from the other methods with respect to the QA laboratories are then evaluated individually and as a group. The threshold for retention within the QA subset is the average uncertainty of the primary pigments must be to within 25% (which is the semiquantitative performance metric) or to within 5% of the reference group. From an overall performance perspective, these thresholds are based on an approximately equal evaluation of both laboratory standards and field samples (Table 9), but because the difference between methods is not always as distinctive with laboratory standards as it is with field samples, much greater weight is given to the results achieved with field samples when it comes to establishing the QA subset. This procedure removes J and M for inclusion in the QA subset.

The objective of the second step is to make sure methods close to the capabilities of quality-assured analysis are carefully considered for inclusion as part of establishing the referencing system. One of the criteria considered is to investigate beyond the primary pigments and look at the convergence of the candidate method with respect to

established QA results with some of the tertiary pigments, like [Neo], [Viola], and Neo+Vio. This is considered important, because method-to-method differences can conceivably produce biases within the referencing system—particularly if the QA subset is dominated by one particular method—and one of the most important objectives of the intercomparison activity is to identify biases and resolve them. As shown in Table 2 and Sect. 1.3, the majority of the methods used during SeaHARRE-3 were based on C_8 columns, and more specifically, were based on a single C_8 method (Van Heukelem and Thomas 2001).

Finally, the remaining laboratories in the QA subset are intercompared. Any method with more than three individual pigments exceeding an uncertainty of 25% is removed from the QA subset (if one of the pigments is TChl *a* the allowed maximum uncertainty is 15%), and a new reference set for all pigments is computed. This procedure removes S_8 from the QA subset, which leaves only C, D, H, and L. All ensuing results presented in the follow-on sections are based on these four laboratories as the quality-assured reference set (and are denoted as the QA subset of laboratories), except as noted for specialized discussions.

A few aspects to note about the four laboratories in the QA subset, in terms of the results that will be presented in subsequent sections are as follows:

- All participated in SeaHARRE-2, and two of them (H and L) participated in SeaHARRE-1;
- All used the same C_8 method (Van Heukelem and Thomas 2001), although three of them (C, D, and L) adopted the method recently as a result of studying the results from SeaHARRE-2; and
- All routinely analyze a large number of samples per year and have significant analytical commitments to an established user base.

The introduction of the QA subset automatically establishes another subset: the laboratories (or methods) that are not part of the QA subset are denoted A^+, which is composed of methods J, M, S_8, and S_{18}. In comparison, for the laboratories not in the QA subset:

- All participated in one or more SeaHARRE activities;
- Both C_8 and C_{18} methods were used, with different methods for both column types; and
- Sample analysis varied for a large number of users (S) at the production level of analysis (thousands of samples per year) to exclusive analyses from a very small user base (J).

The importance of these distinctions are considered in more detail during the subsequent presentations of the results.

1.5.1 Average Pigment Concentrations

One of the most important results is the estimation of the uncertainties in the reported data products. To compute uncertainties, (8) is modified to include only the contributions from the QA subset:

$$\bar{C}_{P_i}^{A'}(S_k) \;=\; \frac{1}{N_L}\sum_{j=1}^{N_L}\bar{C}_{P_i}^{L_j}(S_k), \tag{14}$$

where the A' code indicates the QA subset, and N_L is set to index over laboratories C, D, H, and L. The UPD values (relative uncertainties) are then computed by using (14) in (9):

$$\psi_{P_i}^{L_j}(S_k) \;=\; 100\,\frac{C_{P_i}^{L_j}(S_k) \;-\; \bar{C}_{P_i}^{A'}(S_k)}{\bar{C}_{P_i}^{A'}(S_k)}. \tag{15}$$

The analytical process begins with computing the reference or proxy for truth needed for evaluating the analysis of the field samples by the individual methods, i.e., the average pigment concentrations $\bar{C}_{P_i}^{A'}$ in (14) are the reference values and these are computed from the QA subset. Normally, any pigments with significant coelution or specificity problems are not included in producing the overall averages, but for SeaHARRE-3 no such limitations existed for the QA subset.

The average concentrations for the primary pigments quantitated by the QA subset are presented in Table 10. A recurring objective of field sampling for SeaHARRE activities is to have a wide dynamic range in [TChl *a*], typically two orders of magnitude, and the table shows this was very nearly achieved in SeaHARRE-3. The original plan for SeaHARRE-3 was to collect samples in the oligotrophic ocean, with the specific objective of sampling some of the clearest oceanic surface waters possible, so-called hyper-oligotrophic waters, which were expected to have a [TChl *a*] value of $0.02\,\mathrm{mg\,m^{-3}}$ or less. Combining the Table 3 sampling summary with the Table 10 data shows sample numbers 11, 13, and 14 have the lowest near-surface values.

Table 10 also shows the laboratories not part of the QA subset are typified by higher concentrations on average, about 20.8%, but not in all cases. For example, the results for Allo, Diato, Peri, and Zea are very similar between the two subsets. One of the more notable differences is the higher concentration values for TChl *a*: on average, the A^+ subset is approximately 35.4% higher than the A' subset. Given the objective of average [PPig] uncertainties to within 25% and [TChl *a*] uncertainties to within 15%, these large differences represent significant performance problems.

From a generalized perspective, any pigment with an average concentration less than $0.050\,\mathrm{mg\,m^{-3}}$ can be considered to be at a low concentration. Table 10 shows many of the carotenoids are below this limit with Diato and Allo being notably so. The only two carotenoids with average concentrations above this limit are Fuco and Hex (barely).

Table 10. The average primary pigment concentrations for the QA subset (C, D, H, and L) as a function of the batch sample number for the field samples (in units of milligrams per cubic meter). The overall averages for the individual pigments for the QA subset, $\bar{C}_{P_i}^{A'}$, as well as the range in maximum and minimum values, $\hat{C}_{P_i}^{A'}$ and $\check{C}_{P_i}^{A'}$, respectively, are computed across the 24 samples. Corresponding overall averages for the methods not in the QA subset are presented in the last three rows and are denoted by the A^{+} notations. Exceptions (if any) to the general practice of averaging the concentrations from all methods for each sample are given below the overall averages and ranges.

No.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]
1	0.266	0.032	0.101	0.010	0.047	0.104	0.001	0.018	0.001	0.056	0.008	0.028
2	0.208	0.045	0.073	0.009	0.033	0.083	0.001	0.007	0.001	0.024	0.008	0.018
3	0.091	0.018	0.017	0.009	0.008	0.029	0.001	0.010	0.001	0.005	0.005	0.064
4	0.281	0.074	0.053	0.024	0.026	0.072	0.001	0.008	0.001	0.007	0.011	0.070
5	0.170	0.035	0.033	0.015	0.014	0.050	0.001	0.004	0.001	0.003	0.005	0.045
6	0.036	0.002	0.003	0.004	0.001	0.008	0.001	0.005	0.001	0.001	0.001	0.038
7	0.071	0.010	0.009	0.007	0.005	0.014	0.001	0.002	0.001	0.001	0.002	0.043
8	0.196	0.135	0.050	0.027	0.035	0.047	0.001	0.002	0.001	0.003	0.002	0.032
9	0.169	0.097	0.041	0.020	0.028	0.045	0.001	0.003	0.001	0.003	0.002	0.029
10	0.189	0.102	0.054	0.021	0.036	0.061	0.001	0.004	0.001	0.004	0.003	0.040
11	0.020	0.001	0.003	0.002	0.002	0.005	0.001	0.003	0.001	0.001	0.001	0.011
12	0.180	0.099	0.053	0.021	0.033	0.055	0.001	0.003	0.001	0.003	0.002	0.042
13	0.021	0.001	0.003	0.002	0.001	0.006	0.001	0.004	0.001	0.001	0.001	0.007
14	0.023	0.001	0.003	0.002	0.002	0.006	0.001	0.004	0.001	0.001	0.001	0.010
15	0.272	0.112	0.064	0.028	0.042	0.077	0.001	0.006	0.001	0.007	0.004	0.093
16	0.067	0.005	0.015	0.004	0.008	0.030	0.001	0.010	0.001	0.004	0.003	0.016
17	0.155	0.030	0.042	0.011	0.026	0.060	0.001	0.009	0.001	0.008	0.005	0.049
18	0.290	0.015	0.079	0.012	0.030	0.147	0.001	0.064	0.006	0.013	0.022	0.034
19	0.863	0.030	0.309	0.030	0.027	0.107	0.011	0.102	0.005	0.408	0.031	0.051
20	1.078	0.021	0.420	0.031	0.031	0.123	0.010	0.122	0.006	0.566	0.037	0.035
21	0.622	0.010	0.163	0.019	0.005	0.010	0.009	0.022	0.001	0.294	0.010	0.008
22	0.792	0.332	0.080	0.035	0.005	0.016	0.033	0.013	0.001	0.084	0.027	0.035
23	1.366	0.422	0.199	0.057	0.011	0.043	0.051	0.047	0.001	0.220	0.055	0.058
24	0.805	0.211	0.093	0.037	0.005	0.022	0.024	0.035	0.004	0.143	0.011	0.054
$\hat{C}_{P_i}^{A'}$	1.366	0.422	0.420	0.057	0.047	0.147	0.051	0.122	0.006	0.566	0.055	0.093
$\bar{C}_{P_i}^{A'}$	0.343	0.077	0.082	0.018	0.019	0.051	0.006	0.021	0.001	0.078	0.011	0.038
$\check{C}_{P_i}^{A'}$	0.020	0.001	0.003	0.002	0.001	0.005	0.001	0.002	0.001	0.001	0.001	0.007
$\hat{C}_{P_i}^{A^+}$	1.872	0.548	0.402	0.047	0.093	0.156	0.052	0.141	0.007	0.551	0.055	0.093
$\bar{C}_{P_i}^{A^+}$	0.464	0.098	0.078	0.016	0.024	0.057	0.008	0.024	0.002	0.077	0.012	0.040
$\check{C}_{P_i}^{A^+}$	0.026	0.001	0.002	0.001	0.002	0.006	0.001	0.002	0.000	0.001	0.001	0.008

The persistent low concentrations of Diato and Allo provide a significant opportunity for false positives, i.e., a laboratory reports a pigment is present, when in fact it is not. Alternatively, a seemingly false positive pigment result can occur when a method with exceptional detectability quantifies a result, while other methods cannot detect it; although, the latter is a rare occurrence.

False positives are especially onerous to the computation of uncertainties because the larger false value is differenced with respect to the much smaller reference value, which yields a relatively large number in the numerator of (15). The denominator is the much smaller reference value, so the computed uncertainty is a large number divided by a much smaller number, which yields an uncertainty of many hundreds of percent.

A false negative occurs when a laboratory reports a pigment is not present when in fact it is. False negatives are not as damaging as false positives, because a false small number is differenced with respect to a larger reference value, which yields a relatively large number in the numerator of (15). The denominator is the larger reference value, so the computed uncertainty is a larger number divided by a slightly larger number, which yields an uncertainty a bit less than 100%.

The influence of false positives and negatives on method uncertainty is usually minimal for those pigments that are

Table 11. A subset of the average secondary and tertiary pigment concentrations for the field samples analyzed by the QA subset. The presentation follows the scheme established in Table 10. The [Phytin *a*] entries do not include values from laboratory *C*, because none were reported.

No.	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
1	0.210	0.033	0.023	0.002	0.004	0.006	0.001	0.005	0.001	0.002	0.030
2	0.156	0.040	0.012	0.001	0.004	0.005	0.003	0.005	0.001	0.001	0.019
3	0.058	0.031	0.001	0.002	0.002	0.004	0.001	0.001	0.001	0.002	0.065
4	0.141	0.138	0.002	0.001	0.005	0.007	0.003	0.001	0.001	0.002	0.071
5	0.081	0.088	0.001	0.001	0.002	0.003	0.001	0.001	0.001	0.001	0.046
6	0.016	0.019	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.039
7	0.034	0.036	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.044
8	0.085	0.111	0.001	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.032
9	0.082	0.086	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.030
10	0.100	0.088	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.040
11	0.016	0.004	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.011
12	0.091	0.089	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.042
13	0.018	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.008
14	0.018	0.004	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.010
15	0.145	0.126	0.001	0.002	0.004	0.006	0.004	0.001	0.001	0.002	0.095
16	0.057	0.008	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.017
17	0.110	0.044	0.001	0.002	0.002	0.004	0.001	0.001	0.001	0.002	0.052
18	0.272	0.014	0.004	0.001	0.002	0.006	0.004	0.001	0.001	0.004	0.035
19	0.681	0.002	0.180	0.001	0.002	0.007	0.013	0.103	0.007	0.004	0.052
20	0.859	0.001	0.219	0.001	0.002	0.006	0.011	0.136	0.008	0.003	0.036
21	0.571	0.001	0.051	0.001	0.002	0.003	0.086	0.209	0.008	0.001	0.008
22	0.785	0.001	0.007	0.003	0.038	0.061	0.016	0.025	0.091	0.023	0.038
23	1.338	0.001	0.028	0.008	0.050	0.094	0.019	0.025	0.124	0.044	0.066
24	0.784	0.001	0.021	0.008	0.025	0.063	0.010	0.027	0.069	0.038	0.062
$\hat{C}_{P_i}^{A'}$	1.338	0.138	0.219	0.008	0.050	0.094	0.086	0.209	0.124	0.044	0.095
$\bar{C}_{P_i}^{A'}$	0.280	0.040	0.023	0.002	0.006	0.012	0.008	0.023	0.013	0.006	0.039
$\check{C}_{P_i}^{A'}$	0.016	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.008
$\hat{C}_{P_i}^{A^+}$	1.823	0.169	0.326	0.009	0.040	0.079	0.052	0.137	0.108	0.052	0.095
$\bar{C}_{P_i}^{A^+}$	0.370	0.048	0.035	0.002	0.006	0.012	0.007	0.015	0.013	0.008	0.042
$\check{C}_{P_i}^{A^+}$	0.019	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.008

Note: Both the S_{18} and J methods are not included in the A^+ [DVChl *a*] results, because these methods do not chromatographically separate DVChl *a*.

almost always in an oceanic sample (e.g., Chl *a*), but they are particularly important for those pigments whose presence and abundance changes significantly from sample to sample. The average concentrations of the secondary and tertiary pigments are presented in Table 11, and many of the carotenoids appear in very low concentrations (all have average concentrations below $0.050\,\mathrm{mg\,m^{-3}}$). All of the carotenoids have samples where the abundance is elevated with respect to the average, and as shown in Table 3, the increases are associated with the samples taken in the Chilean upwelling. The Lut values do not exhibit as strong an increase in the upwelling as the other tertiary pigments, so Lut is the second least abundant pigment on average (Diato is the least abundant).

The A' subset results for the secondary and tertiary pigments are characterized by a mix of lower and higher concentrations with respect to the A' subset. The notable exception are the pigments comprising TChl *a*, which have concentrations as high or higher than the corresponding A' values. The net effect is the overall averages are rather similar, but there are notable differences. For example, the A' results for [Phide *a*] are almost 50% higher on average than the corresponding results for the A^+ subset.

1.5.2 Method Precision

Both the J and M laboratories participated in one or more prior SeaHARRE activities and the results were superior to what was achieved with the samples first distributed for SeaHARRE-3. Both laboratories had atypical and significant problems during the initial analysis of samples (Sect. 5.1 and Sect. 7.1) and made corrective mea-

Table 12. The $\bar{\xi}$ values (coefficients of variation in percent) across all 24 batches of field samples as a function of the method for the primary pigments with the last column presenting the overall (horizontal) method average across the pigments. The overall (vertical) QA subset averages (C, D, H, and L) are given in the A' entries, and the methods that were not considered quality assured in the A^+ entries. The lowest values for each pigment in the QA subset constitute a hypothetical *best method* and are shown in bold typeface, which are summarized for all pigments by the A^- entries in the last row. The analyses made by laboratories J and M after the normal analysis, but on a subset of samples (12 each), are given in the J^+ and M^+ entries, respectively.

Meth.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Avg.*
C	6.3	8.8	5.6	10.6	6.7	7.3	1.5	10.7	6.1	9.0	**5.0**	7.0	7.0
D	6.1	7.1	5.9	13.3	6.8	6.5	2.3	9.2	8.1	9.3	11.1	12.5	8.2
H	3.8	7.3	**3.4**	4.3	3.6	**3.3**	**1.1**	**4.1**	**2.3**	4.5	10.6	5.1	4.5
L	**3.2**	**4.1**	3.5	**4.0**	**3.1**	3.7	1.7	4.2	2.9	**4.0**	6.4	**3.8**	**3.7**
J	3.3	5.8	6.2	6.6	7.4	6.2	4.1	8.4	12.1	10.1	7.7	6.8	7.1
M	5.4	13.6	9.4	8.2	5.9	5.6	6.9	6.8	9.2	13.3	9.9	5.7	8.3
S_8	6.4	10.5	10.5	7.9	8.4	6.3	2.8	5.9	11.3	10.7	11.6	4.8	8.1
S_{18}	5.5	16.4	16.1	54.8	18.3	9.0	4.2	7.4	25.8	16.9	36.7	7.4	18.2
A'	4.9	6.8	4.6	8.1	5.1	5.2	1.6	7.0	4.8	6.7	8.3	7.1	5.8
A^+	5.2	11.6	10.6	19.4	10.0	6.8	4.5	7.1	14.6	12.8	16.5	6.2	10.4
A^-	3.2	4.1	3.4	4.0	3.1	3.3	1.1	4.1	2.3	4.0	5.0	3.8	3.4
J^+	*2.8*	*3.4*	*9.7*	*6.2*	*3.5*	*2.4*	*15.8*	*6.4*	*1.2*	*5.7*	*15.2*	*4.8*	*6.4*
M^+	*6.5*	*19.2*	*8.9*	*8.3*	*7.4*	*4.3*	*7.0*	*6.0*	*5.0*	*7.2*	*10.9*	*4.7*	*8.0*

sures after the initial analysis of samples were completed. Consequently, an eighth sample set, which was originally planned for a separate analysis, was split between the two laboratories with each receiving 12 samples spanning approximately the same dynamic range in [TChl *a*]. J received triplicates from batches 1, 3, 4, 8–9, 11–12, 14, 16, 20–21, and 24; while M received triplicates from batches 2, 5–7, 10, 13, 15, 17–19, and 22–23.

The subsequent J and M analyses are designated J^+ and M^+, respectively. Neither of these results are included in any of the A^+ averages or summaries—they are always treated separately. Although detection of the apparently anomalous J and M results occurred during the assessment of method accuracy (Sect. 1.5.3), the usual presentation of results starts with precision. Rather than change the format for this activity, the discussion proceeds as if these data were available at the same time as the other analyses.

Method precision for each pigment is estimated by averaging the values computed from the sample triplicates across all 24 samples, which is denoted by $\bar{\xi}_{P_i}$. Table 12 presents the method precision for the primary pigments plus three types of overall precision: a) the average across all primary pigments for each laboratory (Avg.), b) the average values for the methods in the A' and A^+ subsets, and c) the best precision obtained for each pigment within the A' subset, which is denoted A^-. The latter represent a theoretical *best method* threshold first tested and found to be a satisfactory criteria for a *state-of-the-art method* during SeaHARRE-2 (Hooker et al. 2005).

In most cases, the average method precision results for the primary pigments show the superior precision is associated with [TChl *a*], and there is a general worsening of precision for the other chlorophylls and carotenoids. For SeaHARRE-3, the best average precision in the A' subset was for [Allo], [Diato],[TChl *c*], and then [TChl *a*]. The [Allo] and [Diato] results are somewhat artificial, because much of the data was at the limit of detection, so as long as a laboratory correctly identifies the pigment is absent in those samples, the precision will be excellent. The [TChl *c*] result is atypical of what has been observed in previous SeaHARRE activities, largely because the Chl *c* pigments are associated with more coelution problems than the Chl *a* pigments, plus they usually elute early and can be adversely affected by solvent front as well as baseline disturbances.

With the exception of the S_{18} results, the A^+ average precisions for each primary pigment are either similar to, or slightly worse than, the QA subset. For example, the J and S_8 results are slightly better than the D results, and the M results are only marginally worse. This demonstrates the difficulty of using precision exclusively as a criteria for evaluating method performance. The point to remember here is that precision is the starting point for method performance, and it is very difficult for a method to achieve a suitable accuracy if the precision is poor, but that does not mean a good precision will ensure good accuracy—*it is a necessary, but not sufficient, criteria.*

The further limitations of precision are illustrated by the J^+ and M^+ results in Table 12, which are not notably different from the original J and M results, respectively, although there is a small improvement in both cases. This suggests the problems encountered during the initial analyses were not significantly detrimental to the precision of the

Table 13. The $\bar{\xi}$ values (coefficients of variation in percent) across all 24 batches of field samples for a subset of the secondary and tertiary pigments, following the presentation scheme established in Table 12.

Meth.	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
C	7.2	10.8	**5.2**	**2.0**	12.3	13.7		**2.3**	3.6	5.9	7.2
D	6.4	5.4	21.4	8.9	11.5	13.7	13.7	4.5	2.6	7.5	15.3
H	8.2	4.6	28.3	3.3	5.8	8.2	**11.9**	10.4	2.3	5.7	6.1
L	**3.5**	**2.9**	7.9	4.6	**4.9**	**6.8**	12.1	2.4	**1.2**	**4.8**	**6.0**
J	6.5	5.4†	7.3	10.7	10.7	17.6	8.6		14.8	13.9	12.7
M	5.9	4.8	14.9	3.3					0.7‡	10.0	6.6
S_8	7.6	5.3	12.2	0.9‡	9.0	11.9		2.4	2.2	7.8	4.9‡
S_{18}	7.3	37.6†	25.1	63.5				10.7	27.7	27.9	64.0
A'	6.3	6.0	15.7	4.7	8.6	10.6	12.6	4.9	2.4	6.0	8.7
A^+	6.8	5.2	14.9	19.6	9.9	14.7	8.6	6.6	14.9	14.9	22.0
A^-	3.5	2.9	5.2	2.0	4.9	6.8	11.9	2.3	1.2	4.8	6.0
J^+	*4.9*	*6.9*	*10.3*	*2.6*	*12.8*	*14.3*	*83.2*		*11.3*	*6.5*	*5.5*
M^+	*5.9*	*4.5*	*28.5*	*9.8*					*18.1*	*11.3*	*10.9*

† J and S_{18} are excluded from the A^+ results, because these methods did not chromatographically separate DVChl *a*.
‡ Artificially low, because of a persistent nondetection and not included in the A^+ or A^- results.

methods. There is also the confounding situations where the precision of some subsequent analyses are much worse than the original, e.g., the J^+ [Allo] and [Peri] values, as well as the M^+ [TChl *b*] results.

The individual instances of excellent precision for specific methods and pigments within the QA subset (and also in some of the entries for the other methods), show excellent precision is recurringly possible across all pigments. The precision of the selected best precision in the QA subset (the bold entries in Table 12) ranged from 1.1–5.0%, with an overall average of 3.4%. The narrow range of precision excellence, and the fact that it occurs across three different methods, suggests filter inhomogeneity is not a significant component of the variance, and that methodological differences account for the majority of the diversity in the precision results. The fact that the L method has an overall precision very close to the A^- average shows the best method approach is also achievable.

Table 13 presents the precision obtained by the methods for a subset of the secondary and tertiary pigments using the same format established for Table 12. These results largely confirm the precision conclusions obtained with the primary pigments, but there are some differences. If the results for Phytin *a* are excluded, the best precision of the QA subset occurs over a narrow range (1.2–6.8%) and is spread across three laboratories. The fact that all three QA laboratories that reported [Phytin *a*] have very similar results suggests either a limitation of the method (D, H, and L used the same method) or a variable degradation in the samples. The results for the other degradation pigments do not support the latter, so presumably there is a methodological explanation.

As anticipated from the PPig results, the A^+ results are worse than the A' results—about 60% on average. This is not true for all A^+ methods and all pigments, however. Many of the A^+ results are within the range of the QA subset, with the recurring exception of the S_{18} data. If the S_{18} data are excluded from an A^+ overall average, the remaining methods have an average precision that is very similar to the A' overall average.

The J^+ and M^+ precision results are worse than the original J and M results, respectively. This might suggest some degradation in the filters as a result of storing them for a longer time period, but this is not supported by the improved or similar results achieved with [Chl *a*]. There is some support for such a hypothesis with the [Chlide *a*] and [Phytin *a*] results, but there are other pigments for the J and M results that either were similar or got better or worse with the J^+ and M^+ analyses, so there is no conclusive, broadly-based evidence for degradation.

Table 14 presents the method precision for the primary pigments quantitated for DHI Mix-101. The results show the A' subset has better average precision than the A^+ subset, although many of the individual methods and pigments of the latter are superior and are similar to the former. The range in average precision for the A' subset is not very large (1.1–2.0%), and if the S_8 [Allo] results are ignored, the range in the A^+ results are also not very large (2.4–3.3%). The A^- results (which are a proxy for a theoretical best method) are always less than 2% and have an overall average of 1.1%. This is within the performance metric for state-of-the-art injection, and QA methods satisfy this metric for almost every pigment and all do so on average. The fact that the average result for the L method is very nearly the A^- average is a clear indicator that using the best results from the QA subset produces a realistic and achievable proxy for what a state-of-the-art method should be capable of producing.

Table 14. The $\bar{\xi}$ values (coefficients of variation in percent) for DHI Mix-101 as a function of the method for the primary pigments using the presentation scheme from Table 12. The lowest $\bar{\xi}$ values from the QA subset are given in the last row, A^-. The S_{18} results are from an analysis of Mix-101 performed after the initial reporting of results, because all of the initial distribution of Mix-101 received by S was used during the S_8 analyses.

Meth.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Avg.*
C	1.2	**1.1**	**1.1**	1.5	2.5	1.8	2.1	**0.8**	1.6	1.8	2.5	1.0	1.6
D	1.7	1.2	2.6	2.4	1.8	**1.5**	**1.9**	2.0	2.3	2.7	2.0	2.0	2.0
H	2.1	1.2	1.9	1.6	2.2	1.7	3.3	1.9	2.1	1.5	1.4	1.7	1.9
L	**0.5**	1.2	3.2	**0.8**	**0.5**	1.8	2.5	0.9	**0.4**	**0.7**	**0.8**	**0.2**	**1.1**
J	0.8	0.7	2.0	1.4	4.5	1.4	3.0	5.2	2.4	1.4	5.7	0.9	2.4
M	2.6	2.6	0.5	2.0	4.8	2.8	3.9	1.1	0.7	3.7	3.8	0.8	2.4
S_8	1.6	1.8	4.2	2.4	7.2	3.6	12.1	1.8	2.2	4.8	4.9	2.3	4.1
S_{18}	1.8	1.8	1.7	4.4	1.9	3.1	3.8	2.5	2.2	2.7	2.8	1.8	2.5
A'	1.4	1.2	2.2	1.6	1.8	1.7	2.4	1.4	1.6	1.7	1.7	1.2	1.7
A^+	1.6	1.7	2.2	1.9	5.5	2.6	6.3	2.7	1.8	3.3	4.8	1.4	3.0
A^-	0.5	1.1	1.1	0.8	0.5	1.5	1.9	0.8	0.4	0.7	0.8	0.2	0.9
J^+	*2.7*	*1.6*	*5.6*	*2.7*	*3.4*	*0.1*	*0.1*	*4.0*	*1.4*	*2.3*	*20.5*	*0.3*	*3.7*
M^+	*2.0*	*0.9*	*1.0*	*3.4*	*2.8*	*0.5*	*2.4*	*2.1*	*1.1*	*0.3*	*0.8*	*1.0*	*1.5*

The DHI mix does not have the full complexity of a natural sample and the pigment concentrations are significantly elevated with respect to open ocean surface waters, particularly for many carotenoids. Identifying peaks in the mix is easier than in a natural sample, so false positives or negatives are unlikely. Consequently, the precision obtained with the mix should be closer to the injection precision than the precision of a field sample.

The J^+ chlorophyll results are more imprecise than the J values, but almost all of the carotenoid values are better (Peri is the notable exception). The M^+ results are almost always better than the M values, which results in a significant improvement in overall precision. For the field samples (Table 12), there was a modest improvement in precision for the J^+ and M^+ analyses with respect to the original J and M results, respectively.

1.5.3 Method Accuracy

Method accuracy for each reported pigment product is determined using $|\bar{\psi}|$, the average APD between the reported laboratory values and the average concentrations constructed from the QA subset, that is, (15) is used in (10). Although the limitations already noted in determining method precision (Sect. 1.5.2) are relevant to understanding accuracy, most do not actually change the computations. There are exceptions, however, and the two most important are the aforementioned false positives and false negatives (Sect. 1.5.1), which are both specificity problems.

Note that the primary difference between false negatives and false positives is the uncertainty of a false negative is bounded (it cannot be more than 100%), whereas the uncertainty for a false positive is unbounded (it can be many times more than 100%). In both cases, the actual uncertainty will be somewhat mitigated if the true concentration of the pigment is close to detection limits, because the multiplicative factor n relating y and x will be a small number. The false positive situation, however, will usually be the most serious in terms of average uncertainties.

1.5.3.1 Individual Pigments

The performance metrics for quality-assured (quantitative) results require an average [TChl *a*] and [PPig] uncertainty to within 10% and 15%, respectively (Table 9). Recalling the allowed variance in establishing the QA subset (Sect. 1.5)—no more than three individual primary pigments were permitted to exceed an uncertainty of 25% and the [TChl *a*] uncertainty must be within 15%—the expectation is all QA methods will have PPig uncertainty results satisfying these criteria.

The PPig uncertainties are presented in Table 15, which shows the QA subset (A') satisfies the quantitative performance metric for accuracy—the overall uncertainty is 12.2%. In addition, the overall individual method uncertainties are less than 25%; in fact, they are less than 15% except for C, which is slightly higher than 15%, but within the 2–3% variance anticipated for field sample precision.

The uncertainties in the overall A' individual pigment uncertainties are frequently less than 15%. The notable exceptions are Peri and Diato, although the latter has an overall average less than 25%. The Diato concentrations were likely close to detection limits (Table 10), so the elevated uncertainties are probably the result of false positives and negatives associated with the intercomparison of very small numbers. Although many of the samples were above a reasonable detection threshold for Peri (Table 10), more than half were still at very low concentrations, and false positives and negatives were a significant problem.

Table 15. The $|\bar{\psi}|$ values (average APD in percent) across all 24 batches of field samples for the primary pigments following the presentation scheme established in Table 12.

Meth.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Avg.*
C	**5.2**	23.7	19.9	12.3	26.2	9.0	5.3	6.8	20.5	17.0	46.9	9.7	16.9
D	8.0	**7.7**	**7.8**	16.5	12.9	4.6	7.2	4.3	18.0	8.1	31.8	10.5	11.4
H	5.5	8.8	22.9	17.6	10.5	5.9	2.5	**4.2**	21.0	**7.3**	**18.6**	**5.4**	10.9
L	6.6	15.5	8.9	**6.5**	**8.8**	**4.4**	**1.6**	4.5	**11.6**	9.9	24.6	12.7	**9.6**
J	33.4	31.5	13.6	9.9	24.5	42.2	17.0	18.1	24.9	166.2	356.5	13.3	62.6
M	25.0	27.9	35.9	14.9	387.6	53.6	389.8	34.7	11.6	20.9	36.1	15.7	87.8
S_8	57.4	70.8	29.0	15.2	14.9	9.1	18.9	27.4	38.1	15.7	15.3	7.5	26.6
S_{18}	16.5	17.2	11.1	57.4	23.3	19.7	20.3	7.6	182.4	32.1	40.6	18.4	37.2
A'	6.3	13.9	14.9	13.2	14.6	6.0	4.1	5.0	17.8	10.6	30.5	9.6	12.2
A^+	33.1	36.8	22.4	24.3	112.6	31.1	111.5	22.0	64.3	58.7	112.1	13.7	53.6
A^-	5.2	7.7	7.8	6.5	8.8	4.4	1.6	4.2	11.6	7.3	18.6	5.4	7.4
J^+	*19.1*	30.5	*14.6*	*31.5*	*14.7*	*19.0*	*168.0*	*25.0*	*11.9*	*12.8*	214.4	19.2	*48.4*
M^+	*34.4*	*112.7*	*76.5*	*54.0*	*235.0*	*57.6*	*203.5*	*92.5*	*39.4*	*53.4*	*53.9*	*6.5*	*85.0*

Most of the A' Allo results were likely at the detection limit of most methods, but there was little disagreement between analysts as to whether or not Allo was present, so there were very few false positives or negatives. The overall [PPig] uncertainty is below 15%, and only Diato and Peri exceed this threshold for the individual averages.

The results from the A^+ methods are significantly different from A' subset. The overall average PPig uncertainty (53.6%) does not satisfy the semiquantitative performance metric (Table 9), and neither do any of the individual methods (although the S_8 method is within the 2–3% sampling variance). In addition, the semiquantitative performance metric for TChl *a* is not satisfied as an overall average or by any of the individual methods (although the S_{18} method is within the 2–3% sampling variance).

The individual pigment results for the A^+ subset are further distinguished by anomalously high uncertainties for one or more pigments, e.g., Peri and Fuco for J, Allo and But for M, TChl *a* and TChl *b* for S_8, as well as Caro and Diato for S_{18}. The two S methods have notably opposing large uncertainties. The S_8 method has comparatively large uncertainties for the chlorophylls (52.4% on average), and much smaller uncertainties for the carotenoids (18.0% on average); whereas the S_{18} method has comparatively small uncertainties for the chlorophylls (15.0% on average), and much larger uncertainties for the carotenoids (44.6% on average).

The best possible outcomes from the QA subset, the A^- entries, have an overall average PPig uncertainty of 7.4%, which is within the requirements for a state-of-the-art method. The L method overall PPig average uncertainty also satisfies this metric and the H and D overall average PPig uncertainties are within the 2–3% sampling variance for this metric. Neither the D, H, and L methods satisfy the state-of-the-art metric for TChl *a*, but all are within the 2–3% sampling variance for this metric.

Note that the repeated invocation of adding a margin of 2–3% variance for the performance discussions is simply a reflection that only field samples are being considered and there is a small amount of variance that comes from the preparation of field replicates—they are not perfect replicates—and this variance is beyond the control of the methods being intercompared.

The J^+ and M^+ reanalyses show some overall improvement for the former and almost no improvement for the latter. In both cases, the overall average PPig uncertainty does not satisfy the performance metric for routine analysis, primarily because both methods have multiple individual pigments with large uncertainties. In some cases, the problematic pigments between the original results and the reanalyses are the same (e.g., Peri for J and Allo for M), but both methods have new difficulties with an individual pigment that degrade the effect of the improvements (e.g., Allo for J and TChl *b* for M).

The PPig uncertainties show the overall spread in the A' subset is confined to a relatively narrow range (approximately 10%), and so are many of the individual pigment results (TChl *a*, Caro, Hex, Allo, Diad, Diato, Fuco, and Zea). In addition, the best (A^-) results come from all the A' methods, so each one is contributing towards defining a state-of-the-art method (noting that L and H predominate in this process).

One of the important results from the intercomparison of the PPig analysis is how similar the data within each of the two A' and A^+ subsets are, and how distinctly different the two subsets are with respect to one another. If the referencing system was flawed, at least one of the methods within one of the two groups would appear out of place with a set of results that would not be in keeping with the group in which it was placed. Within this context, the S_8 and S_{18} results exhibit a curious duality: the former has mostly unacceptable uncertainties for the chlorophylls and

Table 16. The $|\bar{\psi}|$ values (average APD in percent) across all 12 batches of field samples for a subset of the secondary and tertiary pigments (following the presentation scheme established in Table 13).

Meth.	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
C	**6.2**	20.0	40.6	27.7	27.9	26.0		33.4	**1.3**	24.3	10.1
D	10.8	**6.9**	**16.7**	**11.2**	**23.5**	16.7	**17.2**	**13.6**	4.5	11.0	10.0
H	8.3	8.0	76.5	13.7	30.4	17.7	18.1	46.6	13.1	**8.9**	**5.3**
L	11.2	7.8	33.8	33.1	24.2	**16.1**	21.6	15.1	12.2	23.4	13.0
J	24.5	39.2	42.3	35.0	58.8	51.7	54.1		175.9	44.1	12.2
M	20.6	16.9	236.9	29.3					19.8	63.2	15.6
S_8	61.5	35.1	34.7	28.5	19.8	15.1		25.9	3.1	20.3	8.5
S_{18}	16.6	106.9	40.9	99.5				26.0	326.4	437.0	19.0
A'	9.1	10.7	41.9	21.4	26.5	19.1	18.9	27.2	7.8	16.9	9.6
A^+	30.8	26.0†	88.7	48.1	39.3	33.4	54.1	26.0	131.3	141.2	13.8
A^-	6.2	6.9	16.7	11.2	23.5	16.1	17.2	13.6	1.3	8.9	5.3
J^+	*11.0*	*1,725.3*	*46.8*	*12.1*	*65.6*	*88.9*	*197.2*		*51.3*	*138.9*	*18.5*
M^+	*41.2*	*28.5*	*37.6*	*21.3*					*143.1*	*46.7*	*5.8*

† Excluding J and S_{18}, because these methods did not chromatographically separate monovinyl and divinyl chlorophyll *a*. J used a dichromatic equation to separate these pigments (Latasa et al. 1996) as a service to the SeaHARRE activity; this approach has not been assessed or used in an operational mode at the JRC and is for informational purposes only.

rather acceptable uncertainties for the carotenoids, while the latter has the opposite.

If the referencing system involved all of the methods, the aforementioned distinctions would become less notable, because the variance between the populations of results would get shared between the individual components. For example, the average uncertainty in PPig for the A' subset would increase to 17.8%, whereas it would decrease to 32.0% for the A^+ subset. So the A^+ subset would now be within the limits for routine analysis, but the A' subset would have degraded from quantitative to semiquantitative status. The effects on TChl *a* would be more dramatic: the uncertainty for the A' subset would increase to 14.2%, but decrease to 15.6% for the A^+ subset. Within the overall variance of sample collections, these two results are indistinguishable and strongly show the importance of selecting and defending a proper referencing system. If the results are blindly pooled together, the methods with reduced performance metrics are improved at the expense of the higher performing methods.

This concept of whether or not the referencing system is properly established is further evaluated by investigating the uncertainties for the secondary and tertiary pigments, which are presented in Table 16. Unlike the PPig results, these data are not supplied by all the methods, so some of the results cannot be used at the same level of applicability. With the exception of the results for Phide *a*, for which only two A^+ methods provided results, the A' uncertainties are significantly lower than the corresponding A^+ values. Part of the reason for this distinction is each of the A^+ methods have one or more pigments for which the uncertainty is anomalously high, both with respect to the A' methods and to the other A^+ methods:

- J Pras, DVChl *a*, Neo and Neo+Vio;
- M Chlide *a* and Viola;
- S_8 Chl *a*; and
- S_{18} DVChl *a*, Lut, Pras, Viola, and (to a certain extent) Zea+Lut.

Although DVChl *a* is listed above, it is important to note the J or S_{18} methods did not chromatographically separate this pigment, so higher uncertainties are expected.

In the case of the J and M methods, the reanalysis results (J^+ and M^+, respectively) also exhibit anomalously high uncertainties, but the pigments involved are usually not the same in the two analysis sets. For example, the J results for Pras are much better in the J^+ reanalysis, but the Viola results are significantly worse. Similarly for M, the reanalysis for Pras is much worse than the original results. In both cases, the uncertainties are at a level that would not be acceptable for a method providing reference values for intercomparisons.

The S_8 method stands out within the A^+ subset, because with the exception of Chl *a* and DVChl *a*, all of the other pigments are within or below the uncertainties exhibited in the A' subset. To a lesser (but still notable) degree, this distinction was also seen in the PPig uncertainties (Table 15). What this shows is the value of a proper referencing system: it highlights which aspects of a method need additional attention and which parts might be performing adequately. The latter qualification is simply a reminder that the S_8 method has been intercompared only once, and additional intercomparisons are needed to verify the findings emerging here.

Within the context of needing more than one intercomparison to provide confidence in the capabilities of a

method, it is worth reviewing the histories of the methods being intercompared here (Tables 1 and 2):

- *C* Participated in SeaHARRE-2, but the results were not part of the QA subset (thus the change in method for SeaHARRE-3).
- *D* Participated in SeaHARRE-2 and the results were part of the QA subset.
- *H* Participated in all prior SeaHARRE activities and has always produced results in keeping with a QA laboratory.
- *J* Participated in SeaHARRE-1 and achieved results in keeping with a QA laboratory.
- *L* Produced QA results for SeaHARRE-1, but not for SeaHARRE-2 (thus the change to a new method for SeaHARRE-3).
- *M* Participated in both prior SeaHARRE activities and achieved results in keeping with a QA laboratory both times, but experienced significant hardware problems during SeaHARRE-3.
- *S* Participated in SeaHARRE-2 with the S_{18} method and qualified as part of the A' subset (but the inability to chromatographically separate Chl *a* and DVChl *a* led to the adoption of the S_8 method for SeaHARRE-3).

Out of all of these methods, *H*, *L*, and *M* are the only ones to have participated in all three SeaHARRE activities; *C*, *D*, *J*, and *S* participated in two intercomparisons, but *C*, *D*, and *S* all used new methods for SeaHARRE-3.

The participation history of the various methods establishes how difficult it is to a) confidently evaluate a method using only one intercomparison, b) maintain a QA status over time, and c) further develop or troubleshoot the capabilities of a method—several analysts simply changed to a proven method rather than try to refine an existing method. For the methods considered here, only *H* has consistently produced QA results over an extensive (many year) time span. This means comparisons to individual *H* results, or the time series of *H* results, help establish confidence in many of the findings presented here.

A separate check on the capabilities of the methods and, in particular, the veracity of establishing two subsets of analyses (A' and A^+), is provided by the results from the DHI mix. With respect to field samples, the DHI mix is distinguished by higher concentrations (larger peaks) and less coeluting contaminants. This means the pigments are easier to quantify and quantitate, so the results should almost be at the highest quality level a method can achieve. In practice what this means is any method should quantitate the DHI mix at one level of performance better than expected for field samples. For the QA subset, the expectation is the results will satisfy the state-of-the-art performance metric on average, and for many of the individual pigments. The A^+ subset is expected to be at a lower level of performance (on average and for many individual pigments), but should be within the 15% threshold.

A separate check on the capabilities of the methods and, in particular, the veracity of establishing two subsets of analyses (A' and A^+), is provided by the results from the DHI mix. In practice any method should be able to quantitate the DHI mix at (approximately) one level of performance better than expected for field samples. A review of the governing equations, (1) and (6), reveals why this is so—fewer variables are needed to compute the concentrations of pigments in the DHI Mix relative to a field sample, because the filtration volume (V_f) and extraction volume (V_x) are not applicable for the former. The variables in common are injection volume (V_c), pigment peak area ($\hat{A}_{P_i}$), and the calibration response factor (R_{P_i}).

The variables used to compute R_{P_i} follow from the procedures used to determine the concentration of pigment standards, which most often are done spectrophotometrically based on principles of the Lambert-Beer Law. The latter states that the fraction of the incident light at a particular wavelength λ that is absorbed by a solution depends on the thickness of the sample, the concentration C of the absorbing compound in the solution, and the chemical nature of the absorbing compound (Segel 1968). This relationship can be expressed as:

$$A_{P_i}(\lambda) \;=\; a_{P_i}(\lambda)\, l_c\, C_{P_i}, \tag{16}$$

where $A_{P_i}(\lambda)$ is absorbance, $a_{P_i}(\lambda)$ is the absorption coefficient (a constant), and l_c is the thickness of the sample in centimeters (the pathlength of the cuvette). To determine concentration from a measured absorbance, (16) is rewritten as

$$C_{P_i} \;=\; \frac{A_{P_i}(\lambda)}{a_{P_i}(\lambda)\, l_c}, \tag{17}$$

where the units for C_{P_i} depend on the expression of $a_{P_i}(\lambda)$. For example, if the concentration is expressed in molarity, a becomes the molar absorption coefficient (ε) and if the concentration is expressed as grams per liter, a is the specific absorption coefficient (α). Absorption coefficients vary depending on wavelength and the solvent in which the compound is suspended, and they are always provided with the solvent and wavelength used.

Primary pigment standards used to calibrate HPLC systems are either a) purchased in solution (with concentrations provided by the manufacturer), b) isolated from natural sources, or c) purchased in solid form. In the latter two cases, pigments are suspended in the solvents specified for use with the selected absorption coefficients, and absorbance is measured spectrophotometrically at the wavelength specified with the absorption coefficient. Assuming the specific absorption coefficient is used, the determination of the concentration of a pigment standard S_i requires the wavelength of maximum absorbance for the particular pigment (which is specified with the absorption coefficient)

and a correction measurement for the absorbance of the pigment at 750 nm:

$$C_{S_i} \;=\; \frac{A_{S_i}(\lambda_m) \;-\; A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c}, \tag{18}$$

where λ_m is the wavelength specified by the absorption coefficient and S_i denotes a pigment standard for pigment P_i.

The calibration process continues with a volume of the standard, V_c, being injected onto the HPLC column. This assumes that the range of peak areas (for the pigment) over which a linear response can be attained has already been determined. With that knowledge, it will be known whether or not the peak area of the standard (when injected undiluted) is within the linear range. For calibration purposes, the standard is injected at the wavelength and bandwidth used for the quantitation of that pigment. Chromatographic purity also needs to be assessed and can be performed at additional wavelengths—most notably the wavelength specified for use with the absorption coefficient or wavelengths permitting detection of other contaminating pigments (an unacceptable situation). It is rare that a pigment standard is chromatographically pure, meaning no isomers, allomers, epimers or other such degradation products are present. It is desirable that the parent peak (or main peak) represents no less than 90% of the total of all peaks (excluding the injection peak). The sum of the parent peak and the area of the alteration products is denoted $\Sigma\hat{A}_{S_i}$.

Returning to the HPLC calibration equation, (1) can be converted to concentration by including the V_c term:

$$C_{S_i} \;=\; \frac{\hat{A}_{S_i}\, R_{P_i}}{V_c}, \tag{19}$$

and remembering that the same pigment is involved for S_i and P_i (the former is used to make it clear that a measurement is made on the pigment standard). The response factor is now accessible, but there are two basic procedures used for determining response factors, which are distinguished by how the peak area information is used and are denoted as follows:

$R^{\Sigma}_{P_i}$ The amount injected onto the column divided by the total peak area (including the sum of the parent peak and degradants).

$R^{\%}_{P_i}$ The purity-corrected amount injected onto the column divided by the area of the main (or parent) peak alone.

With acceptably pure standards, $R^{\Sigma}_{P_i}$ is sufficiently similar to $R^{\%}_{P_i}$ so that either approach is valid. In fact, as will be shown below, they are computationally equivalent.

To derive $R^{\Sigma}_{P_i}$, the definition is applied which produces the following:

$$R^{\Sigma}_{P_i} \;=\frac{V_c C_{S_i}}{\Sigma\hat{A}_{S_i}}, \tag{20}$$

where the numerator on the right side is the amount injected on the column and the denominator is the total peak area. To make use of the spectrophotometric work that began the calibration process, (18) is substituted for C_{S_i} in (20), and the terms rearranged to yield:

$$R^{\Sigma}_{P_i} \;=\frac{A_{S_i}(\lambda_m) \;-\; A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c}\,\frac{V_c}{\Sigma\hat{A}_{S_i}}, \tag{21}$$

and noting that $\Sigma\hat{A}_{S_i}$ can be used directly in (19) for the peak area term, and then (18) and (19) can be equated to provide $R^{\Sigma}_{P_i}$.

To derive $R^{\%}_{P_i}$, a formulation for purity needs to be established, and the typical definition is the ratio of the main (or parent) peak divided by the sum of the parent peak plus degradants:

$$\hat{A}^{\%}_{S_i} \;=\; \frac{\hat{A}_{S_i}}{\Sigma\hat{A}_{S_i}}, \tag{22}$$

where the numerator is the main peak and the denominator is the sum of peaks. The definition for $R^{\%}$ requires the purity-corrected amount injected onto the column, which is the product of $C_{S_i}V_c\hat{A}^{\%}_{S_i}$, where the V_c term converts the concentration to amount, and the purity term provides the needed correction factor. Using the definition for $R^{\%}_{P_i}$ produces

$$R^{\%}_{P_i} \;=\frac{C_{S_i}\, V_c\, \hat{A}^{\%}_{S_i}}{\hat{A}_{S_i}}, \tag{23}$$

but once (22) is substituted into (23) and (18) is applied, the relationship becomes:

$$R^{\%}_{P_i} \;=\frac{A_{S_i}(\lambda_m) \;-\; A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c}\,\frac{V_c}{\Sigma\hat{A}_{S_i}}, \tag{24}$$

which is equivalent to (21).

Given the equivalence of (21) and (24), it is logical to wonder why two techniques for calculating response factors are needed. It is primarily a function of the need to be cognizant of the chromatographic purity of standards, for it is not uncommon for purity to diminish as the standard ages, in which case a change in $R^{\Sigma}_{P_i}$ may be observed and can be a direct result of the absorption coefficient used for the standard not being completely accurate for the alteration products associated with the aged standard. More importantly, the purity-corrected approach must be used when the standard is diluted to the point where the alteration products are not detectable and only the main peak can be integrated. In this case, the amount injected is $C_{S_i}V_c\,\hat{A}^{\%}_{S_i}$ and the peak area of the standard is $\hat{A}_{S_i}$. In these instances, it is important to determine chromatographic purity with an undiluted standard that is near the upper limit of the linear range so that alteration products are detectable.

The formulations for response factors can be combined with a typical quantitation equation for pigment concentration (6) to produce a governing equation involving all the terms associated with computing the concentration of a particular pigment in an individual sample:

$$C_{P_i} = \frac{V_x}{V_f}\,\hat{A}_{P_i}\left[\frac{A_{S_i}(\lambda_m) - A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\,l_c\,\Sigma\hat{A}_{S_i}}\right], \qquad (25)$$

where it is assumed V_c is the same for field samples and laboratory standards, and the terms in brackets represent the response factor.

Additional complexities occur, because an internal standard can be used to improve the determination of V_x. There are two common procedures for using an internal standard, and they are distinguished here by the number of laboratory steps involved: a) the extraction solvent and internal standard are contained together in a mixture (prepared beforehand), which is added to the sample in one step, or b) the extraction solvent and internal standard are added separately in two steps. In the one-step approach, a volume of solvent and internal standard is mixed together in a batch, and a small portion of the mixed volume, V_m, is added to the sample. In the two-step approach, a volume of the extraction solvent, V_e, is added to the sample followed by a small volume of internal standard, V_s. The filter, now soaking in the solvent–standard mixture, is disrupted (most commonly with a sonic probe), clarified (to remove filter debris), and a volume of the clarified sample extract, V_c, is injected onto the HPLC column.

The internal standard permits a correction for the presence of residual water retained on the filter (plus any variations in volume caused by evaporation) by using a) the peak area of the internal standard when it is injected onto the HPLC column ($\hat{A}_c$) prior to its addition to the sample, and b) the peak area of the internal standard in the sample ($\hat{A}_s$). In the one-step approach, $\hat{A}_c$ is determined by injecting the solvent–standard mixture onto the HPLC column, whereas for the two-step approach, the standard is injected directly onto the column. For the one-step approach, the internal standard is diluted by the extraction solvent, so $V_{x_1} = V_m\hat{A}_{c_1}/\hat{A}_{s_1}$, where the "1" in the subscripts indicates the one-step methodology. For the two-step approach, $V_{x_2} = V_s\hat{A}_{c_2}/\hat{A}_{s_2}$ (the "2" in the subscripts indicates the two-step methodology). If an internal standard is not used, an estimate of the volume of water retained on the filter, V_w, is added to the volume of extraction solvent, V_e, so $V_{x'} = V_e + V_w$. For a 25 mm filter, water retention is usually assumed to be 0.2 mL (Bidigare et al. 2003).

Considering a one-step internal standard methodology for determining V_x, (25) becomes

$$C_{P_i} = \frac{\hat{A}_{c_1}}{\hat{A}_{s_1}}\,\frac{V_m}{V_f}\,\hat{A}_{P_i}\left[\frac{A_{S_i}(\lambda_m) - A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\,l_c\,\Sigma\hat{A}_{S_i}}\right], \qquad (26)$$

and now all the terms associated with a rather complicated protocol are accessible (again, assuming V_c is a constant). This means an analyst can begin to assign uncertainties to each term and ultimately estimate a final uncertainty in the quantitated concentrations for each pigment, which is not to imply that any terms not present in the governing equation should be ignored. It is important to scrutinize each step of the protocol to ensure all parts are properly understood.

Given an investigative perspective, uncertainties in V_c, for example, are in part described by the imprecision of replicate injections of the DHI Mix (Sect. 1.5.2 and Table 14). Uncertainty sources affecting $\hat{A}_{P_i}$ differ somewhat between field sample and DHI Mix analyses. For example, effects of filter inhomogeneity and the efficiency with which pigments are extracted from cells are unique to field samples and contribute to interlaboratory differences. With respect to field samples, the DHI mix is distinguished by higher concentrations (larger peaks) and fewer coeluting contaminants, so the pigments are easier to quantify and quantitate. Consequently, DHI Mix results should almost be at the highest quality level a method can achieve. For methods with a restricted limit of linearity (or nonlinear methods), however, the DHI Mix at full strength can produce erroneous results and dilution with validated dilution devices may be needed so peak areas fall within the limit of linearity for the method.

Method uncertainties from the DHI Mix-101 analyses are presented in Table 17. The QA subset has overall PPig and TChl *a* uncertainties of 5.1% and 3.1%, respectively, which are within the performance specifications for state-of-the-art analyses (Table 9). The QA subset is also distinguished by a small range in the overall PPig averages, 4.6%, and the overall best method (L) is within the state-of-the-art precision (3% or less) of the theoretical best method of 1.0%. The overall A' averages are also within the *best method* results established with the field sample analyses (Table 15): 7.4% for PPig and 5.2% for TChl *a*. The difference between the two is 2.1–2.3% and assuming minimal contributions from other sources (e.g., differences in extraction efficiency), establishes an estimate for the variance in the filtering of the samples collected in the field (which is similar to the filtering variance experienced during SeaHARRE-2).

With the exception of Peri, the individual overall primary pigment uncertainties for the DHI mix A' results are always within the state-of-the-art performance metrics. In terms of the individual methods and individual pigments, almost all of the results are within or very close to the state-of-the-art performance metrics and the *best method* entries are spread across all four methods (although L predominates). Notable exceptions are the anomaly in the Caro results, the large But values, and the even larger Peri values. Both of the latter were identified as being problematic in the field sample analyses (Table 15). The fact that these three pigments have anomalously high uncertainties

Table 17. The $|\bar{\psi}|$ values (average APD in percent) for DHI Mix-101 as a function of the HPLC method for the primary pigments using the presentation scheme established in Table 12. The uncertainties are computed using the average concentrations of the pigments determined by the QA subset as the reference in the uncertainty calculation.

Meth.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Average*
C	4.2	8.1	5.5	11.4	**0.9**	3.8	**2.5**	3.2	0.4	2.6	11.7	2.2	4.7
D	6.1	**0.1**	12.8	7.6	14.4	3.7	5.2	7.9	**0.2**	5.9	27.0	2.2	7.8
H	**0.4**	2.6	**0.6**	19.0	1.4	0.1	5.9	5.7	0.3	4.1	10.7	4.8	4.6
L	1.5	5.7	6.6	**0.0**	13.9	**0.1**	3.2	**1.0**	0.8	**0.8**	**4.6**	**0.4**	**3.2**
J	40.5	1.6	15.4	22.8	6.5	13.4	8.0	16.1	1.1	11.1	19.1	18.5	14.5
M	3.6	3.0	7.4	1.5	6.2	15.5	30.7	12.1	7.0	6.1	10.4	5.6	9.1
S_8	97.6	74.1	36.1	50.1	1.7	10.5	22.7	3.8	0.9	14.9	3.4	4.3	26.7
S_{18}	23.1	13.6	5.8	7.8	4.8	18.8	12.8	0.8	3.5	4.0	9.0	10.0	9.5
A'	3.1	4.1	6.4	9.5	7.7	1.9	4.2	4.5	0.4	3.3	13.5	2.4	5.1
A^+	41.2	23.1	16.2	20.6	4.8	14.6	18.5	8.2	3.1	9.0	10.4	9.6	14.9
A^-	0.4	0.1	0.6	0.0	0.9	0.1	2.5	1.0	0.2	0.8	4.6	0.4	1.0
J^+	*17.9*	*0.5*	*11.2*	*2.7*	*10.8*	*3.3*	*4.0*	*8.2*	*5.7*	*1.1*	*47.0*	*4.4*	*9.7*
M^+	*16.2*	*15.1*	*5.6*	*9.2*	*24.2*	*1.1*	*4.2*	*16.0*	*20.1*	*5.9*	*8.4*	*2.1*	*10.7*

for the DHI mix indicates there are analyst-to-analyst interpretation differences for these pigments and, for Caro, deleterious effects from methods using differing absorption coefficients.

The overall A^+ PPig averages for the DHI mix are just within the 15% threshold for PPig quantitative analysis, but not within the metric for routine TChl *a* analysis. There is a large range in the overall A^+ PPig averages, 17.6%, with M and S_{18} having overall performance at the state-of-the-art level, J satisfying quantitative performance, and S_8 meeting the routine analysis requirements. An even larger spread is seen in the TChl *a* uncertainties, 94.0%, with the confounding result that the excellent results for the majority of the pigments achieved by J and S_{18} are not reproduced for TChl *a*.

Another perplexing aspect of the DHI mix results is the duality observed with the field samples for the S_8 and S_{18} methods (the former had mostly unacceptable uncertainties for the chlorophylls and rather acceptable uncertainties for the carotenoids, while the latter had the opposite) is not strongly exhibited. The S_8 results are mostly as expected, except for the anomalously large uncertainties for Caro, Allo, and (to a certain degree) Fuco. The S_{18} results, however, are not as expected: the carotenoid results are significantly better than expected (7.9% on average) and the TChl *a* result is a bit worse than for the field samples and almost does not satisfy routine quality.

The DHI mix results for the J^+ and M^+ reanalyses show a small overall average PPig improvement for the former and a small degradation for the latter (with respect to the original analyses). As was seen with the field samples, there are examples where both methods improved individual pigment uncertainties (e.g., Caro for J and Allo for M), but there are also examples wherein the individual pigment uncertainties did not improve (e.g., Peri for J and Diato for M). All of the J^+ chlorophyll reanalyses are better than the original results, with a significant improvement for TChl *a*, whereas the M^+ chlorophyll results are a combination of improvements and degradations. In both cases, however, the achieved performance for TChl *a* is at the level of routine analysis.

The choice a laboratory makes as to which absorption coefficients to use can introduce large interlaboratory differences, as demonstrated in Hooker et al. (2005). This controllable source of variance is re-evaluated here with a second subset of DHI Mix results from laboratory H, which are denoted H'. The H' subset was created by choosing alternate absorption coefficients based on acetone, which were used for all the carotenoids in the PPig category except for Caro (i.e., eight carotenoids).

All laboratories (including H) used the same ethanol absorption coefficients for Peri, Allo, and Zea, while M used alternate absorption coefficients for all other primary carotenoids; L used alternate absorption coefficients for Diadino, Diato, and Caro; and C and H used an alternate absorption coefficient (in acetone) for $\beta\beta$-Car. Despite the fact that most of the absorption coefficients in use by the marine pigment community do not have known accuracies, if all laboratories choose ones in common, the expectation is the results will improve by approximately one level of performance (simply from enhanced consistency, and not necessarily from improved accuracy). For the QA subset this means the results will satisfy the state-of-the-art performance metric on average (10%); the A' subset is expected to be at a lower level of performance (on average), but should be within the 15% threshold.

The H' and H average uncertainties for the eight PPig carotenoids (Caro excluded) are 14.7% and 4.1%, respectively. Whereas all pigments except Peri had been within

Table 18. The $|\bar{\psi}|$ values (average APD in percent) across the DHI Mix-101 analyses for a subset of the secondary and tertiary pigments (following the presentation scheme established in Table 13).

Meth.	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
C	3.7	3.7	41.3	3.2	**1.0**	**1.9**	26.3	8.9	4.9	2.5	2.7
D	7.5	**0.1**	25.7	2.3	3.8	6.3	**0.8**	2.8	0.9	7.9	**2.3**
H	**1.3**	3.9	**18.7**	**0.2**	7.0	6.4	5.8	**1.1**	**0.1**	6.0	2.4
L	2.5	0.3	48.2	5.4	4.3	2.0	21.3	12.8	3.9	**0.5**	2.5
J	80.5	78.7	73.5	5.3	4.8	3.2	3,760.3		2.5	8.3	11.8
M	2.7	8.2	18.6	13.0					41.0	46.9	9.4
S_8	105.1	60.2	24.5	3.2	6.1	6.1		13.1	9.2	6.0	0.5
S_{18}	11.6	58.4	16.3	7.0	41.8	15.8	29.7	2.8	28.2	0.9	8.4
A'	3.8	2.0	33.5	2.8	4.0	4.2	13.5	6.4	2.5	4.2	2.5
A^+	50.0	34.2†	33.2	7.1	17.6	8.4	1,895.0	7.9	20.2	15.5	7.5
A^-	1.3	0.1	18.7	0.2	1.0	1.9	0.8	*1.1*	0.1	0.5	2.3
J^+	*10.0*	*30.8*	*3.1*	*14.6*	*38.0*	*25.6*	*17.9*		*14.6*	*17.5*	*9.5*
M^+	*14.4*	*29.7*	*0.5*	*0.6*					*54.4*	*16.3*	*0.7*

† Excluding J and S_{18}, because these methods did not chromatographically separate monovinyl and divinyl chlorophyll *a*.

the state-of-the-art category (Table 17), with the use of acetone absorption coefficients, only Peri, Fuco, and Zea are. In fact, H' uncertainties exceed 15% for But, Hex, and Diato. These calculations use the existing referencing system (the C, D, H, and L averages), but had H' and not H been included in the reference calculations, the results would have been only slightly improved—the average uncertainty for the eight carotenoids in question is 11.1% (not 14.7%), and the uncertainties for But, Hex, and Diato still exceed 15%. These findings demonstrate how an otherwise state-of-the-art laboratory can easily change status to the next performance category simply by the choice of absorption coefficients. Unfortunately, the uncertainty in an absorption coefficient is beyond the control of the laboratory using it, and no matter how accurately and precisely a method is otherwise implemented, all quantitative results using that absorption coefficient will be degraded.

The uncertainties for the analysis of secondary and tertiary pigments within DHI Mix-101 is presented in Table 18. These data further reinforce the results already presented with the field samples and the PPig results for the DHI mix: a) the QA subset almost always has significantly lower overall uncertainties than the A^+ subset (Chlide *a* is the notable exception) and is usually at a state-of-the-art level of performance; b) the distribution of the *best method* results are spread across all four QA methods (although H predominates); c) there are numerous examples of A^+ methods achieving state-of-the-art performance, but this is countered by other instances of elevated uncertainties; d) the S_8 chlorophyll uncertainties are larger than the S_{18} values, but the S_8 method has lower overall carotenoid uncertainties.

For the J^+ and M^+ reanalyses, the J^+ chlorophyll results exhibit the most consistent improvement, which had significantly lower uncertainties. The M^+ results are frequently better than the original M values, but there are notable exceptions (e.g., Chl *a*, DVChl *a*, and Pras).

1.5.3.2 Pigment Sums

Prior SeaHARRE activities established a functional relationship in pigment uncertainties: *PPig overall uncertainties decreased significantly as individual pigments were summed.* This is a direct reflection of a) how summing cancels the highs and lows associated with the biases in the individual pigments, and b) more abundant pigments are more important to the sums and they are usually quantitated with lower uncertainties (i.e., smaller biases). If an abundant pigment is also a problematic pigment, summing will frequently not ameliorate the uncertainties involved.

Although there is no performance metric for pigment sums, the expectation is for the uncertainties in pigment sums to be significantly less than the individual pigments. Given a starting perspective of validated methods performing at the semiquantitative quality level, it is reasonable to expect pigment sums to have an uncertainty in keeping with quantitative analysis, i.e., to within 15% on average. Similarly, for a QA laboratory, the expected uncertainty would be to within the next level of quality, that is, 10% on average.

In this cascade towards lower uncertainties, a method having problems with a class of pigments (e.g., the chlorophylls), is expected to have elevated uncertainties for sums predominated by that class of pigments (e.g., TChl). These elevated uncertainties can easily influence other sums if the pigments involved are particularly abundant. For example, chlorophyll *a* is usually the most abundant pigment in marine ecosystems, so if there is a problem with the quantitation of this pigment, there will be elevated uncertainties in the formulation of TChl or TPig.

Table 19. The $|\bar{\psi}|$ values (average APD in percent) across all 24 batches of field samples as a function of the laboratory method for the pigment sums, and following the presentation scheme established in Table 12. The $|\bar{\psi}|$ values of the primary pigments, [PPig], for the field samples (Table 15) are given in the first column for easy comparison. Method averages for the pigment sums are given in the last column.

Meth.	[PPig]	[TChl]	[PPC]	[PSC]	[PSP]	[TCaro]	[TAcc]	[TPig]	[DP]	*Avg.*
C	16.9	6.4	7.5	11.3	7.0	8.3	8.1	6.2	8.9	8.0
D	11.4	6.9	8.2	**5.0**	5.4	**4.5**	**4.2**	5.7	**3.9**	**5.5**
H	10.9	7.2	**6.5**	6.2	6.8	5.3	7.0	6.2	5.0	6.3
L	**9.6**	**5.3**	6.7	5.6	**5.2**	5.7	4.9	**5.2**	6.4	5.6
J	62.6	25.2	8.6	27.7	25.3	12.4	10.2	19.3	14.4	17.9
M	87.8	19.5	18.7	29.1	21.2	22.0	18.5	20.9	19.5	21.2
S_8	26.6	44.7	12.3	7.8	32.6	5.6	9.3	27.5	12.5	19.0
S_{18}	37.2	12.4	9.5	8.7	10.7	5.9	6.2	9.4	9.9	9.1
A'	12.2	6.4	7.2	7.0	6.1	6.0	6.0	5.8	6.0	6.3
A^+	53.6	25.4	12.3	18.3	22.5	11.5	11.0	19.3	14.1	16.8
A^-	7.4	5.3	6.5	5.0	5.2	4.5	4.2	5.2	3.9	3.9
J^+	*48.4*	*14.3*	*20.5*	*10.8*	*13.2*	*9.0*	*7.1*	*9.5*	*6.9*	*11.4*
M^+	*85.0*	*41.4*	*10.1*	*67.2*	*38.4*	*33.0*	*42.1*	*28.1*	*37.9*	*37.3*

In Table 19, the uncertainties in pigment sums for the SeaHARRE-3 field samples are presented. The overall averages from the QA subset show a decrease in average uncertainties from PPig to pigment sums of 12.2% to 6.3%. The range of the uncertainties for the individual sums are frequently rather small, the overall averages for the individual sums are to within 10%, and the range of the individual averages are rather similar (they differ by a maximum of only 1.4%). The *best method* results are spread across three of the QA methods with D predominating. The difference between the overall A^- and A' averages is 2.4%, which is on the order of the variance in preparing the field samples. Although there is no performance metric for pigment sums, the convergence of the A^- and A' overall averages to within the variance in preparing the field samples strongly suggests the A' uncertainties for pigment sums are at the state of the art.

The A^+ subset shows a more significant decrease in uncertainties from the overall PPig average of 53.6% to 16.8%. The individual method decreases are also large with M decreasing the most: 66.6%. Such substantial decreases establish the utility of using sums in databases to minimize uncertainties, particularly if the source data has an unknown quality, but some caution is needed: with the exception of the S_{18} method, the overall averages are not to within 15%, and many of the individual uncertainties exceed 25%. For uncertainties with sums, the latter is an indication of significant biases in the method. For example, the problems with the individual chlorophylls for the S_8 method produce anomalously large uncertainties for TChl, photosynthetic pigments (PSP), and TPig. The more reasonable results for the individual carotenoids for the S_8 method, however, produce acceptable uncertainties for PSC and total carotenoid (TCaro).

Both the J^+ and M^+ overall pigment sum uncertainties are significantly less than the corresponding average PPig uncertainties, with the latter decreasing 47.7%. The J^+ reanalysis results are significantly improved with respect to the original results with only one sum, PPC, exceeding a 15% threshold. The M^+ results, however, are significantly degraded with respect to the original analyses: the overall average exceeds 25% and several of the individual uncertainties exceed, or are very close to, 40%.

1.5.3.3 Pigment Ratios and Indices

The aforementioned functional relationship established by prior SeaHARRE activities extended beyond pigment sums and into higher-order data products: *PPig overall uncertainties decreased as individual pigments were summed, decreased further when pigments were used to form ratios, and then increased slightly with the formation of pigment indices.* The latter are a combination of sums and ratios (Table 5), so the small increase is expected to be on the order of the uncertainty in forming pigment sums (or less).

There are no performance metrics for pigment ratios and indices, but given the prior discussion of expected uncertainties for pigment sums (Sect. 1.5.3.2) and the concept of a cascade in uncertainties, the expectation is the QA subset will have uncertainties to within 5% for pigment ratios, and to within 10% for pigment indices. For other methods, the expectation is the uncertainties for ratios will be to within 10% and the uncertainties for indices to within 15%.

The uncertainties in pigment ratios and indices for the SeaHARRE-3 field samples are presented in Table 20. The QA subset has overall average uncertainties of 3.5% and 6.1% for the ratios and indices, respectively. Both results

Table 20. The $|\bar{\psi}|$ values (average APD in percent) across all 24 batches of field samples as a function of the laboratory method for the pigment ratios and indices (following the presentation scheme established in Table 12).

Lab. Method	$\frac{[\text{TAcc}]}{[\text{TChl } a]}$	$\frac{[\text{PSC}]}{[\text{TCaro}]}$	$\frac{[\text{PPC}]}{[\text{TCaro}]}$	$\frac{[\text{TChl}]}{[\text{TCaro}]}$	$\frac{[\text{PPC}]}{[\text{TPig}]}$	$\frac{[\text{PSP}]}{[\text{TPig}]}$	$\frac{[\text{TChl } a]}{[\text{TPig}]}$	*Avg. Ratio*	[pPF]	[nPF]	[mPF]	*Avg. Index*
C	6.7	3.8	3.1	7.7	5.8	1.4	3.9	4.6	7.9	6.6	15.3	9.9
D	5.3	4.3	5.1	5.0	4.1	0.7	2.9	3.9	4.5	4.8	7.9	5.7
H	4.2	2.4	3.1	**2.9**	4.0	0.9	2.4	2.9	**3.3**	3.2	**5.4**	4.0
L	**4.1**	**1.4**	2.0	3.6	**3.6**	**0.6**	**2.2**	2.5	5.2	**2.9**	6.3	4.8
J	17.6	15.2	13.9	14.6	19.7	5.2	11.9	14.0	9.5	18.4	192.2	73.4
M	10.9	6.6	7.4	10.5	8.1	1.5	6.5	7.4	9.7	73.0	19.5	34.1
S_8	33.2	4.7	9.3	47.7	18.9	4.5	23.8	20.3	18.9	12.3	17.3	16.2
S_{18}	11.1	4.4	6.4	9.8	10.2	1.5	6.7	7.1	8.9	10.7	21.1	13.6
A'	5.1	3.0	3.3	4.8	4.4	0.9	2.9	3.5	5.2	4.4	8.7	6.1
A^+	18.2	7.7	9.2	20.6	14.2	3.2	12.2	12.2	11.7	28.6	62.5	34.3
A^-	4.1	1.4	2.0	2.9	3.6	0.6	2.2	2.4	3.3	2.9	5.4	3.9
J^+	*16.6*	*13.0*	*16.5*	*18.7*	*23.8*	*5.3*	*10.9*	*15.0*	*5.8*	*4.7*	*37.6*	*16.0*
M^+	*65.1*	*20.2*	*18.5*	*33.3*	*30.6*	*8.5*	*25.7*	*28.8*	*15.4*	*24.8*	*31.6*	*23.9*

are to within their respective anticipated thresholds. The individual method uncertainties for the ratios and indices are almost always very similar and span a narrow dynamic range. The notable exception are the C results for mPF, which exceed the 10% threshold. The mPF index is formulated from Fuco, Peri, and DP (Table 5). Recalling the individual C uncertainties for these three constituents (Tables 15 and 19), C had the largest uncertainties within the QA subset, so the large mPF uncertainty is probably a direct reflection of the larger individual uncertainties. Although, the overall average C uncertainty for the indices is to within 10%, the value achieved is notably different from the other QA results.

The uncertainties in the A^+ pigment ratios and indices provide additional clarity about the unique problems with the methods. As a group, the overall average A^+ uncertainties for ratios and indices are much higher than the corresponding QA subset values, particularly for the indices, and the values are above the anticipated thresholds of 10% and 15%, respectively. All of the methods show an individual decrease in overall average uncertainties from sums to ratios, except for the S_8 method, which shows a small increase. The S_8 method also stands out in the progression of uncertainties from ratios to indices—it is the only method for which the uncertainties do not increase. In this regard, the J and M results must also be considered anomalous, because the increases in uncertainty from ratios to indices is excessively large, especially for the J results.

As noted earlier with the C mPF results, problematic pigments can elevate uncertainties in ratios. This is particularly notable if the problematic pigment or pigments appear exclusively in the numerator or the denominator. If the sources of uncertainty appear in both the numerator and denominator, uncertainties can rise, fall, or be nullified depending on the biases the uncertainties represent (i.e., overestimation versus underestimation) and the magnitudes involved.

The more abundant a pigment is naturally, the more important it is to the net uncertainty of a ratio or index, but not in all cases. If a problematic pigment is abundant, the uncertainty from that particular pigment is somewhat nullified if it is present in both the numerator and denominator, e.g., the J results for $[\text{PSP}]/[\text{TPig}]$. If an abundant problematic pigment appears in either the numerator or the denominator (but not both), its presence usually elevates uncertainties, e.g., the S_8 results for $[\text{TChl}]/[\text{TCaro}]$.

The J^+ and M^+ are as noted in earlier discussions: there are examples where the uncertainties in the reanalyses are improved or degraded, with no consistent trend for either method. In both cases, the anticipated thresholds are not achieved, although the J^+ results exhibit the most improvement and are close.

1.6 SUMMARY AND DISCUSSION

The diversity of objectives for the SeaHARRE-3 activity, along with the large number of separate analyses involved, make it appealing to summarize and discuss the most important aspects of the results in one place. The discussion presented here is not intended to be a substitute for reading the greater detail presented in the preceding sections, and follows the original presentation of the objectives (Sect. 1.1) and much of the organizational scheme used to present the results (Sect. 1.5).

1.6.1 Individual Pigment Uncertainties

A recurring aspect of the results presented here has been to discuss the importance of a proper referencing system for establishing method uncertainties. This has been emphasized, because in round robins involving natural samples, there is no *a priori* understanding of truth. A foundation of the SeaHARRE activity is that truth can be estimated by assuming a properly validated HPLC method is capable of providing a good estimate of truth, and the deviations from truth are mostly due to small sources of random errors, so the pooled estimate of truth from several methods should tend towards a realistic proxy value of truth. The potential pitfall in this logic is if a method that has not been properly validated is included in the methods used to estimate the referencing system.

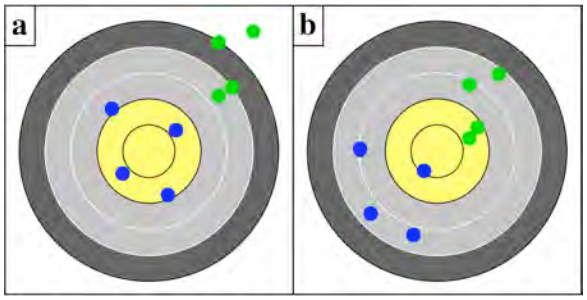

Fig. 2. The targeting results of two archery teams (blue and green) whose shot patterns are exactly the same for two different cases: **a)** with respect to a known bull's-eye, and **b)** with respect to a bull's-eye pattern centered over the average of all the shots.

The severity of this pitfall is illustrated in Fig. 2, which displays the shots of two groups competing for placement on an archery team. With respect to the known bull's-eye (Fig. 2a), all blue archers, but only one green archer, will qualify. For the HPLC problem set, this situation is most similar to the analysis of laboratory standards. Imagine the archers do not aim with respect to a known bull's-eye, because one does not exist (e.g., field samples). Instead, they shoot at a large blank circle and are evaluated by overlaying a target pattern with respect to the average of all the shots (Fig. 2b). In this case, all the archers qualify for the team, and the green archers are slightly more accurate as a group than the blue archers. The latter is a recurring consequence of spreading the variance of potentially degraded results across all of the outcomes.

To minimize the problem of spreading the variance across methods with significantly different capabilities, the approach adopted in SeaHARRE activities is to establish a QA subset (the blue archers in Fig. 2a) as the proxy for truth (the bull's eye). The accuracy of the methods is estimated from the uncertainty of all results with respect to the average results of the QA subset. This cannot be a perfect approach for field samples, because there are so many unknowns and the QA subset can still have unresolved problems that will negatively influence the outcomes. For example, the results of the individual primary pigments (Table 15) reveal there are unresolved analyst-to-analyst interpretation problems with Peri and Diato, and to a lesser extent, But.

Adding in more methods with reduced capabilities will not *beat down the noise* in the quantitation of problematic pigments, i.e., the so-called $\sqrt{N}$ effect. For example, Table 15 shows the inclusion of only one A^+ method can add a significantly unhelpful amount of variance to the aforementioned pigments that need to be scrutinized in the QA subset: 356.5% to Peri, 182.4% to Diato, and 387.6% to But. Adding in more methods with higher variability in the data products will not make it easier to identify why specific pigments have elevated uncertainties under certain circumstances.

Consequently, one of the important attributes of the set of methods used as the referencing system in the computation of uncertainties is that they have rather uniform results across the broadest suite of data products possible. The standard deviation in the uncertainties within the QA subset averaged 1.3% for TChl *a*, 4.9% for PPig, 1.4% for pigment sums, 1.1% for pigment ratios, and 1.6% for pigment indices. The corresponding values for the A^+ subset were 17.6%, 66.3%, 8.0%, 7.6%, and 20.7%, respectively. The latter represents a 5.5- to 13.7-fold increase in variance with respect to the former, and is one of the more distinctive mechanisms of showing the difference between the two subsets of results.

The precision of the individual methods is another important indicator of the distinct differences between the A' and A^+ subsets. Figure 3a shows the average precision of the two subsets as a function of the primary pigments grouped as three chlorophylls, the nine carotenoids, and the overall average. To establish a wider context, summary data are shown for SeaHARRE-1 through SeaHARRE-3. Taken together, the three activities span a wide dynamic range in trophic systems (e.g., the TChl *a* concentration range is approximately 0.020–26.185 mg m^{-3}, or a little more than three decades in concentration): mesotrophic (Mediterranean Sea), eutrophic (Benguela Current), and oligotrophic (South Pacific gyre), respectively. For both of the subsets, the SeaHARRE-3 results are distinguished by being slightly more imprecise on average, which is probably a direct reflection of the difficulties associated with analyzing samples with very low concentrations. Although there are examples for which the two subsets have similar precision, the majority of the data—and the overall average—shows the QA subset has distinctly better precision by about 3.8% on average.

The most significant difference between the two subsets is in the accuracy (uncertainty) of the derived data products, which is shown in Fig. 3b (for all three SeaHARRE activities). A substantial decrease in uncertainties for the QA subset is seen in all data products, but very notably for the individual pigments (the largest decrease

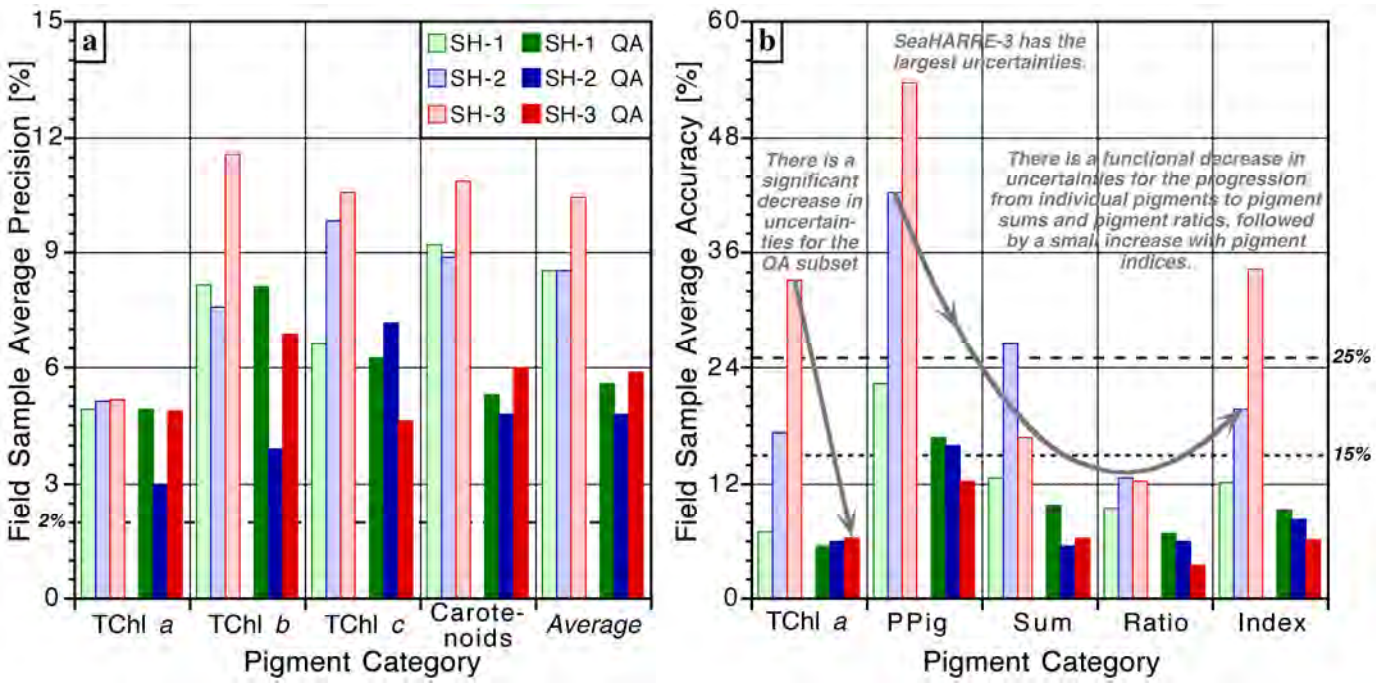

Fig. 3. Overall performance results for the A^+ and A' (QA) subsets (light and dark bars, respectively) for all three SeaHARRE (SH) activities: **a)** method precision, with the approximate 2% variance associated with the preparation of the field samples shown as a dotted line, and **b)** accuracy, with the 15% quantitative and 25% semiquantitative performance metrics for PPig shown as dotted and dashed lines, respectively.

is for the SeaHARRE-3 PPig results). In fact, the QA methods are always within the 2% sampling variance for the 10% and 15% quantitative analysis requirements for TChl *a* and PPig, whereas the A^+ subset frequently does not satisfy the semiquantitative thresholds.

1.6.2 Higher-Order Variables

The uncertainty of the higher-order variables (sums, ratios, and indices) in Fig. 3b are distinguished by lower average uncertainties than the primary (individual) pigments. The average PPig uncertainties for the SeaHARRE-3 QA subset to within the 15% quantitative threshold, and the QA subset for the prior SeaHARRE activities satisfy the threshold to within the 2% sampling variance. The average uncertainties for the QA pigment sums, ratios, and indices are all significantly less than 15% for all SeaHARRE activities. Many the results from the A^+ subset exceed the 15% and 25% thresholds, particularly for SeaHARRE-3. The average A^+ uncertainties for pigment ratios, however, are well within the 15% threshold; they average about 11.4%. These results confirm the possibility of using higher-order variables to minimize uncertainties in large databases derived from a diverse set of contributors (Claustre et al. 2004), especially with pigment ratios.

Although the QA subset has lower uncertainties than the A^+ subset, both sets of uncertainties follow the same functional form: *there is a decrease in the uncertainties for the progression from the primary pigments to the sums and ratios, followed by a small increase with the indices.* This is also seen in the variance of the uncertainties with the two subsets as revealed above—the variance in the results decrease as individual pigments are formed into sums and ratios, but then slightly increases with the formation of indices.

The reproducibility of the uncertainty functional form shown in Fig. 3b allows a revisitation of two anomalies seen with the S_8 method (Sect. 1.5.3.3): there is no decrease in average uncertainties in the progression from sums to ratios, and there is no increase in the subsequent progression of average uncertainties from ratios to indices. During SeaHARRE-2, one of the QA laboratories analyzed a set of unequivocally damaged samples (they were defrosted during shipping). The results showed a QA laboratory analyzing bad samples was superior to a method lacking a proper QA scheme and analyzing good samples. This conclusion was confirmed by the precision data as well. An important aspect of the results, however, was that the functional form of the higher-order variables was not properly reproduced in the damaged samples: the uncertainties steadily decreased with the formation of the higher-order data products, but did not show an increase with the computation of the indices. The lack of this latter signature is interpreted here as suggesting the S_8 method has a problem that is corrupting the natural pigment-to-pigment relationships.

The concept of using the natural relationships between pigments to discern problems with a method is difficult to

apply to the DHI mix results, which are derived from an artificial mixing of pigments from cultured stocks. Nonetheless, the reduction in uncertainties as pigments are summed and then ratioed should occur, and the uncertainties should be considerably lower than in natural samples, i.e., the 15% threshold should always be satisfied and a QA method should have higher-order uncertainties on the order of 5% or less.

A summary of the average uncertainties for the A' and A^+ results for the DHI mix are presented in Table 21. The results for the QA subset satisfy the expected performance thresholds and exhibit a decreasing relationship similar to the functional form presented in Fig. 3b, and includes a small increase in uncertainties associated with the indices. The lowest values are close to the precision of the individual methods, which have an average value of approximately 2% for the DHI mix, so the ability to distinguish them further is not really possible.

Table 21. The average uncertainties in percent for the DHI mix as a function of the A' and A^+ subsets.

Subset	*PPig*	*Sums*	*Ratios*	*Indices*
A'	5.1	2.6	2.7	3.9
A^+	14.9	17.3	14.9	10.5

The A^+ subset has an average precision for the DHI mix of about 3%, so there are no resolution issues for the entries in Table 21. With the exception of the results for the indices, the A^+ subset results are a) close to, or above, the 15% threshold; and b) do not follow the expected functional form, both in terms of the relationships and the magnitudes. (Uncertainties increase in the progression from the primary pigments to the pigment sums, and the decrease for the pigment ratios results in an average uncertainty that is only at the PPig level.)

1.6.3 Ocean Color Requirements

The remote sensing requirements for the *in situ* determination of $\left[\text{TChl } a\right]$ are well satisfied by QA methods, but not for all other methods. The 25% compliance objective was not achieved by all methods during SeaHARRE-3, and the 15% refinement objective was achieved by only the QA methods. Although TChl *a* is currently the primary pigment of interest for ocean color applications, recent inquiries have demonstrated the possibility of deriving pigment indices from satellite observations (Uitz et al. 2006), so the future of remote sensing will include more pigment products than just the chlorophyll *a* concentration. Although no community-wide accuracy objectives exist for any pigments other than Chl *a*, the average uncertainties for the pigment indices for the QA subset are always to within 15%; across all three SeaHARRE activities, the average uncertainty for the QA pigment indices is about 5.4%.

1.6.4 Performance Metrics

The most important individual pigment considered here is TChl *a*, because it is the central pigment in marine bio-optical and ecosystem studies. The average $\left[\text{TChl } a\right]$ uncertainty for the A' subset was 6.3%. This result is very similar to the SeaHARRE-1 and SeaHARRE-2 values of 5.5% and 5.9%, respectively. For an earlier SIMBIOS† pigment round robin, the average uncertainty in determining $\left[\text{TChl } a\right]$ is estimated to be approximately 8.5%‡ (Van Heukelem et al. 2002). These results confirm the 10% accuracy threshold currently being used for quantitative analysis is realistically set, and that the state-of-the-art criterion of 5% is within reach, but only with some extra effort (which is what the state-of-the-art is supposed to represent). Although the A^+ subset was challenged by the quantitative threshold (none satisfied it or the semiquantitative requirement), it is important to remember one of the objectives of the performance metrics is to establish criteria that will help analysts understand where improvements are needed.

1.6.5 Conclusions and Recommendations

The SeaHARRE-3 activity was concerned with estimating the uncertainties in quantifying a diverse set of chlorophyll and carotenoid pigment concentrations for a variety of HPLC methods and related procedures used in the analysis of predominantly oligotrophic waters. Although prior SeaHARRE activities had a wide dynamic range in TChl *a*, only SeaHARRE-1 covered the oligotrophic regime and only for one sample, so oligotrophic waters were poorly represented. Based on the QA subset, the SeaHARRE-3 sampling included 2 eutrophic, 15 mesotrophic, and 7 oligotrophic samples. The partitioning of the uncertainties for TChl *a* within these three regimes averaged 9.6, 5.8, and 6.6%, respectively. These data suggest uncertainties are at a minimum in mesotrophic samples and increase as concentrations become larger and smaller, with the largest increase associated with eutrophic samples. The rather close agreement for the mesotrophic and oligotrophic regimes suggest detection limits were not a significant issue for the QA subset.

Although the SeaHARRE-3 eutrophic samples have the largest average uncertainty, they are based on only two samples, and might be spurious. The SeaHARRE-2 results involved only the eutrophic and mesotrophic regimes, and

† Sensor Intercomparison and Merger for Biological and Interdisciplinary Oceanic Studies

‡ The referencing system for the SIMBIOS round robin was based on comparisons with laboratory H—and not on comparisons with respect to an overall average or a quality-assured subset. The uncertainty in $\left[\text{TChl } a\right]$ is estimated as $\sqrt{7.0^2 + 4.5^2 + 1.5^2}$ or 8.5%, where 7.0% is the average agreement of the equivalent QA methods, 4.5% is the average method precision, and 1.5% is the HPL calibration accuracy.

the average TChl *a* uncertainty for the QA subset was 7.8 and 6.1%, respectively (although the difference is within the variance of the sample preparation). So the higher uncertainty for the eutrophic regime is reproduced. The SeaHARRE-3 result of mesotrophic samples having the lowest uncertainties is also seen in the average PPig uncertainties for the three regimes, which were 18.4, 11.0, and 12.9%, respectively, for the QA subset. Again, the SeaHARRE-2 results confirm this relationship: the average eutrophic and mesotrophic PPig uncertainties were 33.9 and 19.0%, respectively, for the QA subset.

The chlorophyll *a* accuracy requirements for ocean color validation activities (approximately 25% for compliance and 15% for algorithm refinement) can be satisfied in predominantly oligotrophic conditions by a QA method (Table 15). Prior SeaHARRE activities had shown the compliance and refinement thresholds could be satisfied by methods that were not part of the QA subset. For SeaHARRE-3, however, none of the methods that were excluded from the QA subset satisfied the refinement threshold and only one satisfied the compliance threshold, so method performance is a potentially significant issue for oligotrophic analysis.

The performance metrics, which were proposed during SeaHARRE-2 (Hooker et al. 2005), were shown to be well conceived and properly distinguished the QA subset from the other methods at a level in keeping with the expected accomplishments of quantitative analysis. The reference system for computing uncertainties was based on the QA subset and repeatedly revealed significant and statistically significant differences between the two subsets of methods. Based on the robustness of the performance metrics and the close agreement of the QA subset for most data products across all three SeaHARRE activities, it seems warranted to propose performance metrics for the higher-order data products, which are presented in Table 22.

Table 22. The precision and accuracy performance metrics for pigment sums and indices, as well as pigment ratios.

Performance Weight, Category, and Score		*Sums*† $\bar{\xi}$	*Sums*† $\lvert\bar{\psi}\rvert$	*Ratios* $\bar{\xi}$	*Ratios* $\lvert\bar{\psi}\rvert$
1. Routine	0.5	8%	20%	5%	15%
2. Semiquantitative	1.5	5	12	3	9
3. Quantitative	2.5	3	8	2	6
4. State-of-the-Art	3.5	$\leq$2	$\leq$4	$\leq$1	$\leq$3

† Also for pigment indices.

The higher-order associations in pigments (i.e., sums and ratios) repeatedly confirmed the basic conclusions regarding method performance first established with the individual pigments. That is, a method that was classified in the A' or A^+ subset based on individual pigment uncertainties almost always had uncertainties in the higher-order pigment products in keeping with the original classification (especially true for the QA subset). Exceptions were noted, particularly for the S_8 method, which exhibited a duality of responses (poor chlorophyll results, but more acceptable carotenoid results). The strong connection between the efficacy of the individual pigments being represented in the higher-order products, means the uncertainties of the former strongly influence the uncertainty budget for the latter, and this was seen repeatedly with the S_8 results.

All of the QA methods for SeaHARRE-3 were C_8 methods, in fact, they were all the same method: Van Heukelem and Thomas (2001). Given the large disparity in uncertainties between the A' and A^+ subsets, there is a significant statistical difference between the results obtained with the C_8 and C_{18} methods. The two C_{18} methods were significantly challenged and both laboratories involved had achieved QA results in prior SeaHARRE activities. It seems likely that the problems were associated with the procedures used during the SeaHARRE-3 analyses by these laboratories rather than intrinsic deficiencies with the C_{18} method being used (Wright et al. 1991). It is also worth recalling that two of the A^+ methods were C_8 methods and one of them was the same method used by the QA subset (Van Heukelem and Thomas 2001). Consequently, the individual implementation of a method appears to have much more influence over uncertainties than the selected method (noting any exceptions associated with chromatographic separation issues, like DVChl *a*). This means the majority of the burden in controlling uncertainties lies with the practitioners of a method (again, ignoring those limitations in the chromotography that an analyst has no control over).

The laboratory mix proposed during SeaHARRE-2, and now marketed by DHI, is a useful and suitable substitute for a mixed standard. More importantly, it frequently provides the type of insight normally associated with field sample analyses, which means it has the potential of being a powerful analytical tool. Many of the aberrant aspects of method performance were discernible in the DHI mix results. For example, the basic distinctions between the A' and A^+ subsets, as well as the efficacy of the the J^+ and M^+ reanalyses, were reproduced in the mix results (Table 17) as were some of the nuances (Table 21).

The mix does not reproduce the complexity of natural samples, so multiple levels of inquiry are needed for certain types of problems, e.g., the TChl *a* M results for the mix and field samples were not in agreement, but the results for J, S_8, and S_{18} were. Nonetheless, the mix can provide considerable insight into many of the performance aspects of the HPLC method being used and should be used with the broadest range of inquiry possible, and not within a narrowly focused set of criteria.

If a method did not chromatographically separate the monovinyl and divinyl forms of Chl *a*, the SeaHARRE-2 results (Hooker et al. 2005) showed the use of a simultaneous equation (Latasa et al. 1996) produced a recurring

number of false positives, which resulted in large uncertainties for $[\text{DVChl}\,a]$. Although the impact of this on the uncertainties in $[\text{TChl}\,a]$ were not very significant, because the dominant contribution is from $[\text{Chl}\,a]$, a question remained as to whether or not the presence of false positives was somehow restricted to the water types sampled during SeaHARRE-2. Both the J and S_{18} results for SeaHARRE-3 used a simultaneous equation for quantitating $[\text{DVChl}\,a]$. Remembering that J agreed to use the simultaneous equation for information purposes, so this problem could be investigated with a wider diversity of water types, the data presented here show the simultaneous equation does elevate the uncertainties in $[\text{DVChl}\,a]$, but does not appear to significantly effect the uncertainty in $[\text{TChl}\,a]$.

The insensitivity of TChl a to some sources of uncertainties is a unique feature of this robust pigment: it is almost always present and is usually the dominant peak, so it frequently has a high signal-to-noise ratio (SNR) and false positives or negatives are extremely rare. In general, this is not true for most of the other pigments, and elevated uncertainties are a common result at some point in the dynamic range of a particular pigment's concentration. In most cases, uncertainties start to increase rapidly once false positives and false negatives start to materialize. This usually occurs when laboratories cannot agree how to report pigments with low SNRs.

A case study of the relationship between accuracy and detectability is presented in Fig. 4, where individual HPL pigment APDs per sample from SeaHARRE-3 are plotted as a function of the SNR at HPL. This figure excludes the chlorophylls and only features selected carotenoids, including But, Hex, Diato, and Peri, because they are often in low concentrations; Fuco and DHI Mix results are included to extend the dynamic range of the analysis, because their concentrations are most often high. The data are split into two basic groups: all QA laboratories agree a pigment is present (solid symbols), and one or more laboratories do not report a pigment as present (open symbols). A distinction is made for the former between field samples (circles) and the DHI Mix (diamonds). The data show that when all laboratories do not agree a pigment is present (open circles), the HPL SNR is about 50 or less and the average HPL APDs are 29%; but when all QA laboratories agree a pigment is present (solid symbols), the average HPL APDs are 7%.

The reasons for differences among laboratories cannot be discriminated without additional dialogue among laboratories, but the frequency of null values in a laboratory's data set reflects the combined effects of decisions regarding detecting or rejecting a pigment, or whether the concentration reported was simply less than the null value. In SeaHARRE-3, with 24 sites and 12 primary pigments—a total of 216 possibilities—the percentage represented by null values was 31, 17, 15, and 19%, respectively for C, D, H, and L. This is a clear indication of differences between analysts with respect to peak interpretations.

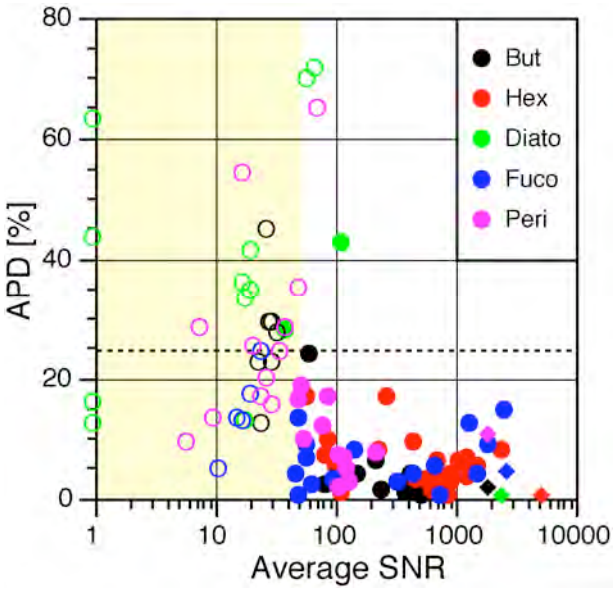

Fig. 4. HPL individual pigment APDs per sample plotted as a function of the SNR for the pigment peak. The circles and diamonds represent SeaHARRE-3 field samples and DHI Mix results, respectively. Solid circles correspond to all QA laboratories reporting a pigment is present, whereas open circles denote one or more QA laboratories not reporting a pigment is present. The yellow area highlights an average SNR of 50 or less, and the dashed line is an APD of 25% (the basic performance metric for QA results.

There are some obvious remedies to the problem of divergence in results among laboratories for pigments with low SNRs. One is for all laboratories to identify the SNR at which they cannot agree with other laboratories regarding the unequivocal presence of a pigment, and then to establish that SNR as their lower limit of quantitation. For HPL, this would approximate an SNR of 50. It is noteworthy that Snyder et al. (2010) estimate the contribution of SNR to method imprecision as being 50/SNR, and cite an SNR of 3 and 10—what has been described for pigments as the LOD and LOQ (Hooker et al. 2005)—as contributing approximately 16 or 5%, respectively, to overall method precision.

Considering state-of-the-art PPig precision, which is less than or equal to 3% (Table 9), filter inhomogeneity of about 2%, and estimating a low imprecision (1%) from other sources, the contribution of SNR to method imprecision must be less than 2%. For the H method, the latter requires an SNR should greater than 25. It is unlikely that laboratories will want to improve accuracy and precision by not reporting pigments quantified with such seemingly high SNRs. In fact, this may not even be desirable, considering that 32% of the SeaHARRE-3 data for the combined

results of But, Peri, Fuco, Hex, and Diato, as observed by H, is less than an SNR of 25, and 44% is less than an SNR of 50.

An alternative strategy, called the *two-sentence rule*, seeks to converge reporting strategies without eliminating any data, in particular peaks with low SNRs. The basic objective of the rule is to try and get analysts to do the same thing when they pass through the threshold of reliable quantitation (approximately an SNR of 50 for Fig. 4) and encounter degraded peak identification caused by problematic absorption spectra.

> *If a peak is good and it can be proved to not be the pigment for that retention time, do not report it; otherwise report it.*
>
> *If a peak is bad and it cannot be disproved to be the correct pigment, report it; otherwise do not report it.*

Notice how the burden of proof switches as the quality of the data changes, but in each case the simpler task is accentuated, so analysts will probably be doing less work. When the data is good, the burden is to prove a peak is not going to be correctly identified, and given the good data available, this task will be rather simple. When the data is poor, the burden is to prove the assumption that the peak is correctly identified is in fact false, but because the data are poor, there will be little chance this will be possible, so the usual outcome will be the simple solution of simply reporting it.

There are purposeful ambiguities in the two-sentence rule: the concept of a *good peak* and a *bad peak* are not defined, although most analysts have an intrinsic opinion or knowledge of what constitutes both of these. The reason they are not defined is the subjective aspects of what individual analysts consider good or bad peaks need to be replaced with objective criteria, and these have not been defined. The inclusion of these concepts in the two-sentence rule is to help promote the discussion and parameterization of what constitutes a good peak and a bad peak. Based on Fig. 4, it might be argued that a bad peak has a SNR of 50 or less.

An unavoidable problem, even with eradication of inconsistencies in interpretation, is a laboratory implementing a method with exceptional detection will have fewer null values, with the unfortunate result that their assessment of accuracy in the context of a round robin will be degraded by results that will appear as false positives, when in fact, they may be more accurate. For example, L should have better SNRs than H because the dimensions of the column used by L increases signal levels, and even though L is using a lower flow rate (0.55 mL), they are still able to use a large injection volume (0.150 mL) because their methanolic extraction solvent is more compatible with the mobile phase than the acetonic extraction solvent of H. (H and L both use the same stationary phase and mobile phases so such comparisons are valid.) Even without knowing the SNRs of L, it is possible to demonstrate their improved detectability by referencing Snyder et al. (2010): the contribution of SNR to method imprecision is characterized by 50/SNR.

Using data in Fig. 4, but limited only to results where both H and L reported pigments as found, the average CV (for triplicate filters) was determined for two pigment groupings: those with an SNR at HPL less than 25, and those with an SNR greater than 25. The average CV for the low and high SNR groups was, respectively, 13 and 5% for H, and 6 and 5% for L. The consistent average CV seen for L with both SNR groups means that whatever the actual SNRs of L are in the lower group, they are not so low as to degrade their method precision. The opposite is true for H—method precision is degraded at SNRs less than 25. It is interesting to note that the frequency of null values across all primary pigments and also including Lut, Neo, and Viola—which is a total of 360 reporting opportunities—L reported 65 null values and H 60 null values. This means L is reporting more null values than H even though the L method exhibits better detectability. This is an indicator of differences in peak interpretations between laboratories.

The ability to present the governing equation for a particular method (26) provides each analyst the opportunity to determine the consequence of the uncertainties in the individual parameters on the final concentration of a pigment. For example, the effects of changing the absorption coefficient (α) can be computed in a straightforward manner. It is also possible to conduct a sensitivity analyses for changing a parameter within a range of plausible values. Although the SeaHARRE-3 exercise did not engage in all aspects of this type of inquiry, the investigations that were presented showed significant differences in methods can be quantified in terms of their effects on uncertainties: for example, the sensitivity of uncertainties to the SNR and the use of alternative absorption coefficients by H (the H' results). The approach for future SeaHARRE activities will be to further dissect the governing equations for the methods involved and try and better understand how individual aspects of a method are contributing to the overall uncertainty budget (e.g., extraction efficiency).

Chapter 2

The CSIRO Method

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Abstract

The CSIRO method is a modified version of the Van Heukelem and Thomas (2001) method. This method has the capability to resolve approximately 35 different pigments with baseline resolution of divinyl and monovinyl chlorophyll *a*, zeaxanthin and lutein, and partial separation of divinyl and monovinyl chlorophyll *b*. Samples are extracted over 15–18 h in an acetone solution before analysis by HPLC using a C_8 column and binary gradient system, with an elevated column temperature. Pigments are identified by retention time and absorption spectra from a photo-diode array (PDA) detector. The method is regularly validated with the use of both internal and external standards and individual pigment calibration. The detection limit of most pigments is within the range of from 0.001–0.005 mg m^{-3}. The method has been used at CSIRO since August 2004, and has proven to offer a good balance between accuracy of pigment composition and concentration, and the number of samples analyzed. The separation of the divinyl and monovinyl forms of chlorophylls *a* and *b* has allowed a complete analysis of samples from the oligotrophic regions of the world ocean.

2.1 INTRODUCTION

The HPLC equipment used at CSIRO is a Waters–Alliance system, comprising a 2695XE separations module with a column heater (set to 55°C) and refrigerated autosampler (set to 4°C), and a 2996 photo-diode array detector. The CSIRO method separates pigments on a C_8 column using a two-solvent gradient system. Using a PDA detector, pigments are verified by the retention time and absorption spectra of each chromatographic peak, and quantified by the detector signal at 436 nm. Analysis time is 30 min per sample plus an additional 5 min injection delay to ensure no carry over between samples.

Separation is achieved for almost all pigments with the exception of the following: a) $\beta\beta$-Car and $\beta\varepsilon$-Car, which coelute, but can be separated by their absorption spectra in samples where either one is dominant, and b) partial separation of Chl *b* and DVChl *b*. Baseline resolution is not achieved for Chl c_2, MgDVP, Chl c_1, and Chlide *a*. It is not common for all four of these pigments to be present in one chromatogram, however, which permits good resolution between Chl c_2 and Chl c_1, and partial resolution between Chl c_1 and Chlide *a* when both are present.

Immediately after samples are collected in the field, they are stored in liquid nitrogen until analysis begins. The SeaHARRE-3 samples were received on 22 March 2005 and were stored in liquid nitrogen until analysis began on 21 June 2005.

2.2 EXTRACTION

To extract the pigments, the thawed filters are cut into small pieces and covered with 100% acetone (3 mL) in a 10 mL centrifuge tube. The scissor and forcep blades are cleaned in between sample handling. The tube is covered with parafilm and vortexed for about 30 s, followed by sonication for 15 min in an ice-water bath in the dark. The samples are then kept in the dark at 4°C for approximately 15 h. After this time period has elapsed, 200 mL of water is added to the acetone to produce an extract mixture of 90:10 acetone:water (vol:vol). The extract is sonicated once more for 15 min in an ice-water bath in the dark.

The extract is transferred to a Biorad column, (a small column containing a scintered glass disc) sitting in a clean centrifuge tube. The original centrifuge tube is rinsed twice with 0.5 mL 90:10 acetone, and the rinsings are added to the column. The column and centrifuge tube are centrifuged for 5 min at 2,500 rpm and −2°C to separate the filter paper from the extract. At this stage, the final extract volumes are recorded from the centrifuge tube graduations. The centrifuged extracts are then passed through a 0.2 µm Teflon† syringe filter, which has been rinsed with

† Identification of commercial equipment to adequately specify or document the experimental problem does not imply recommendation or endorsement, nor does it imply that the equipment identified is necessarily the best available for the purpose.

acetone, directly into a 2 mL amber HPLC vial. The remaining extract from each sample remains in a centrifuge tube, covered with parafilm, and stored at −20°C until the HPLC analysis has been successfully completed.

2.3 HPLC ANALYSIS

Immediately prior to injection, the sample extract is mixed with a buffer solution within the sample loop. The 90:10 (vol:vol) buffer solution is composed of 28 mM aqueous tetrabutyl ammonium acetate (6.5 pH) and methanol, respectively. The injector is programmed by the software to draw up alternating microliter volumes of buffer and sample in the following order (starting and ending with the buffer); 150, 75, 75, 75, and 150.

After injection, pigments were separated using a Zorbax Eclipse XDB-C8 stainless steel 150×4.6 mm^2 ID column with 3.5 µm particle size (Agilent Technologies) and a gradient elution procedure as given in Table 23. The gradient is held in an isocratic mode between 11–15 min to improve the resolution between Viola and But-fuco. The flow rate is 1.1 mL min^{-1} and the column temperature is kept at 55°C. The separated pigments are detected at 436 nm and identified against standard spectra using Waters `Empower` software. The analysis time of each sample is 30 min with an additional 5 min injection delay of the next sample to ensure there is no carry over between samples.

Table 23. The gradient elution system for the CSIRO method. Solvent A is 70:30 (vol:vol) mixture of 28 mM TBAA (6.5 pH) and methanol, respectively. Solvent B is 100% methanol.

Step	*Time* [min]	A [%]	B [%]
Start	0	95	5
2	11	45	55
3	15	45	55
4	22	5	95
5	29	5	95
End	31	95	5

Peak integration and identification is initially performed by the automated features of Waters `Empower` software, which produces an electronic report. Each sample is manually inspected for correct integration markers and identification of pigments. For a few samples, where the pigment concentration is very low, baselines are corrected manually to optimize integration.

2.4 CALIBRATION

Concentrations of Chl *a*, Chl *b*, and *ββ*-Car are determined from Sigma-Aldrich standards, while all other pigment concentrations are determined from DHI standards. The concentrations of all standard stock solutions are determined using a GBC 916 UV/VIS dual-beam spectrophotometer with a 2 nm bandwidth. An absorption spectrum of each pigment is recorded between 350–900 nm. The concentration of each pigment is calculated using the absorption coefficient from the literature (Jeffrey et al. 1997a) together with the absorption measured at the corresponding wavelength. Absorption coefficients, wavelengths, and solvents used for each pigment are listed in Table 24. The absorbance at the wavelength used is corrected for any absorption measured at 750 nm.

Table 24. The α values used with the CSIRO method for the various pigment standards as a function of λ. The units for α are liters per gram per centimeter and the units for λ are nanometers.

Pigment	*Solvent*	λ	α
Perid	100% Ethanol	472.0	132.50†
But-fuco	100% Ethanol	446.0	160.00
Fuco	100% Ethanol	449.0	160.00
Hex-fuco	100% Ethanol	447.0	160.00
Neo	100% Ethanol	439.0	224.30
Pras	100% Ethanol	454.0	160.00
Viola	100% Ethanol	443.0	255.00
Diadino	100% Ethanol	446.0	262.00
Allo	100% Ethanol	453.0	262.00
Diato	100% Ethanol	449.0	262.00
Lut	100% Ethanol	445.0	255.00
Zea	100% Ethanol	450.0	254.00
Asta	100% Acetone	482.0	210.00†
Cantha	100% Ethanol	476.0	207.50
βε-Car	100% Acetone	448.0	270.00
ββ-Car	100% Acetone	454.0	250.00†
Chlide *a*	100% Acetone	664.0	127.00
Chl *b*	100% Acetone	647.0	51.36†
DVChl *a*	100% Acetone	664.3	87.67
MVChl *a*	100% Acetone	664.0	87.67†
Chl c_3	100% Acetone	453.0	346.00
Chl c_2	100% Acetone	443.8	374.00
Phytin *b*	90% Acetone	657.0	31.80†
Phytin *a*	90% Acetone	667.0	51.20†
Phide *a*	90% Acetone	667.0	74.20

† Based on Jeffrey (1997); all other values from DHI.

From these stock solutions, a series of 4–6 standard solutions are prepared and analyzed both spectrophotometrically and by HPLC. Calibration curves are obtained with coefficient of determination (r^2) values never less than 0.99. Response factors (R) for each pigment are determined from these calibration curves.

2.5 VALIDATION

At the start of every set of samples analyzed by HPLC, a pigment mixture is qualitatively analyzed to determine if there is any displacement in the retention time of approximately 30 pigments. A mixture of known concentrations of Fuco, Chl *a*, Chl *b*, and *ββ*-Car is also analyzed to verify

the HPLC system (including the column) is working properly. In the case of the SeaHARRE-3 samples, a mixed pigment standard, supplied by DHI, was analyzed after every sixth sample injection.

Calibrations of Chl *a*, Chl *b*, and $\beta\beta$-Car are done approximately every three months, while calibrations of selected other pigments are done approximately once a year.

2.6 DATA PRODUCTS

Waters `Empower` software creates an electronic file containing each chromatographic peak with its retention time, peak area, and peak height recorded together with the initial pigment identification. Once the chromatograms are manually checked, the peak areas are transferred to an `Excel` spreadsheet in which the concentrations for pigment P_i are calculated using

$$\tilde{C}_{P_i} \;=\; \hat{A}_{P_i}\, R_{P_i}, \tag{27}$$

where $\tilde{C}_{P_i}$ is the amount of pigment injected (in units of nanograms per injection); $\hat{A}_{P_i}$ is the area of the chromatographic peak, and R_{P_i} is the response factor.

The concentration of the pigment P_i in the sample is then determined using the following equation.

$$C_{P_i} \;=\; \frac{V_x}{V_f}\,\frac{\tilde{C}_{P_i}}{V_c}\,D_f, \tag{28}$$

where V_x is the final extraction volume (in units of microliters); V_f is the volume of sample filtered (in units of milliliters); V_c is the volume of sample extract injected onto the HPLC column (in units of microliters); and D_f is the dilution factor. The dilution factor is rarely used and only applied if the color of the extract is dark green and it is likely that the sample Chl *a* concentration will be outside the linear range for Chl *a*.

2.7 CONCLUSIONS

Since the SeaHARRE-2 activity, CSIRO changed its HPLC method for routine analysis of pigments from the Wright et al. (1991) method to a slightly modified version of the Van Heukelem and Thomas (2001) method. The main reason for changing the method was to have a single method capable of analyzing completely different samples from a wide range of regions—tropical and temperate oceanic, coastal, estuarine, and freshwater.

The CSIRO method is now able to resolve the divinyl and monovinyl forms of Chl *a*, as well as Lut from Zea. Accurate identification and quantification of these pigments is important in determining phytoplankton community composition from natural samples. The method has proven to provide a good balance between accuracy of pigment composition and concentration and number of samples analyzed.

Chapter 3

The DHI Method

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Abstract

The HPLC method used at DHI is a somewhat modified version of the HPL method (Van Heukelem and Thomas 2001). In comparison with the DHI method used during SeaHARRE-2 (Wright et al. 1991), the HPL method separates divinyl from monovinyl chlorophyll *a*, and chlorophyll c_1 from chlorophyll c_2. Furthermore, the HPL method provided state-of-the-art results during SeaHARRE-2, so the capabilities of the method were as highly rated as possible. When adapted to the DHI HPLC system, the HPL method, however, did not initially provide the same excellent results for the analysis of the SeaHARRE-3 samples, in terms of the precision of the results. Subsequent troubleshooting identified three problems: a) a fault in the autoinjector, b) an inappropriate tetrabutyl ammonium acetate (TbAA) buffer, and c) the HPLC vials were not completely airtight (so there was some evaporation of the extract while it resided in the autosampler compartment). The poor precision was identified prior to submitting the SeaHARRE-3 results by checking the reproducibility of standardized mixed pigments (from DHI Mix-101), which were distributed to all of the participants as part of the intercalibration exercise. This emphasizes the usefulness of such pigment mixtures and the necessity of quality assurance for detecting method problems, which have an impact on the results.

3.1 INTRODUCTION

For analyzing the SeaHARRE-3 samples, DHI adopted the HPL method developed by Van Heukelem and Thomas (2001 and 2005). This method separates more taxonomically important pigments than the Wright et al. (1991) method (which was used by DHI in SeaHARRE-2), such as divinyl and monovinyl Chl *a*, while divinyl and monovinyl Chl *b* are partly separated. For SeaHARRE-2, the DHI method separated divinyl and monovinyl Chl *a* by using dichromatic equations, but these calculations resulted in false positives in many samples, where divinyl Chl *a* was detected and quantified, while laboratories that were able to separate divinyl from monovinyl Chl *a* chromatographically did not find divinyl Chl *a*. Furthermore, the HPL method separates Chl c_1 and Chl c_2, which are not separated by the Wright et al. (1991) method.

Another reason for choosing the Van Heukelem and Thomas (2001 and 2005) method was that the performance of this method in SeaHARRE-2 was state-of-the-art with particularly excellent results for accuracy and precision. Although the original DHI method was part of the quality assured subset of four laboratories with a precision and accuracy that was significantly better than the overall average in SeaHARRE-2, the precision of the DHI method could be further improved.

3.2 EXTRACTION

The SeaHARRE-3 filters were received in dry shippers on 27 January 2005, and stored at −80°C until analysis (28–30 June 2005). The filters were divided in sets of 18, and extracted in 3 mL 95% acetone containing approximately 0.025 µg mL^{-1} vitamin E acetate internal standard (Van Heukelem and Thomas 2005). The samples were sonicated in an ice-cold sonication bath for 10 min, placed at −4°C for 24 h, and mixed on a Vortex mixer. The filters and cell debris were filtered from the extracts using disposable syringes and 0.2 µm Teflon syringe filters directly into HPLC vials. The vials were then placed in the cooling rack of the HPLC together with a parallel set of vials with the injection buffer composed of a 90:10 (vol:vol) solution of 28 mM aqueous TbAA (6.5 pH) and methanol, respectively.

3.3 HPLC ANALYSIS

The DHI HPLC system is a Shimadzu LC-10ADVP HPLC, which consists of a LC-10ADVP pump, an SPD-M10A VP photodiode array detector, an SCL-10ADVP system controller with `Class VP` software version 5.0, a temperature-controlled autosampler (set at 4°C), a CTO-10ASVP column oven (set at 20°C), and a degasser.

The samples were mixed with buffer using the autoinjector by programming it to withdraw the following sequence: 150 µL buffer, 72 µL sample, 57 µL buffer, 71 µL samples, 150 µL buffer. The entire amount, 500 µL total and 143 µL sample, was injected onto the column. Solvent B was methanol and solvent A was a (70:30) solution of methanol and 28 mM aqueous TbAA (6.4 pH). The column was an Eclipse XDB C8, 4.6×150 mm^2 (Agilent Technologies), the flow rate was 1.1 mL min^{-1}, and the temperature of the column oven was set to 60°C.

Unfortunately, the TbAA originally used was a solid form (Aldrich 33,599-1, 97%) and not the 0.4 M TbAA hydroxide titrant (J.T. Baker HPLC reagent V365-07) used in the original Van Heukelem and Thomas (2001 and 2005) method. The Aldrich TbAA severely affected the DHI method by causing unstable retention times, pressure increase in the column, and suboptimal pigment separations compared with the original method. Furthermore, the pigments in the first part of the chromatogram were distorted, which made it necessary to make several alterations in the gradient program (Table 25).

Table 25. The gradient used with the DHI column organized by the steps involved in the complete analysis of a sample and the percentages of solvents A and B.

Step	*Time* [min]	A [%]	B [%]
Start	0	85	15
2	22	30	70
3	27	5	95
4	31	5	95
5	34	85	15
End	46	85	15

The SeaHARRE-3 samples were run in random order. One vial of mixed pigments (Mix-101) was analyzed approximately every tenth sample (eight in total) followed by a blank (extraction solvent with vitamin E).

Phytin *a*, Phide *a*, DVChl *a*, and MVChl *a* were determined at 665 nm, the internal standard at 222 nm, while the rest of the pigments were determined at 450 nm. Peak identities were routinely confirmed by online PDA analysis. Chl c_1 coeluted with Chlide *a*, while MgDVP coeluted with Chl c_2.

3.4 CALIBRATION

The HPLC system was calibrated with DHI pigment standards prior to analysis of the SeaHARRE-3 field and Mix-101 samples. The concentrations of the calibration standards were determined using a Shimadzu UV-2401PC dual-beam, monochromator-type spectrophotometer, which is subjected to a regular set of quality control procedures. The absorption coefficients (α) used with the DHI method are shown in Table 26. Selected batch numbers of the standards are controlled for purity and concentration by an independent laboratory.

Table 26. The α values used with the DHI method for a variety of pigments as a function of λ. The units for α are liters per gram per centimeter and the units for λ are nanometers.

Pigment	*Solvent*	λ	α
Chl c_3	90% Acetone	452.9	346.00
Chl c_2	90% Acetone	443.8	374.00
Chlide *a*	90% Acetone	664.0	127.00
Perid	100% Ethanol	472.0	132.50
Phide *a*	90% Acetone	667.0	74.20
But-fuco	100% Ethanol	446.0	160.00
Fuco	100% Ethanol	449.0	160.00
Neo	100% Ethanol	439.0	224.30
Pras	100% Ethanol	454.0	160.00
Viola	100% Ethanol	443.0	255.00
Hex-fuco	100% Ethanol	447.0	160.00
Diadino	100% Ethanol	446.0	262.00
Allo	100% Ethanol	453.0	262.00
Myxo	100% Acetone	478.0	216.00
Diato	100% Ethanol	449.0	262.00
Zea	100% Ethanol	450.0	254.00
Lut	100% Ethanol	445.0	255.00
Cantha	100% Ethanol	476.0	207.50
Chl *b*	90% Acetone	646.8	51.36
DVChl *a*	90% Acetone	664.0	87.67
MVChl *a*	90% Acetone	664.3	87.67
Phytin *a*	90% Acetone	667.0	51.20
$\beta\beta$-Car	100% Ethanol	453.0	262.00

3.5 VALIDATION

The performance of the HPLC system was monitored using the DHI Mix-101, for which the reproducibility, calibration, peak identity, peak separations, and the retention time stability of the pigments in the mix were monitored. The precision of the DHI HPLC system was checked just prior to SeaHARRE-3 analyses by injecting a series of four Mix-101 samples; the average CV of 20 pigments was less than 1%. The CV of Mix-101 analyzed as part of the SeaHARRE-3 sample analyses was 2.4%, however, wherein the mixed pigments were injected as discrete injections for every tenth sample.

The degraded precision during the SeaHARRE-3 analyses indicated a change in the precision of the HPLC system. A malfunction in the autoinjector system was subsequently located and corrected, but the CV was still not restored after the repairs were completed. Further investigations revealed the HPLC vials used for the SeaHARRE-3 samples, *brown* vials with silicone and polytetrafluoroethylene (PTFE) slit septa, were not airtight and solvent evaporated. Furthermore, the TbAA used for analyzing the SeaHARRE-3 samples was not suitable (Sect. 3.3). Replacement of the TbAA resulted in a) the retention times

of the individual pigments became stable, b) the pressure in the column was lowered and became stable, and c) the original gradient of Van Heukelem and Thomas (2001 and 2005) was producing narrow peaks without peak distortion on the DHI HPLC. After all these changes, the average precision of repeated injections of the mixed pigments was about 1–2%.

3.6 DATA PRODUCTS

The peak areas and pigment identities were transferred to an `Excel` file, and based on the response factors, the pigment concentrations were calculated:

$$C_{P_i} = \frac{V_x}{V_f} \frac{\hat{A}_c}{\hat{A}_s} \frac{\hat{A}_{P_i} R_{P_i}}{V_c}, \tag{29}$$

where $\hat{A}_{P_i}$ and R_{P_i} are the peak area and the response factor of pigment P_i, respectively; V_x is the extraction volume; V_f is the volume of sample filtered; V_c is the amount of sample injected onto the column; $\hat{A}_c$ is the peak area of the internal standard in the extraction solvent; and $\hat{A}_s$ is the peak area of the internal standard in the sample.

3.7 CONCLUSIONS

The method used at DHI for SeaHARRE-3 analyses was a slightly modified version of the Van Heukelem and Thomas (2001 and 2005) method. The new method was implemented prior to analyzing the samples, but problems were encountered during the analysis of the samples: a) the precision for the mixed pigments analyzed as eight discrete injections over the threes days of analysis was degraded; the retention stability was poorer, and c) the pressure in the column increased. Multiple reasons for these problems were identified: an error was encountered in the autoinjector, an incorrect TbAA buffer was used, and the HPLC vials used were not completely airtight.

The SeaHARRE-3 samples were analyzed in random order, so the problem with the degraded precision affected the results rather equally. The problems encountered in this round robin were located prior to submitting the results by evaluating the performance of the standardized mixed pigments (Mix-101). This emphasizes the usefulness of such pigment mixtures and the necessity of quality assurance for detecting method problems. Unfortunately, the troubleshooting took a long time, and it was not possible to reanalyze SeaHARRE-3 extracts after the HPLC was performing at a restored optimal level.

Acknowledgments

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Chapter 4

The HPL Method

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Abstract

The HPL method was developed for use with a variety of water types. Many pigments important to freshwater, estuarine, and oceanic systems are baseline resolved and quantitatively reported, including divinyl and monovinyl chlorophyll *a*. The method is based on a C_8 HPLC column, a methanol-based reversed-phase gradient solvent system, a simple linear gradient, and an elevated column temperature (60°C). The method can provide quantitative results for up to 25 pigments with qualitative information for additional pigments. Quality assurance measurements are made during sample analysis to confirm that the method performance is within expectations. Investigations into the uncertainties in the method show the 95% confidence limits were estimated as a) 0.5–3.8% for precision of replicate injections within and across sequences, b) 3.2% for chlorophyll *a* calibration reproducibility, and c) 5.1% for chlorophyll *a* method precision, including filter extraction and analysis.

4.1 INTRODUCTION

The HPL HPLC method (Van Heukelem and Thomas 2001) is described in the SeaHARRE-2 report (Hooker et al. 2005) and was modified slightly for SeaHARRE-3. For a complete understanding of the method as it was used during SeaHARRE-3, the SeaHARRE-2 report should be consulted simultaneously with this chapter. The details presented here a) document the modifications applied to the method since SeaHARRE-2, b) provide clarifications not found in the SeaHARRE-2 report, and c) explain additional changes made in response to the lessons learned during SeaHARRE-2.

Two important changes were made in preparation for SeaHARRE-3. First, the extraction volume was reduced to provide a more concentrated extract, thereby increasing sensitivity, because of the emphasis on very low concentrations. Second, to further evaluate the effects of uncommon carotenoid absorption coefficients as a source of bias in field sample results, the HPLC system was calibrated using typical procedures and with carotenoid standards in acetone. For the latter, acetone absorption coefficients were used for pigment quantitation. Consequently, two sets of results for the field samples were submitted. The results produced with the typical calibration procedures were the ones used to assess HPL performance with respect to the other laboratories.

The HPL method uses a C_8 column and a reversed-phase, methanol-based, binary-gradient, solvent system. A linear gradient is used because segmented gradients offered no advantages and contributed to baseline instability, which can interfere with the quantitation of pigments in low concentrations. Elevated column temperature is used to facilitate separation selectivity. High column temperature, however, shortens useful column lifetime and special handling should be considered (Wolcott et al. 2000). The HPL procedures are occasionally modified as new types of samples and activities lead to new understandings, as have been the case with the SeaHARRE activities. This chapter is seen as an opportunity to discuss the many problems encountered during sample analysis in the hopes of stimulating dialogue with other laboratories.

4.2 METHODS

Aspects of the methodology pertain to calculation equations (in which variables affecting pigment concentrations are defined), procedures for extracting samples, and HPLC analysis, which is further subdivided into procedures for injection, pigment separation, detection, calibration, and pigment identification. Data products and data reporting practices during SeaHARRE-3 are also described.

The SeaHARRE-3 samples were received frozen in liquid nitrogen dry shippers the week of 20 December 2004. The filters were immediately placed in a freezer at −80°C until processed in June 2005. DHI Mix-101 standards were received well frozen on dry ice the week of 7 March 2005 and were held at HPL in a −25°C freezer until used.

4.2.1 Calculation Equations

The formulation for determining the concentration (C) of an individual pigment (P_i) begins with the terms describing the calibration of the HPLC system:

$$\tilde{C}_{P_i} \;=\; \hat{A}_{P_i}\, R_{P_i}, \tag{30}$$

where $\tilde{C}_{P_i}$ is the amount of pigment standard injected, $\hat{A}_{P_i}$ is the area of the parent peak and associated isomers, and R_{P_i} is the response factor (amount per area).

Concentration of primary pigment standards are determined spectrophotometrically based on principles of the Lambert-Beer Law, which states that the fraction of the incident light at a particular wavelength λ that is absorbed by a solution depends on the thickness of the sample, the concentration of the absorbing compound in the solution, and the chemical nature of the absorbing compound (Segel 1968). This relationship can be expressed as:

$$A(\lambda) \;=\; a(\lambda)\, l_c\, C, \tag{31}$$

where $A(\lambda)$ is absorbance, $a(\lambda)$ is the absorption coefficient (a constant), l_c is the thickness of the sample in centimeters (the pathlength of the cuvette being used), and C is concentration. To determine concentration from a measured absorbance, (31) is rewritten as

$$C \;=\; \frac{A(\lambda)}{a(\lambda)\, l_c}, \tag{32}$$

where the units for C depend on the expression of $a(\lambda)$. For example, for consistency with current SeaHARRE conventions regarding absorptivity, if the concentration is expressed in molarity, a becomes the molar absorption coefficient (ε) and if the concentration is expressed as grams per liter, a is the specific absorption coefficient (α). If concentration is expressed in percent weight per volume (usually in units of grams per 100 mL), a becomes $a_{1\%}$ (Segel 1968). Usually, a 1 cm pathlength is used, so $l_c = 1$ cm in most cases. Absorption coefficients vary depending on wavelength and the solvent in which the compound is suspended, and they are always provided with the solvent and wavelength used.

The equation for determining the pigment concentration in a natural sample is

$$C_{P_i} \;=\; \frac{V_x}{V_f}\,\frac{\tilde{C}_{P_i}}{V_c}, \tag{33}$$

where V_x is the extraction volume, V_c is the volume of sample extract injected onto the HPLC column, and V_f is the filtration volume.

An internal standard is used to correct V_x for residual water retained on the filter paper (plus any variations in volume caused by evaporation):

$$V_{x_1} \;=\; \frac{\hat{A}_{c_1}}{\hat{A}_{s_1}}\, V_m, \tag{34}$$

where $\hat{A}_{c_1}$ is the peak area of the internal standard (within the internal standard batch mixture) when it is injected onto the HPLC column prior to its addition to the sample (determined using a one-step internal standard methodology), $\hat{A}_{s_1}$ is the peak area of the internal standard in the sample, and V_m is the volume of extraction solvent (with internal standard) added to each sample filter.

If an internal standard is not used, a proxy for V_x is created. This includes an estimate of the average volume of water retained on the filter, V_w, and the volume of extraction solvent added to the extraction tube containing the filter, V_e. In this case, V_e is equivalent to V_m in (34) when solvent contains the internal standard. If an internal standard is used (as it is here), the parameterization used to create a proxy for V_x can be used as a quality assessment tool for V_x (expanded upon in Sect. 4.3.7):

$$V_x \;=\; 0.98\,\big(V_e + V_w\big), \tag{35}$$

where 0.98 approximates for the shrinkage when an organic solvent and water are combined in volume (Sect. 4.3.7).

4.2.2 Extraction Procedures

To create more concentrated sample extracts with the SeaHARRE-3 samples, V_m was changed from 3.0 mL of 95% acetone (used in SeaHARRE-2) to 1.8 mL 100% acetone. Each filter in a set of triplicates was extracted on a different day. Peak splitting of Chl *c* peaks occurred with 15 of the 24 samples extracted on the first day. Because peak splitting can be caused by the acetone content in the sample injection solution being too high, water was added to the sample extraction tubes for the filters extracted on subsequent days.

Batches 9–24 appeared drier than normal, and the lack of water in these filters may have contributed to high acetone concentrations on the first day of extraction. Peak splitting was not encountered with this degree of frequency prior to SeaHARRE-3. The frequency of peak splitting diminished for those sample extracts that had water added to them.

The procedures for extractions were as follows:

1. A frozen filter sample was placed in a 12×75 mm (5 mL) disposable, round-bottomed, polypropylene tube (Falcon, owned by Becton-Dickinson 352002, Franklin, New Jersey).
2. The 1 L solvent bottle containing the extraction solvent (described next) was removed from a −15°C freezer and brought to room temperature. A 1.8 mL volume of room-temperature 100% HPLC-grade acetone (J.T. Baker 9002-03, Phillipsburg, New Jersey) containing DL-α-tocopherol acetate (vitamin E Acetate, Sigma-Aldrich 95250, St. Louis, Missouri) in a concentration of approximately 0.02 mg mL^{-1}†

† This concentration was erroneously stated as 0.02 µg mL^{-1} in the SeaHARRE-2 report.

was added to each tube with a solvent delivery device that had been calibrated gravimetrically with 100% acetone.

3. Deionized water (when added to the extraction tube) was delivered with an automatic pipette.
4. To prevent evaporation, the top of each extraction tube was covered with Parafilm immediately after the acetone (and water) was added.
5. The tubes were placed in an ice bath shielded from the light as soon as the tops were covered with Parafilm. Samples in ice bath were placed in a −25°C freezer for at least 1 h.

After all filters in a set were processed following steps 1–5:

6. Each sample filter was individually disrupted for about 30 s with a Branson 450 Sonifier (Danbury, Connecticut) installed with a 1/8 in microtip sonic probe, and using control settings that resulted in approximately a 30–40 W output.
7. The filter extracts were tightly covered with Parafilm and stored in a −25°C freezer for about 3 h.
8. The filter slurry of each sample was transferred to a clean 5 mL disposable syringe with a Luer-Lok tip (Becton-Dickinson 309603). Each slurry was clarified by pushing it through a PTFE Titan HPLC syringe cartridge filter attached to each Luer-Lok syringe. The pore size of all filters used for clarification was 0.45 µm, but the diameter was either 45 or 25 µm (Sun SRI 44504-NP, Rockwood, Tennessee). The clarified extract was collected in clear, 7 mL scintillation vials with cork-backed, foil-lined screw caps (Fisher Scientific 03-337-26, Pittsburgh, Pennsylvania).

A summary of the extraction details is presented in Table 27.

After all the samples extracted in a day were clarified, the final sample preparation procedures were as follows:

9. Approximately 500 µL of each extract was transferred from the 7 mL receiving vial (see step 8) after the vial and contents were vortexed gently for approximately 2 s. These 500 µL were then put into an amber HPLC vial (National Scientific C4011-6W, Rockwood, Tennessee) and capped with a PTFE and silicone snap cap (National Scientific C4011-54B). Because transfer volumes were not used in quantitations, an autopipette was used. A clean, unused tip was used with each extract.
10. The vials were immediately placed in the HPLC temperature-controlled autosampler (TCAS) compartment, set at 5°C, and analyzed within approximately 24 h.
11. The remaining unused sample extracts were capped tightly with cork-backed, foil-lined screw caps (Fisher Scientific 03-337-26, Pittsburgh, Pennsylvania), and stored in a −15°C freezer until they were used for spectrophotometric analysis.

Table 27. A summary of the SeaHARRE-3 filter extractions. The type of delivery devices used to add solvent to filters (with their calibrated volumes and CV) are given at the top of the table. Whether or not filters appeared to have dried more than normal (i.e., perhaps to have been manually dried during sample collection) is indicated next to the filter triplicate number ("Yes" means dried more than normal). The numeric entries below the extraction days are the volume of water (V_a) in microliters added to the extraction tube. Samples whose chromatograms exhibited peak-splitting with at least one Chl *c* pigment are denoted with the † symbol.

Delivery		*Repipette*[1]		*2.5 mL Syringe*[2]	
Volume [mL]		1.8676	1.8410	1.7862	1.7862
CV [%]		0.82	0.88	0.08	0.08
Triplicate		*June Extraction Day*			
No.	*Dried*	*2*	*6*	*13*	*15*
1	No	0†	100		50
2	No	0	50		50
3	No	0	100†	50	
4	No	0	50		50
5	No	0	100		50
6	No	0†	50†	50	
7	No	0†	100		50
8	No	0	100	50	
9	Yes	0	50†		50
10	Yes	0†	50†	50	
11	Yes	0†	100		50
12	Yes	0†	50		50
13	Yes	0†	50		50
14	Yes	0	50		50
15	Yes	0†	100		50
16	Yes	0†	50		50
17	Yes	0	50†		50
18	Yes	0†	50†		50
19	Yes	0	100		50
20	Yes	0†	50		50
21	Yes	0†	100		50
22	Yes	0†	100		50
23	Yes	0†	100		50
24	Yes	0†	100		50

[1] BrandTech Dispensette, 0–5 mL capacity (Essex, Connecticut).
[2] Hamilton, Co. GASTIGHT glass syringe #1002 (Reno, Nevada).

4.2.3 HPLC Analysis

The filter extracts and standards were analyzed using a fully automated Agilent 1100 HPLC equipped with a quaternary pump, programmable autoinjector, temperature-controlled autosampler, peltier temperature-controlled column oven compartment, PDA detector, and `ChemStation` software revision A.10.02 (all from Agilent Technologies, Inc., Santa Clara, California).

4.2.3.1 Injection

The HPL HPLC is equipped with a metering device capable of drawing up to 900 µL into the sample loop. The injector is programmed to draw successive aliquots of sample extract and buffer into the sample loop, as shown in Table 28. This injection scheme is the same one used in SeaHARRE-2, except the seventh line was added for SeaHARRE-3. In the SeaHARRE-2 report (Hooker et al. 2005), a *mix-in-the-loop* terminology was used to distinguish the injection mode being used at that time from injector programs that combine sample extract with buffer (or water) in a sample vial.

Table 28. The HPLC injector program used during SeaHARRE-3. The metering device speed is in microliters per minute.

Line	*Speed*	*Program Action*	*Vial*
1	500	`DRAW` 150.0 µL from vial 91	Buffer
2	130	`DRAW` 75.0 µL sample	Extract
3	90	`DRAW` 0.0 µL from vial 1	Acetone
4	130	`DRAW` 75.0 µL vial 91	Buffer
5	130	`DRAW` 75.0 µL from sample	Extract
6	90	`DRAW` 0.0 µL from vial 1	Acetone
7	130	`MIX` 350 µL in air†	
8	500	`DRAW` 150.0 µL from vial 91	Buffer
9	250	`INJECT` loop contents onto column	

† The needle is suspended in air while the metering device moves the loop contents backward 350 µL and then forward 350 µL one time.

The mix-in-the-loop terminology used during the execution and documentation of the SeaHARRE-2 activity was technically incorrect and a potential source of confusion, because the injection program did not contain an active `MIX` function. Consequently, an alternative program was created for use during the analysis of SeaHARRE-3 samples to determine the effect of mixing (albeit according to the unique characteristics of the HPL Agilent 1100 injector). This mix function moves the injection loop contents back and forth 350 µL (i.e., in each direction) within the 900 µL loop.

There was no apparent improvement in the results with the addition of the mixing step, as compared with results using the original injector program. When using the same sample extract, the CV for replicate injections was 1.0% with active mixing and 1.1% without mixing. A comparison of pigment concentrations determined with the injector program used in SeaHARRE-3 with those determined for the same extracts analyzed with the previous program used in SeaHARRE-2 showed the RPD values ranged from −0.5 to 3.1% across all pigments quantified, with an overall average APD of 0.9%. After SeaHARRE-3, the mix function was eliminated, because it provided no advantages, took more time, and added unnecessary wear to the metering device.

Examples of procedures used to facilitate injection repeatability included the following:

1. Placing sample and buffer vials in the TCAS at least one hour prior to starting analyses,
2. Disregarding the results of the first injection of a sequence,
3. Maintaining a constant column re-equilibration volume between injections,
4. Using vial and cap combinations that prevent evaporation,
5. Using injection draw-speeds that prevent a vacuum from forming when sample is withdrawn from the vial, and
6. Limiting the vial residence time in the TCAS to approximately 24 h or less.

The ability of the vial and cap combinations used with sample extracts and standards to limit evaporation was evaluated gravimetrically. The loss rates were less than 2 µL d^{-1} per vial for both acetone and ethanol. Typically, approximately 500 µL of sample or standard is placed in a vial (or about 300 µL when volume is limited), so the concentration change due to evaporation is likely to be less than 0.7%.

The injection buffer solution consists of 90:10 (vol:vol), 28 mM TbAA (6.5 pH) and methanol, respectively. The injection buffer is filtered in batches through an Acrodisc 25 mm syringe filter with a 1 µm glass fiber membrane (Pall 4523T, East Hills, New York). Snap caps on the buffer and sample vials are formulated of layered PTFE and silicone, but vial caps used with buffer vials are pre-slit (National Scientific C4011-59) to facilitate accurate draw volumes of the viscous buffer mixture. Snap caps on sample vials are not pre-slit to prevent evaporation of the sample.

HPL HPLC vials contain a maximum volume of 1.8 mL, and each sample analysis requires 375 µL of buffer, so four sample extracts can be analyzed using one HPLC vial of buffer. Ten methods were created, which differ only in the vial positions specified for buffer and 100% acetone (used to rinse the exterior of the injection needle, which is simply lowered into the acetone rinse vial). In the example in Table 28, the vial positions for buffer (lines 1, 4, and 8) and acetone (lines 3 and 6) are specified as numbers 91 and 1, respectively. A subsequent execution of the program would specify numbers 92 and 2, and so on up through vial numbers 100 and 10. Vial positions 11–90 are, therefore, available for samples and standards, and positions 1–10 and 91–100 are used for acetone and buffer, respectively.

The 10 different injection program executions can be used to perform a total of 40 injections, which allows the HPLC to run unattended for about 30 h, after which buffer and acetone vials are replaced with new ones if more samples are to be injected on the same sequence. The acetone rinse vials use snap caps with Teflon septa (National Scientific C4011-52R). The same types of HPLC vials are used for samples, buffer, and acetone. The Table 28 injector

program—and the original program without line 7—yield excellent precision, but are not entirely free of problems. Occasionally, peak-splitting with Chl c_1, Chl c_2, and Chl c_3 is encountered, and MgDVP is similarly affected if present.

4.2.3.2 Separation

The separation conditions used in SeaHARRE-3 were the same as those used in SeaHARRE-2. Solvent A is formulated (vol:vol) from 28 mM TbAA (6.5 pH) and methanol, respectively, in a ratio of 30:70, i.e., 300 mL of 28 mM TbAA (6.5 pH) is measured in a 500 mL graduated cylinder, which is then added to a 1,000 mL graduated cylinder containing 700 mL of HPLC-grade methanol (either J.T. Baker JT9093-3 or Fisher Scientific A452-4, Fair Lawn, New Jersey). The aqueous TbAA solution is made by diluting 0.4 M tetrabutylammonium hydroxide (J.T. Baker V365-7) with high-purity deionized water (HPLC-grade equivalent) and adjusting the pH to 6.5 with acetic acid. Approximately 3–4 L of TbAA solution can be made ahead of time and stored at room temperature in amber bottles (Sect. 4.4.3 provides a discussion of precipitation problems).

After Solvent A is well mixed, it is filtered through a 0.2 µm pore size, 47 mm diameter nylon membrane filter (Millipore GNWP04700, Billerica, Massachusetts). Typically, 4–5 L of Solvent A can be made at a time, and the needed number of batches can be combined in a large glass container, so all Solvent A to be used in a week is homogeneous. This procedure promotes the best day-to-day retention time stability. If Solvent A is not fully consumed within the week, however, precipitate can form. The unused Solvent A can be refiltered and combined with the next batch of Solvent A. If refiltering is not performed, particulate material can clog the column.

Table 29. The HPL pump gradient used during SeaHARRE-3. Solvent A is formulated from (30:70) 28 mM TbAA (6.5 pH) and methanol, respectively, and Solvent B is methanol. The flow rate is at 1.1 mL min^{-1}, and the time is in minutes.

Step	*Action*	*Time*	A [%]	B [%]
1	Inject Sample (Start Run)	0.0	95	5
2		22.0	5	95
3		29.0	5	95
4		31.0	95	5
5	Stop Run	36.0	95	5
6	Start Injection Program	8.9†	95	5

† The indicated time is the duration of the step.

The pump gradient used in SeaHARRE-3 is presented in Table 29. The total run time, from the beginning of one injection to the next, is 44.9 min. Column temperature is set at 60.0 ± 0.8°C. The HPLC column is a Zorbax Eclipse XDB-C8, 3.5 µm particle size, 4.6×150 mm (Agilent 963967-906, Santa Clara, California). The column is used without a guard column or pre-filter. All pigments are eluted before 29 min, and during steps 4–6, 15.2 mL of the solvent of initial conditions flows through the column, which reconditions the column for the next sample.

4.2.3.3 Detection

Chromatograms are plotted using the 450 and 665 nm wavelengths (each with a 20 nm bandwidth), as well as 222 nm with a 10 nm bandwidth. Tungsten and deuterium lamps are both used and absorbance spectra are collected between 350–750 nm with a range step and slit of 2 nm. The flow cell capacity is 13 µL and the pathlength is 1 cm. The wavelength at 222 nm is used exclusively for the internal standard. This wavelength is not associated with the maximum response for vitamin E, but it allows good detection and minimizes baseline drift, which is still approximately -3 mAU min^{-1} caused by solvent effects of the gradient.

The signal height of vitamin E at the concentration used is approximately 600 mAU, and sufficiently high so that baseline drift does not interfere with accurate peak area determinations. The upper limit of linearity for vitamin E has been identified, and a concentration is used that corresponds to a point that is about two-thirds of the threshold where the response becomes nonlinear.

4.2.3.4 Calibration

The HPLC was primarily calibrated with standards isolated at HPL, the procedures for which are described in Van Heukelem and Thomas (2001). Concentrations of pigment standards were determined with the absorption coefficients given in Table 30 [according to (31) and (32)] with alternate absorption coefficients in acetone for selected carotenoids used in experimental applications discussed in Chapter 1. The spectrophotometer used for determining standard concentrations was a Shimadzu 2401-PC (Columbia, Maryland), with bandwidth of either 1 or 2 nm, a medium sampling rate, 1 nm sampling interval, and with correction for turbidity at 750 nm. Absorbance of stock standards is typically between 0.2–0.8, a range recommended by Marker et al. (1980) for best spectrophotometric accuracy.

A few calibration standards were purchased from Fluka (now Sigma-Aldrich) or DHI. The response factors of 17 HPL standards were compared in May 2005 with response factors measured at HPL from DHI standards. All of these standards represented pigments quantified for the SeaHARRE-3 activity, and the average APD between the response factors from HPL and DHI was 2.0%. Consequently, the spectrophotometrically determined concentrations provided by DHI were used to compute the response factors observed from their standards at HPL.

Table 30. The HPL α values (in units of liters per gram per centimeter), used for spectrophotometrically measuring pigment concentrations. Also shown are the solvents and maximum wavelengths (λ_m) specified for use with α values. The units for λ_m are nanometers. Standards isolated at HPL, or purchased from DHI or Sigma-Aldrich are denoted H, D, and S, respectively, in the reference column. The pigment identification (ID) number is from Table 31.

ID	*Pigment*	*Solvent*	λ_m	α	*Ref.*
2	Chl c_3	90% Acetone	453	346.00	[1] H,D
3	Chl c_2	90% Acetone	444	374.00	[1] H,D
5	Chl c_1	90% Acetone	443	318.00	[1] H
6†	Chlide a‡	90% Acetone	664	127.00	[2] H,D
7†	Phide a	90% Acetone	667	74.20	[2] D
10	Perid	100% Ethanol	472	132.50	[3] H,D
10§		100% Acetone	466	134.00	[3] H
14	But	100% Ethanol	447	160.00	[4] H,D
14§		100% Acetone	445	147.00	[5] H
15	Fuco	100% Ethanol	449	160.00	[12] H,D
15§		100% Acetone	443	166.00	[5] H
16†	Neo	100% Ethanol	439	224.30	[7] H,D
18†	Pras	100% Ethanol	438	160.00	[12] H,D
19	Viola	100% Ethanol	443	255.00	[8] H,D
20	Hex	100% Ethanol	447	160.00	[4] H,D
20§		100% Acetone	445	142.00	[5] H
24	Diad	100% Ethanol	446	262.00	[12] H,D
24§		100% Acetone	447	223.00	[9] H
27	Allo	100% Ethanol	453	262.00	[12] H,D
27§		100% Acetone	454	250.00	[10] H
29	Diato	100% Ethanol	449	262.00	[12] H,D
29§		100% Acetone	452	210.00	[9] H
31	Zea	100% Ethanol	450	254.00	[11] H,D
31§		100% Acetone	452	234.00	[6] H
32	Lut	100% Ethanol	445	255.00	[13] H
36	DVChl b	100% Acetone	645	52.50	[10] H
37	Chl b	90% Acetone	647	51.36	[14] H
45	DVChl a	90% Acetone	664	87.67	[1] H
46	Chl a	90% Acetone	664	87.67	[14] S,D
49	Phytin a	100% Acetone	666	¶	[15] H
52†	$\beta\beta$-Car	100% Acetone	454	250.00	[16] H,D

† Calibration procedure differed in SeaHARRE-2.

‡ The R_f of the DHI Chlide a standard matched the R_f of the Sigma-Aldrich Chl a standard (prepared at HPL) adjusted for the molecular weight of Chlide a.

§ Acetone absorption coefficients used with carotenoid standards to generate an alternate, field sample data set.

¶ Based on 46,000 molar absorption coefficient.

[1] Jeffrey (1972)
[2] Lorenzen and Jeffrey (1980)
[3] Jeffrey and Haxo (1968)
[4] Vesk and Jeffrey (1987)
[5] Haugan and Liaaen-Jensen (1989)
[6] Aasen and Liaaen-Jensen (1966b)
[7] Cholnoky et al. (1966)
[8] Davies (1965)
[9] Johansen et al. (1974)
[10] Jeffrey et al. (1997)
[11] Strain (1938)
[12] Bidigare (1991)
[13] Jeffrey (1997) App. E
[14] Jeffrey and Humphrey (1975)
[15] Watanabe et al. (1984)
[16] Hiyama et al. (1969)

Single-point response factors are used, because previously (and also during the SeaHARRE-3 activity) it was demonstrated that the samples being analyzed were within the linear range and, in addition, the linear regressions associated with the multipoint calibration curves exhibited y-intercept values very near zero. The values of the response factors being used are based on averages obtained from using the same method for many years. The validity of the average response factors are checked frequently, and the uncertainties for calibration checks are expected to be within 5%. This uncertainty represents the cumulative effects of spectrophotometric determinations of stock standard concentrations, dilution, and the subsequent HPLC analyses. Uncertainties greater than 5% are investigated and, if necessary, response factors in the calibration table are changed.

Discrete calibration standards were not available for all of the pigments quantitated during the SeaHARRE-3 activity, including the following:

- A Chl c_3-like pigment† which elutes immediately after Chl c_3 and was summed with Chl c_3;
- Chlide a;
- Four pigments with Phide a spectra that elute after the retention time of the Phide a standard purchased from DHI;
- Chl a and DVChl a allomers and epimers; and
- A Phytin a epimer.

The presence of the four pigments with Phide a spectra within natural samples (as identified with in-line visible absorbance spectra) are relied on to update their retention times in the calibration table. The response factor used for these four pigments is determined from the Phide a standard provided by DHI. All of these pigments are described in Table 31.

TChl b was quantified using a different set of procedures during SeaHARRE-3 than what had typically been done in the past, because divinyl form was clearly present and distinguishable from the monovinyl form in many samples ($R_s \approx 0.8$). Consequently, instead of using a quantitation process based on total peak area and a single-point response factor for MVChl b, each Chl b pigment was quantitated by peak height. The DVChl b peak height response factor was used for the DVChl b peak and the MVChl b peak height response factor was used for the MVChl b peak. These two individually-quantified pigments were then summed to produce TChl b. In this case, the individual pigments were not reported.

In some instances, DVChl b was indistinguishable from Chl b, in which case, TChl b was quantitated with the peak height response factor for Chl b. For comparison, TChl b was also quantified with the usual mode of quantitation

† This pigment is described by Garrido and Zapata (1998) as monovinyl Chl c_3.

Table 31. The pigments identified by the HPL method—adapted from Table 50 in Hooker et al. (2005)—listing the pigments individually quantified (shown with a solid circle, •) algal sources and standards used to identify retention times in minutes (t_R) and resolution (R_s). For the latter, NR indicates not resolved, blank entries correspond to $1.0 \leq R_s \leq 1.5$, and the listed values are typical of a new column. The R_s is described for a pigment and the following pigment, and the pairs are identified by their pigment identification (ID) numbers. Potential quantitation problems are encoded as follows: S_p, peak shape subject to distortion from injection conditions; S_s, separation selectivity can change with column age; S_{C_1}, pigments rarely present; S_{C_2}, pigments often present, but in a concentration less than 3% of $\left[\text{TChl } a\right]$; S_{R_1}, R_s adequate, but large first peak can mask second peak (if small); S_{R_2}, $R_s < 1.0$ and an alternate mode of quantitation is required; S_{R_3}, interferences frequently present requiring spectral matching for pigment identity; S_{R_4}, interferences possible, but infrequent; and S_{R_5}, very rare interferences. A quantitation mode denoted with α uses discrete standards (with absorption coefficients in Table 30).

ID	*Pigment*		*Identification*			*Quantitation*	
No.	*Name*	*Source*	t_R		R_s	*Problem(s)*	*Mode*
1•	Chl c_3	C, J, L, N, S, T	3.88			S_p	α
2	MVChl c_3	J	4.14			S_p	Summed with 1
3•	Chl c_2	A–E, H, J, L–O, R–T	5.70	NR	3/4	S_p, S_s	α
4	MgDVP	G, P	5.81	NR	4/a, 4/5	S_p, S_s, [1]	
a	*Unknown*	K	5.92	NR	a/5		
5•	Chl c_1	B, D, E, R	6.05	NR	5/6	S_p, S_s, [1], S_{R_2}	Simultaneous equation
6•	Chlide a	F, H, N, O	6.06	NR	5/6	S_p, S_s, S_{R_2}	Simultaneous equation, part of TChl a
7•	Phide a	DHI†	8.05				α (summed with 8, 9, 12, and 13)
8	Phide a-like		8.29				Part of Phide a
9	Phide a-like		9.19				Part of Phide a
10•	Perid	A, B, M	9.32				α
11	Perid isomer	A, B, M	9.58				
12	Phide a-like		10.60				Part of Phide a
13	Phide a-like		10.78				Part of Phide a
b	*Unknown*	P	11.37				
14•	But	DHI†, C, N, S, T	12.31			S_{R_5}	α
c	*Unknown*	G	12.68	NR	c/15		
15•	Fuco	C, D, E, L, N, R–T	12.63	NR	c/15	S_{R_5}	α
16•	Neo	F, G, P, U	13.29	NR	16/17	S_s, S_{C_2}, S_{R_3}	α
17	4k-Hex-fuco	J	13.31	NR	16/17		
d	*Unknown*	L	13.73	NR	d/18		
18•	Pras	DHI†, G, P	13.74	NR	d/18	S_{R_3}, S_{C_2}	α
19•	Viola	DHI†, F, G, P, Q, U	13.99	1.3	19/20	S_{C_2}	α
20•	Hex	DHI†, C, J, L	14.16	1.3	19/20		α
21	Asta	Shrimp carapace, G	14.53				
e	*Unknown*	P	14.78				
22	Diadchr	M	15.02	NR	22/f		
f	*Unknown*	J	15.09	NR	f/23		
23	*Unknown*	DHI†, K	15.13	NR	23/24	[2]	
24•	Diad	A–E, J, L–N, R–T	15.23	1.4	24/25	S_{R_4}	α
25	Dino	A, M	15.49				
26	Anth	F	15.99				α
27•	Allo	H, O	16.53				α
28	Myxo	DHI†	17.34				α
29•	Diato	C, D, E, M, R, T	17.12	NR	29/30	S_{R_3}, S_{C_2}	α

Table 31. (cont.) The pigments identified by the HPL method.

ID	*Pigment*		*Identification*			*Quantitation*	
No.	*Name*	*Source*	t_R		R_s	*Problem(s)*	*Mode*
30	Monado	H, O	17.22	NR	29/30		
31•	Zea	C, F, G, I, K, P, Q, V	17.79			S_{R_1} wrt Lut	α
32•	Lut	F, G, T, V	17.98			S_{C_2}	α
g	*Unknown*	Q	18.24	NR	g/h		
h	*Unknown*	G	18.32	NR	g/h		
i	*Unknown*	L, N, S, T	18.84				
33	Cantha	W	19.07				
j	*Unknown*	Q	19.23				
34	Gyroxanthin diester	DHI†, C	19.94			S_{C_1}	α
35	Gyroxanthin diester-like	C	21.00				
36•	DVChl *b*	U	21.92	0.8	36/37	S_{R_2}	α (peak height)
37•	Chl *b*	Fluka†, F, G, P	22.03	0.8	38/39	S_{R_2}	α (peak height)
38	DVChl *b*′	U	22.29				
39	Croco	H, O	22.42	NR	39/40		
40	Chl *b*′	Fluka†, F, G, P	22.50	NR	39/40		
41	Vitamin E	Fluka†	22.84				Internal standard
42	Chl *a* allomer	Fluka†, A–T	23.30				α, part of TChl *a*
43	Chl *a* allomer	Fluka†, A–T	23.43	NR	43/k		α, part of TChl *a*
k	*Unknown*	R	23.52	NR	k/l		
l	*Unknown*	R	23.52	NR	l/44		
44	Chl *c*-like	J, R	23.53	NR	l/44		
45•	DVChl *a*	U	23.76				α, part of TChl *a*
m	*Unknown*	L	23.91	NR	m/46		
46•	Chl *a*	Fluka†, A–T	23.96	NR	m/46	S_{R_4}, [3]	α, part of TChl *a*
47	DVChl *a*′	U	24.13				α, part of TChl *a*
48	Chl *a*′	Fluka†, A–T	24.33				α, part of TChl *a*
49•	Phytin *a*	Acidified from Chl *a*	25.43				α
n	*Unknown*	P	25.58				
50	Phytin *a*′		25.62				Summed with 49
51	$\beta\varepsilon$-Car	Sigma†, G, H, J, L, O, P, S	26.65	NR	42/43		Part of Caro
52•	$\beta\beta$-Car	Fluka†, A–G, I–N, P–U	26.71	NR	42/43		α, part of Caro

A *Prorocentrum minimum* B *Gyrodinium uncatenum* C *Karlodinium micrum*
D *Thalassiosira pseudonana* E *Isochrysis* sp. (Tahiti strain) F *Dunaliella tertiolecta*
G *Pycnococcus provasolii* H *Pyrenomonas salina* I *Synechococcus* sp.
J *Emiliania huxleyi* K *Synechococcus* cf. *elongatus* L *Chrysochromulina polylepsis*
M *Amphidinium carterae* N *Pelagococcus subviridis* O *Guillardia theta*
P *Micromonas pusilla* Q *Nannochloropsis* sp. 1 R *Isochrysis galbana*
S *Pelagomonas calceolata* T *Aureococcus anophagefferens* U Mutant corn
V Marigold petals W Gift from Perdue, Inc. † Details are given in Appendix C.

[1] Separation selectivity can cause peak area of MgDVP to be unresolved from either Chl c_2 or Chl c_1.
[2] The same spectra as an Aphanizophyll standard from DHI.
[3] Pigment m is not visible at the wavelength used for Chl *a* quantitation (665 nm).

(TChl b peak area times the MVChl b peak area response factor). These latter results were not submitted, but were used to evaluate bias caused by differing modes of quantitation (Sect. 4.4.8).

4.2.3.5 Pigment Identification

During the analysis of SeaHARRE-3 samples, retention times were documented on a daily basis using standard mixes formulated at HPL, as well as with the DHI Mix-101. These two types of mixtures were injected near the beginning of a sequence and then every twelfth injection thereafter. During the SeaHARRE-3 activity, the criteria for pigment identification were based primarily on retention time, although absorbance spectral match with standards was also used where possible (e.g., where the signal was adequate to produce useable absorbance spectra for peaks in sample extracts). In other words, if a pigment appeared at a retention time for a particular standard, but the absorbance spectrum was clearly inconsistent with what should be apparent for that standard, the pigment was reported as *not present* (i.e., a concentration of zero, $0\,\mathrm{mg\,m^{-3}}$, was assigned).

If a symmetrical, well-shaped peak appeared at the retention time of a standard, but the SNR was too low to confirm pigment identity on the basis of absorbance spectra, the pigment was still quantitated and reported. Small peaks that were clearly contaminated with an interference peak, as evidenced by peak shape distortion, were reported with zero concentrations (this most frequently occurred with Allo and Diato). Pigments with an SNR of 5 or less (at the wavelength used for their quantitation) were considered not present (and zero concentrations were assigned to them).

The retention times of pigments to be quantified, and pigments with a potential to interfere with the quantitation process, were originally documented using algal monocultures and standards (Van Heukelem and Thomas 2001). Table 50 in the SeaHARRE-2 report (Hooker et al. 2005) summarizes this information, which is updated here in Table 31 with additional details of chromatographic problems that can cause large uncertainties for some pigments in natural samples. These chromatographic problems are categorized by the source of the difficulty: peak shape (S_p), separation selectivity (S_s), concentration (S_C), and resolution (S_R).

A classification scheme for these five categories of *potential* problems was produced and coded as follows:

- S_p Affected by injection conditions, for which notable abnormalities are peak splitting and tailing.
- S_s Likely caused by changes in column performance as a result of (prolonged) usage.
- S_C Two subcategories of concentration problems are identified:
  - S_{C_1} Pigment is rarely present; and
  - S_{C_2} Pigment is usually present, but the concentration is less than 3% of $\left[\text{TChl } a\right]$.
- S_R Five subcategories of resolution problems are described:
  - S_{R_1} For a pigment pair with $R_s < 1.5$, if the first pigment in the pair is usually in high abundance relative to the second pigment, the quantitation of the second pigment is degraded (Dolan 2002);
  - S_{R_2} If $R_s < 1.0$, an alternate mode of quantitation is required.
  - S_{R_3} Interfering pigments are frequently present, so spectral matching is required.
  - S_{R_4} Interfering pigments are possible, but infrequent.
  - S_{R_5} Interfering pigments are extremely rare.

As mentioned above, these are potential problems, and all problems do not necessarily occur with all samples. Some chromatographic problems listed in Table 31 were described during sample analyses after SeaHARRE-3, and are included here for completeness. The list of chromatographic problems are expected to be modified as more diverse samples are encountered, more discriminating thought is applied to analyses, and possible remedies to problems emerge.

4.2.4 Data Products

The data products reported with the HPL method include a) individual pigments, b) pigment sums (these are individually quantified pigments summed together), and c) pigments resulting from one or more unresolved pigments that are quantified collectively using a response factor for the most frequently abundant pigment at that retention time (e.g., Caro and Chl c_2). Pigment standards frequently contain small isomers that are usually less than 10% of the total peak area for that standard.

It is rather likely that isomers are also present in natural samples. Most often, however, these isomers are undetectable, because their peak areas are small relative to the parent peak, or they coelute with the main peak of a more dominant pigment than the parent pigment. It is important to note, only the presence of such isomers for MVChl a, DVChl a, and Phytin a are quantitated for the HPL method—although the potential of isomers for coelution is described in Table 11 of Hooker et al. (2005). Isomers for Perid and Chl b are frequently observed, but are not included in the quantitation of the parent pigments in SeaHARRE-3 (although this practice has been changed so they are included when detected).

For the SeaHARRE-3 activity, DVChl a and Chl a were quantified differently from the procedures used during the SeaHARRE-2activity, which are summarized by the formulation details of (34) and (35) in Hooker et al. (2005). In SeaHARRE-3, the total area of all DVChl a and MVChl a

allomers and epimers, plus the main peaks (numbers 42, 43, 45, 46, 47, and 48 in Table 31), were summed and the nanograms per injection was determined by multiplying this total peak area times the Chl *a* response factor. This product—the so-called *sum* Chl *a*, which is denoted $\left[\Sigma\text{Chl } a\right]$—is not reported as a data product, but serves as an intermediate variable for computing $\left[\text{DVChl } a\right]$ and $\left[\text{Chl } a\right]$. To quantify Chl *a*, the main peak area for DVChl *a* was multiplied times the response factor of Chl *a*. The $\left[\text{Chl } a\right]$ was computed as $\left[\Sigma\text{Chl } a\right]$ minus $\left[\text{DVChl } a\right]$. HPL had previously validated that the response factors for Chl *a* were appropriate for use (at 665 nm) with DVChl *a*; otherwise, quantitations are performed according to equations in Sect. 4.2.1.

4.2.4.1 Spectrophotometric Chl *a* (SChl *a*)

Most SeaHARRE-3 sample extracts were analyzed spectrophotometrically for SChl *a* using the trichromatic equation of Jeffrey and Humphrey (1975):

$$\begin{aligned}\tilde{C}_{S_a} &= 11.85\,\tilde{A}(664) - 1.54\,\tilde{A}(647) \\ &\quad - 0.08\,\tilde{A}(630),\end{aligned} \tag{36}$$

where the corrected absorbance values ($\tilde{A}$) at 664, 647, and 630 nm include an absorbance measurement at 750 nm as a turbidity correction, that is, $\tilde{A}(664) = A(664) - A(750)$. To express (36) in units of micrograms per liter of seawater requires:

$$C_{S_a} = \frac{V_{x'}}{V_f}\,\tilde{C}_{S_a}, \tag{37}$$

where $V_{x'}$ is the extraction volume in milliliters, and V_f is the filtration volume in liters. It is important to note, (36) was developed for use with sample extracts in 90% acetone.

Some sample extracts in SeaHARRE-3 were not analyzed spectrophotometrically, because they were too dilute and the absorbance values were expected to be too low for good accuracy. Humphrey and Jeffrey (1975) noted that concentrations less than 0.2 $\mu g\,mL^{-1}$ of extract produced large (up to 60%) uncertainties in recovery, as described in Appendix G of Jeffrey et al. (1997b). Of the 51 extracts analyzed, all but three yielded extract concentrations for Chl *a* greater than 0.2 $\mu g\,mL^{-1}$. Most extracts were analyzed for SChl *a* within 1–2 d of extraction. Matched-glass cuvettes with blackened sides and a 1.5 mL volume capacity were used (Starna Cells 29B-SOG-10, Atascadero, California). The spectrophotometer was a Shimadzu UV-1601 with a 2 nm bandwidth (Columbia, Maryland).

Four of the samples extracted on the first day were turbid, which made them unsuitable for analysis by spectrophotometry. Filters, which were replicates to those that produced turbid extracts on the first day, were extracted on subsequent days when an added volume of water (V_a) was included during extraction. These replicate extracts were not turbid. The source of the turbidity is suspected to be a consequence of the salt on the sample filter being insoluble in the extracts, because the water content was too low (which could have resulted from the addition of 100% acetone to a relatively dry filter). This hypothesis was tested by adding drops of water to the turbid extracts, which caused them to clarify. The latter was only done after HPLC quantitation of these extracts had been performed and extracts so adjusted were not used for quantitation of $\left[\text{SChl}a\right]$.

4.2.4.2 HPLC SChl *a* Equivalent (HSChl *a*)

The spectrophotometric analysis of SChl *a* provides a means to independently evaluate HPLC accuracy. The value of $\left[\text{SChl}a\right]$ does not necessarily equal $\left[\text{TChl } a\right]$ in an HPLC extract, however, because DVChl *a*, Chlide *a*, Phide *a*, and Phytin *a* all absorb strongly at 664 nm, and are not discriminated from SChl *a* in the trichromatic equation. Consequently, the presence of any of the aforementioned pigments causes SChl *a* to be spectrophotometrically overestimated. A more useful approach is to compute the HPLC SChl *a* equivalent, i.e., HSChl *a*, for comparison with SChl *a*. This new HPLC pigment sum requires the following:

- HPLC quantitation of the aforementioned Chl *a*-type pigments (and degradants),
- Determination of the contribution of each pigment to spectrophotometric absorbance at 664 nm, and
- Computation of the equivalent amount of SChl *a* that would result from the total predicted absorbance at 664 nm.

Comparisons between SChl *a* and HSChl *a* were presented in the SeaHARRE-2 report, but the calculation approach for HSChl *a* was in error, so a corrected calculation equation is presented here. The derivation of the formulation begins with the following well-known relationships:

$$1\,\text{mol of } P_i = \frac{1\,\text{g of } P_i}{\Phi_{P_i}}, \tag{38}$$

where Φ_{P_i} is the molecular weight of pigment P_i. The absorbance of the pigment, A_{P_i}, is given as

$$A_{P_i}(\lambda_{P_i}) = C_{P_i}\,\varepsilon(\lambda_{P_i})\,l_c, \tag{39}$$

where C_{P_i} is concentration of the pigment (in units of molarity M, or moles per liter—mol L^{-1}), ε is the molar absorption coefficient, λ_{P_i} is the wavelength specified for use with the selected molar absorption coefficient and specified solvent, and l_c is the pathlength of the cuvette (always 1 cm in the calculations presented here).

Variables in the calculation equation include the molar absorption coefficient of each of the above-described pigments in 90% acetone, the molecular weight of each pigment, and the ratio of the absorbance of the particular

pigment at 664 nm relative to the absorbance of that pigment at the wavelength specified by its molar absorption coefficient (if it differs from 664 nm). Such values used in the computation of HSChl *a* are given in Table 32.

Table 32. The pigments and pertinent variables needed to quantify Chl *a* equivalents for estimating the summed value HSChl *a*. The ε and λ values are from Appendix E in Jeffrey (1997) and refer to 90% acetone solutions; the molecular weight values, Φ_{P_i}, are from Part IV Jeffrey et al. (1997a); and the ratios of the absorbance at 664 nm relative to absorbance of P_i at λ_{P_i} were observed at HPL.

P_i	Φ_{P_i}	ε	λ_{P_i}	*Ratio*
Chl *a*	893.50	78.32	664	1.00
DVChl *a*†			664	1.00
Chlide *a*	614.97	78.30	664	1.00
Phytin *a*	871.21	44.60	667	0.95
Phide *a*	592.67	44.00	667	0.95

† The HPLC quantified amounts for Chl *a* and DVChl *a* are summed when determining their contribution to HSChl *a*. The Φ and α values for Chl *a* are used for both Chl *a* and DVChl *a*.

The following steps exemplify a sample calculation for the contribution of Phide *a* (as measured by HPLC) to spectrophotometric absorbance at 664 nm. This example starts with the concentration of Phide *a* in units of micrograms per milliliter of extract, which is a good starting point because the SChl *a* value computed from the trichromatic equation (36) is in the same units.

1. Convert from micrograms of Phide *a* per milliliter of HPLC extract to grams per liter of extract:

$$\begin{aligned} 0.250\,\mu\mathrm{g\,mL^{-1}} &= 0.250\,\mathrm{mg\,L^{-1}} \\ &= 2.5\times10^{-4}\,\mathrm{g\,L^{-1}}. \end{aligned}$$

2. Phide *a* in grams per liter is converted to moles per liter using (38):

$$4.22\times10^{-7}\,\mathrm{mol\,L^{-1}}\ \text{Phide}\ a \;=\; \frac{2.5\times10^{-4}}{592.67},$$

where the molecular weight of Phide *a* is 592.67.

3. Determine the anticipated absorbance at the appropriate wavelength for a given number of moles per liter based on the molar absorptivity for the pigment and a 1 cm pathlength. Applying (39)

$$\begin{aligned} A(667) &= 4.22\times10^{-7}\,(44.0)\,(1) \\ &= 1.86\times10^{-5} \end{aligned}$$

4. Thus far, $0.250\,\mu\mathrm{g\,mL^{-1}}$ of Phide *a* in the HPLC extract is predicted to produce a spectrophotometric absorbance of 1.86×10^{-5} at 667 nm. The trichromatic equation, however, requires absorbance at 664 nm—not 667 nm—so the predicted absorbance of Phide *a* at 667 nm must be adjusted by the ratio of the absorbance of Phide *a* (in 90% acetone) at 664 nm relative to its absorbance at 667 nm. This has been determined to be approximately 0.95 as evaluated with standards at HPL. Consequently, the predicted absorbance of Phide *a* in this example at 664 nm is:

$$\begin{aligned} A(664) &= \frac{A(667)}{A(664)}\,A(667) \\ &= 0.95\,1.86\times10^{-5} \\ &= 1.76\times10^{-5}. \end{aligned}$$

5. Using (39), the equivalent moles per liter of Chl *a* yielded by the predicted absorbance of Phide *a* at 664 nm can be solved for:

$$1.76\times10^{-3} \;=\; (\mathrm{mol\,L^{-1}}\ \text{of Chl}\ a)\,78.32,$$

so

$$1.38\times10^{-3}\,\mathrm{mol\,L^{-1}}\ \text{of Chl}\ a \;=\; \frac{1.76\times10^{-5}}{78.32}$$

6. Rearranging (38), the Chl *a* equivalent is expressed as weight per volume (rather than moles per volume) to match the units used with the trichromatic equation for SChl *a*:

$$\begin{aligned} \mathrm{g\,L^{-1}}\ \text{of Chl}\ a &= 1.38\times10^{-3}\,(893.5) \\ &= 2.01\times10^{-4}, \end{aligned}$$

and

$$2.01\times10^{-4}\,\mathrm{g\,L^{-1}} \;=\; 0.201\,\mu\mathrm{g\,mL^{-1}},$$

which means $0.250\,\mu\mathrm{g\,mL^{-1}}$ of Phide *a* will yield an absorbance similar to $0.201\,\mu\mathrm{g\,mL^{-1}}$ of Chl *a* when this extract is analyzed on the spectrophotometer at 664 nm.

7. Steps 1–5 are performed for the HPLC quantified values for Chl *a* plus DVChl *a*, Chlide *a*, Phytin *a*, and Phide *a* in the sample extract. The resulting equivalent Chl *a* amounts are then summed to produce the product HSChl *a*. HSChl *a* and SChl *a* are reported in units of micrograms per liter of seawater, according to the following, respectively:

$$\big[\text{HSChl}\ a\big] \;=\; \mu\mathrm{g\,mL^{-1}}\ \text{of HSChl}\ a\,\frac{V_{x_1}}{V_f} \qquad (40)$$

and

$$\big[\text{SChl}\ a\big] \;=\; \mu\mathrm{g\,mL^{-1}}\ \text{of SChl}\ a\,\frac{V_{x'}}{V_f}. \qquad (41)$$

Note that the extraction volumes V_{x_1} and $V_{x'}$ are determined differently in (40) and (41), respectively, to limit the number of variables these equations share in common. The V_{x_1} term is determined according to (34), and $V_{x'}$ according to (42), for which V_w is estimated as in (43), as presented in Sect. 4.3.7.

Table 33. The relationship between SChl *a* and HSChl *a* concentrations or TChl *a* concentration for the *H* results obtained during SeaHARRE-2 (including the unequivocally defrosted—and, therefore, damaged—samples, *Z*) and SeaHARRE-3. Note: *wrt* denotes *with respect to*.

Activity and Data Set	*Total Samples*	*Average Ratio*		*CV of Ratio* [%]	
		SChl *a*/HSChl *a*	SChl *a*/TChl *a*	SChl *a wrt* HSChl *a*	SChl *a wrt* TChl *a*
SeaHARRE-2 (*Z*)	24	1.19	1.27	4.3	6.4
SeaHARRE-2 (*H*)	24	1.11	1.18	5.9	7.4
SeaHARRE-3 (*H*)	51	1.12	1.25	6.7	20.8

Three data sets comparing SChl *a* to both HSChl *a* and TChl *a* show improvement when HSChl *a*, instead of TChl *a*, is used for comparison (Table 33). SeaHARRE-3 data are also shown graphically in Fig. 5, with the corresponding linear regression statistics. While spectrophotometric measurements are perhaps no longer used routinely with field samples, they do have utility for laboratories troubleshooting a method or attempting to independently validate the accuracy of their HPLC measurements outside the context of a round robin.

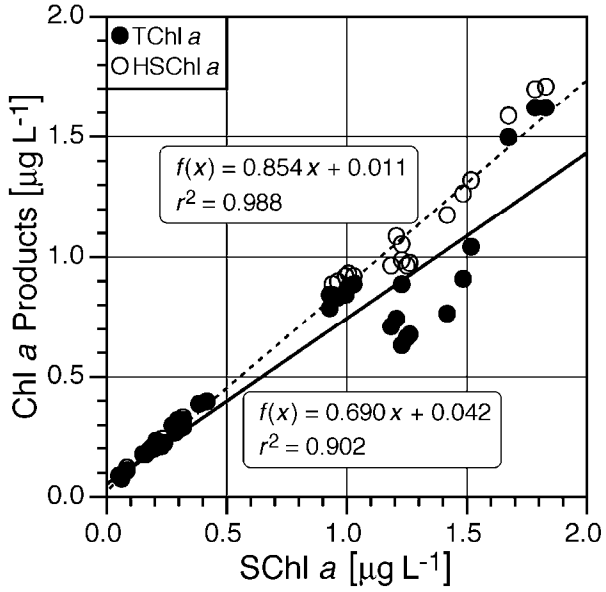

Fig. 5. The concentrations of the HSChl *a* and TChl *a* products with respect to the SChl *a* concentration all determined at HPL for data obtained during the SeaHARRE-3 activity.

4.2.5 Data Reporting

Pigments denoted as *not found* or ones with a SNR less than five were reported with concentrations of zero (0.000 mg m^{-3}). To eliminate insignificant digits to the right of the decimal and establish a reporting precision, the final pigment concentrations were multiplied by 10^5, converted to integers, and then divided by 10^5. For example, a concentration originally given in a spreadsheet cell as 0.0254376930505829, would be reported as 0.02543. This manipulation reduced most unnecessary digits but was not entirely accurate, because, as shown in this example, proper rounding should report a value of 0.02544—not 0.02543. After the completion of the SeaHARRE-3 activity, the `ROUND` function in `Excel` was used, and three digits to the right of the decimal were reported.

4.3 QUALITY ASSESSMENT

Several quality assessment activities were conducted during (or near) the time the SeaHARRE-3 samples were analyzed. Some of these activities are typical QA routines, some were performed to further assess uncertainties with variables in the calculation equation, and some of the validation studies were conducted because of modifications to the extraction procedures used during the analysis of SeaHARRE-3 samples.

4.3.1 LOD and LOQ

Observed values for both the limit of detection (LOD) and limit of quantitation (LOQ) for Fuco and Chl *a* were determined according to (24) and (25) in Hooker et al. (2005). LOD and LOQ values for other quantified pigments were calculated based on the relative response factor (RRF) of the particular pigment in question relative to either Fuco or Chl *a*, the choice of which depended on the wavelength used for quantitation of the pigment in question (e.g., Chl *a* was used as a reference for pigments quantified at 665 nm and Fuco was used as a reference for pigments quantified at 450 nm). These calculations are described with equations (26) and (27) in Hooker et al. (2005). On average, LOQ values were 0.07 ng per injection lower in SeaHARRE-3, and LOD values were 0.03 ng per injection lower.

4.3.2 Replicate Injections of ISTD

The precision of replicate injections of the internal standard (ISTD) is shown in Table 34 for dates during and immediately following the analysis of SeaHARRE-3 field samples. These data considered together revealed an important trend that contributed to the decision to modify the method (Sect. 4.4).

Table 34. The precision (CV) of replicate HPLC analyses of the internal standard, vitamin E acetate, at HPL. The number of replicate injections is given by N_R, and the acetone content of the internal standard solution is given in percent.

Date [2005]	N_R	*Acetone*	*CV*
2 June†	3	100%	0.47%
6 June†	3	100	0.94
13 June†	3	100	1.00
15 June†	2	100	1.51
17 June	2	100	1.74
17 June	2	98	2.96

† The dates for which SeaHARRE-3 samples were analyzed.

4.3.3 Replicate Sample Injections

As part of standard procedures, the extract of the first sample to be analyzed in a sequence of injections is split between two HPLC vials (approximately 500 µL per vial), and both vials are placed in the TCAS at the same time, along with all other sample extracts prepared on that day (referred to here as the daily sample set). The first vial (each of the two vials contain the same sample extract) is the first injection of the daily sample set (but not the first injection of the sequence), and the second vial is injected after all other vials in the daily sample set have been injected.

The timing of the duplicate injections may differ by as much as 24–30 h. For SeaHARRE-3 samples, three different sample extracts were injected two times each as described. The CV (in percent) was determined for TChl *a* and the primary pigments (which were averaged and reported as an overall average PPig CV). These replicate injections represented sample extraction dates of 2, 6, and 15 June, for which the CV of TChl *a* was 0.64, 0.39, and 1.36%; the corresponding CV for PPig was 1.29, 1.37, and 0.88%, for the respective dates. The overall average TChl *a* and PPig CV was 0.80 and 1.18%, respectively.

4.3.4 Replicate DHI Mix-101 Injections

Eight separate vials of the DHI Mix-101 were used for quality assessment. In all cases, when making HPLC injections, the contents of a DHI Mix-101 vial was dispersed into one or more HPLC vials (500 µL per vial) and only one injection was performed from each HPLC vial. When HPLC analysis precision was evaluated with triplicate injections from the contents of one vial of DHI Mix, the analyses are denoted as intra-vial injections.

Uncertainty among differing vials of DHI Mix-101 was determined by one injection each from contents of three different DHI Mix-101 vials, denoted inter-vial injections. Data submitted from *H* represented:

- Three intra-vial injections performed on 2 June 2005, for which average CV% was 0.8% and 0.9%, respectively for TChl *a* and PPig;
- Three inter-vial injections performed on 6 June 2005, for which CV% was 1.4 (TChl *a*) and 1.7% (PPig); and
- Three inter-vial injections performed on 15 June 2009, for which CV% was 1.5 (TChl *a*) and 1.3% (PPig).

To begin the analysis for a particular day, all vials were placed in the TCAS at the same time and the vials resided in the auto-sampler compartment for as little as 2 and as many as 23 h before injection. The interval between subsequent injections was 9 h in all but one instance, where it was 2 h. A final vial of DHI Mix-101 was quantitatively diluted 10 fold in 90% acetone and analyzed in triplicate. The average APD of quantitated amounts (corrected for dilution effect) with respect to the average TChl *a* and PPig concentrations determined from analyses of DHI Mix-101 vials conducted on all three dates was 1.3% (TChl *a*) and 0.9% (PPig). The DHI Mix-101 analyses were performed to evaluate analysis precision according to practices used to analyze sample vials and to validate that the DHI Mix-101 had not exceeded the upper limits of linearity.

4.3.5 Carryover

Carryover was monitored during SeaHARRE-3 by quantifying pigments that appeared in injections of a solution formulated to contain only the internal standard. The internal standard injections used for this purpose immediately followed injections of undiluted DHI Mix-101. This mix was used to evaluate carryover because it was the most concentrated solution injected during the SeaHARRE-3 activity. In fact, except for a few instances where consecutive, replicate injections of Mix-101 were made, Mix-101 was always followed by an injection of the internal standard or an acetone blank to minimize the possibility that carryover would affect the field sample results.

In some instances, carryover from Mix-101 was observed, but only for the middle- to late-eluting pigments, and never for the early-eluting pigments. The results for these analyses are summarized in Table 35, where it is evident on 17 June, that carryover was present and was worse (on a percentage basis) for pigments with increasing order of retention time. The carryover observed on this day was severe enough to cause elevated uncertainties in quantitation if a sample extract (and not the internal standard) had been injected immediately after Mix-101. For example, the Chl *a* carryover on 17 June is 309 times the LOD, and the Caro carryover is about 73 times the LOD for $\beta\beta$-Car. Fortunately, all field sample analyses were completed on the 15 June sequence. This carryover analysis, the increasingly degraded precision with the internal standard shown in Table 34, and the problems that occurred prior to SeaHARRE-3 led to extensive troubleshooting, hardware repairs and modifications to the HPL method (Sect. 4.4).

Table 35. The carryover of selected pigments (shown in order of increasing retention). Dates refer to the HPLC sequence. The top number in a dual entry is carryover in nanograms per injection, and the bottom number is the amount of pigment observed in the internal standard injection expressed as the percent of that pigment observed in the preceding DHI Mix-101 injection. Carryover was not seen for pigments eluting before Diad.

Date	Diad	Allo	Diato	Zea	Lut	Chl *b*	DVChl *a*	Chl *a*	Phytin *a*	Caro
2 June	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.34 0.03%	0.00	0.00
6 June	0.00	0.00	0.00	0.00	0.00	0.00	0.07 0.05%	0.46 0.04%	0.00	0.00
15 June	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
17 June	0.16 0.18%	0.06 0.28%	0.08 0.37%	0.36 0.44%	0.37 0.42%	3.64 0.86%	2.02 1.29%	15.76 1.44%	2.48 3.54%	2.54 3.63%

4.3.6 Validation of Vitamin E Response

For an internal standard to provide an accurate estimate of extraction volume, the response of the internal standard should be stable when suspended in varying acetone–water mixtures (within the range likely to be encountered in sample extracts). The vitamin E acetate response factor was evaluated in varying acetone concentrations, including 98, 96, 94, 92, 90, and 88%, by adding known volumes of internal standard (in 100% acetone) and varying quantitative volumes of water to each of nine different volumetric flasks. Four separate flasks were formulated with 94% acetone in varying internal standard concentrations. All flasks were then brought to volume with 100% acetone.

The contents of each flask were injected in duplicate (for a total of 18 injections, with TCAS residence times of 7.5 h between replicate injections). The stock solution of internal standard (in 100% acetone) used to formulate the above solutions, was also injected in duplicate. The average vitamin E response factor was computed for each acetone concentration and these were then averaged for an overall average response factor across all acetone concentrations. The CV associated with the average response factor was 1.0%, and the average injection precision associated with replicate injections of each solution was 0.9%.

4.3.7 Validation of Extraction Volume

In SeaHARRE-2 and SeaHARRE-3, extraction volumes were calculated with an internal standard according to (34). In SeaHARRE-3 extraction volume was also calculated according to an independent calculation that does not rely on individual HPLC measurements. The latter is calculated as follows:

$$V_{x'} \;=\; 0.98\left(V_m + V_w + V_a\right), \qquad (42)$$

where V_w is the estimated volume of water retained by the filter, V_a is the volume of water added to the extraction tube, and the multiplier 0.98 is a *shrinkage factor* applied to account for the fact that organic solvent and water shrink when combined. This degree of shrinkage has been observed at HPL when formulating 90% acetone (vol:vol) in 100 mL and 1,000 mL graduated cylinders. The purpose for computing $V_{x'}$ is to compare it with V_{x_1}, as a means of quality assuring the V_{x_1} measurement, and to use it as part of the SChl *a* calculation (41).

A limitation of (42) is the accuracy of the value used for V_w and the limited observation that 0.98 is an appropriate shrinkage factor. While 0.2 mL has been stated in the literature as a typical value of V_w for 25 mm filters (Bidigare et al. 2003), this value is expected to be highly variable, especially if filters have been blotted dry (as some were in SeaHARRE-3). An experiment was conducted to evaluate whether measuring V_w according to the following formula was viable and sufficiently accurate:

$$V_w \;=\; \frac{V_{x_1}}{0.98} \;-\; V_m \;-\; V_a, \qquad (43)$$

wherein, V_{x_1} replaces $V_{x'}$ in (42), because they are nearly equivalent measures of the extraction volume, and the terms are rearranged to solve for V_w.

To evaluate (43), V_{x_1} was measured by typical HPLC analyses (34) for tubes containing a constant volume of V_m (1.7862 mL with vitamin E acetate) and variable volumes of V_a within the range of 0.025–0.250 mL per tube, and, because these tubes contained no sample filters, $V_w = 0$ by definition. The combinations of water and acetone evaluated yielded acetone concentrations ranging from 88–100% (vol:vol). In this experiment, if V_{x_1} approximated $V_{x'}$, then (43) could be used to estimate V_w in the analysis of samples for which V_{x_1} values are routinely measured. With enough repetitions of this process—preferably involving multiple investigators—an average V_w (and typical range) could be established.

The linear regression (forced through zero) of $V_{x'}$ (y-axis) versus V_{x_1} (x-axis) for this experiment yielded a slope (m) of 1.0028, $r^2 = 0.975$, with an average absolute percent residual of 0.53%. To attain this relationship, $V_{x'}$ was computed as in (42) for tubes with formulated acetone concentrations between 97–88%. For tubes with acetone concentrations of 99–100%, (42) was used with no shrinkage factor (a leading coefficient of 1.00). This approach appears

reasonable, because the current extraction procedures are unlikely to yield acetone concentrations greater than 97% or less than 88% acetone. These regression statistics predict V_{x_1} and $V_{x'}$ should be within 7 µL for $V_m = 2.5$ mL (the value for V_m used after SeaHARRE-3).

With confidence in (43) established, the value for V_w in the SeaHARRE-3 sample extracts was solved for, and then $V_{x'}$ was solved for using (42). The average V_w for filters that had been blotted dry, samples 9–24 (Table 27), was 0.028 ± 0.030 µL; for those that had not been blotted (samples 1–8), the average V_w value was 0.093 ± 0.046 µL. Consequently, the value of V_w was set at 0.093 µL for samples 1–8 and 0.028 µL for samples 9–24. The RPD and APD of V_{x_1} wrt to $V_{x'}$ were computed for each sample extract. RPDs ranged from −4.81 to 4.04% with an average RPD of 0.004%. The minimum APD value was 0.01%, the maximum APD value was 4.81%, and the average APD value was 1.44%.

4.3.8 Spiked Recovery

So-called *spiked recovery* investigations are used to evaluate the accuracy of the analysis method, and the generalized procedures described by Clesceri et al. (1998) were used here, wherein known amounts of analytes are added to a blank filter (or sample filter), which are then processed through all steps associated with extraction and analysis. Spiked recoveries were performed at HPL by preparing a solution containing known amounts of four pigments and the internal standard in 99% acetone, which was added to extraction tubes (in lieu of the extraction solvent) containing either a blank filter or a SeaHARRE-3 sample filter. All tubes were then carried through the extraction procedures and analyzed as if they were samples.

The observed amounts of pigment per tube, as quantified by HPLC, were compared to the known amounts of pigments added to each tube, and the relationships are expressed as percent recovery. Typically, blank filters are spiked to evaluate accuracy associated with extraction and analysis. Spiked sample filters reflect accuracy of extraction and analysis in the presence of the sample matrix. (It is important to note spiked recoveries do not evaluate the ability of the extraction technique to fully remove pigments from cells, which is usually referred to as *extraction efficiency*.)

4.3.8.1 Spiking Solution

The concentration of pigments in the spiking solution needs to closely match the samples that will be spiked. The spiking solution was formulated after two replicates from each site had already been analyzed, so the approximate pigment concentrations of the remaining unprocessed filters were known. Four of these remaining unprocessed filters were then used for the "spiked sample" recovery experiments. In addition, the acetone content of the spiking solution must closely match the extraction solvent (100% acetone), so stock standards from which the spiking solution was formulated were also in acetone—even carotenoids, which are typically in ethanol. The spiking solution was prepared from 1.00 mL each of stock standards Chl c_1 and Chl a (in 90% acetone, absorption coefficients specified in Table 30) and 1.00 mL and 0.350 mL, respectively, of Fuco and Allo (in 100% acetone, absorption coefficients specified in Table 30).

These four stock standards were dispensed (with calibrated GASTIGHT Hamilton glass syringes) into a 25 mL Class-A, glass volumetric flask, which was brought to volume with the vitamin E internal standard working solution (in 100% acetone). The formulated concentrations of pigments in this spiking solution were 36.513, 73.200, 43.860, and 286.813 ng mL^{-1} for Chl c_1, Fuco, Allo, and Chl a, respectively. The spiking solution was analyzed by HPLC in triplicate to determine the average vitamin E peak area, which served as $\hat{A}_{c_1}$ in determining the extraction volume (34).

Ordinarily, a formulated concentration of a standard within a spiking solution serves as the reference in the calculations of percent recovery, as was done with the formulated concentrations for Chl a and Chl c_1. Because the HPLC is not routinely calibrated with Fuco and Allo standards in acetone, however, their formulated concentrations would not be expected to match their quantified concentrations as determined by HPLC. (Hooker et al. 2005 demonstrated such differences from standards prepared with differing absorption coefficients and solvents.) The reference concentrations for Fuco and Allo in the spiking solution were, therefore, based on their average HPLC-quantified concentrations, as determined from triplicate injections of the spiking solution.

The concentration of pigments in the spiking solution is denoted $\mathscr{S}_{P_i}$, and has units of nanograms per milliliter. This solution was added to all extraction tubes during recovery experiments using the same 2.5 mL gas-tight glass syringe, calibrated at 1.8 mL to deliver 1.786 mL. To eliminate cross-contamination between use, the glass syringe was always rinsed with 10 volumes of acetone. The recovery determinations (Sects. 4.3.8.2 and 4.3.8.3) were based on the total amount of each pigment added to the extraction tube:

$$\mathscr{S}_{P_i}\ \text{ng per tube} \;=\; \left(\mathscr{S}_{P_i}\ \text{ng mL}^{-1}\right) V_m, \qquad (44)$$

where $V_m = 1.786$ mL.

4.3.8.2 Laboratory-Fortified Blanks

For this, an unused dry 25 mm GF/F filter, 0.125 µL of water, and 1.786 mL of the spiking solution were added to each of four, 5 mL extraction tubes. These tubes were then subjected to the process of extraction as though they were sample filters. The pigments in the resulting extracts

were then quantified by HPLC and the percent recovery, Ψ, of each of the four pigments was determined according to

$$\Psi_{P_i} \;=\; 100\,\frac{\vec{\mathcal{B}}_{P_i}}{\vec{\mathcal{S}}_{P_i}}, \tag{45}$$

where amounts are expressed as nanograms per tube, $\vec{\mathcal{S}}_{P_i}$ is determined as in (44) and $\vec{\mathcal{B}}_{P_i}$ is determined as

$$\vec{\mathcal{B}}_{P_i}\text{ ng per tube} \;=\; \left(\vec{\mathcal{B}}_{P_i}\text{ ng per injection}\right)\frac{V_{x_1}}{V_c}, \tag{46}$$

where $V_c = 0.150\,\text{mL}$ and V_{x_1} is determined using (34), for which $V_m = 1.786\,\text{mL}$.

The percent recovery of each pigment in the spiking solution from laboratory-fortified blanks are shown in Table 36. In addition, these analyses provided another opportunity to evaluate the RPD of extraction volumes according to V_{x_1} (34) versus extraction volume according to $V_{x'}$ (42), which is presented in Table 37. The discussion of these results is deferred to the end of Sect. 4.3.8.3.

4.3.8.3 Spiked Recovery From Sample Filters

One filter from each triplicate set of samples from 3, 6, 8, and 10 were subjected to an evaluation of spiked recovery—1.786 mL of the spiking solution (V_m) and 0.050 mL of water (V_a) was added to each sample filter. In addition to determining spiked recoveries, extraction volumes were compared according to $V_{x'}$ and V_{x_1} (42 and 34, respectively) for each sample filter. For $V_{x'}$, the volume used as V_w for filters not blotted dry during sample collection (samples 3, 6, and 8) was 0.093 µL and for sample 10, which was blotted dry, 0.028 µL was used as V_w (see Sect. 4.3.7 for rationale).

Filters were processed through typical extraction and analysis procedures. The percent recovery is based on the total amount of each pigment observed per extraction tube (denoted $\vec{E}_{P_i}$), corrected for the amount of each pigment contributed by the sample filter (denoted E_{P_i}) relative to the amount of pigment per tube originating from the spiking solution (44). The equation for the percent recovery of pigments from sample filters is:

$$\Psi_{P_i} \;=\; 100\,\frac{\vec{E}_{P_i} - \bar{E}_{P_i}}{\vec{\mathcal{S}}_{P_i}}, \tag{47}$$

where the concentrations are expressed as nanograms per extraction tube and

$$\vec{E}_{P_i}\text{ ng per tube} \;=\; \left(\vec{E}_{P_i}\text{ ng per injection}\right)\frac{V_{x_1}}{V_c}, \tag{48}$$

where $V_c = 0.150\,\text{mL}$, and V_m used with $V_{x_1} = 1.786\,\text{mL}$.

The pigment content in unspiked filters is based on average concentrations observed during analysis of the two replicate filters from that site according to the usual procedures:

$$\bar{E}_{P_i}\text{ ng per tube} \;=\; \left(\bar{E}_{P_i}\text{ ng per injection}\right)V_c, \tag{49}$$

where the over-bar symbol ($\bar{}$) indicates an average, $V_c = 0.150\,\text{mL}$, V_{x_1} is the extraction volume observed for each filter on the day of its analysis, and V_m is the calibrated volume of the dispensing device used to add the extraction solvent (with internal standard) to the filter on the day of extraction.

In the follow-on discussion, results of the spiked laboratory blanks and spiked sample filters are discussed in context of each other, although they each contribute distinct information regarding uncertainties with variables in the calculation equation, notably V_{x_1} (34) and $\hat{A}_{P_i}$ in (30). It was demonstrated previously that V_{x_1} was accurate to within 0.3% (see regression statistics of $V_{x'}$ wrt V_{x_1} in Sect. 4.3.7). However, those experiments purposely avoided some processes of extraction (e.g., hold times, sonic disruption, and clarification). So, one purpose of the spiked blank recoveries is to determine the accuracy of quantifying the internal standard (and other pigment standards for that matter) after they have been subjected to all extraction and clarification processes. This is because if pigments or the internal standard are deleteriously affected by such processes, the percent recoveries will deviate from 100% by amounts greater than that simply attributable to analysis imprecision. When comparing V_{x_1} to $V_{x'}$ in spiked blank filters, it is expected that V_{x_1} will be somewhat lower than $V_{x'}$ simply because some evaporation of solvent is expected during sonic disruption and vial storage in the TCAS autosampler compartment (although the latter has been documented in Sect. 4.3.2.1 as $< 0.7\%$ per day at HPL).

With regard to recovery of pigment standards having been subjected to extraction and clarification processes, it is expected that the amount of pigment added to the extraction tube will be very similar to the amount of pigment quantified in that solution after it has been subjected to extraction. In other words, if the peak area of the standard in the spiking solution is not similar to the peak area in the solution after it is subjected to extraction, then one can assume the pigment standard is deleteriously affected by extraction processes and the percent recovery will deviate greatly from the analysis precision for that pigment. Spiked blank filters are a prelude to spiked sample filters, for the latter provides additional information—namely, can the amount of pigment standard added to a sample filter be accurately quantified within the context of a complex sample chromatogram, regardless of whether that pigment is or is not naturally occurring in the sample filter?

The results of spiked blank filters and spiked sample filters are shown in Tables 36 and 37, where the former describes pigment recoveries and the latter describes the RPDs of extraction volume according to V_{x_1} (34) versus

Table 36. For results of the spiked blank filter experiments (left data set), $\vec{\mathscr{S}}_{P_i}$ is the amount of pigment added to each tube in the spiking solution and $\vec{\mathscr{B}}_{P_i}$ is the amount of pigment quantified in each tube after a blank filter (and spiking solution) is subjected to the extraction process. For results of spiked sampler filter experiments (right data set), $\bar{E}_{P_i}$ is the amount of pigment naturally occurring in the sample filter, $\vec{E}_{P_i}$ is the total amount of pigment quantified per tube, and Ψ_{P_i} is the amount of pigment in the spiking solution added to each tube. Four blank filters were analyzed and four sample filters (numbers refer to their SeaHARRE-3 identification designation).

P_i	*Blank*	$\vec{\mathscr{S}}_{P_i}$	$\vec{\mathscr{B}}_{P_i}$	Ψ_{P_i} [%]	*Sample*	$\bar{E}_{P_i}$	$\vec{E}_{P_i}$	$\vec{\mathscr{S}}_{P_i}$	Ψ_{P_i} [%]
Chl c_1	1	65.2128	60.7673	93	6	0.000	63.5320	65.2128	97
Fuco		122.5482	120.5853	98		1.862	122.6960	122.5482	99
Allo		70.8818	69.6216	98		0.000	69.9440	70.8818	99
Chl a		512.2486	510.6248	100		46.634	560.8960	512.2486	100
Chl c_1	2	65.2128	60.4353	93	3	2.394	68.0120	65.2128	101
Fuco		122.5482	120.5995	98		13.230	135.4640	122.5482	100
Allo		70.8818	69.4308	98		0.000	69.3560	70.8818	98
Chl a		512.2486	510.2123	100		184.296	688.9680	512.2486	99
Chl c_1	3	65.2128	61.4479	94	10	2.478	68.6840	65.2128	102
Fuco		122.5482	120.8154	99		10.920	132.0480	122.5482	99
Allo		70.8818	69.8106	98		0.000	71.7360	70.8818	101
Chl a		512.2486	507.5234	99		297.836	804.6080	512.2486	99
Chl c_1	4	65.2128	61.8208	95	8	0.000	67.3960	65.2128	103
Fuco		122.5482	119.9637	98		9.716	129.6680	122.5482	98
Allo		70.8818	69.7249	98		0.000	70.7840	70.8818	100
Chl a		512.2486	509.4351	99		257.026	755.1040	512.2486	97

Table 37. The RPD of extraction volume V_{x_1} with respect to $V_{x'}$.

Blank No.	V_{x_1} [mL]	$V_{x'}$ [mL]	*RPD* [%]	*Sample No.*	V_{x_1} [mL]	$V_{x'}$ [mL]	*RPD* [%]
1	1.838	1.873	−1.9	6	1.935	1.890	2.4
2	1.819	1.873	−2.9	3	1.863	1.890	−1.5
3	1.820	1.873	−2.8	10	1.869	1.827	2.3
4	1.800	1.873	−3.9	8	1.856	1.890	−1.8

extraction volume according to $V_{x'}$ (42). The average pigment recovery from spiked blank filters ranged from 94% (for Chl c_1), to 98–99% for the other three pigments tested. One could assume, then, that Chl c_1 may be somewhat more sensitive to degradation processes during extraction, or there is simply greater uncertainty in the HPLC analysis of Chl c_1. In fact, overall, uncertainties imposed by extraction and clarification procedures, as observed during these spiking experiments—1 to 2% for Fuco, Allo, and Chl a, and 6% for Chl c_1—do not greatly exceed analysis precision, as determined by replicate injections of sample solutions and for which average CV approximates 5% for Chl c_1 and 0.5% for the other three pigments at HPL. From these spiking experiments, one cannot assume these pigments are adversely affected by extraction and clarification processes.

The average percent recovery of pigments from the spiked sample filters is 101% for Chl c_1 and 99% for Fuco, Allo, and Chl a. For Fuco, Allo, and Chl a, the recoveries from spiked blank filters and spiked sample filters differed by no more than 3% and 1% on average (values that are very near the method imprecision for these pigments). Chl c_1 percent recoveries were always higher from spiked sample filters—7% on average, although the percent recovery from spiked sample filters was never higher than 103%, which could be considered still within the analysis imprecision for Chl c_1 (5%). Chl c_1 is subject to coelution problems and peak shape distortions at times, events which undoubtedly contribute to its higher method imprecision and possible differences here between blank filter and sample filter spiked recoveries.

Spiked sample filters are important because recoveries could deviate substantially from method imprecision if the pigments were deleteriously affected by components within the seawater sample (and therefore on the sample filter) that could degrade or enhance peak areas of pigments in the spiking solution. The latter could occur with acidic conditions, coeluting contaminants, interferences from adjacent poorly resolved components, or a resulting solvent extract composition that is either too high or too low in

water content and therefore, not optimal for pigment solubility or peak shape (for example, if the proportion of V_w to V_m in (42) is too high or low).

Spiked blank filters can be used to assess the typical relationship between extraction volume determination by V_{x_1} (34) with respect to $V_{x'}$ (42)—the only variable shared by both is V_m, for which the known uncertainty approximates 0.5% at HPL. In these experiments, $V_m = 1.786$ and referring to (42), $V_a = 0.125\,\mu L$ and $V_w = 0$, so with a shrinkage factor of 0.98, V_{x_1} should equal 1.873 mL if there is no evaporation of solvent during sonic disruption, hold times, and clarification. Using the internal standard in (34), V_{x_1} averaged $1.819\,mL \pm 0.016$ with a CV of 0.85% (data in left column of Table 37).

The average RPD of V_{x_1} with respect to $V_{x'}$ is −2.9%. Considering the imprecision in V_{x_1} (0.85%), it is reasonable to assume that evaporation on the order of 2% (or approximately 40 µL) may have occurred during extraction processes and this caused V_{x_1} to be lower than $V_{x'}$ or the shrinkage factor could be inaccurate, or that both evaporation and an inaccurate shrinkage factor are together contributing to the approximate 2% reduction in V_{x_1}. It would be more alarming if V_{x_1} were routinely higher than $V_{x'}$ because that would mean the volume of extraction solvent had increased, which is an unlikely possibility, but a plausible outcome if factors negatively influencing peak area of the internal standard in (34) had occurred. These types of intricate inquiries are possible because of having conducted spiked blank filter experiments for which V_w in (42) equals 0. Comparing V_{x_1} to $V_{x'}$ is also possible with spiked sample filters, although determination of $V_{x'}$ for these field samples included $V_a = 0.050\,\mu L$ and V_w (42), which was set at 0.093 µL for samples 3, 6, and 8, and 0.028 µL for sample 10 (see Sect. 4.3.7 for rationale). The RPD of V_{x_1} with respect to $V_{x'}$ varied from −1.8 to 2.4% (with an average of −0.4%).

It is important to note that the value set for V_w strongly influences RPDs of V_{x_1} with respect to $V_{x'}$ and if spiked blank filter experiments are not conducted (with V_w set to 0), an independent validation of V_{x_1} is not possible. For example, if a suggested published value for V_w (0.2 mL, Bidigare et al. 2003) was used with these samples, average $V_{x'}$ for these samples would have been 2.044 mL and the average RPD of V_{x_1} with respect to $V_{x'}$ would be −8% (with a range of −5 to −9%). RPDs of this magnitude are cause for concern, but because independent estimates of V_w in these samples were conducted, the value suggested by Bidigare in these instances is known to be too high.

4.4 Discussion

The primary intent of this section is to provide details of how the HPL method changed after SeaHARRE-3 to address problems identified during that activity. Other topics pertinent to understanding uncertainty sources are also presented to encourage analysts to think about such things and agree collectively on procedures that will benefit the marine pigment community at large. HPL is not promoting any one HPLC method, but does promote the concept that quality assurance, method validation, and continuously implemented quality control measurements are the most important factors influencing accuracy and precision with field samples. The topics presented here only touch upon what is possible. It is anticipated that further changes will be made to the HPL method as a result of exposure to the productive practices of other laboratories and to a wider diversity of sample types.

4.4.1 Extraction Procedures

This section is intended to provide details concerning the uncertainties in HPL extraction procedures (pertaining to different volumes of V_m), to provide sufficient detail of the current procedures so another analyst could implement them, to explain an error in calculations pertaining to the calibration of the dispensing device for the variable V_m, and to illustrate the utility of using $V_{x'}$ (42) to identify potential problems with the determination of V_{x_1} according to (34).

4.4.1.1 Variations in V_m Sample Results

An evaluation of $V_m = 2$ mL (100% acetone), with respect to $V_m = 3$ mL (95% acetone), was made prior to SeaHARRE-3 with 15 duplicate pairs of filters collected from the Chesapeake Bay. For this analysis, a filter in each duplicate pair was extracted with $V_m = 2$ mL (100% acetone) and the other duplicate filter was extracted with $V_m = 3$ mL (95% acetone). The established understanding of duplicate filter precision from this sampling location (and field technician) was approximately 4% (on average) for PPig.

During the analysis of extraction procedures, the average APD and RPD (of $V_m = 2$ mL versus $V_m = 3$ mL) between duplicate filters was 5.6 and 2.8%, respectively for PPig and 3.5 and 0.7%, respectively for TChl *a*. The poorest results were for Chlide *a*, for which the average RPD was 6.8%, but the average APD was 52%. Poor precision was also experienced with Chlide *a* in SeaHARRE-3. The CV with triplicate filters was 30%, which is exceptionally poor when considered in context of the overall PPig precision of 4.5% and the 8.2% precision achieved for Chlide *a* in SeaHARRE-2.

4.4.1.2 Current HPL Extraction Procedures

The extraction procedures currently used by HPL were developed to implement good laboratory practices with regard to reduction of uncertainty while keeping in mind a need for high sample throughput. By using the dispensing device with 100% acetone (and adding water in a separate step) the dispensing device can be calibrated daily with the solution of 100% acetone plus internal standard.

(Gravimetric calibration cannot be performed with aqueous organic solvent solutions, because the specific gravity of these solutions would be unknown.) The following procedures produce extracts with an acetone content of 90% or more, which is an important factor for extraction efficiency, as well as the stability of the pigments in solution. This does not imply, however, that this is the best set of procedures possible.

The procedural steps for extracting 25 mm GF/F filters is as follows:

1. Minimize evaporation of the extraction solvent, as well as any exposure to light, during all steps.
2. Label 5 mL polypropylene tubes with the needed sample numbers.
3. Remove the storage bottle containing the solution of extraction solvent plus internal standard from a −15°C freezer, and bring this solution to room temperature before attaching the calibrated bottle-top dispenser designed for organic solvents (Dispensette Organic 0.5–5 mL).
4. Add 100 µL of deionized water (at room temperature), using a calibrated autopipette that measures no more than 1 mL, to each tube. If it is known that the filter was blotted dry, add 200 µL of water. Place each tube in a vial rack in a cooler. The cooler should contain an ice and water bath.
5. Add 2.5 mL of the 100% acetone plus internal standard solution to each tube using the bottle-top dispenser. Immediately cover the tube with Parafilm.
6. Place the cooler and tubes in a −25°C freezer for at least 30 min.
7. Remove the cooler and tubes from the freezer. For each tube, unwrap the Parafilm, add a 25 mm GF/F sample filter (after recording the identification information on an extraction summary sheet), and immediately rewrap with Parafilm.
8. Repeat steps 1–7 until all filters to be extracted for the day are in tubes (up to 33 filters can be extracted and analyzed in one day with a method requiring 38 min for the HPLC part of the analysis).
9. Place the cooler and samples back in a −25°C freezer for at least 1 h.
10. Macerate each filter using an ultrasonic probe. With the digital programmable sonicator now in use, the sonicator is set to pulse for a duration of 8 s (with pulsing on for 0.7 s and off for 0.2 s), for a total of 12 pulses. The amplitude is set to 25%. Prevent cavitation by pausing during sonication to push the filter slurry back down to the bottom of the tube if necessary. Partially submerge the tube in an ice and water bath during sonication to minimize heat accumulation. The condition of the sonicator tip should be periodically monitored and polished to yield a smooth surface, using crocus paper of very fine sandpaper capable of eliminating the fine pitting that can develop on the bottom of the sonic probe tip.
11. After processing a tube, cover it with a fresh piece of Parafilm and return it to the darkened ice bath (in the cooler).
12. Rinse the sonicator tip with 100% acetone and wipe dry between each sample.
13. After all the samples have been processed, empty the cooler of ice and water. Place the samples back in the cooler and return the cooler to a −25°C freezer for 3–6 h. Leave the top of the cooler open, but protect the samples from the light by covering them with a black towel.
14. Remove the cooler containing the samples from the freezer. Filter each sample slurry through a 0.45 µm pore size PTFE syringe cartridge filter attached to a disposable plastic syringe. Collect the pigment extract in a glass scintillation vial with a cork-backed, foil-lined, labeled cap and immediately place the vial in a darkened environment, such as a drawer or under a black towel.
15. After all samples have been processed, vortex each sample for about 2 s and transfer approximately 500 µL of extract to an amber, labeled, HPLC vial. Apply an HPLC cap with a silicone/PTFE septum to the vial, and place it in a prechilled, darkened TCAS compartment.

To modify the procedure for 47 mm filters, use a 12–15 mL thick-walled glass centrifuge tube instead of a polypropylene tube, and add 5 mL of 100% acetone instead of 2.5 mL in step 5. Do not add any water in step 4.

HPLC analysis should be started as soon as the TCAS compartment has been filled with the samples plus the other needed vials to complete the HPLC analysis procedures. Store all the unused extract in a −15°C freezer until the HPLC analyses are successfully completed and the data are reprocessed.

4.4.1.3 Gravimetric Calibration of V_m

Prior to April 2006, the device used to deliver V_m was gravimetrically calibrated approximately every month while in use. This calibration was always performed with 100% acetone, even when 95% acetone was used in the solution of extraction solvent plus internal standard. This procedure involved removing the Dispensette from the bottle containing extraction solvent with internal standard (in 95% acetone) and reattaching it to a bottle containing 100% acetone. After calibration at the setpoint volume, the Dispensette was transferred back to the bottle containing the solution of extraction solvent plus internal standard.

Starting in April 2006, the Dispensette is calibrated every day it is used. To facilitate this process, the setpoint volume is calibrated with the Dispensette attached directly to the bottle containing the solution of extraction solvent plus internal standard, now in 100% acetone. (Note, the solution of extraction solvent plus internal standard is allowed to equilibrate to room temperature before it is used. In addition, HPL has determined that the Vitamin E internal standard solution is stable stored at room temperature in the dark.)

The formulation for the gravimetric calibration is

$$V_m = \frac{W_{fv} - W_{ev} - V_{sp}\left[\text{Vit E}\right]}{sg} \qquad (50)$$

where $\left[\text{Vit E}\right]$ is the concentration of vitamin E (in grams per milliliter) in 100% acetone, W_{ev} is the weight (in grams) of an empty vial and cap, W_{fv} is the weight (in grams) of a vial (with cap) after it is filled with the setpoint volume of extraction solvent plus internal standard, sg is the specific gravity of 100% acetone (specified with the solvent batch, variable to within 0.7%), and V_{sp} is the Dispensette volume (in milliliters). Typically, 2–7 vials are used for this calibration process. The Dispensette is periodically calibrated with water to determine if it meets the manufacturer's specifications for calibration with water. For calibration with water, there is no need for the V_{sp} and $\left[\text{Vit E}\right]$ terms, and the sg of water is used in the denominator.

On 17 days between 29 November 2006 and 26 January 2007, the Dispensette was calibrated according to (50) with a setpoint volume of 2.50 mL. The average daily CV in percent among the number of vials, N_v (2 or 3), was 0.30%). The average calibrated V_m across all 17 days was 2.548±0.0134 mL using the extraction solvent as 100% acetone plus vitamin E. On 25 January 2007, the calibrated volume with water was 2.545±0.0093 mL ($N_v = 5$). The 95% and 99% confidence limits for the expected calibrated V_m can now be defined, and are expressed here as a warning limit and control limit, respectively. The expected V_m (for a setpoint volume of 2.50 mL) should, therefore, be within ±1.1% (the warning limit) and ±1.6% (the control limit) of the average calibrated V_m (2.548 mL).

In practice, the daily calibrated V_m (and not the setpoint volume or average calibrated V_m) is used in determining V_{x_1} and $V_{x'}$. For data reported between 19 April 2006 and 23 January 2007, $\left[\text{Vit E}\right]$ was inadvertently used in (48) with units of milligrams per milliliter and not grams per milliliter when calculating calibrated V_m. This blunder overcorrected the contribution of vitamin E to the total weight of acetone, and reduced V_m by a constant bias of 2.5% for dates 19 April 2006 to 15 November 2006, and a constant bias of 2.3% for dates 28 November 2006 to 23 January 2007.

4.4.1.4 V_{x_1} vs. $V_{x'}$ With Various Filters

During the analysis of about 1,200 HPLC (25 mm GF/F) filter samples from various investigators, V_w was determined according to (43) for each sample set, from which an average V_w ($\bar{V}_w$) for each investigator was determined. Using the unique $\bar{V}_w$ for each investigator in place of V_w in (42) gives a unique estimate of the extraction volume according to $V_{x'}$ per sample set. The APD of the extraction volume can be determined by comparing V_{x_1} with respect to $V_{x'}$. The results of this comparison are shown in Table 38. For the first investigator (Mannino), separate values of $\bar{V}_w$ were determined for three distinctly different sample sets.

Table 38. The average values for V_w determined using (43) for various samples from multiple investigators. The dispersion in V_w per investigator (CV) and the overall average APD of V_{x_1} with respect to $V_{x'}$ are given in units of percent. The standard deviation of V_w is denoted as σ_w. The three different Mannino data sets are distinguished by superscripts 1, 2, and 3.

Sample	n	$\bar{V}_w \pm \sigma_w$	CV	APD
Blotted†	51	0.03 ± 0.030	107	1.2
Unblotted†	25	0.09 ± 0.046	202	1.9
Mannino[1]	122	0.17 ± 0.039	23	1.1
Mannino[2]	72	0.14 ± 0.033	23	0.8
Mannino[3]	175	0.14 ± 0.035	26	0.9
Siegel	200	0.15 ± 0.053	36	1.2
Mitchell	364	0.23 ± 0.039	17	1.2
Nelson	43	0.16 ± 0.022	14	0.8
Subramaniam	197	0.19 ± 0.059	31	2.0

† Samples from SeaHARRE-3.

4.4.2 Injection Procedures

After SeaHARRE-3, the HPL injector program (Table 28) was changed back to the one that was being used during SeaHARRE-2, as described here for an Agilent 1100 injector equipped with a 900 µL metering device. The metering device speed given in units of microliters per minute is the last entry in parentheses:

1. `DRAW` 150.0 µL of buffer from vial number 91 (500).
2. `DRAW` 75.0 µL of sample extract (130).
3. `DRAW` 0.0 µL of acetone from vial number 1 (90)
4. `DRAW` 75.0 µL of buffer vial number 91 (130).
5. `DRAW` 75.0 µL of sample extract (130).
6. `DRAW` 0.0 µL of acetone from vial number 1 (90).
7. `DRAW` 150.0 µL of buffer from vial number 91 (500).
8. `INJECT` loop contents onto the column (250).

On occasion, chromatograms with split peaks are observed for the early-eluting Chl *c* pigments. This is likely to happen when the solvent strength of the sample extract (or

standard) is too high. For example, Chl *c* standards cannot be successfully injected in 100% acetone without severe peak-shape distortion. Chl *c* pigments isolated by HPL and used as standards used to be suspended in 100% acetone, for which corresponding absorption coefficients were used. Subsequently, HPL has changed to absorption coefficients that specify 90% acetone, and peak shape distortion with these standards has been avoided.

The phenomenon of peak splitting can also occur with sample extracts, as illustrated with replicate injections of the same sample extract in Fig. 6. If the distortion is severe enough, the sample is reinjected; otherwise, the split peaks are summed together after confirming their identities with in-line absorbance spectra.

4.4.3 Buffer Precipitate

The HPL method uses deionized water, which is prepared at HPL with a variety of water purification systems capable of producing HPLC grade or equivalent. Recently, the TbAA buffer developed a precipitate within a few days of its formulation, which did not appear to affect the chromatographic results, as long as solvent A was properly filtered (Sect. 4.2.3.2). To investigate the cause of this precipitate, a TbAA solution was formulated using HPLC-grade water (separately purchased), and this solution did not develop a precipitate. Subsequent to this investigation, a failure in the water purification system was identified.

In addition, a fungal growth occasionally appeared in the aqueous TbAA solution, but this problem has been prevented by autoclaving (for 30 min) the high purity water produced by the HPL deionization system. With this step, 4 L of 28 mM TbAA, pH 6.5 can be made in one procedure and stored with no problems in brown 4 L bottles placed in windowless cabinets for as long as two months.

4.4.4 Carryover and Gradient Changes

Immediately prior to SeaHARRE-3, carryover of a magnitude sufficient to interfere with accurate quantitation was observed. Such carryover was evident when an injection of acetone followed the injection of concentrated algal extracts or concentrated individual standards. With Agilent technical support, the injector rotor seal and all injector parts exposed to sample solutions during injection were replaced. This approach reduced, but did not eliminate, carryover.

To rule out the injector as a cause of carryover, a concentrated extract was analyzed, after which all tubing to the injector was disconnected, the column was reconnected directly to the pump, and a blank run (with no injection) was initiated. The blank run also exhibited carryover, which indicated the carryover was coming from a source other than the injector. Changing to a new column reduced carryover to a degree permitting accurate quantitation, so the magnitude of carryover was, in this instance, concluded to be a function of column age.

In the days following completion of the SeaHARRE-3 analyses, substantial carryover from the DHI Mix-101 was noted (Table 35) on a relatively new column. Because the carryover was greatest with highly retained, nonpolar pigments (Chl *a* and Caro) and never occurred with more polar, early-eluting pigments, and because the carryover was not eliminated by changing to a second new column, carryover was suspected to be related to incomplete pigment recovery from the column stationary phase and that a remedy might include adding a strong solvent after elution of Caro and before the next sample was injected.

Modifications to the gradient included the introduction of acetone in line numbers 4, 5, and 6 (Table 39). With this change, carryover became nonexistent or less than 0.1%. Once again, however, after thousands of successful analyses using this pump table, another issue with carryover occurred, but in this case, carryover was severe with polar pigments and nearly nonexistent with nonpolar pigments. After much troubleshooting, this carryover was ultimately eliminated by changing much of the stainless steel tubing to Peek tubing.

Table 39. The pump gradient developed after SeaHARRE-3 to eliminate carryover. Solvents A and B are the same as in Table 8; Solvent C is acetone.

Line No.	*Time* [min]	*% Solvent* A	B	C	*Flow* [mL min^{-1}]
1	Start run 0.00	95	5	0	1.1
2	22.00	5	95	0	1.1
3	24.50	5	95	0	1.1
4	24.75	5	65	30	1.3
5	25.75	5	65	30	1.3
6	25.85	5	65	30	1.1
7	26.10	95	5	0	1.1
8	29.10	95	5	0	1.1
9	Start inject cycle (duration 8.9 min)	95	5	0	1.1

These numerous problems exemplify the need for strict carryover monitoring, which HPL performs multiple times with every sequence of analyses and for which expectations are that carryover should be nonexistent, or at least no greater than 0.1%.

Why carryover became a problem for HPL after so many years of success with using the initial gradient, as published in Van Heukelem and Thomas (2001), is not known. There is a possibility that the stainless steel tubing was damaged during the use of very acidic, aqueous, organic solvent mobile phases used with algal toxin analyses in the 2005 time period. It is possible the acidic organic solvents might have altered the tubing adsorption characteristics. The Hewlett Packard HPLC manual states that such solvents can attack stainless steel tubing (Hewlett Packard Reference Manual 1996), so this remains the most likely explanation.

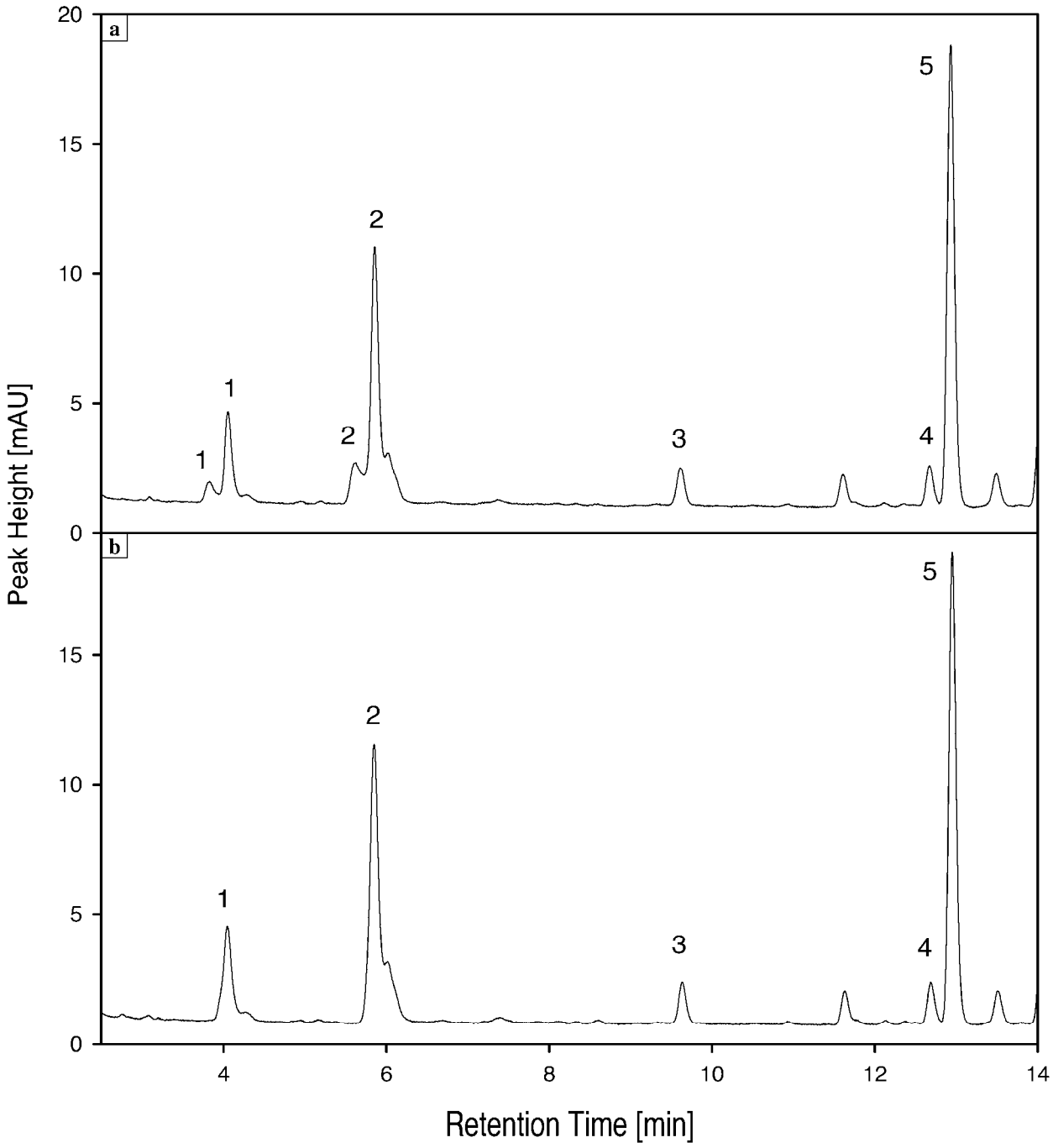

Fig. 6. Chromatograms of early- to mid-eluting pigments from the same extract injected twice on the same day exemplify the occasional phenomenon of peak-splitting for Chl c_3 (peak 1) and Chl c_2 (peak 2) in a) and normal peak shapes in b). Perid (peak 3), But-fuco (peak 4) and Fuco (peak 5) exhibit normal peak shapes in both a) and b).

4.4.5 Solvent Gradient Effects

The HPL method was developed taking into account advice from Lloyd Snyder (pers. comm.) who suggested the *linear gradients (rather than segmented gradients) and gradients that proceed from 5% to 95% solvent B (rather than 0 to 100% solvent B) are more reproducible*. Consequently, the HPL gradient detailed in Table 39, was developed to include a linear gradient from 5–95% solvent B in 22 min (lines 1 and 2) with an isocratic hold for 2.5 min on 95% solvent B (lines 2–3) to allow for elution of pigments (Caro and Phytin *a*) that do not elute during the gradient. This solvent gradient was also developed to minimize run time and maximize the number of analyses that could be performed in one day. Solvent C, acetone, is introduced and used in lines 4, 5, and 6 exclusively for flushing the column to reduce the possibility of cross contamination between analyses. During lines 7, 8, and 9 (a duration of 11.9 min and a total volume of 13.1 mL), the solvent flowing through the column is at "initial conditions," or 95% Solvent A. Thus, the column is thoroughly re-equilibrated before the next sample is loaded onto the column.

In addition to enhanced reproducibility, simple linear gradients (and constant flow rates) during elution of pigments contribute to baseline stability, which is important for accurate quantitation of pigments with low SNRs and for accurate integration of peak areas. To evaluate baseline stability in the context of gradient changes, however, the instrument dwell volume (the volume of solvent that passes through the column before a change in gradient formation at the pump reaches the head of the column) must be considered. Figure 7 shows the interrelationships between baseline stability, gradient changes, and dwell volume. In Fig. 7a, the chromatogram is plotted up to 27.6 min (the typical HPL method stop time for data acquisition) and in Fig. 7b, the time axis is shown from 20–38 min to illustrate the deleterious effects of rapid changes in flow rate and extreme changes in solvent composition on baseline stability.

For further discussions pertaining to these figures, dwell volume is expressed as dwell time: dwell volume (approximately 3 mL) divided by the 1.1 mL min^{-1} flow rate, or about 2.7 min. The percentage of solvent in Fig. 7 is shown at the head of the column (not at the pump) as a function of time, where time is determined as the time the gradient is formulated at the pump, plus the dwell time. For example, the end of the linear gradient at 22 min does not reach the column until about 2.7 min later (24.7 min), at which time the isocratic hold on 95% solvent B begins. At 24.7 min, this transition in solvent composition affects the signal at 450 nm, as expressed by the hump-shaped disturbance between Chl *a* and Caro (Fig. 7a). In addition, the gradient change at 24.7 min causes a pressure change. Acetone (solvent C) is particularly damaging to baseline stability, with its low backpressure (relative to methanol, and aqueous-and-methanol mixtures) and its strong absorbance at wavelengths near the visible wavelengths (less than 330 nm). Solvent C is introduced at line 4 (Table 39) and contributes to the large changes in baseline beginning at about 27.4 min in Fig. 7b.

The gradient detailed in Table 39 was developed with an emphasis on shortened run times and baseline stability during data acquisition, but the close timing of Caro elution (at 26.83 min in this illustration) with gradient changes and detector stop time puts the HPL method at risk of *missing* the Caro peak if it happened to elute even one minute later than is typical. In fact, there is some column-to-column variation with respect to Caro retention, especially with new columns, which are more retentive in general than well-used ones. In such an event, the gradient in Table 39 would need to be modified by adding time to the isocratic hold on 95% solvent B, with subsequent necessary adjustments to lines 4–8 as well. Detector stop time would also need to be extended.

4.4.6 Stability of Separation Selectivity

Column-to-column reproducibility is evaluated by consistency with retention times, separation selectivity, and the R_s between critical pairs (Viola and Hex-fuco plus Zea and Lut are used as critical pairs to monitor the limiting resolution at HPL). Column reproducibility is generally very good, although two new columns in the past nine years (approximately 5% of all columns) exhibited poor R_s (near 1.0) of critical pairs and needed to be exchanged. New columns are more retentive, but this changes with usage. For example, recently a column that was used for about 1,300 analyses exhibited a decrease in Caro and Chl c_2 retention times of approximately 0.37 and 0.57 min, respectively, when first and last injections were compared.

Separation selectivity differences have been observed between columns for Chl c_2, MgDVP, and Chl c_1, as shown in Fig. 8. In the top panel, MgDVP (peak 4) is partially resolved from Chl c_2, which causes it to interfere with Chl c_1 and Chlide *a* (peaks 5 and 6, respectively). Also in the top panel, peak 17 (4k-Hex-fuco) interferes with Neo. Separation selectivity of the column in the bottom panel exhibits less interference by MgDVP and 4k-hex-fuco. It was documented in Van Heukelem and Thomas (2001) that MgDVP interferes with Chl c_2, but the magnitude of MgDVP interference with Chl c_1 quantitation was only recently observed, because the diversity and numbers of samples coming to HPL for analysis has increased. MgDVP is most problematic when prochlorophytes (as indicated by DVChl *a*) are present.

Changes in Chl *c* selectivity can also occur with increased column usage, as seen in Fig. 9 showing chromatograms of replicate injections on the same column separated by approximately 200 injections. These injections were also on different batches of solvent A. The selectivity seen in Fig. 9b, however, was persistent with subsequent batches of solvent A and TbAA buffer.

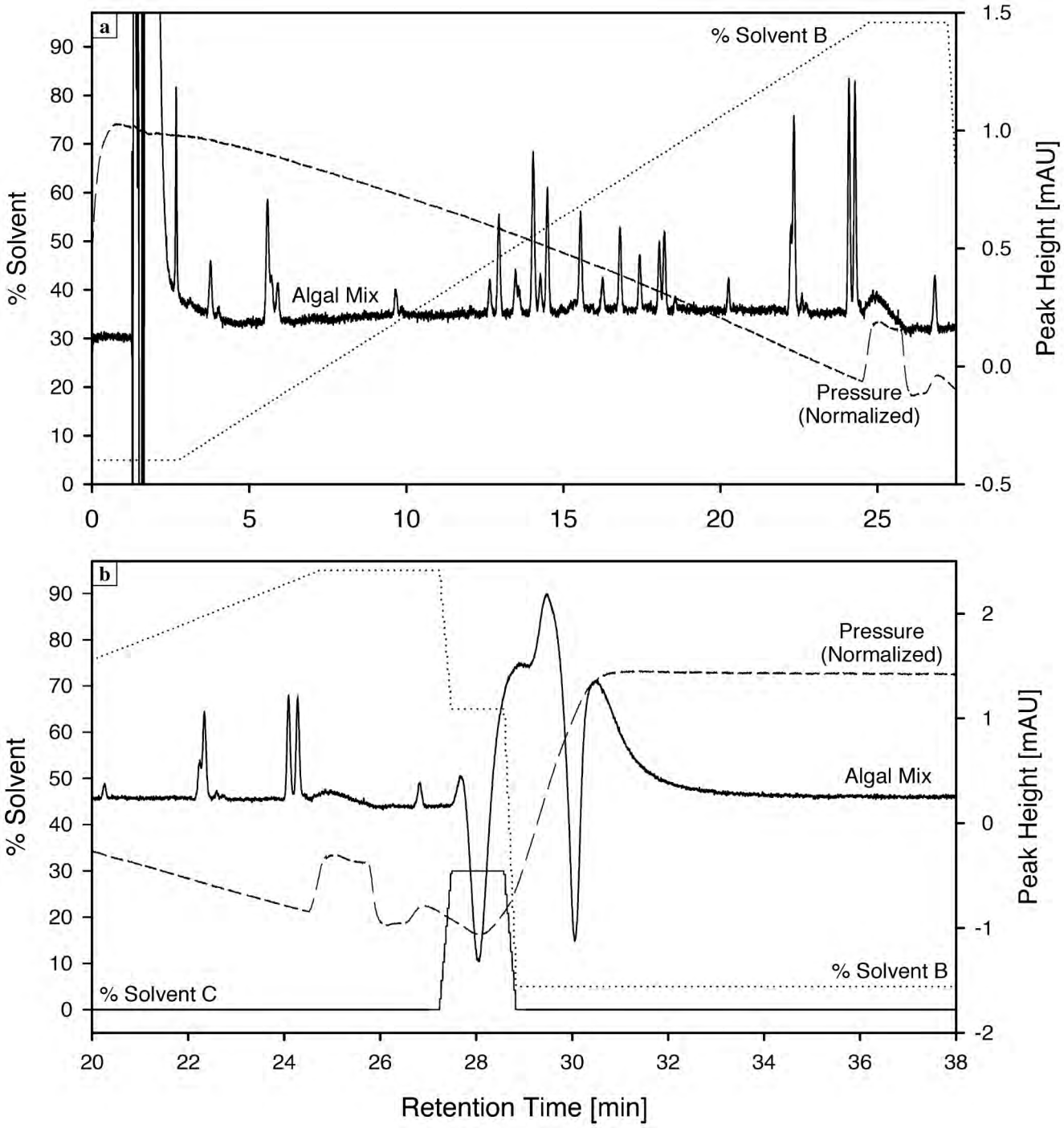

Fig. 7. Chromatograms from a weakly concentrated sample (1 mAU full-scale at 450 nm) shown in the context of gradient changes (primary y-axis) and pressure changes (dashed line) as a function of time. Two baseline stability cases are depicted: **a)** the baseline is relatively stable during pigment elution up to 27 min, and **b)** the baseline is unstable after 27 min. The latter condition is caused by rapid changes in the percentages of solvents B and C, which produce large pressure changes and deflections in the baseline. The pressure is normalized to fit on the y-axis and is shown without units.

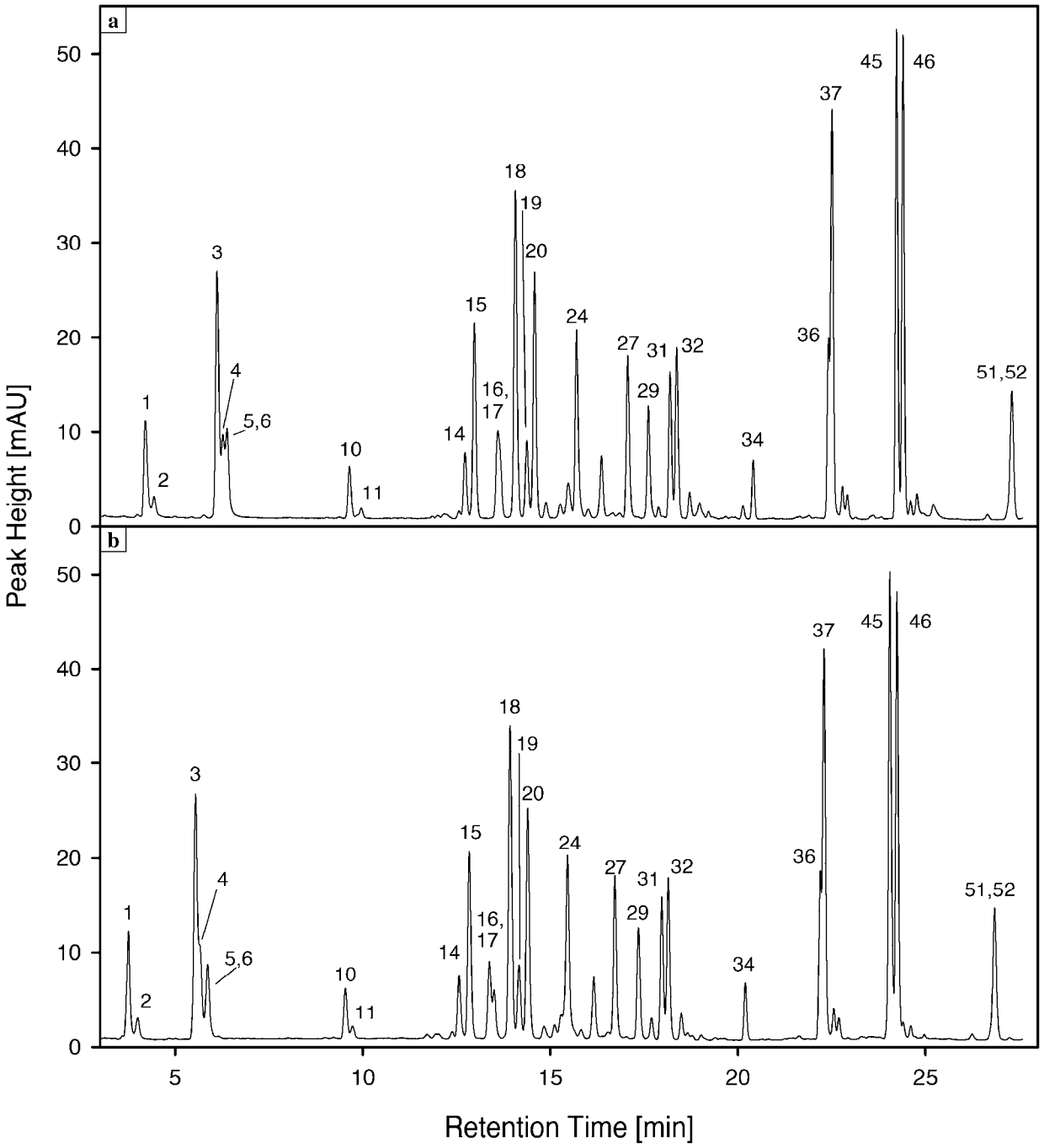

Fig. 8. Chromatograms depicting differences in separation selectivity for peaks 3–6 and 16–17 were observed from the same sample injected on two different Agilent, XDB C_8 HPLC columns: **a)** a newly installed column (serial number 4200), and **b)** a column that had been used for approximately 900 injections (S/N 4048). The peak numbers refer to pigments listed in Table 31: 1 is Chl c_3, 2 is MVChl c_3, 3 is Chl c_2, 4 is MgDVP, 5 is Chl c_1, 6 is Chlide *a*, 10 is Perid, 11 is Perid isomer, 14 is But, 15 is Fuco, 16 is Neo, 17 is 4k-hex-fuco, 18 is Pras, 19 is Viola, 20 is Hex, 24 is Diad, 27 is Allo, 29 is Diato, 31 is Zea, 32 is Lut, 34 is Gyroxanthin diester, 36 is DVChl *b*, 37 is Chl *b*, 45 is DVChl *a*, 46 is Chl *a*, 51 is $\beta\varepsilon$-Car, and 52 is $\beta\beta$-Car.

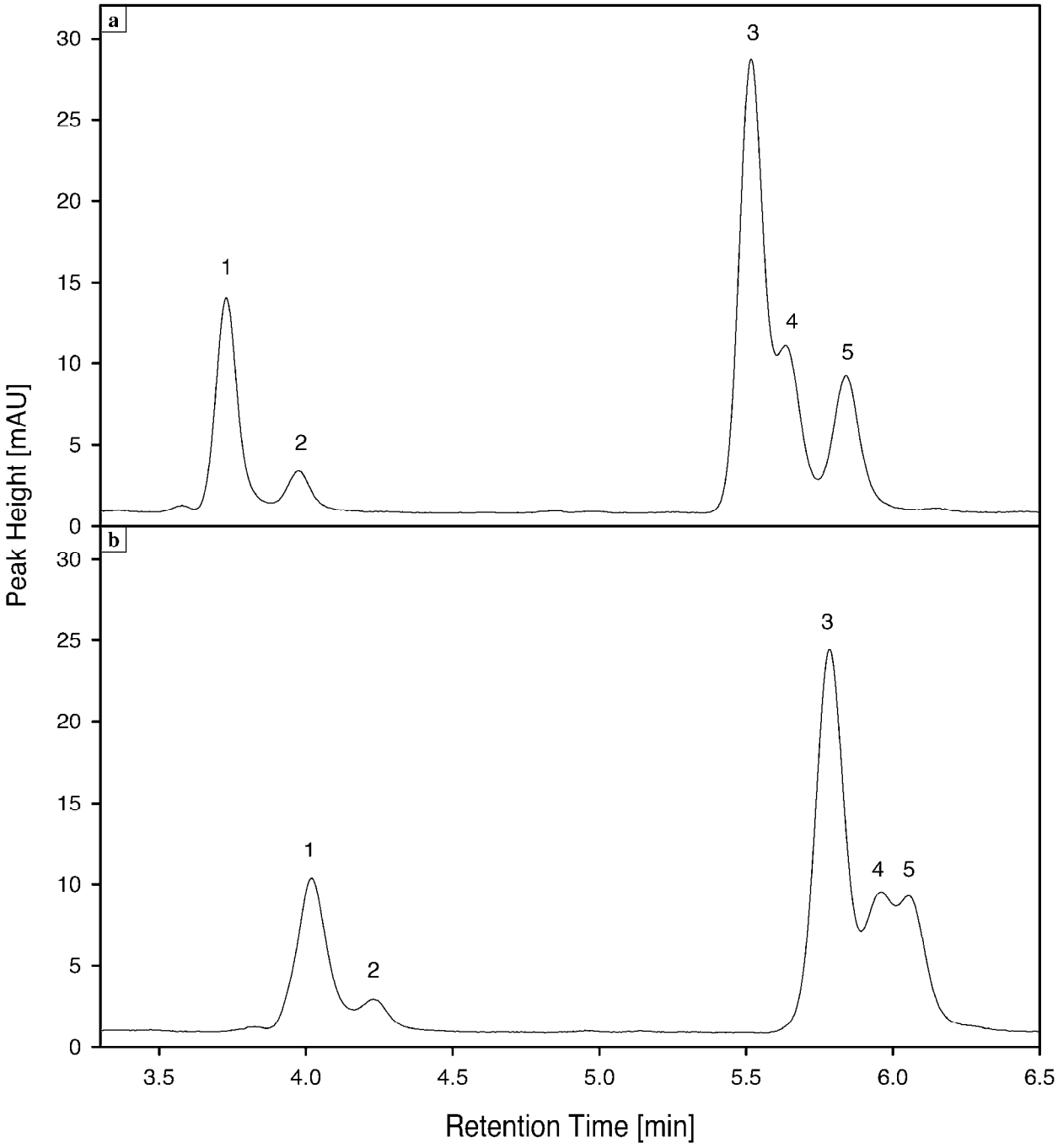

Fig. 9. The change in separation selectivity of Chl c_2 and Chl c_1 relative to MgDVP. The top and bottom panels are replicate injections of the same mixed algal culture on the same column (serial number 4048). Injections were performed two days apart (which represents approximately 200 injections) and on different batches of solvent A. Peak identifications are as follows: 1 is Chl c_3, 2 is MVChl c_3, 3 is Chl c_2, 4 is MgDVP, and 5 is Chl c_1.

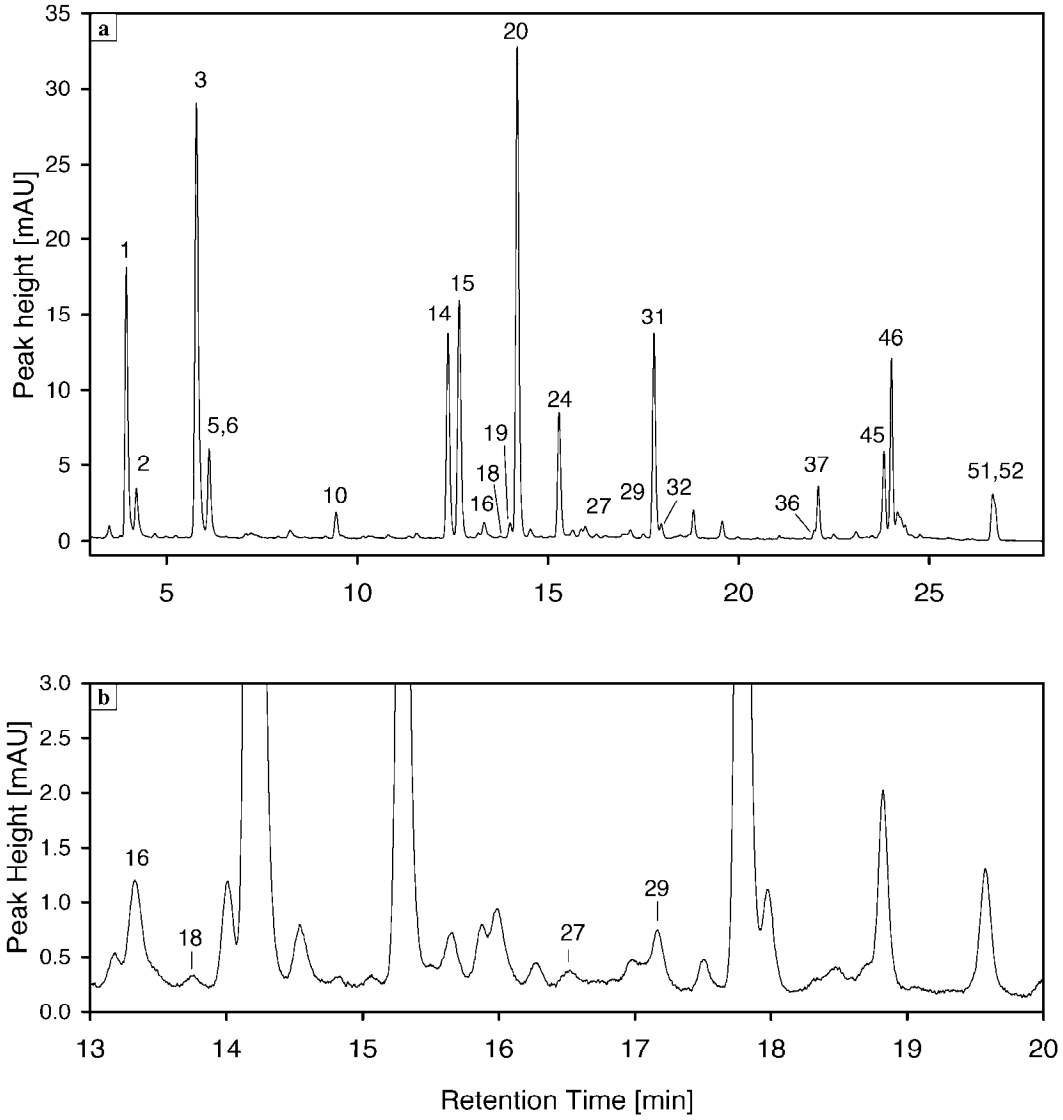

Fig. 10. A sample chromatogram illustrating problems with pigments in low concentrations: **a)** full retention time presentation, and **b)** a magnification of the pigments eluting between 13–20 min. Inconsistency among laboratories regarding the quantitation of such peaks elevates uncertainties and degrades accuracy capabilities. The peak number follows those presented in Table 31; the pigments discussed in the text are as follows: 18 is Pras, 27 is Allo, and 29 is Diato.

4.4.7 Pigment Identification & Reporting

Pigment identification can be based exclusively on retention times or on the combination of retention time and absorbance spectra. Including the latter criterion is not straightforward, because the SNR at which adequate spectra can be obtained for pigment confirmation varies among pigments and these SNRs are much greater than the peak detection threshold (e.g., a SNR of 3). Interpretations of peaks with SNRs between the detection threshold and that required for high quality absorbance spectra, is fraught with interpretation difficulties. The ramifications are very damaging to interlaboratory accuracy, especially because laboratories have not reached a consensus of agreement with respect to what should be used as a rejection threshold: a) the peak detection threshold (in which case absorbance spectra are not used for peak confirmation), or b) the SNR for which proof of a pigment's identity can be attained. Inconsistency and subjectivity in decision making, and the very fact that detection limits among methods vary, all contribute to elevated uncertainties when laboratories results are intercompared.

These problems are discussed in the context of the SeaHARRE-3 sample 1 analyzed at HPL and displayed in Fig. 10, where Fig. 10a depicts the full chromatogram and Fig. 10b, an expanded portion featuring the small peaks at Pras, Allo, and Diato retention times. These peaks with SNRs of 7, 9, and 27, respectively, are subject to

interpretation difficulties. In contrast, But, Fuco, Hex, Diad, and Zea all exhibit SNRs greater than 413, so absorbance spectra would be of high quality, identifications unequivocal, and interpretation differences among laboratories unexpected. This chromatogram was produced from a mesotrophic site, from which 2,800 mL of seawater were filtered. The filter was extracted in 1.9 mL, and 0.150 mL of extract was injected. TChl *a* was quantified as 0.263 mg m^{-3}; however, the SNRs in this chromatogram would be the same if this had been a eutrophic site with a tenfold increase in concentration and filtration volume had been reduced tenfold, to 280 mL. The problems with low SNRs and chromatogram interpretations are not exclusive to trophic regime.

With respect to the sample depicted in Fig. 10, Allo was not reported by any A′ laboratory, Pras was reported only by Laboratory *H* (at 0.0006 mg m^{-3}), and Diato only by Laboratory *D* (at 0.0009 mg m^{-3}). In SeaHARRE-3, if a laboratory reported a pigment as not found (e.g., concentration = 0), or at a concentration < 0.0005 mg m^{-3}, a value of 0.0005 mg m^{-3}(the null value) was assigned as that laboratory's value before APDs were determined. Thus, the null value was used for Allo in sample 1 for all A′ laboratories, and the resulting APD for Allo was 0%. Because the concentration reported by *H* for Pras was so similar to the null value (assigned to *C*, *D*, and *L*), the A′ APD for Pras at this site was 5% and was 10% for *H*.

Inspection of Fig. 10 exemplifies how the peak at the Pras retention time could be interpreted differently among analysts, especially because it is among other rather abundant peaks, and the baseline is frequented with small peaks between the peaks of interest. In all likelihood, a peak with a SNR of 7 in a weakly concentrated chromatogram would not be rejected. With Diato in this sample, the APD of *D* was 47 and the A′ APD was 24%. With sample 1, the APD is higher for Diato than Pras simply because the difference between the concentration reported for Diato and the null value is greater than it is for Pras. In fact, if this chromatogram had been from a eutrophic site, as discussed in the previous paragraph, Pras and Diato SNRs would have been the same, but their APDs for *H* and *D* would have been 216 and 246%, respectively. So, the problem is, the more subject a peak is to differences in interpretation among laboratories (which is largely a function of SNR), and the greater the difference between the observed concentration and the null value, the greater the APD. In the context of the round robin, the damaging effects of these small peaks on method accuracy is disproportionate to their importance in the chromatogram. In fact, the concentrations of Pras and Diato are each less than 0.3% of the TChl *a*.

Adequate peak spectra were unattainable by the *H* method for Pras, Allo, and Diato peaks in the sample extract shown in Fig. 10. To illustrate the concept of *inadequate* absorbance spectra and how poor quality absorbance spectra lead to subjective decisions regarding pigment identification and reporting, the spectrum collected from peak 29 in Fig. 10 is overlaid with a Diato standard in Fig. 11. If peak identification procedures mandated acquisition of adequate absorbance spectra as a prerequisite for quantitation, Pras, Diato, and Allo from the sample shown in Fig. 10 would have been rejected, and all other peaks would have been quantified. However, if the "two sentence rule" had been in effect, *H* would have reported Pras and Allo, and rejected Diato based on peak shape distortion. For such interlaboratory agreement to occur on a regular basis, however, all would have to agree on measurable terms for defining when absorbance spectra are sufficiently adequate to confirm peak identity. This level of agreement among laboratories is not likely to occur in the absence of a clear identification and reporting procedure, such as the *two-sentence rule*. HPL encourages a dialogue with other laboratories regarding such issues.

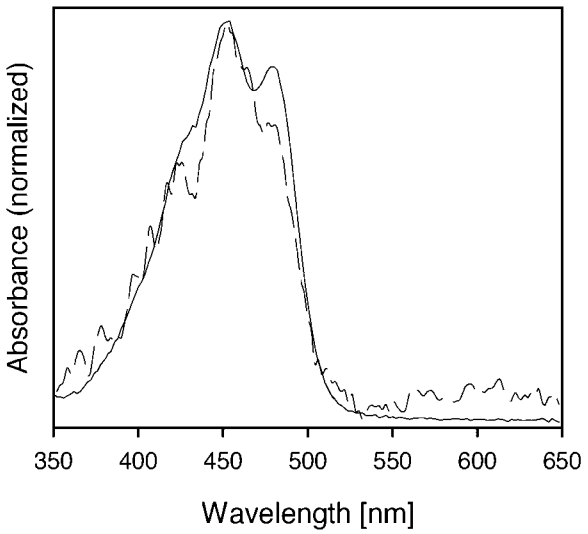

Fig. 11. An example of the effects of poor SNR on HPLC in-line absorbance spectra. HPLC in-line absorbance spectra of a Diato standard (solid line) overlaid with spectra of a peak (with poor SNR) at the Diato retention time (dashed line) from a natural sample.

Pras is very likely to contribute false positive identifications with the *H* method if spectra are not used as part of the pigment identification process, because a peak at its retention time is often present, but spectra rarely match that of the Pras standard. A field sample chromatogram (not from SeaHARRE-3) illustrates this difficulty (Fig. 12), where the absorbance spectrum of the peak at the Pras retention time is slightly shifted relative to the Pras standard (Fig. 12 inset).

To investigate whether this spectral shift could be caused simply by an inability to interpret spectra with low SNRs, several dilutions of an algal mixture, including extracts from *Pycnococcus provasolii* known to contain Pras, were prepared and analyzed. The chromatograms (Fig. 13) and corresponding Pras absorbance spectra (shown in

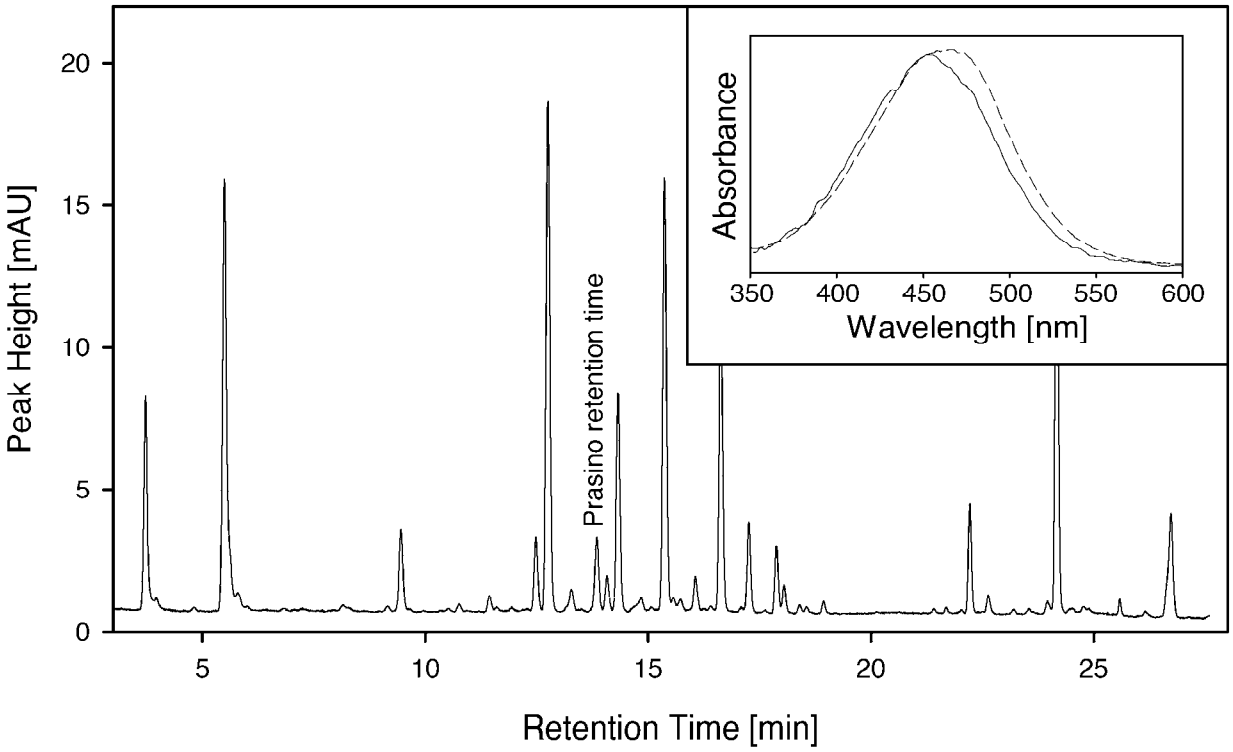

Fig. 12. A chromatogram of a natural sample with a dominant peak at the Pras retention time. HPLC in-line absorbance spectra reveal this peak is not Pras. The inset panel shows the normalized absorbance (dimensionless) spectra of the presumed Pras peak (solid line) overlaid with spectra of a Pras standard (dashed line).

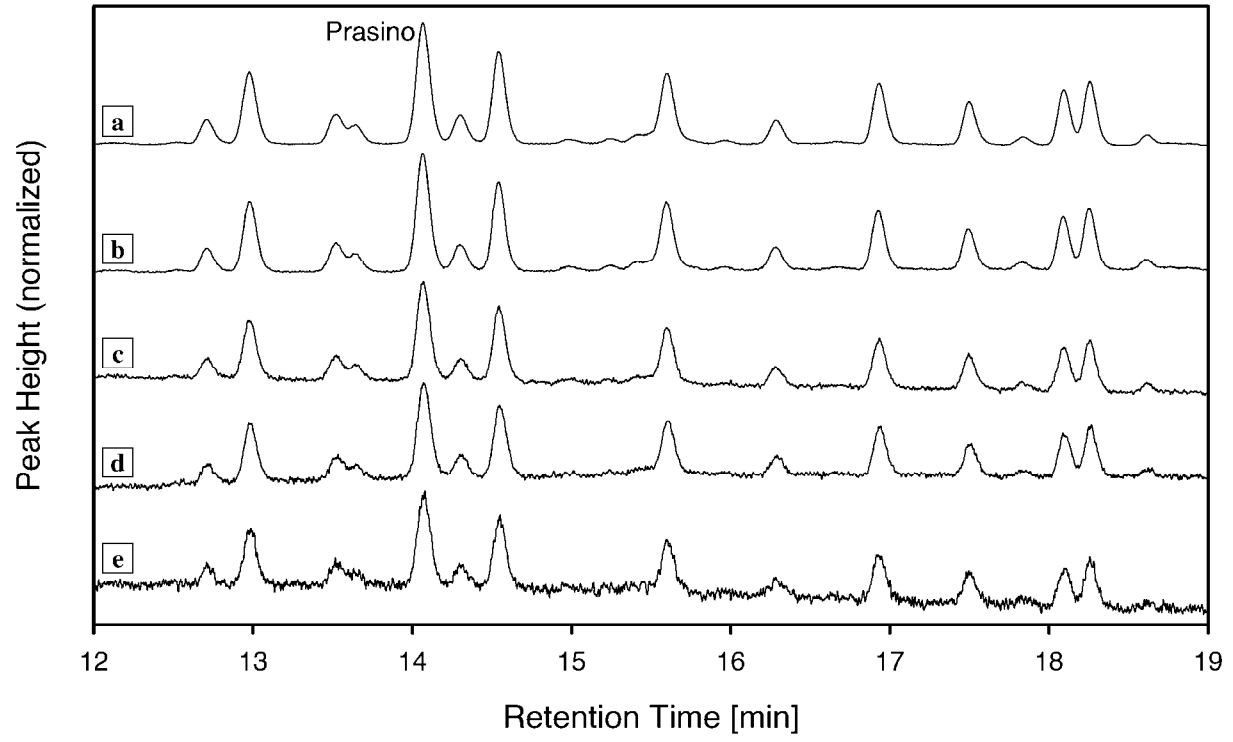

Fig. 13. Chromatograms from absorbance at 450 nm of increasingly dilute solutions of a pigment mixture depicting SNRs for Pras: **a)** 271, **b)** 154, **c)** 50, **d)** 34, and **e)** 17.

Figs. 14a–e) show that as the SNR ratio of Pras decreased from 271 to 17, the integrity of the absorbance spectra was compromised. However, the spectral shape of Pras in the diluted algal culture, even though noisy, was not shifted and was more similar to the Pras standard, as depicted in the spectral overlay in Fig. 15. The overlay in Fig. 15 is distinctly different from what is frequently observed in natural samples (as depicted in Fig. 12 inset). It is concluded the spectral shift seen with the field sample is not simply a function of low SNR.

Many of the chromatograms for the SeaHARRE-3 sample filters revealed substantial DVChl *b* peak heights in comparison to the Chl *b* peak height. Because of this atypical (for HPL) situation, TChl *b* was quantitated using two different techniques:

1. Summing the individual quantitations of DVChl *b* and Chl *b* by peak height (the so-called *summing* procedure and for which DVChl *b* and Chl *b* peak height response factors were used for the individual quantitations); and
2. Using the total peak area and a peak-area response factor determined from a Chl *b* standard (the usual procedure).

In regards to the last technique, HPL considers the summing procedure to be a more accurate means to quantify TChl *b* when DVChl *b* is present, however, it was a less conventional approach to TChl *b* quantitation during the time frame that laboratories analyzed SeaHARRE-3 samples.

4.4.8 Effects of TChl *b* Quantitation

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The two modes of quantitation were compared using linear regression statistics and percent differences. First, the RPD of the concentration of TChl *b* based on summing was computed relative to the usual TChl *b* quantitation. For these comparisons, samples likely to have high uncertainties (e.g., amounts near the LOQ of 0.36 ng) were not included in the statistics. All data (even those not included in the statistics) are presented graphically in Fig. 16 wherein the RPD is plotted as a function of the DVChl *b* to Chl *b* amount ratio for the sample. These results show the $\left[\text{TChl } b\right]$ values estimated by the summing technique produce higher concentrations than the usual $\left[\text{TChl } b\right]$ technique. Furthermore, this effect is exacerbated with increasing proportions of DVChl *b*.

Using the regression equation shown in Fig. 16, for an amount ratio observed in the DHI mix (0.36), the summing procedure produces values of 6.1% higher than the "usual" procedure. However, the DHI Mix A′ average APD was 4.13 and the *H* DHI Mix APD was 2.58, so in the SeaHARRE-3 Field sample data set, it is most likely that the bias observed at HPL for the summing procedure mode of quantitation is overshadowed by the variance in results among laboratories. Since SeaHARRE-3, HPL quantifies Chl *b* by peak area using a Chl *b* peak area response factor, but if DVChl *b* is present, it and Chl *b* are individually quantified by peak height using DVChl *b* and Chl *b* peak height response factors. TChl *b* is reported as the sum of Chl *b* and DVChl *b*, if present. MVChl *b* and DVChl *b* are individually reported, as well.

4.5 CONCLUSIONS

Chapters documenting individual methods in SeaHARRE activities typically describe what was done with that particular set of samples, but in this instance, HPL decided to include quality assurance activities that have been conducted after SeaHARRE-3. The primary perspective of this effort was to understand where uncertainties arise and what their magnitudes are. The governing equation (25) introduced in Sect. 1.5.3.1, will simplify this ongoing task and sets the stage for a collaborative effort among all laboratories to develop efficient and coherent procedures for determining uncertainty budgets. Knowing the magnitude of uncertainties associated with terms in the governing equation is an important step toward improving the accuracy of an individual laboratory and it vastly facilitates understanding the effects of variations in extraction procedures and reporting practices of pigments in low concentrations. It is important to remember the governing equations presented in this report are pertinent to procedures using single-point response factors and the one-step internal standard methodology (26). Other governing equations will need to be derived to address, for example, multipoint calibration, calibration by peak height, and alternate modes of determining extraction volume V_c. The use of a governing equation is in its infancy, and has thus far been successfully proofed against existing calculation equations (Sect. 4.2.1) by HPL for Chl *b* and Zea.

Analysts are commended for electing to participate in SeaHARRE activities, because they entail a great deal of work and risk of being in a potentially uncomfortable situation if performance is below expectations. The feelings of

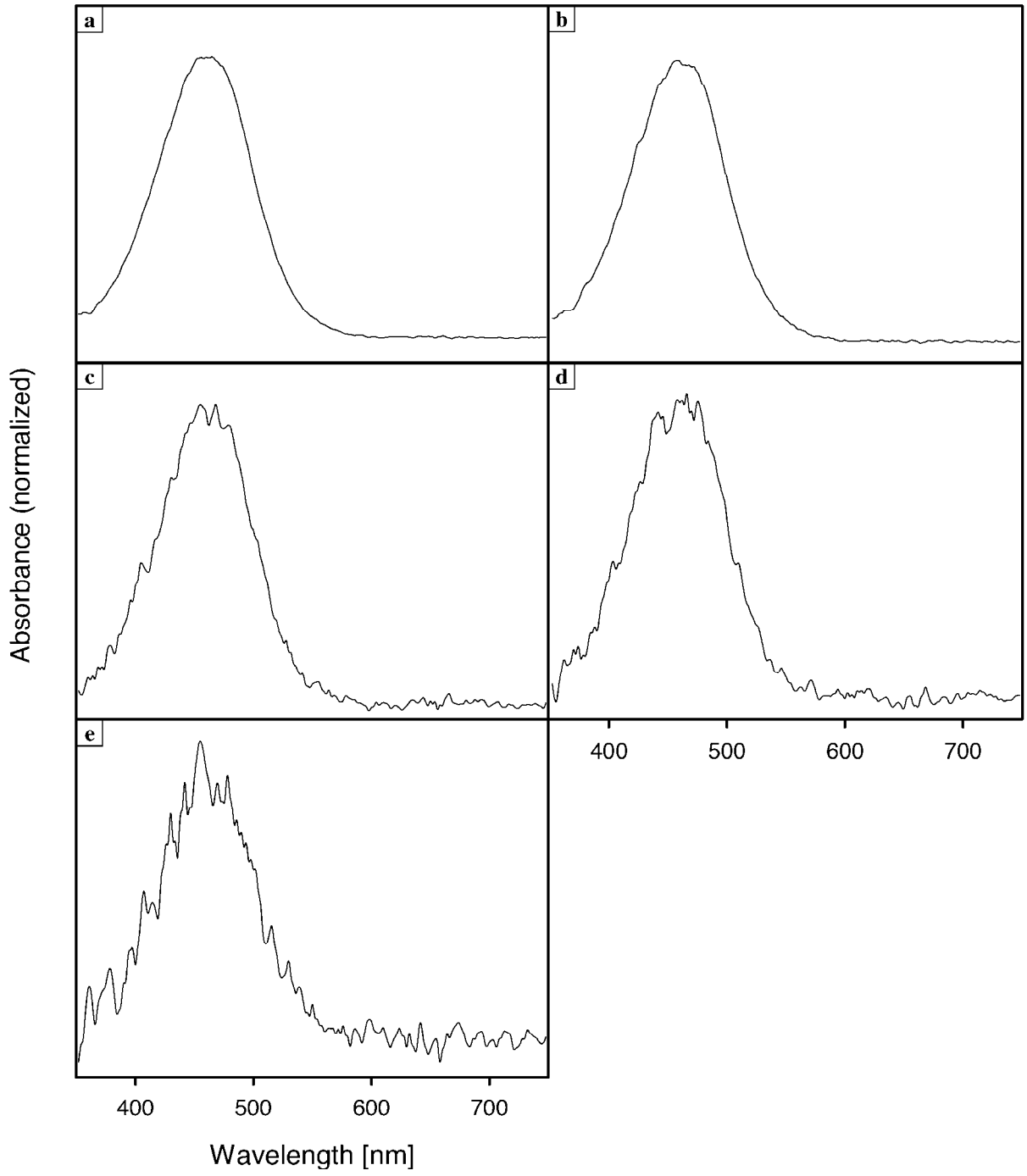

Fig. 14. HPLC in-line absorbance spectra of Pras at various SNRs from chromatograms shown in Fig. 13: **a)** 271, **b)** 154, **c)** 50, **d)** 34, and **e)** 17.

dejection that can arise from the latter are impossible to deny and can cause an analyst to retreat in frustration.

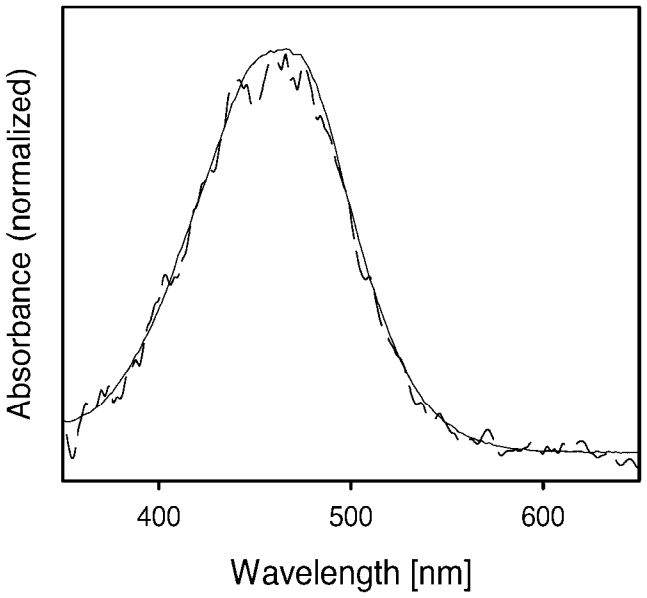

Fig. 15. In-line absorbance spectra of the Fig. 14d Pras peak with an SNR of 34 (dashed line) and a Pras standard (solid line).

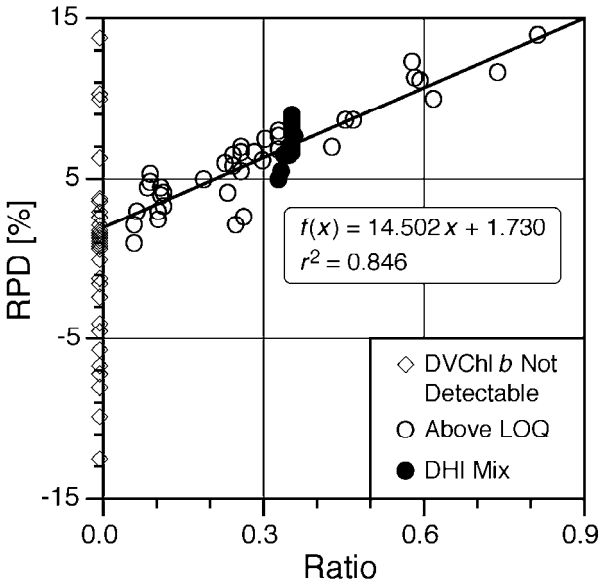

Fig. 16. The RPD comparing TChl *b* quantified by the "summing procedure" with respect to the "usual" procedure as a function of the amount ratio of DVChl *b*:Chl *b* (signal is 450 ± 10 nm). DVChl *b* is discriminated with the former, but not the latter procedure. (See Sect. 4.4.8 for details). The Average RPD and APD between modes of quantitation for samples without DVChl *b* (excluding sample 21) is −1.2 and 2.8%, respectively.

A more productive outcome can be attained, however, if minds come together and alternatives are carefully pursued, such as, when the variables in the calculation equation are individually examined and dissected for sources of uncertainty, when analysts scrutinize their results and other's results for patterns, and when each person voices their ideas, all participants will understand how to do better. Every voice should be heard and should be full of ideas, for the effort to advance the state-of-the-art must be collaborative, and hopefully, without any lasting feelings of rejection, if the results are at one time or another below expectations. One can always choose to improve. This latter point has been impressively demonstrated by laboratory *L* after SeaHARRE-2. Based on the analysis presented in Sect. 1.6.5, where *L* exhibited remarkable consistency of precision across pigments that varied widely in SNR, and *H* results did not, HPL now recognizes a weakness in their method that could not have otherwise been considered in terms of what is possible. In this context, the purpose for SeaHARRE activities cannot be forgotten: *to determine if accuracy capabilities of laboratories contributing to NASA databases are consistent with the needs of remote sensing requirements, and to facilitate a means by which analysts can come together in a cooperative environment to share ideas that will improve not only their own methods, but others' as well.*

It is time for SeaHARRE participants to make a decision regarding the damaging effects of pigments in low concentrations on accuracy. It is unfortunate that the HPLC community does not have standard reference materials with which the accuracy of field samples can be validated independently. Although the DHI Mix is a step in this direction, it does not provide the complexity of a natural sample, so round robins are necessary. It is also unavoidable that one laboratory's results will be degraded by another less capable method, even at the quality assured level. This occurs because of the manner in which the proxy for truth must be determined. However, it is a myopic perspective to think this dilution of capability is only happening in the round robins, because much of the data produced by SeaHARRE analysts are ultimately merged with others in large databases—think of the difficulty in trying to make sense of But data in eutrophic sites after the revelations regarding But uncertainties from SeaHARRE results.

Thinking forward, it would be useful for analysts to start discussing performance according to three criteria: a) across the full dynamic range of the method as is currently done, b) to compute a subset of performance variables including only those sites where all QA laboratories agree the pigment in question is present, and c) to investigate performance for the remainder of sites where one or more (but not all) QA laboratories reported a pigment as present. The first approach is very pertinent, because it describes performance across a range of concentrations and that range of concentrations ultimately ends up in a database. The second approach allows a laboratory to ascertain how their performance changes as a function

of pigments with low SNRs where subjective interpretations and differences in detectability are frequent. The third subset independently describes performance changes that occur when differences between laboratory methods cause disagreement. It also provides a benchmark against which improvements in pigment reporting practices (and detectability) can be evaluated. In this context, a performance metric for SNR may be useful.

It is attractive to think the aforementioned performance changes artificially alter what should be considered the real capability of a laboratory, but such thinking misses an important point: *a rigorously validated HPLC method does not include subjective elements that can significantly degrade the performance of the method at any point within the dynamic range of the method.* This statement admits that there are subjective elements to all methods, because people are an essential part of implementing the procedures, but it emphasizes there is an upper and lower limit of method validation. It will be important for SeaHARRE laboratories to collaboratively establish procedures that will describe where those endpoints are, both in the individual methods and also when data is merged with others.

Chapter 5

The JRC Method

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Abstract

The HPLC method used at the JRC follows the JGOFS protocols (JGOFS 1994) and is a modified version of the method presented in Wright et al. (1991). It does not allow the separation of divinyl chlorophyll *a* and *b* from their respective monovinyl forms. Filters are sonically disrupted, and the pigments are extracted within a 100% acetone solution including an internal standard (*trans*-β-apo-8′-carotenal). The HPLC system used includes a quaternary pump, a three-solvent gradient method, a reversed-phase C_{18} column with an autosampler (both with thermostats), a diode array detector, and a fluorescence detector. The effective limit of detection (computed for the SeaHARRE-3 samples) for the chlorophylls and carotenoids is about $0.0008\,\mathrm{mg\,m^{-3}}$ (for the typical filtration volumes used). In terms of routine sample analysis, this method has been applied almost exclusively to coastal water samples.

5.1 INTRODUCTION

The HPLC method used at the JRC follows the JGOFS protocols (JGOFS 1994) and is a modified version of the method presented in Wright et al. (1991). This method does not allow the separation of divinyl chlorophylls *a* and *b* from monovinyl chlorophylls *a* and *b*, respectively. In addition, chlorophylls c_1 and c_2 are not separated. Until recently, this method has been applied almost exclusively to coastal waters where pigment concentrations are generally elevated, filtration volumes are deliberately chosen high, and a negligible contribution of divinyl chlorophyll *a* and *b* to the total chlorophyll is expected.

Because the initial results demonstrated poor method performance, an attempt was made to improve the signal-to-noise ratio and the resolution of peaks by increasing the mixing loop volume from 100 to 900 mL. A second set of SeaHARRE samples (Sect. 1.5.2) was then analyzed with the new configuration.

5.2 EXTRACTION

The first set of SeaHARRE-3 filters (24 samples in triplicate) were received from the LOV on 5 March 2005 and the DHI Mix-101 on 6 April 2005. The second set of SeaHARRE-3 filters (12 samples in triplicate) were received from DHI on 5 April 2006. Each time, samples were received in dry ice and immediately stored in a −80°C freezer until analysis commenced.

The pigments are extracted in a solution of 100% acetone plus internal standard. The addition of the *trans*-β-apo-8′-carotenal (Fluka 10810, Buchs, Switzerland) internal standard is to correct for a) any evaporation of the extraction solution, and b) water retained by the filter after sampling. The concentration of the internal standard in 100% acetone is chosen in such a way that the chromatographic peak area of the sample chlorophyll *a* and the internal standard are comparable, in general around $0.055\,\mathrm{mg\,m^{-3}}$ (a value established for the analysis of the Adriatic coastal waters samples). This corresponds to an HPLC peak area of approximately 80 mAU (using the peak integration method provided within the Agilent `Chemstation` software) and a maximum absorbance of 0.0143 (at 457 nm as measured with a Perkin Elmer LA12 spectrophotometer).

Empty 14 mL, round-bottom (Falcon™) test tubes were labeled and weighed using a Sartorius Analytic A200S balance. The 100% acetone plus internal standard was stored in a 500 mL amber-colored glass bottle with a 0–10 mL Dispensette (Brand™) dispenser. The first 10 mL were discarded, and then 3 mL of the extraction solution was added to each of the tubes. After hermetically closing the tubes, they were weighed again in order to check the accuracy of the dispenser (the standard deviation for the

3 mL volume was 0.0163 mL). The tray with the filled test tubes was stored in a −18°C freezer until the extraction procedures were started.

The temperature of the test tube refrigerated bath was set to 1°C. Batches of five filter samples were transferred each time from the −80°C freezer to the −18°C freezer. Each of the five filters was then cut using a pair of scissors into about eight slivers, which were put into the corresponding labeled test tube. Each tube was then disrupted by immersing the tip of a sonic probe (Bandelin, Sonopuls GM2070) 5 mm below the surface of the extraction liquid. The probe was activated for two 30 s periods with a power setting of 35 W and a pulse rate of 5 (the latter corresponds to 0.5 s of sonication plus a 0.5 s pause). Between the two sonication periods, the tube was immersed in the refrigerated bath and cooled for 30 s. All debris in the tube was pushed below the solvent surface with a Teflon rod, the tube was hermetically sealed, and then transferred to the sample tray in the −18°C freezer for 24 h. The pair of scissors, the tip of the sonic probe, and all other material that had been in contact with the sample were cleaned with acetone and paper tissue. These procedures permit approximately 20 samples to be prepared per day of analysis. After the 24 h extraction period, the test tubes were thawed one by one and well mixed for 20 s by placing them on a vortex mixer (Heidolph, Reax 2000). The sample slurry was then directly poured into a 10 mL syringe (BD Plastipak) equipped with a filter unit (Millipore, Millex-FH 0.45 µm, 25 mm diameter). The filtered extract was put directly into two labeled 2 mL amber glass vials (Agilent). The vials were immediately flushed with N_2 gas and capped. One of the vials was kept as a duplicate and preserved at −18°C, the other was transferred into the HPLC autosampler tray.

5.3 HPLC ANALYSIS

For the first set of SeaHARRE-3 samples, the temperature controlled autosampler (set at 4°C) was programmed to mix in a 100 µL loop, before injection, 75 µL of the filtrated extract with 22.5 µL of HPLC quality MilliQ water (using a 100 µL syringe plunger). This 97.5 µL solution was then injected into the HPLC system.

For the second set of SeaHARRE-3 samples, a 900 µL loop was added to the system. Consequently, 150 µL of the filtered extract were mixed with 150 µL of HPLC quality MilliQ water (using a 100 µL syringe plunger) by drawing three sequences of 50 µL of extract plus 50 µL of MilliQ water. After mixing the draws in the 900 µL seat, this 300 µL solution was injected into the HPLC system.

The HPLC system used was composed of the following components:

- A reversed phase column (250 × 4.6 mm), 5 µm particle size, ODS-2 C_{18} (Spherisorb) coupled with a (15 × 4.6 mm) ODS-2 C_{18}, 5 µm particle size, (Hichrom) guard column (column temperature is set to 25°C);
- Temperature-controlled autosampler (Agilent 1100);
- Diode array detector (DAD, Agilent 1100);
- Quaternary pump system plus degasser (Agilent 1100);
- Fluorescence detector (Agilent 1100); and
- The data acquisition and analysis software (Agilent Rev. A.10.03).

The solvents in the mobile phase, which had a flow rate of 1 mL min^{-1}, were as follows:

A 80:20 methanol:0.5 M ammonium acetate (7.2 pH);

B 90:10 acetonitrile:water; and

C 100% ethyl acetate.

The JRC gradient is presented in Table 40.

Table 40. The gradient used with the JRC column. The time is in minutes, and the percentages of solvents A, B, and C are given in the last three columns. All steps represent linear gradient conditions except 5 and 8, which are holds.

Step	*Time*	A [%]	B [%]	C [%]
Start	0.0	100	0	0
2	2.0	0	100	0
3	2.6	0	90	10
4	13.6	0	65	35
5	18.0	0	31	69
6	23.0	0	31	69
7	25.0	0	100	0
8	26.0	100	0	0
End	34.0	100	0	0

The diode array detector records the absorption between 350–750 nm (with a 4 nm resolution) as a function of time. Chlorophylls and carotenoids were detected at 436 nm for quantitative analysis, and phaeopigments at 405 nm; other detection wavelengths were 440, 430, and 450 nm. The fluorescence detector was used for qualitative analysis (excitation at 440 nm and detection at 664 nm).

For the second set of SeaHARRE-3 samples, the chromatogram of a blank (90% acetone) was subtracted from the sample chromatogram in order to reduce noise and to eliminate the gradient effect in the baseline (observed mostly for low concentrations). The peak integration was then performed automatically according to the following conditions: 0.09 mAU s area reject, 0.09 min peak width, and 0.09 mAU threshold. When peaks were not significantly separated (this rarely occurred) a manual integration was performed.

5.4 CALIBRATION

Before the analysis of the SeaHARRE-3 samples, the HPLC system was calibrated with a set of 20 chlorophyll and carotenoid pigment standards purchased from DHI

(Hørsholm, Denmark). A new library and calibration table was created in the `Chemstation` software. The initial concentration of each standard was checked using a dual beam monochromator-type Lambda 12 Perkin Elmer spectrophotometer, with a 2 nm bandwidth (using 10 mm path length Hellma Suprasil cells). The absorbance spectrum of each pigment standard (both undiluted and diluted) was recorded from 350–750 nm (with a 2 nm resolution), and dilutions were made by using glass HPLC syringes (Hamilton).

The concentration was calculated from the ratio of the measured absorption at a specific wavelength (corrected by subtracting the 750 nm reading) to the product of the specific absorption coefficient at that wavelength (Table 41) and the cuvette path length. Immediately after the measurement, in order to avoid evaporation, the pigment standard was transferred into a 2 mL amber vial, and flushed with N_2 before sealing. The purity of each standard pigment was checked by HPLC. For every pigment standard, except chlorophyll *a*, a calibration linear fit was performed using multiple (4) single-point regressions forced through zero. For chlorophyll *a*, a linear fit was performed using six measurement points (dilutions) without forcing through zero.

Table 41. The α values used with the JRC method for a variety of pigments as a function of λ. The units for α are liters per gram per centimeter and the units for λ are nanometers.

Pigment	*Solvent*	λ	α
Chlide *a*	90% Acetone	664.0	127.67
Chl c_3	90% Acetone	452.9	346.00
Chl c_2	90% Acetone	630.9	40.40
Perid	100% Ethanol	472.0	132.50
But	100% Ethanol	446.0	160.00
Fuco	100% Ethanol	449.0	160.00
Hex	100% Ethanol	447.0	160.00
Neo	100% Ethanol	439.0	224.30
Pras	100% Ethanol	454.0	160.00
Viola	100% Ethanol	443.0	255.00
Diad	100% Ethanol	446.0	262.00
Allo	100% Ethanol	453.0	262.00
Diato	100% Ethanol	449.0	262.00
Lut	100% Ethanol	445.0	255.00
Zea	100% Ethanol	450.0	254.00
Cantha	100% Ethanol	476.0	207.50
Chl *b*	90% Acetone	646.8	51.36
Chl *a*	90% Acetone	664.3	87.67
Phytin *a*	90% Acetone	667.0	51.20
[$\beta\beta$-Car]	100% Ethanol	453.0	262.00
trans-β-apo-8′-carotenal	100% Ethanol	452.0	254.00†

† Jeffrey et al. (1997a); all other values from DHI.

The HPLC standard pigment chromatograms (at four wavelengths) were obtained by using the same peak integration method as the one used for the analysis of natural samples. Areas of the isomers (allomers and epimers) were included in the total peak area. The different response factors were calculated for purity-corrected pigment standards.

5.5 VALIDATION

For quality check and validation the following tests are performed:

- The correctness of pigment identification is regularly checked (approximately every 30 samples) by injecting a mixture of different standards and comparing the individual retention times with the ones observed for a sample.
- The response factor of the internal standard is verified every three months.
- The variability of the measurements is surveyed by a regular (every four months) performance of three successive injections of the same sample.
- The linearity of the diode array detector (DAD) response is verified every three months by means of caffeine injections (supplied by Agilent) in five different concentrations.

The longevity of the pigment standards is greater than two years from the day of production, under storage conditions recommended by DHI.

5.6 DATA PRODUCTS

For every injection, a file is created where the peaks, with their respective retention times, area, height, and width are listed. The peak areas are transferred into an `Excel` spreadsheet and converted to pigment concentration (in milligrams per cubic meter). The pigment concentrations were calculated as:

$$C_{P_i} = \frac{V_x}{V_f}\,\frac{\hat{A}_c}{\hat{A}_s}\,\frac{\hat{A}_{P_i}}{V_c}\,R_{P_i}, \qquad (51)$$

where $\hat{A}_{P_i}$ and R_{P_i} are the peak area and the response factor of pigment P_i, respectively; V_x is the extraction volume; V_f is the volume of sample filtered; V_c is the amount of sample injected onto the column; $\hat{A}_c$ is the peak area of the internal standard in the extraction solvent; and $\hat{A}_s$ is the peak area of the internal standard in the sample.

5.7 CONCLUSIONS

The JRC method provides measurements of the main pigment concentrations with an effective detection limit of approximately $0.0008\,\mathrm{mg\,m^{-3}}$ for chlorophylls and carotenoids, and an analysis time of about 35 min for the first

SeaHARRE-3 sample set and 45 min for the second set (modified injector program). The method does not chromatographically separate chlorophylls *a* and *b* from their respective divinyl forms. Until recently, the method was applied primarily to European coastal waters, and because the presence of Prochlorophytes is not expected in coastal areas, the inability to separate the divinyl and monovinyl forms has not been considered a relevant handicap.

Chapter 6

The LOV Method

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Abstract

The LOV method is derived from the Van Heukelem and Thomas (2001) technique, and applies a sensitive, reversed-phase HPLC procedure for the determination of chloropigments and carotenoids within 28 min. The different pigments, extracted in 100% methanol, are detected using a diode array detector, which permits automatic pigment identification based on absorption spectra. Optical densities are monitored at 450 nm (chloropigments and carotenoids), 667 nm (chlorophyll *a* and derived pigments) and 770 nm (bacteriochlorophyll *a*). The method provides good resolution between most pigments, but uncertainties may arise because of the partial separation of chlorophyll *b* and divinyl chlorophyll *b*, and for the resolution of chlorophyll *c* pigments. It has proven to be efficient over a wide range of trophic conditions, from eutrophic upwelling waters, to the hyper-oligotrophic South Pacific subtropical gyre. Short- and long-term quality control is monitored regularly to ensure *state-of-the-art* analyses. The injection precision of the method is estimated at 0.4%, and the effective limits of quantitation for most pigments are low (0.0004 mg m^{-3} for chlorophyll *a* and 0.0007 mg m^{-3} for carotenoids, for the typical filtration volumes used).

6.1 INTRODUCTION

The LOV method is derived from the Van Heukelem and Thomas (2001) technique, with modifications made to increase the sensitivity of the method. It is a reversed-phase HPLC procedure (C_8 column), based on a binary gradient, and allows for the determination of most chloropigments (including degradation products) and carotenoids. The pigments are extracted in 100% methanol and analyzed within 28 min. Detection is carried out at three different wavelengths: 450 nm for the carotenoids, as well as for chlorophylls *b* and *c*; 667 nm for chlorophyll *a* and its derived products; and 770 nm for bacteriochlorophyll *a* (BChl *a*).

A very good resolution (less than 1.5) is achieved for most pigments, although only partial resolution (less than 1) is obtained for Chl *b* and DVChl *b*; Chl c_2, Chl c_1, and MgDVP are all only partially resolved. Coeluting pairs include Chlide *a* and Chl c_1 (but they are quantified at different wavelengths), as well as $\beta\beta$-Car and $\beta\varepsilon$-Car. Pigment identification is based on absorption spectra and retention time.

6.1.1 Method Changes

The LOV method underwent a number of changes in the time between the SeaHARRE-2 and SeaHARRE-3 activities. The first type of change involved the protocol being used: as a result of the poor performance of the prior LOV method (Vidussi et al. 1997) during SeaHARRE-2 and the lack of information for certain pigments, the Van Heukelem and Thomas (2001) method was adopted (which had achieved a state-of-the-art performance rating during SeaHARRE-2). Because of particular aspects associated with the experience gained with the Vidussi et al. (1996) method, additional modifications to the new method were implemented with the goal of improving the sensitivity of the method.

The second important change involved the hardware being used: the Thermoquest refrigerated autosampler was replaced by an Agilent Technologies refrigerated autosampler, and this resulted in a significant improvement in injection precision. In addition, an Agilent thermostatted column compartment was added to the system, thereby allowing the use of this new parameter in method improvement.

A summary of the main methodological modifications made to the LOV HPLC methods between SeaHARRE-2 and SeaHARRE-3 is presented in Table 42.

6.1.2 Reasons for Changes

One reason LOV changed methods was the old method experienced calibration problems caused by solvent effects.

Table 42. A summary of the methods used at the LOV between SeaHARRE-2, Vidussi et al. (1997), and SeaHARRE-3, a modified version of Van Heukelem and Thomas (2001), which is denoted VHT (2001). Only the major changes that were made, as well as the most significant final modifications made to the Van Heukelem and Thomas (2001) method, are shown. The flow rate is in units of milliliters per minute.

Method Citation	*Extraction Solvent*	*C_8 Column*	*Solvent A*	*Solvent B*	*Column Temp.*	*Flow Rate*	*Separation Time*
Modifed VHT (2001)	100% MeOH	Zorbax Eclipse 3 × 150 mm	TBAA:MeOH 30:70 (v:v)	100% MeOH	60°C	0.55	28 min
VHT (2001)	90% Acetone	Zorbax Eclipse 4.6 × 150 mm	TBAA:MeOH 30:70 (v:v)	100% MeOH	60°C	1.1	28 min
Vidussi et al. (1997)	100% MeOH	Hypersil 3 × 100 mm	NH_4Ac:MeOH 30:70 (v:v)	100% MeOH	Room	0.4	22 min

Standards in ethanol and acetone that were injected onto the column, for example, resulted in significant peak asymmetry (fronting) and splitting for the polar pigments. This is illustrated in Fig. 17 which presents a comparison of Peri peaks for the old and new methods. Reasons for difficulty with the prior method are probably associated with differences in solvent strength and viscosity between the mobile phase and the extract, as well as a too-large sample injection volume. They resulted in large uncertainties in determining precise and correct response factors during the calibration phase.

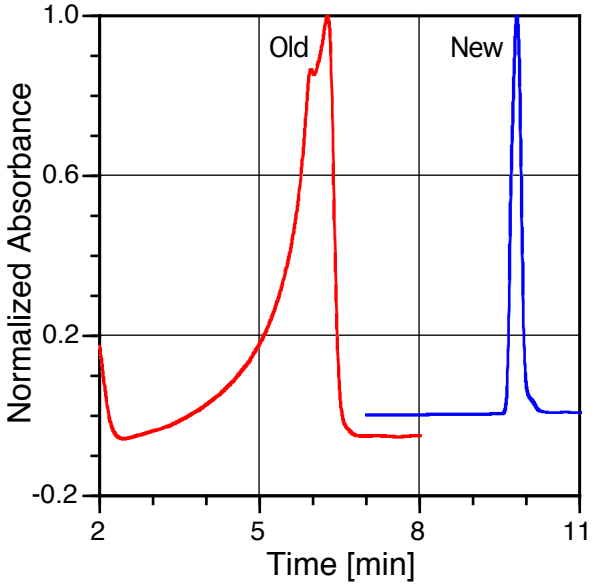

Fig. 17. A comparison of the chromatography of the Peri peak from a DHI standard in 100% ethanol between the old (red) and new (blue) LOV methods. The absorbances have been normalized by the maximum value of the corresponding peak.

Another reason LOV changed methods was coelution problems with the previous method. The main coelution problem involved Hex with Viola, Pras, and Neo. Because of this, the Hex results could be overestimated when the phytoplankton population was rich in green picoeukaryotes. With the present method, this is no longer an issue and a larger number of pigments can now be quantified. Figure 18 presents this part of the chromatogram for both methods.

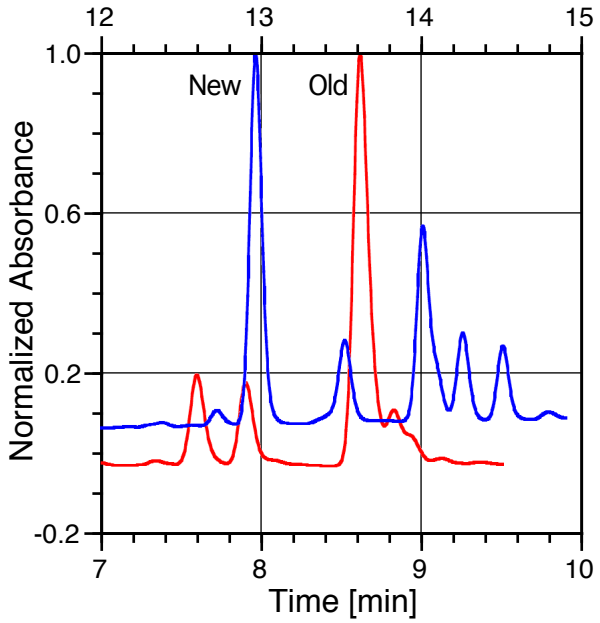

Fig. 18. Chromatograms representing the old (red and bottom x-axis) and new (blue and top x-axis) LOV methods. For the former, the pigments in elution order are But, Fuco, Hex, and Viola; while for the latter, the pigments in elution order are But, Fuco, Neo, Pras, Viola, and Hex. The absorbances have been normalized by the maximum value of the corresponding peak.

With the new method, resolution has also been significantly improved for critical pairs such as Zea and Lut, as well as DVChl *a* and Chl *a*, thus allowing for better precision and accuracy in reporting the concentrations of these pigments

6.1.3 Improved Sensitivity

The sensitivity of the Van Heukelem and Thomas (2001) method was improved by using a narrower column (3 mm

diameter instead of 4.6 mm). A narrower column is known to produce narrower and higher peaks and, thus, a higher SNR for a given concentration. Another difference with the original method is to inject methanol extracts instead of acetone extracts. A large volume of methanol extract can be injected onto the narrow column without the chromatography undergoing peak deformation that would occur if the same volume of acetone had been injected into the methanol-dominated mobile phase. It also appears to increase the robustness of the method as it was not significantly sensitive to differences in the proportion of water in the filter. There are drawbacks to this choice: (a) the manipulation of methanol which has a higher toxicity and requires particular precautions for its manipulation; (b) the risk of rapid degradation of certain pigments in a methanol solution, as was observed during the previous experiment. To avoid the latter, the sample extracts are analyzed within 24 hours after the beginning of the extraction of the filters.

6.1.4 Achievements

In general, the principal objectives in changing the LOV method were achieved, and the analysis and results from the large range of oligotrophic to eutrophic samples from the South Pacific Ocean have been satisfactory. Most notably, there was a very positive improvement of the performance metrics established during SeaHARRE-2. The method has proven to be particularly sensitive, thereby allowing for a reduction in the filtration volumes at sea. The narrow column has also reduced solvent consumption by approximately 50%.

There are, however, certain aspects of the method which might present limitations. These include a) a relatively poor chromatography and separation of the polar Chl *c* pigments, which are probably less accurately and precisely quantified and identified than with the Van Heukelem and Thomas (2001) method; and b) another potential risk is the degradability of pigments in the methanol extraction solvent, especially if, for technical reasons, the analysis of an extract needs to be postponed.

6.2 EXTRACTION

The SeaHARRE-3 *in situ* samples (stored and transported in liquid nitrogen) were stored at −80°C until analysis. The filters were extracted and analyzed between 25–27 July 2005. The extraction process involved the following steps:

1. The 25 mm GF/F filter was placed into a 10 mL disposable (Falcon) tube.
2. A pipette (Ependorf) was used to add 3 mL of 100% methanol, including a vitamin E acetate (Sigma-Aldrich) internal standard, to each tube, while making sure the filter was completely covered; the tube was closed with an airtight cap.
3. The samples were placed in a −20°C freezer for a minimum of 30 min.
4. The filters were disrupted using an ultrasonic probe (Ultrasons-Annemasse) for a maximum of 10 s. The probe was rinsed with methanol and wiped between each sample. To protect the pigments from the heat caused by ultrasonication, the tubes were placed in an ice-filled beaker.
5. The samples were returned to the −20°C freezer for a minimum of another 30 min.
6. The samples were clarified by vacuum filtration with GF/F (0.7 µm particle retention size, 25 mm diameter) filters using a Millipore™ filtration unit. A glass tube (cleaned with methanol and wiped between each sample) was used to apply pressure to the sample slurry. The filtrate was collected in 10 mL (Falcon™) tubes, and closed with airtight caps.
7. Clarified extracts were stored at −20°C until analyzed by HPLC (within 24 h).

6.3 HPLC ANALYSIS

Pigment analyses were carried out on a complete Agilent Technologies 1100 series HPLC system with the following components: a) a degasser, b) a binary pump, c) an automated sampler, including a Peltier temperature control (set at 4°C) plus a programmable autoinjector with sample preparation prior to injection, d) a programmable column oven compartment, e) a diode array detector, and f) `Chemstation for LC` software (A.09.03).

The sample extracts and standards were transferred into 2 mL glass vials using disposable glass Pasteur pipettes. The vials were then placed in the autosampler at 4°C for less than 24 h. The sample preparation—a mixture between the buffer (28mM aqueous TbAA) and the sample on a one-to-one basis—was carried out in the 500 µL loop just before injection. Alternating volumes of buffer and sample were used to aid mixing. The total injection volume was 250 µL. Although this injection mixture is optimized for methanolic extracts, standard solutions in ethanol or acetone, which are used for calibration, do not present the peak fronting problems encountered during the analysis of the SeaHARRE-2 samples.

In order to increase sensitivity, the modifications to the Van Heukelem and Thomas (2001) method included injection onto a narrow diameter, *solvent saving*, Zorbax Eclipse XDB-C_8 column (3×150 mm^2, 3.5 µm particle size) and a 0.55 mL min^{-1} flow rate. The fact that the pigments are in methanol also allows for a larger injection volume. The column temperature was maintained at 60°C.

Separation was based on a linear gradient established between a 70:30 methanol:28 mM TbAA mixture and a 100% methanol solution (solvents A and B, respectively). The gradient ranged from 10–95% of solvent B in 22 min,

followed by an isocratic hold at 95% of solvent B for 5 min (Table 43). At the end of the run, the mobile phase returned to initial conditions (10% of solvent B) for a column equilibration time of 5 min.

Table 43. The gradient elution system used with the LOV method. Solvent A is 70:30 (vol:vol) mixture of 28 mM TbAA and methanol, respectively. Solvent B is 100% methanol.

Step	*Time* [min]	A [%]	B [%]
Start	0	90	10
2	22	5	95
3	27	5	95
4	28	5	10
End	33	90	10

Detection was carried out at three different wavelengths (each with 10 nm bandwidths): 450 nm for all the carotenoids, Chl *c* and Chl *b*; 667 nm for DVChl *a*, Chl *a* plus associated allomers and epimers, as well as Chlide *a* and phaeopigments; and 770 nm for BChl *a*. The internal standard was detected at 222 nm, where there is no interference from phytoplankton pigments. For all signals, a reference at 850 nm was applied to compensate for fluctuations caused by baseline absorbance.

The off-line version of the `Chemstation` software was used for verification and eventual correction of the peak integrations in each chromatogram. Automatic spectral identification was also applied. The individual pigment concentrations were calculated using a `Visual Basic` program executed within `Excel`, but only after the automatic identification step was manually checked.

6.4 CALIBRATION

A calibration was performed in July 2005, shortly before the analysis of the SeaHARRE-3 samples. The concentrations for 15 pigment standards† provided by DHI and Sigma-Aldrich were determined by spectrophotometry using a Perkin Elmer Lambda 19 dual-beam spectrophotometer (2 nm slit, 400–800 nm spectral range, with a correction at 700 nm). The multipoint calibration curves were composed of 4–10 points, and the corresponding response factors at 440, 667, and 770 nm were determined by HPLC analysis of each standard solution.

The response factors for divinyl chlorophyll *a* and divinyl chlorophyll *b* were computed from the following:

- Knowing the specific absorption coefficients of either Chl *a* or Chl *b*;
- Accounting for the absorption of Chl *a* and DVChl *a* (or Chl *b* and DVChl *b*) at 667 nm when the spectra are both normalized at their red maxima; and
- Considering that both pigments have the same molar absorption coefficient at this red maximum.

The same process was used for determining the absorption coefficient for Chlide *a* relative to Chl *a*. Because $\beta\beta$-Car and $\beta\varepsilon$-Car coelute, the peak was first identified spectrally. The spectral shape pointed to either one being the dominant pigment, and it was quantified as such. For the remaining pigments, their specific absorption coefficients were either derived from previous calibrations or from the literature (Jeffrey et al. 1997a). The absorption coefficients for the LOV standard pigments are listed in Table 44. Because $\beta\varepsilon$-Car and $\beta\beta$-Car coelute, the peak was first identified spectrally. The spectral shape pointed to the dominant pigment and it was quantified as that pigment.

Table 44. The α values used with the LOV method for a variety of pigments, which are listed in the same order as their retention times, as a function of λ. The units for α are liters per gram per centimeter and the units for λ are nanometers. Absorption coefficient values are from DHI, unless otherwise noted.

Pigment	*Solvent*	λ	α
Chl c_3	90% Acetone	452.9	346.00
Chl c_2	90% Acetone	630.9	40.40
MgDVP	90% Acetone	630.9	40.40 [1]
Chl c_1	90% Acetone	630.9	40.40 [2]
Chlide *a*	90% Acetone	667.0	87.67 [3]
Phide *a*	90% Acetone	667.0	87.67 [3]
Perid	100% Ethanol	472.0	132.50
But	100% Ethanol	446.0	160.00
Fuco	100% Ethanol	449.0	160.00
Neo	100% Ethanol	439.0	224.30
Pras	100% Ethanol	454.0	160.00
Viola	100% Ethanol	443.0	240.00
Hex	100% Ethanol	447.0	255.00
Diad	100% Ethanol	446.0	160.00
Allo	100% Ethanol	453.0	262.00
Diato	100% Ethanol	449.0	262.00
Zea	100% Ethanol	450.0	254.00
Lut	Diethyl Ether	445.0	248.00 [4]
BChl *a*	100% Acetone	664.3	87.67 [5]
DVChl *b*	90% Acetone	646.8	51.40 [6]
Chl *b*	90% Acetone	646.8	51.36
DVChl *a*	90% Acetone	664.0	87.67
Chl *a*	90% Acetone	664.3	87.67
Phytin *a*	90% Acetone	667.0	51.20
$\beta\varepsilon$-Car	100% Acetone	454.0	250.00 [7]
$\beta\beta$-Car	100% Acetone	454.0	250.00

[1] The same as Chl c_1.
[2] The same as Chl c_2.
[3] Derived from Chl *a*.
[4] Jeffrey et al. (1997a).
[5] Oelze (1985).
[6] Derived from Chl *b*.
[7] The same as $\beta\beta$-Car.

† The standards were Peri, But, Pras, Hex, Diato, Diad, Allo, Zea, Fuco, Neo, Viola, $\beta\beta$-Car, Chl *b*, Chl *a*, and bacteriochlorophyll *a*.

Table 45. The performance metrics for validating the determination of marine pigments using an HPLC method: concentration (average precision and accuracy for TChl *a* and PPig); separation (minimum resolution and average retention time precision); injection precision (the average of an early- and late-eluting pigment standard, e.g., Perid and Chl *a*); and calibration (average residual, $|\bar{\psi}|_{\mathrm{res}}$, for Chl *a* and the precision of the dilution devices, $\bar{\xi}_{\mathrm{cal}}$).

Performance Weight, Category, and Score		*TChl* a		*PPig*		*Separation*		*Injection* ($\bar{\xi}_{\mathrm{inj}}$)		*Calibration*	
		$\bar{\xi}$	$\lvert\bar{\psi}\rvert$	$\bar{\xi}$	$\lvert\bar{\psi}\rvert$	$\check{R}_s$	$\bar{\xi}_{t_R}$	*Perid*	*Chl* a	$\lvert\bar{\psi}\rvert_{\mathrm{res}}$	$\bar{\xi}_{\mathrm{cal}}$
LOV	3.7	1.9	6.6	1.8	9.6	1.4	0.01	0.3	0.4	1.6	0.3
State-of-the-Art	*3.5*	≤2.0	≤5.0	≤3.0	≤10.0	≥1.5	≤0.04	≤2.0	≤1.0	≤1.0	≤0.5

6.5 VALIDATION

Short-term quality control (during a sequence run) was monitored using the methanol plus internal standard solution, which was injected twice at the beginning of a sequence and once after 10 sample injections. This was done to verify retention time reproducibility, peak area precision (which should be less than 1%), and instrument stability during the analytical sequence. The first two injections of a sequence, were discarded as they generally tended to lack reproducibility. For troubleshooting purposes, the pressure signal was also monitored during the analyses.

The identification of individual pigments was manually checked by retention time comparison and observation of the absorption spectra using the `Chemstation` spectral library. This pigment library comprises the retention times and spectral information of different pigments obtained from the analysis of standard solutions or identified phytoplankton cultures.

Long-term quality control is carried out using a mixed pigment standard supplied by DHI. This standard is regularly injected, at least in triplicate, to monitor the quality of the column and of the instrument performance. This is represented by a number of parameters, including the following: a) initial backpressure; b) noise level at 450 and 667 nm; c) injection precision of Chl *a* and Fuco; d) accuracy of these pigments; e) plate numbers, peak widths, and retention times; and f) the resolution of two critical pairs (DVChl *a* and Chl *a*, plus But and Fuco). Signs of column deterioration can, therefore, be rapidly detected. Generally, a column is changed every 2,000 samples, although they have lasted even longer. The calibration of the volumetric measuring devices (pipettes, syringes, etc.) is carried out annually.

These data are used to provide the performance metrics, which were determined during the SeaHARRE-2 exercise (Table 9). The objective is to evaluate and maintain a state-of-the-art level of analysis at the LOV. The performance metrics measured at the time of the SeaHARRE-3 HPLC analyses are summarized in Table 45.

If a technical problem prevents the analysis of samples that have already been extracted within the 24 h limit, the extracts are stored under nitrogen gas and placed in a −80°C freezer until a routine analysis capability is reestablished.

6.6 DATA PRODUCTS

The `Chemstation for LC` program produces a spreadsheet file for each sample comprising the pigment identification, retention times, peak areas, peak heights, peak widths, and other chromatographic information. This file is used in a `Visual Basic` program to extract the peak areas and names, and then to calculate the internal standard corrected concentrations, C_{P_i} (in milligrams per cubic meter) for each pigment P_i:

$$C_{P_i} = \frac{\hat{A}'_{P_i}}{V_f} R_{P_i}, \tag{52}$$

where $\hat{A}'_{P_i}$ is the corrected peak area (in units of milli-absorbance units seconds), R_{P_i} is the pigment response factor (in units of milligrams per milli-absorbance unit per second), and V_f is the volume of water filtered (in cubic meters). The $\hat{A}_{P_i}$ term is computed as:

$$\hat{A}'_{P_i} = \frac{\hat{A}'_{c_1}}{\hat{A}_{s_1}} \hat{A}_{P_i}, \tag{53}$$

where $\hat{A}_{P_i}$ is the uncorrected peak area, $\hat{A}_{c_1}$ is the reference area of the internal standard (established as the average of internal injections over a single day), and $\hat{A}_{s_1}$ is the area of the internal standard in the sample.

6.7 CONCLUSIONS

During this exercise, the LOV method proved to be well adapted for a large range of trophic conditions, from eutrophic upwelling waters, to the hyper-oligotrophic waters of the South Pacific Subtropical Gyre where sensitivity was the main concern. Generally, a very good resolution was obtained for most pigments, although uncertainties may arise for the resolution of chlorophyll *c* pigments or $\beta\beta$-Car and $\beta\varepsilon$-Car which tend to coelute, as well as DVChl *b* and Chl *b*, which are still partially separated. The improvements related to sensitivity comprise the use of a narrower column diameter (3 mm instead of 4.6 mm) and of methanol as an extraction solvent, which allows for a larger quantity of sample to be injected. The method was adjusted,

however, so standard solutions in ethanol or acetone, which are used for calibration, do not present the peak fronting problems encountered during SeaHARRE-2. The effective limits of quantitation for a filtered 2.8 L volume of seawater are estimated to be $0.0004\,\mathrm{mg\,m^{-3}}$ for chlorophyll *a* and $0.0007\,\mathrm{mg\,m^{-3}}$ for carotenoids.

Chapter 7

The MCM Method

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Abstract

The MCM method is a reversed-phase HPLC technique using a binary solvent system following a step linear gradient on a C_8 chromatography column. Baseline separation of monovinyl and divinyl chlorophyll *a* and of lutein and zeaxanthin, partial separation of monovinyl and divinyl chlorophyll *b*, and resolution of other key chlorophylls and carotenoids are achieved in an analysis time of approximately 30 min. The use of *trans*-β-apo-8′-carotenal as an internal standard improves the accuracy of pigment determinations. Providing a pragmatic balance between good analyte resolution and acceptable sample throughput, the method is suitable for the analysis of a wide range of oceanographic seawater samples.

7.1 INTRODUCTION

For the MCM method, pigments were analyzed by a reversed-phase HPLC procedure using a binary solvent system following a stepwise, linear gradient on a C_8 chromatography column (Barlow et al. 1997). Baseline separation of mono- and divinyl chlorophyll *a* and of lutein and zeaxanthin, partial separation of mono- and divinyl chlorophyll *b*, and resolution of other key chemotaxonomic chlorophylls and carotenoids were achieved in an analysis time of 30 min. It was found that chlorophylls c_1 and c_2, as well as $\beta\beta$-Car and $\beta\varepsilon$-Car, were not well separated by this technique. Prior to analysis, samples were stored either in liquid nitrogen or in a −85°C freezer.

For SeaHARRE-3, poor performances were experienced with the UV6000 detectors. Prior to the start of HPLC analysis, an electronic board in the MCM detector was damaged by electrical power surges, so a detector from another institute was borrowed as a substitute. This detector was not very well maintained and problems were encountered in tuning the diodes to the desired levels. Nonetheless, the best results possible were obtained. The MCM detector had been repaired by the time the M^+ samples were analyzed, and the tuning and the detector response all seemed to be nominal. Only later during a service call did the agent detect problems with the new board in the detector and it looked like this had affected the data output for the M^+ data, as well as other sample sets that had been analyzed. Consequently, the board was replaced a second time.

7.2 EXTRACTION

The SeaHARRE-3 samples were stored at −80°C until analysis. Extraction began with 2 mL of the 100% acetone containing *trans*-β-apo-8′-carotenal internal standard being added to the frozen filter samples in centrifuge tubes. The 25 mm filters contained approximately 0.2 mL of water after filtration, and the final extraction solution was, therefore, approximately 90% acetone. Samples were disrupted with the aid of ultrasonication for 30 s and were then allowed to extract for another 30 min in the dark. The extracts were then clarified by centrifugation in a refrigerated centrifuge.

7.3 HPLC ANALYSIS

Extracts in dark glass vials were loaded into a Thermo Separations AS3000 autosampler and cooled to 2°C. The autosampler incorporates a column compartment containing either a 3 µm Hypersil MOS2 C_8 column or a 5 µm Hypersil HyPURITY C_8 column (SeaHARRE-3 repeat samples), plus an autoinjector, and both were heated to 25°C. Prior to injection, the autosampler was programmed to vortex mix 300 mL of extract with 300 mL of 1 M ammonium acetate buffer, and 100 mL of the extract-buffer solution was injected onto the chromatography column. Pigments were separated at a flow rate of 1 mL min^{-1} by a stepwise, linear gradient using a Varian ProStar tertiary pump programmed as shown in Table 46.

Table 46. The gradient used with the MCM column. The time is in minutes, and the percentages of solvents A and B are given in the last two columns.

Step	*Time*	A [%]	B [%]
Start	0	75	25
2	1	50	50
3	20	30	70
4	25	0	100
End	32	0	100

7.4 CALIBRATION

Three single-point calibrations were run with all of the standards, except for chlorophyll *a* and *trans*-β-apo-8′-carotenal, for which multipoint calibrations were conducted. *Trans*-β-apo-8′-carotenal (Fluka) was purchased from Sigma-Aldrich, Ltd. The chlorophyll *a* standard was purchased from Sigma-Aldrich, and a stock solution was prepared at approximately 1 mg in 100 mL of 100% acetone. A 10% working standard was then prepared in 100% acetone from the stock solution. All other additional standards (2.5 mL) were purchased from DHI, and are noted in Table 47.

Table 47. The α values used by the MCM method for a variety of pigments as a function of λ. The units for α are liters per gram per centimeter and the units for λ are nanometers.

Pigment	*Solvent*	λ	α
trans-β-apo-8′-carotenal†	100% Acetone	462	254.00
Chl *a*†	100% Acetone	663	88.15
DVChl *a*	90% Acetone	664	88.87
Chlide *a*	90% Acetone	664	127.00
Chl *b*	90% Acetone	645	51.36
Chl c_2	90% Acetone	630	40.40
Chl c_3	90% Acetone	453	346.00
Perid	100% Ethanol	475	132.50
Fuco	100% Ethanol	452	152.50
But-fuco	100% Ethanol	450	134.90
Hex-fuco	100% Ethanol	448	130.00
Prasino	100% Ethanol	454	250.00
Neo	100% Ethanol	438	227.00
Viola	100% Ethanol	443	255.00
Diadino	100% Ethanol	448	241.30
Diato	100% Ethanol	452	248.10
Allo	100% Ethanol	454	262.00
Zea	100% Ethanol	453	254.00
Lut	100% Ethanol	447	255.00
$\beta\beta$-Car	100% Ethanol	454	250.00

† The pigment standard was purchased from Sigma-Aldrich; all other pigment standards were purchased from DHI (the chlorophyll standards were shipped in 90% acetone and the carotenoid standards in 100% ethanol).

The concentrations of all external standards and the *trans*-β-apo-8′-carotenal internal standard were determined and checked from absorbance measurements, scanning between 400–750 nm, using a monochromatic, double-beam Hitachi U-2000 spectrophotometer with a 2 nm bandwidth. Red and blue wavelength maxima were used for the chlorophylls and carotenoids, respectively, with the absorption coefficients (Table 47) estimated from data given in Jeffrey et al. (1997a).

7.5 VALIDATION

Pigments were identified by retention time comparison with external and internal standards and from diode array spectra. Response factors were calculated relative to the internal standard, and a quality control check was run by injecting the chlorophyll *a* working standard and *trans*-β-apo-8′-carotenal on a daily basis. Variability of the chlorophyll *a* response factor ranged from 2–7% of the original calibration. The concentration of the *trans*-β-apo-8′-carotenal internal standard was checked from time to time by absorbance measurement, as described above, to ensure stability under working and storage conditions, and the response factor was checked from a chromatographic run. Variability of the *trans*-β-apo-8′-carotenal response factor ranged from 1–6% of the original calibration.

7.6 DATA PRODUCTS

Chromatographic results were reported files containing the pigment identification, retention times, peak areas, and peak heights. Response factors were computed from the peak areas and pigment standard concentrations, and a relative response factor was calculated by relating individual pigment response factors to the *trans*-β-apo-8′-carotenal response factor. The weight of the *trans*-β-apo-8′-carotenal internal standard added in each 2 mL extraction was known from the concentration of *trans*-β-apo-8′-carotenal in the acetone extract solution and was approximately 400–500 ng mL^{-1}.

Pigment concentrations of samples were calculated using the following formulation:

$$C_{P_i} \;=\; \frac{W_s/\hat{A}_{s_1}}{V_f}\,\hat{A}_{P_i}\,R'_{P_i}, \tag{54}$$

where the units of C_{P_i} are nanograms per liter, $\hat{A}_{P_i}$ is the pigment area, W_s is the carotenal weight, $\hat{A}_{s_1}$ is the carotenal area in the sample, V_f is the volume of water filtered, and R'_{P_i} is the relative response factor (the inverse response factor of the internal standard divided by the inverse response factor of the pigment P_i).

Because chlorophyllide *a* and chlorophyll c_1+c_2 coelute, their individual concentrations were estimated using the following simultaneous equations:

$$\big[\text{Chlide } a\big] \;=\; \frac{\hat{A}_1\,R_{y_2} \;-\; \hat{A}_2\,R_{y_1}}{R_{x_1}\,R_{y_2} \;-\; R_{x_2}\,R_{y_1}} \tag{55}$$

and

$$\left[\mathrm{Chl}c_{1+}c_2\right] \;=\; \frac{\hat{A}_2\,R_{x_1} \;-\; \hat{A}_1\,R_{x_2}}{R_{x_1}\,R_{y_2} \;-\; R_{x_2}\,R_{y_1}}, \qquad (56)$$

where $\hat{A}_1$ and $\hat{A}_2$ are the respective chromatographic areas at 665 nm and 440 nm; R_{x_1} and R_{x_2} are the response factors for chlorophyllide *a* at 665 nm and 440 nm, respectively; and R_{y_1} and R_{y_2} are the response factors for chlorophyll $c_{1+}c_2$ at 665 nm and 440 nm, respectively.

7.7 CONCLUSIONS

The reversed-phase HPLC method using a C_8 column provided good resolution of mono- and divinyl chlorophylls *a* and *b*, as well as lutein and zeaxanthin. It also satisfactorily separated other key pigments within 30 min. Providing a pragmatic balance between good analyte resolution and acceptable sample throughput, the method is suitable for the analysis of a wide range of oceanographic samples.

Chapter 8

The SDSU (CHORS) Method

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Abstract

The CHORS method was developed to provide HPLC phytoplankton pigment analysis support for the NASA MODIS program. The method is a modified version of Van Heukelem and Thomas (2001) and uses a reversed-phase C_8 column, with a binary solvent gradient. A temperature-controlled autosampler provides continuous sample injection to maintain the quota of 4,000 samples per year run by CHORS. System calibration is monitored and recorded to ensure repeatability and consistency of data products

8.1 INTRODUCTION

The current focus of HPLC analysis at CHORS is to characterize phytoplankton pigments from water column samples taken to support the NASA MODIS Project. The method is a modified version of Van Heukelem and Thomas (2001) and chromatographically separates monovinyl from divinyl chlorophyll *a*. Because of the large number of samples processed (more than 4,000 per year), the CHORS HPLC laboratory procedures have been streamlined to accommodate the variety and quantity of water samples collected by the MODIS research community. By using larger HPLC solvent reservoirs, multiple sample tube racks, and running the system 24 h a day, the CHORS laboratory can run continuously over a 3–4 week period and accommodate 500 samples a month.

8.2 EXTRACTION

The SeaHARRE-3 samples were stored in liquid nitrogen pending extraction and analysis. The 25 mm GF/F filters were extracted in 4.0 mL of 95% acetone (using a Brinkmann Bottletop Dispenser (with a coefficient of variation of 0.321%). An internal pigment standard, *trans*-β-apo-8′-carotenal (which is normally not found in natural samples), was added to the acetone extract, prior to pipetting the 4.0 mL, to correct for any extraction volume changes during sample processing. After 24 h of extraction in a −20°C freezer, the samples were sonicated for 10 s using an ultrasonic microprobe tip at a 60% duty cycle. The samples were then extracted for an additional 24 h (stored in a freezer at −20°C). Glass-fiber particles, generated during sonication, were removed from the extract by centrifuging the samples at 5,100 rpm, for 4 min, and subsequent filtration using 0.2 µm PTFE in-line filters.

8.3 HPLC ANALYSIS

Following is the equipment used with the CHORS method:

- ThermoQuest HPLC system with membrane degasser and P4000 quaternary pump;
- AS3000 temperature-controlled autosampler (maintained at 4°C);
- UV6000 PDA detector (scanning 400–700 nm at 1 nm resolution);
- Alltech (model 631) column heater (60°C set point); and
- System controller with `ChromQuest` (V4.1) software.

Pigments were separated on a reversed-phase, Agilent ZORBAX Eclipse XDB (3.5 µm) C_8 column (150 mm length and 4.6 mm internal diameter) using a two-solvent gradient system—which was modified from Van Heukelem and Thomas (2001) to accommodate the CHORS HPLC system (Table 48)—with a 1.1 mL min^{-1} flow rate. Each sample extract was mixed (510 µL:290 µL, buffer:extract), and then vortexed for 0.1 min, with 200 µL of this mix injected onto the column. The autosampler buffer was 90:10, 28 mM TbAA and methanol, respectively. The TbAA was made using 0.4 M tetrabutylammonium hydroxide (J.T. Baker V365-07), titrated to 6.5 pH using glacial acetic acid, and was then volumetrically adjusted with HPLC grade H_2O to bring the final concentration to 28 mM TbAA.

The separation of the various pigments requires 28 min with the pigment peaks being detected by an absorption detector. Pigments were monitored at 436, 450, and 664 nm with peak retention time or spectrophotometric recognition used to determine peak identification. Accuracy for

each pigment compound was based on availability of pigment standards and the selection of pigment-specific absorption coefficients.

Table 48. The gradient elution system used with the CHORS method. Solvent A is 70:30 (vol:vol) mixture of methanol and 28 mM TbAA, respectively. Solvent B is 100% methanol.

Step	*Time* [min]	A [%]	B [%]
Start	0	95	5
2	22	5	95
3	26	5	95
End	28	95	5

8.4 CALIBRATION

The calibration standards were purchased from Sigma Chemical Company and from DHI. System calibrations were performed to determine individual standard response factors for each compound using multipoint calibrations. Concentrations of the standards were provided by DHI, or verified spectrophotometrically (for monovinyl chlorophyll *a* and monovinyl chlorophyll *b*) using published absorption coefficients (Latasa et al. 1996 and Jeffrey et al. 1997a). The internal standard was purchased from Fluka and diluted to appropriate concentration levels (with the approximate peak size equal to 100,000 mAU).

8.5 VALIDATION

Peak integration was performed using the `ChromQuest` (V4.1) software, and manually checked to ensure proper integration of each peak. Retention time and spectral signatures of the standards were used to verify peak identification. Peak areas were quantified using software-aided integration. Response factors generated during the system calibration were used for final concentration calculations. If no standard was available, the closest associate pigment response factor was used (e.g., for divinyl chlorophyll *b*, the response factor for monovinyl chlorophyll *b* was used to estimate the concentration of the pigment).

8.6 DATA PRODUCTS

Each sample data file included the integrated peak area, which was used with the pigment response factors to compute pigment concentration:

$$C_{P_i} = \frac{V_m}{V_f}\,\frac{V_c}{V_s}\,\frac{\hat{A}_{c_1}}{\hat{A}_{s_1}}\,\hat{A}_{P_i}/F_{P_i}, \qquad (57)$$

where V_m is the volume of extract (solvent plus standard) added (in milliliters); V_f is the volume of sea water filtered (in liters); V_c is the volume of sample extract injected onto the column; V_s is the volume of standard injected to determine the inverse response factor, F_{P_i}; $\hat{A}_{c_1}$ is the average peak area of 25 internal standard injections; $\hat{A}_{s_1}$ is the peak area of the internal standard in the sample; and $\hat{A}_{P_i}$ is the peak area of pigment P_i. For the CHORS method, $V_c = V_s$, so (57) can be simplified to:

$$C_{P_i} = \frac{V_m}{V_f}\,\frac{\hat{A}_{c_1}}{\hat{A}_{s_1}}\,\hat{A}_{P_i}/F_{P_i}. \qquad (58)$$

Table 49 is a list of the absorption coefficients for the CHORS standard pigments. All pigment standards were purchased from DHI, except monovinyl chlorophylls *a* and *b*, which were sometimes purchased from Sigma-Aldrich.

Table 49. The α values used with the CHORS method for the reported pigments as a function of λ (in order of elution time). The units for α are liters per gram per centimeter, and the units for λ are nanometers.

Pigment	*Solvent*	λ	α
Chl c_3	90% Acetone	452.9	346.00†
Chl c_2	90% Acetone	630.9	40.40†
Chlide *a*	90% Acetone	664.0	127.00†
Chl c_1	90% Acetone	630.9	40.40†
Phide *a*	90% Acetone	664.0	127.00†
Peri	100% Ethanol	472.0	132.50†
But	100% Ethanol	446.0	160.00†
Fuco	100% Ethanol	449.0	160.00†
Neo	100% Ethanol	439.0	224.30†
Pras	100% Ethanol	454.0	160.00†
Viola	100% Ethanol	443.0	255.00†
Hex	100% Ethanol	447.0	160.00†
Diad	100% Ethanol	446.0	262.00†
Allo	100% Ethanol	453.0	262.00†
Diato	100% Ethanol	449.0	262.00†
Zea	100% Ethanol	450.0	254.00†
Lut	100% Ethanol	445.0	255.00†
Gyro	100% Ethanol	445.0	255.00†
DVChl *b*	90% Acetone	646.6	51.36‡
MVChl *b*	90% Acetone	646.6	51.36‡
Chl a'	90% Acetone	663.5	87.67‡
DVChl *a*	90% Acetone	664.3	87.67†
MVChl *a*	90% Acetone	663.5	87.67‡
Chl a''	90% Acetone	663.5	87.67‡
Phytin *a*	90% Acetone	667.0	51.20†
$\beta\varepsilon$-Car	100% Acetone	448.0	270.00†
$\beta\beta$-Car	100% Ethanol	453.0	262.00†

† DHI Water and Environment
‡ Jeffrey et al. (1997a)

8.7 CONCLUSIONS

The CHORS method provides consistent and repeatable HPLC and fluorometric pigment concentrations, and accommodates the high number of samples, and timely turnaround of final data products. Overall, bulk pigment properties of the community structure can be determined for a variety of oceanographic water types.

Acknowledgments

The SeaHARRE-3 activity was sponsored by the SeaWiFS and MODIS Projects along with the individual institutes representing the seven laboratories that participated. The expertise of the DHI shipping office was instrumental in the successful delivery of many sample sets, and the professional contributions of Bo Guttmann was particularly helpful.

Appendices

A. The SeaHARRE-2 Science Team
B. The SCOR WG 78 Pigment Abbreviations
C. Commercial HPLC Manufacturers and Pigment Suppliers

Appendix A

The SeaHARRE-3 Science Team

The science team is presented alphabetically.

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Appendix B

The SCOR WG 78 Pigment Abbreviations

The chlorophyll pigments used in this report and their SCOR WG 78 abbreviations are presented alphabetically:

Chl *a*	Chlorophyll *a*,
Chl *a*′	Chlorophyll *a* epimer,
Chl *b*	Chlorophyll *b*,
Chl *b*′	Chlorophyll *b* epimer,
Chl c_1	Chlorophyll c_1,
Chl c_2	Chlorophyll c_2,
Chl c_3	Chlorophyll c_3,
Chlide *a*	Chlorophyllide *a*,
DVChl *a*	Divinyl chlorophyll *a*,
DVChl *a*′	Divinyl chlorophyll *a* epimer,
DVChl *b*	Divinyl chlorophyll *b*,
DVChl *b*′	Divinyl chlorophyll *b* epimer,
Phide	Phaeophorbide *a*, and
Phytin *a*	Phaeophytin *a*.

The carotenoid pigments and their SCOR WG 78 abbreviations are presented alphabetically (with their trivial names in parentheses):

Allo	Alloxanthin,
Anth	Antheraxanthin,
Asta	Astaxanthin,
But-fuco	19′-Butanoyloxyfucoxanthin,
Cantha	Canthaxanthin,
Croco	Crocoxanthin,
Diadchr	Diadinochrome (Diadinochrome I and II),
Diadino	Diadinoxanthin,
Diato	Diatoxanthin,
Dino	Dinoxanthin,
Fuco	Fucoxanthin,
Hex-fuco	19′-Hexanoyloxyfucoxanthin,
Lut	Lutein,
MgDVP	Mg 2,4-divinyl phaeoporphyrin a_5 monomethyl ester,
Monado	Monadoxanthin,
Myxo	Myxoxanthophyll,
Neo	Neoxanthin,
Perid	Peridinin,
Pras	Prasinoxanthin,
Viola	Violaxanthin,
Zea	Zeaxanthin,
$\beta\beta$-Car	$\beta\beta$-Carotene (β-Carotene), and
$\beta\varepsilon$-Car	$\beta\varepsilon$-Carotene (α-Carotene).

Appendix C

Commercial HPLC Manufacturers and Pigment Suppliers

The commercial HPLC manufacturers and pigment suppliers discussed in this report are presented alphabetically.

Agilent Technologies, Inc.[1]
2850 Centreville Road
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Voice: 800–227–9770
Fax: 800–519–6047
Net: `http://www.agilent.com/chem`

Branson Ultrasonics Corporation
41 Eagle Road
Danbury, CT 06810
Voice: 203–796–0400
Fax: 203–796–0320
Net: `http://www.bransoncleaning.com`

Carl Roth GmbH and Company
Schoemperlenstraße
1-5 D-76185 Karlsruhe
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Fax: 49–721–560–6149
Net: `http://www.carl-roth.de`

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Fax: 45–45–16–9292
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Fluka Chemical Corporation[3]
1001 West St. Paul Avenue
Milwaukee, WI 53233
Voice: 414–273–3850
Fax: 414–273–4979
Net: `flukausa@sial.com`

Hewlett-Packard Company
3000 Hanover Street
Palo Alto, CA 94304–1185
Voice: 650–587–1501
Fax: 650–857–5518
Net: `http://www.hp.com`

Hitachi Instruments, Inc.
5100 Franklin Drive
Pleasanton, CA 94588-3355
Voice: 925–218–2800
Fax: 925–218–2900
Net: `http://www.hitachi-hta.com`

Scientific Resources, Inc.
P.O. Box 957297
Duluth, GA 30095–7297
Voice: 800–637–7948
Fax: 770–476–4571

Shimadzu Scientific Instruments
7102 Riverwood Drive
Columbia, MD 21046–1245
Phone Number: 800–477–1227
Fax Number: 410–381–1222

[1] Formerly the Hewlett-Packard Analytical Division.

[2] Formerly the VKI Water Quality Institute.

[3] Part of Sigma-Aldrich.

Sigma-Aldrich Company[4]
3050 Spruce Street
St. Louis, MO 63103
Voice: 314–771–5765
Fax: 314–771–5757
Net: sigma@sial.com

ThermoQuest[5]
355 River Oaks Parkway
San Jose, CA 95134–1991
Voice: 408–526–1100
Fax: 408–965–6810
Net: http://www.thermoquest.com

Glossary

APD Absolute Percent Difference

BIO Bedford Institute of Oceanography (Canada)
BIOSOPE Biogeochemistry and Optics South Pacific Experiment

CHORS Center for Hydro-Optics and Remote Sensing
CSIRO Commonwealth Scientific and Industrial Research Organisation (Australia)
CTD Conductivity, Temperature, and Depth
CV Coefficient of Variation

DAD Diode Array Detector
DHI DHI Water and Environment Institute (Denmark)
DP (Total) Diagnostic Pigments

GF/F Not an acronym, but a type of glass fiber filter.
GSFC Goddard Space Flight Center

HP Hewlett-Packard
HPL Horn Point Laboratory
HPLC High Performance Liquid Chromatography

JGOFS Joint Global Ocean Flux Study
JRC Joint Research Centre

ID Identification (pigment)
ISTD Internal Standard

LOD Limit of Detection
LOQ Limit of Quantitation
LOV *Laboratoire d'Océanographie de Villefranche* (Oceanographic Laboratory of Villefranche, France)

MCM Marine and Coastal Management (South Africa)
MODIS Moderate Resolution Imaging Spectroradiometer
MPF Microplankton Proportion Factor

NASA National Aeronautics and Space Administration
NPF Nanoplankton Proportion Factor
NR Not Resolved

PDA Photo-Diode Array
PML Plymouth Marine Laboratory (United Kingdom)
PPC Photoprotective Carotenoids
PPF Picoplankton Proportion Factor
PPig Primary Pigments
PSC Photosynthetic Carotenoids
PSP Photosynthetic Pigments
PTFE Polytetrafluoroethylene

QA Quality Assurance

RPD Relative Percent Difference
RRF Relative Response Factor

SCOR Scientific Committee on Oceanographic Research
SDSU San Diego State University
SeaHARRE SeaWiFS HPLC Analysis Round-Robin Experiment
SeaHARRE-1 The first SeaHARRE
SeaHARRE-2 The second SeaHARRE
SeaHARRE-3 The third SeaHARRE
SeaWiFS Sea-viewing Wide Field-of-view Sensor
SIMBIOS Sensor Intercomparison and Merger for Biological and Interdisciplinary Oceanic Studies
SNR Signal-to-Noise Ratio

TAcc Total Accessory Pigments
TbAA Tetrabutyl Ammonium Acetate
TCaro Total Carotenoids
TCAS Temperature-Controlled Autosampler
TChl Total Chlorophyll
TPig Total Pigments

UMCES University of Maryland Center for Environmental Science
UPD Unbiased Percent Difference

WG Working Group
wrt With Respect To

Symbols

a Specific absorption coefficient.
A Used to denote the average of all the methods.
$A(\lambda)$ Absorbance.
$\tilde{A}$ The corrected absorbance values.
A' Used to denote the QA subset.
A^+ The average across the D, H, M, and S methods.
A^- The set of best results (e.g., lowest uncertainties or precisions) from a group of methods.
$\hat{A}_1$ The chromatographic area at 665 nm.
$\hat{A}_2$ The chromatographic area at 440 nm.
$\hat{A}_c$ The peak area of the internal standard when it is injected onto the HPLC column.
$\hat{A}_{c_1}$ $\hat{A}_c$ determined using a one-step internal standard methodology.
$\hat{A}_{c_2}$ $\hat{A}_c$ determined using a two-step internal standard methodology.
A_{P_i} Absorbance of the pigment.
$\hat{A}_{P_i}$ The area of the parent peak and associated isomers for pigment P_i.
$\hat{A}'_{P_i}$ The corrected peak area (in units of milli-absorbance units seconds).
$a_{P_i}(\lambda)$ Absorption coefficient of a pigment (a constant).

[4] Formerly Sigma Chemical.

[5] Formerly Thermo Separation Products.

$\hat{A}_s$ The peak area of the internal standard in the sample.
$\hat{A}_{s_1}$ $\hat{A}_s$ determined using a one-step internal standard methodology.
$\hat{A}_{s_2}$ $\hat{A}_s$ determined using a two-step internal standard methodology.

b_i The y-intercept of a linear equation.
$\vec{B}_{P_i}$ The amount of pigment quantified in each tube after a blank filter (and spiking solution) is subjected to the extraction process.

C The CSIRO method or the concentration of a pigment (depending on usage).
$\bar{C}$ The average concentration of a particular pigment.
C_a The concentration of chlorophyll *a*.
C_A The concentration of alloxanthin.
C_{P_i} The concentration of a particular pigment.
$\tilde{C}_{P_i}$ The amount of pigment injected for pigment P_i, usually in units of nanograms.
$\bar{C}^A_{P_i}$ The average concentration of a particular pigment across all methods.
$\hat{C}^{A'}_{P_i}$ The maximum concentration of pigment P_i across all 24 samples.
$\bar{C}^{A'}_{P_i}$ The overall averages for the individual pigments for the QA subset.
$\bar{C}^{A^+}_{P_i}$ The overall averages for the individual pigments for the methods not in the QA subset.
$\hat{C}^{A^+}_{P_i}$ The average concentration of a particular pigment across all methods for the not in the QA subset.
$\check{C}^{A^+}_{P_i}$ The minimum concentration of pigment P_i across all 24 samples not in the QA subset.
$\check{C}^{A'}_{P_i}$ The minimum concentration of pigment P_i across all 24 samples.
C_B The concentration of 19′-butanoyloxyfucoxanthin.
C_C The concentration of the carotenes.
C_{Ca} The concentration of chlorophyllide *a*.
C_{Da} The concentration of divinyl chlorophyll *a*.
C_{Dd} The concentration of diadinoxanthin.
C_{Dt} The concentration of diatoxanthin.
C_F The concentration of fucoxanthin.
C_H The concentration of 19′-hexanoyloxyfucoxanthin.
C_L The concentration of lutein.
C_N The concentration of neoxanthin.
C_{N+V} The concentration of neoxanthin plus violaxanthin.
C_P The concentration of peridinin.
C_{Pb_a} The concentration of phaeophorbide *a*.
C_{P_i} The concentration of a particular pigment.
$\tilde{C}_{P_i}$ The amount of pigment injected for pigment P_i, usually in units of nanograms.
$\bar{C}_{P_i}$ The average concentration for pigment P_i.
C_{Pr} The concentration of prasinoxanthin.
C_{Pt_a} The concentration of phaeophytin *a*.
C_{T_a} The concentration of total chlorophyll *a*.
C_{T_b} The concentration of total chlorophyll *b*.
C_{T_c} The concentration of total chlorophyll *c*.
C_V The concentration of violaxanthin.
C_Z The concentration of zeaxanthin.
C_{Z+L} The concentration of zeaxanthin plus lutein.

D The DHI method.
D_c Column diameter.
D_f A dilution factor.

E_{P_i} Each pigment contributed by the sample filter.
$\bar{E}_{P_i}$ The amount of pigment naturally occurring in the sample filter.
$\vec{E}_{P_i}$ The total amount of each pigment observed per extraction tube.

F_{P_i} Inverse response factor for pigment P_i, i.e., $1/R_{P_i}$.

H The HPL method.

i An array index.

j An array index.
J The JRC method.

k An index indicating the station ID.

l An index indicating the replicate number.
l_c The pathlength of the cuvette.
L_c Column length.
L_j The laboratory or method code.
L The LOV method.

M The MCM method.
m_i The slope of a linear equation (equating change in peak area with change in amount).

N_L The number of laboratories quantitating a pigment.
N_R The total number of replicates: 3 for M and 2 for all other methods.
N_S The number of samples.
N_v The number of vials.

P_i A particular pigment (referenced using index i).
P_s Column particle size.

r^2 Coefficient of determination.
R The response factor (from a generalized perspective).
R_{P_i} The response factor for pigment P_i, usually expressed as the amount of pigment divided by the peak area.
$R^{\Sigma}_{P_i}$ The amount injected onto the column divided by the total peak area (including the sum of the parent peak and degradants).
$R^{\%}_{P_i}$ The purity-corrected amount injected onto the column divided by the area of the main (or parent) peak alone.
R'_{P_i} The relative response factor for pigment P_i.
R_s The resolution (or separation) between peaks.
$\check{R}_s$ The minimum resolution determined from a critical pair for which one of the pigments is a primary pigment.
R_{x_1} The response factor for chlorophyllide *a* at 665 nm.
R_{x_2} The response factor for chlorophyllide *a* at 440 nm.
R_{y_1} The response factor for chlorophyll $c_1{+}c_2$ at 665 nm.
R_{y_2} The response factor for chlorophyll $c_1{+}c_2$ at 440 nm.

S The (SDSU) CHORS method.
S_{C_1} The pigments are rarely present (potential quantitation problem).
S_{C_2} The pigments are often present, but in a concentration less than 3% of [TChl *a*] (potential quantitation problem).
sg The specific gravity of 100% acetone (specified with the solvent batch, variable to within 0.7%).
S_i The pigment standard for pigment P_i.
S_k The k^{th} station or sample ID.

$S_{k,l}$ The station or sample ID set by k, and the replicate number set by l.
S_p Peak shape subject to distortion from injection conditions (potential quantitation problem).
$\vec{\mathcal{S}}_{P_i}$ The concentration of pigments in the spiking solution.
S_R Resolution problems.
S_{R_1} For a pigment pair with $R_s < 1.5$, if the first pigment in the pair is usually in high abundance relative to the second pigment, the quantitation of the second pigment is degraded (potential quantitation problem).
S_{R_2} If $R_s < 1.0$, an alternate mode of quantitation is required (potential quantitation problem).
S_{R_3} Interfering pigments are frequently present, so spectral matching is required (potential quantitation problem).
S_{R_4} Interfering pigments are possible, but infrequent (potential quantitation problem).
S_{R_5} Interfering pigments are extremely rare (potential quantitation problem).
S_s Separation selectivity; likely caused by changes in column performance as a result of (prolonged) usage (potential quantitation problem).

T_c Column temperature.
t_R The retention time.

V_a The volume of water.
V_c The volume of sample extract injected onto the HPLC column.
V_e The volume of the extraction solvent.
V_f The volume of water filtered in the field to create the sample.
V_m The volume of extraction solvent (containing internal standard) added to a filter.
V_s Internal standard.
V_{sp} The setpoint volume (in milliliters), or the Dispensette volume depending on usage.
V_x The extraction volume.
$V_{x'}$ The extraction volume in milliliters.
V_{x1} Replacement for $V_{x'}$ in (40), because they are equivalent measures of the extraction volume.
V_w The volume of water retained on the filter, usually assumed to be 0.2 mL for a 25 mm filter.
$\bar{V}_w$ The average V_w.
[Vit E] The concentration of vitamin E (in grams per milliliter) in the extraction solvent plus internal standard.

W_{ev} The weight of an empty vial and cap.
W_{fv} The weight of a vial (with cap) after it is filled with the setpoint volume of extraction solvent plus internal standard.
W_s The carotenal weight.

α Specific absorption coefficient a.

ε The molar absorption coefficient at the specified wavelength for the pigment λ_{P_i}.

λ The spectral wavelength.
λ_m The maximum wavelength.
λ_{P_i} The specified wavelength for the pigment.

ξ The coefficient of variation (12).
$\bar{\xi}$ The average precision (13).
$\bar{\xi}_{\text{cal}}$ The average CV for gravimetric calibration of dilution devices.
$\bar{\xi}_{\text{inj}}$ The injector precision.
$\bar{\xi}_{t_R}$ The CV of retention time.

σ The standard deviation (12).
σ_w The standard deviation of V_w.
$\Sigma\hat{A}_{S_i}$ The total peak area.
$[\Sigma\text{Chl}\,a]$ The total peak area times the Chl a response factor.

Φ_{P_i} The molecular weight of pigment P_i (407).

Ψ The percent recovery.
Ψ_{P_i} The percent recovery of each of the four pigments.
ψ The UPD (9).
$|\psi|$ The absolute UPD (10).
$|\bar{\psi}|$ The average APD (10).
$|\bar{\psi}|_{\text{res}}$ The average of the absolute residuals.

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14. ABSTRACT

Seven international laboratories specializing in the determination of marine pigment concentrations using high performance liquid chromatography (HPLC) were intercompared using in situ samples and a mixed pigment sample. The field samples were collected primarily from oligotrophic waters, although mesotrophic and eutrophic waters were also sampled to create a dynamic range in chlorophyll concentration spanning approximately two orders of magnitude (0.020–1.366 mg m^{-3}) The intercomparisons were used to establish the following: a) the uncertainties in quantitating individual pigments and higher-order variables (sums, ratios, and indices); b) the reduction in uncertainties as a result of applying quality assurance (QA) procedures; c) the importance of establishing a properly defined referencing system in the computation of uncertainties; d) the analytical benefits of performance metrics, and e) the utility of a laboratory mix in understanding method performance. In addition, the remote sensing requirements for the in situ determination of total chlorophyll a were investigated to determine whether or not the average uncertainty for this measurement is being satisfied.

15. SUBJECT TERMS

SeaWiFS, High Performance Liquid Chromatography, HPLC, analysis, round-robin, pigment, chlorophyll, method

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