NASA/TM-2012-217503

The Fifth SeaWiFS HPLC Analysis Round-Robin Experiment (SeaHARRE-5)

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National Aeronautics and
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November 2012

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Level of Review: This material has been technically reviewed by technical management

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Abstract

Eleven 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. Although prior SeaHARRE activities conducted in open-ocean waters covered a wide dynamic range in productivity, and some of the samples were collected in the coastal zone, only SeaHARRE-4 involved exclusively coastal samples and the uncertainties were significantly elevated for many pigments. Consequently, SeaHARRE-5 was organized and executed as a strictly coastal activity and the field samples were collected from primarily eutrophic waters within the coastal zone of New England and Tasmania. The latter samples were expected to produce less cluttered baselines than the former, which was a desirable analysis distinction, because the SeaHARRE-4 samples had complicated baselines. The more restrictive perspective limited the dynamic range in chlorophyll concentration to approximately one and a half orders of magnitude (previous activities covered more than two orders of magnitude). The method intercomparisons were used for the following objectives: a) estimate the uncertainties in quantitating individual pigments and higher-order variables formed from sums and ratios; b) confirm if the chlorophyll *a* accuracy requirements for ocean color validation activities (approximately 25%, although 15% would allow for algorithm refinement) can be met in coastal waters; c) establish the reduction in uncertainties as a result of applying QA procedures; d) show the importance of establishing a properly defined referencing system in the computation of uncertainties; e) quantify the analytical benefits of performance metrics; and f) demonstrate the utility of a laboratory mix in understanding method performance.

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). The dynamic range in the total chlorophyll *a* (TChl *a*) concentration, denoted $\left[\text{TChl } a\right]$, spanned 0.044–2.089 mg m^{-3}.

Field sampling for SeaHARRE-2 took place in 2002 (Hooker et al. 2005) and emphasized mesotrophic and eutrophic regimes (Benguela Current), involved eight laboratories using eight different methods (four C_8 and four C_{18}), although some of the methods were based on the same general method resulting in five rather distinct methods. Sample distribution involved 12 duplicates (12 triplicates were planned) spanning a $\left[\text{TChl } a\right]$ dynamic range of 0.357–26.185 mg m^{-3}.

Field sampling for SeaHARRE-3 took place in 2004 (Hooker et al. 2009) and emphasized the oligotrophic waters of the South Pacific Ocean central gyre. Additional samples were collected in mesotrophic waters and in the higher productivity of the Chilean upwelling. A total of 24 samples were distributed in triplicate covering a $\left[\text{TChl } a\right]$ dynamic range of 0.020–1.366 mg m^{-3}. Seven laboratories participated, but one of them executed two different methods, so a total of eight methods were intercompared.

Field sampling for SeaHARRE-4 took place in 2006 (Hooker et al. 2010) and was the first activity to emphasize coastal sampling, in particular the shallow waters associated with near-shore ecosystems. Unlike prior SeaHARRE field sampling campaigns, which took advantage of already scheduled oceanographic expeditions, the field sampling for SeaHARRE-4 was executed as a separate activity. The samples were collected from the fjords, estuaries, and bays within the coastal zone of Denmark. Sample distribution involved 12 triplicates spanning a $\left[\text{TChl } a\right]$ dynamic range of 1.896–42.704 mg m^{-3}. Ten laboratories participated, but one of them analyzed two different sample sets, because a change in extraction procedures degraded results.

The results from SeaHARRE-4 established the challenges with analyzing coastal samples, which have more complicated baselines and more unknown pigments than prior open-ocean round robins. Frequently, the content in the samples were most likely a mixture of freshwater and marine algae, which means they were more complicated to analyze than samples from high salinity areas. Consequently, for SeaHARRE-5 the sampling was expanded to include two types of coastal regimes: the more polluted coastal environment of the rivers and bays of the Gulf of Maine, and the more pristine environment of Tasmania.

Eleven international laboratories agreed to participate in SeaHARRE-5 with so-called *validated* HPLC methods:

1. The Australian Commonwealth Scientific and Industrial Research Organisation (CSIRO);
2. The Danish DHI Institute for Water, Environment and Health (DHI);
3. The Norwegian University of Nordland (UN), formerly the Bodø University College (BUC);

4. The American National Aeronautics and Space Administration (NASA) Goddard Space Flight Center (GSFC);
5. The American Horn Point Laboratory (HPL) University of Maryland Center for Environmental Science (UMCES);
6. The Indian National Institute of Oceanography (NIO);
7. The American Scripps Institution of Oceanography (SIO);
8. The French *Laboratoire d'Océanographie de Villefranche* (LOV);
9. The Canadian Dalhousie University (DalU);
10. The Portuguese *Centro de Oceanografia, Universidade de Lisboa* (COUL); and
11. The American University of Southern Mississippi (USM).

The participants who were involved in the SeaHARRE-5 Science Team are given in App. A.

The other laboratories that have participated in SeaHARRE activities are as follows:

12. The Canadian Bedford Institute of Oceanography (BIO);
13. The American University of South Florida (USF) Florida Institute of Oceanography (FIO);
14. The European Joint Research Centre (JRC), which is located in Italy;
15. The South African Marine and Coastal Management (MCM);
16. The British Plymouth Marine Laboratory (PML);
17. The American Center for Hydro-Optics and Remote Sensing (CHORS) at the San Diego State University (SDSU); and
18. The American University of South Carolina (USC).

The participants associated with these other institutes are documented in the prior four SeaHARRE technical reports as documented by Hooker et al. (2000, 2005, 2009, and 2010, respectively).

Of all the laboratories involved with SeaHARRE activities, only HPL and LOV participated in every exercise, although CSIRO and DHI participated in four each (Table 1). There are advantages in having a recurring set of *core* participants within the overall SeaHARRE activity, most notably because they provide an established capability and knowledge base that can be counted on during data analysis and workshop discussions (there have been almost as many workshops as round robins). The experience of the core analysts has provided invaluable learning opportunities for new analysts and they have helped steer the evolving objectives of each activity. In addition, however, emphasis is placed during the planning stages of each SeaHARRE activity to try and recruit new practitioners because another objective of the SeaHARRE activity is to provide the broadest investigation of community capabilities as possible.

Table 1. The laboratories, with their corresponding countries and assigned one-letter codes that participated in the five SeaHARRE activities executed to-date. New participants are shown in slanted typeface.

Laboratory and Country		*Code*	*SeaHARRE*				
BIO	Canada	*B*		*2*			
CSIRO	Australia	*C*		*2*	3	4	5
DHI	Denmark	*D*		*2*	3	4	5
UN§	Norway	*E*					*5*
FIO	United States	*F*				*4*	
GSFC	United States	*G*				*4*	*5*‡
HPL	United States	*H*	1†	2	3	4	5
NIO	India	*I*					*5*
JRC	Italy	*J*	1†		3	4	
SIO	United States	*K*					*5*
LOV	France	*L*	*1*	2	3	4	5
MCM	South Africa	*M*	*1*	2	3		
DalU	Canada	*N*				*4*	5
COUL	Portugal	*O*					*5*
PML	United Kingdom	*P*		*2*			
SDSU	United States	*S*		2†	3	4	
USM	United States	*T*					*5*
USC	United States	*U*				*4*	
	New Participants		2	4	0	4	6

§ Formerly BUC.

† Participated in a prior round robin, but not SeaHARRE.

‡ A new analyst for an established laboratory.

The aforementioned concept of a validated method requires some additional explanation, because there is no external process or independent agency that certifies an HPLC phytoplankton pigment 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 data products, i.e., individual pigment concentrations, sums, ratios, and indices. 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

Table 2. The methods used by all the laboratories in the five HPLC round-robin intercomparisons executed to-date as part of the SeaHARRE activity. The laboratory codes under the citations indicate the method used by a particular laboratory as a function of the five round robins.

Round Robin No.	*Gieskes and Kraay (1989)*[1]	*Wright et al. (1991)*[1]	*Egeland et al. (1995)*[1]	*Pinckney et al. (1996)*[1]	*Vidussi et al. (1996)*[2]	*Barlow et al. (1997)*[2]	*Zapata et al. (2000)*[2]	*Van Heukelem and Thomas (2001)*[2]
1		*J*			*L*	*M*		*H*
2	*B*	*C D S*			*L*	*M P*		*H*
3		*J* S^3				*M*		*C D H L* S^3
4		*N* S^4		*F U*				*C D G H J L*
5		*I N* T^5	*E*				*K O*	*C D G H L* T^5
Total	*1*	*11*	*1*	*2*	*2*	*4*	*2*	*19*

[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 former is denoted S_{18} and the latter S_8).

[4] SDSU switched back to the Wright et al. (1991) method.

[5] USM executed two methods, the Wright et al. (1991) method and the Van Heukelem and Thomas (2001) method, so they could be compared (the former is denoted T_{18} and the latter T_8).

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), which have been continuously reviewed and updated (Hooker et al. 2009), since their inception. The evaluation of the performance metrics—in particular the corresponding accuracy and precision parameters—resulted in some participants replacing the methods they were initially using for a single (more modern) method with superior performance parameters: the Van Heukelem and Thomas (2001) method (VHT). In the history of SeaHARRE activities, the maximum diversity in the combination of laboratories and methods was achieved during SeaHARRE-2, and the least during SeaHARRE-3. Consequently, an emphasis was placed on recruiting new laboratories and methods for SeaHARRE-4, particularly methods that would help counterbalance the evolving predominance of C_8 methods over C_{18} methods, and the same emphasis was a central part of planning SeaHARRE-5 (Table 2).

The reduction in method diversity over time was not expected. There was a strong feeling in the early planning for SeaHARRE-1 that the approach used in the joint Global Ocean Flux Study (JGOFS 1994)—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. Two principal concerns with having too many practitioners using the same method was a) a bias in the predominant method would go undetected, and b) the state of the art would not continue to evolve. The practical benefit of adopting a proven method instead of investing an unknown amount of time and resources in trying to improve a method, however, was simply too alluring.

The allure is frequently a consequence of practical considerations. Many laboratories do not have the luxury of pursuing pure research, and funding and resource requirements demand a method that works, so that output on projects is maintained at a high level. Many analysts would like to evolve their chosen method, but instrument downtime while doing so is frequently not an option. In addition, for certain separations, no amount of extra effort with a particular method might prove fruitful. For example, a C_{18} method usually cannot separate the monovinyl and divinyl forms of Chl *a*, and if this separation is needed, the method typically must be changed (however, for the UN method, separation of the monovinyl and divinyl forms of Chl *a* was achieved by using a double C_{18} system). 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-5 are presented in Chap. 1 and the individual methods of the 11 laboratories are presented in Chaps. 2–12, respectively. A summary of the material presented in each chapter is given below.

1. SeaHARRE-5 Methods, Data, and Analysis

The focus of this study was the estimation of uncertainties in quantitating a diverse set of chlorophyll and carotenoid pigment concentrations for a variety of HPLC

methods and related procedures used in the analysis of predominantly coastal waters. Samples were collected from two different environments, which were anticipated to have very different HPLC baselines. The first set of 12 samples were taken from the New England (United States) coastal waters around Portsmouth, New Hampshire. The second set of 12 samples were obtained in the near vicinity of the coastal waters around Hobart, Tasmania (Australia). The latter were expected to have simpler baselines than the former, because the environmental conditions of the Tasmanian watersheds were expected to be less influenced by anthropogenic sources (as established by prior analyses). The SeaHARRE-5 activity was designed to investigate the following objectives: a) estimate the uncertainties in quantitating individual pigments and higher-order variables formed from sums and ratios; b) confirm if the chlorophyll *a* accuracy requirements for ocean color validation activities (approximately 25%, although 15% would allow for algorithm refinement) can be met in coastal waters; c) establish the reduction in uncertainties as a result of applying QA procedures; d) show the importance of establishing a properly defined referencing system in the computation of uncertainties; e) quantify the analytical benefits of performance metrics, f) demonstrate the utility of a laboratory mix in understanding method performance, and g) determine whether or not noisy baselines elevate pigment uncertainties. All of these objectives were satisfied and the results associated with each are presented.

2. *The CSIRO Method*

The CSIRO method is a modified version of the Van Heukelem and Thomas (2001) or HPL method and can resolve about 35 different pigments with baseline resolution of divinyl and monovinyl Chl *a*, as well as Zea and Lut. Partial separation of divinyl and monovinyl Chl *b*, as well as Chl c_1 and Chl c_2 is also achieved. The method used for SeaHARRE-5 was the same method used for the prior two SeaHARRE activities with one exception. 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 spectrum from a photo-diode array (PDA) detector. The method is regularly validated with the use of internal and external standards and individual pigment calibration. The detection limit of most pigments is within the range 0.001–0.005 mg m^{-3}. This method is applicable to the study of pigment composition and concentration in samples from all water types including freshwater, estuarine, upwelling coastal regions, the oligotrophic open ocean, as well as in the microphytobenthos of shallow coastal regions.

3. *The DHI Method*

The DHI HPLC method is a modified version of the HPL method. The method provides good separation of more than 30 of the most important pigments in freshwater, estuarine, and oceanic environments. Validation steps include four injections of a chlorophyll *a* standard to verify the calibration of the HPLC, use of an internal standard for correcting evaporation errors, and injection of a mixture of pigments to verify correct elution and retention times, as well as for documenting the precision of the HPLC and response factor stability. The DHI two-sentence rule is used to guide the analysis of pigments in very low concentrations: i) the relative retention time shall be identical to the pigment in question, and ii) if the spectrum of a small peak matches the spectrum for the pigment in question, quantitate it; otherwise do not quantitate it. When reporting results, the concentration of such small peaks are often lower than the limit of quantitation (LOQ) and are consequently replaced by LOQ values.

4. *The UN Method*

A new HPLC method for analyzing oceanographic field samples was developed by the University of Nordland (formerly known as Bodø University College) and used for the analysis of the SeaHARRE-5 samples. The method was based on a regularly used method for carotenoid analysis of cultivated algal species. To obtain improved resolution of the large number of pigments that may be present in oceanographic samples, the new method used two C_{18} columns and a lower solvent flow. The new method provided a good separation, identification, and quantitation for carotenoids of all polarities, with the exception of lutein and zeaxanthin. For the chlorophyll pigments, the usefulness of the method is limited, because it did not separate either the polar chlorophyll c_1, chlorophyll c_2, and MgDVP, or the divinyl and monovinyl forms of chlorophyll *b*. Further method adjustment would be needed to obtain separation of these pigments, or other methods must be used. For chlorophylls *a* and *b*, the method showed very good separation of chlorophyll allomers and epimers from the parent pigments, which is a capability not observed with common HPLC methods.

5. *The GSFC Method*

The GSFC Method uses an Agilent 1200 Series Rapid Resolution LC system and a single quadrupole mass spectrometer, which was purchased in August 2008. The purpose of the system was to establish protocols to a) reduce the amount of time needed to analyze an HPLC sample, and b) improve the detection and quantitation of marine pigments. The latter is the most important, because a significant limitation with the current protocols is the inability to unequivocally identify and accurately quantitate pigments in low concentrations. The SeaHARRE-5 activity was the first operational test of the system, however, the aforementioned configuration could not be used because of method incompatibility issues that are still unresolved. Instead, a configuration akin to a standard Agilent 1200 series system (quaternary pump and standard vacuum degasser) was used. A new method (to the chromatographer) applied simultaneously to a new instrument

proved to be a significant challenge. This chapter includes a discussion on the method and analysis tools used during participation. Moreover, the challenges met and overcome to achieve quality-assured status is also discussed.

6. The HPL Method

The HPL method employed by Horn Point Laboratory 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, elevated column temperature (60°C), and separation of all pigments to be quantitated with a simple linear gradient. 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 are estimated as follows: 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.

7. The NIO Method

The NIO method is a slightly modified version of the method developed by Wright et al. (1991) and further adapted by Bidigare and Charles (2002). The NIO method was developed for use in connection to many pigments important to freshwater, estuarine, and oceanic systems. The method is based on a C_{18} reversed-phase column with a standard Beckman guard column. The method can provide quantitative estimates for more than 16 pigments in an analysis time of 34 min. The method does not separate Chl c_1, and divinyl Chl *a* and *b* are not separated from their respective monovinyl forms. In such cases, a C_8 column was used with the same procedure to separate the divinyl and monovinyl forms of Chl *a*. The retention times of Zea and Lut are sufficiently close to each other such that standard pigments are frequently used to confirm the peaks.

8. The SIO (Vernet Laboratory) Method

The SIO (Vernet Laboratory) method for HPLC separation of phytoplankton pigments was developed for use in processing field samples during expeditions in Antarctica. The procedure uses an Agilent 1100 system and follows the basic protocol established by Zapata et al. (2000). Analysis includes a reversed-phase C_8 column with a binary gradient, a temperature-controlled column, and both a diode array detector (DAD) and a visible wavelength detector, with peak quantification done at 440 nm. Sample extraction is in 90% acetone (with ultrasonication) and extracts are cleaned using Puradisc syringe-tip filters before injection onto the HPLC column. Samples for SeaHARRE-5 were processed on a similar Agilent 1100 system at Horn Point Laboratory in Cambridge, Maryland. The SIO method successfully separates most chlorophylls and carotenoids commonly seen in coastal and oceanic Antarctic waters.

9. The LOV Method

The LOV method is derived from the technique described by Van Heukelem and Thomas (2001), and applies a sensitive reversed-phase HPLC analysis for the determination of chloropigments and carotenoids within a run time of 28 min. The different pigments, extracted in methanol, are detected by DAD, which allows for automatic identification to be carried out on the basis of absorption spectra. Optical densities are monitored at 450 nm (chloropigments and carotenoids), 667 nm (chlorophyll *a* and derived pigments), 770 nm (bacteriochlorophyll *a*) and 222 nm (vitamin E acetate, the internal standard). The method provides good resolution between most pigments, but uncertainties may arise because of the partial separation of monovinyl and divinyl forms of Chl *b*, for the resolution of Chl *c* pigments, and for the separation of βε-Car and ββ-Car. 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 so-called *state-of-the-art* analyses. At the time the SeaHARRE-5 samples were analyzed, the injection precision of the method was estimated at 0.4% and the limits of detection for most pigments were low ($0.016\,\mathrm{ng\,inj^{-1}}$ for Chl *a* and $0.034\,\mathrm{ng\,inj^{-1}}$ for the carotenoids).

10. The DalU Method

The HPLC pigment technique used at Dalhousie University follows the methodology developed by Wright et al. (1991). It is a reversed-phase HPLC procedure (C_{18} column), based on a tertiary gradient and includes a cooled autosampler, plus photodiode array and fluorescence detectors. Samples are disrupted with a sonic probe in 100% methanol including an internal standard (vitamin E acetate). The method is validated with the use of internal and external standards. The method does not allow the separation of divinyl chlorophylls *a* and *b* from their respective monovinyl forms. In addition, chlorophylls c_1 and c_2 are not separated, and neither is lutein from zeaxanthin. It offers good separation of a variety of pigments, however, in a relatively short period of time (29 min).

11. The COUL Method

The COUL HPLC method is based on a reversed-phase OS C_8 column and a pyridine-containing mobile phase, developed for the simultaneous resolution of chlorophyll and carotenoid pigments. This method allows for the separation of pigment pairs such as Chl c_1 and Chl c_2, as well as MVChl *a* and DVChl *a*. Pigments are identified from absorbance spectra and retention times, and concentrations calculated from the signals in the PDA or fluorescence

detectors. Pigment calibration is regularly performed using commercial standards. The method has been used at COUL since 2002, and was chosen as the standard method for pigment analysis of phytoplankton samples of coastal and oceanic waters. After SeaHARRE-5, some modifications were introduced, namely the control of column temperature (25°C), which has improved the resolution of critical pigment pairs (e.g., Lut and Zea), the use of longer extraction times, and the inclusion of an internal standard.

12. The USM Method

The USM laboratory uses two different HPLC methods to analyze phytoplankton pigments; the first is a modified version of the method from Wright et al. (1991), and the second method is adapted from Van Heukelem and Thomas (2001). At USM, pigment samples from lakes, estuaries, and shallow coastal ocean to oligotrophic ocean waters are routinely analyzed, for which approximately 25 pigments are quantitatively separated. The methods are regularly validated using commercially available standards and individual pigment calibration. Having two identical systems running two different methods allows cross validation and efficient analysis to address specific research goals for different water types. During the SeaHARRE-5 activity, USM encountered problems in identifying small peaks, particularly for Peri and Diato; use of a specific spectral library for small peaks alleviated some of the problems, but not all.

Chapter 1

SeaHARRE-5 Methods, Data, and Analysis

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Abstract

The focus of this study was the estimation of uncertainties in quantitating a diverse set of chlorophyll and carotenoid pigment concentrations for a variety of HPLC methods and related procedures used in the analysis of predominantly coastal waters. Samples were collected from two different environments, which were anticipated to have very different HPLC baselines. The first set of 12 samples were taken from the New England (United States) coastal waters around Portsmouth, New Hampshire. The second set of 12 samples were obtained in the near vicinity of the coastal waters around Hobart, Tasmania (Australia). The latter were expected to have simpler baselines than the former, because the environmental conditions of the Tasmanian watersheds were expected to be less influenced by anthropogenic sources (as established by prior analyses). The SeaHARRE-5 activity was designed to investigate the following objectives: a) estimate the uncertainties in quantitating individual pigments and higher-order variables formed from sums and ratios; b) confirm if the chlorophyll *a* accuracy requirements for ocean color validation activities (approximately 25%, although 15% would allow for algorithm refinement) can be met in coastal waters; c) establish the reduction in uncertainties as a result of applying QA procedures; d) show the importance of establishing a properly defined referencing system in the computation of uncertainties; e) quantify the analytical benefits of performance metrics, f) demonstrate the utility of a laboratory mix in understanding method performance, and g) determine whether or not noisy baselines elevate pigment uncertainties. All of these objectives were satisfied and the results associated with each are presented.

1.1 INTRODUCTION

The results obtained in the first four SeaHARRE activities established a continuing interest in estimating and understanding the sources of 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. More recently, the SeaHARRE-4 results showed the added difficulties associated with the more complex chromatograms obtained during the analyses of coastal samples, so the SeaHARRE-5 activity was planned to further investigate the problems involved.

1.2 THE DATA SET

The SeaHARRE-5 analyses were derived from two sets of field samples, 12 each for a total of 24 samples in triplicate, plus a laboratory mixture of natural pigments. The activity involved 11 laboratories (Table 1) using four different types of methods (Table 2). A total of eight C_8 and four C_{18} methods were executed. One laboratory, T, executed both method types denoted T_8 and T_{18}, respectively.

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-5 sampling plan emphasized coastal waters. The samples were collected in two different coastal environments, which were anticipated to have very different HPLC baselines. The first set of 12 samples were

taken from the New England (United States) coastal waters around Portsmouth, New Hampshire. The second set of 12 samples were obtained in the near vicinity of the coastal waters around Hobart, Tasmania (Australia). The latter were expected to have simpler baselines than the former, because the environmental conditions of the Tasmanian watersheds were expected to be less influenced by anthropogenic sources (as established by prior analyses by CSIRO). The difference in baseline noise was a desirable feature of the sampling, because the SeaHARRE-4 samples had complicated baselines and the hypothesis was that the noisy baselines were elevating pigment uncertainties. The two different types of baselines in the SeaHARRE-5 samples would prove or disprove this theory. The locations of the sampling stations are shown in Fig. 1. Details concerning individual stations that were occupied to collect the 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, all of which 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*, ββ-Car, and βε-Car. The 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 for 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-5 is denoted DHI Mix-105. Each laboratory received multiple ampules of the mix and was requested to make at least three analyses of the mix, and to use it 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. 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. Table 4 also provides

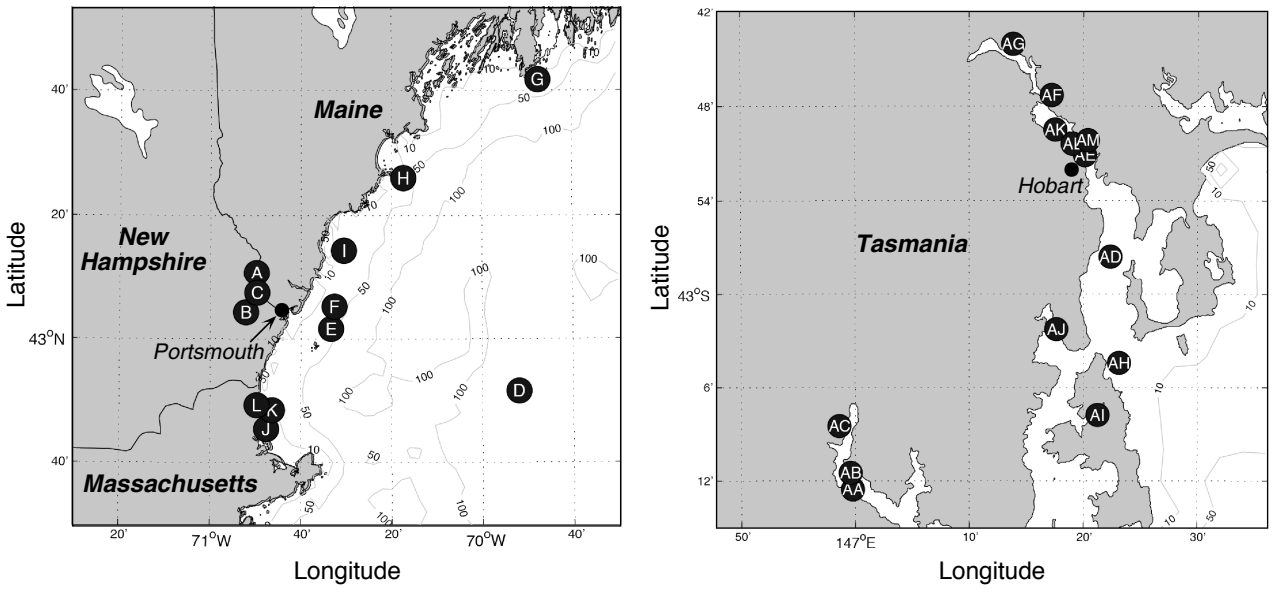

Fig. 1. Station locations for SeaHARRE-5 samples collected in **a)** New England (US), and **b)** Tasmania (Australia). For the former, the 1–12 numeric bullets correspond to stations `A`–`L`, respectively, whereas for the latter they correspond to `AA`–`AK` and `AM`, respectively. Depth contours are in meters.

Table 3. The SeaHARRE-5 logs for field sample batches, which were all collected in triplicate. Samples were collected for 12 methods, so each batch contained 36 filters. The individual filters were identified alphabetically from `A` to `L` for the New England samples and `AA` to `AM` for the Tasmanian samples, but the `AL` samples were not part of the distribution to the participants (they were retained for another purpose). All samples are from the surface. The water depth is shown with the volume of water filtered, V_f, for all filters within a batch. A blank entry indicates no data.

Station Code and Name		*Date and Time* [GMT]		*Longitude* [°]	*Latitude* [°]	V_f [mL]	*Salinity* [PSU]	*Water Depth* [m]
`A`	Cocheco River	09 Oct 2008	1256	-70.8296	43.1799	200		4.3
`B`	Great Bay	09 Oct 2008	1735	-70.8687	43.0721	400		5.8
`C`	Dover Point	09 Oct 2008	2032	-70.8276	43.1248	300		4.6
`D`	Wilkinson Basin 7	10 Oct 2008	1402	-69.8654	42.8598	900		254.0
`E`	Wilkinson Basin 2	10 Oct 2008	2043	-70.5558	43.0277	600		60.0
`F`	Boon Island	11 Oct 2008	1210	-70.5437	43.0875	550		58.0
`G`	Sequin Island	11 Oct 2008	1652	-69.7903	43.6961	400		28.0
`H`	Wood Island	11 Oct 2008	1935	-70.2895	43.4340	300		60.0
`I`	Bald Head Cliff	11 Oct 2008	2108	-70.5090	43.2404	300		45.0
`J`	Plum Island	12 Oct 2008	1253	-70.7937	42.7569	300		5.1
`K`	Merrimack River	12 Oct 2008	1628	-70.7722	42.8076	300		24.0
`L`	Black Rocks Creek	12 Oct 2008	1727	-70.8289	42.8213	200		6.4
`AA`	Killala Bay	10 Nov 2008	1140	146.9963	-43.2093	1,000	34.5	6.1
`AB`	Wheatleys	10 Nov 2008	1115	146.9932	-43.1916	400	34.1	12.5
`AC`	Castle Forbes Bay	10 Nov 2008	1045	146.9769	-43.1413	300	34.5	2.5
`AD`	Taroona	11 Nov 2008	0935	147.3728	-42.9592	1,200	33.7	24.0
`AE`	Cornelian Bay	11 Nov 2008	1020	147.3353	-42.8518	700	29.5	16.0
`AF`	Dogshear Point	11 Nov 2008	1040	147.2869	-42.7883	500	17.0	5.0
`AG`	Bridgewater Bridge	11 Nov 2008	1100	147.2308	-42.7341	250	8.2	5.4
`AH`	Storm Bay	12 Nov 2008	0930	147.3855	-43.0738	1,000	33.8	20.0
`AI`	Barnes Bay	12 Nov 2008	1030	147.3541	-43.1296	500	34.2	14.0
`AJ`	North West Bay	12 Nov 2008	1100	147.2938	-43.0378	600	34.2	13.0
`AK`	Prince of Wales Bay	13 Nov 2008	1250	147.2922	-42.8247	300	26.5	0.5
`AL`	New Town Bay	21 Nov 2008	0900	147.3172	-42.8397	300	28.9	3.5
`AM`	Geilston Bay	04 Dec 2008	1130	147.3398	-42.8360	250	26.0	2.0

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 presence of [Neo+Vio] and [Zea+Lut] is to maintain continuity with prior SeaHARRE activities, for which these sums were important.

Variable	*Method*	*Secondary Pigment*	*Calculation*
[Chl *a*]	*C D E G H I K L N O* T_8 T_{18}	Chlorophyll *a* (Chl *a*)	Plus allomers and epimers
[DVChl *a*]	*C D E G H K L O* T_8	Divinyl chlorophyll *a* (DVChl *a*)	
[Chlide *a*]	*C D E G H K L N O* T_8 T_{18}	Chlorophyllide *a* (Chlide *a*)	
[Chl *b*]	*C D E G H I K L N O* T_8 T_{18}	Chlorophyll *b* (Chl *b*)	
[Chl c_{1+2}]	*C D E G H I K L N O* T_8 T_{18}	Chlorophyll c_{1+2} (Chl c_{1+2})	[Chl c_1] + [Chl c_2]
[Chl c_3]	*C D E G H K L N O* T_8 T_{18}	Chlorophyll c_3 (Chl c_3)	
[βε-Car]	*E G I K N* T_{18}	β,ε-Carotene† (βε-Car)	
[ββ-Car]	*E G I K L N O* T_{18}	β,β-Carotene‡ (ββ-Car)	
Variable	*Method*	*Tertiary Pigment*	*Calculation*
[Chl c_1]	*C G K*	Chlorophyll c_1 (Chl c_1)	
[Chl c_2]	*C D E G I K L N O* T_8 T_{18}	Chlorophyll c_2 (Chl c_2)	
[Lut]	*C D G H K L* T_8 T_{18}	Lutein (Lut)	
[Neo]	*C D E G H I K L O* T_8 T_{18}	Neoxanthin (Neo)	
[Neo+Vio]	*C D E G H I K L O* T_8 T_{18}	Neoxanthin and Violaxanthin (Neo+Viola)	[Neo] + [Viola]
[Phide *a*]	*C D E G H L O* T_8 T_{18}	Phaeophorbide *a* (Phide *a*)	
[Phytin *a*]	*C D E G H L O* T_8 T_{18}	Phaeophytin *a* (Phytin *a*)	
[Pras]	*C D E G H I K L O* T_8 T_{18}	Prasinoxanthin (Pras)	
[Viola]	*C D E G H I K L O* T_8 T_{18}	Violaxanthin (Viola)	
[Zea+Lut]	*C D E G H I K L N O* T_8 T_{18}	Zeaxanthin and Lutein (Zea+Lut)	[Zea] + [Lut]
[Gyro]	*C E H*	Gyroxanthin (Gyro)	
Variable	*Method*	*Ancillary Pigment*	*Calculation*
[DVChl *b*]	*C H*	Divinyl chlorophyll *b* (DVChl *b*)	
[Myxo]	*C D*	Myxoxanthophyll (Myxo)	
[MgDVP]	*C G*	Mg 2,4-divinyl phaeoporphyrin a_5 monomethyl ester (MgDVP)	
[BChl *a*]	*L*	Bacterial chlorophyll *a* (BChl *a*)	
[Pyro]	*C*	Pyro-phaeopigments¶	
[Phytin *b*]	*C E*	Phaeophytin *b* (Phytin *b*)	
[Anth]	*C D E K*	Antheraxanthin (Anthera)	
[Cantha]	*C*	Canthaxanthin (Cantha)	
[Croco]	*D*	Crocoxanthin (Croco)	
[Monado]	*K*	Monadoxanthin (Monado)	

† Also referred to as α-Carotene.

‡ Also referred to as β-Carotene.

¶ Pyro-phaeophorbide *a* and pyro-phaeophytin *a*.

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_{C_a} 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 THE GOVERNING EQUATIONS

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:

$$\tilde{C}_{P_i} \;=\; \hat{A}_{P_i}\, R_{P_i}, \tag{1}$$

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

Table 5. The (individual) primary pigments, sums, ratios, and 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. 1997). Abbreviated forms for the pigments are shown in parentheses.

Variable	*Primary Pigment (PPig)*	*Calculation*
[TChl *a*]	Total chlorophyll *a*† (TChl *a*)	[Chlide *a*] + [DVChl *a*] + [Chl *a*]
[TChl *b*]	Total chlorophyll *b*† (TChl *b*)	[DVChl *b*] + [Chl *b*]
[TChl *c*]	Total chlorophyll *c*† (TChl *c*)	[Chl c_1] + [Chl c_2] + [Chl c_3]
[Caro]	Carotenes† (Caro)	[ββ-Car] + [βε-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)	[TChl *a*] + [TChl *b*] + [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]
[TCaro]	Total Carotenoids (TCaro)	[PPC] + [PSC]
[TAcc]	Total Accessory Pigments (TAcc)	[TCaro] + [TChl *b*] + [TChl *c*]
[TPig]	Total Pigments (TPig)	[TAcc] + [TChl *a*]
[DP]	Total Diagnostic Pigments (DP)	[PSC] + [Allo] + [Zea] + [TChl *b*]
Variable	*Pigment Ratio*	*Calculation*
[TAcc]	The [TAcc] to [TChl *a*] ratio	[TAcc]/[TChl *a*]
[TChl *a*]	The [TChl *a*] to [TPig] ratio	[TChl *a*]/[TPig]
[TChl]	The [TChl] to [TCaro] ratio	[TChl]/[TCaro]
[PPC]	The [PPC] to [TPig] and [TCaro] ratios	[PPC]/[TPig] and [PPC]/[TCaro]
[PSC]	The [PSC] to [TCaro] ratio	[PSC]/[TCaro]
[PSP]	The [PSP] to [TPig] ratio	[PSP]/[TPig]
Variable	*Pigment Index*	*Calculation*
[mPF]	Microplankton Proportion Factor‡ (MPF)	$\frac{[\text{Fuco}] + [\text{Peri}]}{[\text{DP}]}$
[nPF]	Nanoplankton Proportion Factor‡ (NPF)	$\frac{[\text{Hex}] + [\text{But}] + [\text{Allo}]}{[\text{DP}]}$
[pPF]	Picoplankton Proportion Factor‡ (PPF)	$\frac{[\text{Zea}] + [\text{TChl } b]}{[\text{DP}]}$

† Considered as individual pigments, although computed as sums by some methods; *L* and *P* only quantitate [ββ-Car].
‡ As a group, also considered as *macrovariables*.

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.

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.

† A milli-absorbance unit is denoted mAU.

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, which is measured in milliliters. A variety of filters are used for the filtration protocol, but a common selection is a 25 mm glass-fiber filter with a 0.7 µm pore size. The latter have been used for all of the SeaHARRE activities.

The variables used to compute R_{P_i} in (1), or the terms in brackets within (3), 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:

$$A_{P_i}(\lambda) \;=\; a_{P_i}(\lambda)\, l_c\, C_{P_i}, \tag{7}$$

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, (7) is rewritten as

$$C_{P_i} \;=\; \frac{A_{P_i}(\lambda)}{a_{P_i}(\lambda)\, l_c}, \tag{8}$$

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 are also referred to as extinction coefficients in the established literature (Jeffrey et al. 1997) and vary depending on wavelength and the solvent in which the compound is suspended. Consequently, 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:

$$C_{S_i} = \frac{A_{S_i}(\lambda_m) - A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c}, \tag{9}$$

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 properly determined and documented. 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:

$$C_{S_i} = \frac{\hat{A}_{S_i}\, R_{P_i}}{V_c}, \tag{10}$$

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 from above is applied, which produces the following:

$$R^{\Sigma}_{P_i} = \frac{V_c C_{S_i}}{\Sigma\hat{A}_{S_i}}, \tag{11}$$

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, (9) is substituted for C_{S_i} in (11), and the terms rearranged to yield:

$$R^{\Sigma}_{P_i} = \frac{A_{S_i}(\lambda_m) - A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c}\, \frac{V_c}{\Sigma\hat{A}_{S_i}}, \tag{12}$$

and noting that $\Sigma\hat{A}_{S_i}$ can be used directly in (10) for the peak area term, and then (9) and (10) 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:

$$\hat{A}^{\%}_{S_i} = \frac{\hat{A}_{S_i}}{\Sigma\hat{A}_{S_i}}, \tag{13}$$

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

$$R^{\%}_{P_i} = \frac{C_{S_i}\, V_c\, \hat{A}^{\%}_{S_i}}{\hat{A}_{S_i}}, \tag{14}$$

but once (13) is substituted into (14), and (9) is applied, the relationship becomes:

$$R^{\%}_{P_i} = \frac{A_{S_i}(\lambda_m) - A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c}\, \frac{V_c}{\Sigma\hat{A}_{S_i}}, \tag{15}$$

which is equivalent to (12).

Given the equivalence of (12) and (15), it is logical to wonder why two techniques for calculating response factors are desirable. 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:

$$C_{P_i} = \frac{V_x}{V_f} \hat{A}_{P_i} \left[\frac{A_{S_i}(\lambda_m) - A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c\, \Sigma \hat{A}_{S_i}} \right], \qquad (16)$$

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

Added 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 internal 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, (16) becomes

$$C_{P_i} = \frac{\hat{A}_{c_1}}{\hat{A}_{s_1}} \frac{V_m}{V_f} \hat{A}_{P_i} \left[\frac{A_{S_i}(\lambda_m) - A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c\, \Sigma \hat{A}_{S_i}} \right], \qquad (17)$$

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. This is not to imply that any terms not present in the governing equation should be ignored (for example, the cancellation of V_c for the assumptions presented here). It is important to scrutinize each step of the protocol to ensure all parts are properly understood.

1.4 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 HPLC methods presented here (Chaps. 2–11) are based on diverse objectives, but are most commonly used with samples from a variety of environmental regimes:

- *C* Based on the VHT method and used predominantly with temperate water samples;
- *D* Based on the VHT method and used mostly with samples from freshwater estuaries and coastal areas;
- *E* Based on the Egeland et al. (1995) method and used with primarily for research purposes;
- *G* Based on the VHT method and used primarily for methodological research with a wide diversity of aquatic samples;
- *H* Based on the VHT method and used with a wide variety of water samples from freshwater lakes, estuarine ecosystems, and the oligotrophic ocean;
- *I* Based on the Wright et al. (1991) method and used with a wide range of pigment concentrations from water types within the Indian Ocean and its environs;
- *K* Based on the Zapata et al. (2000) method and used with a wide range of pigment concentrations from water types in the Southern Ocean and eastern Pacific Ocean;
- *L* Based on the VHT method and used initially with Case-1 (open ocean) samples, but also successfully with Case-2 (coastal) waters;
- *N* Based on the Wright et al. (1991) method and used with a wide range of oceanic samples;

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. For this and subsequent presentations, 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.

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, 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
E	1.0	100% Acetone	None	None	≥ 18	0.2 µm Anotop syringe filter
G	1.8	95% Acetone	Vitamin E acetate	Sonic probe ∼15 s	3–4	0.45 µm Teflon syringe filter
H	2.6	95% Acetone	Vitamin E acetate	Sonic probe ∼6 s	4	0.45 µm Teflon syringe filter
I	10.0	90% Acetone	Vitamin E acetate	Sonicating bath ∼10 min	24	0.45 µm Teflon syringe filter
K	2.96	90% Acetone	None	Sonic probe 12 s	24	0.45 µm Teflon syringe filter
L	3.0	100% Methanol	Vitamin E acetate	Sonic probe 10 s	2†	0.7 µm GF/F filter
N	2.0	100% Methanol	Vitamin E acetate	Sonic probe 20 s	2	Centrifuge 3 min (13,000 rpm)
O	2.5	95% Methanol	*trans*-β-apo-8′-carotenal	Sonic probe 60 s	0.5	Centrifuge 5 min 0.2 µm Teflon filter
T_8	1.0	100% Acetone	Cantha-xanthin	Sonic probe ∼30 s	0.5	Centrifuge 1 min 0.2 µm Teflon filter
T_{18}	1.0	100% Acetone	Cantha-xanthin	Sonic probe ∼30 s	0.5	Centrifuge 1 min 0.2 µm Teflon filter

† The sum of soaking for 1 h, sonicating, and then soaking for another 1 h.

‡ Plus a 0.2 µm Teflon membrane filter.

O Based on the Zapata et al. (2000) method and used with a wide range of pigment concentrations from coastal and oceanic waters;

T_8 Based on the VHT method and tested initially with coastal water samples;

T_{18} Based on a modification of the Wright et al. (1991) method and used with a wide range of pigment concentrations from open-ocean, estuarine, and freshwater environments.

Note there are 11 laboratories and 12 methods, but essentially only 3 distinctly different methods. It is important to remember, however, that the implementation of a common method always results in differences that will distinguish 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*, *N*, and *O* used methanol. Sonic disruption predominated, although one method (*E*) used none. The soak time for the extract ranged from 0.5 h to 24 h, and clarification was an almost equal combination of centrifugation and filtration. The internal standard used by *C*, *D*, *G*, *H*, *L*, and *N* was vitamin E acetate; whereas *E* and *K* did not use one, *O* used *trans*-β-apo-8′-carotenal, and T_8 and T_{18} used Cantha.

A summary of the HPLC column separation procedures and solvent systems used by the SeaHARRE-5 participants are given in Tables 7 and 8, respectively. The type of stationary phase divides the 10 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 except *E* used an injection buffer, and the majority of the methods are two-solvent systems, except *N* used a three-solvent system, and *E* and T_8 used a four-solvent system. The flow rates of the methods are very similar except for laboratory *L*, which had a flow rate that is half as much as the others (a lower flow rate is re-

Table 7. A summary of the HPLC separation procedures used by the SeaHARRE-5 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. Photo-diode array (PDA) or diode array detector (DAD) systems were used.

Meth.	*Stationary*	*Column*				*Detector and Monitoring Wavelength*	
Code	*Phase*	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 2996	436
D	C_8	3.5	150	4.6	60°C	Shimadzu SPD-M10A VP-DAD	222†, 450, and 665
E	C_{18}	5.0	250§	4.6	25°C	Agilent 1100	390, 420, 450, and 480
G	C_8	3.5	150	4.6	60°C	Agilent 1200	222†, 450, and 665
H	C_8	3.5	150	4.6	60°C	Agilent 1100	222†, 450, and 665
I	C_{18}	5.0	150	4.6	25°C	Agilent 1100	436 and 450
J	C_8	3.5	150	4.6	60°C	Agilent 1100	222†, 450, and 665
K	C_8	3.5	150	4.6	25°C	Agilent 1100	440
L	C_8	3.5	150	3.0	60°C	Agilent 1100	222†, 440, 667, and 770
N	C_{18}	5.0	150	4.6	Room	Agilent 1100	436 and 450
O	C_8	3.5	150	4.6	Room	Shimadzu SPD-M10A VP-DAD	436 and 450
T_8	C_8	5.0	250	4.6	30°C	Waters PDA 996	409–480
T_{18}	C_{18}	5.0	250	4.6	30°C	Waters PDA 996	409–480

† Used for monitoring vitamin E (the internal standard).
§ Two columns are used, both with the same specifications.

Table 8. A summary of the HPLC solvent systems used with the SeaHARRE-5 methods: MeCN is acetonitrile, NH_4Ac is ammonium acetate, EtOAc is ethyl acetate, MeOH is methanol, TbAA is tetrabutyl ammonium acetate, and BHT is butylated hydroxytoluene (an antioxidant). Acetone, hexane, and pyridine are abbreviated ace., hex., and pyr., respectively. The flow rate is in units of milliliters per minute. The last column presents the initial conditions for the gradients, which are all based on solvents A and B, except method T_{18}. Individual method chapters should be consulted for documentation regarding the complete gradient programs.

Meth.	*Injection*	*Flow*	*Mobile Phase Solvent*				*Initial*	
Code	*Buffer*	*Rate*	A	B	C	D	*A*	*B*
C	TbAA:MeOH†	1.1	70:30 28 mM TbAA:MeOH	MeOH			95	5
D	TbAA:MeOH†	1.1	70:30 28 mM TbAA:MeOH	MeOH			95	5
E	None	0.5	1 M NH_4Ac	MeOH	Ace.	Hex.	20	80
G	TbAA:MeOH†	1.1	70:30 28 mM TbAA:MeOH	MeOH			95	5
H	TbAA:MeOH†	1.1	70:30 28 mM TbAA:MeOH	MeOH			95	5
I	H_2O	1.0	80:20 MeOH: 0.5 M NH_4Ac + 0.01% BHT	87.5:12.5 MeCN: H_2O + 0.01% BHT	EtOAc		100	
K	H_2O	1.0	50:25:25 MeOH:MeCN:0.24 M aq. Pyr.	20:60:20 MeOH:MeCN:Ace.			100	
L	TbAA¶	0.55	90:10 28 mM TbAA:MeOH	MeOH			95	5
N	0.5 M NH_4Ac	1.0	80:20 MeOH:0.5 M NH_4Ac	90:10 MeCN:H_2O	EtOAc		100	
O	0.5 M NH_4Ac	1.0	50:25:25 MeOH:MeCN:0.24 M aq. Pyr.	20:60:20 MeOH:MeCN:Ace.			100	
T_8	TbAA:MeOH†	1.1	70:30 28 mM TbAA:MeOH	MeOH			95	5
T_{18}	H_2O	1.1	MeOH:NH_4Ac:BHT	MeCN:BHT	EtOAc	H_2O	80	20§

† 28 mM TbAA:MeOH in a 90:10 (v:v) mixture, and the TbAA has a 6.5 pH.
‡ Variable flow rate of 0.8–1.5 mL min^{-1}.
§ Initial conditions for T_{18} are 80% A and 20% D.
¶ 28 mM aqueous TbAA with 6.5 pH.

quired with the narrower internal diameter column used by L).

1.5 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 contributing laboratories or methods:

$$\bar{C}_{P_i}^{L_j}(S_k) \;=\; \frac{1}{N_R}\sum_{l=1}^{N_R} C_{P_i}^{L_j}(S_{k,l}), \tag{18}$$

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 (18), the i index represents an arbitrary ordering of the pigments, and the j index is used for summing over the 10 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, $j = 1, 2, \ldots, 10$ corresponds to the C, D, E, G, H, I, K, L, N, O, T_8, and T_{18} methods, respectively (which is based on a simple alphabetic ordering of the method 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 (or batches). 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):

$$\bar{C}_{P_i}^{A}(S_k) \;=\; \frac{1}{N_L}\sum_{j=1}^{N_L} \bar{C}_{P_i}^{L_j}(S_k), \tag{19}$$

where the superscript A denotes an average across all (applicable) methods, and N_L is the number of laboratories or methods quantitating a pigment. For the primary pigments, $N_L = 10$, but for the secondary and tertiary pigments N_L is frequently less than 10 (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

$$\psi_{P_i}^{L_j}(S_k) \;=\; 100\,\frac{C_{P_i}^{L_j}(S_k) \;-\; \bar{C}_{P_i}^{A}(S_k)}{\bar{C}_{P_i}^{A}(S_k)}. \tag{20}$$

Note that the formulation in (20) 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 $\bar{C}_{P_i}^{A}$ 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:

$$|\bar{\psi}|_{P_i}^{L_j} \;=\; \frac{1}{N_S}\sum_{k=1}^{N_S} \left|\psi_{P_i}^{L_j}(S_k)\right|, \tag{21}$$

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 or methods (N_L) reporting the pigment involved:

$$|\bar{\psi}|_{P_i}^{A} \;=\; \frac{1}{N_L}\sum_{j=1}^{N_L} |\bar{\psi}|_{P_i}^{L_j}, \tag{22}$$

where the A code indicates all the laboratories were averaged (and $\bar{\psi}_{P_i}^{A}$ values are formed in a similar fashion from the UPD values). In general, (22) is only computed for the primary pigments, so $N_L = 10$.

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

$$\xi_{P_i}^{L_j}(S_k) \;=\; 100\,\frac{\sigma_{P_i}^{L_j}(S_k)}{\bar{C}_{P_i}^{L_j}(S_k)}, \tag{23}$$

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:

$$\bar{\xi}_{P_i}^{L_j} \;=\; \frac{1}{N}\sum_{k=1}^{N_S}\xi_{P_i}^{L_j}(S_k). \tag{24}$$

Subsets of the laboratories (or methods) involved are also used for different aspects of the analysis. One of the most important results is the estimation of the uncertainties in the reported data products, which requires an estimate or *proxy* for the truth. From the perspective of pigment concentration, there is no *a prior* understanding of truth with a field sample, so a procedure must be adopted to determine it. For the SeaHARRE activity, the performance metrics established during SeaHARRE-2 (Hooker et al. 2005) and expanded during SeaHARRE-3 (Hooker et al. 2009) and SeaHARRE-4 (Hooker et al. 2010) are used to determine the quality of the results from the participating laboratories. The methods satisfying the quantitative level of performance (Table 9) are considered to have uncertainties sufficiently low to be considered quality-assured (QA) methods and, thus, acceptable representatives of truth. The results from the other methods are considered to be not validated at the QA level (NV) and are not used in determining truth.

To compute uncertainties, (19) is modified to include only the contributions from the QA subset:

$$\bar{C}_{P_i}^{A'}(S_k) \;=\; \frac{1}{N_L}\sum_{j=1}^{N_L}\bar{C}_{P_i}^{L_j}(S_k), \tag{25}$$

where the A' code indicates the QA subset, and N_L is set to index over the laboratories within the QA subset. The UPD values (relative uncertainties) are then computed by using (25) in (20):

$$\psi_{P_i}^{L_j}(S_k) \;=\; 100\,\frac{C_{P_i}^{L_j}(S_k) \;-\; \bar{C}_{P_i}^{A'}(S_k)}{\bar{C}_{P_i}^{A'}(S_k)}, \tag{26}$$

where the reference value for truth in each calculation is given by $\bar{C}_{P_i}^{A'}(S_k)$. APD values are determined using (21).

Summary averages for the performance of the QA and NV subsets are derived from (22) by setting N_L to reflect the number of laboratories (or methods) within each subset. Although it is a notable achievement to be included in the QA subset—which is associated with a data quality level in keeping with calibration and validation activities—there are many research inquiries that do not require this level of expertise. A significant advantage of not initially being in the QA subset is all subsequent evaluations are truly independent, so a laboratory (or method) that is evaluated against the QA subset and found to have data products in keeping with the quantitative level of performance has achieved a singularly remarkable result. Indeed, one of the most useful aspects of the SeaHARRE activity is to provide the opportunity for any method to be evaluated against established performance capabilities and criteria.

The formulations presented in (18)–(26) 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.

1.6 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.

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 12 samples, $|\bar{\psi}|$, and the average precision is given as the average coefficient of variation (CV) across the 12 samples, $\bar{\xi}$. These are the principal parameters for determining method performance and the uncertainties in the methods.

Table 9. The current 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); pigment sums (also for pigment indices); pigment ratios; 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* during SeaHARRE-2 are given as an example (selected because SeaHARRE-2 represented an exhaustive inquiry into method performance). The overall performance of *H* is considered "state-of-the-art," because the average score of the weights is 3.6, $(4+4+4+3+4+3+4+3+3+4+4+4+3+4)/14$.

Performance Weight, Category, and Score	*TChl* a		*PPig*		*Sums*		*Ratios*		*Sep.*†		*Inj.*‡ ($\bar{\xi}_{\text{inj}}$)		*Cal.*§	
	$\bar{\xi}$	$\lvert\bar{\psi}\rvert$	$\bar{\xi}$	$\lvert\bar{\psi}\rvert$	$\bar{\xi}$	$\lvert\bar{\psi}\rvert$	$\bar{\xi}$	$\lvert\bar{\psi}\rvert$	$\check{R}_s$	$\bar{\xi}_{t_R}$	*Perid*	*Chl* a	$\lvert\bar{\psi}\rvert_{\text{res}}$	$\bar{\xi}_{\text{cal}}$
1. Routine 0.5	8%	25%	13%	40%	8%	20%	5%	15%	0.8	0.18%	10%	6%	5%	2.5%
2. Semiquantitative 1.5	5	15	8	25	5	12	3	9	1.0	0.11	6	4	3	1.5
3. Quantitative 2.5	3	10	5	15	3	8	2	6	1.2	0.07	4	2	2	0.9
4. State-of-the-Art 3.5	≤2	≤5	≤3	≤10	≤2	≤4	≤1	≤3	≥1.5	≤0.04	≤2	≤1	≤1	≤0.5
Method H	*1*	*5*	*2*	*12*	*2*	*5*	*1*	*5*	*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 ββ-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 to include 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.

The first step in the analysis of the SeaHARRE data is to establish the QA subset using the performance metrics (Table 9). Although is initially based on the precision obtained with the field samples, there is also the automatic exclusion of any method not providing results for all samples and all primary pigments (*C* and *I*). 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 (*E*, possibly *K*, and *O*). In addition, a laboratory with three or more primary pigments with a precision exceeding routine capabilities (13%) is considered for exclusion, but no other methods are considered for exclusion based on this criterion.

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 average for the reference group. From an overall performance perspective, these thresholds are based on an approximately equal evaluation of both laboratory standards and field samples, but because the difference between methods is not always as distinctive with laboratory standards as it is with field samples, greater weight is given to the results achieved with field samples when it comes to establishing the QA subset. This procedure possibly removes *K*, but definitely removes *N*, T_8, and T_{18}, 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. These more extended inquiries suggest the exclusion of *K*. This process is considered cautiously, 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. 1.4, the majority

of the methods used during SeaHARRE-5 were based on C_8 columns, and more specifically, were based on a single C_8 method (VHT).

Finally, the remaining laboratories in the QA subset are intercompared. Any method with more than three individual pigments exceeding an uncertainty of 25% is normally 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 excludes K and leaves D, G, H, and L as the QA subset. 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 of them, except G, participated in SeaHARRE-2 and SeaHARRE-3, and two of them (H and L) participated in all SeaHARRE activities.
- All used the same C_8 method (VHT).
- With the exception of G, all routinely analyze a large number of samples per year and have significant analytical commitments to an established user base.
- The participation of G was designed to be a demonstration of what a laboratory with HPLC analysis experience (SeaHARRE-4) was capable of achieving if the VHT method and the performance metrics for quantitative analysis were strictly followed on a brand new HPLC system.

The latter point is particularly relevant, because it shows the power of the performance metrics. The analyst in this case was an experienced HPLC analyst, but who had not used the selected method or the new hardware.

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 C, E, I, K, N, O, T_8, and T_{18}. In comparison, for the laboratories not in the QA subset:

- Only C and N participated in one or more prior 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 (C) at the production level of analysis (thousands of samples per year) to exclusive analyses for research purposes (E).

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

Because of problems with a newly adopted extraction protocol, method C analyzed a second set of samples using the established extraction protocol and these results are denoted C'. The first set of results are part of the A^+ subset as already noted, and the C' results are evaluated independently and are not assigned to a particular subset, which follows prior SeaHARRE practice (Hooker et al. 2009). The C' analyses were done prior to the workshop where all the results were discussed (Sect. 1.7), but were not finalized until after the workshop.

1.6.1 Average Pigment Concentrations

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 (25) 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-5 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 $\left[\text{TChl } a\right]$, typically two orders of magnitude, and the table shows this was partially achieved for SeaHARRE-5. Although the dynamic range in chlorophyll is not as large as desired, the dynamic range in several of the other primary pigments exceeds what has been experienced in prior SeaHARRE activities. The dynamic range in Peri, for example, is more than two orders of magnitude with elevated concentrations across the entire range.

Table 10 also shows the laboratories not part of the QA subset are typified by lower concentrations, which can exceed 30% on average (e.g., Caro). The most notable case of overestimation is Zea, which is exceeds the A' average value by more than 30%. There are many cases of similar concentrations between the two subsets (e.g., TChl *a*, Hex, Diad, and Peri), but given the objective of average $\left[\text{PPig}\right]$ uncertainties to within 25% and $\left[\text{TChl } a\right]$ uncertainties to within 15%, the occurrence of large differences represents significant performance challenges.

From a generalized perspective, any pigment with an average concentration less than $0.050\,\text{mg}\,\text{m}^{-3}$ can be considered to be at a low concentration. Table 10 shows some of the carotenoids are below this limit with But and Diato being notably so, and Zea being nearly so. The other carotenoids have average concentrations above this limit and usually do not fall below it. The persistent low concentrations of But and Diato provide a significant opportunity for false positives, i.e., a pigment is reported 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,

Table 10. The average individual PPig concentrations for the QA subset (D, G, H, and L) as a function of the batch sample code 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. Overall values for the second set of results from the C method and the methods not in the QA subset (C, E, I, K, N, O, T_8, and T_{18}) are presented in the corresponding pair of three rows and are denoted by the C' and A^+ notations, respectively.

Code	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]
A	0.711	0.043	0.049	0.042	0.002	0.001	0.095	0.022	0.003	0.091	0.029	0.012
B	1.459	0.148	0.112	0.069	0.001	0.003	0.191	0.052	0.002	0.194	0.054	0.009
C	0.912	0.087	0.067	0.045	0.001	0.001	0.106	0.029	0.003	0.135	0.038	0.010
D	1.682	0.163	0.403	0.070	0.052	0.346	0.132	0.138	0.018	0.337	0.027	0.037
E	1.846	0.258	0.352	0.102	0.053	0.274	0.097	0.171	0.023	0.333	0.044	0.170
F	1.549	0.220	0.281	0.095	0.040	0.249	0.091	0.113	0.014	0.237	0.024	0.139
G	3.876	0.242	0.464	0.147	0.013	0.072	0.377	0.182	0.020	0.717	0.176	0.018
H	2.353	0.260	0.433	0.102	0.057	0.246	0.107	0.259	0.045	0.715	0.024	0.090
I	3.522	0.471	0.700	0.164	0.108	0.497	0.211	0.371	0.051	0.869	0.080	0.127
J	5.243	0.810	0.880	0.295	0.052	0.787	0.355	0.299	0.032	0.780	0.123	0.198
K	5.198	0.740	0.886	0.267	0.088	0.708	0.332	0.536	0.064	0.820	0.330	0.236
L	2.143	0.346	0.172	0.109	0.014	0.096	0.169	0.100	0.016	0.309	0.051	0.087
AA	4.232	0.045	0.846	0.072	0.001	0.003	0.010	0.425	0.008	0.422	2.514	0.006
AB	11.662	0.083	2.369	0.205	0.001	0.001	0.036	1.284	0.014	0.531	8.287	0.010
AC	8.899	0.132	1.719	0.171	0.001	0.001	0.042	0.888	0.011	0.639	5.640	0.011
AD	1.465	0.081	0.272	0.035	0.004	0.028	0.010	0.129	0.007	0.252	0.561	0.019
AE	7.792	0.101	1.547	0.148	0.001	0.011	0.051	0.689	0.026	1.085	3.242	0.015
AF	1.248	0.132	0.153	0.045	0.001	0.002	0.059	0.085	0.004	0.142	0.287	0.038
AG	1.015	0.141	0.053	0.048	0.001	0.001	0.089	0.037	0.002	0.056	0.075	0.090
AH	5.191	0.084	1.030	0.103	0.008	0.030	0.014	0.612	0.023	0.302	3.612	0.022
AI	1.547	0.202	0.247	0.049	0.006	0.047	0.019	0.137	0.005	0.134	0.525	0.035
AJ	2.574	0.144	0.472	0.059	0.004	0.030	0.018	0.315	0.009	0.116	1.457	0.019
AK	9.051	0.240	1.323	0.255	0.048	0.006	0.062	0.476	0.037	2.951	0.741	0.025
AM	4.158	1.259	0.404	0.136	0.001	0.013	0.053	0.278	0.024	0.198	1.102	0.050
$\hat{C}_{P_i}^{A'}$	11.662	1.259	2.369	0.295	0.108	0.787	0.377	1.284	0.064	2.951	8.287	0.236
$\bar{C}_{P_i}^{A'}$	3.722	0.268	0.635	0.118	0.023	0.144	0.114	0.318	0.019	0.515	1.210	0.061
$\check{C}_{P_i}^{A'}$	0.711	0.043	0.049	0.035	0.001	0.001	0.010	0.022	0.002	0.056	0.024	0.006
$\hat{C}_{P_i}^{C'}$	11.527	1.160	2.606	0.300	0.142	0.735	0.337	1.458	0.052	2.425	8.423	0.244
$\bar{C}_{P_i}^{C'}$	3.403	0.252	0.740	0.124	0.027	0.138	0.104	0.356	0.014	0.469	1.218	0.056
$\check{C}_{P_i}^{C'}$	0.686	0.001	0.033	0.030	0.001	0.001	0.001	0.020	0.001	0.048	0.001	0.001
$\hat{C}_{P_i}^{A^+}$	15.157	1.548	2.853	0.293	0.137	0.951	0.535	1.528	0.086	3.617	8.320	0.609
$\bar{C}_{P_i}^{A^+}$	3.568	0.285	0.502	0.079	0.020	0.137	0.100	0.311	0.017	0.486	1.154	0.084
$\check{C}_{P_i}^{A^+}$	0.373	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.027	0.001	0.001

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 frequently yields an uncertainty of many hundreds of percent. So the large overestimation of Zea within the A^+ subset is a likely source of large uncertainties with respect to the relatively smaller A' values. A false positive can occur if a method is more sensitive than other methods, so the ability to detect the pigment below a particular concentration permits quantitation, but the threshold is sufficiently high to cause recurring instances of null values by the other less sensitive methods. A more common cause of false positives is coelution, which causes an elevated peak area. The DVChl *a* results for the A^+ subset exhibit false positives, which are associated with more than one method.

A false negative occurs when a method reports a pigment is not present when in fact it is. False negatives 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.

Code	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
A	0.697	0.001	0.013	0.010	0.001	0.004	0.056	0.074	0.002	0.003	0.022
B	1.422	0.001	0.037	0.022	0.015	0.037	0.049	0.088	0.018	0.022	0.032
C	0.891	0.001	0.022	0.012	0.011	0.021	0.036	0.086	0.014	0.010	0.022
D	1.567	0.001	0.115	0.006	0.020	0.041	0.012	0.138	0.042	0.021	0.043
E	1.693	0.001	0.153	0.018	0.033	0.071	0.015	0.161	0.055	0.038	0.188
F	1.452	0.001	0.097	0.013	0.030	0.060	0.016	0.153	0.048	0.029	0.152
G	3.774	0.001	0.102	0.011	0.027	0.056	0.046	0.180	0.041	0.029	0.029
H	2.097	0.001	0.257	0.019	0.035	0.073	0.018	0.308	0.079	0.038	0.110
I	3.102	0.001	0.420	0.034	0.059	0.125	0.022	0.304	0.115	0.066	0.161
J	4.909	0.001	0.334	0.055	0.094	0.204	0.040	0.324	0.106	0.110	0.254
K	4.808	0.001	0.390	0.069	0.086	0.196	0.042	0.273	0.114	0.110	0.304
L	2.069	0.001	0.074	0.065	0.038	0.097	0.105	0.168	0.043	0.060	0.152
AA	4.120	0.001	0.113	0.001	0.008	0.019	0.065	0.063	0.005	0.010	0.007
AB	11.537	0.001	0.125	0.001	0.014	0.039	0.129	0.091	0.006	0.026	0.010
AC	8.766	0.001	0.133	0.003	0.019	0.047	0.104	0.088	0.024	0.028	0.014
AD	1.394	0.001	0.071	0.005	0.009	0.018	0.015	0.086	0.012	0.009	0.024
AE	7.161	0.001	0.631	0.005	0.018	0.044	0.100	0.229	0.025	0.027	0.021
AF	1.220	0.001	0.028	0.026	0.019	0.051	0.058	0.038	0.021	0.032	0.064
AG	0.998	0.001	0.017	0.045	0.025	0.066	0.048	0.020	0.002	0.041	0.135
AH	4.998	0.001	0.193	0.001	0.014	0.036	0.056	0.052	0.017	0.022	0.023
AI	1.533	0.001	0.014	0.002	0.024	0.054	0.017	0.014	0.049	0.030	0.037
AJ	2.558	0.001	0.016	0.001	0.017	0.036	0.026	0.008	0.030	0.020	0.019
AK	7.074	0.001	1.977	0.022	0.038	0.087	0.211	1.939	0.051	0.049	0.047
AM	4.107	0.001	0.051	0.070	0.134	0.326	0.078	0.033	0.309	0.192	0.120
$\hat{C}_{P_i}^{A'}$	11.537	0.001	1.977	0.070	0.134	0.326	0.211	1.939	0.309	0.192	0.304
$\bar{C}_{P_i}^{A'}$	3.498	0.001	0.224	0.021	0.033	0.075	0.057	0.205	0.051	0.043	0.083
$\check{C}_{P_i}^{A'}$	0.697	0.001	0.013	0.001	0.001	0.004	0.012	0.008	0.002	0.003	0.007
$\hat{C}_{P_i}^{C'}$	11.527	0.001	0.001	0.071	0.124	0.385	0.001	1.535	0.376	0.262	0.309
$\bar{C}_{P_i}^{C'}$	3.403	0.001	0.001	0.017	0.026	0.070	0.001	0.064	0.068	0.044	0.073
$\check{C}_{P_i}^{C'}$	0.686	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
$\hat{C}_{P_i}^{A^+}$	14.734	0.130	2.405	0.086	0.131	0.743	0.280	3.367	0.425	0.250	0.609
$\bar{C}_{P_i}^{A^+}$	3.370	0.006	0.143	0.016	0.020	0.062	0.044	0.145	0.043	0.031	0.091
$\check{C}_{P_i}^{A^+}$	0.373	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

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 (26). 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%. A false negative can occur if a method is less sensitive than other methods, so the ability to detect the pigment below a particular concentration prevents quantitation, but the threshold is sufficiently high to cause recurring instances of null values by the less sensitive method. The Chlide *a* and Phytin *a* results for C' are examples of false negatives.

The influence of false positives and negatives on method uncertainty is usually minimal for pigments that are almost always in an oceanic sample (e.g., Chl *a*), but they are particularly important for 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. There are many pigments with average concentrations below $0.050\,\mathrm{mg\,m^{-3}}$, most notably Lut, Neo, and Viola. The A^+ subset results for the secondary and tertiary pigments are characterized by usually lower average concentrations with respect to the A' subset, but the magnitude of the differences are frequently greater than was seen with the PPig results (Table 10).

Table 12. The method precision results for the individual PPig $\bar{\xi}$ values (coefficients of variation in percent) across all 24 batches of field samples. The data are presented as a function of the laboratory (or method) with the last column presenting the overall (horizontal) method average across the pigments. The overall (vertical) QA subset averages are given in the A' entries, and the methods that were not validated at the QA level in the A^+ entries. The lowest precision 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 bottom row. The repeat analyses made by laboratory C are indicated by the C' results, which are not included in the determination of either subset or any of the summary averages; they are presented separately and are shown in slanted typeface. A blank entry indicates a particular laboratory (or method) did not quantitate the pigment indicated; none of the averages include contributions from blank entries.

Method	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Avg.*
D	4.6	4.5	6.3	7.1	5.4	5.3	7.2	8.0	13.8	5.2	9.6	10.6	7.3
G	5.8	6.4	6.3	7.3	**2.2**	3.1	5.5	6.5	**9.2**	5.1	9.7	6.1	6.1
H	4.5	4.4	**4.9**	**4.6**	3.7	4.9	**4.7**	**5.6**	14.1	4.9	**8.3**	**5.6**	5.8
L	**4.1**	**4.3**	5.5	7.2	3.6	**2.3**	4.8	6.4	10.7	**4.5**	8.5	6.1	**5.7**
C	6.0	5.7	5.9	5.8	2.1	3.7	5.1	7.1	5.3	6.7	4.1	4.2	5.1
E	7.8	14.8	34.3	13.7	5.1	3.3	10.1	18.1	18.5	8.0	19.1	11.2	13.7
I	12.6	35.1	25.7	73.0	23.1	18.3	6.0	23.7		44.1	35.3	28.1	29.5
K	6.3	14.5	11.1	8.0	10.6	2.9	5.4	6.0	20.7	5.2	7.0	9.3	8.9
N	5.6	6.0	7.4	7.7	6.0	8.2	5.6	10.1	11.6	5.3	11.8	6.3	7.6
O	8.8	7.5	10.5	44.0	2.3	7.0	23.0	8.1	6.7	13.0	11.5	17.1	13.3
T_8	5.8	6.1	4.4	4.5	1.4	1.9	4.5	6.7	5.2	5.1	5.5	4.9	4.7
T_{18}	8.4	8.5	6.5	5.6	1.2	2.6	4.4	6.6	7.7	5.6	6.5	4.3	5.7
C'	*5.1*	*5.1*	*4.7*	*5.6*	*1.7*	*2.9*	*5.2*	*6.1*	*6.7*	*5.2*	*5.3*	*3.7*	*4.8*
A'	4.7	4.9	5.7	6.6	3.7	3.9	5.6	6.6	12.0	4.9	9.0	7.1	6.2
A^+	7.9	13.2	14.3	22.4	7.1	6.3	8.4	11.3	11.7	12.3	13.8	11.6	11.7
A^-	4.1	4.3	4.9	4.6	2.2	2.3	4.7	5.6	9.2	4.5	8.3	5.6	5.0

1.6.2 Method Precision

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 (Avg.) across all primary pigments for each laboratory, 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 prior SeaHARRE activities.

In most cases, the average method precision results for the primary pigments show superior precision is associated with [TChl *a*], and there is a general worsening of precision for the other chlorophylls and carotenoids—this is particularly true for the A^+ subset. The best average precision in both the A' and A^+ subsets are for But and Hex, which were two of the lowest primary pigments in the average concentration (Table 10). As shown in SeaHARRE-3 (Hooker et al. 2009), the good results for But is 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 or minimally observed in those samples, the precision will be very good.

The A' average precisions fall within a narrow range of outcomes, 5.7–7.3%. Despite the tight grouping within the A' averages, each individual method exhibits at least one anomalously high precision value, with Diato exhibiting double-digit precision for all but one of the methods. These distinctions are important indicators as to where additional work needs to be done by the individual analysts in particular, and the SeaHARRE activity in general.

The individual instances of excellent precision for specific methods and pigments within the QA subset—and also in many entries for the other methods—show excellent precision is a recurring outcome across all pigments. The precision of the selected best results in the QA subset (the bold entries in Table 12) ranged from 1.4–4.2%, with an overall average of 5.0% (the overall average for the previous SeaHARRE activity was 3.4%). The narrow range of precision excellence, and because it occurs across three methods (G, H, and L), 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 G, H, and L results have an overall precision to within almost

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]
D	4.9		20.0	13.0	13.0	8.9	12.1	12.0	5.0	10.5	10.7
G	7.3	0.0	15.9	10.2	6.3	5.6	10.6	15.8	**5.0**	6.9	5.9
H	5.3	0.0	15.0	5.1	**5.3**	**5.4**	**5.9**	**8.0**		**6.2**	**5.6**
L	**4.0**	0.0	**12.8**	**5.1**	8.0	6.7	10.6	16.0	5.2	6.7	6.0
C	6.0	0.0	0.5	3.0	4.2	4.1	2.1	3.6	4.1	5.4	4.1
E	7.8	6.5	0.0		8.7	9.1	27.4	8.6	10.1	7.1	11.2
I	12.6				16.3	16.3			36.1		28.1
K	6.5	24.1	13.0	19.9	19.8	16.5			9.7	12.9	13.7
N	5.9		11.7								6.3
O	8.4	7.1	10.5		8.6	11.0	13.2	17.3	4.8	3.6	17.1
T_8	6.0	0.0	1.2	5.9	6.7	5.7	6.4	20.4	4.6	7.5	4.6
T_{18}	9.2		6.9	7.5	7.8	5.1	11.1	6.4	4.9	7.1	4.6
C'	*5.1*	*0.0*	*0.0*†	*3.7*	*5.8*	*5.3*	*0.0*†	*0.3*†	*5.4*	*7.0*	*3.4*
A'	5.4	0.0	15.9	8.3	8.1	6.6	9.8	12.9	5.1	7.6	7.1
A^+	8.1	9.4	7.2	11.1	11.3	10.6	14.5	13.2	11.7	7.6	12.2
A^-	4.0	0.0	12.8	5.1	5.3	5.4	5.9	8.0	5.0	6.2	5.6

† Artificially low precision, because of a preponderance of false negatives.

1% of the A^- average shows the best method approach is achievable and a useful indicator of the state of the art.

The A^+ average precisions for each primary pigment show a larger range of outcomes, but not in all cases. Although the E, I, and O results have degraded precision with averages above 10%, the C, C', T_8, and T_{18} results are within the range of the A' averages, and the N results are nearly so. Like N, the K results stand out as only requiring small improvements to establish a precision in keeping with the QA subset. This shows the difficulty of using precision alone as a criteria for evaluating method performance. The point to remember here is that precision is the starting point, and it is very difficult for a method to achieve a suitable accuracy if method precision is poor, but that does not mean a good precision will ensure good accuracy—*it is a necessary, but not sufficient, criteria.*

Table 13 presents the precision obtained by the methods for a subset of the secondary and tertiary pigments using the format established for Table 12. These results largely confirm the precision conclusions obtained with the primary pigments, but there are some differences. The best precision of the QA subset occurs over a slightly larger range and is associated primarily with H. The worst results are seen with [Chlide *a*], which was present in reasonably high abundances (Table 11). Two other pigments with poor precision are Phytin *a* and Phide *a*, for which only H achieved somewhat reasonable results. As noted in Sect. 1.5, a precision value of 10% or more is a concern.

As anticipated from the PPig results, the A^+ results are usually worse than the A' results. 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 E, I, and O data. Although the C, C', T_8, and T_{18} results are in keeping with the A' results, with a few exceptions, the K results are frequently not and the N results are too incomplete for comment. The degradation of the K results for the secondary and tertiary pigments was one reason K was not included in the QA subset (Sect. 1.5). Once again, areas for additional research can be identified by anomalously poor precision for individual methods, e.g., Chlide *a*, Phytin *a*, and Phide *a* for both the A' and A^+ methods.

Table 14 presents the method precision for the DHI Mix-105 primary pigments. The results show the A' subset has better average precision than the A^+ subset, although some of the individual methods and pigments of the latter are superior, or are similar to, the former. For example, C, K, N, and T_8 are all distinguished by excellent precision, and the results for E and T_{18} are only slightly worse. The range in average precision for the A' subset is larger than what was seen in the last coastal SeaHARRE (which was 0.5–1.1%), but only because of the slightly poorer precision obtained by G and the poor precision in the L results for TChl *c*. The best and worst results include both C_8 and C_{18} methods, so there is no indication that one column is superior to the other in terms of precision.

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 (note, however, the false negatives obtained by N for Zea). Consequently, the precision obtained with the mix should be closer to the injection precision than the precision of a field sample. Nonetheless,

Table 14. The $\bar{\xi}$ values (coefficients of variation in percent) for DHI Mix-105 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^-.

Meth.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Avg.*
D	0.7	2.3	1.0	1.0	0.7	0.8	0.5	1.1	0.7	**0.5**	0.6	0.8	0.9
G	2.2	4.5	2.3	2.9	2.5	3.0	3.6	5.6	5.2	2.4	3.3	2.6	3.4
H	**0.1**	**0.2**	**0.5**	**0.3**	**0.4**	**0.7**	**0.5**	**0.6**	**0.0**	0.7	**0.3**	**0.2**	**0.4**
L	0.8	1.0	14.0	2.2	1.2	0.8	1.2	1.3	3.8	1.3	1.3	1.3	2.5
C	0.5	0.8	1.4	0.3	1.6	1.1	2.1	1.8	1.6	1.0	1.4	0.7	1.2
E	2.4	3.1	4.3	2.9	2.6	2.7	2.7	2.6	2.7	3.1	3.7	2.7	3.0
I	8.7	9.1	29.9	5.1	10.5	10.3	9.3	5.1		9.5	10.2	8.8	10.6
K	1.3	0.3	2.3	1.1	1.3	0.7	4.5	3.9	1.6	0.7	1.6	1.8	1.7
N	0.3	0.4	0.6	0.2	0.5	0.5	0.5	0.4	0.5	0.8	0.2	0.0†	0.4
O	1.4		5.3	6.4	8.8		11.1	13.0	7.1	16.1	8.3		8.6
T_8	1.2	3.1	1.3	1.2	1.8	2.3	1.0	1.9	1.5	3.8	1.3	2.2	1.9
T_{18}	1.6	2.1	2.6	5.8	4.1	4.7	2.6	5.6	4.4	3.7	6.2	2.9	3.9
A'	1.0	2.0	4.4	1.6	1.2	1.3	1.4	2.2	2.4	1.2	1.4	1.2	1.8
A^+	2.4	3.0	6.6	3.2	4.2	3.5	4.5	4.7	3.0	5.4	4.5	3.7	4.1
A^-	0.1	0.2	0.5	0.3	0.4	0.7	0.5	0.6	0.0	0.5	0.3	0.2	0.3

† Artificially low precision, because of persistent false negatives—not included in averages.

as will be shown later, the precision obtained with the mix is an important QA opportunity, which can provide a lot of information about method performance.

The A^- results (which are a proxy for a theoretical best method) are always to within 0.7% (which was also obtained for SeaHARRE-4) and have an overall average of 0.3%. This means they are within the 2% performance metric for state-of-the-art injection (Table 9), and two of the QA methods satisfy this metric, with one satisfying it for every pigment—as does the N method (except for Zea). Notably, the C, K, and T_8 methods satisfy the 2% threshold on average. The fact that so many methods achieved precision results close to or within this value is a notable indicator that using the best results from the QA subset produces a realistic and achievable metric for what a method should be capable of achieving.

1.6.3 Method Accuracy

Method accuracy for pigment products are determined using $|\bar{\psi}|$, the average APD between the reported laboratory values and the average concentrations constructed from the QA subset, that is, (26) is used in (21). Although the limitations already noted in determining method precision (Sect. 1.6.2) 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 negatives (Sect. 1.6.1), 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 the observation to the reference value (Fig. 2) will be a small number. The false positive situation, however, will usually be the most serious in terms of average uncertainties.

$$\begin{aligned} \psi &= 100\,\frac{C_O - C_R}{C_R} \\ &= 100\,\frac{nx - x}{x} \\ &= 100(n-1) \\ &\approx n100 \end{aligned} \qquad \begin{aligned} \psi &= 100\,\frac{C_O - C_R}{C_R} \\ &= 100\,\frac{x - nx}{nx} \\ &= 100(1-n)/n \\ &\approx -100 \end{aligned}$$

Fig. 2. The approximate RPD values, ψ, for a false positive (left) and false negative (right) observed concentration, C_O, with respective to a reference concentration, C_R. The terms of greatest magnitude are assumed to be n times larger than the smallest value x and are shown in red.

1.6.3.1 Individual Pigments

The performance metrics for QA (quantitative) performance 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. 1.6)—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.

Table 15. The $|\bar{\psi}|$ values (average APD in percent) across all 12 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.*
D	**3.3**	6.0	9.5	14.8	17.8	28.3	7.8	8.7	34.0	8.8	26.8	12.9	14.9
G	5.9	**5.0**	**7.4**	**11.1**	21.5	27.4	**7.5**	**8.0**	31.7	4.3	12.0	8.5	**12.5**
H	8.6	10.2	23.1	29.0	28.5	19.0	10.2	16.8	48.2	7.0	15.2	**6.4**	18.5
L	3.9	12.1	17.8	16.7	**17.4**	**16.8**	8.4	14.9	**27.3**	**3.6**	**9.1**	11.8	13.3
C	33.6	32.5	22.0	44.3	44.6	40.5	41.6	42.2	62.7	30.4	45.3	45.8	40.5
E	28.0	67.8	48.9	61.5	43.9	26.7	22.2	29.9	79.3	15.2	41.0	89.0	46.1
I	30.4	49.8	57.6	82.5	64.2	44.9	71.5	58.4		38.4	77.2	92.0	60.6
K	8.2	14.5	26.4	12.7	30.4	29.7	10.8	11.4	44.8	3.5	27.6	52.7	22.7
N	4.7	13.8	13.4	9.5	139.0	251.0	7.6	30.6	55.3	12.0	16.3	71.5	52.0
O	18.5	10.6	30.7	87.6	44.5	47.1	27.1	35.9	74.6	17.2	56.2	229.8	56.6
T_8	12.7	23.8	15.6	21.0	38.4	29.5	20.2	8.0	90.4	6.5	45.0	36.6	29.0
T_{18}	14.0	13.5	51.1	28.2	43.9	30.8	15.6	17.6	73.0	7.5	42.6	36.4	31.2
C'	*10.7*	*11.3*	*15.8*	*14.3*	*39.7*	*22.4*	*21.1*	*10.3*	*52.1*	7.5	*15.5*	*38.7*	*21.6*
A'	5.4	8.3	14.4	17.9	21.3	22.9	8.5	12.1	35.3	5.9	15.8	9.9	14.8
A^+	16.6	27.7	34.8	43.3	57.8	65.7	25.0	27.4	69.6	14.3	43.7	86.8	42.7
A^-	3.3	5.0	7.4	11.1	17.4	16.8	7.5	8.0	27.3	3.6	9.1	6.4	10.2

The individual and summary PPig uncertainties are presented in Table 15 and shows the overall average for the QA subset (A') is 14.8%, which satisfies the refinement threshold. Three of the QA methods have overall average uncertainties below 15%, which is a significant improvement over the SeaHARRE-4 results wherein only one method was within the 25% threshold (semiquantitative performance). The results for [TChl *a*] show all the QA methods are well within the 10% refinement threshold, and the average is one of the lowest TChl *a* uncertainties for all SeaHARRE activities. Furthermore, the overall A' average and almost all the individual A' methods have TChl *a* uncertainties within the 2–3% sampling variance for the state-of-the-art performance metric.

The overall averages for each pigment for the QA subset are frequently to within 15%, and with the exception of Diato, to within 25%. But and Hex are close to the 25% threshold, and significantly improved with respect to the SeaHARRE-4 results, wherein the uncertainties were 87.2 and 57.5%, respectively. Diato has a large uncertainty and was present in very low concentrations (the lowest of the primary pigments), which resulted in many false negatives and positives. Diato, But, and Hex have been problematic pigments in the past, and the challenges go beyond a single simplistic explanation, but if it were possible to get analysts to be more consistent in dealing with the challenges involved, the uncertainties would improve.

The results from the A^+ methods are frequently very different from the A' subset and are characterized by higher or significantly higher uncertainties. There are exceptions, however, the most notable of which are the K and C' results, and then the T_8 and T_{18} results. The former two have overall average PPig uncertainties to within 25%, and the latter two are almost so. The E, I, N, and O methods exceed the 40% routine performance metric (Table 9), but in some cases, only a few pigments are responsible for a disproportionate amount of uncertainty (e.g., But and Hex for N, and Zea for O). The semiquantitative performance metric for TChl *a* is nearly satisfied as an overall average, and the K and N methods satisfy the quantitative performance requirements (the C' results satisfy the latter to within a 2–3% sampling variance).

The problematic pigments within the A^+ subset are the same ones seen with the A' subset with the addition of Zea, for which multiple methods had anomalously poor results. The individual pigment results for the A^+ subset are further distinguished by all methods contributing large uncertainties to one or more of the problematic pigments (recalling that the results from K, T_8, T_{18}, and C' do not have significantly elevated uncertainties, which is reflected in the overall averages from these methods).

The best possible outcomes from the QA subset, the A^- entries, have an overall average TChl *a* and PPig uncertainty of 3.3 and 10.2%, respectively. These results are within the requirements for state-of-the-art and quantitative performance, respectively. The D, G, and L overall PPig average uncertainty also satisfies this metric, and if a 2–3% sampling variance is included, the G and very nearly the L methods can be considered state of the art for both TChl *a* and PPig. Note that the 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.

As noted earlier, the K and C' methods stand out within the A^+ subset, because both have the lowest overall average uncertainty in the A^+ subset. If the But and Zea results were not so anomalous (i.e., closer to the A' average), the overall PPig average uncertainty for K and C' would be within the range of uncertainties for the A' subset (i.e., 18.4 and 17.7%, respectively). What this shows is the value of a proper referencing system: it highlights which parts of a method need additional attention and which parts are performing adequately. The latter qualification is applicable to both methods, because SeaHARRE-5 is the first intercomparison of the K method and the C' results are being used to establish that the C method suffered from the change in extraction procedures and nothing else. In both cases, greater confidence will come from additional intercomparisons 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 then switched to the VHT method; all results from SeaHARRE-3 and SeaHARRE-4 were part of the QA subset.
- D participated in SeaHARRE-2, but switched to the VHT method for the SeaHARRE-3 activity; all results were part of the QA subset.
- E, I, K, O, and T have not participated in a prior SeaHARRE activity.
- G participated in SeaHARRE-4 as a novice analyst and results were part of the QA subset, but participated in SeaHARRE-5 as an experienced analyst using a new method and new hardware.
- H participated in all prior SeaHARRE activities and always produced results in the QA subset.
- L produced results that were part of the QA subset for SeaHARRE-1, but not for SeaHARRE-2; all subsequent results were part of the QA subset after switching to the VHT method for SeaHARRE-3.
- N participated in SeaHARRE-4.

Of these laboratories, H and L are the only ones to have participated in all prior SeaHARRE activities. Laboratories C and D have participated in four round robins, but are not using their original methods (and neither is L).

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 (recalling that it is not always possible to refine a method to achieve a desired separation, e.g., a C_{18} method cannot separate the monovinyl and divinyl Chl *a* forms). For the methods considered here, H has consistently produced QA results over an extensive (many year) time span, and C, D, and L have now emerged with sufficiently long time series of quality control (QC) variables. Comparisons to individual established methods, or the time series of the results from those methods, help establish confidence in many of the findings presented here.

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. For example, each method in the A^+ subset has at least one pigment with uncertainties that are large (exceeding 50%) or very large (exceeding 100%). The A' subset has much lower uncertainties, although H has a nearly large value for Diato. 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. From this perspective K, and perhaps T_8 and T_{18}, have results that suggest they should have been included in the QA subset. This dichotomy is addressed further below.

The 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. These data are usually not supplied by all the methods, so some of the results cannot be used at the same level of efficacy. The A' uncertainties are usually significantly lower than the corresponding A^+ values, but not in all cases. For example, note the T_8 and T_{18} results for Pras are within the range established by the A' results, as is the C' Pras result.

Despite some good results within the A^+ subset, the general distinction between the A' and A^+ results is almost all of the A^+ methods have one or more pigments for which the uncertainty is anomalously degraded, both with respect to the A' methods and to the other A^+ methods:

C	Chlide *a*, Phytin *a*, and Phide *a*;
E	Chlide *a* and Phide *a*;
I	Neo, Neo+Vio, and Pras;
K	DVChl *a* and Lut;
O	DVChl *a*, Viola, and Zea+Lut;
T_8	Chlide *a* and Pras; and
T_{18}	Neo+Vio.

Like the C results, the C' results exhibit anomalous uncertainties for Chlide *a*, Phytin *a*, and Phide *a*.

The presence of the anomalously high uncertainties in the A^+ subset, many of which are caused by false negatives, further reinforces the need for a properly established referencing system. 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

Table 16. The $|\bar{\psi}|$ values (average APD in percent) across all 24 batches of field samples for a subset of the secondary and tertiary pigments (following the presentation scheme established in Tables 12, 13, and 15).

Meth.	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
D	**3.4**		**14.4**	**19.0**	18.2	16.3	14.5	59.5	51.3	**16.2**	11.5
G	7.4	0	22.0	36.5	**9.2**	**12.1**	11.6	29.2	77.9	16.5	11.4
H	8.2	0	26.0	25.2	15.6	13.7	**9.2**	**24.1**	95.6†	17.9	**8.1**
L	4.7	0	34.2	27.4	15.1	12.8	12.2	39.6	**42.0**	17.4	11.8
C	31.0	0	90.1†	65.2	49.5	46.0	201.3	81.9†	51.2	48.1	46.5
E	35.2	39	99.0†		36.1	28.9	46.8	95.8†	46.1	33.4	37.4
I	26.5				92.0†	96.7†			83.4†		85.8
K	11.6	4,929	47.8	176.9	44.4	34.2			47.9	34.1	48.8
N	4.7		47.4								26.7
O	16.8	432	73.7		77.6	79.7	30.3	39.0	84.6	103.5	126.4
T_8	12.6	0	98.9†	37.0	25.3	20.2	48.3	69.1	57.9	15.7	38.9
T_{18}	13.2		64.4	54.6	33.3	138.9	38.2	63.6	41.8	28.9	40.2
C'	*7.4*	*0*	*99.0*†	*48.1*	*33.3*	*23.2*	*98.5*†	*95.7*†	*45.5*	*18.6*	*39.9*
A'	6.0	0	24.2	27.0	14.5	13.7	11.9	38.1	66.7	17.0	10.7
A^+	17.2	1,350	71.9	89.5	51.4	66.4	40.9	66.9	60.3	43.1	57.7
A^-	3.4	0	14.4	19.0	9.2	12.1	9.2	24.1	42.0	16.2	8.1

† Elevated uncertainty from persistent false negatives.

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.

A review of the governing equations, (1) and (6), reveals why results for the DHI Mix should be better: 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 to 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}). 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.

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. 1.6.2 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 identify and quantitate. Consequently, DHI Mix results should almost be at the highest quality level a method can achieve.

Method uncertainties for the DHI Mix-105 analyses are presented in Table 17. The QA subset has overall TChl *a* and PPig uncertainties of 2.6% and 9.6%, 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 of 1.8%. The low value of the theoretical best method, 6.8%, shows how many high-quality results were achieved for the individual pigments, but this is higher than what was achieved for SeaHARRE-4 (1.2%). The increase is caused by problems with the quantitation of TChl *b*, TChl *c*, and Diad, with the first two posing the most challenges. The TChl *b* are the worst and are caused by *D* and *G* not reporting any DVTChl *b*, whereas *H* and *L* do and at levels above MVTChl *b*.

The increased uncertainties in the QA subset are the result of the results splitting into two groups for the three identified pigments. For TChl *b*, *D* and *G* obtained similar results, whereas *H* and *L* were similar but different from *D* and *G*. This also occurred for TChl *c*, except *D* and *H* grouped together versus *G* and *L*. For Diato the results were more similar, except *G* was a bit more different than *D*, *H*, and *L*. The remaining A' average PPig uncertainties for DHI Mix-105 are within the state-of-the-art performance metric (Table 9). In terms of the individual methods and individual pigments, virtually all of the results are within, or close to, the state-of-the-art performance metric, except for the three pigments already noted. The best method (A^-) entries are spread across all four methods.

The difference between the A' uncertainties for robust field samples (i.e., those without quantitation problems) and their corresponding uncertainties in the DHI Mix-105

Table 17. The $|\bar{\psi}|$ values (average APD in percent) for DHI Mix-105 as a function of the primary pigments using the presentation scheme established in Tables 12, 13, and 15.

Meth.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Avg.*
D	3.5	51.5	18.6	2.6	**2.7**	2.0	4.5	12.1	**0.0**	**1.3**	1.7	3.6	**8.7**
G	**0.1**	51.3	**13.7**	0.4	8.8	6.4	**2.3**	12.5	6.4	3.0	**1.5**	4.9	9.3
H	1.7	58.4	22.3	1.9	4.2	**0.1**	4.0	**11.4**	3.7	2.0	8.2	**3.2**	10.1
L	5.2	**44.4**	27.2	**0.3**	7.3	4.4	2.8	13.2	2.6	2.3	11.4	5.3	10.5
C	1.2	61.8	57.8	5.7	4.2	0.4	2.5	6.5	8.6	2.2	11.0	27.0	15.7
E	4.7	89.8	8.0	42.7	17.1	5.8	11.3	17.5	19.4	43.7	12.2	118.9	32.6
I	5.3	88.9	57.8	3.9	9.0	1.0	37.1	47.9		32.2	14.1	32.8	30.0
K	6.5	63.3	5.1	4.6	29.9	3.5	27.4	6.9	29.1	2.5	4.2	21.0	17.0
N	9.7	61.3	6.4	10.7	29.7	33.1	0.6	4.3	5.7	6.4	15.2	99.9†	23.6
O	6.3	100.0†	90.4	47.4	76.5	99.9†	32.3	69.3	50.3	67.4	85.3	99.9†	68.7
T_8	4.7	63.6	13.1	7.7	7.4	5.8	5.6	1.4	2.0	3.2	5.8	2.9	10.3
T_{18}	7.0	75.4	30.7	15.3	3.6	3.8	4.0	12.0	2.3	72.4	8.0	3.9	19.9
A'	2.6	51.4	20.4	1.3	5.7	3.2	3.4	12.3	3.2	2.1	5.7	4.3	9.6
A^+	5.7	75.5	33.7	17.3	22.2	19.2	15.1	20.7	16.8	28.8	19.5	50.8	27.1
A^-	0.1	44.4	13.7	0.3	2.7	0.1	2.3	11.4	0.0	1.3	1.5	3.2	6.8

† Elevated uncertainty from persistent false negatives.

Table 18. The $|\bar{\psi}|$ values (average APD in percent) across the DHI Mix-105 analyses for a subset of the secondary and tertiary pigments (following the presentation scheme established in Tables 12, 13, and 15).

Meth.	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
D	3.5	2.6	19.9	**2.3**	9.7	8.8	19.9	15.0	3.6	8.2	3.0
G	**0.1**	**0.1**	14.7	2.4	4.6	**0.1**	10.5	14.0	8.7	**3.2**	3.6
H	0.5	9.2	**2.1**	3.6	3.0	4.2	**4.3**	**1.7**	**1.7**	5.0	**0.2**
L	3.0	11.9	36.6	8.3	**2.0**	4.5	13.7	30.7	10.6	6.3	6.8
C	3.8	21.8	98.0†	2.1	4.2	2.5	38.4	97.7†	1.4	1.4	14.6
E	3.6	5.3	98.0†		3.6	3.6	22.8	97.7†	24.3	3.5	9.9
I	36.4			104.7	12.0	53.3			9.1		68.6
K	6.3	19.3	98.0†	22.0	21.9	15.0			11.4	10.1	21.5
N	40.3		15.0								99.9†
O	6.6	1.9	98.0†		53.0	80.4	99.4†	97.7†	99.7†	99.7†	99.9†
T_8	2.7	14.9	98.0†	5.2	4.4	6.3	8.8	33.1	4.0	7.6	4.1
T_{18}	15.7		9.3	9.5	7.8	83.4	24.2		4.0	12.8	6.7
A'	1.8	6.0	18.3	4.2	4.9	4.4	12.1	15.3	6.1	5.7	3.4
A^+	14.5	12.7	73.4	28.7	15.3	34.9	38.7	81.6	22.0	22.5	40.7
A^-	0.1	0.1	2.1	2.3	2.0	0.1	4.3	1.7	1.7	3.2	0.2

† Elevated uncertainty from persistent false negatives.

analyses can be used to estimate the contribution of sampling variance to the uncertainty budgets. For example, the difference for TChl *a* is 2.8% and the difference for Fuco is 3.8% (the corresponding values for SeaHARRE-4 were 2.2% and 3.4%, respectively). This range of differences establishes a variance estimate of approximately 2–3% in field sample filtering (which is similar to the filtering variance experienced during SeaHARRE-2 and SeaHARRE-3).

The overall A^+ averages for the DHI Mix-105 analyses are 5.7% for TChl *a* and 27.1% for PPig; the former is within expectations, but the latter is not. Closer inspection shows the T_8 method is performing as expected, and the *C*, *K*, and T_{18} methods nearly so. For the latter, the Fuco results are a primary reason for elevated uncertainties, which is unusual, because Fuco is usually a robust pigment (as noted above). The remaining A^+ methods also have at least one pigment with anomalously high uncertainties. Again, these kinds of distinctions reinforce why a proper referencing system is so important.

The uncertainties for the analysis of secondary and tertiary pigments within DHI Mix-105 are 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 sig-

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 (following the presentation scheme established in Tables 12, 13, and 15). The PPig $|\bar{\psi}|$ values for 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.*
D	14.9	**3.0**	10.3	13.3	5.2	11.3	9.3	5.6	9.9	8.5
G	**12.5**	5.0	**6.7**	5.4	4.4	4.6	**4.2**	4.3	3.7	**4.8**
H	18.5	10.1	16.6	9.8	10.0	11.3	12.8	10.5	9.6	11.3
L	13.3	4.9	11.0	**4.5**	**3.9**	**4.2**	5.6	**4.0**	**3.6**	5.2
C	40.5	30.2	34.1	31.2	30.0	31.5	27.6	30.3	29.6	30.6
E	46.1	20.5	22.5	13.5	17.0	13.2	12.0	13.7	15.4	16.0
I	60.6	31.4	56.2	38.7	30.6	35.1	36.5	32.2	30.0	36.3
K	22.7	9.1	12.8	6.0	7.8	7.3	6.5	6.7	7.5	8.0
N	52.0	4.9	23.2	14.7	5.8	16.2	12.8	6.8	11.9	12.0
O	56.6	16.2	25.2	19.6	11.1	17.6	13.8	10.8	19.1	16.7
T_8	29.0	11.9	7.3	13.7	7.8	11.6	7.5	7.4	8.7	9.5
T_{18}	31.2	8.4	15.8	8.6	7.8	11.2	14.9	8.8	6.4	10.2
C'	*21.6*	*8.2*	*9.6*	*7.6*	*7.9*	*6.1*	*5.6*	*7.4*	*6.9*	*7.4*
A'	14.8	5.7	11.1	8.2	5.9	7.9	8.0	6.1	6.7	7.4
A^+	42.7	14.6	23.3	16.4	12.6	16.0	14.9	12.3	14.2	15.5
A^-	10.2	3.0	6.7	4.5	3.9	4.2	4.2	4.0	3.6	4.3

nificantly lower overall uncertainties than the A^+ subset (Chlide *a* and Phytin *a* are notable exceptions) and is usually at a state-of-the-art level of performance; b) the distribution of the *best method* results are spread across more than one QA method (although H predominates); and c) there are many examples of A^+ methods achieving state-of-the-art performance, but this is countered by many other instances of elevated uncertainties, some of which are significant (e.g., Lut for I and the many cases of false negatives).

1.6.3.2 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), because they are not detection limited. If an abundant pigment is also a problematic pigment, summing will frequently not ameliorate the uncertainties involved.

In this cascade towards lower uncertainties, a method having problems with a class of pigments (e.g., the carotenoids), is expected to have elevated uncertainties for sums predominated by that class of pigments (e.g., PPC). 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 total pigments (TPig).

The uncertainties in the pigment sums for the field samples are presented in Table 19. The overall averages from the QA subset show a decrease in average uncertainties from PPig to pigment sums of 14.8% to 7.4%. The range of the uncertainties within the individual sums is frequently rather small, with the exception of some of the H results. The uncertainties in the individual pigment sums are usually close to, or less than, 8%, as are the overall averages for the individual sums or laboratories (except for H), which is the performance level for quantitative analysis. The range of the individual averages is not as similar as in SeaHARRE-4, which differed by a maximum of only 2.5%. The *best method* results are spread across three of the QA methods with L predominating. The difference between the overall A^- and A' averages is 3.1%.

The A^+ subset shows a more substantial decrease in uncertainties from the overall PPig average of 42.7% to 15.5% for the average of the pigment sums. The individual method decreases are also large with N and O decreasing the most. 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: the overall average for each pigment sum is not to within 12%—the threshold for semi-quantitative analysis (Table 9)—although a few are rather close. In addition, many of the individual uncertainties and laboratory averages exceed 20%, the threshold for routine analysis. For uncertainties with sums, the latter is an indication of significant biases in the method. For example, the problems with the individual carotenoids for the

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 Tables 12, 13, and 15).

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*
D	6.9	3.0	4.2	9.5	**5.8**	0.6	3.2	4.7	12.2	**6.2**	4.0	7.5
G	6.5	2.1	**3.8**	6.2	6.0	**0.5**	2.9	4.0	5.5	7.4	2.1	5.0
H	**3.9**	**2.0**	5.8	**2.5**	6.6	0.6	**1.8**	**3.3**	**3.7**	7.2	**1.9**	**4.3**
L	4.4	3.1	8.4	4.7	8.6	0.8	1.9	4.6	9.0	7.9	3.1	6.7
C	20.5	8.8	16.9	16.8	24.1	2.9	8.7	14.1	30.3	20.8	8.8	20.0
E	29.2	6.5	14.1	34.7	29.6	3.6	15.4	19.0	48.4	22.7	10.4	27.2
I	12.8	33.3	38.9	15.5	39.4	5.1	5.7	21.5	53.7	53.3	29.9	45.7
K	6.1	4.5	7.8	11.4	16.8	2.1	2.6	7.3	12.2	11.2	4.6	9.3
N	12.9	3.0	8.5	13.6	17.9	1.8	5.6	9.0	9.6	24.2	4.1	12.6
O	33.4	8.2	10.1	27.1	29.1	3.8	13.3	17.8	17.9	31.5	8.5	19.3
T_8	13.7	3.9	9.2	21.7	7.8	1.1	6.9	9.2	22.9	17.6	6.7	15.7
T_{18}	15.9	5.3	7.5	12.2	10.0	1.4	8.7	8.7	13.0	14.9	3.8	10.6
C'	*12.1*	*2.8*	*7.4*	*6.1*	*10.8*	*1.1*	*5.3*	*6.5*	*11.9*	*13.7*	*3.1*	*9.5*
A'	5.4	2.5	5.6	5.7	6.7	0.6	2.5	4.2	7.6	7.2	2.8	5.8
A^+	17.7	9.2	13.7	19.4	21.5	2.7	8.3	13.2	25.4	25.1	9.7	20.1
A^-	3.9	2.0	3.8	2.5	5.8	0.5	1.8	2.9	3.7	6.2	1.9	3.9

C and I methods produce anomalously large uncertainties for PPC and total carotenoids (TCaro).

The K, N, T_8, and T_{18} methods frequently have individual pigment sum results within the corresponding range of the QA subset, as does the C' method. This is a strong indication these methods are performing at a level very close to the quantitative level of performance, and a few small improvements for specific pigments would more than likely ensure compliance.

1.6.3.3 Pigment Ratios and Indices

The aforementioned functional relationship in uncertainties established by all 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 pigment ratios were formed, and then increased slightly with the formation of pigment indices.* The latter are a combination of sums and ratios (Table 6), so the small increase is expected to be on the order of the uncertainty in pigment sums (or less).

The uncertainties in pigment ratios and indices for the SeaHARRE-5 field samples are presented in Table 20. The QA subset has overall average uncertainties of 4.2% and 5.8% for the ratios and indices, respectively (which is comparable to the respective SeaHARRE-4 values of 4.1% and 7.4%). Both results satisfy their respective anticipated performance thresholds for quantitative analysis, which are to within 6% and 8%, respectively (Table 9). The individual A' method uncertainties for the ratios are almost always within or close to quantitative performance limits and span a narrow range. The notable exception are the D results for TChl/TCaro, which exceeds the 6% threshold. The individual method uncertainties for the indices are quite similar and are within, or close to, expectations, except for the pPF results for D and L. The latter might be due to anomalously high Zea and DP uncertainties for D, but there appears to be no straightforward explanation for the L pPF result.

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 average index values are above the performance thresholds even for routine analysis. The overall A^+ average decreases in the progression from PPig, to sums, to ratios, and correctly increases with the ratios in all cases–although the I increase is quite large. The lack of deviation from the expected functional form of the uncertainties indicates there are no significant methodological (Hooker et al. 2009) or sampling problems (Hooker et al. 2005).

The individual uncertainties in the A^+ pigment ratios and indices provide additional insight regarding the unique problems with the methods. The K, T_8, N, and T_{18} results are within the sampling variance for the quantitative level of analysis of pigment ratios. Only small improvements are needed to bring the results firmly within quantitative analysis. For these methods, needed improvements are quickly ascertained by investigating the pigments involved (Table 5). For example, for the N method, improvements in the PPC uncertainty will benefit both the

pigment sums and ratios, and ultimately the nPF ratio (although for the latter, improvements in But and Hex uncertainties will likely make the most difference).

The index uncertainties for the A^+ subset are frequently very large, but the K, T_{18}, N, and T_8 results have the lowest values. The former three are very nearly within the sampling variance for the quantitative level of analysis, but the latter is a bit outside that range. The recurring most-problematic index variable is pPF, even for the A' subset. The larger uncertainty is probably driven by anomalously large uncertainties in Zea with additional contributions from the uncertainty in TChl *b* for some methods.

The C' ratio results are also within the sampling variance for quantitative analysis, and small improvements with the TAcc/TChl *a* and PPC/TPig data products will bring the overall average to within the range of the A' results. The C' index results are nearly as expected for quantitative analysis except for a high nPF result. The latter is probably due to high uncertainties in But and Allo.

1.7 THE SeaHARRE-5 WORKSHOP

The SeaHARRE-5 Workshop took place from 12–16 April 2010 and was hosted by Lesley Clementson at the CSIRO facility in Hobart, Tasmania. The attendees consisted of both veteran and new participants. The primary objectives of the workshop were to introduce new participants to the concepts associated with achieving and maintaining QA in marine pigment analysis and quantitation (the performance metrics, accuracy, and precision); discuss method similarities and differences that influence the aforementioned QA parameters; and address outstanding concerns and issues associated with marine pigment quantitation during the working groups.

To counter the trend towards a predominant method, SeaHARRE emphasizes international participation and a diversity of specialized analyses to understand how uncertainties are influenced by the full complexity of the methods being used. The latter has included a) new hardware introduced to an established method; b) new analysts, both experienced or novice, executing an established method; c) unequivocally damaged (defrosted) samples being analyzed by a QA laboratory; d) reanalyses of replicate samples to better understand analysis anomalies; and e) the use of two simultaneous methods by one laboratory. For SeaHARRE-5, the majority of the HPLC analysts were new to the activity (Table 21) and a variety of sample storage and extraction efficiency experiments were conducted.

The major questions and concerns that were posed during the workshop included the following:

- How should pigments quantitated below the limit of quantitation (LOQ) be reported?
- How should small peaks be identified and quantitated?
- What are the methodological factors causing anomalously high uncertainties in certain primary pigments (e.g., diatoxanthin, 19′-hexanoyloxyfucoxanthin and 19′-butanoyloxyfucoxanthin)?
- Is the high uncertainty for prasinoxanthin caused by the interference of another carotenoid, e.g., 4-keto-19′-hexanoyloxyfucoxanthin?
- Would improvements in pigment quantitation or reporting practices (or both) decrease uncertainties?
- Should a new null value be used, so it can be unequivocally identified as a substitution value (e.g., a value with more digits of precision)?
- Would developing a common protocol for summing multiple peaks reduce uncertainties for certain pigments (e.g., when to sum the phaeophorbides and the peridinin main peak and associated cis-isomer)?

The agenda for the workshop was constructed to facilitate the resolution of these questions and is presented in Fig. 3. Much of the discussion for the questions occurred during the break-out sessions for working groups or in the plenary sessions associated with synthesizing the material covered by the working groups.

1.7.1 Working Group One

Working group one was concerned primarily with recommendations for improving the reporting practices for pigment products. It has been suggested that improving and homogenizing the reporting practices of pigments may avoid some uncertainty. Common discrepancies occur, particularly when pigment concentration is at, or near, the instrument LOQ, i.e., when peaks are small or are not confidently identified. The participants were divided into smaller subgroups to discuss three topics of interest: the first subgroup discussed method differences in reporting; the second subgroup discussed the determination of a new null value; and the third subgroup discussed problem pigments.

The first subgroup concluded that the so-called *two-sentence rule*, developed during the SeaHARRE-3 activity (Hooker et al. 2009) and refined during SeaHARRE-4 (Hooker et al. 2010), is difficult for non-native English speakers and needs additional clarification:

> If a peak is *good* and it can be proved to be the incorrect pigment for that retention time (e.g., the absorption spectrum does not match), do not report it; otherwise report it.
>
> If a peak is *bad* and it cannot be proved to be the incorrect pigment, report it; otherwise do not report it.

The point of the two-sentence rule is to have the burden of proof switch as the quality of the data changes, but in each case to have the simpler task emphasized, so analysts will more likely be doing the same thing while doing less

Time	13 April (Tue)	14 April (Wed)	15 April (Thu)	16 April (Fri)
0830	Welcome (L. Clementson)	Welcome (L. Clementson)	Welcome (L. Clementson)	Welcome (L. Clementson)
0840	Workshop Agenda (S. Hooker)	Workshop Agenda (S. Hooker)	Workshop Agenda (S. Hooker)	Workshop Agenda (S. Hooker)
0850	SeaHARRE Overview, the Governing Equation, and the NASA Perspective (S. Hooker)	Calculating Uncertainties (S. Hooker)	CHEMTAX (Simon Wright, Australian Antarctic Divison, Kingston)	Estimation of Performance Metrics (S. Hooker, L. Clementson, C. Thomas, A. Neeley, and L. Schlüter)
0900				
0910		Overview of SeaHARRE-5 Analysis Results (S. Hooker)		
0920				
0930	The CSIRO Method (L. Clementson)		Questions and Answers	
0940			New Pigments (Shirley Jeffrey, CSIRO, Hobart)	
0950	The USM Method (S. Lohrenz)	SeaHARRE-5 Results Discussion (S. Hooker)		
1000				
1010	The Scripps Method (W. Kozlowski)	SeaHARRE Technical Reports (S. Hooker)		
1020			Questions and Answers	
1030	*Break*	*Break*	*Break*	*Break*
1100	The Bodø Method (E. Egeland)	Reduced Performance from Reporting Issues (S. Hooker)	IMOS (Simon Allen, CSIRO, Hobart)	Future Plans for the SeaHARRE Activity (S. Hooker)
1110				
1120	The HPL Method (C. Thomas)			
1130		Recommendations for Improving Reporting Practices of Pigment Products (S. Hooker, L. Clementson, C. Thomas, A. Neeley, and L. Schlüter)	Ocean Color Validation (Thomas Schroeder, CSIRO, Canberra)	
1140				
1150	The FURG Method (V. Garcia)			
1200			Algal Cultures and Pigments (Susan Blackburn, CSIRO, Hobart)	
1210	The IO Method (V. Brotas)			
1220				
1230	*Lunch*	*Lunch*	*Lunch*	*Lunch*
1300				
1330				
1400	The Dalhousie Method (C. Normandeau)	Estimation of Uncertainties for the Terms Within the HPLC Governing Equation (S. Hooker, L. Clementson, C. Thomas, A. Neeley, and L. Schlüter)	Tour of CSIRO (L. Clementson)	Local Field Trip (L. Clementson)
1410				
1420	The GSFC/CVO Method (A. Neleey)			
1430				
1440				
1450	Quantitation Problems with Prasinoxanthin (M. Maddox)			
1500				
1530	*Break*	*Break*	*Break*	
1600	The DHI Method (L. Schlüter)	Performance as a Function of Concentration and SNR (S. Hooker)	HPLC Issues Identified by SeaHARRE Activities (S. Hooker)	
1610				
1620				
1630	Methods Discussion (C. Thomas)	Reducing Uncertainties in the Quantitation of Small Peaks (S. Hooker, L. Clementson, C. Thomas, A. Neeley, and L. Schlüter)	Round Table Discussion with Invited Speakers	
1640				
1650				
1700				
1710				
1720				
1730	*Adjourn*	*Adjourn*	*Adjourn*	

Fig. 3. The agenda for the SeaHARRE-5 workshop showing informal meeting times (blue), plenary sessions (green), break-out sessions for working group discussions (yellow), invited presentations (orange), and alternative scheduling (purple). Individual method presentations were the primary focus of the first day, followed by working group meetings in break-out sessions, invited presentations, final discussions and future plans, and a local field trip.

Table 21. The workshop participants organized according to the organizations they represented (acronyms are defined in the glossary). Core participants (HPLC analysts who have participated in three or more SeaHARRE activities) are shown in bold face typeface, and new participants (HPLC analysts for which SeaHARRE-5 was their first intercomparison) are shown in slanted typeface (Table 2).

Organization	*Country*	*Participant*	*E-mail Address*
CSIRO	Australia	**Lesley Clementson**§	lesley.clementson@csiro.au
	Australia	Tasha Waller	tasha.waller@csiro.au
	Australia	Shirley Jeffrey†	shirley.jeffrey@csiro.au
	Australia	Simon Allen†	simon.allen@csiro.au
	Australia	Thomas Schroeder†	thomas.schroeder@csiro.au
	Australia	Susan Blackburn†	susan.blackburn@csiro.au
AAD	Australia	Simon Wright†	simon.wright@aad.gov.au
FURG	Brazil	*Virginia Garcia*	docvmtg@furg.br
	Brazil	*Raphael Mendes*	rmendes@fc.ul.pt
Dalhousie University	Canada	Claire Normandeau	c.normandeau@dal.ca
DHI	Denmark	**Louise Schlüter**	lsc@dhigroup.com
	Denmark	**Merete Allerup**	mea@dhigroup.com
LOV	France	**Hervé Claustre**‡	claustre@obs-vlfr.fr
	France	**Joséphine Ras**‡	jras@obs-vlfr.fr
NIO	India	*S.G.P. Matondkar*‡	sgpm@nio.org
	India	*Shuma Parab*‡	psushma@nio.org
BUC (now UN)	Norway	*Einar Skarstad Egeland*	einar.skarstad.egeland@uin.no
University of Lisbon	Portugal	*Vanda Brotas*	vbrotas@fc.ul.pt
	Portugal	*Paolo Cartaxana*	pcartaxana@fc.ul.pt
NASA/GSFC	USA	Stanford Hooker§	stanford.b.hooker@nasa.gov
	USA	*Aimee Neeley*	aimee.neeley@nasa.gov
HPL	USA	**Crystal Thomas**	cthomas@hpl.umces.edu
	USA	Meg Maddox	mmaddox@umces.edu
SIO	USA	*Wendy Kozlowski*	wkozlowski@ucsd.edu
USM	USA	*Steve Lohrenz*	steven.lohrenz@usm.edu
	USA	*Sumit Chakraborty*	sumit.chakraborty@usm.edu

§ A meeting organizer.
† An invited speaker.
‡ Did not attend the workshop, but participated in the data analysis.

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 disprove the assumption that the peak is correctly identified, but because the data are poor, there will be little chance this will be possible, so the usual outcome will be the straightforward solution of simply reporting it.

The four cases of the two-sentence rule can also be summarized in terms of absorption spectra matching (the most common means for accepting or rejecting peak identities) as follows:

1. If a peak is *good* and it matches the spectrum, report it;
2. If a peak is *good* and it does not match the spectrum, do not report it;
3. If a peak is *bad* and it cannot be proved to not match the spectrum, report it; and
4. If a peak is *bad* and it can be proved to not match the spectrum, do not report it.

The language of the two-sentence rule is purposely vague, in terms of the definitions of a "good" versus "bad" peak. The idea was to use it as a motivator for analysts to start establishing what these definitions should be. Although aspects of the definitions were discussed, these terms are expected to be more completely defined in a future working group.

The second subgroup determined that all quantitated values will have three digits of precision and the null value will have six digits, which will flag the pigment as not quantitated. The *new* null value will be 0.000999.

The third subgroup was assigned the task of addressing the topic of problematic pigments, or those pigments that consistently produce high uncertainties. The subgroup suggested that one recurring problem that may contribute to high uncertainties was associated with the rules for reporting coeluting pigments and summing pigments.

For example, there are discrepancies among analysts on whether to sum all identified phaeophorbide peaks (usually three or more) or quantitate them separately. Moreover, there is a lack of agreement on whether to include the peridinin cis-isomer that follows the main (parent) peridinin peak in the final quantitation, or quantitate them separately.

The third subgroup also suggested that the problem of uncertainties might be associated with coeluting peaks, which are method dependent. The question was posed as to whether or not there should be a threshold that determines when to report these pigments as a sum and when to report them as individual pigments. It was also suggested that a list of the limitations of each method should be compiled and then determine what can be done to optimize these methods through modifications, although it is understood that some of these issues are hardware dependent.

One concern of the participants was the reporting practices used to provide data products. For example, if the presence of a pigment is uncertain, particularly when it is at a low concentration where spectra matching can become dubious, how should it be reported? At low concentrations, the spectral matches are noisy, so the ambiguity in reporting practices increases significantly. One suggestion was to devise a similarity index, particularly for those pigments that possess similar spectra. To accomplish this task, a separate spectral library would be constructed using diluted pigment standards to simulate low concentrations observed in some field samples.

1.7.2 Working Group Two

Working group two was concerned primarily with defining a *good* and *bad* peak. Following the first discussion about the reporting practices associated with the clarifications to the two-sentence rule, it was obvious that objective definitions of 'good' and 'bad' peaks should be determined. These questions prompted a second working group that was divided into the following three subgroups: the first subgroup was to define a good peak; the second subgroup was to define a bad peak; and the third subgroup was to assess software applications that are available for defining these parameters.

The first subgroup proposed the following criteria for a good peak: on the basis of the peak itself, a good peak should have a symmetrical shape, a good signal-to-noise ratio (SNR), and the same spectrum all over the peak. In comparison with the reference standard, a good peak should have the same peak width, retention time, spectral wavelength(s), and spectral shape.

The second subgroup defined a bad peak as having a retention time outside the normal range; an abnormal elution order; an asymmetric or distorted peak—asymmetry includes peak doubling, peak tailing, unexpected shouldering, and peak broadness in relation to height; poor resolution (e.g., coelution); low SNR; and baseline distortion or anomaly.

The bad peak spectral characteristics were defined as low similarity to candidate library spectra; low SNR of the spectrum (the spectral form is not clear because of excessive noise); multiple spectrum matches with library spectra; an incomplete absorption spectrum (i.e., a partial match); and inconsistencies of the spectrum in different regions of the peak.

The second subgroup also developed a method that may be used to decide when a peak is bad:

- If the peak is big and does not exhibit any of the bad peak characteristics defined above, move to assessing the spectral characteristics.
- For small peaks (as defined by SNR) the peak shape characteristics are often bad, so they should be evaluated based on SNR and spectra.

Future considerations for defining bad peaks that must be discussed further include establishing how many characteristics define a peak as bad; determining whether good peaks are only defined by peak characteristics other than spectra, and whether bad peaks are defined by both shape and spectral characteristics; and investigating if there is a gradient between the good and bad peak. For the latter, this involves discerning whether or not there is a breaking point between the two, is it always in the same place, or does the pigment determine it?

The third subgroup assessed software features that may help with defining good and bad peaks and decided that SNR, spectra match (a numerical value), and tailing factor are features of most software packages that may help to characterize good and bad peaks. Thresholds may be set using these parameters to determine good and bad peaks. Next, the software manufacturers should be contacted to elucidate other possible features of the software to meet these parameters. The automation of these values into the reporting system would be ideal.

1.7.3 Working Group Three

Working group three was concerned primarily with applying definitions of good and bad peaks to chromatograms. After the second working group discussion concluded, the participants were given the opportunity to review their chromatograms from the SeaHARRE-5 activity and choose three examples of what they considered good and bad peaks. Each participating laboratory presented these chosen peaks to the rest of the group to test the applicability of the previously determined definitions of good and bad to the identification and quantification of marine pigments.

1.7.4 Working Group Four

Working group four was concerned primarily with the estimation of performance metrics. The participants discussed the current version of the performance metrics and whether it should be revised, i.e., should parameters be removed or added for further refinement. All agreed that

software-dependent parameters, such as peak symmetry, should not be used because of differences among software manufacturers on how these values are calculated. Moreover, it was acknowledged that the performance metrics are based (in part) on the analysis of standards, which are more representative of analyzing samples from cultures rather than the field. The current weighting is more towards the latter than the former, because the latter represents a more complex sample set and the majority of the SeaHARRE samples are field samples.

It was suggested that the performance metrics should be more explicitly weighted based on the analysis type. For example, if 50% of the phytoplankton samples from a particular laboratory are cultured and 50% are sourced from the field, then the performance metrics for each analytical environment would be equally weighted (multiplied by 0.5) and summed to produce a more representative overall performance; or, the two different performance capabilities could be reported separately (in which case, there would be no weighting).

It was suggested that the exchange of field samples with other laboratories would act as a periodic quality check of precision and accuracy. In this same context, DHI could collect field samples and sell them for this type of comparison. If this idea were adopted, the samples would need to cover all water types (oligotrophic, mesotrophic, eutrophic, and coastal waters). Another suggestion was to use 13 mm filters instead of 25 mm filters to decrease filtration time and extraction volume. It was also suggested that zeaxanthin and lutein individual standards could be mixed together and then analyzed to assess resolution.

1.7.5 Future SeaHARRE Activities

One of the objectives of the SeaHARRE activities is to address problem sets frequently encountered by the pigment community. Previous activities have examined the effects of damaged samples, storage methods, and extraction efficiency on data quality; however, many other scenarios exist and should be addressed. The participants were asked to list topics or problem sets they would like to address, and the resulting suggestions for the future include the analysis of the following:

- Samples from regions of high chlorophyll *a* (i.e., bloom conditions with chlorophyll *a* concentrations approaching or exceeding $50\,\mathrm{mg\,m^{-3}}$);
- Samples from harmful algal blooms;
- Samples from algal cultures;
- Samples from freshwater ecosystems;
- Samples from marshland communities at the land–sea boundary;
- Samples from coastal waters;
- Samples from both the Arctic and Antarctic regions;
- Samples from anywhere, but with the inclusion of microscopy;
- Samples from a large-scale Arabian Sea bloom of the dinoflagellate *Noctiluca miliaris*;
- Samples consisting of a mixture of phytoplankton cultures that reflect a field sample scenario, which should include such problematic pigments as 4-keto-19′-hexanoyloxyfucoxanthin and prasinoxanthin;
- Samples from the Chesapeake Bay; and
- Samples selected to emphasize the comparison of extraction methods.

1.8 SUMMARY AND DISCUSSION

The diversity of objectives for the SeaHARRE-5 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. 1.6).

1.8.1 Precision

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. There is no evidence in the results presented that the QA subset was corrupted by an improperly validated method.

Consequently, an important attribute of the subset of methods used as the referencing system for computing 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 2.4% for TChl *a*, 5.0% for PPig, 3.5% for pigment sums, 1.3% for pigment ratios, and 1.8% for pigment indices. The corresponding values for the A^+ subset were 10.8%, 28.1%, 10.9%, 7.7%, and 12.9%, respectively. The latter represents approximately a 2- to 7-fold increase in variance with respect to the former, and is one of the distinctive mechanisms for showing the difference between the two subsets. This is not to say all methods within the A^+

subset are associated with the same levels of variance. As has already been shown, there are numerous examples of excellent results from a subset of A^+ methods and for a subset or variety of pigment data products.

The precision of the individual methods is another important indicator of the distinct differences between the A' and A^+ subsets. Figure 4a 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 PPig average. To establish a wider context, summary data are shown for all five SeaHARRE activities executed to-date. The precision of the A' subset for SeaHARRE-5 is largely indistinguishable from the first four activities. The SeaHARRE-5 A^+ results are characterized by being more imprecise on average and for all cases.

There are examples for which the two subsets in Fig. 4a have similar precision, for example, the TChl *a* results are not widely different. The majority of the data, however, shows the QA subset has distinctly better precision, and the overall A' average is about 5.5% better, on average, than the A^+ average. The latter represents almost the entire performance budget for the PPig precision metric for quantitative analysis (5%). Given that precision is the first step in the uncertainty budget, it also represents one-third of the PPig accuracy metric for quantitative analysis, so it is a significant difference.

1.8.2 Accuracy

The most significant difference between the A' and A^+ subsets is in the accuracy (uncertainty) of the derived data products, which is shown in Fig. 4b for all five SeaHARRE activities. A substantial decrease in uncertainties from the A^+ to A' subset is seen in all data products, but very notably for the individual pigments (the largest SeaHARRE-5 decrease is for the individual PPig results). In fact, the QA methods are within the approximately 2% sampling variance for the 10% and 15% quantitative analysis requirements for TChl *a* and PPig, except for SeaHARRE-4, for which the results are a bit above this threshold. The A^+ subset frequently does not satisfy the TChl *a* and PPig semiquantitative thresholds (15% and 25%, respectively), particularly for the SeaHARRE-3 and SeaHARRE-4 results.

With the exception of the PPig results obtained during SeaHARRE-4 (the only other coastal SeaHARRE activity), the A' accuracies for all pigment categories within each SeaHARRE activity are rather similar. The closest agreement is seen for TChl *a*, which is important, because it is currently the most significant pigment for ocean color remote sensing. The A^+ results are typified by much greater variability with each pigment category—even for TChl *a*.

The most troubling results are the SeaHARRE-4 A^+ results, because of the absence of the established functional form of the uncertainties—that is, the decrease in uncertainties from PPig to sums to ratios, followed by a small increase with the indices—is seen in all SeaHARRE activities, but not in SeaHARRE-4. Prior SeaHARRE investigations suggested this represents a corruption of the natural relationships between the pigments (Hooker et al. 2005 and 2009). A likely explanation for the corruption is the presence of significant biases in one or more of the pigment data products, which can occur if a method is put into service without proper validation or if a quality assurance plan is not implemented and rigorously applied over time. The presence of the functional form for SeaHARRE-5 indicates the A^+ results are not corrupted, but merely have more and greater sources of variance.

As already noted, the five SeaHARRE activities executed to-date span a wide dynamic range in trophic systems, e.g., the TChl *a* concentration range is approximately 0.020–42.704 $\mathrm{mg\,m^{-3}}$, which represents a little more than four decades in concentration. The sampling for the first three activities involved oceanic regimes emphasizing the mesotrophic Mediterranean Sea, the eutrophic Benguela Current, and the oligotrophic South Pacific gyre, respectively. The last two activities emphasized coastal waters: SeaHARRE-4 sampled Danish coastal waters (fjords and estuaries) and SeaHARRE-5 sampled New England and Tasmanian rivers and bays. Note the international extent of the sampling that was conducted.

It is also important to remember that the composition of each SeaHARRE activity changes over time as the overall objectives evolve. The largest number of new analysts participating in a round robin occurred during the second, fourth, and fifth exercises, and the greatest diversity in the individual methods occurred during SeaHARRE-2 (Table 1). Some qualifications regarding the results need to be remembered, because the largest variances in the A^+ results frequently come from a minority of methods. For some activities, this was caused by known hardware problems (e.g., SeaHARRE-3), but in other cases it was simply caused by methods whose performance was compromised by unknown or improperly investigated problems, which led to degraded data products (e.g., SeaHARRE-4).

Additional details regarding the uncertainty in individual primary pigments for the A' and A^+ subsets across all four SeaHARRE activities are presented in Table 21, and within the perspective of a generalized overview, some notable results are as follows (recalling that the first three round robins used open-ocean samples and the last two used coastal samples):

- The lowest uncertainty for both the A' and A^+ subsets is for TChl *a*, which is largely invariant to water type for the A' data.
- The only carotenoids with an uncertainty to within state-of-the-art PPig performance (to within 10%), are Fuco and Diad for the A' subset, and they are also largely invariant to water type; TChl *b* is close to being compliant.
- The two highest uncertainties for the A' subset are for Diato and But.

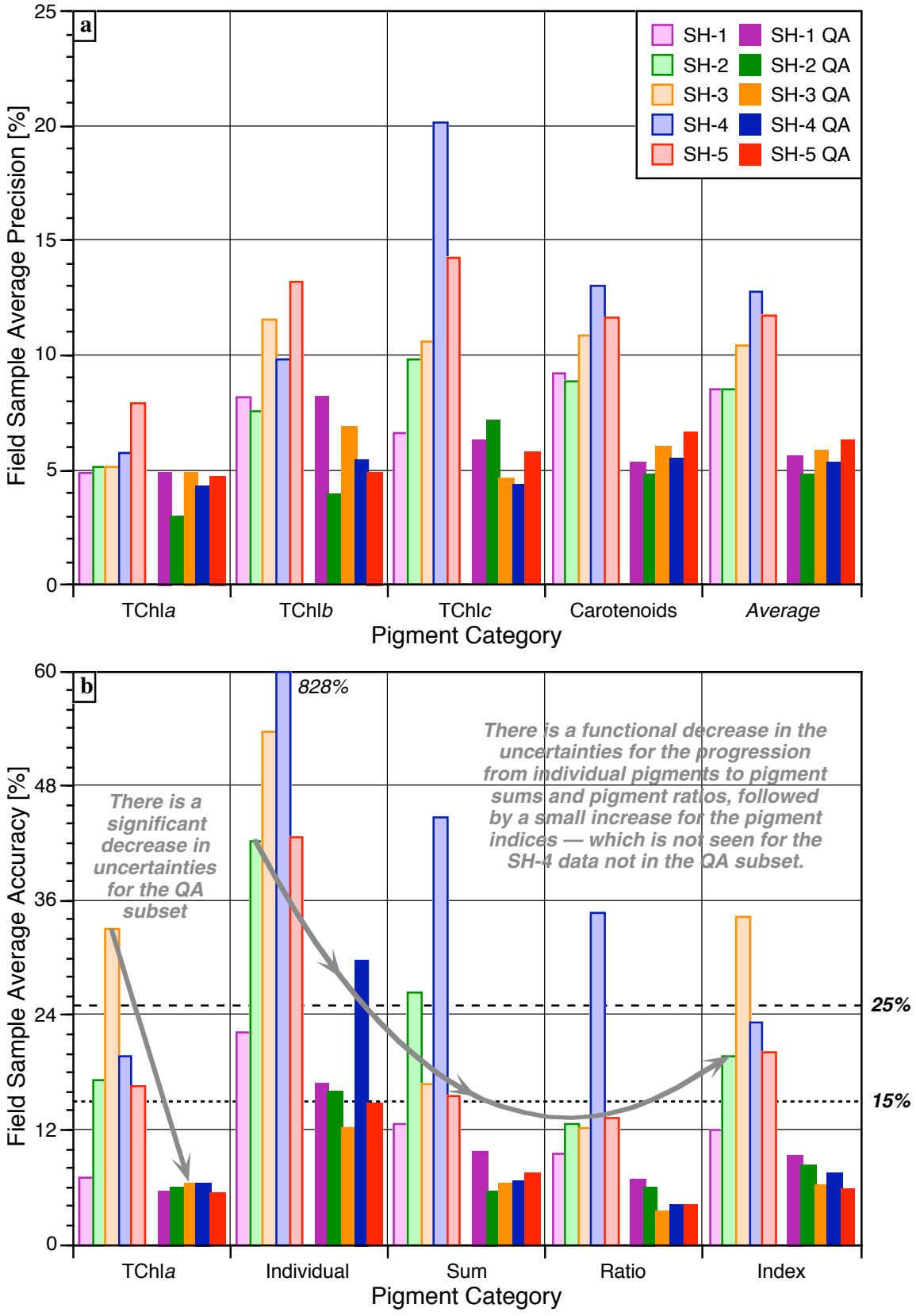

Fig. 4. Overall performance results for the A^+ and A' (QA) subsets (light and dark bars, respectively) for all five SeaHARRE (SH) activities: **a)** method precision, and **b)** method accuracy, with the 15% quantitative and 25% semiquantitative performance metrics for PPig shown as dotted and dashed lines, respectively.

Table 22. The $|\bar{\psi}|$ values (average APD in percent) for field samples across all five SeaHARRE activities for the primary pigments following Tables 12, 13, and 15. The lowest individual pigment uncertainties across all the SeaHARRE activities and within the A' subset are shown in bold typeface and summarized in the A^- entries. Exceptionally large uncertainties with truncated digits of precision are shown in a red typeface.

Meth.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Avg.*
A' SH-1	7.0	14.0	26.5	17.6	23.6	24.8	38.9	16.0	55.8	8.8	**13.0**	11.4	21.5
A' SH-2	5.9	16.5	21.8	16.8	30.6	9.5	20.3	8.7	20.6	**4.7**	15.4	21.4	16.0
A' SH-3	6.3	13.9	14.9	13.2	**14.6**	**6.0**	**4.2**	**5.0**	**17.8**	10.6	30.5	**9.6**	**12.2**
A' SH-4	6.4	12.4	20.4	**9.6**	87.2	57.5	13.0	9.3	49.8	6.6	66.6	19.0	29.8
A' SH-5	**5.4**	**8.3**	**14.4**	17.9	21.3	22.9	8.5	12.1	35.3	5.9	15.8	9.9	14.8
A^+ SH-1	7.9	18.0	32.7	20.2	29.5	24.8	38.9	24.9	59.0	11.5	32.3	21.5	26.8
A^+ SH-2	17.2	20.8	25.6	23.5	63.7	40.4	95.4	30.5	49.3	39.1	56.9	44.3	42.2
A^+ SH-3	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^+ SH-4	19.8	19.0	288.2	345.9	8,078	843.6	24.8	20.7	62.0	20.1	119.8	89.2	827.6
A^+ SH-5	16.6	27.7	34.8	43.3	57.8	65.7	25.0	27.4	69.6	14.3	43.7	86.8	42.7
A' Avg.	6.2	13.0	19.6	15.0	35.4	24.1	17.0	10.2	35.9	7.3	28.2	14.3	18.9
A^+ Avg.	18.9	24.5	80.7	91.5	1,668	201.1	59.1	25.1	60.8	28.8	73.0	51.1	198.6
A^-	5.4	8.3	14.4	9.6	14.6	6.0	4.2	5.0	17.8	4.7	13.0	9.6	9.4

- In between the highest and lowest uncertainty for the A^+ subset, Hex, Peri, and TChl *c* emerge as problematic pigments.
- The A^+ subset includes the A' problematic pigments plus Caro, Allo, and Zea.
- The chlorophyll uncertainty rankings are the same for the A' and A^+ subsets; the lowest uncertainty is for TChl *a*, followed by TChl *b*, and TChl *c*.
- The lowest average uncertainties for the A' and A^+ subsets are associated with oligotrophic and mesotrophic waters, respectively. The latter corresponds to SeaHARRE-1, which had the fewest number of participants and the closest agreement between the A' and A^+ classifications.
- The highest average uncertainties for both the A' and A^+ subsets are associated with coastal waters.
- The lowest individual pigment uncertainties within the A' subset are spread across all five SeaHARRE activities, but SeaHARRE-3 predominates with the greatest number of best results.
- The lowest individual pigment uncertainties within the A' subset and across the five SeaHARRE activities establish a *best method* average of 9.4%, which is within the state-of-the-art performance metric for PPig accuracy (Table 9).
- The problematic pigments within the A^- results are Diato, But, TChl *c*, and Peri.
- The difference between the A^- overall average uncertainty (9.8%) and the best A' overall result for an individual SeaHARRE activity (12.2%) is within the 2–3% sampling variability that has been estimated for each round robin.

The importance of some of these findings are discussed in the following sections.

1.8.3 DHI Mix-105

The concept of using natural relationships between pigments to discern problems with a method is difficult to apply to the DHI Mix-105 results, which are produced by using an artificial mixing of pigments from cultured stocks. Nonetheless, the reduction in uncertainties as pigment sums and then ratios are computed should occur, and the uncertainties should be considerably lower than in natural samples because of the (mostly) artificially high abundance of the pigments in the mix, plus V_x and V_f do not interfere, 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 DHI Mix-105 is presented in Table 23. The results for the QA subset do not satisfy the expected performance thresholds for sums and ratios, because of the problems noted with TChl *b*, which also degrades the uncertainty value for the indices. In addition, the A' results do exhibit a decreasing relationship similar to the functional form presented in Fig. 2b, and the expected small increase in uncertainties associated with the indices is in fact quite large. These anomalies point the importance of the referencing system being as consistent as possible.

Table 23. The average uncertainties in percent for DHI Mix-105 as a function of the A' and A^+ subsets.

Subset	*PPig*	*Sums*	*Ratios*	*Indices*
A'	9.6	10.2	8.5	23.9
A^+	27.1	24.5	18.1	39.3

The A^+ subset has an average precision for the DHI Mix of about 4.1%, so there are no resolution issues for the entries in Table 23. The A^+ subset results exceed the 20% and 15% routine thresholds for the sums and ratios (Table 9). Although the A^+ results follow the expected functional form, in terms of the relationships, the magnitude of the increase for the indices is exaggerated. As for the A' subset, the aberrant magnitude of the uncertainty of the indices is evidence of one or more pigments having significant influence within the A^+ subset.

1.8.4 Ocean Color Requirements

The invariance of $\left[\mathrm{TChl}\,a\right]$ uncertainties to water type for the A' subset and the fact that it has the lowest overall uncertainty for both the A' and A^+ subsets, are important results for the remote sensing problem set. Equally important is the average uncertainty for the A' and A^+ subsets are less than 10% and almost to within 15% plus a 2–3% sampling variance, respectively. This implies much of the worldwide contributions of $\left[\mathrm{TChl}\,a\right]$ values are, on average, compliant with the SeaWiFS calibration and validation specification, and contributions from QA laboratories can probably be used in algorithm refinement exercises.

The difficulty with the above compliance statement is the range of variance that has been established for methods that are not part of the QA subset can be significant, and most algorithm validation work—particularly in the early stages of validating a new algorithm—proceed more effectively with higher-quality data. The same is true for the very important work of first establishing a new algorithm. Being able to partition databases between A' and A^+ contributions would make establishing and refining an algorithm easier to accomplish.

1.8.5 Performance Metrics

The participation of laboratory G was an experiment to demonstrate what an experienced analyst—who had never executed the VHT method—could accomplish on new HPLC equipment that the analyst had never used if the performance metrics for quantitative analysis were strictly followed. The analyst in this case was familiar with the concept of following *best practices* in the laboratory. The fact that the G results were in the A' subset and were repeatedly some of the best results within that subset demonstrates how adherence to protocols and performance metrics can result in significantly lower uncertainties in the production of data products from field samples, even when using a new method and new equipment. It also shows the performance metrics are achievable and realistically set.

1.8.6 Conclusions and Recommendations

Although previous SeaHARRE activities conducted in open-ocean waters covered a wide dynamic range in productivity and some of the samples were collected in the coastal zone, only the SeaHARRE-4 activity involved exclusively coastal samples. The SeaHARRE-4 uncertainties were significantly elevated for many pigments. Consequently, SeaHARRE-5 was organized and executed as a strictly coastal activity and the field samples were collected from primarily eutrophic waters within the coastal zone of New England and Tasmania. The latter samples were expected to produce less cluttered baselines than the former, which was a desirable analysis distinction, because the SeaHARRE-4 samples had complicated baselines.

The SeaHARRE-5 activity was designed to make investigations regarding the following objectives: a) estimate the uncertainties in quantitating individual pigments and higher-order variables formed from sums and ratios; b) confirm if the chlorophyll a accuracy requirements for ocean color validation activities (approximately 25%, although 15% would allow for algorithm refinement) can be met in coastal waters; c) establish the reduction in uncertainties as a result of applying QA procedures; d) show the importance of establishing a properly defined referencing system in the computation of uncertainties; e) quantify the analytical benefits of performance metrics; and f) demonstrate the utility of a laboratory mix in understanding method performance. All of these objectives were satisfied and the most salient aspects involved are as follows:

- Estimates of the uncertainties in quantitating individual pigments and higher-order variables formed from sums and ratios show the latter provide a significant opportunity to reduce uncertainties in larger databases (Fig. 4).
- The chlorophyll a accuracy requirements for ocean color validation activities are significantly satisfied in coastal waters, but some additional investigation is needed to reduce uncertainties when detection by absorption spectra is sufficiently degraded that analysts cannot reasonably be expected to make similar choices as to the presence or absence of a pigment (the so-called *two-sentence rule* is a starting point for this work).
- The reduction in uncertainties as a result of applying QA procedures is demonstrated by the significantly lower uncertainties associated with the QA subset across all five SeaHARRE activities executed to-date (Fig. 4 and Table 22).
- The importance of establishing a properly defined referencing system in the computation of uncertainties is revealed by the large difference between the A' and A^+ subsets. This is because if the partition had not been properly defined, the larger variance of the A^+ subset would have been spread across all the methods. It would, therefore, have been very difficult to distinguish the excellent results achieved by not only the QA methods, but also the methods that very nearly complied with QA subset (K, N, T_8, T_{18}, and C').

- The analytical benefits of the performance metrics are shown by the excellent results achieved with method *G* (Tables 15–20), which involved an experienced HPLC analyst using a new method (VHT) on new hardware that was guided primarily by strict adherence to the method protocols and the performance metrics.
- The utility of the laboratory mix was demonstrated by how the differences between the A' and A^+ subsets are still seen in the average uncertainties for DHI Mix-105 (Table 23) even though the mix is significantly easier to analyze than a field sample (no extraction procedures).

The SeaHARRE-4 chromatograms were more difficult to integrate, because they contained more unknown pigments than prior SeaHARRE activities. This was probably due to the stations being in eutrophic and estuarine environments. Frequently, the content in the samples were most likely a mixture of freshwater and marine algae, which means they were more complicated to analyze than samples from high salinity areas. By itself this is a challenging analysis scenario, but for an analyst who is not used to these samples, it is doubly challenging, because interpretation is usually more reliable if experience is also a factor. Consequently, a likely reason for the QA subset increase in uncertainties for SeaHARRE-4 was having so many analysts confronting such a complex sample set for the first time.

This conclusion was factored into the field sampling for SeaHARRE-5 and resulted in two sets of samples from different environments being collected: the presumably more anthropogenically influenced coastal waters of New England, and the presumably more pristine waters of Tasmania (Table 3). The hypothesis that the former would be more difficult to analyze than the latter, and thus the former would have higher uncertainties is examined in Table 24, which shows the partition of the uncertainties for the two sampling regimes. Unexpectedly, the uncertainties for both the A' and A^+ subsets are higher for the Tasmanian samples than the New England samples.

Table 24. The average uncertainties in percent for the A' and A^+ subsets as a function of the pigment categories and the two sampling regimes.

Pigment	*New England*		*Tasmania*	
Category	A'	A^+	A'	A^+
TChl *a*	4.2	14.9	6.7	18.4
PPig	12.9	41.6	16.8	43.9
Sums	4.9	15.4	9.9	15.6
Ratios	2.4	12.9	5.9	13.6
Indices	2.9	16.4	8.8	23.7

The Tasmanian field samples have an average PPig concentration that is twice that of the New England field samples (Table 10), but that does not mean all the individual primary pigments are more abundant in the former versus the latter. In fact, seven of the primary pigments are less abundant and five are more abundant. The uncertainty in the less abundant Tasmanian pigments is slightly higher than the corresponding New England pigments. The rest of the increase in uncertainty for the Tasmanian samples comes from the more abundant pigments of which Diad accounts for much of the increase. Consequently, the Table 24 results suggest the larger source of uncertainty in coastal samples is not complicated baselines, but rather the difficulty of analysts following different practices when the identification of pigments becomes problematic. This topic is considered in more detail within the individual method chapters.

It is also worth noting that although the Tasmanian waters are considered more pristine in terms of anthropogenic inputs from industry, the Derwent and Huon estuaries from where the samples were primarily collected are within shipping areas and do have very high colored dissolved organic matter (CDOM) concentration. It may be that some small part of the humics and tannins within the system extract with the pigments and make the baseline a little more complex than it would be otherwise.

As part of documenting their individual HPLC methods (Chaps. 2–12), several of the SeaHARRE-5 participants included a discussion of little peaks and the application of the two-sentence rule to determine the identity and concentration of little peaks. From a generalized perspective, the analysis of pigment results is usually from one of two perspectives: remote sensing applications, or pigment diversity as an indicator of phytoplankton community composition. Whether a pigment is identified or not, if the identification is equivalent to a very small concentration (e.g., less than $0.010\,\mathrm{mg\,m^{-3}}$), the result is sometimes considered inconsequential and replaced by the LOQ value. Whether or not the "inconsequential" label was correct or not can be a function of the water type and the filtering protocols used in the field.

For water types associated with very low productivity or for situations wherein an inadequate volume of seawater was filtered in the field, there can be many pigments at low concentration such that determining community composition from very low pigment concentrations is a challenging exercise. Consequently, there will frequently be circumstances wherein the proper quantitation of little peaks are important to the validity of the scientific conclusions derived from the pigment analyses. Another circumstance wherein the quantitation of small peaks are important is in intercomparison exercises, such as the SeaHARRE activity. The accurate identification of small peaks takes on greater importance during round-robin intercomparisons, because of the potentially significant consequences of false positives and false negatives on method accuracy (Sect. 1.6.3).

For the SeaHARRE-5 laboratories, there was a general consensus that the crux of the little peak problem was the

level of subjectivity in deciding whether a peak was a) good or bad, and b) able to be identified accurately. If a threshold or rule based on objective factors could be established, then a reduction in the reporting of false positives and false negatives was believed to be achievable. This perspective was the basis for the two-sentence rule, which was designed to be the starting point for a discussion on objective criteria, and not necessarily the *a priori* solution. Indeed, the anticipated follow-on discussion to the two-sentence rule was expected to include the consideration of objective elements, like the SNR.

HPL was the only group to attempt to quantify an objective threshold (Chap. 6). Initially, HPL used the spectral match value of 0–1,000 from their `ChemStation` software package, wherein a value of 1,000 was a perfect match between the absorption spectrum of a known pigment standard and the absorption spectrum of a tentatively identified pigment within a field sample. A spectral match value of greater than or equal to 996 as a sufficient indicator of predictive accuracy and this value was equivalent to an SNR value over a range of 34–43, depending on the individual pigments. HPL decided that spectral matching could not be relied on to determine peak identity when a peak had an SNR value of 15 or less.

The Dalhousie group also suggested that identification and quantitation of a peak should only occur if the SNR value of the peak is greater than or equal to 10 (Chap. 10). The CSIRO method showed that slight deterioration of the absorption spectrum occurred for peaks with an SNR between 30–50, when compared with the absorption spectrum of the same pigment peak, but with a higher SNR. This is in general agreement with the spectral matching threshold value of 996 established by the HPL group. The CSIRO method showed that deterioration in the absorption spectra of pigment standards occurred as the SNR value of the peak was lowered and suggested that a library of absorption spectra from peaks with an SNR of 10 and less should be established to aid in the identification of small peaks (Chap. 2). The USM group have already established such a library and use it routinely to determine the identification of small peaks (Chap. 12).

In summary, both the more formal approach as was taken by the HPL group and the visual assessment between the absorption spectra for peaks of different SNR as done by CSIRO, Dalhousie, and USM, suggest that accurate pigment identification of a chromatographic peak is increased if the peak has an SNR value of 10 or more. The establishment of a spectral library based on peaks with an SNR value of 10 and less would also enhance the identification process. Interestingly, the suggested threshold of an SNR value of greater than or equal to 10 for identifying peaks is identical to the SNR used to calculate the LOQ, which is presently used to replace very small concentrations by many analysts.

Chapter 2

The CSIRO Method

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Abstract

The CSIRO method is a modified version of the Van Heukelem and Thomas (2001) or HPL method and can resolve about 35 different pigments with baseline resolution of divinyl and monovinyl Chl *a*, as well as Zea and Lut. Partial separation of divinyl and monovinyl Chl *b*, as well as Chl c_1 and Chl c_2 is also achieved. The method used for SeaHARRE-5 was the same method used for the prior two SeaHARRE activities with one exception. 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 spectrum from a photo-diode array (PDA) detector. The method is regularly validated with the use of internal and external standards and individual pigment calibration. The detection limit of most pigments is within the range 0.001–0.005 mg m^{-3}. This method is applicable to the study of pigment composition and concentration in samples from all water types including freshwater, estuarine, upwelling coastal regions, the oligotrophic open ocean, as well as in the microphytobenthos of shallow coastal regions.

2.1 INTRODUCTION

For the SeaHARRE-5 activity,12 triplicate samples, collected from the New England coastal waters, were received (filters folded in half and wrapped in aluminum foil) on dry ice on 9 February 2009. The samples were immediately transferred to liquid nitrogen for storage until analysis. The DHI mixed standards were received at the same time and were stored at −20°C. The 12 triplicate samples from Tasmanian coastal waters (filters folded in half and wrapped in aluminum foil) were stored in liquid nitrogen directly after filtration (November 2008). Both sets of samples were analyzed between 26 May and 3 June 2009. Because of problems incurred during the analysis of this first set of samples, a complete second set of samples was received on 9 February 2010 in a liquid nitrogen dry shipping dewar. This second set of samples was analyzed between 24 March and 1 April 2010.

When the analysis of the first set of samples was started, it was noted that the filters were very pale in color. In an attempt to increase the detection level of the individual pigments, the volume of acetone used to extract each filter was reduced from 3 to 2 mL. Although all other additions to the extract were adjusted in the same proportion as the reduction in the volume of acetone, the final results for the primary pigments were approximately 22% lower than the average concentrations for the QA subset, although there was a difference between the US and Australian sample sets. Discussion of this problem is presented below.

The CSIRO method separates pigments on a C_8 column using a two-solvent gradient system. Pigments are verified by the retention time and absorption spectra (using a PDA detector) of each chromatographic peak and quantitated by the detector signal at 436 nm. Following a 9 min injection cycle, analysis time is 31 min per sample with a further 5 min injection delay to ensure no carryover between samples. Separation is achieved for most pigments, the exceptions being ββ-Car and βε-Car, which coelute, but can be separated by their absorption spectra in samples where one of the carotenes is dominant. Baseline resolution is not achieved between Chl c_1, Chl c_2, MgDVP, and Chlide *a*, however, it is not common for all four pigments to be present in the one chromatogram, allowing for good resolution between Chl c_1 and Chl c_2, as well as partial resolution between Chl c_1 and Chlide *a* when both are present. While baseline resolution of divinyl and monovinyl Chl *a* is achieved, only partial separation of divinyl and monovinyl Chl *b* can be achieved.

2.2 EXTRACTION

To extract the pigments, the thawed filters are cut into 3–4 pieces and covered with 3 mL of 100% acetone in a 10 mL centrifuge tube. Scissors and forceps are cleaned between samples. 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, 200 µL of water is added to the acetone, such that the extract mixture is 90:10 acetone:water (vol:vol), including the water held in the filter from the sample filtering process. The sample extract is then sonicated once more for 15 min in an ice-water bath in the dark.

The extract is transferred to a small Biorad 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:water with the rinse solution 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 extract volumes are recorded from the centrifuge tube graduations. The centrifuged extracts are then passed through a 0.2 µm Teflon syringe filter (Advantec), which has been rinsed with acetone and air, directly into a 2 mL amber HPLC vial. Any remaining extract is kept in a centrifuge tube, covered with Parafilm, and stored at −20°C until the HPLC analysis has been successfully completed.

2.3 HPLC ANALYSIS

CSIRO uses a Waters-Alliance HPLC system with a 2695XE separations module, column heater, refrigerated autosampler, and 2996 PDA detector. The autosampler is set to 4°C. Immediately prior to injection, the sample extract is mixed with a buffer solution (90:10 28 mM aqueous TbAA, 6.5 pH:methanol) within the sample loop. The injector is programmed to draw up alternating microliter volumes of buffer and sample in the following order: 150, 75, 75, 75, and 150 starting with the buffer. After injection, pigments are separated using a Zorbax Eclipse XDB-C_8 stainless steel 150×4.6 mm (interior diameter) column with 3.5 µm particle size (Agilent Technologies) and a gradient elution procedure as shown in Table 25.

Table 25. The gradient elution program for the CSIRO laboratory method as executed during the SeaHARRE-5 activity. Solvent A is 70:30 28 mM TbAA (6.5 pH):methanol, and solvent B is 100% methanol.

Time [min]	A [%]	B [%]
0	95	5
11	45	55
15	45	55
22	5	95
29	5	95
31	95	5

The gradient was held in an isocratic mode between 11–15 min to improve the resolution between Viola and Hex. The flow rate was 1.1 mL min^{-1}, and the column temperature was 55°C. The separated pigments are detected at 436 nm and identified against standard spectra using Waters `Empower` software. Peak integration and identification is initially performed by the automated features of the 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 in sample chromatograms were determined from Sigma-Aldrich standards, while all other pigment concentrations were determined from DHI standards. The concentration of all standard stock solutions were determined using a Cintra 404 UV/VIS dual-beam spectrophotometer with a 2 nm band width. An absorption spectrum of each pigment was recorded between 350–900 nm. The concentration of each pigment was calculated using the absorption coefficient from the literature (Jeffrey et al. 1997) together with the absorption measured at the corresponding wavelength. The absorption coefficients, wavelengths, and solvents used for each pigment are listed in Table 26.

Table 26. Absorption coefficient (α) values in liters per gram per centimeter for the CSIRO method as a function of wavelength (λ). The literature reference is in the rightmost column.

Pigment	*Solvent*	λ	α	*Ref.*
Peri	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	§
Gyro. diester	100% Ethanol	445.0	262.00	§
Asta	100% Acetone	482.0	210.00	†
βε-Car	100% Acetone	448.0	270.00	§
ββ-Car	100% Acetone	454.0	250.00	†
Chlide *a*	90% Acetone	664.0	127.00	§
Chl *b*	90% Acetone	647.0	51.36	†
DVChl *a*	90% Acetone	664.3	87.67	§
MVChl *a*	90% Acetone	664.0	87.67	†
Chl c_3	90% Acetone	453.0	346.00	§
Chl c_2	90% 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	§

† Jeffrey et al. (1997b) § DHI (Hørsholm, Denmark)

The absorbance at the wavelength used is corrected for any absorption measured at 750 nm. From these stock solutions, a series of 4–6 standard solutions were prepared and analyzed both spectrophotometrically (using the equations of Jeffrey and Humphrey 1975) and by HPLC. Calibration curves were obtained with r^2 values always greater than 0.99, and the response factors for each pigment were determined from the calibration curves.

2.5 VALIDATION

At the start of every set of samples analyzed by HPLC using the CSIRO method, a pigment mixture is qualitatively analyzed to determine if there is any movement in the retention time of approximately 30 pigments. A mixture of known concentrations of Asta, Chl *a*, Chl *b*, and ββ-Car is also analyzed to determine that the HPLC system, including the column, is working appropriately. Between 26 May and 16 September 2009 this pigment mixture was analyzed 74 times yielding a precision (as quantified using the CV in percent) of 1.87, 0.97, 1.12, and 1.57% for Asta, Chl *a*, Chl *b*, and ββ-Car, respectively. Multipoint calibrations of Chl *a*, Chl *b*, and ββ-Car are done approximately every 6 months, while multipoint calibrations of all pigments are done approximately every 18 months.

2.5.1 DHI Mix-105

For the SeaHARRE-5 activity, a mixed pigment standard, supplied by DHI as Mix-105, was analyzed three times within each set of 12 samples. This resulted in a total of nine analyses of Mix-105. The precision (as quantified using the CV in percent) of the nine injections ranged from 0.32–3.35%, with a mean value of 1.58%, and was less than 1.0% for all major pigments.

2.5.2 Carryover

After having analyzed several thousand field samples, CSIRO had never seen carryover between samples. The concentration of the pigments in the DHI Mix-105 were so much higher than the field samples that very small peaks of DVChl *a*, MVDVChl *a*, and ββ-Car were seen in the chromatogram of the injection immediately following the mixed standard.

To avoid contamination of the first of the triplicate field sample injections, the DHI mixed standard injection was always followed by the injection of an acetone blank. Percent carryover from the standard to the blank was less than 0.25% for both DVChl *a* and MVChl *a*, and less than 0.5% for ββ-Car of the concentration of the individual pigments in the DHI mix.

If the injection of the blank had not followed the injection of the standard, the carryover would have equated to small increases in concentration of up to 4% for the following sample. Because DVChl *a* was not present in any of the SeaHARRE-5 samples, the carryover of this pigment into a following sample would have resulted in large errors for DVChl *a*, because false positives would have been reported for several samples.

2.6 DATA PRODUCTS

Waters `Empower` software created an electronic file in which each chromatographic peak had its corresponding retention time, peak area, and peak height recorded together with initial pigment identification. Once the chromatograms had been manually inspected, the peak areas were transferred to an `Excel` spreadsheet in which the amount of pigment injected was calculated using the appropriate response factor:

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

where $\tilde{C}_{P_i}$ is the amount of pigment P_i injected (in units of nanograms per injection); $\hat{A}_{P_i}$ is the area of the chromatographic peak corresponding to the pigment; and R_{P_i} is the response factor for the pigment. The concentration of the pigment in the sample is determined using the following equation:

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

where V_x is the final extraction volume (in microliters), V_f is the volume of sample filtered (in milliliters), V_c is the volume of sample extract injected onto the HPLC column (in 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 $\big[\text{Chl } a\big]$ value would be outside the linear range that was used for the calibration of Chl *a*.

For pigments MgDVP and Chl c_1, the response factor determined from the calibration curve for Chl c_2 is used for quantitation.

2.6.1 Extract Volume Exception

As indicated above (Sect. 2.1), after reducing the extract volume, the concentrations of the primary pigments were significantly lower than the average concentration of the QA subset, although there were differences between the sample sets between the US and Australia. The concentration of MVChl *a* and Fuco determined by the CSIRO method using both a 2 mL and a 3 mL extract volume is compared to the MVChl *a* and Fuco concentrations determined by the respective averages from the A' subset (DHI, HPL, and LOV) methods in Table 27.

Table 27 shows that when the C method used a 2 mL extract volume, $\big[\text{Chl } a\big]$ was lower on average by 48% than the average concentration determined by the DHI, HPL, and LOV methods for the US sample set as compared to only 15% lower for the Australian sample set. Similar results were obtained for $\big[\text{Fuco}\big]$, with the C method producing results that were lower on average by 41% and 11%

Table 27. A comparison of Chl *a* and Fuco concentrations between the A' (quality assured subset) methods and the C results using a 2 mL versus 3 mL extract volume (noting that 3 mL follows the usual C extraction procedure, and 2 mL was used as an exception for the SeaHARRE-5 activity). Concentrations are in micrograms per liter and the RPD is computed as $100(C\text{-}A')/A'$ (i.e., A' is the reference value, so a negative RPD means the C concentration is less than the A' reference value). Blank entries correspond to original omissions in the C results.

Sample	C [Chl *a*]		A'	RPD [%]		C [Fuco]		A'	RPD [%]	
Code	2 mL	3 mL	Avg.	2 mL	3 mL	2 mL	3 mL	Avg.	2 mL	3 mL
A	0.373	0.686	0.724	−48.5	−5.2	0.044	0.082	0.089	−50.6	−7.9
B	0.772	1.360	1.483	−47.9	−8.3	0.087	0.181	0.197	−55.8	−8.1
C	0.516	0.877	0.933	−44.7	−6.0	0.069	0.129	0.138	−50.0	−6.5
D	0.967	1.747	1.733	−44.2	0.8	0.240	0.353	0.343	−30.0	2.9
E	0.906	1.669	1.875	−51.7	−11.0	0.218	0.321	0.335	−34.9	−4.2
F	0.810	1.397	1.573	−48.5	−11.2	0.140	0.224	0.236	−40.7	−5.1
G	2.481	3.458	3.933	−36.9	−12.1	0.495	0.701	0.710	−30.3	−1.3
H	1.219	2.069	2.396	−49.1	−13.6	0.483	0.720	0.724	−33.3	−0.6
I	1.522	3.154	3.582	−57.5	−11.9	0.548	0.825	0.879	−37.7	−6.1
J	2.619	4.385	5.312	−50.7	−17.5	0.456	0.686	0.786	−42.0	−12.7
K	2.569	4.262	5.255	−51.1	−18.9	0.521	0.772	0.822	−36.6	−6.1
L	1.238	1.802	2.182	−43.3	−17.4	0.145	0.285	0.310	−53.2	−8.1
A–L			*Avg.*	−47.8	−11.0			*Avg.*	−41.3	−5.3
AA	3.766	4.517	4.305	−12.5	4.9	0.426	0.424	0.420	1.4	1.0
AB	10.356	11.527	11.953	−13.4	−3.6	0.498	0.473	0.527	−5.5	−10.2
AC	8.666	8.958	8.801	−1.5	1.8	0.627	0.611	0.624	0.5	−2.1
AD	1.352	1.319	1.487	−9.1	−11.3	0.207	0.210	0.254	−18.5	−17.3
AE	7.263	7.171	7.839	−7.3	−8.5	0.925	0.958	1.076	−14.0	−11.0
AF		1.017	1.319		−22.9		0.137	0.142		−3.5
AG		0.739	1.053		−29.8		0.048	0.055		−12.7
AH	5.147	4.937	5.277	−2.5	−6.4	0.277	0.265	0.301	−8.0	−12.0
AI	1.112	1.268	1.556	−28.5	−18.5	0.107	0.129	0.131	−18.3	−1.5
AJ	2.025	2.332	2.606	−22.3	−10.5	0.097	0.109	0.115	−15.7	−5.2
AK	6.240	7.506	9.353	−33.3	−19.7	2.001	2.425	3.022	−33.8	−19.8
AM	3.295	3.513	4.062	−18.9	−13.5	0.184	0.179	0.179	2.8	0.0
AA–AM			*Avg.*	−14.9	−11.5			*Avg.*	−10.9	−7.9

than the A' average concentration determined for the US and Australian sample sets, respectively.

When the C method used a 3 mL extract volume the results were improved significantly, especially for the US sample set (Table 27); the M[Chl *a*] results were only lower on average by 11% than the average concentration determined by the A' methods, as compared to 48% when a 2 mL extract volume was used. Similarly, the [Fuco] results for a 3 mL extract volume were lower on average by 5% compared to 41% when a 2 mL extract volume was used. The results for both [Chl *a*] and [Fuco] were both improved by approximately 3% for the Australian samples.

Because the extract volume and all other additions to the extract (rinses, etc.) were kept in proportion, the only difference between the 2 mL and 3 mL analyses is the water retained in the filter after filtration of the sample water. To determine the volume of water retained in a 25 mm GF/F filter, 100 mL of pure (Milli-Q) water was filtered through each of six preweighed GF/F filters and the weight measured after filtration. The six weights ranged from 214–257 mg with a mean value of 230.6 mg. Including this value in the determination of the percent ratio of acetone to water in the final extract volume for the 2 mL extract volume, the ratio of acetone to water would be 86:14, while for the 3 mL extract volume, the ratio of acetone to water would be 88:12. If, however, the volume of water retained in the filter was greater than 230.6 mg, then the ratios would have a greater proportion of water.

These results suggest there is a threshold of the acetone-to-water ratio, and if the ratio is reduced below this threshold, then there is a significant effect on the concentration of the pigments. It would seem that the threshold is close to the ratio of 88:12 (acetone:water). One operator filtered all of the Australian samples for the SeaHARRE-5 activity (plus the aforementioned pure water test), so it appears reasonable to assume that the same approximate volume of water would have been retained in all of these

filters. The US sample set was prepared by a different operator, and it is possible that more than 230.6 mg of water was retained within these filters and, therefore, the ratio of acetone-to-water fell below 86:14 for the 2 mL extract volume analysis.

The Australian samples indicate that if the acetone-to-water ratio falls between 88:12 and 86:14 acetone:water, there is a slight reduction in the concentration of all pigments; while the US samples tend to suggest that if the ratio falls below 86:14, then the concentration of the pigments can reduce significantly.

Further tests need to be undertaken, but the results from the CSIRO method suggest that the ratio of acetone to water in the final volume of the pigment extract is quite critical and attention should be paid to the amount of water that can be retained within a filter after filtration of samples. In the CSIRO method, the vacuum is left on while removing the filter, but it could be that *filters should be blotted with a tissue to absorb excess water after removal from the filtration cup whether the vacuum is left on or not*†. Discovering this possibly critical step in the extraction method would almost certainly not have happened without an intercalibration exercise such as the SeaHARRE activity.

2.6.2 Quantitating Small Peaks

The SeaHARRE activities represent a unique set of HPLC pigment analyses, wherein different laboratories using different extraction and analysis methods are compared through their quantitation of a common set of samples. In the normal day-to-day running of any of these laboratories, the identification of very small peaks in their individual chromatograms would not be a major issue. For any "internal" analysis of pigment results, be it for remote sensing calculations or just pigment diversity as an indicator of phytoplankton community composition, whether a pigment is identified or not, if the identification is equivalent to a very small concentration (e.g., less than 0.010 mg m^{-3}), the result is frequently inconsequential.

In the SeaHARRE activity, however, the ability to identify small peaks takes on greater importance, because of the negative consequences of false positives and false negatives on method accuracy (Sect. 1.6.3). Identification of a pigment when few of the other participating labs makes the same identification (or the reverse where non-identification when several of the other laboratories make an identification) can cause high uncertainties for that particular laboratory and its associated methods for that specific pigment. This is one of the mechanisms by which the vulnerabilities of a method—or community protocols—are exposed in round-robin intercomparisons, and why they are so valuable to the individual analyst and the community at large.

To promote the investigation of the false reporting problem and to help reduce the high uncertainties for some laboratories, it was decided at the SeaHARRE-4 workshop to establish some rules that could guide analysts to make a common decision. The so-called *two sentence rule* was purposely established to be as simple as possible to ensure subsequent discussion and debate amongst the SeaHARRE analysts (Hooker et al. 2009):

> 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.

The first step, therefore, is to decide whether a peak is "good" or "bad," that is, to establish what constitutes a good or bad peak.

Figure 5 shows two chromatograms with very small peaks. Although the peaks are small, they have good peak shape and are distinct from the baseline, therefore, the *C* method would classify them as "good" peaks. The second step for these peaks, is to prove whether or not the retention time matches the identified pigment. This is done with the *C* method by comparing the absorption spectrum of the specified peak with the absorption spectrum of a known standard that has the same retention time as the specified peak.

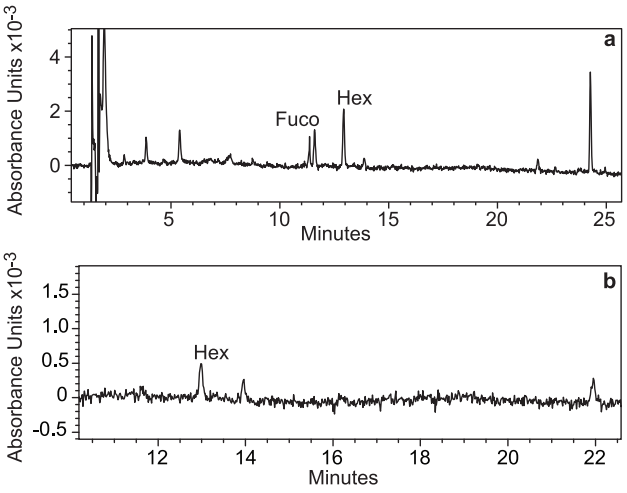

Fig. 5. Sample chromatograms with very small peaks for **a)** Fuco and Hex (top), and **b)** for Hex (bottom).

From Fig. 5a, if the peak labelled Fuco is considered, the concentration of Fuco in the sample is 0.014 mg m^{-3}, which is equivalent to 1.05 ng inj^{-1}, and the peak has an SNR value of 6. Figure 6 shows the absorption spectra for three Fuco standards as well as the absorption spectrum for

† Some HPLC practitioners place the *wet* filter in the crease of a larger GF/F filter (created by having already folded the larger filter over), and then refold the large filter, thereby folding the smaller sample filter and *blotting* it dry in one step.

the peak labelled Fuco in Fig. 5a. The absorption spectrum for a peak of Fuco standard with an SNR value of 37 shows the spectral shape is maintained at the top of the peak, but there is a slight widening of the peak and a lifting of the baseline as compared to the absorption spectrum of a peak with an SNR value of 469 (Fig. 6). Further comparison with a peak with an SNR value of 6 shows the baseline to have lifted significantly, the width of the absorption spectrum has increased considerably, and the spectral shape at the top of the peak has deteriorated. The absorption spectrum of the peak with an SNR value of 6, although poor, does *not* prove the chromatogram peak is not Fuco, so the peak would have to be reported as Fuco.

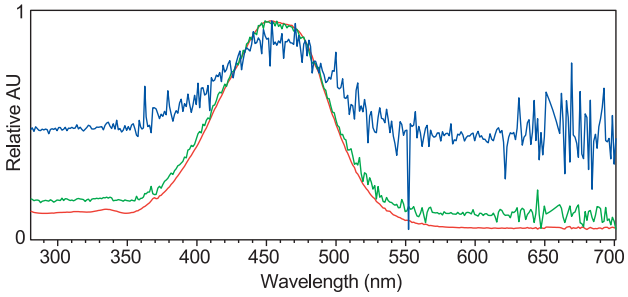

Fig. 6. Absorption spectra for a Fuco standard with the following characteristics: $53\,\mathrm{ng\,inj^{-1}}$ and an SNR value of 469 (red); $4.42\,\mathrm{ng\,inj^{-1}}$ and an SNR value of 37 (green); and $1.05\,\mathrm{ng\,inj^{-1}}$ and an SNR value of 6 (blue), which also corresponds to Fig. 5a.

Similarly, Fig. 7 shows the absorption spectra for two Hex standards as well as the absorption spectrum for the peak labelled Hex in Fig. 5a. For the peak labelled Hex, the concentration of Hex in the sample is $0.02\,\mathrm{mg\,m^{-3}}$, which is equivalent to $1.5\,\mathrm{ng\,inj^{-1}}$, and the peak has an SNR value of 10. The same deterioration in spectral shape is seen with a reduction in the SNR values of the Hex peaks as was seen for the Fuco peaks.

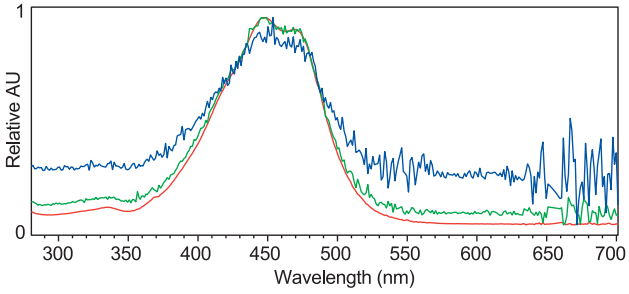

Fig. 7. Absorption spectra for a Hex standard with the following characteristics: $67\,\mathrm{ng\,inj^{-1}}$ and an SNR value of 580 (red); $5.58\,\mathrm{ng\,inj^{-1}}$ and an SNR value of 48 (green); and $1.5\,\mathrm{ng\,inj^{-1}}$ and an SNR value of 10 (blue), which also corresponds to Fig. 5a.

Figure 8 shows the same absorption spectra as in Fig. 7, but with the addition of another absorption spectrum from a peak with an SNR value of 2.5 from the peak labelled Hex in Fig. 5b. Figure 8 shows significant increase in deterioration of the spectral shape between peaks with an SNR value of 10 and 2.5.

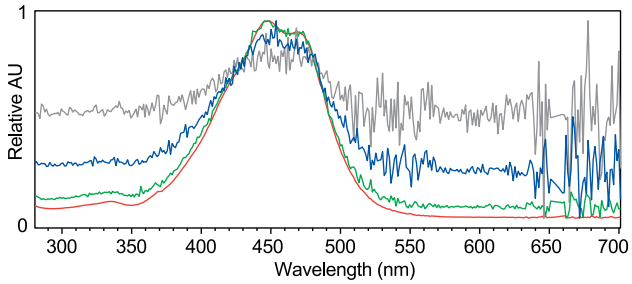

Fig. 8. Figure 7 plus an additional absorption spectrum from Fig. 5b, which has the following characteristics: $0.45\,\mathrm{ng\,inj^{-1}}$ and an SNR value of 2.5 (gray).

In all the examples shown above, the absorption spectra of the peaks with low SNR values are very poor. In all the examples, however, the poor absorption spectra do *not* prove the chromatogram peaks not to be pigments that would be reported for that retention time.

During the SeaHARRE-5 workshop (Sect. 1.7), the two-sentence rule was discussed, and the degree of subjectivity in deciding what constituted a good or bad peak was considered in detail. On the basis of the peak itself, a good peak should have the following: a) a symmetrical shape, b) a good SNR, and c) the same spectrum across the extent of the peak. In comparison with the reference standard, a good peak should have the same peak width, retention time, spectral wavelength(s), and spectral shape.

These workshop discussions also noted that the two-sentence rule is difficult for non-native English speakers who would, therefore, benefit from additional clarifications. Consequently, a *four-sentence rule* was agreed upon (Sect. 1.7.1) as follows (repeated here for convenience):

1. If a peak is *good* and it matches the spectrum, report it;
2. If a peak is *good* and it does not match the spectrum, do not report it;
3. If a peak is *bad* and it cannot be proved to not match the spectrum, report it; and
4. If a peak is *bad* and it can be proved to not match the spectrum, do not report it.

Considering now the four-sentence rule and the three examples above for which the absorption spectra of the peaks with low SNR values are not the same as the corresponding absorption spectra of known standards: the three examples have different peak widths and spectral shapes, so they would not be identified as the pigments that would be reported for that retention time. This is the opposite

result from implementing the two-sentence rule; thus the four-sentence rule is not simplifying the two-sentence rule.

Because the four-sentence rule suggests that a good peak has to have the same peak width and spectral shape as the standard pigment at that retention time and that it must also match the absorption spectrum for it to be reported, then from the examples shown in Figs. 5–8, peaks with an SNR value of 37 or 48 show some increase in peak width compared to peaks with an SNR greater than 400. This result questions where the cut off for discarding a peak should be set.

Perhaps more than one library of absorption spectra should be established. One library should be of absorption spectra from standard peaks that have SNR values less than 10, so the changes in spectral shape and peak width can be compared to the absorption spectra of very small peaks. There should be less discrepancy between shape with this library, which would aid in the accurate identification of small peaks.

2.7 CONCLUSIONS

Since the middle of 2004, CSIRO changed the HPLC method it was using 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 one method that could completely analyze samples from different regions, i.e., tropical and temperate oceanic, coastal, estuarine, and freshwater. The CSIRO method is now able to resolve the divinyl and monovinyl forms of *a*, and Lut from Zea. Resolution between Chl c_1 and Chl c_2 still remains problematic. Accurate identification and quantification of all pigments is important in determining phytoplankton community composition from pigment composition. The new CSIRO method has proven to provide a good balance between accuracy of pigment composition and quantitated concentration versus sample throughput (the number of filters analyzed per unit time).

Chapter 3

The DHI Method

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Abstract

The DHI HPLC method is a modified version of the Van Heukelem and Thomas (2001) method. The method provides good separation of more than 30 of the most important pigments in freshwater, estuarine, and oceanic environments. Validation steps include four injections of a chlorophyll *a* standard to verify the calibration of the HPLC, use of an internal standard for correcting evaporation errors, and injection of a mixture of pigments to verify correct elution and retention times, as well as for documenting the precision of the HPLC and response factor stability. The DHI two-sentence rule is used to guide the analysis of pigments in very low concentrations: i) the relative retention time shall be identical to the pigment in question, and ii) if the spectrum of a small peak matches the spectrum for the pigment in question, quantitate it; otherwise do not quantitate it. When reporting results, the concentration of such small peaks are often lower than the limit of quantitation (LOQ) and are consequently replaced by LOQ values.

3.1 INTRODUCTION

The DHI mix of cultured pigments have been a part of the quality assurance of the pigment method at DHI, since DHI implemented and validated the Van Heukelem and Thomas (2001) method, which occurred prior to the analysis of the SeaHARRE-3 samples. DHI mixed pigments are well suited for documenting the precision of the HPLC, and are used in the validation procedure for controlling the elution order, the separation, and that the retention times and response factors are stable.

DHI holds a Danish Accreditation and Metrology Fund (DANAK) accreditation for carrying out accredited measurements of pigment concentration in aquatic environments. DHI performs pigment analyses by HPLC in accordance with International Organization for Standardization (ISO) 17025† accredited by DANAK, which handles the administration of accreditation and metrology in Denmark.

3.2 EXTRACTION

The SeaHARRE-5 filters were stored in a freezer at −80°C until analysis by HPLC. The filters were extracted in 3 mL of 95% acetone containing vitamin E acetate (Van Heukelem and Thomas 2001) as the internal standard. The samples were sonicated in a sonication bath, precooled with ice to approximately 4°C 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 0.45 µm Teflon syringe filters directly into HPLC vials, and the vials were placed in the cooling rack of the HPLC together with a parallel set of vials with the injection buffer (90:10, 28 mM aqueous TbAA, pH 6.5:methanol).

3.3 HPLC ANALYSIS

The DHI HPLC system is a Shimadzu LC-10ADVP HPLC composed of the following:

- One pump (LC-10ADVP),
- PDA detector (SPD-M10A VP),
- System controller (SCL-10ADVP) equipped with `LC Solution` software version 1.24 SP1,
- Temperature-controlled autosampler (TCAS) set at 4°C,
- A column oven (CTO-10ASVP) set at 60°C, and
- A degasser.

The samples were mixed with buffer by the autoinjector by programming it to withdraw 150 µL buffer, 72 µL sample, 57 µL buffer, 71 µL samples, 150 µL buffer, and inject the entire amount (500 µL in total, sample volume injected

† ISO 17025 is an international standard (published by the International Organization for Standardization) that specifies the general requirements for the competence to carry out tests and calibrations.

was 143 µL) onto the column. The method used for HPLC analysis was the VHT method, where solvent B was methanol, and solvent A was (70:30) methanol:28 mM aqueous TbAA, pH 6.4 (Table 28). The column was an Eclipse XDB C8, 4.6×150 mm (Agilent Technologies), the flow rate was 1.1 mL min^{-1}, and the temperature of the column oven was set to 60°C.

Table 28. The gradient system used with the DHI HPLC method. Solvent A is 70:30 methanol:28 mM aqueous TbAA, 6.4 pH, and solvent B is 100% methanol.

Step	*Time*	A [%]	B [%]
Start	0	95	5
2	5	95	5
3	27	5	95
4	34	5	95
5	35	0	100
6	38	0	100
End	39.5	95	5

A total of five mixed pigment samples taken from DHI Mix-105 were analyzed with the SeaHARRE-5 field samples, and they were randomly distributed in between the samples, followed by a blank (extraction solvent with vitamin E).

The identification and integration of pigment peaks occurred at three different wavelengths: chlorophyllide *a*, phaeophytin *a*, phaeophorbide *a*, divinyl chlorophyll *a*, and monovinyl chlorophyll *a* were determined at 665 nm; the internal standard was determined at 222 nm; and the rest of the pigments were determined at 450 nm. Peak identities were routinely confirmed by online PDA analysis.

3.4 CALIBRATION

The HPLC was calibrated with pigment standards obtained from DHI (Hørsholm, Denmark), prior to analysis of the SeaHARRE-5 samples. Four pigment standards were mixed, and a series of six dilutions were made of each standard mixture: three different concentrations at the range of expected concentrations of the natural samples, and three different concentrations at low concentrations near the limit of quantitation (LOQ) and limit of detection (LOD). The linearity of the series was verified, and the response factors were calculated from the regression forced through zero.

The concentrations of the calibration standards for each pigment were determined using a Shimadzu model UV-2401PC dual-beam, monochromator-type spectrophotometer. The performance capabilities of the spectrophotometer are maintained using a routinely applied set of quality control procedures. The absorption coefficients used are shown in Table 29. Selected batch numbers of the standards are controlled for purity and concentration by an independent laboratory.

Table 29. Absorption coefficient (α) values in liters per gram per centimeter used with the DHI method for the pigments listed as a function of wavelength (λ).

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
Peri	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
Diad	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
ββ-Car	100% Ethanol	453.0	262.00

3.5 VALIDATION

DHI holds a DANAK accreditation for carrying out accredited measurements of HPLC pigment concentration in the aquatic environment. DHI performs pigment analyses by HPLC in accordance with ISO 17025 (accredited by DANAK). Validation follows the DHI Standard Operating Procedure (SOP) No. 30/852:01. Accredited measurements of pigment concentrations in the aquatic environment. DHI validation steps include the following:

- Four injections of a chlorophyll *a* standard to verify the calibration of the HPLC,
- Use of an internal standard for correcting evaporation errors, and
- Injection of a mixture of pigments (DHI mixed pigments) to verify correct elution, retention times, and for documenting the precision of the HPLC and response factor stability.

The identification of the individual pigments in each sample is confirmed by comparing the relative retention time with the retention time in DHI mixed pigments and by comparing the absorption spectra to spectra from standards loaded in the `LC Solution` software library.

Concerning the high uncertainties introduced when reporting very small peaks, the two-sentence rule (Sect. 1.7.1)

was posed as a mechanism to guide analysts toward consistency in reporting and quantitating practices (Hooker et al. 2009). The purpose of the rule is to lower the high uncertainties arising when pigments are weakly present and to obtain consistent reporting practices between laboratories. This rule was discussed at both the SeaHARRE-4 and SeaHARRE-5 workshops which included discussion on how to define a "good" or a "bad" peak. At DHI, the spirit of having a guiding principle to determine whether or not a peak would be quantitated was retained, but the specifics of the two-sentence rule was modified as follows:

1. The relative retention time shall be identical to the pigment in question.
2. If the spectrum of a small peak matches the spectrum for the pigment in question, quantitate it; otherwise, do not.

Examples on the use of the DHI two-sentence rule are shown in Fig. 9 for sample `A`. A peak with the retention time of But is detected in front of Fuco. There is hardly a spectrum for this peak and the library search function of the `LC Solution` software shows that the spectrum does not match the inserted spectrum of a But standard. Consequently, the peak was not included when calculating the individual concentrations of pigments in the sample. The three peaks after Fuco in Fig. 9 have retention times matching Neo, Pras, and Viola, respectively, and the spectra of these pigments match the spectra according to the library search function of the `LC Solution` software. Consequently, the pigments were included.

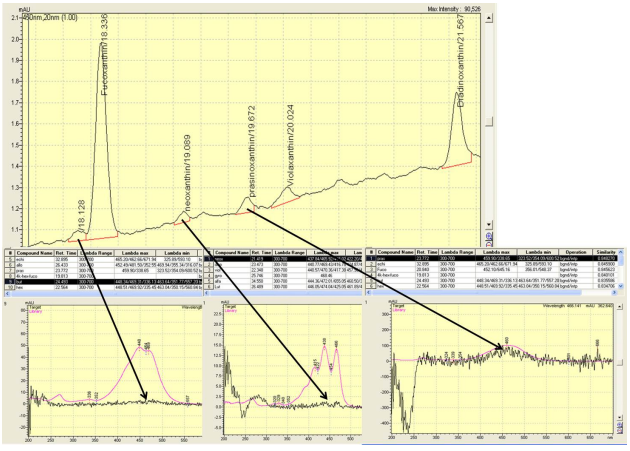

Fig. 9. A portion of the chromatogram (upper part) for sample `A` showing examples of small peaks, where the DHI two-sentence rule was applied. It was determined to exclude But (lower left), but to include Neo (lower middle), Pras (lower right), and Viola (not shown).

Once the decision to include a small peak is made by the HPLC analyst, the peak area of the pigment is transferred to the spreadsheets where the corresponding pigment concentration is calculated. When reporting results, the concentration of the small peaks are often below the LOQ threshold and are consequently replaced by LOQ values calculated as described in the SeaHARRE-2 report (Hooker et al. 2005). This was the case for Neo, Pras, and Viola in Fig. 9, which were all close to their respective LOD values (and, thus, below their respective LOQ values).

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:

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

where the $\hat{A}_{P_i}$ and R_{P_i} terms 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 HPLC 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.

The SeaHARRE-5 pigment quantitation results were submitted in separate spreadsheets with zero values in one sheet, and results where LOQ values were inserted when the concentrations were lower than LOQ. An established level of precision (i.e., reported number of digits) was used for all reported concentrations.

3.7 CONCLUSIONS

The method used at DHI for the SeaHARRE-5 analyses was the VHT method, which in the SeaHARRE exercises has been shown to provide state-of-the-art results, in terms of the SeaHARRE performance metrics (Table 9). The VHT method has been used at DHI since 2005, and during the intervening years, considerable knowledge regarding the capabilities of the VHT method has been acquired and applied in the laboratory during sample analyses. For example, long-term information on response factors, retention time stability, pressure on the column, and knowledge on the duration of the buffer have all been acquired and applied over time. The temporal characteristics of these parameters yield valuable information, which is used in the validation procedures applied during sample analyses.

Chapter 4

The UN Method

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Abstract

A new HPLC method for analyzing oceanographic field samples was developed by the University of Nordland (formerly known as Bodø University College) and used for the analysis of the SeaHARRE-5 samples. The method was based on a regularly used method for carotenoid analysis of cultivated algal species. To obtain improved resolution of the large number of pigments that may be present in oceanographic samples, the new method used two C_{18} columns and a lower solvent flow. The new method provided a good separation, identification, and quantitation for carotenoids of all polarities, with the exception of lutein and zeaxanthin. For the chlorophyll pigments, the usefulness of the method is limited, because it did not separate either the polar chlorophyll c_1, chlorophyll c_2, and MgDVP, or the divinyl and monovinyl forms of chlorophyll *b*. Further method adjustment would be needed to obtain separation of these pigments, or other methods must be used. For chlorophylls *a* and *b*, the method showed very good separation of chlorophyll allomers and epimers from the parent pigments, which is a capability not observed with common HPLC methods.

4.1 INTRODUCTION

For the purpose of the SeaHARRE-5 samples, a new HPLC method was developed. This method was based on the regular HPLC method for carotenoid analysis at the Faculty of Biosciences and Aquaculture (FBA), University of Nordland (UN), which was developed for the analysis of cultivated algal species. Despite this, it has commonly been used for carotenoids from aquatic animals, except where non-esterified astaxanthin has been the main pigment (e.g., salmon flesh).

The method developed for SeaHARRE-5 samples was based on the so-called *HPLC method 4* as published by Egeland et al. (1995). The method used a C_{18} column, diode array detection, as well as a quaternary gradient composed of 1 M ammonium acetate, methanol, acetone, and hexane. To obtain improved resolution of the large number of pigments that may be present in oceanographic samples, the new method used two C_{18} columns and a lower solvent flow. No effort was made to obtain a good separation of chlorophylls, because these pigments are not prioritized at FBA.

The new method gave a good separation of carotenoids of all polarities, from glycosides to fatty acid esters, with the exception of lutein and zeaxanthin. Some polar chlorophylls were not resolved, while various chlorophyll *a* and *b* allomers and epimers were well resolved from intact chlorophylls *a* and *b*.

4.2 EXTRACTION

The samples were stored in their original aluminum foil packets at −32°C for 10 months, before the start of the analyses. The analyses were performed in dim light, by turning off all light in the laboratory except a single light at the other end of the room, which did not provide direct illumination onto the samples.

All filters were removed from the aluminum foil packets and transferred to labelled 6 mL vials covered with a lid without septum (opening in each lid) and lyophilized. The lyophilized filters were stored at −32°C until extraction (0–14 days later). Because only 12 filters can be analyzed each day using the developed HPLC method, filters were scheduled for extraction every day from Monday to Thursday, yielding enough extracts to analyze until the following day, or the entire weekend.

For the extraction, each filter was torn by a pair of pliers and added to 1 mL of −32°C 30% methanol in acetone kept in 2 mL vials. The vials were flushed gently under a stream of nitrogen, immediately capped, and kept overnight at −32°C. The next day, the extract solvent was transferred with a Pasteur pipette to a syringe with an 0.2 µm Anotop 10 syringe filter (Millipore) and filtered into an amber 2 mL HPLC vial, flushed with nitrogen and capped. The batch of 12 extracts (or more, if analysis took place on a Friday) were kept in the HPLC autosampler at 4°C until analysis.

4.3 HPLC ANALYSIS

The HPLC analyses were performed using an Agilent 1100 HPLC instrument with vacuum degasser, thermostatted autosampler with enlarged injection loop, quaternary pump, thermostatted column compartment, and diode array detector. Two identical C_{18} columns were used (ACE 5 C_{18} part no. ACE-121-2546, 4.6×250 mm each, with 5 µm packing) with a separate guard column (ACE). The column was kept at a stable temperature of 25°C. As eluent, a gradient with 1 M ammonium acetate (AmAc), methanol (MeOH), acetone, and hexane were used, as given in Table 30. The flow was kept constant at 0.5 mL min^{-1}.

Table 30. The gradient system used with the BUC HPLC method. Solvent A is 1 M AmAc, solvent B is MeOH, solvent C is acetone, and solvent D is hexane.

Step	*Time*	A [%]	B [%]	C [%]	D [%]
Start	0	20	80	0	0
2	60	0	70	30	0
3	100	0	30	50	20
4	110	0	0	40	60
5	120	0	100	0	0
End	130	20	80	0	0

The same volume of extract, 50 µL, was injected from each extract vial (without mixing), with the accidental exception of five random samples wherein only 25 µL was injected. The detection wavelengths were 390, 420, 450, and 480 nm. All pigments were identified on the basis of retention time compared with an external standard and their visible spectra. Quantification was done on the basis of the calibration performed just before analyzing the samples.

4.4 CALIBRATION

For the calibration, the dry pigment (either solid or dry after evaporation of the solvent) was dissolved in a pure solvent of known volume (using a 1.00 mL measuring flask). The spectrum was recorded by a common spectrophotometer, and the standard solution was injected in 0.2, 1, 5, 10, and 25 µL quantities onto the HPLC column. A linear calibration line was made using the HPLC computer program.

Solvents and absorption coefficients are given in Table 31. All quantities were measured using the peak of the spectrum, for wavelengths obtained from the spectrometer, see the table. For diatoxanthin, it was observed that the calibration was not correct for the small amounts present in the sample, because the calculated concentration for a few samples were negative. Pigments used for calibration were either donated, purchased from a commercial entity (CaroteNature, DHI, DSM, Sigma-Aldrich, and VWR), or isolated from a known source.

Table 31. Absorption coefficient (α) values in liters per gram per centimeter used with the UN method for the pigments listed as a function of wavelength (λ). For pigments with no measured absorption coefficients, a hypothesized value based on similarities to other pigments has been used.

Pigment	*Solvent*	λ	α
Allo	Benzene	463.5	216.00
Anthera	Ethanol	447.0	235.00
But-fuco	Acetone	443.5	147.00
ββ-Car	Hexane	450.5	259.00
βε-Car	Hexane	445.0	270.00
εε-Car	Hexane	439.5	312.00
ψψ-Car	Hexane	471.0	347.00
Chl *a*†	Acetone	663.0	88.20
Chl *b*†	Acetone	456.0	132.00
Chl c_2	Acetone	630.0	37.20
Chl c_3	1% pyr. in ace.‡	452.9	346.00
Chlide *a*	90% Acetone	665.5	127.00
Croco	Hexane	445.5	237.00
Crypto	Hexane	450.0	246.00
Diad	Acetone	446.5	224.00
Diato	Acetone	453.5	272.00
DVChl *a*	Acetone	663.5	88.20
Fuco	Acetone	446.0	166.00
Gyro diester	Acetone	446.0	183.00
Hex-fuco	Acetone	444.5	142.00
Lut	Ethanol	446.0	255.00
MgDVP	90% Acetone	628.5	45.80
Myxo	Acetone	477.5	216.00
9′-*cis*-Neo	Ethanol	438.0	233.00
Phide *a*	90% Acetone	666.5	74.20
Phytin *a*	90% Acetone	666.0	51.20
Peri	Acetone	473.0	134.00
Pras	Acetone	453.0	182.00
Viola	Acetone	442.5	240.00
Zea	Acetone	453.5	234.00

† Entries are also used for allomers and epimers.
‡ 1% pyridine in acetone.

4.5 VALIDATION

The 72 field samples, 5 DHI Mix-105 samples, and two pure solvents (blanks) were analyzed in random order. All peaks were automatically labeled and quantified relative to the external standards. All peak identities were manually controlled to avoid misidentification or lack of identification because of small shifts in retention time.

In addition, spectra for each pigment peak were checked manually, to ensure that no incorrect identities were reported, or severely impure peaks were assumed to be a pure pigment. Both spectral shape and wavelength were checked to detect any impurities (although any coelution of another pigment with identical spectrum could not be detected). In case of spectrum distortion by noise due to

small amounts of pigment, these were accepted, unless different shapes or absorption maximum were seen.

Many peaks were observed for the derivatives of both chlorophylls *a* and *b* with similar retention times as their parent pigment. This may be due to the long storage time before the analysis, but may also be due to other, unknown factors. The degradation of chlorophylls *a* and *b* should be taken as a warning that degradation of other pigments may also have occurred.

For the derivatives of chlorophylls *a* and *b*, the absorption coefficients for intact chlorophylls *a* and *b* were used. Because these pigments will probably have different absorption coefficients, the quantitation of these pigments are not accurate. Note that the quantitation of chlorophyll will be about just as wrong, when using methods not separating these chlorophyll derivatives, whenever they are present.

To check if these derivatives were caused by the HPLC method, pure chlorophyll *a* was purchased from Sigma-Aldrich and DHI. The DHI chlorophyll *a* solvent was evaporated, and the pigment redissolved in case the solvent itself could cause degradation. For the DHI chlorophyll *a*, only one peak was observed. In comparison, for a total of eight samples received from Sigma-Aldrich, a number of peaks were observed for all samples, despite one of these samples only having a single peak when analyzed by the HPL laboratory.

4.6 DATA PRODUCTS

The amount injected onto the column for each identified pigment was automatically calculated for each sample by the HPLC program. The value given was inserted in a `Numbers` spreadsheet and the concentration of each pigment per liter of seawater was calculated, taking into consideration the volume of injected sample relative to the total volume, and the reported volume of water filtered. Because of the large uncertainty in the absorption coefficients, all quantities were reported with only one or two significant digits.

4.7 CONCLUSIONS

The UN HPLC method developed for the SeaHARRE-5 activity was good for the identification and quantification of algal carotenoids, with the exception of lutein and zeaxanthin. For chlorophylls, the usefulness of the method is limited, because it did not separate either the polar chlorophyll c_1, chlorophyllc_2, and MgDVP, or the divinyl and monovinyl forms of chlorophyll *b*. Further method adjustment would be needed to obtain separation of these pigments, or other methods must be used. For the chlorophylls *a* and *b* pigments, the method showed very good separation of chlorophyll allomers and epimers, not observed by common HPLC methods.

Chapter 5

The GSFC Method

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Abstract

The GSFC Method uses an Agilent 1200 Series Rapid Resolution LC system and a single quadrupole mass spectrometer, which was purchased in August 2008. The purpose of the system was to establish protocols to a) reduce the amount of time needed to analyze an HPLC sample, and b) improve the detection and quantitation of marine pigments. The latter is the most important, because a significant limitation with the current protocols is the inability to unequivocally identify and accurately quantitate pigments in low concentrations. The SeaHARRE-5 activity was the first operational test of the system, however, the aforementioned configuration could not be used because of method incompatibility issues that are still unresolved. Instead, a configuration akin to a standard Agilent 1200 series system (quaternary pump and standard vacuum degasser) was used. A new method (to the chromatographer) applied simultaneously to a new instrument proved to be a significant challenge. This chapter includes a discussion on the method and analysis tools used during participation. Moreover, the challenges met and overcome to achieve quality-assured status is also discussed.

5.1 INTRODUCTION

For the purposes of SeaHARRE participation, the configuration of the Agilent 1200 series system included a quaternary pump, standard vacuum degasser, temperature controlled autosampler (TCAS) SL with a 900 µL metering head, thermostatted column compartment SL, and a diode array detector (DAD) SL (80 Hz sampling rate). The GSFC laboratory used an adaptation of the Van Heukelem and Thomas (2001) method. The SeaHARRE-5 samples were not processed until the instrument performance was at the state of the art level, as described by the performance metrics table (Hooker et al. 2005). The resolution achieved for the critical pairs of Zea/Lut and Viola/Hex were 1.51 and 2.04, respectively.

5.2 EXTRACTION

All the SeaHARRE-5 samples were delivered to GSFC by February 2009. The regular SeaHARRE-5 samples were extracted and analyzed from 25–27 August 2009. The extraction procedure was adapted from that used by HPL and was as follows:

1. A mixture of 2.5 mL 100% acetone and vitamin E acetate was added to polypropylene tubes using an automatic pipette. The vitamin E mixture was stored in a glass amber bottle at 15°C and warmed to room temperature prior to pipetting; 100 µL water was manually pipetted into each tube.
2. The tubes were covered with Parafilm (to stop evaporation) and placed in a −15°C freezer for 30 min.
3. The tubes were removed from the freezer, frozen filters were added to each, and they were placed back in the freezer for approximately 1 h. The samples were sonicated using a Branson Sonifier Cell Disruptor with a probe (on ice) for 25–30 s (or until the filter was pulverized), and the tubes were returned to the cooler (on ice) after sonication. The sonicator settings were a) pulse on for 1 s, b) pulse off for 0.5 s, and c) amplitude of 25%.
4. The tubes were returned to the freezer for 3–6 h.
5. The tubes were removed from the freezer and the extract was filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter and a plastic syringe.
6. Extracts were stored in amber glass scintillation vials with foil-lined caps and were then either placed in a −15°C freezer or immediately analyzed.

5.3 HPLC ANALYSIS

The GSFC HPLC analysis is organized in terms of the separation, detection, and injection procedures used.

5.3.1 Separation

Pigments were separated using a 4.6×150 mm Agilent Eclipse XDB C_8 column, 3.5 µm particle size, heated at

a set temperature of 60°C. The method was based on Van Heukelem and Thomas (2001), 70:30 methanol:28 mM TbAA 6.5 ph, solvent A; and 100% HPLC-grade methanol (Fisher A452-4), solvent B. The TbAA was made by diluting 70 mL of 0.4 M HPLC-grade tetrabutyl ammonium hydroxide (J.T. Baker V365-07) in a beaker of 500 mL of HPLC-grade water (Fisher W5-4) on a stir plate. The pH was adjusted with concentrated acetic acid until the desired pH was achieved (pH 6.5 ± 0.02). After adjustment of the pH, the TbAA was transferred to a 1,000 mL graduated cylinder. The beaker was rinsed three times with the HPLC-grade water and the solution was brought to a final volume of 1 L. The solution was mixed, the pH was rechecked again, and it was stored in a 1 L amber bottle.

Solvent A was made by measuring 300 mL of TbAA and 700 mL of methanol in separate glass 1,000 mL graduated cylinders. The TbAA was then added to the methanol. Typically, a 3–4 L batch of solvent A was made at one time to maintain consistency of analysis. The solvent was then vacuum filtered (at approximately 5–7 psi) through a 0.2 µm pore-size polycarbonate filter (Gelman 66602).

Solvent C was 100% HPLC-grade acetone (Fisher A949-4) and was used as a flush at a 1.3 mL min^{-1} flow rate, the purpose of which was to prevent carryover between samples. The method was extended 5 min longer than the original Van Heukelem and Thomas (2001) method to improve separation of critical pairs, and also to maintain the peak shape of the Chl *c* pigments (Table 32).

Table 32. The GSFC pump gradient used during SeaHARRE-5. Solvent A is 70:30 methanol:28 mM TbAA (pH 6.5), solvent B is methanol, and solvent C is an acetone rinse to help alleviate carryover.

Time [min]	A [%]	B [%]	C [%]	*Flow* [mL min^{-1}]
0	95	5	0	1.1
27.00	5	95	0	1.1
29.50	5	95	0	1.1
29.75	30	65	5	1.3
30.85	30	65	5	1.3
31.10	95	5	0	1.1
34.10	95	5	0	1.1

The injection buffer was produced by measuring 90 mL of 28 mM TbAA in a 100 mL graduated cylinder. A 10 mL glass volumetric pipette was then used to add 10 mL of HPLC-grade methanol to the cylinder. The buffer was filtered through a PTFE syringe filter, 0.45 µm pore size, and stored in a 100 mL glass amber bottle with a Teflon-lined cap. The samples were kept at 4°C in the TCAS until HPLC analysis.

5.3.2 Detection

Most pigments were analyzed using the 665 nm (20 nm bandwidth) or 450 nm (20 nm bandwidth) wavelengths. In addition, Chl *b* was analyzed at 474 nm (10 nm bandwidth), and analyzed and reported on both the 450 and 474 nm channels. Vitamin E was analyzed at 222 nm (10 nm bandwidth). The response time was 0.5 s, and the slit width was 2 nm. The DAD SL (80 Hz) contained a standard size flow cell (10 mm nominal path length, 9.80 ± 0.7mm actual) with a 13 µL cell volume.

5.3.3 Injection

The injector procedure used by GSFC is presented in Table 33. The needle wash was a programmed dip of the needle into a vial of acetone that was changed once per day. The TCAS was allowed to equilibrate to 4°C for 1 h or more prior to the samples being placed into sample trays. The adapted method of Van Heukelem and Thomas (2001) added 25 µL of buffer at the beginning and end of the injection sequence, which allowed for better peak shape of the Chl *c* pigments and better separation of critical pairs.

Table 33. The HPL injector program used during SeaHARRE-5, with amounts in microliters and speeds in microliters per minute.

Step	*Amount*	*Source*	*Speed*
1. `DRAW`	175	Buffer	130
2. `DRAW`	75	Sample	130
3. `DRAW`	0	Acetone	130
4. `DRAW`	75	Buffer	130
5. `DRAW`	75	Sample	130
6. `DRAW`	0	Acetone	130
7. `DRAW`	175	Buffer	130
8. `INJECT`	575		250

5.4 CALIBRATION

Calibration of the GSFC HPLC took place from 23 June to 24 August 2009, beginning with the Chl *a* calibration. For Chl *a*, the system was calibrated using a crystallized spinach Chl *a* standard from Sigma-Aldrich (C5753). The crystals were dissolved in 90% acetone at room temperature, and under darkness, overnight. The concentration of the Chl *a* extraction was measured spectrophotometrically the following day. A five-point dilution series was made; the amount injected ranged from 2–180 ng inj^{-1}.

For all other pigments, standards were purchased from DHI (Table 34). The DHI concentrations for each standard were used for computing calibration curves. Five-point calibration curves were computed for each standard. Standards were diluted with 90% acetone using calibrated glass, gas-tight syringes, and stainless steel needles. The syringes were rinsed thoroughly with acetone after use. If needed, one point was eliminated from the calibration to improve residuals, and acceptance or rejection of a calibration curve was based on a 2.0% threshold for residuals. The calibration was repeated if this requirement was not met.

The average residuals for all calibrations met the 2.0% or less threshold, except for Neo and Phytin *a*. For these two standards, although calibrations curves were repeated, it was necessary to delete two points from the calibration curve to achieve average residuals below 2.0%.

Table 34. The α values used by the GSFC method as a function of the solvents and maximum wavelengths (λ_m) specified for use with α values.

Pigment	*Solvent*	λ_m	α
Chl c_3	90% Acetone	453	346.00
Chl c_2	90% Acetone	444	374.00
Chlide *a*	90% Acetone	664	127.00
Phide *a*	90% Acetone	667	74.20
Peri	100% Ethanol	472	132.50
But	100% Ethanol	446	160.00
Fuco	100% Ethanol	449	160.00
Neo	100% Ethanol	439	224.30
Pras	100% Ethanol	454	160.00
Viola	100% Ethanol	443	255.00
Hex	100% Ethanol	447	160.00
Diad	100% Ethanol	446	262.00
Allo	100% Ethanol	453	262.00
Diato	100% Ethanol	449	262.00
Zea	100% Ethanol	450	254.00
Lut	100% Ethanol	455	255.00
Chl *b*	90% Acetone	647	51.36
DVChl *a*	90% Acetone	664	87.67
Chl *a*	90% Acetone	664	87.67
Phytin *a*	90% Acetone	667	51.20
βε-Car	100% Acetone	448	270.00
ββ-Car	100% Acetone	454	250.00

The *y*-intercept was forced through zero for all curves after calculating and establishing that the *y*-intercept was close to zero and negligible. Percent purity was calculated for each standard, and concentrations were adjusted accordingly. The mean response factor (R_{P_i}) from each curve was used for pigment quantitation. A spectral library was created from these standards for the purpose of the SeaHARRE-5 activity.

5.5 VALIDATION

The validation of the GSFC HPLC system took place from 23 June to 24 August 2009. Quality assurance was part of the validation activity, and was also part of the daily analysis of the SeaHARRE-5 samples. At the beginning of each analysis day, a blank was run (90% acetone injection) first to condition the system. One to two injections of several standards (DHI Mix-105, Chl *a*, and vitamin E) were performed prior to running samples to confirm that the system was running optimally. In order to monitor instrument precision and monitor changes in retention time or injection failures, between every six samples, injections of vitamin E standard and either five-fold diluted retention time mix (DHI Mix-105) or a DHI Chl *a* were performed.

The mean area of vitamin E during the analysis of the regular SeaHARRE-5 samples was 3166.89, with a CV of 0.47%. The mean DHI Chl *a* concentration was $1901.20\,\mu g\,L^{-1}$ with a CV of 1.04%. It was later determined that the precision for the retention time mix was lower because separate batches of diluted (five-fold) retention mix, rather than one large batch, were made. For the DHI retention time mix, the highest CV occurred for Peri, Chl *b*, Diato, and Diad (9.6, 4.5, 4.0, and 5.6%, respectively). The CV for all other pigments in the mix ranged from 2–3%.

5.5.1 Discussion

The reasons GSFC purchased the Rapid Resolution LC were threefold: a) to expand upon the foundation of the pigment analysis method of Van Heukelem and Thomas (2001), b) to apply the GSFC modification of this method to the analysis of SeaHARRE-5 samples, and c) to eventually develop and establish new and innovative techniques for lowering the uncertainties in pigment quantitation. The configuration of the Rapid Resolution LC system includes a high-pressure binary pump, microvacuum degasser, and a 200 µL metering head.

The smaller metering head capacity, smaller volume of the microvacuum degasser (1 mL per channel instead of 12 mL per channel in standard vacuum degassers), and smaller tubing internal diameter (and eventually shorter, smaller bore size columns) were necessary to reduce dwell volume, so that the system could accommodate higher pressures as part of the rapid resolution capability. Because of the small metering head capacity, a multidraw loop would be required to accommodate the large injection volume. The original injector program with this configuration required multiple draws of sample and buffer, which were ejected into the multidraw loop until injection of the full volume could occur.

5.5.2 System Issues

Although all hardware components of the GSFC system were received in October 2008, the system was not installed until 27 January 2009. The system, in its entirety, was installed for operation in the traditional mode (for immediate use), not in the rapid resolution mode. Unfortunately, pressure instability problems during HPLC analysis were immediately observed.

With a brand new column and fresh mobile phases, the pressure at initial conditions (95% mobile phase A and 5% mobile phase B) was too high (over 200 bar). Moreover, after every injection of sample, the pressure would incrementally increase, and never stabilize as shown in Fig. 10 (note that the actual *x*- and *y*-axis values are unimportant). The chromatography of the pigments was acceptable (e.g., the resolution of the Zea/Lut critical pair was

greater than 1.45); however, the instability of the pressure was troubling. It was considered plausible that the rapid resolution configuration, which allows a lower dwell volume (i.e., smaller internal diameter of the tubing, etc.), may result in higher pressures than those observed with the traditional configuration. This explanation would not account for the observed instability, however. The problem required further investigation and a solution, because pressure instability in chromatography can ultimately result in loss of resolution, peak distortion, and degradation of the column.

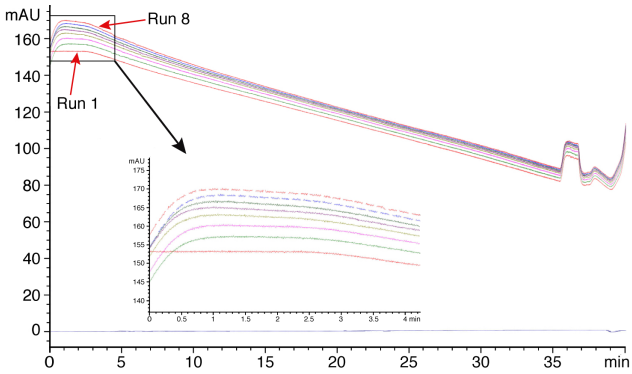

Fig. 10. Increasing pressure over eight runs; the system configuration is with a binary pump and microvacuum degasser. The shape of the pressure trace at 35 min is due to the acetone rinse and is normally observed for this method.

The first investigative hypothesis was that the problem was occurring in the mixing chamber. The problem persisted, however, with 100% solvent A (TbAA and MeOH). The second hypothesis was that precipitates from the TbAA/MeOH solution were aggregating on the column, however, after installing a prefilter, the situation was not resolved (although the aggregates could be smaller than the prefilter pore size). Yet another hypothesis was that the compressibility compensation needed to be adjusted for TbAA, but that also did not solve the issue.

5.5.2.1 The VHT Method

After the system was reconfigured with a quaternary pump and standard vacuum degasser instead of the binary pump and microvacuum degasser, the system performed adequately, so the analyses of the SeaHARRE-5 field and laboratory samples proceeded. The system was calibrated and validated over a two-month time period from mid-June to mid-August 2009. On 4 September 2009, sample analysis was complete.

During the analysis of the SeaHARRE-5 samples, although performance was improved, the pressure was unstable (Fig. 11). After five days of continuously running, the pressure increased 40 bar, from 116 to 156 bar, at initial conditions. In addition, after only 115 injections, the column showed other signs of degradation, such as peak broadening and retention time shifting.

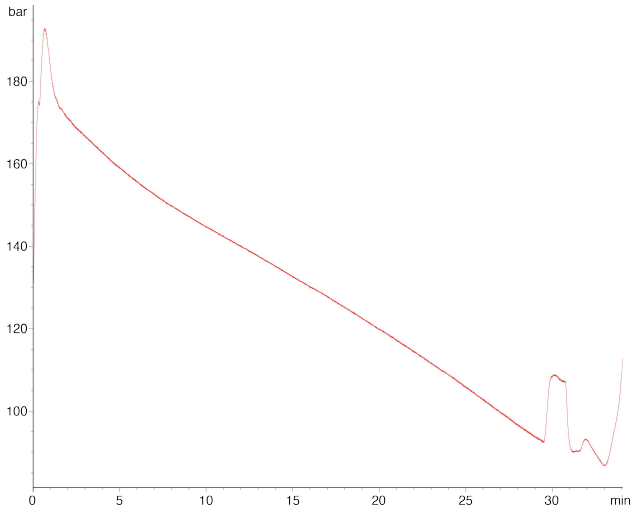

Fig. 11. The pressure trace after 115 injections with the VHT method. Note the large peak in pressure after injection; the system configuration is with a quaternary pump and a standard vacuum degasser.

After a new column was installed, the pressure trace returned to expected values (Fig. 12). Column degradation after 100 injections was not considered normal or acceptable. For example, it is not unusual for a practitioner of the VHT method to get approximately 1,000 injections before a column must be replaced, and at least one practitioner gets about 2,000 injections per column. Although the pressure problems persisted, it was not at the severity of the previous configuration. Moreover, the pressure issues and short life of the column apparently did not negatively impact the SeaHARRE-5 analyses and results.

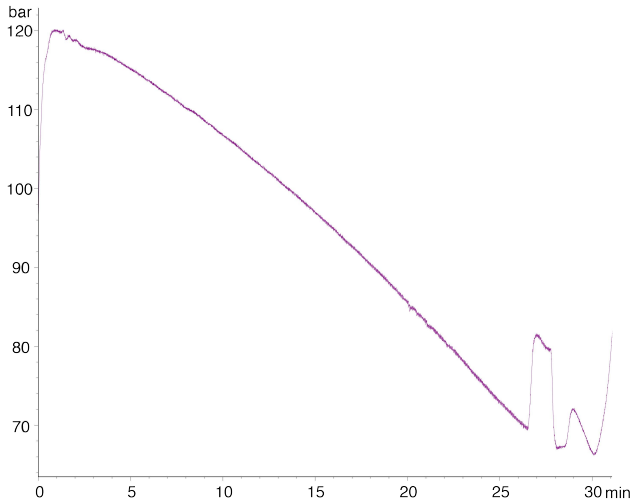

Fig. 12. The pressure trace for the VHT method after a new column was installed. Note the peak in pressure after injection is absent; the system configuration is with a quaternary pump and a standard vacuum degasser.

After the SeaHARRE-5 analyses were completed, the focus was shifted to troubleshooting the hardware, not only to determine why the VHT method was apparently not compatible with the Agilent 1200 Rapid Resolution system hardware, but also to determine whether or not other established marine pigment chromatographic methods could be used on the system, perhaps to greater efficacy. It was also discovered that TbAA is not compatible with mass spectrometric analysis, because TbAA volatilizes, creating a very large background noise. On 1 October 2009, a meeting with Agilent representatives took place to determine the necessary steps to completely resolve the Agilent 1200 series Rapid Resolution LC system pressure issues. The following steps were agreed upon:

1. A compliance test of all hardware (completed 19 October 2009);
2. Repeated injection of reserpine standard, with a mobile phase of 70% ammonium formate and 30% water, over a 48 h time period (completed November 2009); and
3. Incorporate a completely different pigment chromatography method that does not contain TbAA to test for pressure instability and run it for three consecutive days (completed 11 November 2009).

The purpose of the compliance test was to confirm that all of the hardware associated with the Agilent 1200 series Rapid Resolution LC system was fully functional. The microvacuum degasser and binary pump were reintroduced into the configuration, because they are necessary for the maturation of future applications (i.e., rapid resolution).

According to the Equipment Qualification Report, a document provided by Agilent containing the results of the compliance test, all of the hardware passed the rigorous testing. It can be stated confidently that the Agilent hardware did not play a direct role in the repeated occurrence of the pressure instability (i.e., the hardware itself was not faulty).

To test the system when analyses are performed over a consecutive number of days, Agilent implemented a method to run the quality control standard reserpine over two days, using a mixture of ammonium formate and methanol as the mobile phase. The column used for this test was a Zorbax 3.5 µm SB-C-18 2.1×30 mm. The mean pressure over 1,084 injections was 59.5 bar at the beginning of a run, and was 65.8 bar at the end of a run. The pressure ranged from 58.8–59.7 bar at the beginning of the run, and from 64.8–66.6 bar at the end of the run.

5.5.2.2 The Zapata et al. (2000) Method

The system was reconfigured to incorporate the binary pump and microvacuum degasser to test the pressure stability of the system using the Zapata et al. (2000) method. In this method, the buffered solvent contains aqueous pyridine. The intent of this test was to determine if the pressure instability would also be observed in methods that do not contain TbAA. For the purposes of the test, a Zorbax C_8 column was used (the same as used in the VHT method).

Over the three-day period of 5 November and 9–10 November 2009, approximately 108 blank injections were run using the Zapata et al. (2000) method. During the analyses, the pressure ranged from 125–127.9 bar (with a mean of 126.5 bar) at the beginning of the run, and 121.1–122.9 bar (with a mean of 121.9 bar) at the end of the run. The pressure trace shown in Fig. 13 is an overlay of six separate runs (again, the actual x- and y-axis values are unimportant). Overall, there was no significant increase in pressure over time.

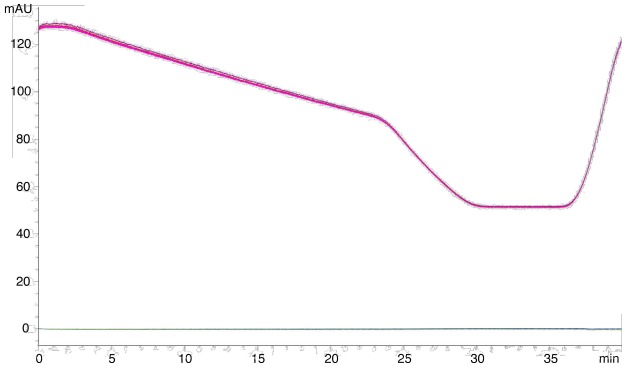

Fig. 13. The pressure trace for the Zapata et al. (2000) method; the system configuration is with a binary pump and microvacuum degasser.

5.6 DATA PRODUCTS

The concentration, C_{P_i}, of each pigment in the standard mix and samples was calculated using the following equation:

$$C_{P_i} = \frac{V_x}{V_f}\frac{\hat{A}_c}{\hat{A}_s}\frac{\hat{A}_{P_i}}{V_c}R_{P_i}, \tag{30}$$

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, $\hat{A}_c$ is the peak area of the internal standard in the extraction solvent (an average of the multiple injections as part of the validation procedure during sample processing), and $\hat{A}_s$ is the peak area of the internal standard in the sample. The latter two terms provide a correction for the residual water left in the filter.

The SNR was also calculated for all samples. The noise was calculated according to procedures outlined in Hooker et al. (2005). First, the noise was determined for each channel (450, 474, and 665 nm) using seven injections of vitamin E during sample analysis, because its signal is detected only at 222 nm. The `ChemStation` software was used to calculate noise at 3 min intervals from 3–30 min of each chromatogram. The highest and lowest noise values were excluded, and the mean of the noise from each chromatogram was calculated.

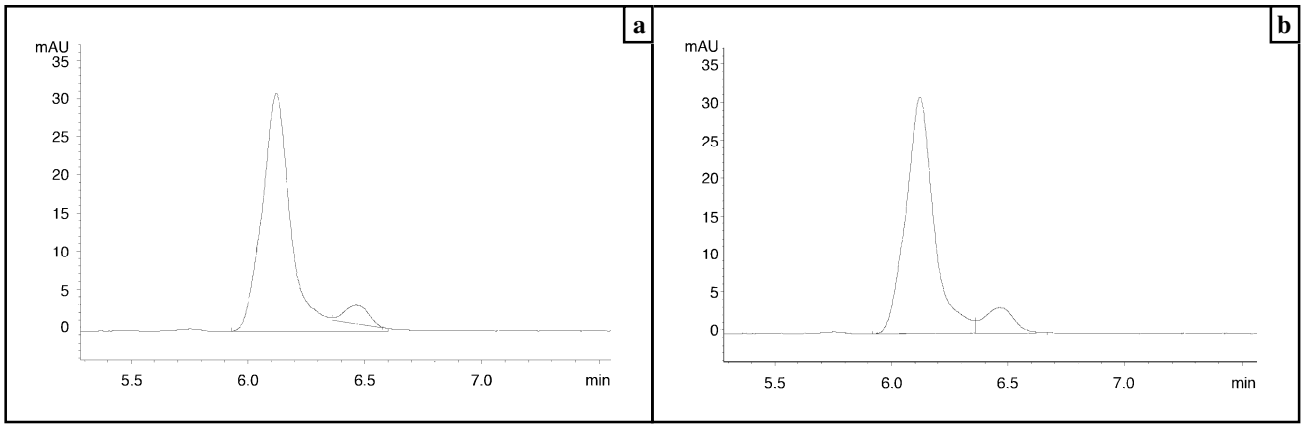

Fig. 14. Two different techniques for integrating the Chlide *a* interference peak from the Chl c_2 peak: **a)** the tangent skim integration technique, and **b)** the perpendicular drop integration technique.

For each sample, the peak height for every pigment was divided by the mean noise to give the SNR. Reporting practices for most pigments are consistent with definitions put forth in Hooker et al. (2005). The Peri isomer that elutes immediately after Peri, although detected and quantitated, was not reported. Four peaks of Phide *a* were detected and quantitated using the Phide *a* response factor and were reported separately.

5.7 CONCLUSIONS

This was the second time GSFC participated in a SeaHARRE activity; many lessons were learned and many questions were answered. Some questions, however, have yet to be answered, if an answer actually exists. First, it was learned that meticulously following the performance metrics significantly improved performance and, in fact, was instrumental in placing the results in the quality assured (QA) subset. Second, using the proper dilution devices was shown to be important, e.g., using a syringe for diluting the retention time mix (and making larger batches of the dilution) improves accuracy and precision of the mixed standards.

Lastly, proper integration of small peaks and coeluting peaks became a topic of consideration during the analysis of the SeaHARRE-5 samples. The decision of when to use a tangent skim or a perpendicular (baseline) method can ultimately affect the uncertainties in the quantitation of those pigments. When two pigments are coeluting or one acts as a small shoulder to another larger peak, the peak pairs will have some overlap, but it is almost impossible to distinguish this quantity. A perpendicular drop could significantly over- or under-integrate one of the peaks, depending on the peak height ratio of the two or more peaks that are coeluting. Dolan (2009) advocated using the following criteria:

> If the minor peak is less than 10% of the height of the major peak, then the tangent skim should be used.
>
> If the minor peak is greater than 10% of the height of the major peak, then the perpendicular drop should be used.

These criteria were used when integrating the Chlide *a* interference peak from the Chl c_2 peak. All samples were integrated at the baseline from the beginning tail of Chl c_2 to the ending tail with Chlide *a*. The tangent skim method was then used to subtract the Chlide *a* peak from the entire area (Fig. 14a), rather than the perpendicular drop alone (Fig. 14b).

Successfully calibrating and validating a new HPLC instrument is an arduous task. A practitioner must be vigilant with regards to the behavior of the instrument, so patience is an essential quality of the chromatographer. Strict adherence to the performance metrics was the driving force behind the validation processes used for the GSFC method. Although a state-of-the-art ranking may not always be achieved, adhering to the performance metrics will ensure the highest potential results. By diligently following the metrics, state-of-the-art resolution of the critical pairs and precision of primary pigments were attained by GSFC during this activity.

Chapter 6

The HPL Method

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Abstract

The HPL method employed by Horn Point Laboratory 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, elevated column temperature (60°C), and separation of all pigments to be quantitated with a simple linear gradient. 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 are estimated as follows: 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.

6.1 INTRODUCTION

The HPL HPLC method (Van Heukelem and Thomas 2001) and modifications to it have been extensively described in previous SeaHARRE reports (Hooker et al. 2005, 2009, and 2010). The HPL method uses a C_8 column and a reversed-phase, methanol-based, 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.

The SeaHARRE-5 samples were received frozen in liquid nitrogen dry shippers on 2 February 2009. The filters were immediately placed in a freezer at −80°C until processed in March 2009. DHI pigment standards, including the Mix-105 standard, were received well frozen on dry ice the week of 2 February 2009, and held at HPL in a −25°C freezer until analyzed.

The HPL methodology presented in the following sections includes descriptions of sample extraction procedures, calculation equations (in which variables affecting pigment concentrations are defined), and HPLC analysis, which is further subdivided into procedures for injection, pigment separation, detection, calibration, and pigment identification. Data products and data reporting practices specific to the SeaHARRE-5 activity are also described.

6.2 EXTRACTION

At HPL, the *default* volumes used during sample extraction are 2.5 mL of 100% acetone and 100 µL of water. These volumes are sometimes adjusted depending on the individual sample set. Several different extraction volumes have been previously tested and validated. The procedures used for extraction of the SeaHARRE-5 sample sets were as follows:

1. The extraction solvent, 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} was kept in a 1 L brown glass bottle. The bottle was removed from a −15°C freezer and brought to room temperature. A 2.5 mL aliquot of room-temperature extraction solvent was added to a polypropylene tube (Becton Dickinson 352002, Franklin Lakes, New Jersey) using a bottle-top dispenser designed for organic solvents (Dispensette Organic, Brandtech Scientific, Essex, Connecticut), which had been calibrated gravimetrically with 100% acetone.
2. An automatic pipette was used to deliver 0.1 mL of deionized water to the tube.

3. To prevent evaporation, the top of the extraction tube was covered with Parafilm immediately after the acetone and water were added, and then the tube was placed in an ice bath.

After all the tubes in a set were processed using steps 1–3, the following steps were executed:

4. Extraction tubes in the ice bath were placed in a −25°C freezer for at least 30 min.
5. The extraction tubes were removed from the freezer, and a frozen filter sample was placed in each tube and the tube was rewrapped with Parafilm.
6. Each sample filter was individually disrupted for approximately 6 s on a pulse setting ("on" 0.7 s, "off" 0.2 s) with a Branson 450 Digital Sonifier (Danbury, Connecticut) equipped with a 1/8 in microtip sonic probe, using control settings that resulted in approximately a 30–40 W output.
7. After each sonication, the tube containing the filter extract was once again rewrapped with Parafilm.
8. Once all samples had been sonicated, the extraction tubes were returned to the −25°C freezer for an additional (approximately) 3 h.
9. The samples were removed from the freezer and the filter slurry of each sample was transferred to a clean 5 mL disposable polypropylene syringe with a Luer-Lok tip (Becton-Dickinson 309603). The filter slurry was clarified by pushing it through a 17 mm PTFE Titan HPLC syringe filter with a 0.45 µm pore size (Sun SRI 44513-NP, Rockwood, Tennessee) attached to each Luer-Lok syringe. 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).

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

10. The sample extract (contained in a 7 mL scintillation vial) was vortexed gently for approximately 1 s. Using an autopipette, approximately 0.5 mL was transferred to an amber HPLC vial (National Scientific C4011-6W, Rockwood, Tennessee) and capped with a snap cap with a PTFE and silicone septa (National Scientific C4011-54B). A clean, unused tip was used with each extract.
11. The vials were placed in the HPLC TCAS compartment, which was set to a temperature of 4°C, and HPLC analysis occurred within approximately a 24 h time period.
12. The remaining unused portion of the sample extracts was recapped and stored at −15°C until all quantitation calculations were completed for all of the samples.

6.3 HPLC ANALYSIS

The filter extracts and standards during SeaHARRE-5 were analyzed using a fully automated Agilent 1100 HPLC equipped with a quaternary pump, programmable autoinjector, TCAS, Peltier temperature-controlled column oven compartment, PDA detector, and `ChemStation` software revision A.10.02 (all from Agilent Technologies, Inc., Santa Clara, California).

6.3.1 Injection

The HPL HPLC system has been modified with a metering device and sample loop capable of drawing up to 900 µL. The injector is programmed (Table 35) to draw successive aliquots of sample extract and buffer into the sample loop, for final total volumes of 150 µL and 375 µL, respectively.

Table 35. The HPL injector program used during SeaHARRE-5, with amounts in microliters and speeds in microliters per minute.

Step	*Amount*	*Source*	*Speed*
1. `DRAW`	150	Buffer	500
2. `DRAW`	75	Sample	130
3. `DRAW`	0	Acetone	90
4. `DRAW`	75	Buffer	130
5. `DRAW`	75	Sample	130
6. `DRAW`	0	Acetone	90
7. `DRAW`	150	Buffer	500
8. `INJECT`	525		250

Several steps have been identified at HPL that contribute to excellent injector precision, and are therefore always implemented:

- Place sample and buffer vials in the TCAS at least 1 h prior to starting analyses;
- Disregard at least the first injection of a sequence;
- Between injections, maintain a sufficient and constant column re-equilibration volume;
- Use vial and cap combinations that have been tested and proven to prevent evaporation (Hooker et al. 2009);
- Use injection draw speeds that prevent a vacuum from forming when a sample is withdrawn from the vial; and
- Limit the vial residence time in the TCAS to approximately 24 h or less.

The injection buffer solution is 0.028 M TbAA (6.5 pH) and methanol, mixed 90:10 (vol:vol), respectively. This solution is filtered through an Acrodisc 25 mm syringe filter with a 1 µm glass fiber membrane (Pall 4523T, East Hills, New York). Snap-cap septa on the buffer and sample vials are formulated of layered PTFE and silicone, but the septa

used with buffer vials are pre-scored with a star-shaped slit (National Scientific C4011-59) to facilitate accurate draw volumes of the more viscous buffer mixture. Snap caps on the sample vials are not prescored to prevent evaporation of the sample.

Rather than use a large reservoir from which to draw buffer, buffer is put in the TCAS in the same type of HPLC vials as samples. Each HPLC vial contains enough buffer for four separate injections. Using these smaller buffer reservoirs helps control potential contamination of the buffer by sample extract, and it minimizes the impact of this type of carryover if it were to occur. The needle is also dipped in a vial of 100% acetone (covered with a cap with PTFE septa, National Scientific C4011-52R) after each time the needle draws from a sample vial, in order to help control carryover. Occasionally, peak-splitting with Chl c_1, Chl c_2, and Chl c_3 is encountered, and MgDVP is similarly affected if present.

6.3.2 Separation

The separation conditions used during SeaHARRE-5 were the same as those used in the last three SeaHARRE activities. Solvent A is formulated (vol:vol) from 0.028 M TbAA (6.5 pH) and HPLC-grade methanol (Fisher Scientific A452-4, Fair Lawn, New Jersey) in a ratio of 30:70, respectively. The aqueous TbAA solution is made by diluting 0.4 M tetrabutyl ammonium 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.

Solvent A is well mixed and then filtered through a 0.2 µm pore size, 47 mm diameter nylon membrane filter (Millipore GNWP04700, Billerica, Massachusetts). The quantity of solvent A needed to complete a week of analyses is combined in a large glass carboy, so it is homogeneous. This container is kept on a stir plate at a very low setting to prevent layering from occurring in the solvent container over the course of the week. This procedure promotes the best day-to-day retention time stability. Any solvent A not fully consumed within the week is either discarded or combined with the next batch of solvent A and refiltered to avoid potential precipitates forming and clogging the column.

The pump gradient used in SeaHARRE-5 is presented in Table 36. The total run time, including the injector program, 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 prefilter, because both have been tested at HPL and shown to offer no advantages. A total of 15.2 mL of the solvents of initial conditions flows through the column in between the time of the completion of one sample injection and the injection of the next sample.

Table 36. The HPL pump gradient used during SeaHARRE-5. Solvent A is 70:30 methanol:28 mM TbAA (pH 6.5), solvent B is methanol, and solvent C is acetone. (After SeaHARRE-3, Solvent C was added as a rinse to help alleviate carryover.)

Time [min]	A [%]	B [%]	C [%]	*Flow* [mL min^{-1}]
0	95	5	0	1.1
22.00	5	95	0	1.1
24.50	5	95	0	1.1
24.75	5	65	30	1.3
25.75	5	65	30	1.3
25.85	5	65	30	1.1
26.10	95	5	0	1.1
29.10	95	5	0	1.1

6.3.3 Detection

Chromatograms are plotted using the 450 and 665 nm wavelengths (each with a 20 nm bandwidth), as well as at 222 nm with a 10 nm bandwidth. Tungsten and deuterium lamps are both used and absorbance spectra are collected from 350–750 nm, with a range step and slit of 2 nm. The flow cell capacity is 13 µL and the path length is 1 cm.

The wavelength at 222 nm is used exclusively for the quantitation of the internal standard (vitamin E acetate). This wavelength is not associated with the maximum response for vitamin E, but it allows for more than sufficient detection while minimizing baseline drift (which is still approximately -3 mAU min^{-1}, and principally caused by solvent effects of the gradient). The signal height of vitamin E at the concentration used is approximately 600 mAU, which is sufficiently high so that baseline drift does not interfere with accurate peak area determinations. The vitamin E solution is formulated at a concentration such that the resulting peak height corresponds to about two-thirds of the upper limit of linearity.

6.4 CALIBRATION

The retention times of pigments to be quantitated, as well as pigments with a potential to interfere with the quantitation process, were originally documented using algal monocultures and laboratory standards (Van Heukelem and Thomas 2001). This information is summarized in Table 31 in the SeaHARRE-3 report (Hooker et al. 2009), as well as additional details on chromatographic problems that can cause elevated uncertainties for some pigments in natural samples.

The spectrophotometer used for determining the concentrations of standards isolated at HPL or purchased from Sigma-Aldrich or CaroteNature and dissolved at HPL was a Shimadzu 2401-PC (Columbia, Maryland) with bandwidth set at 1 nm, medium sampling rate, 1 nm sampling interval. A turbidity correction was made at 750 nm, and the concentrations of the stock standards were adjusted

to keep the absorbance between 0.2–0.8, a range recommended by Marker et al. (1980) to promote spectrophotometric accuracy.

The HPLC system was calibrated with both purchased standards and those isolated at HPL. Concentrations of pigment standards were determined with the absorption coefficients given in Table 37. For standards that were purchased from DHI, the spectrophotometrically determined concentrations provided by DHI were used to compute the response factors observed from their standards when injected at HPL.

Table 37. 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. Absorption coefficients and reference wavelengths used at HPL match those used at DHI. The units for λ_m are nanometers. Standards isolated at HPL, or purchased from DHI, Sigma-Aldrich or CaroteNature, are denoted H, D, S, and C, respectively, in the source column.

Pigment	*Solvent*	λ_m	α	*Source*
Chl c_3	90% Acetone	452.9	346.00	D
Chl c_2	90% Acetone	444.0	374.00	H
Chl c_1	90% Acetone	443.0	318.00	H
Chlide a	90% Acetone	664.0	127.00	D
Phide a	90% Acetone	667.0	74.20	D
Peri	100% Ethanol	472.0	132.50	D
But	100% Ethanol	446.0	160.00	D
Fuco	100% Ethanol	449.0	160.00	H
Neo	100% Ethanol	439.0	224.30	H
Pras	100% Ethanol	454.0	160.00	H
Viola	100% Ethanol	443.0	255.00	D
Hex	100% Ethanol	447.0	160.00	D
Diad	100% Ethanol	446.0	262.00	H
Allo	100% Ethanol	453.0	262.00	H
Diato	100% Ethanol	449.0	262.00	H
Zea	100% Ethanol	450.0	254.00	C
Lut	100% Ethanol	445.0	255.00	C
Chl b	90% Acetone	647.0	51.36	S
DVChl a	90% Acetone	664.0	87.67	D
Chl a	90% Acetone	664.0	87.67	S
Phytin a	100% Acetone	667.0	51.20	D
ββ-Car	100% Ethanol	453.0	262.00	D

Single-point response factors are generally used at HPL for calibration, because it was previously demonstrated that these samples being analyzed were within the linear range. In addition, the linear regressions associated with multipoint calibration curves analyzed at HPL 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 is 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-5 activity, including the following:

- A Chl c_3-like pigment that elutes immediately after Chl c_3 and was summed with Chl c_3;
- Four pigments with Phide a spectra that elute after the retention time of the Phide a standard purchased from DHI (quantitated with the response factor from the DHI Phide a standard);
- Chl a and DVChl a allomers and epimers (quantitated as part of TChl a); and
- A Phytin a epimer (quantitated with the Phytin a response factor).

Because HPL had no discrete standards for these pigments, retention times were updated as they were observed in the DHI retention time mix or in natural samples (as identified with in-line visible absorbance spectra).

6.5 VALIDATION

Quality assurance and validation at HPL are closely linked and rigorously applied. The two most frequent measurements to validate performance are the analysis of replicate injections and carryover. For the former, the average daily precision of replicate injections of the internal standard was 0.29% during the analysis of SeaHARRE-5 field samples (three each day).

6.5.1 Replicate Injections

As part of HPL standard HPLC procedures, duplicate sample extract injections are performed daily. Aliquots of the extract of the first sample to be analyzed in a sequence of injections are added to two HPLC vials, then 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 (of the two vials that 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 samples in the daily sample set have been injected.

The timing of the duplicate sample injections may differ by as much as 20–30 h. The CV (in percent) was determined for TChl a and the primary pigments (which were averaged and reported as an overall average PPig CV). For these replicate injections, which represent samples extracted on 10, 11, 12, 18, and 19 March 2009, the CV of TChl a was 0.13, 0.10, 0.32, 0.0, and 0.05%, respectively;

the corresponding CV for PPig was 4.91, 1.91, 2.64, 1.72, and 1.97% for the respective dates. The overall average CV for TChl *a* was 0.12% and 2.63% for PPig.

When making HPLC injections of DHI Mix-105, the contents of the vial was dispersed into one or more HPLC vials (500 µL per vial) and only one injection was performed from each HPLC vial. Three intravial injections (multiple injections from the contents of one vial of DHI Mix were performed on 18 March 2009, for which the CV was 0.09% for TChl *a* and 0.44% for PPig.

6.5.2 Carryover

In the HPL SeaHARRE-3 chapter (Hooker et al. 2009), it was noted that HPL experienced substantial carryover immediately following the SeaHARRE-3 activity, which was alleviated by adding an acetone rinse to the solvent gradient (Table 36). During SeaHARRE-5, carryover for pigments was monitored by inspecting chromatograms of any internal standard or individual standard injection for extraneous peaks. Carryover of internal standard was monitored by inspecting chromatograms that followed an internal standard injection, if the following injection would not normally contain internal standard (such as a Chl *a* QC injection or a retention time mix injection). No carryover was observed during SeaHARRE-5.

6.6 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 quantitated collectively using a response factor for the most frequently abundant pigment at that retention time (e.g., Caro and Chl c_{1+2}).

Pigment standards frequently contain small isomers, which are usually less than 10% of the total peak area for that standard. It is 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. Only isomers for MVChl *a*, DVChl *a*, Phytin *a*, and Peri are routinely quantitated for the HPL method—although the potential of isomers for coelution is described in Table 11 of Hooker et al. (2005). Isomers for Chl *b* have also been occasionally observed.

In SeaHARRE-5, the total area of all MVChl *a* allomers and epimers, plus the main Chl *a* peak, were summed and the nanograms per injection was determined by multiplying the resulting total peak area times the Chl *a* response factor. No DVChl *a* was observed in the SeaHARRE-5 samples, but DVChl *a* was present in DHI Mix-105.

The chromatographic peaks representing Chl c_2, Chl c_1, and MgDVP were quantified as a single entity, which is referred to here as Chl c_{1+2}, because MgDVP, also a Chl *c* pigment, is frequently found in oligotrophic samples and, when present with the HPL method, interferes with accurate quantitation of both Chl c_2 and Chl c_1. This summing represents a change in practice from previous SeaHARRE activities for HPL.

6.6.1 Data Reporting

During the analysis of SeaHARRE-5 samples, retention times were documented on a daily basis using DHI Mix-105. This mix was injected near the beginning of a sequence and then approximately once every 20 h thereafter. Pigment identification in natural samples was based primarily on retention time; absorbance spectra matched with known standards was also used where the signal was adequate to produce usable absorbance spectra for peaks in sample extracts (see Sect. 6.6.3 for an extended discussion).

If a symmetrical, well-shaped peak appeared at the retention time of a standard, with an SNR value of 4 or more, but the SNR was too low to confirm or reject 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 or spectral distortion, were reported with a limiting value.

Following SeaHARRE-4, some changes in the reporting practices of HPL were made; these new procedures were used during SeaHARRE-5. Digits of precision being reported were reduced from four places to three. Pigments are rarely quantifiable at concentrations less than $0.0005\,\mu g\,L^{-1}$, and, in fact, these low concentrations are often associated with uncertainties of similar magnitude. As an example, the average standard deviation of pigments in mesotrophic and oligotrophic samples is on the order of $0.0005\,\mu g\,L^{-1}$, as determined from replicate injections of sample extracts—a quality control analysis performed daily with all samples.

Pigments with an SNR value of 4 or less (at the wavelength used for their quantitation) are now considered not present and a limiting value is assigned for their concentration. A null value of $0.0009999\,\mu g\,L^{-1}$ is used to represent pigments "not detected" as well as pigments present, but at concentrations less than $0.0005\,\mu g\,L^{-1}$. To put this in perspective, for primary pigments, the average limits of detection (SNR value of 3) and quantitation (SNR value of 10) for filtration and extraction volumes typical of oligotrophic samples are 0.0004 and $0.0013\,\mu g\,L^{-1}$, respectively.

6.6.2 Governing Equation

The HPL calculation of pigment concentration in a natural seawater sample can be expressed in one governing equation, the derivation of which is described more fully in the SeaHARRE-4 report (Hooker et al. 2010):

$$C_{P_i} = \frac{\hat{A}_{c_1}}{\hat{A}_{s_1}} \frac{V_m}{V_f} \hat{A}_{P_i} \left[\frac{A_{S_i}(\lambda_m) - A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c\, \Sigma \hat{A}_{S_i}} \right], \qquad (31)$$

where C_{P_i} is the concentration of a pigment in a natural sample, $\hat{A}_{c_1}$ 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, V_m is the volume of extraction solvent (containing internal standard) added to each sample filter, V_f is the filtration volume, $\hat{A}_{P_i}$ is the area of the parent peak (and associated isomers), A_{S_i} is the absorbance value of the pigment standard (as measured by the spectrophotometer and including a correction measurement for the absorbance of the pigment at 750 nm), λ_m is the reference wavelength associated with the absorption coefficient, α_{S_i} is the absorption coefficient for the pigment of interest, l_c is the path length of the spectrophotometer cuvette (usually 1 cm), and the sum of the parent peak and the area of the alteration products is denoted as $\Sigma\hat{A}_{S_i}$.

It may be noticed that certain variables that are relevant to sample extraction and calculation (e.g., HPLC injection volume) do not appear in the governing equation. It is not that the variables are not of importance, but rather that the variables *drop out* once multiple formulas are combined into a master formula. Quality control measurements are still made to ensure the accuracy and precision of such variables.

6.6.3 Discussion

The discussion presented here investigates two aspects of quantitation in greater detail: a) filter precision, and b) a quantitative approach to reprocessing. The latter includes some in-depth details of an alternative approach that HPL undertook toward chromatographic interpretation during SeaHARRE-5.

6.6.3.1 Filter Precision

The average imprecision observed at HPL among filter triplicates from Australian sites was higher than what HPL usually observes for SeaHARRE triplicates, and was noticeably higher than was observed for the US samples analyzed during this SeaHARRE activity (Table 38). When comparing the precision of these two sets, amounts injected that were less than the noninstrumental LOQ were replaced with the effective LOQ, so that uncertainties would not be unduly influenced by pigments with low concentrations.

Table 38. The average HPL CV (in %) observed at HPL for SeaHARRE (SH) samples, categorized by collection location and separated into TChl *a*, PPig, and the tertiary pigments.

Data Set	*TChl a*	*PPig*	*Tertiary*
SH-5 US Sites	2.1	4.1	4.0
SH-5 Australia	6.8	6.8	4.9
Overall SH Avg.	*4.1*	*4.9*	*5.8*

Peri was a dominant pigment in almost all of the Australian SeaHARRE-5 samples. When HPL chromatograms of triplicate filters from Australian stations were overlaid, several sites (`AC`, `AG`, `AI`, and `AJ`) exhibited more disparate peak heights with regards to pigments that correlate with dinoflagellates containing Peri (i.e., Diad, Chl *a*, TChl *c*, and Caro in addition to Peri). Abnormally poor precision was also observed with triplicates from station `AK`, possibly because this sample appears to be dominated by diatoms, (Fuco and Chlide *a* are abundant, with the latter 23–40% of TChl *a*). HPL often has difficulty with TChl *a* precision with samples that are dominated by diatoms, because of the conversion of Chl *a* to Chlide *a* during the extraction procedure.

One filter in the triplicate set HPL received from site `AM` contained much higher amounts of pigments indicative of chlorophytes (Chl *b*, Neo, Viola, and Lut) than the other two filters. The average CV among these pigments was 30%, but among other pigments (Peri, Fuco, TChl *c*, and Diad) the CV was 4%. (All pigments mentioned are well above a non-instrumental LOQ.)

Given the previously stated replicate injection precision for HPL (Section 6.3.1) and the fact that HPL imprecision issues did not occur at all sites or with all pigments at a particular site, the imprecision values do not necessarily point to a problem with the HPL HPLC. It should be noted that poor precision did not always translate to poor accuracy, nor did the apparent presence of a bloom automatically cause HPL to have issues with precision (e.g., site AH).

It is not known what caused the differences in HPL precision in the SeaHARRE-5 sample sets. From the perspective of considering everyday practices associated with HPLC pigment work, however, it is possible that factors such as filtration volume (Bidigare et al. 2003) and the formation of algal colonies might have negatively affected the filtration precision. It is also possible for a laboratory to have contributory limitations in regards to algal classes or naturally-occurring interferences, which are not normally encountered. These atypical aspects of HPLC analysis can be specific to the extraction procedure, or the HPLC method itself, and negatively affect quantitation performance. The types of issues being considered here become more important to assess and resolve as pigment analysts try to work closer to the previously stated goal of 15% agreement among laboratories necessary for algorithm refinement.

6.6.3.2 Quantitative Reprocessing

When interpreting chromatograms at HPL, the normal procedure is to implement an interpretation of the so-called *two-sentence rule*, as described previously in Hooker et al. (2009 and 2010). The results obtained using the normal approach were submitted for the SeaHARRE-5 quantitations. In the discussion presented here, a description of

an experimental procedure with a more quantitative approach to chromatographic interpretation during analysis of SeaHARRE-5 chromatograms is presented. The objective is to stimulate discussion among analysts with the hope that, through a combined effort, the community can begin to converge upon what steps can be taken during chromatographic analysis to reduce uncertainties, particularly for those pigments most at risk of subjective interpretation.

The procedures described here should be considered as an initial contribution to a subsequent more detailed and collaborative effort to develop more quantitative means of chromatographic analysis. The goal of this developing discussion is to improve the accuracy and precision of pigment reporting. This presentation is not necessarily a definitive solution to the problem of subjective interpretation of chromatograms or one that HPL will implement on a permanent basis. It is intended to explore the problem and encourage an exchange of ideas and relevant results within the community of HPLC analysts.

When examining any peak in a chromatogram, the decisions that a chromatographer makes surround two main variables: identity and quantity. Identity is simply whether or not a particular peak represents a particular pigment; quantitation concerns proper integration of the peak, as well as contamination within the peak.

HPL wanted to build on the foundation put in place with the so-called *two-sentence rule*, previously put forth during SeaHARRE-3 (Hooker et al. 2009), and began to apply quantitative criteria when implementing the rule. The objective of this exercise was to remove some of the subjectivity in interpretation of both the rule and of chromatograms, as well as perceived ambiguity in terminology. To reiterate, the two-sentence rule is as follows (Sect. 1.7.1):

> If a peak is *good* and it can be proved to be the incorrect pigment for that retention time (e.g., the absorption spectrum does not match), do not report it; otherwise report it.
>
> If a peak is *bad* and it cannot be proved to be the incorrect pigment, report it; otherwise do not report it.

Differences in implementation of this rule among laboratories arise because analysts have varying concepts of what is a "good" or "bad" peak, as well as what is necessary to prove or disprove the identity of a pigment. HPL has begun to develop a quantitation scheme as to what constitutes good and bad, and what establishes proof of the identity of a pigment.

First, a peak must be deemed to be a peak and not simply a mistake of the integration software. Because software simply integrates baseline variations based on pre-entered parameters, there are instances when baseline dips, noise, or other disturbances are integrated when they are not *true* peaks. At HPL, the retention time of any unknown peak was compared to retention times of pigments in the DHI reference mixture and other pigment standards documented during the same sequence of analysis as the unknown.

To determine if a chromatographic peak was a peak of interest, the retention time of the tentatively identified unknown had to fall within three standard deviations† of the retention time of the positively identified pigment in the nearest QC injection (except for Chl c_3 and Caro). The variance in Chl c_3 retention time is greater than other pigments, presumably because it is the first eluted peak, and therefore, its retention is most affected by small variations in initial conditions. Because βε-Car and ββ-Car coelute in the HPL method, the retention time of Caro varies according to their relative abundance. Consequently, Chl c_3 and Caro were excluded from this metric.

Pigment spectra to be used for references were compiled into a library at HPL from algal monocultures or discrete standards analyzed using the same conditions with a wavelength range spanning 350–750 nm (which is greater than that needed for absorbance spectra of carotenoids). The `ChemStation` software used by HPL calculates a spectral match value by comparing the acquired absorbance spectrum of the compound under investigation to spectra of various reference pigments in the spectral library. (Other chromatography software packages typically have a similar quantitative matching function.) The magnitude (between 0–1,000 in `ChemStation`) of the spectral match value can be degraded by poor SNR, contamination of the peak, and mismatched identity.

The relationship between (peak apex) spectral match value and SNR was evaluated with serial dilutions of the DHI retention time mix (Fig. 15). HPL identified the lowest SNR for each known pigment that produced a spectral match value of 996, which is a threshold value HPL considered a good indicator of predictive accuracy regarding pigment identity. This SNR, which was called the spectra limited threshold, varied somewhat depending on the pigment, and ranged from 34–43 (the shaded area in Fig. 15). Peaks with SNRs above the spectra-limited threshold exhibit spectral match values higher than 996, and peaks with SNRs below the spectra-limited threshold exhibit spectral match values lower than 996. The dashed line indicates the functional form of the data, which was used as a guide by HPL when considering the identity of a peak. HPL expected that corresponding pigments in field samples should follow the same functional form, if the unknown pigments in field samples were indeed the pigments they were identified to be.

† Standard deviations of retention time for individual pigments were determined from a set of SeaHARRE-5 samples bracketed by DHI reference mix injections.

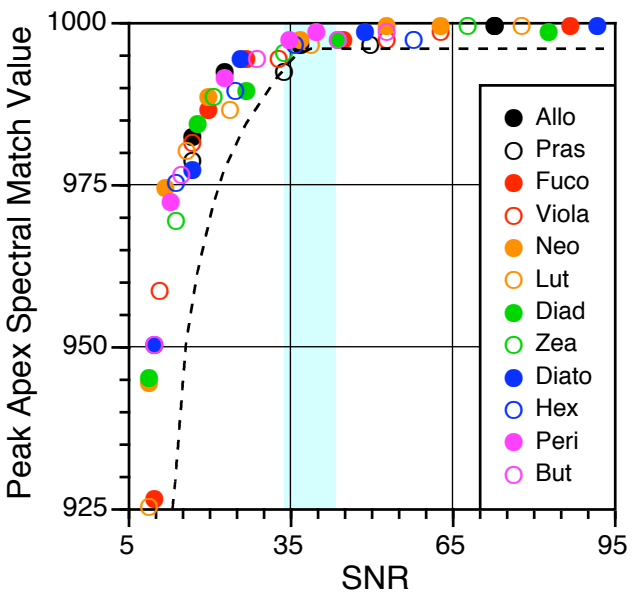

Fig. 15. Spectral match values for xanthophylls in dilutions of the DHI retention time mix as a function of SNR. The blue shaded area encompasses the SNR range within which all assessments yielded a spectral match value of 996, a value indicating sufficient quality such that HPL considers the identity of a peak is confirmed. The dashed line indicates the functional form used as a guide by HPL when considering the identity of a peak; a pigment to the right and below this black dashed line was considered not positively identified.

The average spectral match value (and associated CV for a pigment in a set of SeaHARRE-5 triplicates) was plotted as a function of the average SNR for individual pigments in samples (example shown in Fig. 15). Spectral match value uncertainty increased markedly as SNRs approached 15. Because the spectral match value began to drop off rapidly with the decrease in SNR, HPL decided that spectral matching could not be relied on as a criterion for determining pigment identity when a peak had an SNR less than or equal to 15. The SeaHARRE-5 xanthophyll results were characterized as exhibiting low (SNR values up to 15), middle (SNR from 15 up to the spectra-limited threshold), or high SNR values (SNRs greater than the spectra-limited threshold). Determination of peak identity was determined by which SNR category it was in and whether its spectral match value followed the functional form described by the DHI results in Fig. 15.

For all peaks of interest, the peak was then designated either "good" or "bad." To be designated good, a peak needed be in the high SNR category, exhibit expected resolution (R_s) from adjacent peaks, have a symmetrical peak shape, and be quantifiable by either peak height or area. (R_s was quantified, but peak shape was evaluated by visual inspection. Software programs can produce a peak symmetry value, so this parameter is quantifiable; visual inspection was used in the interest of time.) There were some pigments that were classified as good by these criteria, but were rarely proven to be the peak for that retention time. Pras, for example, fell in this category for HPL.

To be designated bad, a peak needed to exhibit only one of the following properties: an SNR in the low or middle categories, asymmetrical peak shape, (which sometimes can be improved by manual integration), or poor R_s from adjacent peaks. In this set of chromatograms evaluated, it happened that peaks that could be classified as bad by peak shape or R_s could also be classified as bad by their SNR alone. In addition, peaks that had a high SNR were not rejected from the good category based on the other criteria. While in general these patterns have been seen to persist with other sample sets, the convenient grouping seen here does not always occur.

Absorbance spectra were acquired at each peak apex using either manual or automatic wavelength referencing, whichever yielded the best spectral match. Most HPLC reprocessing software packages have the capability to override automatic referencing during spectral analysis with a manual referencing function. The ability to manually reference when checking low level spectra is a highly useful tool, but should be used with prudence (see the HPL chapter in Hooker et al. 2010 for further discussion). Sometimes, a poor spectral match value for a sample can be improved by editing the wavelength range used for the matchup, so it encompasses the absorbance spectrum of the pigment in question more specifically. This technique can be useful, for example, if there is considerable noise above 500 nm, or if there is an interfering peak at a wavelength not shared by the pigment under investigation and not used for quantitation. The approach applied by HPL to incorporate a more quantitative use of spectral matching to prove or disprove the identity of a pigment for purposes of data reporting is explained according to the following sequence.

A determination about whether a peak identification is correct or if the peak is instead something other than what is expected for that retention time needs to be made using absorbance spectra. Consequently, once a peak was identified as a pigment of interest based on retention time, and given a designation of good or bad (which in followed the classification into SNR categories), the next test of peak identity involved comparison of spectra from the peak in question to that of spectra compiled in the spectral library. Sufficient absorbance spectrum was needed to be able to assess the spectral match value, therefore, this type of test could only be executed for peaks that fell within the middle or high SNR category. Because absorbance spectra acquired from peaks below critical SNR values yield little diagnostic information, identity of peaks in the low SNR category (which would have already been designated "bad") could not be proved or disproved by spectral matching and the pigment identity could not be rejected.

If the peak was in the middle SNR category and exhibited severe departure from the expected spectral match

value and SNR functional form, it was rejected. Usually the pigments that were rejected also were affected by coelution or other factors, such as baseline disturbance. It is usually difficult to remove subjectivity for peaks in the middle SNR category.

An example of the decision-making procedure is shown in Fig. 16 for But. The solid symbols are from dilutions of the DHI retention time mix (data also shown in Fig. 15). By this reprocessing method, if a sample had an SNR above its spectra limited threshold but the spectral match value were to fall below the functional form line (as indicated by the data points from the DHI retention time mix dilutions, Fig. 16) it would be considered a rejection of peak identity. The peak labeled But in sample AK (red data point) had a spectral match of 979, which is well below the spectral match required for this peak to be considered But. No other But peaks with an SNR larger than the spectra limited threshold were candidates for rejection.

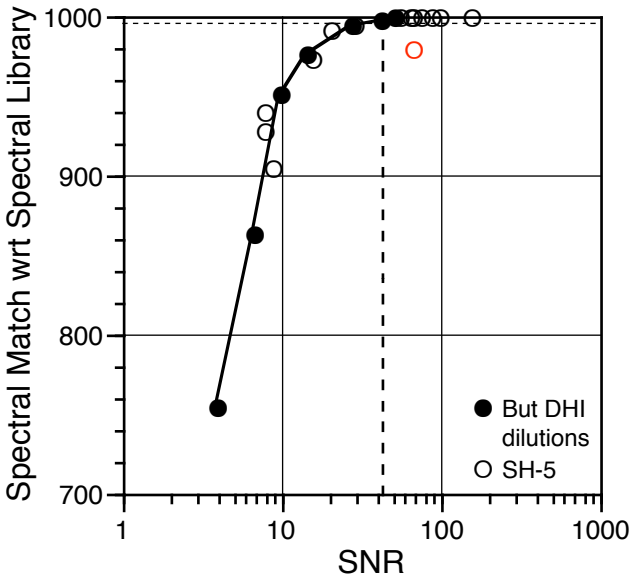

Fig. 16. Spectral match values of But in DHI dilutions and SeaHARRE-5 (SH-5) samples as a function of SNR. The spectra limited threshold for But is shown by the vertical dashed line (SNR equals 44). The top horizontal dotted line is for a peak apex spectral match value of 996. The red data point is from sample AK.

To illustrate the But in sample AK example in terms of visual spectra, Fig. 17 shows the spectrum from the peak that had been labeled as But in sample AK overlaid with a But reference spectrum (Fig. 17a), as well as a But spectrum from the DHI retention time mix diluted to a similar SNR as the sample, also overlaid with the But reference spectrum (Fig. 17b). With visual inspection of these spectra, it is evident that the two spectra in Fig. 17a do not match as well as those in Fig. 17b, and reemphasizes the conclusion to reject the identity of the peak in AK as But.

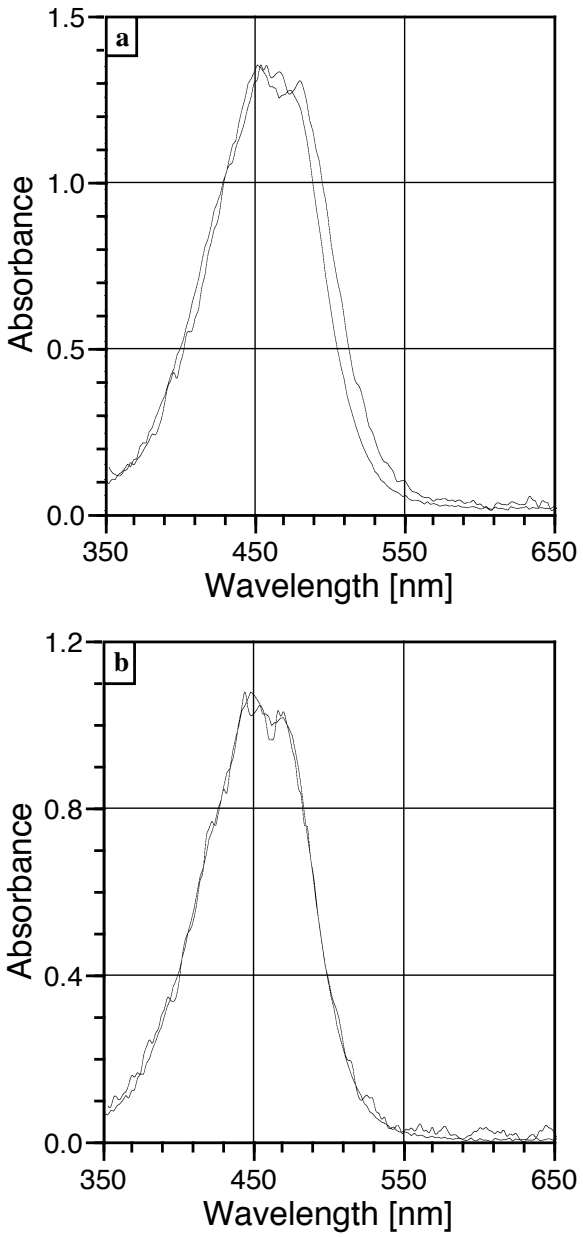

Fig. 17. The absorbance spectrum of But from the spectral library overlaid with **a)** the absorbance spectrum of the peak at the retention time of But (SNR value of 70 and a spectral match of 979), and **b)** the absorbance spectrum of But from a dilution of the DHI retention time mix for comparison purposes (SNR equals 55 and the spectral match is 998).

This quantitative approach yielded results that were very similar to the regular approach, yet reduced subjectivity in the decision-making process during chromatographic interpretation. With refinement, certain aspects could be implemented on a regular basis, allowing chromatographic interpretation to become faster and more consistent, both among chromatographers and even by the

same chromatographer. In addition, this approach has the possibility of making it simpler to train new chromatographers.

6.7 CONCLUSIONS

The HPLC method used at HPL has been in use since 1991 and is capable of providing quantitative results for up to 25 different phytoplankton pigments. HPL has documented the elution position and absorbance spectra of 58 pigments in a spectral library that can be used during the analysis of samples to assist pigment identification. HPLC pigment analysis is conducted at HPL under the guidance of a detailed Quality Assurance Plan. Limitations of the method are regularly reassessed as more diverse water types are analyzed. Such limitations cannot be known until an analysis method is used with all possible combinations of samples, a process that is not possible during initial method validation. As such, unforeseen coelution problems have been encountered that required some pigments originally intended for quantitation to be disregarded.

Chapter 7

The NIO Method

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Abstract

The NIO method is a slightly modified version of the method developed by Wright et al. (1991) and further adapted by Bidigare and Charles (2002). The NIO method was developed for use in connection to many pigments important to freshwater, estuarine, and oceanic systems. The method is based on a C_{18} reversed-phase column with a standard Beckman guard column. The method can provide quantitative estimates for more than 16 pigments in an analysis time of 34 min. The method does not separate Chl c_1, and divinyl Chl *a* and *b* are not separated from their respective monovinyl forms. In such cases, a C_8 column was used with the same procedure to separate the divinyl and monovinyl forms of Chl *a*. The retention times of Zea and Lut are sufficiently close to each other such that standard pigments are frequently used to confirm the peaks.

7.1 INTRODUCTION

For the determination of chlorophylls and carotenoids, the NIO method separates pigments on a Beckman Coulter reversed phase C_{18} column (Ultrasphere ODS, 5 µm spherical 80Å pore, 4.6 mm×15 cm) attached with a guard column (Ultrasphere ODS, 4.6 mm×4.5 cm). The HPLC method is based on Wright et al. (1991) as further modified by Bidigare and Charles (2002). The method does not separate Chl c_1, and divinyl Chl *a* and *b* are not separated from their respective monovinyl forms.

7.2 EXTRACTION

The SeaHARRE-5 samples arrived at NIO on 22 January 2009 and were stored in liquid nitrogen until the extraction and analysis were done. The filters were removed from the liquid nitrogen and placed in plastic vials for extraction in acetone. Prior to the HPLC analysis, each filter was immersed in 10 mL of 90% acetone, sonicated in darkness at 0°C, allowed to extract at −20°C for 24 h, and then, using a glass syringe, filtered through a 0.45 µm 13 mm PTFE filter (Pall, Acrodisc PSF, PTFE, Premium syringe filters) to remove particulate debris. Aliquots of 1 mL of the pigment extract was then mixed with 0.3 mL of distilled water in a 2 mL amber vial and allowed to equilibrate for 5 min prior to injection onto the HPLC column.

7.3 HPLC ANALYSIS

The NIO uses an Agilent 1100 HPLC system equipped with a diode array detector, fluorescence detector, thermostatted autosampler, micro-vacuum degasser, quaternary pump, and thermostatted column compartment. The vials of samples were placed in the autosampler compartment tray maintained at 10°C. From each vial, 500 µL were injected using the injection program presented in Table 39 at a flow rate of 1 mL min^{-1}.

Table 39. The gradient elution program used with the NIO method (Wright et al. 1991). The time is in minutes, and the percentages of solvents A, B, and C are given in the last three columns, where solvent A is 80:20 100% methanol:0.5 M ammonium acetate (pH 7.2) + 0.01% BHT; solvent B is 87.5:12.5 100% acetonitrile:H_2O + 0.01% BHT; and solvent C is 100% ethyl acetate.

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

HPLC-grade solvents were used with the NIO method and the volumes of solvents were measured before mixing. A solvent-resistant 0.2 µm filter paper (Pall Life Sciences, Ultrapor Nylon 6, 6 membrane filter) was used to filter the solvents before use.

Chlorophylls and carotenoids were detected at 436 nm. Peak identities were routinely confirmed by comparing the retention time of standard pigments from DHI and Sigma-Aldrich. The analysis time of each sample is 34 min with an additional 5 min injection delay of the next sample to ensure there is no carryover between samples.

7.4 CALIBRATION

The HPLC system was calibrated with pigment standards from DHI and Sigma-Aldrich before the analysis of the SeaHARRE-5 samples. The system calibrations were performed to determine the individual standard response for each compound. The concentration of the standards were provided by DHI or verified spectrophotometrically for Chl *a* and Chl *b*. Once standards failed spectrophotometric verification, the batch was rejected, and a new batch for the standard was used.

7.5 VALIDATION

Pigment retention times were checked daily by injecting a mixture of pigment standards and comparing retention times with those of the known and unknown sample. Injections of a Chl *a* standard were made two times immediately after running a set of samples and were routinely used to control the accuracy of the response factor.

7.6 DATA PRODUCTS

The Agilent `ChemStation` software was used to create an electronic file in which each chromatographic peak is recorded with its retention time, peak area, and peak height together with an initial pigment identification. Once the chromatograms were manually inspected, the peak areas were transferred to a spreadsheet in which the pigment concentrations were calculated using the appropriate response factor:

$$C_{P_i} = \frac{V_x}{V_f} \frac{\hat{A}_{P_i}}{V_c} R_{P_i}, \tag{32}$$

where C_{P_i} is the concentration of the pigment, V_x is the extraction volume (in milliliters), V_f is the volume of sample water filtered (in liters), $\hat{A}_{P_i}$ is the area of the chromatographic peak (in milliabsorbance units), R_{P_i} is the response factor (i.e., the inverse slope calculated from standard curve regressions), and V_c is the volume of sample extract injected onto the HPLC column (in milliliters). The response factor was calculated using

$$R_{P_i} = \frac{C_{S_i}/D_{S_i}}{\hat{A}_{S_i}}, \tag{33}$$

where C_{S_i} is the actual concentration of the pigment standard S_i from DHI, D_{S_i} is the dilution factor for the pigment standard, and $\hat{A}_{S_i}$ is the peak area for the standard as detected on the HPLC system.

7.7 CONCLUSIONS

The NIO method provides the resolution and quantitation of more than 16 pigments with detection ranging from 0.034–107.26 mg m^{-3} within an analysis time of 34 min. One disadvantage of the method is that separation of the monovinyl forms of Chl *a* and Chl *b* cannot be chromatographically separated from their divinyl forms. There are some unknown peaks that elute very close to Chl *a* and Chl *b*, which are unidentified. Both Zea and Lut can be separated by this method.

Chapter 8

The SIO (Vernet Laboratory) Method

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Abstract

The SIO (Vernet Laboratory) method for HPLC separation of phytoplankton pigments was developed for use in processing field samples during expeditions in Antarctica. The procedure uses an Agilent 1100 system and follows the basic protocol established by Zapata et al. (2000). Analysis includes a reversed-phase C_8 column with a binary gradient, a temperature-controlled column, and both a diode array detector and a visible wavelength detector, with peak quantification done at 440 nm. Sample extraction is in 90% acetone (with ultrasonication) and extracts are cleaned using Puradisc syringe-tip filters before injection onto the HPLC column. Samples for SeaHARRE-5 were processed on a similar Agilent 1100 system at Horn Point Laboratory in Cambridge, Maryland. The SIO method successfully separates most chlorophylls and carotenoids commonly seen in coastal and oceanic Antarctic waters.

8.1 INTRODUCTION

Since 1994, a major portion of the HPLC sample analyses performed by the Vernet Laboratory (SIO) has been done in the field, either in the laboratories at Palmer Station, Antarctica, or on board research vessels during field campaigns. Beginning in 2000, an Agilent 1100 system and methods based on the Zapata et al. (2000) protocols were employed. Approximately 1,000–1,500 samples were run annually until 2008, when the field campaigns ended. SIO was invited to participate in SeaHARRE-5, and processed the test samples using the Agilent 1100 system at Horn Point Laboratory in Cambridge, Maryland. For the Vernet Laboratory, the SeaHARRE-5 exercise served two purposes: first, as an evaluation of the SIO methodology in comparison with other participating laboratories, and second, as a test of the portability of the SIO method to a system with a similar setup but that had not previously run the Zapata et al. (2000) protocol.

The SIO technique for HPLC pigment analysis uses a reversed-phase C_8 column and a pyridine-containing mobile phase with a binary gradient. Prior to the start of the SeaHARRE-5 activity, no effort had been made to separate or quantify DVChl *a* or DVChl *b*, because both are considered rare pigments in the laboratory's usual sampling region in cold waters south of the Polar Front (Waterbury et al. 1986, Fouilland et al. 1999, and Fiala et al. 2003). The method does separate most chloropigments and carotenoids, and all SeaHARRE primary and secondary pigments, and several tertiary pigments were quantitated and reported.

8.2 EXTRACTION

Samples were hand delivered (in liquid nitrogen) to HPL from the NASA Goddard Space Flight Center (GSFC) on 2 February 2009 and were kept at −80°C until processing. Beginning 3 February 2009, the HPLC system was set up for the SIO method and the column calibrated. Filter extraction and analysis was completed between 6–9 February 2009, in batches of 24 samples each.

The sample extraction protocol included the following steps, all of which were completed as efficiently as possible and under dim light to minimize the degradation of any of the pigments:

1. The filter was removed from storage, examined for any notable problems (e.g., improper folding, tears, etc.), and placed in a disposable polypropylene tube that contained 3.0 mL of 4°C, 90% HPLC-grade acetone.
2. The sample was ultrasonicated with 6–7 2 s pulses, the tube capped tightly, and stored at −20°C for 24 h. Before injection, extracts were clarified using a Puradisc brand 25 mm, 0.45 µm, PP syringe-tip filter.
3. Immediately (up to 5 h) prior to injection, 500 µL of sample was mixed with 400 µL of HPLC-grade water in 2.0 mL amber glass sample vials and capped with a split silicone/teflon septa.
4. The remaining (uninjected) extract was stored in polycarbonate cryovials at −80°C until analysis and sample processing was complete.

8.3 HPLC ANALYSIS

Sample analysis was carried out on an 1100-series Agilent HPLC, which consisted of a vacuum degasser, a quaternary pump, an autosampler equipped with a 900 µL sample loop, a column thermostat (set to 25°C), and a diode array detector (DAD). The injection volume was 200 µL, but re-injection volumes were adjusted as high as 600 µL or as low as 75 µL, depending on peak size, in order to obtain peak areas within the calibrated range of the pigments. Peaks were separated on a Waters Symmetry C_8 column (4.6×150 mm with a 3.5 µm particle size, and a 100 Å pore size), which was protected by a Waters Symmetry Sentry Guard column (3.9×20 mm with a 5 µm particle size). The flow rate was maintained at a constant 1 mL min^{-1} and the solvent gradient for the SIO method is presented in Table 40.

Table 40. The two-solvent gradient program used with the SIO HPLC method. Solvent A is 50:25:25 methanol:acetonitrile:0.25M aqueous pyridine; and solvent B is 20:60:20 methanol:acetonitrile:acetone. Solvent A was filtered through a 45 mm GF/F filter before use.

Step	*Time*	A [%]	B [%]
Start	0	100	0
2	18	60	40
3	22	0	100
4	38	0	100
5	40	100	0
End	45	100	0

Detection was carried out at 440 nm (10 nm bandwidth) for all pigments, and peaks were identified by a combination of retention time and spectral analysis between 400–800 nm. Integration and peak identification was done using the Hewlett-Packard `ChemStation` Software (Rev. A.10.02) and the pigment concentrations were calculated using Microsoft `Excel`.

8.4 CALIBRATION

The instrument and column were calibrated immediately prior to the analysis of the SeaHARRE-5 samples. Powdered extracts (Sigma Chemicals) of Chl *a* and Chl *b* were dissolved in 90% HPLC-grade acetone, and standard absorbance spectra were measured between 400–800 nm (1 nm slit, 0.5 nm sampling interval) on a Shimadzu 2401-PC spectrophotometer. Concentrations for Chl *a* and Chl *b* were calculated using the absorption coefficients provided in Table 41. The Chl c_3, Chlide *a*, Chl c_2, Peri, But, Fuco, Pras, Hex, Diad, Allo, Diato, Zea, Lut, βε-Car and ββ-Car standards were all purchased from DHI, and the spectrophotometrically determined concentrations provided by DHI were used to compute response factors for these pigments. In addition to the standards listed above, DVChl *a* and Viola were quantified and conditionally reported using previously measured response factors.

Table 41. Absorption coefficient (α) values in liters per gram per centimeter used with the SIO method for the pigments listed as a function of wavelength (λ).

Pigment	*Solvent*	λ	α
Chl *a*	90% Acetone	664.0	87.67
Chl *b*	90% Acetone	646.0	51.36

Four different standard mixtures were made with the standards, grouping pigments by retention times. Four-to-five point curves were injected (volumes ranging from 10–400 µL, equating to masses of pigment expected to be measured) and the linearity of detector response was verified. Response factors were calculated from the regressions, without forcing the intercept through zero (all *y*-intercept values equated to values of less than ±0.0003 µg, or ±0.03% of the value of the slope). Duplicate injections were made of all mixtures to confirm reproducibility of the autosampler and injection system. LOQ was defined as the area of the lowest mass injected for each pigment, rather than 10 times the SNR.

One point to note regarding the SeaHARRE-5 activity in comparison to samples typically run on the SIO Agilent 1100 system used in the field, is the difference in the detector systems that were used for instrument calibration. Because of large baseline noise observed in the DAD, particularly while running the instrument at sea, peak areas during "normal" SIO HPLC sample processing are determined using the signal at 440 nm from a variable wavelength detector (VWD). For the samples run at HPL for SeaHARRE-5, peak areas were calculated from a DAD data-stream, because a VWD was not available. A comparison of the response factors from the VWD to those of the DAD for SeaHARRE-5, however, showed no greater difference than those historically observed with the SIO system.

8.5 VALIDATION

Each sequence of HPLC samples that were analyzed also contained three types of QA/QC injections. After the first 12 samples, one acetone injection was made to confirm that no pigment carryover was occurring. This was followed by one injection of a mixed standard solution to ensure reproducibility of retention times and peak separations (for the SeaHARRE-5 activity, this was the Mix 105 sample set, provided by DHI). After the second set of 12 samples was run, another acetone and mixed standard injection was made; and finally, a Chl *a* standard was injected to verify consistency in retention time, as well as response factor of the column as compared to the initial calibration. System pressure while running each solvent was also tracked daily to monitor column quality.

8.6 DATA PRODUCTS

Chromatogram information, including the injection volume, retention time, and peak area for all peaks (identified or not), was exported from `ChemStation` to `Excel` using a Dynamic Data Exchange (DDE) based macro written by Don Grothen (Agilent Technologies). Pigment concentrations (in micrograms per liter) were calculated as:

$$C_{P_i} \;=\; \frac{V_x}{V_f}\,\frac{\hat{A}_{P_i}}{V_c}\,R_{P_i}, \tag{34}$$

where C_{P_i} is the concentration of the pigment, V_x is the extraction volume (in milliliters), V_f is the volume of sample water filtered (in liters), $\hat{A}_{P_i}$ is the area of the chromatographic peak (in milliabsorbance units), R_{P_i} is the response factor (i.e., the inverse slope calculated from standard curve regressions), and V_c is the volume of sample extract injected onto the HPLC column (in milliliters). The V_c term was calculated as:

$$V_c \;=\; V_i\,\frac{V_s}{V_s \;+\; V_w}, \tag{35}$$

where V_i is the injection volume (in milliliters), V_s is the volume of the pigment sample (0.500 mL), and V_w is the volume of water mixed in the sample vial (0.400 mL).

The $\left[\text{TChl}\,a\right]$ was reported as the sum of $\left[\text{Chlide}\,a\right]$, plus any allomers and epimers of $\left[\text{Chl}\,a\right]$ when present, plus $\left[\text{DVChl}\,a\right]$. The $\left[\text{TChl}\,b\right]$ was reported as the sum of $\left[\text{Chl}\,b\right]$ plus any epimers when present.

8.7 CONCLUSIONS

The SIO method tested in the SeaHARRE-5 activity has been used for over 10 years for the analysis of long-term samples collected in Southern Ocean waters. The method results in good separation and accurate quantitation of most chlorophylls and carotenoids, with specific areas of improvement possible in the identification and quantification of Diato, plus the resolution of the Zea and Lut, as well as the βε-Car and ββ-Car pairs.

Future incorporation of an internal standard (such as vitamin E), more rigorous decision-making processes regarding small peaks (as discussed during SeaHARRE-5 meetings) and use of SNR, LOD, and LOQ parameters will likely further improve method results. This exercise was successful in showing that the SIO method is consistent and portable enough to be used with minimal adaptation on HPLC instrumentation other than the original SIO hardware, while continuing to produce reliable and accurate data products for the Antarctic and other global oceanic waters.

Chapter 9

The LOV Method

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Abstract

The LOV method is derived from the technique described by Van Heukelem and Thomas (2001), and applies a sensitive reversed-phase HPLC analysis for the determination of chloropigments and carotenoids within a run time of 28 min. The different pigments, extracted in methanol, are detected by DAD, which allows for automatic identification to be carried out on the basis of absorption spectra. Optical densities are monitored at 450 nm (chloropigments and carotenoids), 667 nm (chlorophyll *a* and derived pigments), 770 nm (bacteriochlorophyll *a*) and 222 nm (vitamin E acetate, the internal standard). The method provides good resolution between most pigments, but uncertainties may arise because of the partial separation of monovinyl and divinyl forms of Chl *b*, for the resolution of Chl *c* pigments, and for the separation of βε-Car and ββ-Car. 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 so-called *state-of-the-art* analyses. At the time the SeaHARRE-5 samples were analyzed, the injection precision of the method was estimated at 0.4% and the limits of detection for most pigments were low (0.016 ng inj^{-1} for Chl *a* and 0.034 ng inj^{-1} for the carotenoids).

9.1 INTRODUCTION

The present method used by LOV is derived from the method described by Van Heukelem and Thomas (2001), with modifications aimed at increasing sensitivity. It is a reversed-phase HPLC technique (C_8 column), based on a binary gradient and allows for the determination of most chloropigments (including degradation products) and carotenoids, which are extracted in 100% methanol and analyzed within 28 min. Detection is carried out at four wavelengths:

- 450 nm for carotenoids, Chl *b*, and Chl *c*;
- 667 nm for Chl *a* and derived products;
- 770 nm for BChla *a*; and
- 222 nm for the internal standard (vitamin E acetate).

A very good resolution is achieved for most pigments (less than 1.5), although only partial resolution (less than 1) can be obtained for the monovinyl and divinyl forms of Chl *b*, as well as Chl c_1, Chl c_2, and MgDVP. Coeluting pairs include Chlide *a* and Chl c_1 (but they are quantified at different wavelengths), plus ββ-Car and βε-Car. Pigment identification is based both on absorption spectra and retention time.

9.2 EXTRACTION

The SeaHARRE-5 *in situ* samples (stored and transported in liquid nitrogen) were stored in a −80°C freezer at LOV once they were received and until analysis commenced. The filters were extracted and analyzed between the 4–19 May 2009. The extraction process involved the following steps:

1. The 25 mm GF/F filter was placed into a 10 mL disposable Falcon tube.
2. Next, 3 mL of 100% methanol, including an internal standard (vitamin E acetate, Sigma), was added to each tube using an Eppendorf pipette, while making sure that 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 1 h.
4. The filters were then disrupted using an ultrasonic probe (Bandelin Sonopuls HD2200) for 10 s. The probe was rinsed with ethanol, and then wiped between each sample. In order to protect the pigments from heating during sonication, the tubes were placed in an ice-filled beaker.
5. The tubes were returned to the freezer for a minimum of another 1 h.

6. The samples were clarified using vacuum filtration through 25 mm GF/F filters (0.7 µm particle retention size) using a Millipore filtration unit. A glass tube (cleaned with ethanol and wiped between each sample) was used to press the sample slurry. The filtrate was collected in 10 mL Falcon tubes, and closed with airtight caps.
7. The clarified extracts were stored at −20°C until HPLC analysis (which occurred within 24 h).

9.3 HPLC ANALYSIS

Pigment analyses were carried out on a complete Agilent Technologies 1100 series HPLC system, with the following components:

- A degasser;
- A binary pump;
- An automated sampler, including Peltier temperature control (set at 4°C) and a programmable autoinjector with sample preparation prior to injection;
- A programmable column oven compartment (set at 60°C);
- A diode array detector; and
- `ChemStation for LC` software (A.09.03).

The sample extracts and standards were transferred to 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. Sample preparation (mixture between buffer (28 mM aqueous TbAA) and sample on a 1:1 basis) was carried out in the 500 µL loop just before injection, with alternating volumes of buffer and sample in order to enhance mixing in the loop. 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 that were encountered during the SeaHARRE-2 activity.

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

Separation was based on a linear gradient between a 70:30 methanol:TBAA 28 mM mixture and a 100% methanol solution (solvent A and B, respectively), ranging from 10–95% B in 22 min, followed by an isocratic hold at 95% solvent B for 5 min. At the end of the run, the mobile phase returned to initial conditions (10% solvent B) for a column equilibration time of 5 min (Table 42). If a technical problem should prevent the analysis of already extracted samples within the 24 h limit, the extracts are stored under nitrogen gas and placed in a −80°C freezer until routine analysis is re-established.

Table 42. The gradient used for the LOV method. The time is in minutes, and the percentages of solvents A and B are given in the last two columns. The flow rate is 0.55 mL min^{-1}.

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

Detection was carried out at three different wavelengths (10 nm bandwidths): 450 nm for all carotenoids, Chl *c*, and Chl *b*; 667 nm for DVChl *a*, Chl *a* and 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.

9.4 CALIBRATION

A calibration was performed in March 2009, two months before the analysis of the SeaHARRE-5 samples. The concentrations for 11 pigment standards (Chl c_2, Peri, But, Fuco, Hex, Allo, Zea, Chl *b*, Chl *a*, ββ-Car, and BChl *a*) purchased from DHI and Sigma-Aldrich, were determined by spectrophotometry using a PerkinElmer 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 5–12 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 DVChl *a* and DVChl *b* were computed using the following information:

- Knowing the specific absorption coefficients of 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 Chlide *a* absorption coefficient relative to Chl *a*.

For the remaining pigments, their specific absorption coefficients were either derived from previous calibrations or from the literature (Jeffrey et al. 1997). Because βε-Car and ββ-Car coelute, the peak was first identified spectrally.

Table 43. The α values (in liters per gram per centimeter), associated with their respective maximum wavelengths, λ_m (in nanometers), used in the LOV method for a variety of pigments, which are listed in the same order as their retention times, t_R (units in minutes). Source references for the α values are given in the rightmost column.

Pigment	*Solvent*	λ_m	α	t_R	*Reference*
Chl c_3	90% Acetone	630.6	42.60	4.33	As for Chl c_2
Chl c_2	90% Acetone	630.6	42.60	5.83	DHI
Mg DVP	90% Acetone	630.6	42.60	5.86	As for Chl c_2
Chl c_1	90% Acetone	630.6	42.60	5.90	DHI
Chlide *a*	90% Acetone	664.0	127.00	5.90	Jeffrey et al. (1997)
Phide *a*	90% Acetone	667.0	74.20	7.30	Jeffrey et al. (1997)
Peri	100% Ethanol	472.0	132.50	8.97	DHI
But	100% Ethanol	446.0	160.00	11.91	DHI
Fuco	100% Ethanol	449.0	160.00	12.16	DHI
Neo	100% Ethanol	437.0	224.30	12.86	DHI
Pras	100% Ethanol	454.0	160.00	13.10	DHI
Viola	100% Ethanol	441.0	255.00	13.49	DHI
Hex	100% Ethanol	447.0	160.00	13.75	DHI
Diad	100% Ethanol	446.0	262.00	14.80	DHI
Allo	100% Ethanol	453.0	262.00	16.30	DHI
Diato	100% Ethanol	452.0	262.00	16.80	DHI
Zea	100% Ethanol	450.0	254.00	17.34	DHI
Lut	Diethyl Ether	445.0	248.00	17.53	Jeffrey et al. (1997)
BChl *a*	100% Acetone	770.0	54.67	21.60	Oelze (1985)
DVChl *b*	90% Acetone	646.8	51.47	21.61	Derived from Chl *b*
Chl *b*	90% Acetone	646.8	51.36	21.72	DHI
DVChl *a*	90% Acetone	664.3	87.87	23.49	Derived from Chl *a*
Chl *a*	90% Acetone	664.3	87.67	23.68	DHI
Phytin *a*	90% Acetone	667.0	51.20	25.22	Jeffrey et al. (1997)
βε-Car	100% Ethanol	453.0	262.00	26.32	As for ββ-Car.
ββ-Car	100% Ethanol	453.0	262.00	26.38	DHI

The spectral shape therefore pointed to either one dominant pigment or the other and it was quantified as such. The absorption coefficients for the LOV standard pigments are listed in Table 43.

9.5 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 the 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 the sequence, however, 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:

- The initial back-pressure;
- The noise level at 450 and 667 nm;
- The injection precision of Chl *a* and Fuco;
- The accuracy of these pigments;
- Their plate numbers, peak widths, and retention times; and
- The resolution for two critical pairs (DVChl *a* and Chl *a*, as well as But and Fuco).

Signs of deterioration of the column can, therefore, be rapidly detected and corrective actions applied. Generally, a column is changed every 2,000 samples, although they have shown to last even longer.

Table 44. The original performance metrics established during the SeaHARRE-2 activity (Hooker et al. 2005) for five of the seven categories presently in use for validating the determination of marine pigments using an HPLC method (compare to Table 9). In this abbreviated approach, the overall performance of L is considered "state-of-the-art," because the average score of the weights is 3.5, $(3+4+4+3+3+4+4+4+3+3)/10$; $\bar{\xi}_{t_R}$ is the average CV retention time.

Performance Weight, Category, and Score		*TChl* a $\bar{\xi}$	*TChl* a $\lvert\bar{\psi}\rvert$	*PPig* $\bar{\xi}$	*PPig* $\lvert\bar{\psi}\rvert$	*Separation*† $\check{R}_s$	*Separation*† $\bar{\xi}_{t_R}$	*Injection*‡ ($\bar{\xi}_{\text{inj}}$) *Perid*	*Injection*‡ ($\bar{\xi}_{\text{inj}}$) *Chl* a	*Calibration*§ $\lvert\bar{\psi}\rvert_{\text{res}}$	*Calibration*§ $\bar{\xi}_{\text{cal}}$
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 L		*2.5*	*3.9*	*2.6*	*11.7*	*1.3*	*0.03*	*1.3*	*0.9*	*1.3*	*0.8*

The calibrations of the volumetric measuring devices (pipettes, syringes, etc.) are carried out annually. The combination of these quality control data are used to contribute to the computation of the performance metrics, which were first established during the SeaHARRE-2 activity (Hooker et al. 2005) and also provided in Table 9. In this way, the objective is to evaluate and maintain the *state-of-the-art* level of analysis at the LOV. The performance metrics measured at the time of the SeaHARRE-5 HPLC analyses are summarized in Table 44 and show the LOV method performing at the *state-of-the-art* level of analysis.

9.6 DATA PRODUCTS

The `ChemStation for LC` program produces a spreadsheet file for each sample comprising the pigment identifications, 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 concentrations with the internal standard corrections applied, C (in milligrams per cubic meter) of each pigment P_i, as in the following equation:

$$C_{P_i} = \frac{\hat{A}'_{P_i}}{V_f} R_{P_i}, \tag{36}$$

where $\hat{A}'_{P_i}$ is the corrected peak area (in units of mAU), R_{P_i} is the pigment response factor (in units of milligrams per mAU), and V_f is the volume of water filtered (in units of cubic meters).

The $\hat{A}'_{P_i}$ term is computed as:

$$\hat{A}'_{P_i} = \frac{\hat{A}_{c_1}}{\hat{A}_{s_1}} \hat{A}_{P_i}, \tag{37}$$

where $\hat{A}_{P_i}$ is the uncorrected peak area (in mAU units), $\hat{A}_{c_1}$ is the reference area (in mAU units) 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 (in mAU units).

9.7 CONCLUSIONS

During the SeaHARRE-5 activity, the LOV method proved to be well adapted to the complex, high chlorophyll samples from US and Australian coastal waters. Generally, a very good resolution was obtained for most pigments, although uncertainties may arise for the resolution of Chl *c* pigments or βε-Car and ββ-Car, which tend to coelute. Problems did occur because of the presence of many unidentified small peaks that sometimes interfered with integration.

Indeed, the quantitation of small peaks (e.g., Lut or Pras), was a difficult task because of a baseline rendered noisy from small peaks. The two-sentence rule (Sect. 1.7.1) could not completely solve this problem, although it did reduce the number of false negatives and false positives. The limits of detection are estimated to be $0.016\,\text{ng inj}^{-1}$ for Chl *a* and $0.034\,\text{ng inj}^{-1}$ for carotenoids. Effective LOD is not mentioned this time because of the high variability in filtration volumes for these samples.

Chapter 10

The DalU Method

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Abstract

The HPLC pigment technique used at Dalhousie University follows the methodology developed by Wright et al. (1991). It is a reversed-phase HPLC procedure (C_{18} column), based on a tertiary gradient and includes a cooled autosampler, plus photodiode array and fluorescence detectors. Samples are disrupted with a sonic probe in 100% methanol including an internal standard (vitamin E acetate). The method is validated with the use of internal and external standards. The method does not allow the separation of divinyl chlorophylls *a* and *b* from their respective monovinyl forms. In addition, chlorophylls c_1 and c_2 are not separated, and neither is lutein from zeaxanthin. It offers good separation of a variety of pigments, however, in a relatively short period of time (29 min).

10.1 INTRODUCTION

The Dalhousie University (DalU) protocol follows the method detailed in Wright et al. (1991). It is a reversed-phase HPLC procedure (C_{18} column), based on a tertiary gradient. Chl c_1 and Chl c_2 are not separated, and neither is Lut from Zea. Chlide *a* coelutes with Chl c_2, but the identification and quantification can be done on different detectors, therefore, the interference between the two pigments is avoided.

The DalU method does not allow the separation of divinyl Chl *a* and Chl *b* from the respective monovinyl forms. This does not interfere with the routine analysis conducted at Dalhousie University, however, because most phytoplankton samples analyzed come from temperate coastal waters in which the producer of divinyl chlorophylls, *Prochlorococcus*, is not abundant. *Prochlorococcus* has not been found in the well-studied Nova Scotian inlet, Bedford Basin (Li and Dickie 2001).

10.2 EXTRACTION

The SeaHARRE-5 samples were received on 5 February 2009, stored immediately at −80°C, and analyses were started on 31 March 2009. All procedures were carried out under dim light, and samples were kept on ice. Only 12 samples per batch were extracted at a time, which minimized the risk for pigment degradation while samples sat in the autosampler. Filters were cut in three pieces and put in a 4 mL amber glass vial. Next, 2 mL of extraction solvent was added to the vials with a gravimetrically calibrated automatic pipette. This solvent consisted of 100% methanol with approximately 0.125 mg L^{-1} of vitamin E acetate internal standard.

After tightly capping the vials, the samples were stored for 2 h in a −20°C freezer. After this time period, samples were transferred into a 10 mL BD syringe (Becton, Dickinson and Co., Franklin Lakes, New Jersey) with the tip closed with parafilm. The samples were disrupted using a sonicator probe set to pulse mode for a duration of 20 s. The samples were vortexed shortly thereafter, transferred into a 2.5 mL Eppendorf centrifuge tube, and clarified by centrifugation at 13,000 rpm for 3 min. The supernatant was transferred into a 2 mL autosampler vial and stored at −20°C until all samples were prepared and ready to be analyzed.

10.3 HPLC ANALYSIS

The HPLC system, an Agilent 1100, consisted of a temperature-controlled autosampler (cooled to 4°C), quaternary pump, and photodiode array and fluorescence detectors. Immediately prior to injection, 231 µL of sample was mixed three times with 69 µL of buffer solution within the 500 µL sample loop. The buffer solution consisted of 0.5 M ammonium acetate (7.2 pH). A total volume of 300 µL was injected in the HPLC system.

Pigments were separated with a Zorbax Eclipse XDB-C_{18} 150 mm×4.6 mm HPLC column with a 5 µm particle size (Agilent Technologies). The column temperature was not controlled by thermostat and the pigments were separated at ambient temperature. The flow rate was 1 mL min^{-1} and the gradient elution program is given in Table 45.

Table 45. The gradient elution program for the DalU method (Wright et al. 1991). The time is in minutes, and the percentages of solvents A, B, and C are given in the last three columns where solvent A is 80:20 100% methanol:0.5 M ammonium acetate (pH 7.2); solvent B is 90:10 100% acetonitrile:Milli-Q water; and solvent C is 100% ethyl acetate.

Step	*Time*	A [%]	B [%]	C [%]
Start	0	100	0	0
2	4	0	100	0
3	18	0	20	80
4	21	0	100	0
5	24	100	0	0
End	29	100	0	0

Peak integration was initially performed using Agilent `ChemStation` software. All peaks were manually identified by retention time and spectra signature comparison with known pigment standards. Absorption peaks of Chl *a* and phaeopigments were detected at 436 nm and carotenoids were detected at 450 nm.

10.4 CALIBRATION

All standards were purchased from DHI, and the concentrations provided with each standard were used to calculate concentrations for the 4–6 points dilution series. Dilutions were prepared with gravimetrically calibrated automatic pipettes.

More dilutions at low concentrations for Chl *a* and Fuco permitted calculation of the relative LOD and LOQ for all pigments (Hooker et al. 2005). SNR values of 3 and 10 were used for the LOD and LOQ, respectively. The detection limit for most pigments ranged from 0.14–0.40 ng inj^{-1} and 0.4–1.3 ng inj^{-1} for the LOQ. All calibration curves were linear regressions of area versus concentrations forced through zero. Their correlation coefficients (r^2) were always better than 0.99 and generally, absolute percent residuals were no more than 2%, as recommended by Hooker et al. (2005).

10.5 VALIDATION

At the beginning of an HPLC sequence run, four injections preceded the sample set. The first injection was 100% methanol. This first chromatogram was discarded and served as instrument *warmup*. Next, a Chl *a* standard solution was used to verify the stability in the response factor of this pigment. Then, a mixed standard solution provided by DHI was injected to ensure good retention time and pigment separation. Finally, the vitamin E acetate internal standard used in the quantification equation was used. After 12 samples had been injected, the internal standard, mixed pigment standard, and Chl *a* standard were reinjected to ensure that no degradation or retention time shift occurred during the sequence.

10.6 DATA PRODUCTS

The concentration of each pigment in the sample, C_{P_i} (in micrograms per liter), was determined using the following equation:

$$C_{P_i} = \frac{V_x}{V_f} \frac{\hat{A}_c}{\hat{A}_s} \frac{\hat{A}_{P_i}}{F_{P_i}}, \tag{38}$$

where the inverse response factor F_{P_i} is the slope obtained by plotting the peak area with the concentrations of a specific pigment standard injected; $\hat{A}_{P_i}$ is the peak area; V_x is the extraction volume (in milliliters); V_f is the volume of sample filtered (in milliliters); $\hat{A}_c$ is the peak area of the internal standard when it is injected onto the HPLC column; and $\hat{A}_s$ is the peak area of the internal standard in the sample.

Standard deviation and unbiased percent differences were calculated on all replicates and used as quality control measures. High deviants were revisited to ensure that no errors in pigment identification, integration, or quantitation were present.

During routine analysis, concentration values less than the LOQ are not reported and are replaced by the respective LOQ values for the pigment. For the purpose of the SeaHARRE-5 activity, however, peak areas down to the LOD (SNR value of 3) were considered, and absent pigments were reported as 0 (zero). The reason for this was to implement and evaluate the so-called *two-sentence rule,* which was proposed during the SeaHARRE-4 workshop, with the purpose of lowering high uncertainties, especially in association with the quantitation of very small peaks.

The two sentence rule is presented in Sect. 1.7.1 and is repeated here for convenience:

> If a peak is *good* and it can be proved to be the incorrect pigment for that retention time (e.g., the absorption spectrum does not match), do not report it; otherwise report it.
>
> If a peak is *bad* and it cannot be proved to be the incorrect pigment, report it; otherwise do not report it.

Despite the application of the two-sentence rule by many of the SeaHARRE-5 analysts, large errors were associated with the reported results for many peaks that had very small amplitude. The problematic pigments were mostly Hex, But, and Diato.

As an example of a problematic analysis associated with peak identification and quantitation, Fig. 18 shows a small peak (with an SNR value of 6.8) detected at the Hex retention time. This would be considered a bad peak because of the low resolution from Viola. According to the two sentence-rule, however, a value for Hex would be reported, because the peak could not be proved to be the incorrect pigment, because of an unrecognizable or absent spectrum. Nevertheless, the APD between the DalU reported result and the averaged value obtained by the A' group was 2,521%. *This dramatically illustrates why an*

objective or rule-based set of pigment identification and quantitation criteria are needed.

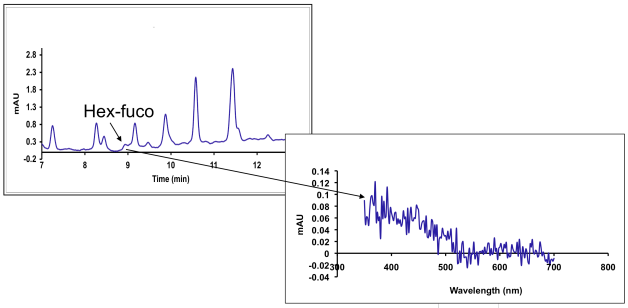

Fig. 18. An example of a small peak (SNR value of 6.8) in sample `AG` and absent spectrum.

High APD values in small peaks are explained by higher differences in interpretation of a peak among laboratories, and greater difference between the observed concentration and the null value (Van Heukelem 2009). The different interpretations of a peak, in this case, could have been caused by the different understanding of the two-sentence rule, different criteria for accepting or disqualifying a peak depending on spectra, or by different detection thresholds among methods. It is important to note, however, that in the context of the round robin, the damaging effects of these small peaks on method accuracy is usually disproportionate to their importance in the chromatogram (Van Heukelem 2009).

During the SeaHARRE-5 workshop, it appeared that most participants were hesitant to identify peaks with an SNR less than 10. For SNR higher than 10, spectra are less noisy and easier to identify. Disagreement is still possible, however, because of the subjective aspect of spectra matching. The question is,

> *What criteria should be used to accept or reject a peak based on its spectrum?*

As an example, Fig. 19 shows a small peak (SNR value of 10) identified by DalU as Hex. The standard spectrum is compared to the sample spectrum. The APD between DalU and the A' subgroup for the peak in Fig. 19 was 214%. Many participants agreed that this was not Hex and that both spectrum sides needed to follow each other. This was also the conclusion of Van Heukelem and Thomas (2009) for an investigation done on the spectrum of Pras to verify if a spectral shift could be due to a low SNR value; because the SNR value of Pras in a diluted algal culture decreased from 271 to 17, the integrity of the absorbance spectrum was compromised, but the spectrum was not shifted.

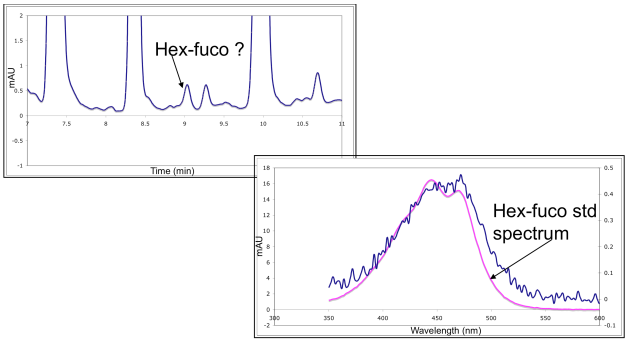

Fig. 19. An example of a small peak (SNR value of 10) in sample `AA` and absent spectrum.

Based on the foregoing discussion and the Fig. 19 example, *it is recommended that identification and quantitation of a peak should only occur if the SNR is higher than 10.* The intent of the two-sentence rule could still be maintained, but criteria for accepting and rejecting a peak based on spectrum match should be better defined (which the two-sentence rule was designed to promote).

10.7 CONCLUSIONS

The SeaHARRE-5 activity demonstrated that the C_{18} reversed-phase HPLC method used by Dalhousie University is a useful method for routine analysis of pigments, as long as the separation of divinyl Chl *a* and Chl *f* from their respective monovinyl forms, and Lut from Zea, are not critical to the research objectives. Indeed, the DalU method provides good resolution of most marine pigments, as well as consistent and repeatable pigment compositions and concentrations. In order to improve the accuracy of each laboratory, however, dialogue must continue regarding common practices and procedures of identifying and quantitating pigments.

Chapter 11

The COUL Method

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Abstract

The COUL HPLC method is based on a reversed-phase OS C_8 column and a pyridine-containing mobile phase, developed for the simultaneous resolution of chlorophyll and carotenoid pigments. This method allows for the separation of pigment pairs such as Chl c_1 and Chl c_2, as well as MVChl a and DVChl a. Pigments are identified from absorbance spectra and retention times, and concentrations calculated from the signals in the PDA or fluorescence detectors. Pigment calibration is regularly performed using commercial standards. The method has been used at COUL since 2002, and was chosen as the standard method for pigment analysis of phytoplankton samples of coastal and oceanic waters. After SeaHARRE-5, some modifications were introduced, namely the control of column temperature (25°C), which has improved the resolution of critical pigment pairs (e.g., Lut and Zea), the use of longer extraction times, and the inclusion of an internal standard.

11.1 INTRODUCTION

For the analysis of the SeaHARRE-5 samples, COUL used the C_8 method developed by Zapata et al. (2000). Contrary to the C_{18} method described by Kraay et al. (1992) and adapted by Brotas and Plante-Cuny (1996), which COUL uses regularly for microphytobenthic samples, the C_8 method allows the separation of Chl c_1 and Chl c_2, as well as the separation of Chl a and DVChl a (Mendes et al. 2007). For this reason, since 2002, the C_8 method was chosen as the standard method for pigment analysis of phytoplankton samples of coastal and oceanic waters. The SeaHARRE-5 samples were received frozen in dry ice and immediately transferred to −80°C until extraction. Analyses were performed between 20 February 20 and March 3, 2009.

11.2 EXTRACTION

Photosynthetic pigments in the filters were extracted with 2.5 µL of 95% cold-buffered methanol (2% ammonium acetate), using a ramrod for filter grinding. Samples were sonicated (Bransonic, model 1210) for 60 s, mixed in a Vortex mixer, and kept at −20°C for 30 min. The samples were centrifuged at 1,100 g for 5 min, at 4°C, and the extracts were filtered using Fluoropore PTFE filter membrane filters with a 0.2 µm pore size. Vials were placed in the cooling rack of the auto-analyzer and injected within 10 min. The remaining filtered extract was stored in an Eppendorf at −80°C until the analysis was completed.

11.3 HPLC ANALYSIS

Pigment extracts were analyzed using a Shimadzu VP Series HPLC (Tokyo, Japan), with the following components:

- A solvent delivery module (LC-10ADVP),
- A system controller (SCL-10AVP) with `Class VP` software version 5.0,
- A membrane degasser (DGU-14A),
- A refrigerated autosampler (SIL-10A), plus
- A photodiode array (SPD-M10ADVP), as well as a fluorescence (RF-10AXL), detector.

The chromatographic separation of pigments was achieved using the method of Zapata et al. (2000), with a monomeric octylsilica (OS) C_8 column (Symmetry, 150×4.6 mm dimensions, 100 Å pore size, 3.5 µm particle size, 337 $m^2\,g^{-1}$ surface area, and 12.27% carbon load).

The solvent gradient used a flow rate of 1 mL min^{-1}, an injection volume of 100 µL, and a run duration of 40 min. Details on the gradient profiles and the mobile phase composition are presented in Table 46. Acetic acid was used to set the the solution containing pyridine to 5.0 pH. Organic solvents employed to prepare the mobile phases were HPLC grade.

Table 46. The two-solvent gradient used for the COUL HPLC method. Solvent A is 50:25:25 methanol:acetonitrile:aq. pyridine, and solvent B is 20:60:20 methanol:acetonitrile:acetone. Time is in minutes.

Step	*Time*	A [%]	B [%]
Start	0	100	0
2	20	60	40
3	26	5	95
4	38	5	95
End	40	100	0

Pigments were identified from absorbance spectra and retention times. Concentrations were calculated from the signals in the photodiode array detector or fluorescence detector (excitation at 430 nm and emission at 670 nm). Absorption spectra were compared with a previously created spectral library. Peak integrations were performed automatically by the automated `Class VP` software, but each peak was inspected manually, and when necessary, `Class VP` tools were used to optimize integration.

Resolution (R_s) between a peak and the preceding one was calculated (for critical pigment pairs) using the following:

$$R_s \;=\; 2\,\frac{t_{R_2} \;-\; t_{R_1}}{\hat{w}_{B_2} \;+\; \hat{w}_{B_1}}, \tag{39}$$

where t_{R_1} and t_{R_2} are the retention times of peaks 1 and 2, respectively, and $\hat{w}_{B_1}$ and $\hat{w}_{B_2}$ are the corresponding peak widths at their bases.

11.4 CALIBRATION

Calibration of the HPLC peaks was performed using Chl *a*, Chl *b*, and ββ-Car standards from Sigma-Aldrich (St. Louis, MO); all other pigments were calibrated using standards from DHI (Hørsholm, Denmark). In the case of Sigma-Aldrich standards, the concentrations of standard stock solutions were checked using a Shimadzu UV-1603 spectrophotometer. The absorption spectra were recorded and the concentration of each pigment calculated using the absorption coefficient (Table 47) available in the literature (Jeffrey et al. 1997b). For DHI standards, the concentrations supplied with the standards were used.

Table 47. The absorption coefficient (α) values in liters per gram per centimeter used with the COUL method for the pigments listed as a function of wavelength (λ).

Pigment	*Solvent*	λ	α
Chl *a*	90% Acetone	664.3	87.67
Chl *b*	90% Acetone	646.8	51.36
ββ-Car	100% Acetone	454.0	250.00

A series of 4–5 standard solutions were prepared and analyzed by HPLC. Multipoint calibration curves were established for each pigment standard. The pigment response factor, R_{P_i}, was derived from the slope of the linear fit of the data (forced through zero) equating pigment amount to peak area as follows:

$$R_{P_i} \;=\; \frac{\Delta\tilde{C}_{P_i}}{\Delta\hat{A}_{P_i}}, \tag{40}$$

where $\Delta\tilde{C}_{P_i}$ is the change in the amount of pigment injected onto the column (usually in units of nanograms) and $\Delta\hat{A}_{P_i}$ is the corresponding change in peak area. Calibrations are done at or close to the wavelength of maximum absorption (e.g., Chl *a* at 430 nm, Fuco at 448 nm, and Zea at 454 nm).

11.5 VALIDATION

The COUL method is regularly validated with the use of commercial standards for pigment calibration. In the case of SeaHARRE-5, a mixed pigment standard supplied by DHI was analyzed. Unfortunately, the analysis of the mixed pigment standard was problematic, especially in the definition of early eluting peaks. Samples in acetone may suffer peak distortion in methods with mobile phases based on methanol because of different viscosities, and require sample dilution in water.

The limit of detection (LOD) and quantification (LOQ) were calculated as the amount of pigment (in nanograms) resulting in SNR values of 3 and 10, respectively, as described by Hooker et al. (2005). The SNR is the signal amplitude (or peak height) divided by the observed noise (characterized using a two standard deviation formulation) at the same wavelength for which the pigment signal was determined.

After the conclusion of the SeaHARRE-5 workshop, *trans*-β-apo-8′-carotenal at a concentration of 0.056 mg L^{-1} was included in the extraction solvent as an internal standard, for correcting pigment concentrations.

11.6 DATA PRODUCTS

After checking the chromatograms and correcting integrations manually, peak areas were transferred to an `Excel` spreadsheet and the pigment concentrations in the sample calculated as follows:

$$C_{P_i} \;=\; \frac{V_x}{V_f}\,\frac{\hat{A}_{P_i}}{V_c}\,R_{P_i}, \tag{41}$$

where C_{P_i} is the concentration of the pigment (usually in units of micrograms per liter), $\hat{A}_{P_i}$ is the area of the chromatographic peak (including isomers) in the same units as used in (40), V_x is the extraction volume, V_c is the volume of sample extract injected onto the column (in the same units as V_x), V_f is the volume of sample filtered (in liters), and R_{P_i} is the response factor of the calibration curve.

11.7 CONCLUSIONS

After the SeaHARRE-5 workshop, some modifications were introduced in the COUL method. First, the acquisition of a column oven allowed column temperature control and the improvement of resolution of critical pigment pairs, such as Lut and Zea. Secondly, the underestimation of Chl *a* detected for the COUL samples led to the use of longer extraction times (60 min instead of 30 min). Finally, the internal standard *trans*-β-apo-8′-carotenal was included in the extraction solvent for correcting pigment concentrations.

Chapter 12

The USM Method

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Abstract

The USM laboratory uses two different HPLC methods to analyze phytoplankton pigments; the first is a modified version of the method from Wright et al. (1991), and the second method is adapted from Van Heukelem and Thomas (2001). At USM, pigments samples from lakes, estuaries, and shallow coastal ocean to oligotrophic ocean waters are routinely analyzed, for which approximately 25 pigments are quantitatively separated. The methods are regularly validated using commercially available standards and individual pigment calibration. Having two identical systems running two different methods allows cross validation and efficient analysis to address specific research goals for different water types. During the SeaHARRE-5 activity, USM encountered problems in identifying small peaks, particularly for Peri and Diato; use of a specific spectral library for small peaks alleviated some of the problems, but not all.

12.1 INTRODUCTION

The USM laboratory uses two different HPLC separation methods for analysis of photosynthetic pigments. The first is a modification of the Wright et al. (1991) method, referred to as the T_{18} method, which uses a C_{18} column and a reversed-phase gradient elution. This method provides good separation of major pigments and works well for samples from lakes, estuaries, and shallow coastal environments. The method does not separate Chl c_1 from Chl c_2, Zea from Lut, or monovinyl Chl *a* and Chl *b* from their respective divinyl forms.

A second HPLC approach was adapted from the Van Heukelem and Thomas (2001) method, referred to as the T_8 method, that involves a binary gradient separation on a C_8 column. The latter method is capable of separating monovinyl and divinyl Chl *a*, Zea and Lut, plus partial separation between Chl c_1 and Chl c_2, as well as the monovinyl and divinyl forms of Chl *b*. The method does not separate ββ-Car and βε-Car. The method was originally developed for open ocean samples from tropical and subtropical environments.

12.2 EXTRACTION

Prior to HPLC analysis, filters were lyophilized (freeze-dried) at −47 to −52°C and 0.100 mbar for 12–15 h (Labconco FreeZone 6) to remove all the water from the filters. After lyophilization, the filters were extracted in 90% acetone (3 mL), vortexed, and weighed. The filters were then soaked overnight at −19°C, vortexed for 1 min, and reweighed. The weighing was done to quantify any weight loss due to evaporation. Long-term comparisons at USM have shown that this loss is usually negligible.

The next step was to transfer the sample slurry to a 5 cc glass syringe and filter the slurry through a 13 mm diameter 0.2 µm PTFE HPLC syringe filter (Alltech, catalog item 2164) The clarified extract was collected in disposable microcentrifuge tubes (2 mL) and stored at −19°C for a up to 8 h. In cases where longer storage was necessary, the samples were immediately stored in a freezer at −80°C until analysis.

12.3 HPLC ANALYSIS

Pigments were separated using two different methods using two identical HPLC systems. The HPLC systems were composed of a Waters 600 pump and a photodiode array UV detector Model 2996 (T_{18}) and 2996 (T_8), with a wavelength resolution of 1.2 nm. The photodiode arrays were set to record spectra from 350–650 nm (T_8) and 350–700 nm (T_{18}) every second. The Waters proprietary software package, `MaxPlot`, was used to acquire a chromatogram, the amplitude of which represented the largest absorbance of each 1 s spectrum between 408–480 nm. The integration software was set to only report peaks greater than 0.0005 AU.

12.3.1 Injection

Immediately prior to injection, a 50:50 mixture was prepared using 350 µL of sample extract and 350 µL of ei-

ther 0.05 M ammonium acetate as an ion-pairing agent for the T_{18} method or TbAA adjusted to 6.5 pH for the T_8 method. The mixture was then injected to flush and fill a 500 µL injection loop, the contents of which were then injected onto the column.

12.3.2 Gradient

The gradient for the T_{18} method was modified from the Wright et al. (1991) method. The column was an Altima HP C_{18} 5 µm reversed-phase 250×4.6 mm column. The column was maintained at 21°C. Solvent A consisted of 80% methanol and 20% ammonium acetate adjusted to a 7.2 pH; solvent B was 100% acetonitrile; solvent C was 100% acetone, and solvent D was 100% water. In addition, 0.1% of butylated hydroxytoluene (BHT) was added to solvents A and B. Table 48 shows the gradient sequence for the T_{18} method.

Table 48. The gradient system used with the USM T_{18} method. Time is in minutes and the flow rate is 1.0 mL min^{-1}.

Step	*Time*	A [%]	B [%]	C [%]	D [%]
Start	0	100	0	0	0
2	2	0	90	0	10
3	4	0	90	0	10
4	14	0	66	25	7
5	15	0	32	65	3
6	17	0	32	65	3
7	30	0	25	75	0
8	34	100	0	0	0
9	38	100	0	0	0
End		100	0	0	0

The gradient for the T_8 method was essentially identical to the VHT method as documented in prior SeaHARRE reports. The runtime was 31 min, the column used was a Zorbax Eclipse XDB C_8 with a particle size of 3.5 µm and 150×4.6 mm internal dimensions.

12.4 CALIBRATION

Prior to the SeaHARRE-5 activity, the systems were calibrated using pigments from algal cultures or from standards purchased from DHI. All regression coefficients (r^2) for major pigment standards were 0.99 or higher. The coefficients of variation for replicate injections were approximately 2%. Pigment concentrations and the purity of each pigment were examined using a dual-beam UV-visible spectrophotometer (Cary 300 Bio). The absorption coefficients used for pigment quantitation were those reported by DHI in the SeaHARRE-2 report (Hooker et al. 2005). All solvents and chemicals used for the execution of the method were of HPLC-grade (Fisher Scientific) and analytical grade.

12.5 VALIDATION

Efforts were taken at USM to assure quality control of the data and system performance within expectations. Because the USM HPLC pigment analysis methods involve manual injection, the injection loop was routinely cleaned with acetone to avoid contamination between samples. In addition, acetone blanks were run following idle periods of more than two weeks to rule out the presence of residual contamination.

Multi-point calibrations of all pigments are performed every 2–3 yr, whereas multi-point Chl *a* and Fuco calibrations are done more frequently, i.e., approximately every 6 mos. During the SeaHARRE-5 activity, DHI Mix-105 was used to determine fluctuations in retention time for all pigments, and a minimum variation in retention time was observed.

Periodic checks of the stability of the HPLC systems are also made approximately every 6 mos at USM. Assessments of the long-term stability of the systems, expressed as the average noise calculated according to the methods presented in the SeaHARRE-2 report (Hooker et al. 2005), yielded a coefficient of variation (CV) in for replicate injections of 13.7% for the T_8 method and 34.0% for the T_{18} method.

12.6 DATA PRODUCTS

The Waters proprietary software package (`Empower Pro`, Service Pack D) was used to create American Standard Code for Information Interchange (ASCII) files that included information about the retention time, peak area, and peak height for each analyzed sample. Each peak of an individual chromatogram was visually inspected and matched with library spectra before final reporting. Pigments were reported to be present only if the spectrum matched USM criterion of peak identification (based on peak shape and retention time) and whether or not concentration was above the LOD (SNR value of 3). Otherwise, concentrations were reported as 0.0005 µg L^{-1}.

12.6.1 Pigment Concentrations

Pigment concentrations were calculated using the following equation:

$$C_{P_i} = \frac{V_x}{V_f}\frac{\tilde{C}_{P_i}}{V_c}, \tag{42}$$

where C_{P_i} is the concentration of the pigment (in units of micrograms per liter), V_x is the extraction volume (in milliliters), V_c is the volume of sample extract injected onto the HPLC column (in microliters), V_f is the volume of sample filtered (in milliliters), and $\tilde{C}_{P_i} = \hat{A}_{P_i} R_{P_i}$, where $\hat{A}_{P_i}$ is the area under the chromatographic peak for pigment P_i, and R_{P_i} is the response factor for the pigment.

12.6.2 Small Peaks

A principal challenge for any HPLC analysis is successfully resolving those problems associated with small peaks. To minimize the ambiguity associated with small peaks, USM laboratories use a spectral library that was constructed with pigment spectra having very low concentrations (Fig. 20). In the Fig. 20 example, when the Peri library spectrum for higher concentration was compared with the sample there was some ambiguity (the SNR was 7.7). When the sample was compared with the library spectrum at a much lower concentration, however, a better match was observed. Consequently, peridinin was reported to be present and the APD was 11.7%.

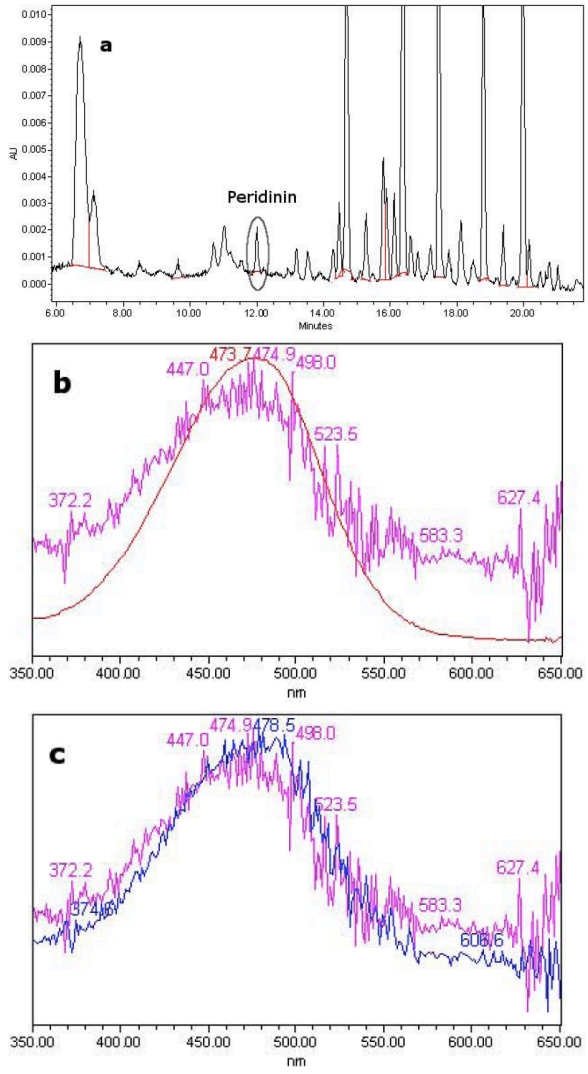

Fig. 20. An example small peak analysis for Peri using sample `F`: **a)** a typical chromatogram, **b)** the Peri library spectrum for a high concentration of $129.9\,\mathrm{ng\,\mu L^{-1}}$ (red smooth curve) compared to a sample `F` spectrum (pink noisy spectra), and **c)** the sample `F` spectrum (pink) compared with a library spectrum for a low concentration (blue) of $6.6\,\mathrm{ng\,\mu L^{-1}}$.

The USM spectra library has aided in the identification and quantitation of low concentration samples on numerous occasions. During the SeaHARRE-5 activity, the USM laboratory also proposed the idea of using the similarity index (SI) to assist in the identification of ambiguous pigments. Despite the advantages of the spectra library, USM encountered problems with misidentification or lack of identification of small peaks during the analysis of SeaHARRE-5 field samples, particularly for the problematic pigments Diato and Peri. USM reported a large number of false negatives (i.e., rejecting the presence of a pigment, when a majority of laboratories reported it as present) for both of these pigments, especially in the case of Diato.

One specific case of a false negative identification and quantitation for Diato is well illustrated with the Australian sample set `AE` (Figs. 21 and 22). High APD values of 98.1% were observed for both the T_8 and T_{18} methods for the `AE` sample set. Additionally, for both methods, the SNR values were below the LOQ (defined with an SNR value of 10); for the T_8 method the SNR for the peak was 9.8, and for the T_{18} method the SNR for the peak was 8.9. Although the retention time of the peak was within the expected range for Diato, 19.6–19.7 min for the T_8 method and 19.7–19.8 min for the T_{18} method, the noisy nature of the spectra along with small offsets (Figs. 21b and 22b) were the contributing factors that led to rejecting the peak as Diato.

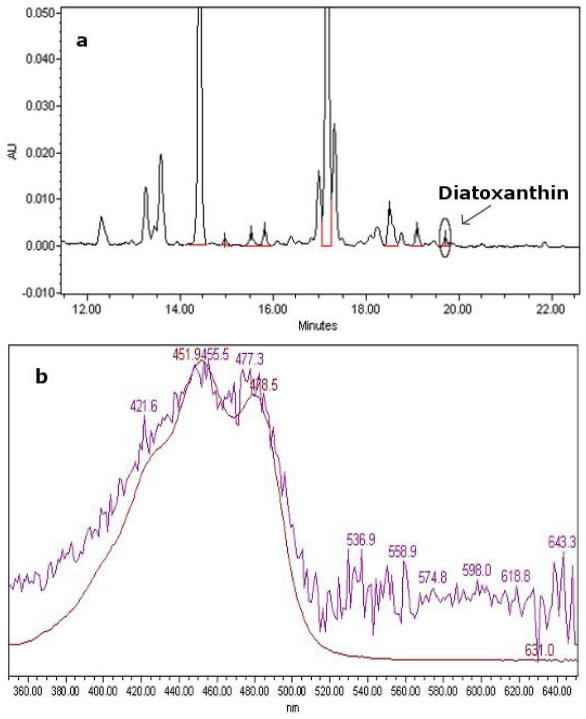

Fig. 21. An example of a small Diato peak in sample `AE` for the T_8 method: **a)** a representative chromatogram, for which the APD for Diato was 98.1%; and **b)** a comparison of the smooth library spectra and the noisy spectra from the field sample.

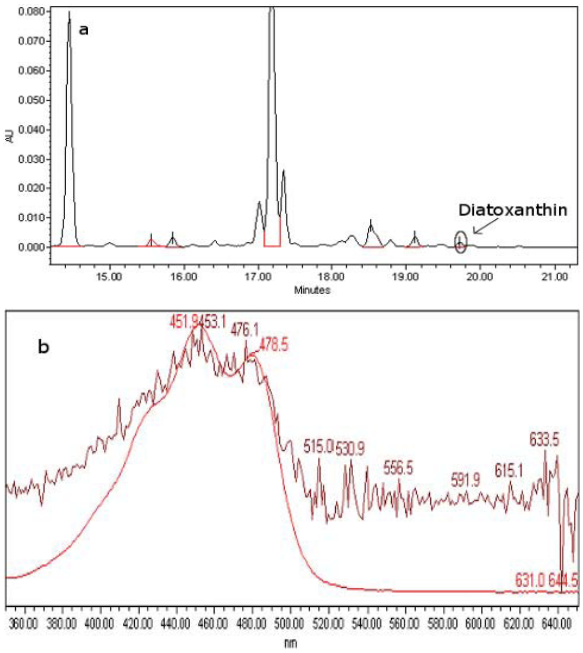

Fig. 22. A small T_{18} Diato peak in sample AE: **a)** a representative chromatogram, for which the APD was 98.1%; and **b)** a comparison of the smooth library spectra and the noisy field sample spectra.

A contrasting case occurred with sample L where the T_8 method (Fig. 23) resulted in a false negative for Diato with a peak SNR value of 2.6. With the T_{18} method for the same sample (Fig. 24), Diato was reported present, and had an SNR value of 17. The peak retention times were within the range for Diato in all cases.

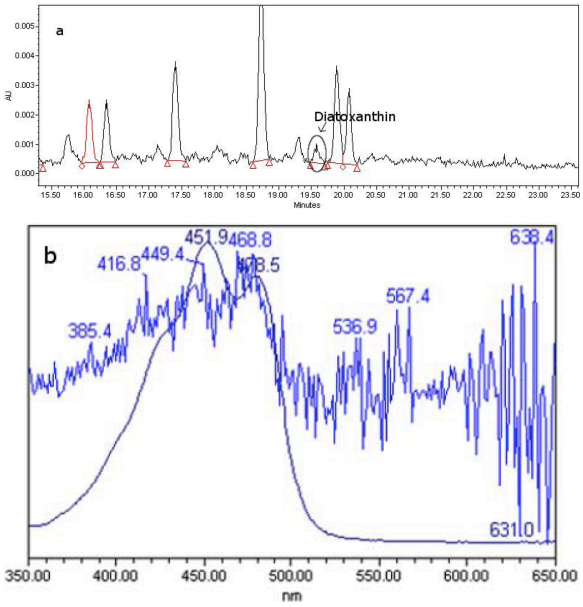

Fig. 23. A small T_8 Diato peak in sample L: **a)** a representative chromatogram, for which the APD was 96.9%; and **b)** a comparison of the smooth library spectra and the noisy field sample spectra.

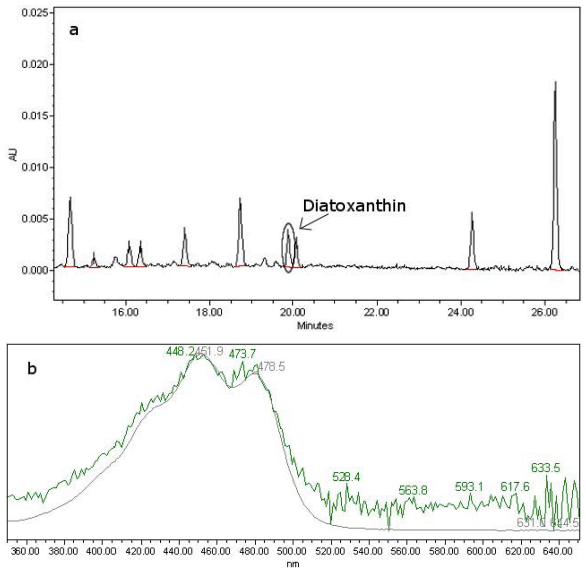

Fig. 24. An example of a small Diato peak in sample L for the T_{18} method: **a)** a representative chromatogram, for which the APD for Diato was 36%; and **b)** a comparison of the smooth library spectra and the noisy spectra from the field sample.

The very small SNR below the LOD for the T_8 method results, and spectral offsets plus noise in the spectrum resulted (Fig. 23b) in rejection using the T_8 method. In contrast, the high SNR and close match of the spectrum (Fig. 24b) satisfied criteria for acceptance with the T_{18} method.

12.7 CONCLUSIONS

The use of the two methods at USM provides the advantage of cross-checking pigment concentrations and identification. Each of the methods has its own advantages and disadvantages, and investigators have the option of choosing the method that best suits their specific application. The high level of uncertainty associated with small peaks is also a consequence of the subjective nature of peak identification and is dependent on the analyst's discretion. It is recommended that future SeaHARRE activities dedicate efforts to examine this issue in greater detail.

Acknowledgments

The SeaHARRE-5 activity was sponsored by NASA HQ and the MODIS Project along with the individual institutes representing the eleven 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-5 Science Team
B. The SCOR WG 78 Pigment Abbreviations
C. Commercial HPLC Manufacturers and Pigment Suppliers

Appendix A

The SeaHARRE-5 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,
ββ-Car	ββ-Carotene (β-Carotene), and
βε-Car	βε-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
Wilmington, DE 19808
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
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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

Sigma-Aldrich Company[4]
3050 Spruce Street
St. Louis, MO 63103
Voice: 314–771–5765
Fax: 314–771–5757
Net: `sigma@sial.com`

[1] Formerly the Hewlett-Packard Analytical Division.

[2] Formerly the VKI Water Quality Institute.

[3] Part of Sigma-Aldrich.

[4] Formerly Sigma Chemical.

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
ASCII American Standard Code for Information Interchange

BHT Butylated Hydroxytoluene
BIO Bedford Institute of Oceanography (Canada)
BUC Bodø University College

CHORS Center for Hydro-Optics and Remote Sensing
CDOM Colored Dissolved Organic Matter
COUL *Centro de Oceanografia, Universidade de Lisboa* (Portugal)
CSIRO Commonwealth Scientific and Industrial Research Organisation (Australia)
CTD Conductivity, Temperature, and Depth
CV Coefficient of Variation

DAD Diode Array Detector
DalU Dalhousie University (Canada)
DANAK Danish Accreditation and Metrology Fund
DDE Dynamic Data Exchange
DHI DHI Water and Environment Institute (Denmark)
DP (Total) Diagnostic Pigments
DV Divinyl

FBA Faculty of Biosciences and Aquaculture
FIO Florida Institute of Oceanography

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

ISO International Organization for Standardization

JGOFS Joint Global Ocean Flux Study
JRC Joint Research Centre

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
MV Monovinyl

NASA National Aeronautics and Space Administration
NIO National Institute of Oceanography (India)
nPF Nanoplankton Proportion Factor
NR Not Resolved
NV Not Validated

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, or quality assured, depending on usage
QC Quality Control

RPD Relative Percent Difference

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
SeaHARRE-4 The fourth SeaHARRE
SeaHARRE-5 The fifth SeaHARRE
SeaWiFS Sea-viewing Wide Field-of-view Sensor
SI Similarity Index
SIO Scripps Institution of Oceanography
SNR Signal-to-Noise Ratio
SOP Standard Operating Procedure

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
UN University of Nordland
USC University of South Carolina
USF University of South Florida
UPD Unbiased Percent Difference
USM University of Southern Mississippi

VHT Van Heukelem and Thomas (2001) method
VWD Variable Wavelength Detector

WG Working Group

Symbols

a The specific absorption coefficient.
A This is used to denote the average of all the methods.
$A(\lambda)$ Absorbance.
A' The methods within the QA subset.
A^+ The methods not within the QA subset, which are also referred to as the NV methods (not validated at the QA level of performance).
A^- The set of best results (e.g., lowest uncertainties or precisions) from a group of methods.
$\hat{A}_c$ The peak area of the internal standard when it is injected onto the HPLC column.

[5] Formerly Thermo Separation Products.

$\hat{A}_{c_1}$ The $\hat{A}_c$ determined using a one-step internal standard methodology.
$\hat{A}_{c_2}$ The $\hat{A}_c$ determined using a two-step internal standard methodology.
A_{P_i} The 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).
$a_{P_i}(\lambda)$ The absorption coefficient of a pigment (a constant).
$\hat{A}_s$ The peak area of the internal standard in the sample.
$\hat{A}_{s_1}$ The $\hat{A}_s$ determined using a one-step internal standard methodology.
$\hat{A}_{s_2}$ The $\hat{A}_s$ determined using a two-step internal standard methodology.
A_{S_i} The absorbance of the internal standard measured on the spectrophotometer.
$\hat{A}_{S_i}$ The peak area of the internal standard measured on the HPLC.

b_i The y-intercept of a linear equation.

C The CSIRO method, or the concentration of a pigment (depending on usage).
C' A second set of samples analyzed with method C using the established extraction protocol.
$\bar{C}$ The average concentration of a particular pigment.
C_a The concentration of chlorophyll *a*.
C_A The concentration of alloxanthin.
C_O The observed concentration.
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.
C'_{P_i} The amount pigment injected.
$\tilde{C}_{P_i}$ 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_R The reference concentration.
C_{S_i} The concentration of pigment standard for pigment P_i.
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 The column diameter.
D_f A dilution factor.
D_{S_i} The dilution factor for the pigment standard.

e Extract.

F The FIO method.
F_{P_i} The inverse response factor for pigment P_i, i.e., $1/R_{P_i}$.

G The GSFC method.

H The HPL method.

i An array index.

j An array index.
J The JRC method.

k An index indicating the station number.

l An index indicating the replicate number.
L The LOV method.
L' The histoprep analyses.
l_c The pathlength of the cuvette.
L_c The column length.
L_j The laboratory or method code.

m_i The slope of a linear equation (equating change in peak area with change in amount).

N The DalU method.
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.

P_i A particular pigment (referenced using index i).
P_s The column particle size.

r^2 The coefficient of determination.
R The response factor (from a generalized perspective).
$R^{\%}$ The purity corrected response factor.
R_I The ratio of the volume of IP solution plus V_m divided by V_m.
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_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.

s The sample.
S The (SDSU) CHORS method.
S_{18} The (SDSU) CHORS C_{18} method.
S_8 The (SDSU) CHORS C_8 method.
S_i The pigment standard for pigment P_i.
S_k The k^{th} station or sample number.
$S_{k,l}$ The station or sample number set by k, and the replicate number set by l.

T The USM method.
T_{18} The USM C_{18} method.
T_8 The USM C_8 method.
T_c The column temperature.
$[\text{TChl } a]$ The concentration of total chlorophyll a.
t_R The retention time.
t_{R_1} The retention time of peak 1.
t_{R_2} The retention time of peak 2.

U The USC method.

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_i The injection volume.
V_m The volume of extraction solvent (containing internal standard) added to a filter.
V_s The internal standard.
V_w The volume of water.
V_x The extraction volume.
$V_{x'}$ The extraction volume in milliliters.
V_{x1} The extraction volume computed using a one-step internal standard methodology.
V_{x2} The extraction volume computed using a two-step internal standard methodology.
[Vit E] The concentration of vitamin E (in grams per milliliter) in the extraction solvent plus internal standard.

$\hat{w}_{B_1}$ The corresponding peak width of t_{R_1} at its base.
$\hat{w}_{B_2}$ The corresponding peak width of t_{R_2} at its base.

α Specific absorption coefficient a.
α_{S_i} The absorption coefficient for the pigment of interest.

$\Delta\hat{A}_{P_i}$ The change in peak area.
$\Delta\tilde{C}_{P_i}$ The change in the amount of pigment injected onto the column.

ε The molar absorption coefficient at the specified wavelength for the pigment λ_{P_i}.

λ The spectral wavelength.
λ_m The maximum wavelength.

ξ The coefficient of variation.
$\bar{\xi}$ The average precision.
$\bar{\xi}_{\text{cal}}$ The average CV for gravimetric calibration of dilution devices.
$\bar{\xi}_{\text{inj}}$ The injector precision.
$\bar{\xi}_{P_i}$ The average precision for pigment P_i.
$\bar{\xi}_{t_R}$ The CV of retention time.

σ The standard deviation.
$\Sigma\hat{A}_{S_i}$ The sum of the parent peak and the area of the alteration products.
ψ The UPD.
$\bar{\psi}^A_{P_i}$ The average of the RPD values for a particular pigment across the number of laboratories or methods (N_L) reporting the pigment involved.
$|\psi|$ The absolute UPD.
$|\bar{\psi}|^A_{P_i}$ The average of the APD, $|\bar{\psi}|$, values for a particular pigment across the number of laboratories or methods (N_L) reporting the pigment involved.
$|\bar{\psi}|$ The average APD.
$|\bar{\psi}|_{\text{res}}$ The average of the absolute residuals.

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