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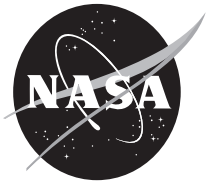

The Second SeaWiFS HPLC Analysis Round-Robin Experiment (SeaHARRE-2)

Stanford B. Hooker, Laurie Van Heukelem, Crystal S. Thomas, Hervé Claustre, Joséphine Ras, Ray Barlow, Heather Sessions, Louise Schlüter, Jason Perl, Charles Trees, Venetia Stuart, Erica Head, Lesley Clementson, James Fishwick, Carole Llewellyn, and James Aiken

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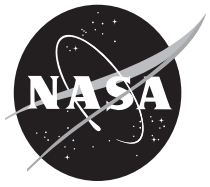

The Second SeaWiFS HPLC Analysis Round-Robin Experiment (SeaHARRE-2)

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Preface

The Second SeaWiFS HPLC Analysis Round-Robin Experiment (SeaHARRE-2) is the most comprehensive pigment round robin I know of and has built upon the previous experiences derived from SeaHARRE-1 (Hooker et al. 2000) plus the SIMBIOS fluorometry and HPLC intercomparison† (Van Heukelem et al. 2002). The fact that SeaHARRE-2 was a broadly-based international collaboration must be applauded as an exceptional achievement, especially because it was a strictly voluntary effort with no significant funding augmentations. The results highlight the requirement for continuing intercomparison activities that are needed to maintain consistency in the pigment data sets as new methodologies develop and as additional laboratories become sources for calibration and validation data. What these experiments have shown is that the uncertainty in the field measurements of chlorophyll *a* can constitute a major fraction of the overall satellite uncertainty goal of ±35%. On the other hand, it is also clear that *in situ* measurement uncertainties can be significantly reduced if state-of-the-art methodologies are implemented. More importantly, the SeaHARRE-2 results demonstrate metrics on quality can be established, and with the availability of so much quantitative analysis of pigment uncertainties, it may be prudent for the remote sensing community to reevaluate the original uncertainty goal in terms of performance-based metrics.

SeaHARRE-2 covered a pigment range that spans the concentrations usually encountered in the open ocean, but—as stated—is not sufficient for more complex coastal regimes. Consequently, future SeaHARRE activities will be needed for coastal data sets. Additionally, analogous experiments will be required to deal with other dissolved and particulate constituents that contribute to the carbon cycle or impact remote sensing observations (e.g., colored dissoloved organic matter, dissolved organic carbon, particulate inorganic carbon, and particulate organic carbon). Consequently, NASA and the other space agencies need to plan for supporting these experiments in the future, especially now that SeaWiFS and SIMBIOS are over and major missions like MODIS, MERIS, and GLI are at or nearing an end.

Greenbelt, Maryland
October 2004

—C. R. McClain
SeaWiFS Project Scientist

† High performance liquid chromatography (HPLC) round robins have been sponsored by the Sea-viewing Wide Field-of-view Sensor (SeaWiFS) and Sensor Intercomparison and Merger for Biological and Interdisciplinary Oceanic Studies (SIMBIOS) projects, although other international intercomparison activities have taken place, they are not the subject of the results presented here.

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Abstract

Eight international laboratories specializing in the determination of marine pigment concentrations using high performance liquid chromatography (HPLC) were intercompared using *in situ* samples and a variety of laboratory standards. The field samples were collected primarily from eutrophic waters, although mesotrophic waters were also sampled to create a dynamic range in chlorophyll concentration spanning approximately two orders of magnitude (0.3–25.8 mg m^{-3}). The intercomparisons were used to establish the following: a) the uncertainties in quantitating individual pigments and higher-order variables (sums, ratios, and indices); b) an evaluation of spectrophotometric versus HPLC uncertainties in the determination of total chlorophyll *a*; and c) the reduction in uncertainties as a result of applying quality assurance (QA) procedures associated with extraction, separation, injection, degradation, detection, calibration, and reporting (particularly limits of detection and quantitation). In addition, the remote sensing requirements for the *in situ* determination of total chlorophyll *a* were investigated to determine whether or not the average uncertainty for this measurement is being satisfied. The culmination of the activity was a validation of the round-robin methodology plus the development of the requirements for validating an individual HPLC method. The validation process includes the measurements required to initially demonstrate a pigment is validated, and the measurements that must be made during sample analysis to confirm a method remains validated. The so-called *performance-based metrics* developed here describe a set of thresholds for a variety of easily-measured parameters with a corresponding set of performance categories. The aggregate set of performance parameters and categories establish a) the overall performance capability of the method, and b) whether or not the capability is consistent with the required accuracy objectives.

PROLOGUE

Whether for biogeochemical studies or ocean color validation activities, high performance liquid chromatography (HPLC) is an established reference technique for the analysis of chlorophyll *a* and associated phytoplankton pigments. The emphasis of HPLC methods in marine studies has also been promoted because the international Joint Global Ocean Flux Study (JGOFS) program recommended HPLC techniques in the determination of chlorophyll *a* (JGOFS 1994) and, more precisely, from 1991, to use the Wright et al. (1991) method. More recently, a set of HPLC protocols for marine pigment analyses were codified into the *Ocean Optics Protocols for Satellite Ocean Color Sensor Validation* (Bidigare and Trees 2000), which have been updated more or less on an annual basis (Bidigare et al. 2002 and 2003), and are hereafter referred to as the Protocols.

As part of the *Productivité des Systèmes Océaniques Pélagiques*† (PROSOPE) JGOFS-France cruise, which occurred from 4 September to 4 October 1999, four laboratories, using four different HPLC methods, participated in an intercomparison exercise based solely on natural (field) samples (Hooker et al. 2000). This exercise was called the first Sea-viewing Wide Field-of-view Sensor (SeaWiFS) HPLC Analysis Round-Robin Experiment (SeaHARRE-1), and the samples were collected over a large gradient of trophic conditions (based on the chlorophyll *a* concentration) ranging from the high productivity (upwelling) conditions off the northwestern coast of Africa (2.2 mg m^{-3}) to the oligotrophic regime of the Ionian Sea (0.045 mg m^{-3}).

Despite the diversity in trophic conditions and HPLC methodologies, the agreement between laboratories during SeaHARRE-1 was approximately 7.0% for total chlorophyll *a*, which is well within the 35% accuracy objective for remote sensing validation purposes (Hooker and Esaias 1993). For other pigments (mainly chemotaxonomic carotenoids), the agreement between methods was 21% on average (ranging from 11.5% for fucoxanthin, to 32.5% for peridinin), and inversely depended on pigment concentration (with large disagreements for pigments whose concentrations were close to the methodological detection limits).

Although every effort was made to make SeaHARRE-1 as complete as possible (e.g., all analyses were based on replicates), there were deficiencies in the work plan. Consequently, a follow-on activity to specifically address recommendations from the first round robin was developed: a) a more concerted effort to sample oligotrophic and eutrophic regimes (from a remote sensing perspective, data from these two concentration levels are also where the most new data are needed); and b) the inclusion of standard pigment samples, so a control data set is available for analysis. The use of standard pigment samples was deemed particularly important, because several sources of uncertainty are best quantified if the concentration of the samples are independently known.

The planning for SeaHARRE-2 coincided with an anticipated field deployment to the onshore and deep water environments of the Benguela upwelling system. With

† Translated as the Productivity of Pelagic Oceanic Systems cruise, which is documented at the following Web address: `http://www.obs-vlfr.fr/jgofs/html/prosope/home.htm`.

the opportunity for a substantial dynamic range in chlorophyll concentration, eight international laboratories agreed to participate in SeaHARRE-2 with so-called *validated* HPLC methods (the scientists involved are given in Appendix A):

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

Of these laboratories, only HPL, LOV, and MCM participated in SeaHARRE-1.

The concept of a *validated* method requires some additional explanation, because there is no external process or independent agency that certifies an HPLC method is validated. The validation process is currently conceived and executed by the individual laboratory based on the sampling requirements and research objectives associated with the method. Consequently, validation occurs largely in isolation and relies heavily on a temporal evaluation of the calibration procedures, although some laboratories use more sophisticated evaluation criteria (Sect. 5.5). The SeaHARRE-1 activity was the first time a multitude of HPLC methods were intercalibrated, which is a more robust mechanism for demonstrating the degree of validation for a particular method.

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

The National Aeronautics and Space Administration (NASA) has funded several marine pigment round robins under the sponsorship of the SeaWiFS and Sensor Intercomparison and Merger for Biological and Interdisciplinary Oceanic Studies (SIMBIOS) Projects (Hooker et al. 2000 and Van Heukelem et al. 2002, respectively), and the European Space Agency (ESA) has sponsored a chlorophyll *a* round robin‡. These activities have supplied sufficient detail about HPLC methods to provide the basis for proposing a fully mature method validation process, with the end goal that each laboratory will then be able to describe the measurement uncertainty associated with various trophic regimes regardless of when filter samples were analyzed or data reported. This approach to validation will provide the end user (data manager, research scientist, etc.) a continuity of understanding across all methods.

Three aspects of this validation process need to be developed: a) the measurements required to initially demonstrate a method is validated, b) the measurements that must be made during the analysis of samples to confirm a method remains validated, and c) the level of method performance an analyst should expect to achieve in order to meet specific accuracy objectives. The latter requires a set of criteria that must be available before the validation process begins. What is imagined here are a set of thresholds for a variety of easily-measured parameters, and if an analyst achieves equality with a particular threshold, then the corresponding performance category is assigned for that parameter. The aggregate set of performance parameters and categories establish the overall performance capability of the method. This approach is referred to hereafter as *performance-based metrics*.

The overall results of SeaHARRE-2 are presented in Chapter 1 and the individual methods of the eight laboratories are presented in Chapters 2–9, respectively. A summary of the material presented in each chapter is given below.

1. SeaHARRE-2 Methods, Data, and Analysis

The focus of this study was the estimation of a variety of chromatographically determined pigments from eight HPLC methods (and laboratories). More than 30 individual pigments or pigment associations were intercompared. An initial analysis of the data showed the average uncertainty in the determination of total chlorophyll *a* for the

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

‡ The results of the European round robin were distributed in a report by K. Sørensen, M. Grung, and R. Röttgers in 2003 titled *An Intercomparison of in vitro Chlorophyll-a Determinations—Preliminary Results.*

eight methods was 10.9%, although a four-laboratory subset (denoted A') had an average uncertainty of 7.8%. The average uncertainty of the so-called *primary pigments* (a mixture of total chlorophylls and carotenoids typically analyzed by most laboratories) had an average uncertainty of 29.7%. Development and application of quality assurance procedures showed the A' subset was the most appropriate reference data for computing uncertainties, and ultimately reduced the average A' uncertainties to 5.9% for total chlorophyll *a* and 16.0% for the primary pigments. From the perspective of ocean color remote sensing and the definitions adopted in this study, the accuracy requirement for determining the concentration of total chlorophyll *a* in support of vicarious calibration activities should be changed from the current "routine" value of 25% to a "semiquantitative" value of 15% (with a precision to within 5%). The "semiquantitative" performance category should also be adopted as the minimum QA level for all pigment data being used in validation activities. This means the primary pigments must be determined within an accuracy of 25% and a precision of 8%.

2. The BIO Method

The BIO HPLC method is similar to the method described by Gieskes and Kraay (1989), using a C_{18} HPLC column in combination with a methanol-based, reversed-phase binary gradient system. This method effectively separates the major key pigments in an analysis time of 30 min, but does not permit the separation of mono- and divinyl chlorophylls *a* and *b*. Separation of the divinyl pigments can, however, be achieved by acidifying a second sample, and quantifying the divinyl phaeophytin-like pigments. Acidification of samples with dilute HCL effectively separates divinyl phaeophytin *a* and *b* from phaeophytin *a* and *b*, respectively. Application of the latter procedure to chromatographically separate the divinyl forms requires each sample to be analyzed twice.

3. The CSIRO Method

The HPLC method used by CSIRO is a modified version of the Wright et al. (1991) method. This method has the capacity to separate 50 different pigments and is used routinely in the CSIRO laboratory to separate approximately 35 different pigments, but does fail to separate monovinyl chlorophyll *a* and chlorophyll *b* from their divinyl forms. CSIRO has continued to use this method, because until recently, it was the recommended method of SIMBIOS (Mueller et al. 2003), and for many years the work of this laboratory has been in temperate waters for which the method works well. More recently, CSIRO has occasionally been working in tropical waters where divinyl chlorophyll *a* is often the predominant form of chlorophyll, and in these cases, the laboratory has started using the Zapata et al. (2000) method. For the SeaHARRE-2 activity, the Wright et al. (1991) method was used exclusively. Samples are extracted over 15–18 h in an acetone solution before analysis by HPLC using a reversed-phase C_{18} column and ternary gradient system with a photodiode array (PDA) detector. The method is regularly validated with the use of external standards and individual pigment calibration. The detection limit of most pigments is within the range of 0.001–0.005 mg m^{-3}. The method has proven to offer a good balance between accuracy of pigment composition and concentration and number of samples analyzed; an important factor in an applications based laboratory.

4. The DHI Method

The HPLC method used at DHI is a slightly modified version of the method developed by Wright et al. (1991). This method does not separate chlorophyll c_1 and chlorophyll c_2; furthermore, divinyl chlorophylls *a* and *b* are not separated from monovinyl chlorophylls *a* and *b*, respectively. Dichromatic equations were used to separate divinyl chlorophyll *a* from monovinyl chlorophyll *a* (Latasa et al. 1996). The results of the dichromatic equations, however, showed in several instances that divinyl chlorophyll *a* was present although this pigment was not detected by the laboratories that were able to separate the two pigments chromatographically. The dichromatic equation method was apparently giving erroneous results, and should therefore be used with caution. Phaeophorbide *a* was not separated from fucoxanthin, but because these pigments have distinctly different absorption spectra, they could subsequently be separated.

5. The HPL Method

The HPL method was developed for use with a variety of water types. Many pigments important to freshwater, estuarine, and oceanic systems are baseline resolved and quantitatively reported, including divinyl and monovinyl chlorophyll *a*. The linear dynamic range for chlorophyll *a* extends approximately from 0.3–700 ng per injection. The method is based on a C_8 HPLC column, a methanol-based reversed-phase gradient solvent system, a simple linear gradient, and an elevated column temperature (60°C). The method can provide quantitative results for up to 25 pigments with qualitative information for additional pigments. Quality assurance measurements are made during sample analysis to confirm method performance is within expectations. Investigations into the uncertainties in the method show the 95% confidence limits were estimated as a) 0.5–3.8% for precision of replicate injections within and across sequences, b) 3.2% for chlorophyll *a* calibration reproducibility, and c) 5.1% for chlorophyll *a* method precision, including filter extraction and analysis.

6. The LOV Method

The LOV method applies a sensitive reversed-phase HPLC technique for the determination of chloropigments and carotenoids within approximately 24 min. The different pigments are detected by a DAD which allows for automatic identification to be carried out on the basis of absorption spectra. Optical densities are monitored at

440 nm (chloropigments and carotenoids) and at 667 nm (chloropigments only). The method provides a good resolution between chlorophyll *a* and divinyl chlorophyll *a*, but uncertainties may arise for the partial separation of chlorophyll *b* and divinyl chlorophyll *b*, and for the resolution of chlorophyll *c* pigments. The problem of coelution between 19′-hexanoyloxyfucoxanthin and prasinoxanthin may also be an issue in coastal Case-2 waters where the latter can be present in more significant concentrations. Effective limits of quantitation for most pigments are low ($0.0005\,\mathrm{mg\,m^{-3}}$ for chlorophyll *a* and $0.0008\,\mathrm{mg\,m^{-3}}$ for carotenoids).

7. The MCM Method

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

8. The PML Method

The PML method successfully resolves and quantifies over 20 chlorophyll and carotenoid pigments from a variety of water types, ranging from estuaries to oligotrophic oceans. A C_8 column is used in combination with a methanol-based binary solvent system following a linear gradient. The method separates monovinyl and divinyl chlorophylls *a* and *b*, and the carotenoids lutein and zeaxanthin. A DAD is configured at 440 nm to determine chlorophylls and carotenoids and at 667 nm for the determination of chlorophyllide *a* and phaeopigments. The method provides good resolution with a run time of approximately 30 min excluding an additional 15 min to restore the column and solvent gradient prior to the next injection. The internal standard, *trans*-β-apo-8′-carotenal, improves the accuracy of the analysis.

9. The SDSU (CHORS) Method

The CHORS method was developed to provide HPLC phytoplankton pigment analyses for the NASA SIMBIOS program, following the protocols presented in the *Ocean Optics Protocols for Satellite Ocean Color Sensor Validation* (Bidigare et al. 2002). The method (Wright et al. 1991) was designed to support a wide range of pigment concentrations from waters sampled throughout the world ocean, but does not chromatographically separate the divinyl chlorophylls *a* and *b* from their corresponding monovinyl forms. The Latasa et al. (1996) dichromatic equations were used to spectrally resolve divinyl chlorophyll *a* from monovinyl chlorophyll *a*. The method uses a reversed-phase C_{18} column, with a tertiary solvent gradient. In addition, a temperature-controlled autosampler provides continuous sample injection to maintain a quota of analyzing 4,000 samples per year. System calibration is routinely monitored and recorded each month to ensure repeatability and consistency of data products.

Chapter 1

SeaHARRE-2 Methods, Data, and Analysis

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Abstract

The focus of this study was the estimation of a variety of chromatographically determined pigments from eight HPLC methods (and laboratories). More than 30 individual pigments or pigment associations were intercompared. An initial analysis of the data showed the average uncertainty in the determination of total chlorophyll *a* for the eight methods was 10.9%, although a four-laboratory subset (denoted A') had an average uncertainty of 7.8%. The average uncertainty of the so-called *primary pigments* (a mixture of total chlorophylls and carotenoids typically analyzed by most laboratories) had an average uncertainty of 29.7%. Development and application of quality assurance procedures showed the A' subset was the most appropriate reference data for computing uncertainties, and ultimately reduced the average A' uncertainties to 5.9% for total chlorophyll *a*, and 16.0% for the primary pigments. From the perspective of ocean color remote sensing and the definitions adopted in this study, the accuracy requirement for determining the concentration of total chlorophyll *a* in support of vicarious calibration activities should be changed from the current "routine" value of 25% to a "semiquantitative" value of 15% (with a precision to within 5%). The "semiquantitative" performance category should also be adopted as the minimum QA level for all pigment data being used in validation activities. This means the primary pigments must be determined within an accuracy of 25% and a precision of 8%.

1.1 INTRODUCTION

Because phytoplankton concentration is an important variable in the study of marine biogeochemical cycles, the accurate quantification of its biomass is a fundamental requirement. Phytoplankton have a unique characteristic: they possess chlorophyll *a*, which is a colored molecule with specific light reaction properties. As a light-harvesting pigment, chlorophyll *a* absorbs radiation in the blue and red parts of the visible spectrum, and fluoresces in the red when stimulated in the blue. These absorption and fluorescence properties have been the starting point in the development of a large variety of methods for quantifying the concentration of the chlorophyll *a* pigment, $\left[\text{Chl}\,a\right]$, from the single cell to the synoptic scale.

Examples of techniques using the fluorescence properties of chlorophyll *a* to quantify phytoplankton biomass, either discretely (with collected samples) or *in toto* (directly *in situ*) include *in vitro* fluorescence (Yentsch and Menzel 1963); *in vivo* fluorescence, either excited (Lorenzen 1966) or natural (Chamberlin et al. 1990); lidar (Hoge and Swift 1981); flow cytometry (Olson 1983); epifluorescence microscopy (Li and Wood 1988); and spectrofluorometry (Neveux and Lantoine 1993). The absorption properties of chlorophyll *a* allow its quantification by spectrophotometric techniques on acetonic extracts (Parsons and Strickland 1963), on filters (Kishino et al. 1985), or *in situ* using recent instrumental advances (Claustre et al. 2000). Finally, remote measurements of the light emerging from the ocean surface, the so-called water-leaving radiance, can be used to estimate $\left[\text{Chl}\,a\right]$ by taking advantage of the (at least partial) dependence of water color on phytoplankton (chlorophyll *a*) absorption (O'Reilly et al. 1998), particularly in the open ocean where the optical properties in the upper

part of the water column are determined almost exclusively by the resident phytoplankton populations (Morel 1988).

The quality of phytoplankton biomass—that is, its taxonomic composition—influences many biogeochemical processes and is a fundamental property to be documented. The functional specialization of phytoplankton is a critical component in the biogeochemical description of a marine ecosystem, so it is essential to simultaneously determine phytoplankton biomass, as well as its composition over the continuum of phytoplankton size (about 0.5–100 μm). The determination of chlorophyll and carotenoid pigment concentrations by HPLC fulfills most of these requirements. Indeed, many carotenoids and chlorophylls are taxonomic markers of phytoplankton taxa, which means the composition of the phytoplankton community can be evaluated at the same time that $\left[\text{Chl } a\right]$ is accurately quantified.

Since the initial methodological paper of Mantoura and Llewellyn (1983), the possibility of determining community composition and biomass has resulted in HPLC rapidly becoming the technique of choice in biogeochemical and primary production studies. The emphasis of the HPLC method in marine studies was promoted because the international Joint Global Ocean Flux Study (JGOFS) program recommended from 1991 to use the Wright et al. (1991) method in the determination of $\left[\text{Chl } a\right]$. Since the start of the JGOFS decade in the 1980s, HPLC techniques evolved considerably (Jeffrey et al. 1999), and some JGOFS contributors decided to adopt the new methods. In particular, the C_8 method of Goericke and Repeta (1993) was an important improvement, because it allowed the separation of divinyl chlorophyll *a* from its monovinyl form.

Because the marine pigment analysis by HPLC was a new and rapidly changing research field, it was necessary to carefully check the performance between the evolving methods and, if necessary, propose corrections. Consequently, the Scientific Committee on Oceanographic Research (SCOR) United Nations Educational, Scientific, and Cultural Organization (UNESCO) Working Group 78 for the determination of photosynthetic pigments in seawater (Appendix B) was established in 1985, which culminated with the publication of a monograph with many methodological recommendations (Jeffrey et al. 1997). Similarly, JGOFS sponsored an intercomparison exercise involving the distribution and analysis of pigment standards among several laboratories which also resulted in some analytical recommendations (Latasa et al. 1996).

More recently, HPLC has become the reference method for calibration and validation activities of chlorophyll *a* remote sensing measurements, for which accuracy is an essential requirement. For example, the SeaWiFS Project requires agreement between the *in situ* and remotely sensed observations of chlorophyll *a* concentration to within 35% over the range of 0.05–50.0 mg m^{-3} (Hooker and Esaias 1993). The 35% performance criteria was based (in principle) on inverting the optical measurements using a bio-optical algorithm, so the *in situ* pigment observations will always be one of two axes to derive or validate the pigment relationships (Hooker and McClain 2000). Given this, it seems appropriate to reserve approximately half of the uncertainty budget for the *in situ* measurements. The sources of uncertainty are assumed to combine independently (i.e., in quadrature), so an upper accuracy range of 25% is acceptable, although 15% would (presumably) allow for significant improvements in algorithm refinement.

The SeaHARRE-1 activity was planned as the first of multiple round robins using *in situ* samples, and it was based on the voluntary participation of the participants. Consequently, it was not necessary, nor financially feasible, to anticipate all elements useful to the entire process within a single work plan. It was anticipated that subsequent round robins with more extensive budgets and sampling opportunities would evolve to deal with deficiencies identified in the initial investigations. The primary motivation of the work, and the principal evaluation criteria, was the accuracy requirement of remote sensing algorithms for chlorophyll *a* determination. Given the aforementioned importance of taxonomic composition—which is anticipated to be an objective for remote sensing projects in the near future—and the absence of any separate accuracy objectives for the other pigments, the chlorophyll *a* accuracy requirements were simply applied to all the other pigments.

The follow-on experiment, SeaHARRE-2, was designed to address the sampling and analysis recommendations from the first round robin, which suggested eutrophic concentration levels and standard pigment samples should be considered in any subsequent work plan. The latter were deemed especially useful, because several aspects of estimating uncertainties are best determined by intercomparing the analysis of samples with known concentrations (but unknown to the analysts at the time of HPLC analysis).

The first round robin also generated a strong interest in understanding the uncertainties associated with the principal terms in the equation governing the calculation of the concentration (C) of an individual pigment (P_i) from a field sample. Ignoring the specific details of the basic HPLC processes, because they are presented in detail by Jeffrey et al. (1997) 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 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

† A milli-absorbance unit is denoted mAU.

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 given in (2) can be expressed to follow (1) as follows:

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

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

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

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

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

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

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

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

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

An inquiry into HPLC uncertainties is naturally guided by the terms and underlying processes associated with quantifying the parameters given in (6). The factors influencing proper pigment identification and quantification of peak areas, are an explicit part of understanding HPLC uncertainties. More subtle aspects are discerned from the procedures associated with the individual methods or parameters. For example, a methodology requiring multiple measurements with a pipette is more prone to enhanced uncertainties than one that requires only one, and a method that uses a calibrated and highly precise pipette to measure volume is expected to have lower uncertainties than one that relies on reading a meniscus from a graduated cylinder (whether calibrated or not).

The consideration of these types of refinements resulted in the following goals for SeaHARRE-2:

1. Estimate the uncertainties in quantifying a diverse set of chlorophyll and carotenoid pigment concentrations for a variety of HPLC methods and related procedures, and compare the uncertainties to the 25% compliance and 15% refinement thresholds established for ocean color remote sensing.
2. Determine whether or not the chlorophyll *a* accuracy requirements for ocean color validation activities (approximately 25%, although 15% would allow for algorithm refinement) can be met in predominantly eutrophic conditions.
3. Establish how higher-order associations in individual pigments (i.e., sums and ratios) influence the uncertainty budget, while also determining how this information can be used to minimize the variance within larger pigment databases.
4. Compare the uncertainties in the determination of chlorophyll *a* concentration using HPLC methods and the spectrophotometric (trichromatic equation) technique (Jeffrey and Humphrey 1975).
5. Investigate how procedures that can be subjected to a quality assurance (QA) protocol (e.g., extraction, injection, and calibration) plus the parameters involved (e.g., detection limits, separation, extinction coefficients†, and stability) influence uncertainties.

† The term "extinction coefficient" refers to the quantitative relationship between the absorption at a specific wavelength of a known amount of sample in a known volume of solvent, as clearly described in Jeffrey et al. (1997) for spectrophotometrically determining concentrations of pigment standards. It is now recommended that the term "extinction coefficient" not be used and that "absorption coefficient" be used instead. Absorptivity (α), expressed in liters per gram per centimeter, is the *internal absorbance divided by the product of sample pathlength and mass concentration of the absorbing material*, and molar absorptivity (ε), expressed in liters per mole per centimeter, is the *internal absorbance divided by the product of sample pathlength and mole concentration of the absorbing material* (Guide to Authors, *Journal of Applied Spectroscopy* Vol. 57, Number 1, 2003). Following such recommendations, the terms "absorption coefficient" and "molar absorption coefficient" are used in this report.

6. Validate the data analysis method for determining uncertainties with field samples by applying the same data analysis method to laboratory standards and comparing the results.
7. Propose a set of performance-based metrics for evaluating HPLC methods used to quantitate marine pigments with an emphasis on sampling or analysis procedures that could be easily incorporated into existing methods.

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

Setting arbitrary accuracy thresholds, because none are available, is not a satisfactory state of affairs. Consequently, a significant part of the SeaHARRE-2 activity was to establish performance-based metrics independent of any special emphasis from a particular subset of the community (like remote sensing). The philosophy here is to rely on a representative set of methods to describe different categories of performance spanning what is routinely achievable and what is state of the art. Once the categories are established, anyone requiring pigment concentration at a particular performance level can readily determine whether their requirements can be satisfied or not.

1.2 THE DATA SET

An HPLC intercomparison requires samples with a variety of pigment types and relative concentration levels, which is a diversity in the amount of one pigment with respect to another. This is needed to ensure the expression of coelution problems plus whatever processes influence peak shape (height and area) and pigment identification. Field samples, by definition, provide the most realistic set of factors influencing these parameters. The disadvantage of field samples is the concentrations are unknown. Laboratory standards have known concentrations, but the pigment diversity and chromatographic complexity of mixed standards is usually unrealistic; furthermore, the absolute and relative concentration levels are frequently artificial.

To capitalize on the advantages of both sample types, the SeaHARRE-2 data set was derived from *in situ* samples from an oceanographic field campaign and two types of laboratory standards. One set of standards was from DHI, which specializes in the commercial distribution of pigment standards, and the other set was from HPL. At the time of HPLC analysis, the analysts did not know the concentration of the laboratory standards (this information was only revealed after all the results were collected for synthesis and statistical analysis).

1.2.1 The Field Samples

The filtered seawater used for SeaHARRE-2 was collected during the Benguela Calibration (BENCAL) cruise on board the South African Fisheries Research Ship (FRS) *Africana* (Barlow et al. 2003). BENCAL was an ocean color calibration and validation cruise in the Benguela upwelling ecosystem which took place from 4–17 October 2002. The cruise was staged in the southern Benguela Current system between Cape Town and the Orange River within the region 14–18.5°E,29–34°S (Fig. 1).

The initial analysis plan was for each laboratory to analyze 12 samples, with each sample provided in triplicate (for a total of 36 filters). Because eight laboratories were participating, 24 replicates were collected from various depths at 12 different stations over a large range of phytoplankton biomass. Seawater was obtained from several Niskin bottles, or from the surface using a pump or bucket. Once the seawater was on deck, it was transferred into a 75 L carbuoy and thoroughly mixed to preserve the homogeneity between the 24 replicate samples. Volumes ranging from 0.25–2.8 L were filtered through 25 mm GF/F filters, such that a visually-similar amount of phytoplankton was retained on filters from different sites. The filters were placed in cryovials or in aluminum foil jackets and immediately frozen and stored in liquid nitrogen.

A total of 288 samples were distributed, with each laboratory receiving three replicates from each station. A set of replicates from a particular station is referred to hereafter as a *batch*, so a total of 12 batches were collected. The replicates are used to report a *sample* value for each pigment for each batch (or station). The sample value is simply the average of the replicates within a batch. Despite being carefully monitored over a two-week period to ensure the functionality of the dry shippers (by weighing them), several defrosted during transport, resulting in the degradation (to an unknown degree) of three complete sets of replicate batches (108 filters).

The samples not distributed to isolated locations were redistributed between laboratories such that each laboratory received two frozen replicates instead of the original set of triplicates (except MCM retained a set of triplicates). The replicates were analyzed by each laboratory and the average of the replicates within each batch were reported as the *final sample value* for the batch. The intercomparison of the final sample values is used to determine the accuracy of the methods, and the variance of the samples within a batch is used to estimate method precision.

Three sets of so-called *residence time* samples were also collected. These samples were intended to be used to investigate how the amount of time sample extracts reside in a temperature-controlled autosampler (TCAS) compartment, or a storage freezer, affects HPLC uncertainties. Unfortunately, these samples were transported in one of the liquid nitrogen dry shippers that arrived defrosted, so two larger sets of replacement samples were provided by

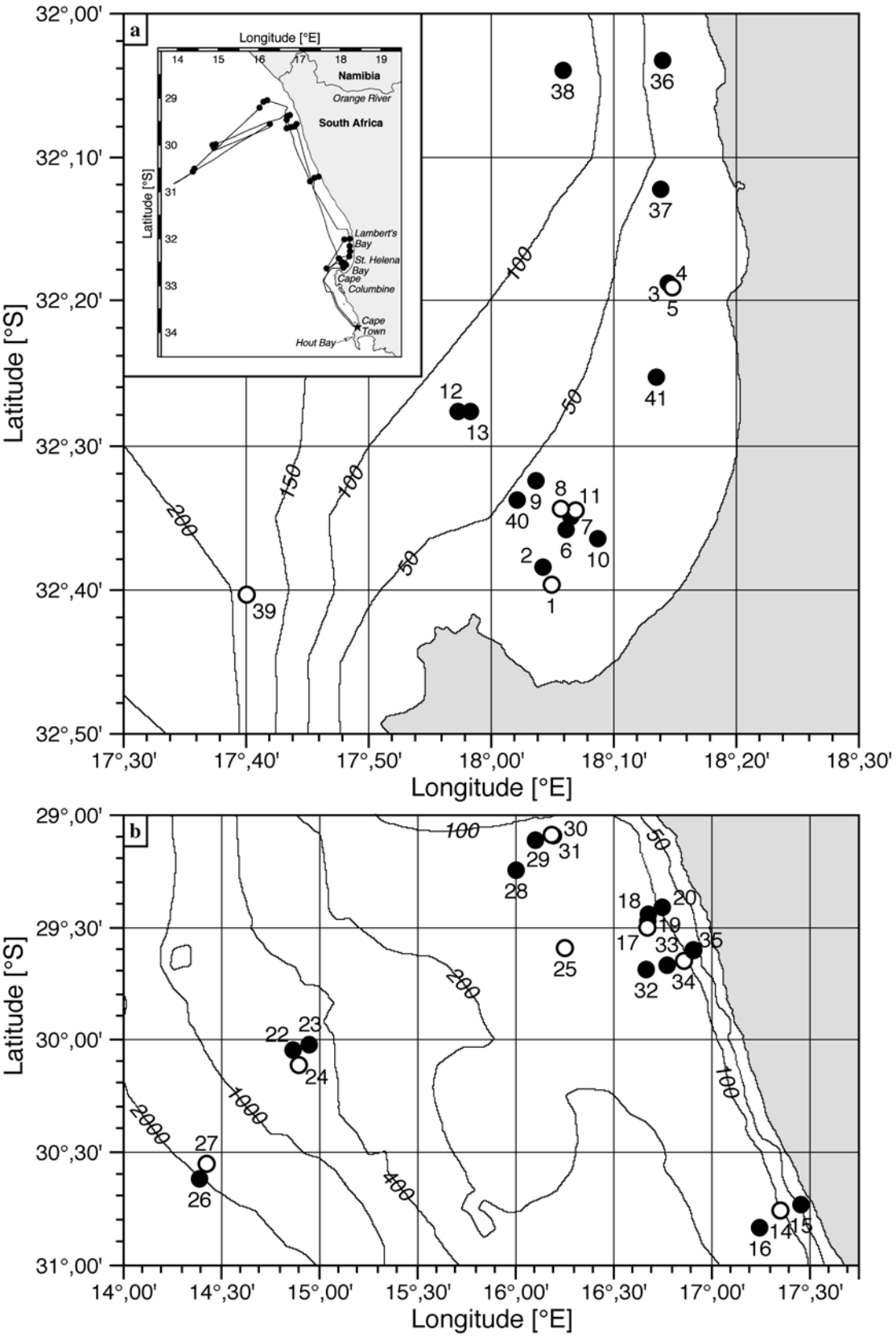

Fig. 1. Overall BENCAL station locations (inset panel) plus specific stations and bathymetry in the **a)** St. Helena Bay, and **b)** Orange River regions. The numbers indicate the actual station number and the water depth is in meters. The SeaHARRE-2 sample stations are shown as open circles.

LOV and MCM after the BENCAL cruise from mesotrophic (Mediterranean Sea) and eutrophic (Benguela Current) waters, respectively. The replacement samples ensured the specialized experiments associated with the original samples could still be conducted.

The *in situ* field samples were extracted per the usual procedures for the individual methods. To evaluate the effects of residence time in the TCAS compartment, one of the filter extracts from station 11 and another from station 9 were injected three times each at the beginning of the sequence and then three times each after the extracts had resided in the TCAS for 24 h. The extracts analyzed at the beginning of the sequence resided in the TCAS for at least 1 h before analysis. A separate vial was usually used for each injection to minimize the evaporation that can result from a vial having been pierced by the injection needle. If there were sufficient extract volumes, the analyses were performed in triplicate, otherwise duplicate injections were done before and after the 24 h residence time.

A summary of the primary *in situ* sampling aspects for the 12 field samples, organized by the identification (ID) and station number, is presented in Table 1. Most of the samples were collected during conductivity, temperature, and depth (CTD) profiles; more complete details are available in Barlow et al. (2003).

Table 1. The sampling log for the 12 field samples. The ID number for each of the 24 filters collected at each station was constructed as `RR2-nnnl`, where `RR2` denotes a SeaHARRE-2 sample, `nnn` is the number from the first column of the table, and `l` is a sequential letter of the alphabet (`a`–`x`). The station number entry includes the sequential day of the year (SDY) in parentheses. The sampling depth is shown with the filtration volume, V_f.

ID No.	*Station No.* (SDY)	*Time* [GMT]	*Sample Type*	*Depth* [m]	V_f [L]
1†	1 (278)	0817	CTD 1	2.0	0.50
2	5 (279)	1405	CTD 5	2.9	0.50
3‡	8 (280)	1309	CTD 8	3.3	0.50
4	11 (281)	1053	Bucket	0.0	0.50
5	14 (282)	0800	CTD 13	3.0	0.50
6	17 (283)	0715	Pump	0.0	0.25
7	24 (284)	1336	CTD 21	10.0	2.00
8	25 (285)	0757	CTD 23	30.0	1.00
9	27 (286)	1447	CTD 26	40.0	2.80
10	30 (287)	1435	Pump	0.0	1.00
11	34 (288)	1245	Pump	0.0	0.50
12	39 (290)	0617	CTD 36	3.2	1.00

† `RR2-001m` Complete filtration took a very long time.
‡ `RR2-003x` $V_f = 0.44$ L (loss of 60 mL).

1.2.2 The Laboratory Standards

Most of the laboratories were also sent a batch of unknown standard samples from DHI and HPL for HPLC analysis plus one sample from HPL for spectrophotometric analysis. The DHI standards were six individual standards shipped and kept separate in solitary vials, whereas the HPL standards were composed primarily of three multiple standards, wherein each standard was a combination of individual standards mixed together in one vial. The fourth HPL sample was a separate (chlorophyll *a*) standard, but the analysis objective for this standard was to analyze it spectrophotometrically. In all cases, the laboratory standards were the only samples with known concentrations for the constituent pigments, and they were taken from the same production lots to ensure any differences between lots could not contribute to the uncertainties associated with the analysis of the individual methods.

The DHI standards were intended for HPLC analysis and are hereafter referred to as Mix D. The three HPL standards intended for HPLC analysis are referred to as Mix A, Mix B, and Mix C, or generically as Mix ABC as a group. The HPL solitary chlorophyll *a* standard intended for spectrophotometric analysis is called Mix E. The properties and composition of the standards differed, which permit a variety of problems to be considered:

A. A mixture of six pigments provided in ethanol whose individual pigment concentrations were determined using common§ (ethanol) absorption coefficients.

B. A mixture of seven pigments (Mix A plus chlorophyll *a*) provided in 90% acetone whose individual concentrations were based on uncommon (acetone) absorption coefficients, except a common absorption coefficient was used for chlorophyll *a*.

C. A complex mixture of 20 pigments whose individual concentrations were determined using primarily uncommon carotenoid (acetone) absorption coefficients and provided almost completely in 90% acetone (two of the pigments were mixed in while suspended in ethanol, so the final concentration was 88% acetone, 8% water, and 4% ethanol).

D. Six solitary pigments provided in either acetone or ethanol (three pigments each) whose individual concentrations were determined using common acetone and ethanol absorption coefficients, respectively.

E. A single pigment, chlorophyll *a*, provided in 90% acetone whose concentration was determined using a common absorption coefficient.

The original work plan envisioned the laboratory standards being analyzed at the same time as the field samples, but shipping and equipment problems did not permit this in some cases.

§ For the purposes of the SeaHARRE-2 activity, if a majority of the marine community uses a particular absorption coefficient, it is considered a "common" value; all other values are considered "uncommon." This concept is considered in more detail in Sect. 1.3.4.

Table 2 shows the number of replicate field samples that were analyzed by each laboratory, along with the number of injections associated with the analysis of the laboratory standards. The table also presents the one-letter code for each laboratory, plus two special codes associated with initially questionable (Y) and unequivocally defrosted (Z) samples. PML analyzed the Y samples and determined they could participate with the remaining duplicates, which are reported as the P results. HPL analyzed the Z samples at the same time as a set of properly frozen samples (the latter are reported as the H results).

Table 2. The laboratories that participated in SeaHARRE-2 along with their one-letter identification codes, and the samples they analyzed. The latter is indicated by the number of field replicates or the number of injections for the laboratory standards. Blank entries indicate no participation.

Laboratory		*Samples Analyzed*			
Name	*Code*	*Field*	*Mix ABC*	*Mix D*	*Mix E*
BIO	*B*	2			
CSIRO	*C*	2	3	3†	2
DHI	*D*	2	3	3	1
HPL	*H*	2	3	3	2
LOV	*L*	2	3	2	1
MCM	*M*	3	3	3	1
PML	*P*	2		3	
SDSU	*S*	2	3	2	
PML	*Y*	1‡			
HPL	*Z*	2§			

† The 19′-Hexanoyloxyfucoxanthin vial arrived broken.
‡ An initial analysis to verify the efficacy of the samples.
§ An analysis of unequivocally defrosted samples.

The Mix A, B, and C standards were distributed in two vials each; the vials denoted "1" contained approximately 2.5 mL and the vials denoted "2" contained about 1.5 mL. Two HPLC analyses were performed on the A1, B1, and C1 vials, and one HPLC analysis for vials A2, B2, and C2. The DHI standards were shipped in solitary vials and usually analyzed in triplicate. The HPL Mix E standard was distributed in a single vial, and the spectrophotometric analysis was done once or twice depending on the laboratory.

Despite the best effort of everyone involved, it proved difficult to ship both the field and laboratory standards. Shipment of the field samples started on 18 October 2002, shortly after the *Africana* docked in Cape Town, but did not conclude until the delivery of the CSIRO samples on 22 January 2003. The primary difficulty was convincing commercial airlines to accept nonstandard cargo during a period of heightened security concerns.

The deliveries of the laboratory standards were equally problematic, because the solvents in which the pigments were suspended, along with the use of a dry shipper, classified the cargo as hazardous. Shipment of the laboratory samples was initiated in the middle of November 2002, and was not completed until the receipt of the MCM sample set on 11 February 2003. In some cases, the DHI and HPL laboratory samples were combined into one shipment, and the entire shipment was handled by DHI. The expertise of the DHI shipping office was instrumental in the successful shipment of many sample sets, which included a replacement delivery of samples to PML, because the first delivery was held in customs and defrosted.

Table 3 presents the details associated with sample delivery, storage, extraction, and analysis. Most laboratories analyzed the field samples on or within the same day of extraction, but there was a 3–4 day lag between extraction and HPLC analysis for laboratory S and an 8-day lag between extraction and spectrophotometric analysis for laboratory L. The completion of all aspects of the work plan was delayed, because of scheduling problems created by the late arrival of the shipments and technical problems with some equipment. The final delivery of all data products did not take place until 29 July 2003.

1.2.3 The Pigments

In some parts of this document, abbreviations from the 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.

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 laboratories 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);

Table 3. A summary of the dates samples were received (top portion), storage conditions and extraction dates (middle portion), and the dates samples were analyzed (bottom portion). If a laboratory did not particiapte in a particular analysis, the entry is denoted not applicable (N/A).

Sample Procedure	*Laboratory*							
	B	*C*	*D*	*H*	*L*	*M*	*P*	*S*
Field Samples	16 Jan 03	22 Jan 03	17 Dec 02	30 Oct 02	5 Nov 02	18 Oct 02	30 Oct 02	19 Nov 02
DHI Standards	*N/A*	4 Feb 03	*N/A*†	5 Dec 02	3 Dec 02	11 Dec 02	5 Dec 02	5 Dec 02
HPL Standards	*N/A*	4 Feb 03	16 Dec 02	10 Dec 02‡	17 Dec 02	11 Feb 03	*N/A*	19 Nov 02
Field Storage	−80°C	Liquid Nitrogen	−80°C	−80°C	−80°C	Liquid Nitrogen	Liquid Nitrogen	Liquid Nitrogen
Standards Storage	*N/A*	Liquid Nitrogen	−80°C	−15°C	−80°C	−20°C	−20°C	−20°C
Field Extractions	17–24 Jan 03	15 Mar 03	29 Jan 03	7–8 Nov 02	10 Feb 03	20–21 Nov 02	3–4 Feb 03	5 Dec 02
Field HPLC	17–24 Jan 03	15 Mar 03	30–31 Jan 03	7–8 Nov 02	10 Feb 03	20–21 Nov 02	3–4 Feb 03	8–9 Dec 02
Field Spectro.	*N/A*	15 Mar 03	30 Jan 03	7–8 Nov 02	18 Feb 03	20–21 Nov 02	*N/A*	*N/A*
DHI Standards	*N/A*	17 Mar 03	29 Jan 03	11 Dec 02	13 Feb 02	28 Jul 03	28 Jan 03	8–9 Dec 02
HPL Standards	*N/A*	17 Mar 03	29 Jan 03	11 Dec 02	13 Feb 02	29 Jul 03	*N/A*	9 Dec 02

† The DHI standards were analyzed directly by DHI without any shipping.
‡ To simulate the effects of transportation, HPL stored the samples in a dry shipper for one week.

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

All laboratories quantitated the individual primary pigments, except $\left[\text{Zea}\right]$ for *B*, which are used to create 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. (2005) to derive size-equivalent pigment indices which roughly correspond to the biomass proportions of pico-, nano-, and micro-phytoplankton, 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).

Table 4. The secondary (top portion) and tertiary (middle portion) pigments shown with their variable forms, names, and calculation formulas (if applicable). The laboratories that quantitated 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. 1997). Abbreviated pigment forms are shown in parentheses. The ancillary pigments (bottom portion) were quantitated by the indicated laboratories, 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 laboratory.

Variable	*Laboratory*	*Secondary Pigment*	*Calculation*
[Chl *a*]	*B C D H L M P S*	Chlorophyll *a* (Chl *a*)	Including allomers and epimers
[SChl*a*]	*C D H L M*	Spectrophotometric chlorophyll *a* (SChl *a*)	Jeffrey and Humphrey (1975)
[DVChl *a*]	*B* ¶ *H L M* ¶	Divinyl chlorophyll *a* (DVChl *a*)	
[Chlide *a*]	*B C D H L M P S*	Chlorophyllide *a* (Chlide *a*)	
[Chl *b*]	*B C D H L M P S*	Chlorophyll *b* (Chl *b*)	
[Chl c_{1+2}]	*B C D H L M P S*	Chlorophyll c_{1+2} (Chl c_{1+2})	[Chl c_1] + [Chl c_2]
[Chl c_3]	*B C D H L M P S*	Chlorophyll c_3 (Chl c_3)	
[$\beta\varepsilon$-Car]	*B C D* *S*	$\beta\varepsilon$-Carotene† ($\beta\varepsilon$-Car)	
[$\beta\beta$-Car]	*B C D L S*	$\beta\beta$-Carotene‡ ($\beta\beta$-Car)	
Variable	*Laboratory*	*Tertiary Pigment*	*Calculation*
[Lut]	*C D H L M P S*	Lutein (Lut)	
[Neo]	*B D H P*	Neoxanthin (Neo)	
[Neo+Vio]	*B D H L P*	Neoxanthin and Violaxanthin (Neo+Viola)	[Neo] + [Viola]
[Phide *a*]	*B D H L*	Phaeophorbide *a* (Phide *a*)	
[Phytin *a*]	*B C D H*	Phaeophytin *a* (Phytin *a*)	
[Pras]	*B D H S*	Prasinoxanthin (Pras)	
[Viola]	*B D H M P S*	Violaxanthin (Viola)	
[Zea+Lut]	*B C D H L M P S*	Zeaxanthin and Lutein (Zea+Lut)	[Zea] + [Lut]
Variable	*Laboratory*	*Ancillary Pigment*	*Calculation*
[DVChl *b*]	*B*	Divinyl chlorophyll *b* (DVChl *b*)	
[Chl c_1]	*H*	Chlorophyll c_1 (Chl c_1)	
[Chl c_2]	*H*	Chlorophyll c_2 (Chl c_2)	
[Anth]	*D*	Antheraxanthin (Anth)	
[Asta]	*B*	Astaxanthin (Asta)	
[Cantha]	*H* §	Canthaxanthin (Cantha)	
[Croco]	*L*	Crocoxanthin (Croco)	
[Phide *c*]	*B*	Phaeophorbide *c* (Phide *c*)	
[Phy c_1]	*L*	Phytollated c_1 (Phy c_1)	
[Phy c_2]	*L*	Phytollated c_2 (Phy c_2)	
[DVPhytin *a*]	*B*	Divinyl Phaeophytin *a* (DVPhytin *a*)	
[Phytin *b*]	*B C*	Phaeophytin *b* (Phytin *b*)	
[DVPhytin *b*]	*B*	Divinyl Phaeophytin *b* (DVPhytin *b*)	
[Pyro *a*]	*P*	Pyro-phaeophytin *a* (Pyro *a*)	

¶ Laboratories *D* and *S* used the Latasa et al. (1996) simultaneous equations to estimate [DVChl *a*].

† Also referred to as α-Carotene.

‡ Also referred to as β-Carotene.

§ Laboratory *S* used Cantha as an internal standard.

Table 5. The (individual) primary pigments, pigment sums, pigment ratios, and pigment indices shown with their variable forms, names, and calculation formulas (if applicable). All methods produced all of these variables, but the *B* method quantitated zeathanthin and lutein together, and not separately, so the data analysis approach was modified accordingly (Sect. 1.5). 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)	[$\beta\beta$-Car] + [$\beta\varepsilon$-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]
[TAcc]	Total Accessory Pigments (TAcc)	[PPC] +[PSC] + [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]/[TChl *a*]	The [TAcc] to [TChl *a*] ratio	[TAcc]/[TChl *a*]
[TChl *a*]/[TPig]	The [TChl *a*] to [TPig] ratio	[TChl *a*]/[TPig]
[PPC]/[TPig]	The [PPC] to [TPig] ratio	[PPC]/[TPig]
[PSC]/[TPig]	The [PSC] to [TPig] ratio	[PSC]/[TPig]
[PSP]/[TPig]	The [PSP] to [TPig] ratio	[PSP]/[TPig]
Variable	*Pigment Index*	*Calculation*
[mPF]	Microplankton Proportion Factor§ (MPF)	$\frac{[\text{Fuco}] + [\text{Peri}]}{[\text{DP}]}$
[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 [$\beta\beta$-Car].

‡ Actually [Zea+Lut] for method *B*, but used as [Zea] because Zea is a primary pigment and Lut is usually a minor contributor.

§ As a group, also considered as *macrovariables*.

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_{T_a} 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 the statistical analysis of the results, are as follows:

C_a Chlorophyll *a*,
C_{S_a} Spectrophotometric determination of C_a (which includes the contribution of all chlorophyll *a*-like pigments),
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_P Prasinoxanthin,
C_V Violaxanthin, and
C_{Z+L} Zeaxanthin plus lutein.

These 12 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.

The pigment composition of the laboratory standards is presented in Table 6. Mixes A and B were very similar and only differed by the presence of Chl *a* in the latter. Mix C was the most representative of a field sample, in terms of pigment diversity, but the concentration levels were rather uniform and usually above the levels found in the open ocean. Mix D provided a good balance between chlorophylls and carotenoids, and most importantly, included Chlide *a*, so $\left[\text{TChl } a\right]$ could be computed. Mix E contained only one pigment and was included so the spectrophotometric determination of $\left[\text{TChl } a\right]$ in field samples could have a laboratory standard counterpart.

Table 6. A summary of the pigment composition for the laboratory standards used in SeaHARRE-2. The solvent given with the absorption coefficients used to measure pigment concentrations is either 100% or 90% acetone (Ace.), for carotenoids and chlorophylls, respectively, or ethanol (Eth.).

Pigment Standard	*Laboratory Standard* Mix A	Mix B	Mix C	Mix D	Mix E
Chl *a*†		Ace.	Ace.	Ace.	Ace.
DVChl *a*†			Ace.	Ace.	
Chlide *a*†				Ace.	
Chl *b*			Ace.		
Chl c_2			Ace.		
Chl c_3			Ace.		
$\beta\beta$-Car			Ace.		
Allo‡	Eth.	Ace.	Ace.		
But-fuco‡			Eth.		
Diad‡	Eth.	Ace.	Ace.		
Diato‡			Ace.		
Fuco‡	Eth.	Ace.	Ace.	Eth.	
Hex-fuco‡	Eth.	Ace.	Ace.	Eth.	
Perid‡	Eth.	Ace.	Ace.	Eth.	
Zea‡	Eth.	Ace.	Ace.		
Lut			Ace.		
Neo			Eth.		
Phide *a*			Ace.		
Pras			Ace.		
Viola			Ace.		
Cantha§			Ace.		

† Taken all together, can be used to determine TChl *a*, a primary (chlorophyll) pigment.
‡ A primary (carotenoid) pigment.
§ An internal standard for some methods (e.g., *S*).

Table 7. A summary of the pigments used in the higher-order variables (pigment sums, ratios, and indices). The number of times the pigments are used in each variable is given by N_T. Note that only the numerator terms for the [mPF], [nPF], and [pPF] indices are shown so their distincitive aspects are made clearer ([DP] appears in the denominator, so the number of pigments for each index is more complicated and exactly the same).

Higher-Order Variable	*Primary Pigments*												
	Chlorophylls			*Carotenoids*									
[TChl]	[TChl *a*]	[TChl *b*]	[TChl *c*]										
[PPC]				[Caro]	[Allo]		[Diad]	[Diato]					[Zea]
[PSC]						[But]			[Fuco]	[Hex]	[Peri]		
[PSP]	[TChl *a*]	[TChl *b*]	[TChl *c*]			[But]			[Fuco]	[Hex]	[Peri]		
[TCaro]†				[Caro]	[Allo]	[But]	[Diad]	[Diato]	[Fuco]	[Hex]	[Peri]	[Zea]	
[TAcc]		[TChl *b*]	[TChl *c*]	[Caro]	[Allo]	[But]	[Diad]	[Diato]	[Fuco]	[Hex]	[Peri]	[Zea]	
[TPig]	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[Allo]	[But]	[Diad]	[Diato]	[Fuco]	[Hex]	[Peri]	[Zea]	
[DP]		[TChl *b*]			[Allo]	[But]			[Fuco]	[Hex]	[Peri]	[Zea]	
[TAcc]/[TChl *a*]	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[Allo]	[But]	[Diad]	[Diato]	[Fuco]	[Hex]	[Peri]	[Zea]	
[PSC]/[TCaro]				[Caro]	[Allo]	[But]	[Diad]	[Diato]	[Fuco]	[Hex]	[Peri]	[Zea]	
[PPC]/[TCaro]				[Caro]	[Allo]	[But]	[Diad]	[Diato]	[Fuco]	[Hex]	[Peri]	[Zea]	
[TChl]/[TCaro]	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[Allo]	[But]	[Diad]	[Diato]	[Fuco]	[Hex]	[Peri]	[Zea]	
[PPC]/[TPig]	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[Allo]	[But]	[Diad]	[Diato]	[Fuco]	[Hex]	[Peri]	[Zea]	
[PSP]/[TPig]	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[Allo]	[But]	[Diad]	[Diato]	[Fuco]	[Hex]	[Peri]	[Zea]	
[TChl *a*]/[TPig]	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[Allo]	[But]	[Diad]	[Diato]	[Fuco]	[Hex]	[Peri]	[Zea]	
[mPF]									[Fuco]		[Peri]		
[nPF]					[Allo]	[But]				[Hex]			
[pPF]		[TChl *b*]											[Zea]
N_T	*8*	*11*	*9*	*11*	*13*	*14*	*11*	*11*	*14*	*14*	*14*	*13*	

† The total concentration of the carotenoids within the primary pigment grouping.

The importance of the laboratory standard mixtures to the higher-order variables (pigment sums, ratios, and indices) is discerned by comparing Tables 6 and 7. The latter shows the individual pigment composition for the higher-order variables (which are presented more compactly in Table 5). From the point of view of the data products, and without placing any importance based on concentration levels, the most recurring carotenoids for the data products discussed here are Allo, But-fuco, Fuco, Hex-fuco, Perid, and Zea. The weighted contribution of the pigments is discussed in Sect. 1.5, but note that the higher-order variables cannot be formed without the contributions of all of the so-called primary pigments.

1.3 LABORATORY METHODS

For SeaHARRE-2, it was always considered 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 methods presented here (Chapts. 2–9) are based on diverse objectives, but are most commonly used with samples from a variety of environmental regimes:

B Based on modifications to the Gieskes and Kraay (1989) method and used with a wide range of sample types (acidification is required if the water temperature during field collection is above about 10°C and if zeaxanthin is present);

C Based on the Wright et al. (1991) method and used predominantly with temperate water samples;

D Based on the Wright et al. (1991) method and used mostly with samples from freshwater estuaries and coastal areas;

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

L Based on the Vidussi et al. (1996) method and used initially with Case-1 (open ocean) samples, but also successfully with Case-2 (coastal) waters;

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

P Based on the Barlow et al. (1997) method and used with samples from a large diversity of water types ranging from estuaries to oligotrophic oceans; and

S Based on the Wright et al. (1991) method and used with a wide range of pigment concentrations from water types throughout the world ocean.

Note there are eight laboratories, but only five methods are completely different (recognizing that the implementation of a common method always results in differences that will distinguish the methods from one another over time).

The basic HPLC process involves the collection and storage of field samples, extraction of the pigments, analysis of the extract, and quantitation of individual pigments. There are additional details within this broad description, however, and the ones explicitly presented here are a) the extraction procedures, b) the separation procedures and solvent systems, c) the injection procedures, and d) the calibration procedures, including the quantitation parameters involved (e.g., absorption coefficients).

It is important to recognize early on that procedural differences in the methods represent a source and a hinderance for understanding the uncertainties or capabilities of the methods. This is a consequence of the complexity of the methods in terms of how many parameters are needed to describe and, thus, compare them. In a complex array of possibilities, there is always the chance that the statistical behavior of a particular result might correlate with a variety of parameters. This correlation might be simply fortuitous—and, therefore, erroneous—and it must be separated from a genuine explanation for the result.

Discerning the underlying explanations for method uncertainties and correlating them with specific methodological procedures is a primary objective of this study. For the methods to be completely accessible to the analytical process (and to the reader), however, a large amount of detail needs to be considered (and presented). This is not so much an apology as a warning: the body of information is very diverse, and it is not possible to present all the desired linkages in one place using one mechanism. Consequently, the information is separated into the basic groups associated with the HPLC process.

1.3.1 Extraction Procedures

Only two laboratories (*B* and *C*) did not use an internal standard. Of the methods that did use an internal standard, *D*, *L*, *M*, and *P* used *trans*-β-apo-8′-carotenal; *H* used vitamin E acetate; and *S* used canthaxanthin. For the purposes of describing extraction procedures, the sample is considered to be the original filter collected in the field and contained within a vessel (usually a glass centrifuge tube). There are two common procedures associated with the use of 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 volume, V_m (typically 3 mL), is added to the sample. In the two-step approach, a volume of the extraction solvent, V_e, is added to the sample (e.g., 3 mL) followed by a small volume of internal standard, V_s (e.g., 50 µL). 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 advantage of using an internal standard is V_x (6) can be computed more precisely by correcting for the presence of residual water retained on the filter paper (plus any variations in volume caused by evaporation) by using a) the peak area of the internal standard when it is injected onto the HPLC column ($\hat{A}_c$) prior to its addition to the sample, and b) the peak area of the internal standard in the sample ($\hat{A}_s$). In the one-step approach, $\hat{A}_c$ is determined by injecting the solvent–standard mixture onto the HPLC column, whereas for the two-step approach, the standard is injected directly onto the column.

For the one-step approach, the internal standard is diluted by the extraction solvent, so (6) is rewritten as

$$C_{P_i} = \frac{\hat{A}_{c_1}}{\hat{A}_{s_1}} \frac{V_m}{V_f} \frac{\tilde{C}_{P_i}}{V_c}, \tag{7}$$

that is, $V_x = V_m \hat{A}_{c_1} / \hat{A}_{s_1}$, where the c_1 and s_1 subscripts indicate the one-step methodolocy. For the two-step approach, (6) is rewritten as

$$C_{P_i} = \frac{\hat{A}_{c_2}}{\hat{A}_{s_2}} \frac{V_s}{V_f} \frac{\tilde{C}_{P_i}}{V_c}, \tag{8}$$

and $V_x = V_s \hat{A}_{c_2} / \hat{A}_{s_2}$ (the c_2 and s_2 subscripts indicate 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 (6) is rewritten as

$$C_{P_i} = \frac{V_e + V_w}{V_f} \frac{\tilde{C}_{P_i}}{V_c}, \tag{9}$$

and $V_x = V_e + V_w$. For a 25 mm filter, water retention is usually assumed to be 0.2 mL (Bidigare et al. 2003).

A summary of the filter extraction procedures is presented in Table 8. All of the methods used acetone as an extraction solvent, except *L* used methanol. Sonic disruption predominated, although one method relied on a mechanical homogenizer (*B*). The soak time for the extract ranged from 0 to more than 24 h, and clarification was an almost equal combination of centrifugation and filtration. For methods using an internal standard, the HPLC column

Table 8. A summary of the filter extraction procedures used by the SeaHARRE-2 laboratories. The extraction solvent entries (in milliliters) specify the volume of solvent added to the sample filter for extraction, and the HPLC column specifies the volume of solvent used in the calculation equations (for B and C) or with laboratories using an internal standard, the CV of the extraction volumes observed, $\bar{\xi}_{V_x}$. The column showing the solvent added describes the solvent composition as it was added to the filter, and the net percentage entries describe the percent organic solvent after adding to the filter (and assuming a constant 0.2 mL of water is contributed by the sample filter). Filter disruption procedures, time allowed for the filter to soak in the solvent, and the clarification process of the filter homogenate are also given.

Lab. Code	*Extraction Solvent Added*	*HPLC*	*Solvent Added*	*Net Percent*	*Mode and Time of Disruption*	*Soak Time* [h]	*Clarification Procedure(s)*
B	1.5 [1]	1.7 ($V_e + V_w$)	90% Acetone	79% Acetone	Teflon homogenizer	*None*	Centrifuge 2 min (12,000 *g*)
C	4.2 [2]	4.0 ± 0.1 [3]	97.5% Acetone	90% Acetone	Sonicating bath 15 min	15–18	Centrifuge and 0.2 µm Teflon syringe filter
D	3.099	$\bar{\xi}_{V_x} = 3.3\%$ [4]	100% Acetone	94% Acetone	Sonicating bath ∼ 10 min	24	0.45 µm Teflon syringe filter
H	3.078	$\bar{\xi}_{V_x} = 1.4\%$ [5]	95% Acetone	89% Acetone	Sonic probe ∼ 60 s	4	0.45 µm Teflon syringe filter
L	3.0	$\bar{\xi}_{V_x} = 4.2\%$ [5]	100% Methanol	94% Methanol	Sonic probe ≤ 10 s	1 [6]	1.3 µm GF/C filter
M	2–6 [7]	$\bar{\xi}_{V_x} = 4.6\%$ [5]	100% Acetone	91–97% Acetone	Sonic probe 30 s	0.5	Centrifuge 10 min (3,500 rpm)
P	2.0	$\bar{\xi}_{V_x} = 3.6\%$ [5]	90% Acetone	82% Acetone	Sonic probe 30 s	1 [6]	Centrifuge 2 min (14,000 rpm)
S	4.0	$\bar{\xi}_{V_x} = 3.8\%$ [8]	100% Acetone	95% Acetone	Sonic probe < 15 s	≥ 24	Centrifuge 4 min (5,100 rpm) [9]

[1] V_e is added in three steps.

[2] The sum of 3.0 mL 100% acetone, 0.2 mL water, and 2×0.5 mL 90% acetone.

[3] The extract volume is measured by observing the meniscus in a graduated tube to verify it is 4.0±0.1 mL; if it is not, the volume measured is recorded for subsequent calculations.

[4] $\bar{\xi}_{V_x}$ is determined from $V_x = V_s \hat{A}_{c_2} / \hat{A}_{s_2}$.

[5] $\bar{\xi}_{V_x}$ is determined from $V_x = V_m \hat{A}_{c_1} / \hat{A}_{s_1}$.

[6] The sum of soaking for 0.5 h, sonicating, and then soaking for another 0.5 h.

[7] Sample dependent: 2.0 mL for samples 1–10, 6.0 mL for sample 11, and 4.0 mL for sample 12.

[8] $\bar{\xi}_{V_x}$ is (incorrectly) determined from $V_x = (V_m + V_w) \hat{A}_{c_1} / \hat{A}_{s_1}$, where $V_w = 0.22$ mL.

[9] Plus a 0.2 µm Teflon membrane filter.

entries in Table 8 give the average coefficient of variation (CV) in extraction volume determinations†. Some variability is expected, because different filters retain varying amounts of water, and evaporation may occur. If evaporation is minimized, and because solvent mixed with internal standard is added quantitatively with calibrated pipets, pigment concentrations can be calculated using the internal standard or ignoring it as if the extraction volume was estimated as solvent volume plus 0.2 mL of water from the filter (as B did). In such a comparison, if the precision in replicate filters is not improved using an internal standard, the internal standard may be a source of variability.

† The coefficient of variation for a set of values is the standard deviation, σ, divided by the average, and expressed in percent. It is denoted in this study by ξ, and is also known as the relative standard deviation (RSD), or the %RSD.

1.3.2 Separation Procedures

Pigment quantitation by HPLC is possible if pigments are first retained by the stationary phase, and then eluted individually (in the mobile phase) as discrete, gaussian-shaped peaks, which exhibit minimal (or no) overlap and whose peak apex (retention time) is unique. Separation occurs because pigments have different properties, e.g., polarity, molecular shape, and degree of ionization. Such differences, in combination with separation conditions, cause pigments to migrate through the column at different average speeds. This is called *differential migration*, and it is affected by the type of mobile phase (or solvent system), the physical characteristics of the stationary phase, column temperature, column re-equilibration time between analyses and, for early eluting pigments especially, injection conditions.

Table 9. A summary of the HPLC separation procedures used by the SeaHARRE-2 laboratories. 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.

Lab. Code	*Stationary Phase*	*Column* P_s	L_c	D_c	T_c	*Detector and Monitoring Wavelength* *Manufacturer† and Model*	λ [nm]
B	C_{18}	3.0	70	4.6	Room	Beckman 168 UV-VIS	430
C	C_{18}	5.0	250	4.6	30°C	Waters PDA 996	436
D	C_{18}	5.0	250	4.6	20°C	Shimadzu SPD-M10A VP-DAD	436, 450, and 665
H	C_8	3.5	150	4.6	60°C	Hewlett-Packard 1100	222‡, 450, and 665
L	C_8	3.0	100	3.0	Room§	Hewlett-Packard 1100	440 and 667
M	C_8	3.0	100	4.6	25°C	ThermoQuest UV6000	440 and 665
P	C_8	3.0	100	4.6	25°C	ThermoQuest UV6000	440
S	C_{18}	5.0	250	4.6	Room¶	ThermoQuest UV6000	436 and 450

† A list of HPLC and related equipment manufacturers is given in Appendix C.
‡ Used for monitoring vitamin E (the internal standard).
§ Approximately 25°C.
¶ Maintained at 18°C by a specialized air conditioner.

Table 10. A summary of the HPLC solvent systems used with the SeaHARRE-2 methods: MeCN is acetonitrile, NH_4Ac is ammonium acetate, EtOAc is ethyl acetate, MeOH is methanol, and TBAA is tetrabutyl ammonium acetate. The flow rate is in units of milliliters per minute.

Lab. Code	*Injection Buffer*	*Flow Rate*	*Mobile Phase Solvent* A	B	C	*Initial Conditions*
B	0.5 M NH_4Ac	1.0	80:20 MeOH:0.5 M NH_4Ac	70:30 MeOH:EtOAc		100% A
C	*None*	1.0	80:20 MeOH:0.5 M NH_4Ac	90:10 MeCN:Water	EtOAc	100% B†
D	Water	1.0	80:20 MeOH:0.5 M NH_4Ac‡	90:10 MeCN:Water	EtOAc	100% A
H	TBAA:MeOH§	1.1	70:30 28 mM TBAA:MeOH	MeOH		95% A:5% B
L	1.0 M NH_4Ac	0.5	70:30 MeOH:0.5 M NH_4Ac	MeOH		80% A:20% B
M	1.0 M NH_4Ac	1.0	70:30 MeOH:1.0 M NH_4Ac	MeOH		75% A:25% B
P	1.0 M NH_4Ac	1.0	70:30 MeOH:1.0 M NH_4Ac	MeOH		75% A:25% B
S	Water	1.0	80:20 MeOH:0.5 M NH_4Ac	90:10 MeCN:Water	EtOAc	100% A

† Changes from 100% A to 100% B, when the injection procedure starts.
‡ With a 7.2 pH.
§ 28 mM TBAA:MeOH in a 90:10 (v:v) mixture, and the TBAA has a 6.5 pH.

A summary of the HPLC column separation procedures and solvent systems used by the SeaHARRE-2 participants are given in Tables 9 and 10, respectively. The type of stationary phase divides the eight laboratories into two equal groups: C_{18} and C_8. Additional distinction can be seen with a) column temperature (*B* and *L* do not control column temperature and *H* uses high temperature control), and b) multiple- versus single-wavelength monitoring systems (*H*, *L*, *M*, and *S* versus *B*, *C*, *D*, and *P*, respectively). Note also the diversity in equipment manufacturers. All methods use an injection buffer, except *C*, and the majority of the methods are two-solvent systems, with *C*, *D*, and *S* using three-solvent systems. The flow rates of the methods are very similar except for laboratory *L*, which has a flow rate that is half as much as the others.

Achieving reproducible retention of pigments that elute as well-resolved, symmetrical peaks requires a fine balance of these many variables. Once the separation and retention of pigments has been described, the consequences of changing any separation variable should be well quantified. Understanding the factors affecting peak area integration is important because peak area is a recurring variable in the calculation equations, (1)–(3) and (7)–(8). Broad, asymmetrical peaks yield poorer results than sharp narrow peaks, in part because the separation between pigments is compromised, and in part because differentiation of the peak beginning and ending from the baseline slope is more difficult to determine.

The separation or resolution, R_s, between peaks is commonly calculated based on peak widths at half-height or at the baseline. For this example, the calculation is based on the baseline:

$$R_s = 2\,\frac{t_{R_2} - t_{R_1}}{\hat{w}_{B_2} + \hat{w}_{B_1}}, \tag{10}$$

where t_{R_1} and t_{R_1} are the retention times of peaks 1 and 2, respectively, and $\hat{w}_{B_1}$ and $\hat{w}_{B_2}$ are the corresponding widths at their bases (Wright 1997).

Table 11. A summary of the pigment coelutions for the SeaHARRE-2 methods. The presentation is arbitrarily ordered to represent the most commonly occurring to the least occuring number of coelutions, in terms of the number of methods involved. The tertiary pigments are shown underlined.

Pigment Coelution(s)	*B*	*C*	*D*	*H*	*L*	*M*	*P*	*S*
Chl c_1 and Chl c_2	✓	✓	✓		✓	✓	✓	✓
DVChl *b* and Chl *b*		✓	✓	✓	✓	✓	✓	✓
Zea and Lut	✓	✓	✓		✓			✓
$\beta\varepsilon$-Car and $\beta\beta$-Car		✓		✓	✓	✓	✓	
Chl $c_{1+}c_2$ and Chlide *a*		✓			✓	✓	✓	
DVChl *a* and Chl *a*		✓	✓					✓
Fuco and Phide *a*			✓			✓	✓	
Hex-fuco and Pras					✓	✓	✓	
Diadino (isomer) and Allo				✓	✓			
Neo and Viola						✓	✓	
Chl c_3 and Chlide *a*			✓					
Chl c_1 and Chlide *a*				✓				
DVChl *a* (epimer) and Chl *a*				✓				
But-fuco (isomer) and Hex-fuco				✓				
Fuco (isomer) and Diadino				✓				
Perid and Phide *a*					✓			
Neo, Viola, and Hex-fuco					✓			
Pras and Cis-Hex								✓

For quantitative analysis, the key pigments must be distinguishable from their nearest eluting neighbors (described by separation selectivity), and they must also be *adequately* separated. The latter requires the peaks to be narrow enough to ensure $R_s \geq 1$ (Bidigare et al. 2003). Table 11 presents a summary of the pigment coelutions with an emphasis on the primary pigments (or individual pigments that are summed to form a primary pigment) for which $R_s < 1$ or for which another type of separation problem was determined important. A summary of the most pigments that coelute is presented in Table 11. The majority of the coelution problems are between primary, secondary, and ancillary pigments, but many involve tertiary pigments (Tables 4 and 5).

All laboratories used reversed-phase gradient HPLC methods. In such methods, analytes with a weak attraction to the stationary phase elute earlier than those having a strong affinity for the stationary phase. Pigments poorly retained by the column are, therefore, easily eluted with a weak mobile phase (containing a high proportion of solvent A). In contrast, late-eluting pigments are considered well-retained and require a strong solvent (solvent B or C) to elute from the column. Usually, C_{18} phases and cool column temperatures favor stronger pigment retention than C_8 columns and high temperatures. In both cases, retention depends on the strength of the injection solvent and mobile phase at initial conditions.

Solvent strength is described by the so-called *polarity index* (PI) and denoted, P_I. The higher the P_I value, the weaker, or more polar, the solvent in relation to the type of stationary phase being used. When a mobile phase is composed of more than one solvent, P_I is calculated as in the following example for a 70:30, methanol:buffer solution (Bidlingmeyer 1992):

$$\begin{aligned} P_I &= (0.70 \times 6.6) + (0.30 \times 9.0) \\ &= 7.3, \end{aligned} \tag{11}$$

where 6.6 is the PI of pure methanol, and 9.0 is the PI of water.

1.3.3 Injection Procedures

A summary of the HPLC injection procedures used by the participating laboratories is given in Table 12. With the exception of *B*, which used manual injection, all of the laboratories used an automated injection procedure. For the latter group, the primary distinction was that laboratory *C* did not mix the sample extract and buffer, and *H* was the only method that mixed them in the sample loop. For the laboratories that used automated injectors, the vials of sample extract were placed in the TCAS compartment (generally at 4°C) where they resided for as short as 7 min and as long as 24 h before being analyzed (the maximum residence time of laboratory *C* was 12 h).

Table 12. A summary of injection procedures used with the SeaHARRE-2 methods. TCAS indicates the temperature of the injector auto-sampler compartment where samples reside before injection; sample to buffer ratio indicates the volume of sample extract added to the volume of buffer (if used) prior to injection; and sample load indicates the volume of sample extract actually loaded onto the column. The polarity index is given for a) the HPLC solvent at the initial conditions (when the injection is made), b) the field sample extract, c) standards suspended in ethanol, and d) standards suspended in 90% acetone. Polarity indices for the latter three were determined according to the corresponding sample-to-buffer ratio.

Laboratory Code	*TCAS Temp.* [°C]	*Sample to Buffer Ratio*	*Injection Volume* [µL]	*Sample Load* [µL]	*Polarity Index*			
					HPLC Solvent	*Field Sample*	*Standard in Ethanol*	*Standard in 90% Acetone*
B	*N/A*	0.5:1	250	83.3	7.1	8.1	7.7	7.9
C	6–7	90% Ace.	200	200.0	7.1	5.8	5.2	5.8
D	4	3.35:1	300	231.0	7.1	6.4	6.1	6.5
H	5	0.4:1	525	150.0	7.3	8.1	7.9	8.1
L	4–6	2:1	200	133.0	7.2	7.5	6.5	6.9
M	2	1:1	100	50.0	7.1	7.3	7.1	7.4
P	0	1:1	100	50.0	7.1	7.5	7.1	7.4
S	4	3.1:1	100	75.6	7.1	6.4	6.1	6.6

With the exception of laboratory C, the injectors were programmed to mix sample (or standard) with buffer (or water) immediately before injection. Laboratories L, M, P, and S performed the mixing steps as follows:

1. Empty vials, vials (or bottles) containing buffer, and vials containing sample extract were placed in the TCAS compartment.
2. The autoinjector withdrew a specified volume of buffer (from the buffer container) plus sample extract (from the specified sample vial), and expelled the combined contents into an empty vial, where mixing was performed.
3. Then the autoinjector withdrew a volume from the mixing vial for injection onto the column.

A similar approach was used by H, but instead of mixing the buffer and sample in a separate, empty vial, the buffer and sample were drawin into the sample loop, and the entire volume was injected onto the column.

Laboratory D manually filled vials with 1 mL of sample extract (using a calibrated pipette) and then programmed the autoinjector to withdraw buffer (from a buffer vial) and expel the contents into a specified vial containing sample, mix the contents, and then withdraw a specified volume for injection. Laboratory B performed these mixing steps manually with calibrated pipettes and injected 250 µL through a 500 µL sample loop. Laboratory C programmed the autoinjector to inject samples and standards directly without modification.

The injection solutions (and large injection volumes) used with pigment analyses create changes in the composition of the initial mobile phase such that pigment retention and peak shapes are different from what would occur relative to an ideal scenario: when the injection solvent matches the initial mobile phase and small injection volumes are used (such as 25 µL or less). Because of the great diversity in pigment polarity and frequent low concentrations, extreme injection conditions are necessary to effect retention of the earliest eluting pigments, such as chlorophyll *c* compounds, chlorophyllide *a*, and early eluting carotenoids.

Poor retention or peak distortion can occur if the solvent in which pigments are suspended has a low polarity index relative to the initial mobile phase. It is, therefore, necessary to adjust the strong (extraction or suspension) solvent (with buffer or water), so the P_I of the resulting injection solution either exceeds, or is roughly equal to, the P_I of the initial mobile phase. This effect on retention and peak distortion is exacerbated when injection volumes are large relative to the initial mobile phase flow rate. Injection conditions are given in Table 12. Included is the polarity index of the initial mobile phase and the injection solution when field sample extracts and standards (either suspended in 90% acetone or ethanol) are injected.

1.3.4 Calibration Procedures

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

$$A(\lambda) \;=\; a(\lambda)\, l_c\, C, \tag{12}$$

where $A(\lambda)$ is absorbance, $a(\lambda)$ is the absorption coefficient (a constant), l_c is the thickness of the sample in centimeters

(the pathlength of the cuvette being used), and C is concentration. To determine concentration from a measured absorbance, (12) is rewritten as

$$C = \frac{A(\lambda)}{a(\lambda)\, l_c}, \tag{13}$$

where the units for C depend on the expression of $a(\lambda)$. For example, for consistency with current suggestions regarding absorptivity (Sect. 1.1), if the concentration is expressed in molarity, a becomes the molar absorption coefficient (ε) and if the concentration is expressed as grams per liter, a is the specific absorption coefficient (α). If concentration is expressed in percent weight per volume (usually in units of grams per 100 mL), a becomes $a_{1\%}$ (Segel 1968). Usually, a 1 cm pathlength is used, so $l_c = 1$ cm in most cases. Absorption coefficients vary depending on wavelength and the solvent in which the compound is suspended, and they are always provided with the solvent and wavelength used.

For determining the concentrations of pigment standards, the wavelength of maximum absorbance for the particular pigment (which is specified for use with the absorption coefficient), is corrected by the absorbance of that pigment at 750 nm:

$$C_{P_i} = \frac{A(\lambda) - A(750)}{a(\lambda)\, l_c}, \tag{14}$$

where λ is the corresponding wavelength of maximum absorbance.

Primary pigment standards used by participants to calibrate their HPLC systems were either a) purchased from DHI in solution (with concentrations provided by DHI), b) isolated from natural sources, or c) purchased in solid form. In the latter two cases, pigments were suspended in the solvents specified for use with the absorption coefficients selected by the laboratory involved, absorbance was measured spectrophotometrically at the wavelength specified with the selected absorption coefficient, and the concentrations were computed using

$$C_{P_i} = \frac{A(\lambda) - A(750)}{\alpha(\lambda)\, l_c}. \tag{15}$$

Laboratories B, L, M, and P purchased several standards from DHI, but analyzed these standards spectrophotometrically, and used the observed concentrations rather than using the concentrations provided with the standards by DHI. The difference in concentrations provided by DHI and observed by B, L, M, and P are presented in Table 13. Although many of the differences in Table 13 are small (approximately ±5% or less), laboratory M is a notable exception. When remeasuring the concentrations of the DHI standards, laboratory M recorded absorbance at the observed wavelength maximum rather than using the wavelength published for use (although these values were very similar), and then calculated concentrations using absorption coefficients which, in many cases, differed from those originally used by DHI.

Table 13. The relative percent difference (RPD) in primary pigment concentrations observed spectrophotometrically for DHI standards. The RPD values are expressed in percent and computed relative to the concentrations provided by DHI. All the comparisons are based on absorption coefficients used by DHI.

Pigment	*B*	*L*	*M*	*P*
Chl *a*		0.7		−5.0
DVChl *a*	−14.7		−0.5	4.9
Chl *b*		−12.1	1.0	5.7
Chl c_2			14.0	17.2
Chl c_3			−21.9	0.2
Phytin *a*				6.7
But-fuco		3.1	−15.0	2.8
Fuco		0.1	−25.6	0.2
Hex-fuco	−10.9	1.0	−21.4	−0.4
Perid		0.5	−15.0	2.2
Diad			−30.2	1.1
Allo		−1.8	−27.1	−4.6
Diato			−20.4	3.7
Lut			−32.0	−0.9
Zea	3.0	0.3	−23.3	3.9
$\beta\beta$-Car			−20.8	1.3
Neo				3.9
Pras				−0.6
Viola			−31.7	−5.2

A summary of the absorption coefficients used to determine the concentrations of carotenoid standards is given in Table 14, and a summary of absorption coefficients used with the chlorophyll family of pigments is given in Table 15. The tables show a wide diversity of absorption coefficients (in particular, laboratory C for all pigments and laboratory M for carotenoids), although some pigments and laboratories were frequently the same (e.g., D and S). It should be noted that laboratory C, which used many absorption coefficients based on dissolution of the pigment in 100% acetone, adjusted the standard (9:1) with water so the resulting injection solvent was 90% acetone. This was done to eliminate abnormal peak shapes caused by the injection of 100% acetone solutions. Such adjustments were performed using calibrated pipettes.

The HPLC calibration procedures used by each laboratory are given in Table 16. In many cases, the primary pigment standards were used to generate single-point response factors, in which case R (1) was taken as the average observed for the number of injections involved. In other cases, a dilution series was created, so the pigment calibration factors were based on multipoint linear regression statistics (2). Single-point response factors were used for

Table 14. A summary of the $\alpha(\lambda)$ values used for determining the concentrations of carotenoid standards as a function of the laboratories. The three entries for each corresponds to the absorption coefficient (top line), the solvent (middle line), and the specified wavelength (bottom line). Blank entries indicate the laboratory involved did not calibrate the indicated pigment. Absorption coefficients and solvents that are different than the ones being used by the majority of the laboratories—so-called "uncommon" values—are shown in bold typeface, and any qualification for the indicated pigment is given in slanted typeface. With respect to the latter, Zea and Lut coelute for the *B* method.

Pigment	*B*	*C*	*D*	*H*	*L*	*M*	*P*	*S*
$\beta\varepsilon$-Car	**262.0** **Ethanol** 446 nm	270.0 100% Ace.† 448 nm	270 100% Ace. 448 nm		270 **Ethanol** 448 nm			270.0 100% Ace. 448 nm
$\beta\beta$-Car	262.0 Ethanol 452 nm	**250.0** **100% Ace.** 454 nm	262.0 Ethanol 453 nm	262.0 Ethanol 453 nm	**250.0** **100%Ace.** 454 nm	262.0 Ethanol 452 nm	262.0 Ethanol 453 nm	262.0 Ethanol 453 nm
Allo	262.0 Ethanol 454 nm	**250.0** **100% Ace.** 454.5 nm	262.0 Ethanol 453 nm	262.0 Ethanol 453 nm	262.0 Ethanol 453 nm	262.0 Ethanol 454 nm	262.0 Ethanol 453 nm	262.0 Ethanol 453 nm
But-fuco	160.0 Ethanol 446 nm	**147.0** **100% Ace.** 445 nm	160.0 Ethanol 446 nm	160.0 Ethanol 447 nm	160.0 Ethanol 446 nm	**134.9** Ethanol 450 nm	160.0 Ethanol 446 nm	160.0 Ethanol 446 nm
Diadino	262.0 Ethanol 448 nm	**223.0** **100% Ace.** 448 nm	262.0 Ethanol 446 nm	262.0 Ethanol 446 nm	**223.0** **100% Ace.** 447.5 nm	**241.3** Ethanol 448 nm	262.0 Ethanol 446 nm	262.0 Ethanol 446 nm
Diato	*Same* *R_f as* *Diadino*	**210.0** **100% Ace.** 452 nm	262.0 Ethanol 449 nm	262.0 Ethanol 449 nm	**210.0** **100% Ace.** 452 nm	**248.1** Ethanol 452 nm	262.0 Ethanol 449 nm	262.0 Ethanol 449 nm
Fuco	**150.7** **100% Ace.** 446 nm	**166.0** **100% Ace.** 447 nm	160.0 Ethanol 449 nm	160.0 Ethanol 449 nm	160.0 Ethanol 449 nm	**152.5** Ethanol 452 nm	160.0 Ethanol 449 nm	160.0 Ethanol 449 nm
Hex-fuco	160.0 Ethanol 445 nm	**142.0** **100% Ace.** 445 nm	160.0 Ethanol 447 nm	160.0 Ethanol 447 nm	160.0 Ethanol 447 nm	**130.0** Ethanol 448 nm	160.0 Ethanol 447 nm	160.0 Ethanol 447 nm
Perid	**134.0** **100% Ace.** 468 nm	**134.0** **100% Ace.** 466 nm	132.5 Ethanol 472 nm	132.5 Ethanol 472 nm	132.5 Ethanol 472 nm	132.5 Ethanol 475 nm	132.5 Ethanol 472 nm	132.5 Ethanol 472 nm
Zea	*254.0* *Ethanol* *450 nm*	**234.0** **100% Ace.** 454 nm	254.0 Ethanol 450 nm	254.0 Ethanol 450 nm	254.0 Ethanol 450 nm	254.0 Ethanol 453 nm	254.0 Ethanol 450 nm	254.0 Ethanol 450 nm
Lut		255.0 **100% Ace.** 447.0 nm	255.0 Ethanol 445 nm	255.0 Ethanol 445 nm	**248.0** **D. Ether**‡ 445 nm	255.0 Ethanol 447 nm	255.0 Ethanol 445 nm	255.0 Ethanol 445 nm
Neo	227.0 Ethanol 438 nm	227.0 **100% Ace.** 438 nm	**224.3** Ethanol 439 nm	227.0 Ethanol 438 nm	227.0 Ethanol 438 nm		**224.3** Ethanol 439 nm	**224.3** Ethanol 439 nm
Pras	160.0 Ethanol 454 nm	**250.0** **100% Ace.** 450 nm	160.0 Ethanol 454 nm	160.0 Ethanol 454 nm	**250.0** **D. Ether**‡ 446 nm		160.0 Ethanol 454 nm	160.0 Ethanol 454 nm
Viola	*Same* *R_f as* *Neo*	**240.0** **100% Ace.** 442 nm	255.0 Ethanol 443 nm	255.0 Ethanol 443 nm	240.0 **100% Ace.** 442 nm	255.0 Ethanol 443 nm	255.0 Ethanol 443 nm	255.0 Ethanol 443 nm

† Acetone ‡ Diethyl Ether

Table 15. A summary of the $\alpha(\lambda)$ values used for determining the standards concentrations of chlorophyll and its associated pigments (except as noted) following the presentation scheme established in Table 14. The DVChl *a* entries for the *D* and *S* methods are for use with the Latasa et al. (1996) simultaneous equations, and are not used as a part of chromatographic separation, so they are shown in slanted typeface. The Chl c_1 and Chl c_2 entries for method *D* are averaged to provide the results for Chl $c_{1+}c_2$ (method *D* does not chromatographically separate Chl c_1 and Chl c_2, so they are shown in slanted typeface). The *H* method is the only one to chromatographically separate Chl c_1 and Chl c_2, and *B* is the only method to chromatographically separate the monovinyl and divinyl forms of chlorophyll *b*. Note that the *B* method calculates Phide *a* and Phytin *a* as chlorophyll equivalents. Blank entries indicate the laboratory involved did not calibrate the indicated pigment.

Pigment	*B*	*C*	*D*	*H*	*L*	*M*	*P*	*S*
MVChl *a*	87.67 90% Ace. 664 nm	**88.15** **100% Ace.** 665 nm	87.67 90% Ace. 664.3 nm	87.67 90% Ace. 664.3 nm	87.67 90% Ace. 664.3 nm	**88.15** **100% Ace.** 663 nm	87.67 90% Ace. 664.3 nm	87.67 90% Ace. 663.5 nm
DVChl *a*	87.67 90% Ace. 664.3 nm		*87.67* *90% Ace.* *664.3 nm*	**88.15** **100% Ace.** 663 nm	**74.2** 90% Ace. 664 nm	87.87 90% Ace. 664 nm	87.67 90% Ace. 664.3 nm	*87.67* *90% Ace.* *664.3 nm*
Chlide *a*	*Same* *as* *MVChl* a	127.0 **100% Ace.** 664 nm	127.0 90% Ace. 664 nm	*Same* *as* *MVChl* a†	127.0 90% Ace. 664 nm	127.0 90% Ace. 664 nm	*Same* *as* *MVChl* a	127.0 90% Ace. 664 nm
Phide *a*	*Calc.* *as Chl.* *Equiv.*		74.2 90% Ace. 667 nm	*46,000*‡	74.2 90% Ace. 667 nm			
Phytin *a*	*Calc.* *as Chl.* *Equiv.*	51.2 90% Ace. 667 nm	51.2 90% Ace. 667 nm	*46,000*§ *100% Ace.* *665.9 nm*			51.2 90% Ace. 667 nm	51.2 90% Ace. 667 nm
MVChl *b*	51.4 90% Ace. 646 nm	51.36 **100% Ace.** 646 nm	51.36 90% Ace. 646.8 nm	51.36 90% Ace. 646.8 nm	51.36 90% Ace. 646.8 nm	51.36 90% Ace. 645 nm	51.36 90% Ace. 646.8 nm	51.36 90% Ace. 646 nm
DVChl *b*	51.4 90% Ace. 650.0 nm				28.03 90% Ace. 646.8 nm			
Chl c_1				44.8 90% Ace. 631 nm				
Chl c_2			*374.0* *90% Ace.* *443.8 nm*	40.4 90% Ace. 631 nm			*346.0* 90% Ace. 452.9 nm	
Chl $c_{1+}c_2$	**38.2** **100% Ace.** 630 nm	**37.2** **100% Ace.** 628.9 nm	*Average* *of Chl* c_1 *and Chl* c_2		**42.6** 90% Ace. 630.6 nm	40.4 90% Ace. 630.9 nm	40.4 90% Ace. 630.9 nm	40.4 90% Ace. 630.9 nm
Chl c_3	*Same* *as* *Chl* $c_{1+}c_2$	**334.5** **100% Ace.** 453 nm	346.0 90% Ace. 452.9 nm	*Average* *of Chl* c_1 *and Chl* c_2	**42.6** 90% Ace. 630.6 nm	346.0 90% Ace. 453 nm	346.0 90% Ace. 452.9 nm	346.0 90% Ace. 452.9 nm

† Then corrected for the molecular weight of chlorophyllide *a*.

‡ The molar absorption coefficient of phaeophytin *a*; the value is then corrected for the molecular weight of phaeophorbide *a*.

§ The molar absorption coefficient.

Table 16. A summary of calibration procedures used with the SeaHARRE-2 methods. Results for dilution devices indicate set point volume, the RPD of gravimetrically observed volumes with respect to the set point volume, and the CV of replicate observations (in units of percent).

Lab. Code	*Chlorophyll* a *Calibration*	*y-intercept* [ng/injection]	*Injection Carry Over*	*Dilution Devices for Standards Calibrated with Organic Solvent*		
B	Single Point (6 injections)	*N/A*	None	300 µL 250 µL 150 µL	0.67 0.74 0.86	0.28 0.28 0.50
C	Dilution Series ($r^2 = 0.999$)	Forced Through 0	None	1.000 mL 0.100 mL	2.22 −12.40	0.56 2.85
D	Single Point (4 injections)	*N/A*	None	*No Dilutions*		
H	Dilution Series ($r^2 > 0.999$)	0.06	0.85%	10 mL 100 µL 500 µL	0.14 −1.00 0.60	0.01 0.36 0.15
L	Dilution Series ($r^2 > 0.999$)	1.3	None	250 µL 500 µL	0.80 2.40	0.34 0.65
M	Dilution Series† $r^2 > 0.999$)	Forced Through 0	None	2 mL	0.35	0.60
P	Single Point (3 injections)	*N/A*	None	*No Dilutions*		
S	Dilution Series ($r^2 = 0.999$)	Forced Through 0	<0.3% Area Carryover‡	1.000 mL 0.100 mL	2.10 1.00	0.73 0.86

† Dilution series also for *trans*-β-apo-8′-carotenal (the internal standard).

‡ For areas greater than 600,000 mAU.

all pigments by laboratories D and P (and for Chl a by B), for all pigments except Chl a by H, and all pigments except Chl a and the internal standard, by M (which quantified the internal standard by weight). Multipoint calibration curves were used by L, C, and S to quantitate Chl a (and carotenoids by L and S). If a simultaneous equation is used for coeluting pigments, calibration variables do not permit a y-intercept, so they are computed from single-point response factors or slopes forced through $y = 0$.

For SeaHARRE-2, several of the laboratories relied on simultaneous equations for pigment quantitation:

D [DVChl a] and [Chl a],

H [Chl c_1] and [Chlide a],

M [Chl $c_{1+}c_2$] and [Chlide a], and

S [DVChl a] and [Chl a].

Quality assurance measures applied by all laboratories included using gravimetrically-calibrating measuring devices for diluting standards, adding internal standard to filters, or adding sample extract to the HPLC vials. Such gravimetric calibrations were conducted with 100% acetone or with methanol (L). Although individual CV estimates for gravimetric calibration of dilution devices are presented in Table 16, a more useful parameter for summarizing the importance of this type of precision to an individual method is the average CV, which is denoted $\bar{\xi}_{\text{cal}}$. Cross-contamination between the analysis of samples (or standards) and blanks or internal standards was not observed, but in the case of H and S, carryover was quantified.

1.3.5 Spectrophotometric Methods

After completing all HPLC analyses of the field sample extracts, the remainder of each filter extract was subjected to spectrophotometric analysis by five of the laboratories (Table 3). The objective of this part of the work plan was to determine the total chlorophyll a concentration (in units of micrograms per liter) using the trichromatic equation of Jeffrey and Humphrey (1975):

$$C_{Sa} = 11.85\,\tilde{A}(664) - 1.54\,\tilde{A}(647) - 0.08\,\tilde{A}(630), \tag{16}$$

where the corrected absorbance values ($\tilde{A}$) at 664, 647, and 630 nm include a 750 nm turbidity correction, that is, $\tilde{A}(664) = A(664) - A(750)$. The trichromatic equation (16) was developed for use with sample extracts in 90% acetone. Although L performed the spectrophotometric measurements, their field samples were extracted in methanol.

The spectrophotometers had bandwidths that were no greater than 2 nm, and were first *zeroed* with a 90% acetone blank (the readings from the blank were subtracted

from all subsequent readings). The extracts were brought to room temperature before the analysis began, and the filter extracts were not diluted or altered prior to spectrophotometric analysis (except laboratory C diluted the sample if the absorbance was greater than 0.6 and reported these results in addition to the undiluted results).

1.4 DATA ANALYSIS METHODS

This study uses laboratory and field samples. Although the former were distributed with a known concentration (but unknown to the analysts at the time of HPLC analysis), the latter—by definition—have 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 for each laboratory for each batch of replicates (which constituted a sample), even though multiple (2 or 3) replicates were analyzed by each laboratory within each batch. To ensure a consistency in reporting, all values were converted to concentrations of milligrams per cubic meter. The solitary (or sample) concentrations were the averages of the individual replicates analyzed for each batch.

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 for each pigment from each sample as a function of the eight contributing laboratories:

$$\bar{C}_{P_i}^{L_j}(S_k) \;=\; \frac{1}{N_R}\sum_{l=1}^{N_R} C_{P_i}^{L_j}(S_{k,l}), \tag{17}$$

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 station ID and replicate number, using the k index for the former (following Table 1) and l for the latter ($l = 1$, 2, or 3 for M, and $l = 1$ or 2 for all other laboratories); and N_R is the total number of replicates (3 for M and 2 for all others).

In (17), the i index represents an arbitrary ordering of the pigments, and the j index is used for summing over the eight laboratory (or method) codes. Although any ordering for the pigments and laboratories is permissible, the former are ordered following their presentation in Table 2; for the latter, $j = 1, 2, \ldots, 8$ corresponds to the B, C, D, H, L, M, P, and S laboratories, respectively (which is based on a simple alphabetic ordering of the one-letter codes).

Only one value for each pigment is reported for each station, and this is generically referred to as a "sample," so the number of samples equals the number of stations. This was done to deal with the fact that M received triplicate filters for each batch, but all the other laboratories received duplicates, and to mimic the usual practice of having only one realization of a pigment per station (understanding that in most field campaigns, the charaterization of a station usually includes data from different depths, but, again, each depth is usually characterized with a solitary value).

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):

$$\bar{C}_{P_i}^{A}(S_k) \;=\; \frac{1}{N_L}\sum_{j=1}^{N_L} \bar{C}_{P_i}^{L_j}(S_k), \tag{18}$$

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

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

$$\psi_{P_i}^{L_j}(S_k) \;=\; 100\,\frac{C_{P_i}^{L_j}(S_k) \;-\; \bar{C}_{P_i}^{A}(S_k)}{\bar{C}_{P_i}^{A}(S_k)}. \tag{19}$$

Note that the formulation in (19) provides an 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:

$$|\bar{\psi}|_{P_i}^{L_j} \;=\; \frac{1}{N_S}\sum_{k=1}^{N_S} \left|\psi_{P_i}^{L_j}(S_k)\right|, \tag{20}$$

where S_k is the k^{th} station ID number (Table 1) associated with pigment P_i. For the analysis of field samples, $N_S = 12$, but when the analysis is extended to laboratory standards, N_S is less and depends on how the standards are combined (if at all).

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 values, so it is important to ensure they are not underestimated.

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

$$|\bar{\psi}|^{A}_{P_i} = \frac{1}{N_L}\sum_{j=1}^{N_L}|\bar{\psi}|^{L_j}_{P_i}, \tag{21}$$

where the A code indicates all the laboratories were averaged (and $\bar{\psi}^{A}_{P_i}$ values are formed in a similar fashion from the UPD values). In general, (21) is only computed for the primary pigments, so $N_L = 8$.

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

$$\xi^{L_j}_{P_i}(S_k) = 100\,\frac{\sigma^{L_j}_{P_i}(S_k)}{\bar{C}^{L_j}_{P_i}(S_k)}, \tag{22}$$

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

$$\bar{\xi}^{L_j}_{P_i} = \frac{1}{N}\sum_{k=1}^{N_S}\xi^{L_j}_{P_i}(S_k). \tag{23}$$

The formulations presented in (17)–(23) 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.

1.4.1 Coelution and Quantitation

Based on the documentation provided by the HPLC analysts prior to the analysis of the SeaHARRE-2 data set, several of the methods had coelution or quantitation problems associated with some primary pigments:

- *B* Zea coelutes with Lut, so [Zea] is not available chromatographically separated;
- *D* DVChl *a* is not chromatographically separated, so the Latasa et al. (1996) simultaneous equations are used;
- *L* [Caro], which requires both $\beta\varepsilon$-Car and $\beta\beta$-Car, is based only on the quantitation of the latter;
- *P* [Caro], which requires both $\beta\varepsilon$-Car and $\beta\beta$-Car, is based only on the quantitation of the latter; and
- *S* DVChl *a* is not chromatographically separated, so the Latasa et al. (1996) simultaneous equations are used.

In these cases, the average concentrations for the affected pigments were determined only with the results provided by methods with no coelution or quantitation problems, e.g., the *B* results for [Zea+Lut] were excluded in the determination of the average concencentration for [Zea].

The aforementioned pigments are sufficiently important, however, that their values cannot be completely ignored. The value of [Zea], for example, is needed in many higher-order associations or variables (Table 7). Consequently, these slightly compromised concentrations were used in all subsequent determinations of uncertainties for the individual methods and averages. Whenever the most representative statistical value was needed, the slightly compromised methods were ignored, but these cases are highlighted and indicated in the analytical descriptions or tables.

In most cases, these irregularities are ultimately rather insignificant, but there are aspects of the results where they are not, so some appreciation of the methodologicial differences is needed. For example, Lut frequently occurs at a much lower concentration than Zea, so once Zea is summed into higher-order variables, the contamination from Lut is usually very minor.

1.4.2 Specificity Problems

Specificity refers to the ability to accurately determine the concentration of a pigment in the presence of other sample components (e.g., other pigments, impurities, or degradation products), which are expected to interfere with the identification and quantitation of the pigment. Coelution is the most common form of a specificity problem, and it occurs over a wide range of severity if all pigments and methods are considered. In terms of the effect on the uncertainty budget, the most significant specificity problem usually occurs when a pigment that is not present is identified and quantitated, or a pigment that is present in a sample is not detected (this usually only occurs for the minor so-called tertiary pigments, but not always). The former is referred to here as a *false positive*, and the latter as a *false negative*.

Although a false negative can be associated with a detection threshold not being satisfied, it can also be because the pigment was not properly identified and an incorrect peak was quantitated. The latter is also a frequent explanation for false positives. None of the SeaHARRE-2 laboratories submitted any information regarding the detection thresholds for the reported pigments, so the specificity problems are initially assumed to be the result of incorrect peak identification and inappropriate quantitation.

The methods that had significant specificity problems and the pigments involved are as follows (the type of specificity problem is shown in parentheses):

- *C* [Phytin *a*] (false negatives),
- *D* [DVChl *a*] (false positives),

L [Phide a] (false negatives),

M [Viola] (false negatives),

P [Neo] (false negatives), and

S [DVChl a] (false positives).

Note that the P specificity problem with [Neo] also affected the [Neo+Vio] results.

When specificity failures occur, there are some rather significant consequences to some of the statistical parameters that need to be appropriately considered. For example, if a pigment is persistently and incorrectly not detected, the precision of the method for that pigment will be artificially low (to within 0%), and if the persistently low precision values are included in the average concentration for that pigment, the average will be significantly biased low. In the approach adopted here, any time a pigment was not routinely detected by a method when other methods did detect it, the false negative results were not included in the calculation of the average pigment concentration. Furthermore, the results were not used in determining the precision of the method for that pigment, or in the overall precision.

It is important to remember that if a pigment is persistently and correctly not detected, the precision of the method for that pigment will be to within 0%. This will not produce an anamolous precision result, because the other methods should also be indicating a nondetection for that pigment, and they too will have a precision to within 0%. The only time a problem will occur is if one of the methods reports a false positive. In the approach adopted here, any time a pigment was routinely detected by a method when other methods did not detect it, the false positive results were not included in the calculation of the average pigment concentration. Furthermore, the results were not used in determining the precision of the method for that pigment, or in the overall precision.

Note that the concepts of false positives and false negatives are only possible with intercomparison exercises or with laboratory standards. In the absence of having a value (or proxy) for truth to compare a reported value with, an individual laboratory cannot determine if it is incorrectly reporting a pigment that is not present, or wrongly indicating a pigment is not present when it actually is. The only way an individual laboratory can maintain proper selfconsistency is to know the concentration level for which the uncertainties in the method start to create the possibility of erroneously reporting the presence or absence of a pigment. This threshold is usually quantified by determining the detectability limits of the entire method, i.e., the skill of the analyst, the capabilities of the hardware, the calibration of any labware, etc. Two common thresholds analysts rely on are the limit of detection (LOD) and the limit of quantitation (LOQ). Both are used in this study as part of a quality assurance investigation into minimizing uncertainties.

1.4.3 Quality Assurance

Although each participating SeaHARRE-2 laboraotory should have reported their pigment concentrations along with an LOD or LOQ analysis, no laboratory actually did this—in fact, most laboratories providing marine pigment analysis do not normally report such information. To ensure consistency in reporting during SeaHARRE-2, any *no detection* (null) results were replaced with a constant value of $0.0005\,\mathrm{mg\,m^{-3}}$. This value is not a proxy for the value that would be assigned from a proper detection or quantitation analysis, and was only used to minimize the statistical consequences of having absolute zeroes in the full data matrix.

A more complete inquiry into specificity problems requires more sophisticated detection concepts than simply accepting null results and replacing them with an agreed upon (but largely arbitrary) constant. There are a variety of procedures that can be used to estimate detection limits, e.g., the mimimum detection limit (MDL) documented by Bidigare et al. (2003), which is based on the precision associated with the analysis of replicate filters. The resulting MDL values are variable, however, because they depend on the concentrations of the pigments in the sample filters used for the analysis. The philosophy adopted here is to quantify the limitations of the various methods in terms of a common detection principle, the signal-to-noise ratio (SNR), but to use a procedure that is easy to implement and a part of common practices. The latter is particularly important, because the most important aspect of advocating a new procedure is to make sure analysts will routinely use it and report it.

The process is based on determining the amount (in nanograms) of an injected pigment that fulfills a specified SNR. The amount of pigment that results in an SNR of 3 is defined here as the LOD and the amount of pigment that results in an SNR of 10 is defined as the LOQ. After a more detailed discussion of the use of the LOD and LOQ as QA thresholds, the basic steps required to quantify the LOD and LOQ values are presented in the subsequent sections. The steps include a) estimating the noise, b) measuring the signal (or peak height), and c) determining the nanograms of pigment that result in SNR values of 3 and 10.

1.4.3.1 QA Thresholds

The list of pigments an HPLC method is capable of quantifying is an integral part of properly describing the method. When the method is applied to samples of unknown content and concentration, pigments capable of being quantified, but not actually found in the samples, still need to be correctly reported. If a quantifiable pigment is not detected, most HPLC analysts assign the pigment a concentration value of zero or no value at all (i.e., the spreadsheet cell corresponding to the pigment is left blank). These null concentrations are a ubiquitous feature of the

data given to the end user, and the reporting of zero or blank values is referred to hereafter as *null reporting*.

Although alternative reporting schemes exist, most results are not screened, regardless of the concentration of the individual pigments and whether or not the values were acquired close to an established or estimated detection limit. In this method of reporting, values insignificantly different from zero cannot be distinguished from quantifiably meaningful values, so the net results are not significantly different from those achieved with null reporting.

The consequences of omitting a quality assurance step in the reporting process is considered here by comparing uncertainties in replicate filter results produced with a null reporting approach to the results achieved after applying three different detection thresholds:

1. Zero or blank values are replaced with a *limiting value* or threshold,
2. Concentrations less than the LOD are replaced by a *limiting value*, and
3. In a more rigorous approach, data less than the LOQ are replaced by a *limiting value*.

The choices for the limiting values for the three QA scenarios above are somewhat arbitrary, but in practical terms there are only a few logical possibilities to choose from.

The limiting value, in its most generalized sense, is defined here as the concentration below which a result is determined substantially—but not exclusively—by the uncertainty in the method. In its most rigorous application, the limiting value is the lowest concentration wherein a value is still statistically distinguishable from measurement noise to within the QA objectives. The latter is an important point, because a more stringent QA criteria is usually associated with a greater limiting value (and a higher statistical confidence).

For the three quality assurance scenarios considered here, the limiting values considered within the analytical approach are as follows: a) a small nonzero constant (here $0.0005\,\mathrm{mg\,m^{-3}}$), b) the *effective LOD*, and c) the *effective LOQ*. Note that the latter two scenarios require a rigorous investigation and corresponding calculations, whereas the former is simply set to a value representative of the most conservative estimate of detectability to remove the statistical problems associated with zeroes (which means it is usually close to the noise amplitude estimate, but it is not a proper surrogate for the noise amplitude).

In fact, the use of a constant for the limiting value is motivated by more than just a desire to remove statistical problems in HPLC databases. In some cases, analysts want to discern the faintest indications of relationships and correlations within the results, and the more aggressive the QA process, the less likely these faint patterns will emerge. The basic rationale behind looking at the rawest data possible varies, but in most cases, it is derived from a) a strong belief that the skill of the analyst is sufficiently expert to properly resolve and quantitate very low pigment concentrations, and b) the observations are an incremental contribution to a larger database, and the emergence of any faint signal is expected to be clarified over time as the number of observations, N, is increased (the so-called $\sqrt{N}$ noise-reduction effect). Note that if the noise is not adequately reduced, any patterns or correlations that appear in the data might not be statistically robust.

There are several problems with relying on an expert-based reporting system once data are shared beyond the confines of an individual laboratory. First of all, the expertise of analysts is variable, so subjective assumptions about the reporting skills of analysts, and thus the uncertainties in the pigment concentration data, are inevitable. The danger exists that the analyst's skill is overestimated and uncertainties are underestimated, which can lead to poor decision making and spurious scientific results. When databases from several sources are combined, the capabilities of the analysts (whether expert or not) are not transported with the data, so again (unfounded) assumptions are made about the uncertainties in the data.

The only way to remove the subjective aspects of data reporting is to produce a completely objective reporting system. Because uncertainties are ultimately of interest to all database users, it makes sense to construct a QA procedure based on uncertainty analyses that are easily implemented and documented. In addition to the advantage of having database contributions accompanied by uncertainty values, this approach allows the analyst to temporally monitor uncertainties and, thus, confirm method performance at any point in time. Consequently, an analyst can make corrections or alterations to laboratory procedures to bring uncertainties within the limits—or performance metrics—established by the QA objectives.

The effectiveness of reducing uncertainties is evaluated here by computing the precision of pigment concentrations in duplicate filters for four reporting schemes: no QA screening (null reporting), null values replaced by a constant ($0.0005\,\mathrm{mg\,m^{-3}}$), LOD thresholds applied, and LOQ thresholds applied. It is worth remembering that in most cases, the limiting value is zero, so null reporting largely mimics general reporting practices (including the usual procedures of the SeaHARRE-2 participants).

1.4.3.2 The Noise Amplitude

There are many ways to measure the noise associated with an HPLC baseline. To be consistent among laboratories, the definition for noise used here is termed the *very short-term detector noise* and is described by a statistical parameterization of the baseline fluctuations at times approximating the elution position of the pigments of interest. Many HPLC software packages provide a measure of these random amplitudes automatically (wherein the time duration and interval are specified by the user). Alternatively, it can be accurately and easily estimated using commercial software applications as long as the parameters

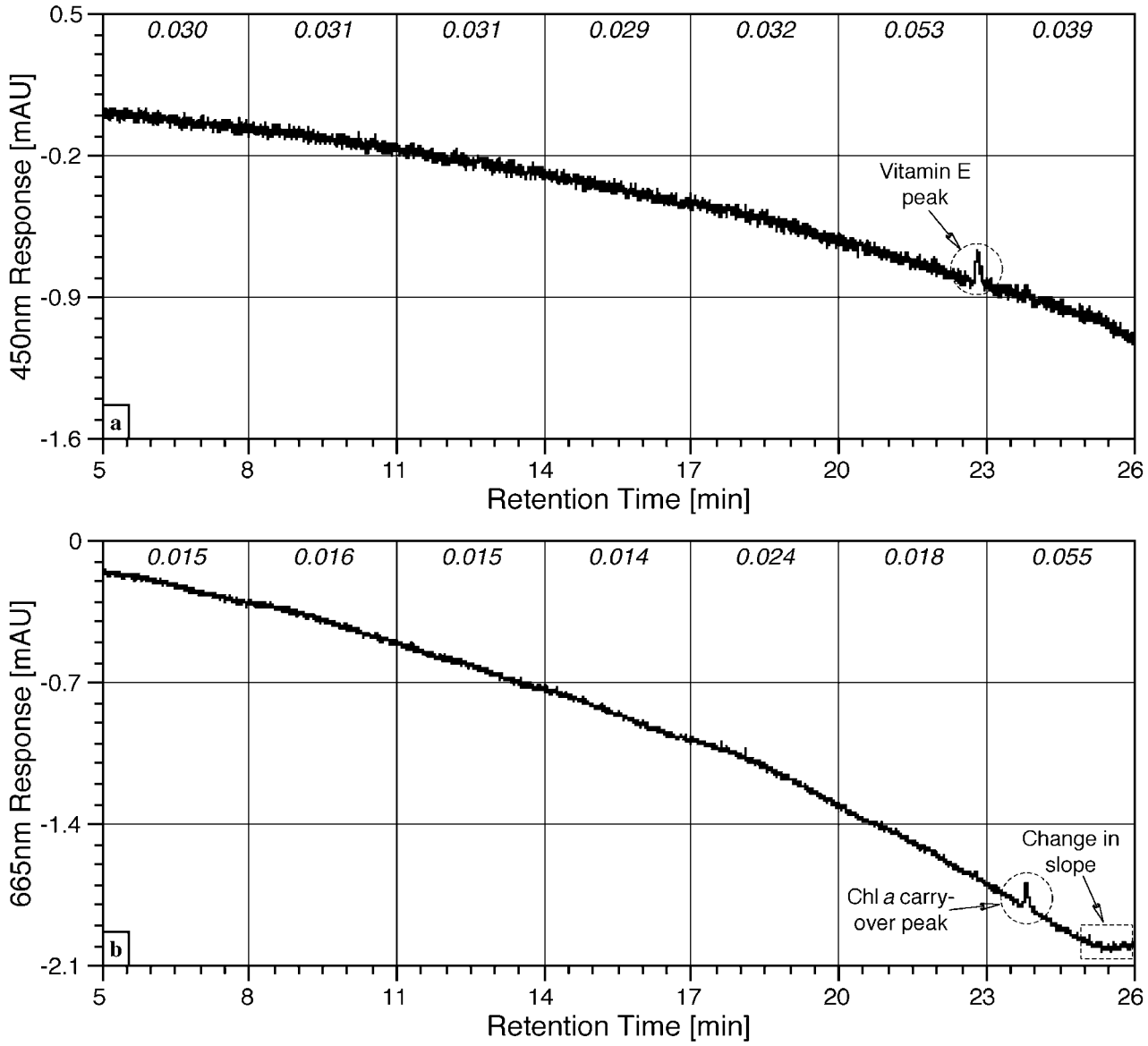

Fig. 2. The detector response for the analysis of an acetone solution containing the internal standard (vitamin E) as a function of retention time, for **a)** 450 nm, and **b)** 665 nm. Vitamin E absorbs poorly at 450 and 665 nm, so this analytical process is particularly useful for estimating noise. At 222 nm, the wavelength used to quantify the vitamin E peak area, the SNR is approximately 34,000). The x and y axes span the same ranges for both panels, and the noise estimate for each 3 min time interval is given along the top of each panel. The dashed circles and square highlight departures from the regular behavior of the baseline noise.

defining a chromatogram can be exported as a tabular set of numbers, for example, in a spreadsheet configuration of columns and rows.

It is best to average the noise over a minimum of at least 1 min. In the example shown in Fig. 2a, the noise at 450 nm was measured using Hewlett-Packard (HP) ChemStation (Agilent Technologies†) software over seven consecutive, 3 min intervals and from chromatograms depicting the analysis of an acetone solution with typical injection and analysis procedures at HPL. Because the H method uses a second wavelength (665 nm) for quantitation of chlorophyll a and like pigments, noise was also measured at 665 nm (Fig. 2b). Although the average noise at 665 nm is less than at 450 nm, the basic behavior is very similar except for a large change in baseline slope at 25–26 min. With the exception of the problematic intervals in Fig. 2 (the dashed circles and square), the average noise in each 3 min interval is consistent over all time positions. This

† Summary information regarding commercial manufacturers and suppliers described in this report is presented in Appendix C.

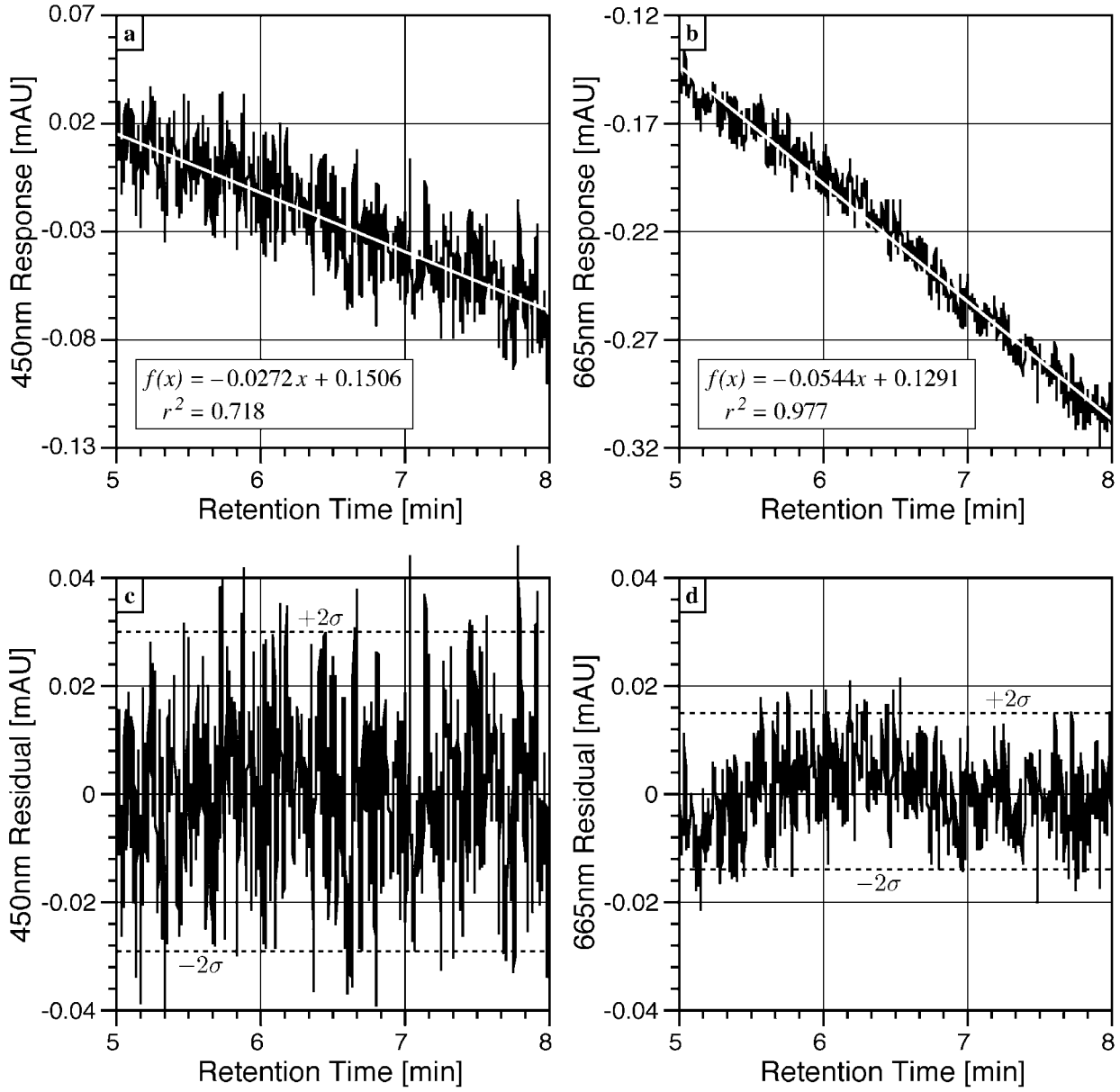

Fig. 3. Magnified displays and linear curve fits of the data presented in the first time interval (5–8 min) in Fig. 2: **a)** 450 nm, and **b)** 665 nm. Linear fits to the data are indicated by the white lines, and the regression formulas and determination coefficients are given in the inset panels. Plots of the residuals to the fits are given in **c)** 450 nm, and **d)** 665 nm, respectively. The dashed lines represent the $\pm 2\sigma$ lines of the residuals.

suggests an overall average or summary noise estimate is appropriate for the quantitation of SNRs of pigments for the H method, regardless of the elution position.

The noise calculation involves the following steps:

1. A linear fit is made to the data in the selected time interval.
2. Residuals of the data with respect to the fit are computed as the observed value at each time minus the fitted value (well-behaved data have approximately equal portions of positive and negative residuals).
3. The standard deviation, σ, of the residuals is computed, and the noise amplitude is approximated as 2σ (the 2σ criteria assumes a histogram of the residuals approximates a gaussian distribution).

Figure 3 shows this entire process for the data from the 5–8 min time interval in Fig. 2. Note that for well-behaved baselines (the majority of the baseline intervals in Fig. 2), the 2σ lines can be estimated rather well using graphical or visual fitting techniques if there was no way to export the data from the chromatography software (but this is only recommended as a last resort). Intervals with problematic baselines need to be excluded from the fitting process, because they have anomalously elevated noise levels.

Table 17. The (2σ) noise observations at 450 and 665 nm (in units of milli-absorbance units) as determined by laboratory H in a set of calibrations identified by a sequence and injection (S/I) number. The latter is encoded as sequence number first, followed by the injection number. The bold entries are the maximum values across all the intervals for the indicated S/I number, and the last line shows the average of all the maximum values with the coefficient of variation given in parentheses (and expressed in percent). The numeric entries shown in slanted typeface denote intervals with carryover or baseline distortion problems and were ignored in the noise analysis. The interval number sets the time range in minutes: 1 for 5–8, 2 for 8–11, 3 for 11–14, 4 for 14–17, 5 for 17–20, 6 for 20–23, and 7 for 23–26 min. Sequences 1 and 2 were executed approximately one year apart, and sequences 2 and 3 were done about one month apart.

S/I	*450 nm Interval Number*							*665 nm Interval Number*						
No.	1	2	3	4	5	6	7	1	2	3	4	5	6	7
1–1	0.024	**0.026**	0.023	0.024	0.023	0.022	*0.062*	0.013	0.012	0.013	0.012	0.013	**0.014**	*0.033*
1–2	**0.033**	0.024	0.023	0.021	0.023	0.025	*0.063*	0.012	0.012	**0.013**	0.012	0.013	0.012	*0.030*
1–3	0.023	**0.025**	0.024	0.024	0.022	0.024	*0.061*	0.012	0.013	**0.013**	0.012	0.013	0.012	*0.031*
2–1	0.024	0.024	0.022	0.023	**0.024**	0.022	*0.055*	0.013	0.014	0.015	0.017	**0.018**	0.013	*0.038*
2–2	0.022	0.023	0.023	0.023	0.023	**0.026**	*0.056*	0.013	0.014	0.014	0.012	**0.015**	0.015	*0.060*
3–1	0.030	0.031	0.031	0.029	0.032	*0.053*	**0.039**	0.015	0.016	0.015	0.014	**0.024**	0.018	*0.055*
3–2	**0.038**	0.031	0.031	0.029	0.030	*0.054*	*0.059*	**0.020**	0.014	0.017	0.014	0.017	0.016	*0.107*
Ave.	**0.030 (21.3%)**							**0.015 (23.0%)**						

If an HPLC software package is used to estimate the 2σ noise value (rather than the steps described above), confirmation is needed that the estimate is based on the residuals to the linear trend and that problematic areas are excluded. In some cases, the software might properly compute the statistics with respect to the detrended and filtered data, but use a definition for noise that is much greater than 2σ (e.g., 6σ). In these cases, a simple scaling factor might have to be applied.

The utility of using SNRs as part of a QA threshold approach requires the noise measurements to be relatively stable over time, so performance degradation can be properly quantified and detected (i.e., not confused with a change in the baseline noise). Consequently, the noise measurements described above were performed with at least two separate injections in the same sequence, and a measure of the average noise over the replicate injections was computed. This procedure was repeated for three different sequences conducted over a 13-month time period to include a long-term sensitivity to the analysis. The results of this QA process are shown in Table 17 for both the 450 and 665 nm detectors as a function of the seven 3 min intervals displayed in Fig. 2.

A parameterization of method noise must also be statistically robust, so the results can be used with confidence. In general, the preferred criteria will have a small probability that the data are not properly characterized by the noise estimate. Consequently, the approach adopted here is to a) use 2σ values, because 2σ parameterizes a sufficiently significant majority of the variance (assuming a gaussian noise distribution), and b) establish overall values using the average of the maximum noise estimates (the bold entries in Table 17) while ignoring intervals with carryover or baseline distortion problems (the slanted entries in Table 17). As noted earlier, the latter need to be filtered out of the noise estimates, because they represent artificial increases in the baseline noise (almost any method is going to have portions of the baseline with such problems, so this is not an exceptional requirement).

For the data presented in Table 17, the averages of the maximum values are only a little larger than the averages of all the nonproblematic data: 0.030 mAU versus 0.026 mAU for 450 nm, and 0.015 mAU versus 0.014 mAU for 665 nm, respectively. This good agreement shows the noise is minimal in amplitude and basically stable, both in terms of the interval number and the sequence number (i.e., stable over short- and long-term time periods). The data in Table 17 also show the noise estimates are consistent irrespective of the time interval, which means the overall maximum averages are representative of all the time intervals. The noise at 450 nm is greater than the corresponding noise at 665 nm, but the CV values are approximately the same.

1.4.3.3 The Signal Amplitude and SNR

The measurements of signal amplitude used here are simply the peak heights measured by the HPLC system integrator. For very low SNR values, the beginning and ending of a particular peak can be somewhat difficult to identify. In such instances, it is recommended to measure the peak height using the best estimate of the average baseline noise as the starting and ending point, as illustrated in Fig. 4 for a peak with an SNR of approximately 6. Note that this process requires a substantially linear trend in the baseline noise (as shown in Fig. 4 by the linear fit to the baseline slope).

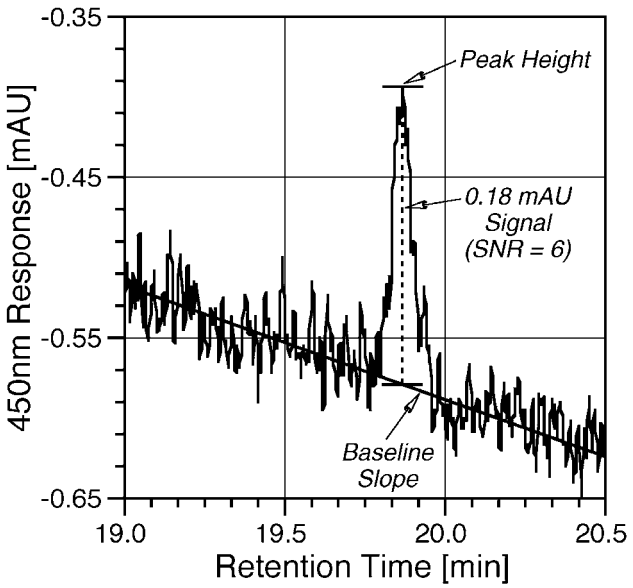

Fig. 4. A schematic showing the computation of the signal height (dashed line) and the SNR for a pigment peak. The diagonal line is a linear fit to the baseline slope.

The SNR, as used in this study, is the signal amplitude (or peak height) divided by the observed noise (characterized using a 2σ formulation) at the same wavelength from which the pigment signal was determined. As shown in Figs. 2 and 3 (and verified by Table 17), the overall noise (averaged over all seven of the 3 min time intervals) is suitable for noise measurements with the H method, regardless of the retention time. If noise measurements vary greatly with retention time, it may be more appropriate to use a noise determination in close retention time proximity to the pigment whose SNR is being determined.

1.4.3.4 The LOD and LOQ

Although alternative SNR thresholds for the LOD and LOQ could be assigned, the values chosen here (3 and 10, respectively) are in keeping with traditional choices and provide sufficient discrimination to discern the effect of using increasingly aggressive QA thresholds. The LOD and LOQ values are determined experimentally for a given pigment by performing injections of dilute standards of known concentrations such that an SNR at 3 and 10 can be well defined (which means the experimental methodology must bracket these SNR levels). At HPL, this was accomplished by preparing a standard mixture of 16 carotenoids in ethanol, and a separate mixture of 7 chlorophylls and 1 carotenoid in 90% acetone. Several dilutions of these stock mixtures were prepared, so the resulting analyses yielded SNRs typically less than 30 for the highest concentrations, and SNRs of approximately 2–3 for the lowest.

For illustrative purposes, chromatograms from analyses of carotenoids in ethanol are shown in Fig. 5 for four different dilutions (panels a–d). The actual dilution levels, along with the SNR and concentration of Fuco for each dilution are given in Table 18. The Viola and Hex-fuco plus the Zea and Lut critical pairs (adjacent pigments exhibiting the poorest resolution, but nonetheless quantitated based on chromatographic separation) are also shown in Fig. 5, so their properties as a function of decreasing SNR can be discerned. Also shown is the evolution of Gyr diester, which has an SNR range of 30, 15, 6, and 3 for panels a–d, respectively. Note the Gyr diester peak in Fig. 5d, which has an SNR of 3, is almost indistinguishable from the elevated baseline noise next to the peak. This is a good example of why an SNR of 3 is a reasonable choice for establishing a detectability limit.

Table 18. The dilution series used with Fig. 5, where the identifiers correspond to the figure panels. The dilution factor is the percentage of the stock solution used, with the number in parentheses giving the amount of Fuco in the dilution.

Series Identifier	*Dilution Factor* [%]	*Fuco SNR*
a	9.1 (1.40 ng)	64
b	4.8 (0.74 ng)	33
c	2.0 (0.31 ng)	14
d	1.0 (0.15 ng)	8

In the methodology developed here, LOD and LOQ computations are expected to be an explicit part of the calibration process. The response factors for each pigment are determined using a dilution series composed of two repartitions of the dynamic range of the anticipated concentrations of the samples to be analyzed. One repartition is established to ensure that standards sufficiently dilute to adequately describe the detection limits are included in the calibration process, and the other repartition is set to bracket the expected range of concentrations in the samples to be analyzed. For most field samples and methods, this means there will be a set of concentrations that are rather high, and another set that are very low. The main peak area is linearly regressed against the amount (or mass) of the purity-corrected pigment injected onto the column, M_{P_i}, with the regression line forced through zero. The inverse of the regression slope is R_{P_i} (4).

A graphical presentation of the calibration procedure for MVChl *a* is given in Fig. 6. More points are used than usual, to show how well the data correspond to a fit through zero at very low pigment amounts (inset panel). Although the $r^2 = 1.000$ result indicates an excellent fit, a more appropriate measure is the RPD between the observed and fitted values (i.e., the residuals), $\psi_{\rm res}$. The average RPD of the fit, $\bar{\psi}_{\rm res}$, is -1.2% and the maximum deviation is 7.0%. By comparison, if the regression is not forced through zero, the average RPD is 5.7%, and the maximum deviation is 33.2%. A better measure of the dispersion with respect to the fitted line is the average of the

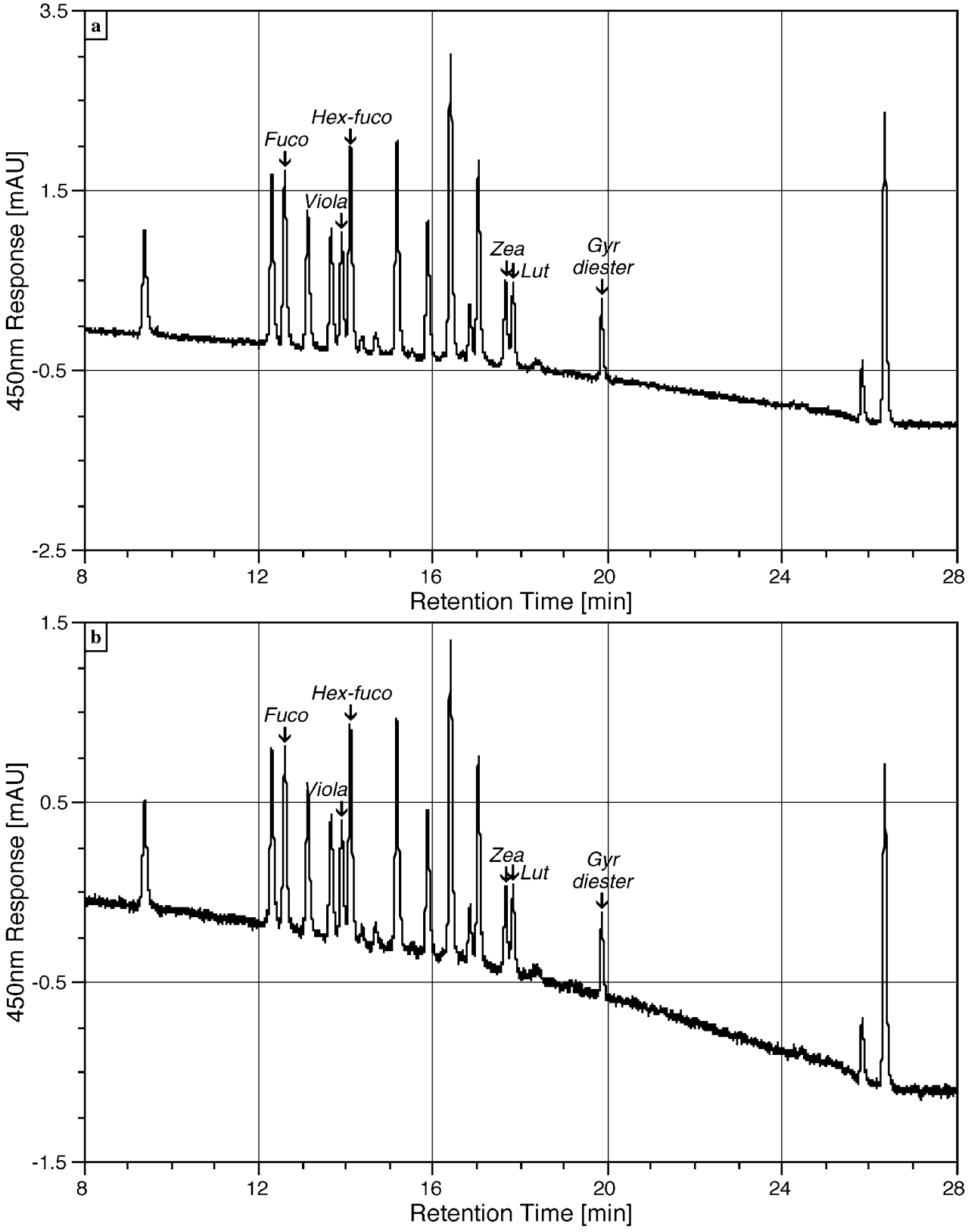

Fig. 5. The chromatograms from analyses of the carotenoids in ethanol for two different stock dilution levels: **a)** 9.1%, the most concentrated solution, and **b)** 4.8%. Individual pigment peaks are identified by the bold arrows. Note the change in the y-axis scale between the two panels.

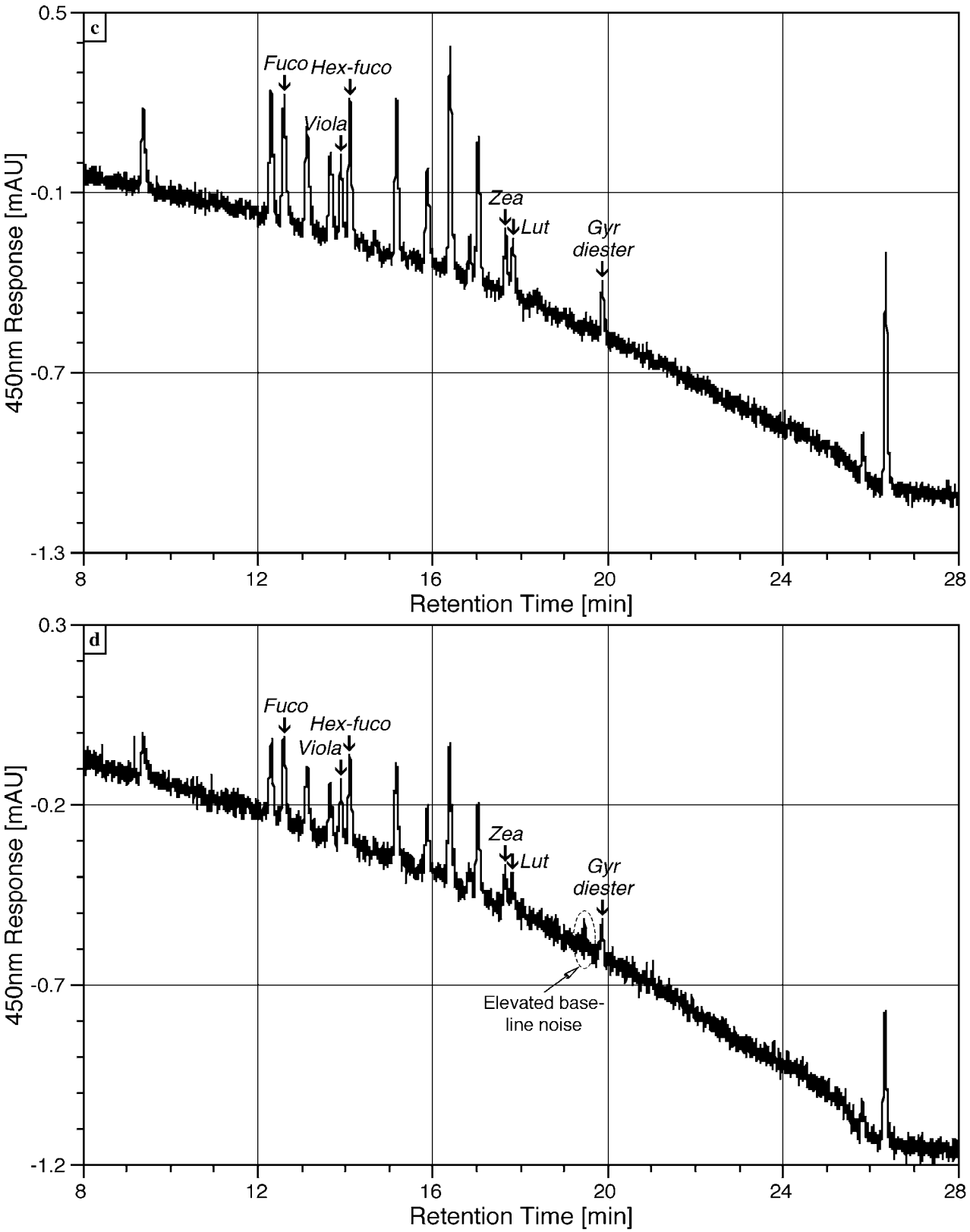

Fig. 5. (cont.) The chromatograms from analyses of the carotenoids in ethanol for two different stock dilution levels: **c)** 2.0%, and **d)** 1.0%, the most dilute solution. Individual pigment peaks are identified by the bold arrows. Note the change in the y-axis scale between the two panels.

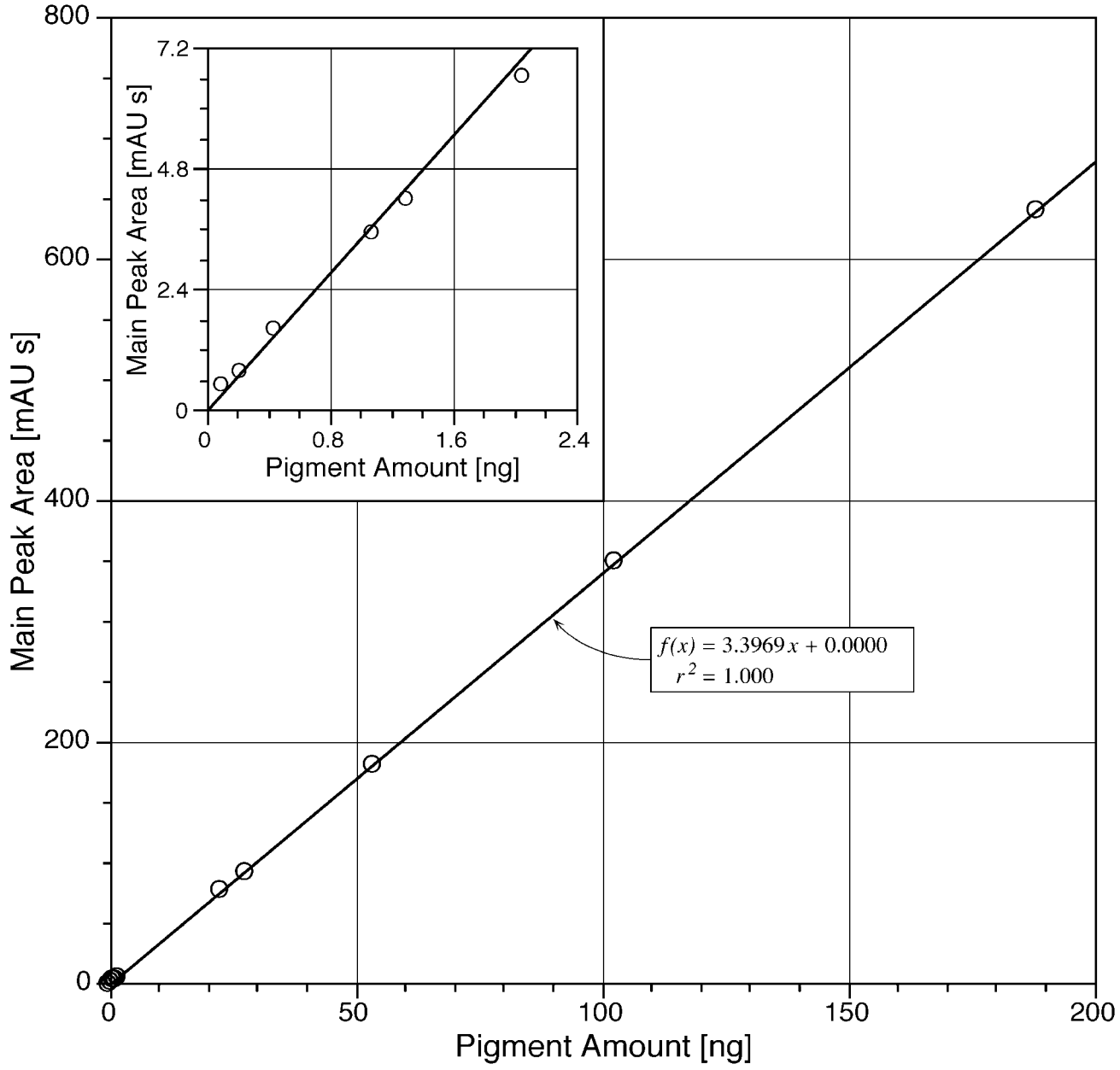

Fig. 6. The calibration of MVChl *a* at 665 nm showing the regression of main peak area versus pigment amount (M_{P_i}). The (upper left) inset panel shows the values at very low pigment amounts. The linear fit is forced through zero, but still has a coefficient of determination (r^2) that is equal to 1.000.

absolute residuals, $|\bar{\psi}|_{\rm res}$, because there is no fortuitous cancellation of positive and negative values. For Fig. 6, $|\bar{\psi}|_{\rm res}$=2.8% (these data are over sampled for illustrative purposes, so the results are slightly larger than usual).

The reason for forcing the fit through zero deserves additional comment, because some analysts do not do this. Instead, the y-intercept is used as a QA parameter wherein if the y-intercept is too far from zero (either positively or negatively), the calibration process is flagged as substandard. Such a QA opportunity is not lost in the method proposed here, because, the residuals to the fit increase whenever a poor calibration process is executed (as would the y-intercept). The advantage of forcing through zero is the likelihood of negative results at low concentrations (which are nonphysical) are minimized. In addition, many analysts are already effectively relying on fits forced through zero, because they use single-point calibrations (perhaps with multiple injections at a single concentration). The net result is there is no loss in applicability by forcing the fit through zero.

Assuming the baseline noise has been determined from a solvent blank, the main SNR values for each pigment

in each analysis can now be computed using the corresponding pigment peak heights. (Sect. 1.4.3.2). The LOD and LOQ values are determined using a linear regression of M_{P_i} (in units of nanograms of pigment), as the x variable, and SNR as the y variable. The linear regression is forced through the *noise origin* (0,1), which is most easily accomplished by subtracting 1 from all the SNR values and forcing the regression through zero. The slope of the linear regression equation, $S_{P_i}(\lambda_d)$, is then used to estimate the LOD and LOQ, respectively:

$$D_{P_i}(\lambda_d) \;=\; \frac{3}{S_{P_i}(\lambda_d)} \tag{24}$$

and

$$Q_{P_i}(\lambda_d) \;=\; \frac{10}{S_{P_i}(\lambda_d)}, \tag{25}$$

where, again, λ_d sets the detector wavelength.

1.4.3.5 The Relative LOD and LOQ

It is not routinely practical to experimentally determine the LOD and LOQ for all pigments to be quantitated. An alternate approach is proposed here based on characterizing the LOD and LOQ of a few so-called *reference* pigments, and then using the response factors of the other pigments that match the same detector settings as the reference pigments to estimate a relative LOD and LOQ. For example, if the LOD and LOQ for Fuco are experimentally determined, and the response factors of Fuco and the pigment under investigation (P_i) are known, the relative LOD and LOQ values (D' and Q', respectively) are then calculated as follows:

$$D'_{P_i}(\lambda_d) \;=\; \frac{R_{P_i}}{R_F}\, D_F(\lambda_d) \tag{26}$$

and

$$Q'_{P_i}(\lambda_d) \;=\; \frac{R_{P_i}}{R_F}\, Q_F(\lambda_d), \tag{27}$$

where the *prime* ($'$) notation denotes a relative parameter, R_F is the response factor for Fuco, D_F is the LOD for Fuco, and the pigment P_i is quantified using the same detector settings and wavelength (λ_d) as Fuco.

In the particular case of the H method, Fuco was used as the reference for all pigments quantified with the same detector settings as Fuco, and MVChl *a* was the reference for all pigments quantified at the detector settings used for MVChl *a*. In principle, if only one wavelength is used for pigment quantification, either Fuco or MVChl *a* could be used as the reference.

The LOD and LOQ for 21 pigments were experimentally measured at HPL and are presented in Table 19. LOD values varied between 0.03–0.15 ng, and LOQ varied between 0.12–0.50 ng (Chl *b*, Phide *a*, and Phytin *a* all exhibited the highest LOD and LOQ values.) The accuracy of the relative LOD and LOQ values was assessed by comparing the experimental and relative values. Relative LOQ values ranged between −24.0 to 22.9% of experimentally determined LOQ values, and usually were within ±12.5% (particularly the primary pigments). Given that LOD and LOQ values are expected to change over time, the differences between the experimental and relative values are mostly insignificant within the context of their intended use. Consequently, it seems entirely appropriate to use relative LOD and LOQ values when applying QA thresholds to a database of pigment concentrations, particularly for retroactive QA analysis.

Table 19. The LOD and LOQ for 21 pigments determined experimentally at HPL. Also shown are the RPD† values between the experimental values and the relative values. For the latter, Fuco and MVChl *a* were the reference pigments for the other pigments quantitated with a detector wavelength (λ_d) of 450 and 665 nm, respectively. Average values across the primary pigments and all pigments are given in slanted typeface (the RPD averages do not include MVChl *a* and Fuco).

Pigment P_i	λ_d [nm]	D_{P_i} [ng]	Q_{P_i} [ng]	ψ_{P_i} [%]
MVChl *a*	665	0.08	0.25	0.0
Chl *b*	450	0.15	0.49	4.1
Chl c_1	450	0.06	0.19	−12.2
Chl c_2	450	0.05	0.16	−12.4
Chl c_3	450	0.05	0.15	−0.4
$\beta\beta$-Car	450	0.03	0.12	−12.6
Allo	450	0.04	0.13	7.4
But-fuco	450	0.06	0.21	3.4
Diadino	450	0.04	0.14	−3.1
Diato	450	0.04	0.12	7.9
Fuco	450	0.06	0.21	0.0
Hex-fuco	450	0.06	0.20	6.6
Perid	450	0.10	0.34	−12.2
Zea	450	0.04	0.13	11.7
Primary Pigment Avg.		*0.06*	*0.20*	*−1.0*
Neo	450	0.07	0.22	−3.2
Pras	450	0.07	0.22	1.1
Viola	450	0.06	0.21	−18.6
Anth	450	0.05	0.16	1.1
Myxo	450	0.09	0.30	−5.8
Lut	450	0.04	0.14	0.5
Gyr diester	450	0.04	0.13	22.9
Cantha	450	0.06	0.20	−2.7
Phide *a*	665	0.15	0.50	−24.0
Phytin *a*	665	0.15	0.50	0.7
Overall Average		*0.07*	*0.23*	*−1.8*

† The RPD values (denoted ψ) are computed using the formulation $\psi = 100(Y - X)/X$, where Y is the relative (computed) value and X is the experimental value. Only one RPD value for the LOD and LOQ is reported, because they are equal (the LOD and LOQ formulations differ by a constant multiplier).

1.4.3.6 The Effective LOD and LOQ

Ultimately, the information from a detectability analysis is most accessible if it is available in units of concentration. These so-called *effective* values are computed from the LOD and LOQ values using the volumetric information associated with the analysis of the samples:

$$D_{P_i}^{\text{eff}} = \frac{D_{P_i}}{V_c}\frac{V_x}{V_f} \tag{28}$$

and

$$Q_{P_i}^{\text{eff}} = \frac{Q_{P_i}}{V_c}\frac{V_x}{V_f}, \tag{29}$$

where V_x is usually set to $V_e + V_w$ for simplicity regardless of whether or not an internal standard is used in the extraction process (Sect. 1.3.1).

Relative LOD and LOQ values can be used in place of D_{P_i} and Q_{P_i} in (28) and (29), respectively, if the LOD and LOQ values are only explicitly determined for a subset of the chlorophylls or carotenoids (e.g., if only D_a, Q_a, D_F, and Q_F are computed). This substitution is only valid, however, if the reference pigments are determined at the same detector settings as the pigments of interest. In addition, it is very important the pigments of interest do not a) elute at a retention time associated with abnormal baseline behavior, or b) normally have significant elution problems (peak fronting, asymmetric peaks, poor resolution, excessive peak width, etc.). Any pigment exhibiting these problems should be calibrated and quality assured using an explicit dilution series.

1.4.3.7 A QA and Calibration Protocol

A step-by-step protocol for computing the LOD and LOQ can be combined with the calibration process to provide a complete QA procedure for any HPLC method:

1. Measure the noise of a blank, solvent-only injection (Sect. 1.4.3.2) by a) splitting the chromatogram into approximately 3 min intervals, b) calculating residuals to linear regressions of each interval as the observed value minus the fitted value, and c) computing the 2σ values for the residuals in each interval. Estimate the noise amplitude as the maximum 2σ value across all intervals after excluding any intervals with abnormal baselines (carryover, large slope changes, etc.). Note, it may be acceptable to use the noise estimate from the HPLC analysis software rather than computing the noise as described here, but this will require a separate set of experimental trials to confirm the two are comparable over a wide range of conditions.
2. Establish a dilution series for each pigment to be calibrated by setting: a) three concentrations in the repartition expected during sample analysis, and b) three concentrations in the LOD and LOQ repartition. For each of these repartitions, a point is needed below the minimum, above the maximum, and approximately in the middle. For the LOD and LOQ repartition, the distribution of points should equate approximately to an SNR of about 2, 10, and 20. Any values less than an SNR of 2 will not be very useful, because the points are composed primarily of noise.
3. After analyzing the dilution series, linearly regress (with the fit forced through zero) the main peak area versus the purity-corrected amount of pigment for the entire dilution series, and compute the response factor as the inverse of the regression slope (Sect. 1.4.3.4).
4. Estimate the signal amplitude of each point in the dilution series as peak height, and compute the SNR (Sect. 1.4.3.3) as the signal amplitude divided by the noise amplitude.
5. Linearly regress the amount of pigment (the x-axis) versus the SNR (the y-axis) for the entire dilution series, with the fit forced through the noise origin (0,1), and compute a) the LOD as 3 divided by the slope, and b) the LOQ as 10 divided by the slope. A simple way to force the fit through the noise origin is to reduce all the SNR values by 1, and then force the fit through zero (Sect. 1.4.3.4).
6. If the multipoint (dilution series) calibration approach is only used for a small number of reference pigments (e.g., MVChl *a* and Fuco), with the remaining pigments calibrated using single-point calibrations, the remaining pigments should have their LOD and LOQ values computed using the relative LOD and LOQ formulations (Sect. 1.4.3.5), respectively. It is important to make sure the reference pigments are selected such that they have the same detector settings as the pigments of interest, and the latter do not have elution or resolution problems that would invalidate the relative LOD and LOQ formulations.
7. Determine the effective LOD and LOQ using the relevant volumetric information from method execution and sample preparation (Sect. 1.4.3.6).

The culmination of the LOD and LOQ process depends on the QA objectives of the HPLC method. Any quantitated pigment with a concentration a) less than the LOD should be replaced with the effective LOD, or b) less than the LOQ should be replaced with the effective LOQ. The higher SNR used with the LOQ means there will always be greater confidence in these results than those obtained with an LOD approach (again, consider the Gyr diester peak in Fig. 5d, which has an SNR of 3, versus the adjacent elevated noise peak).

Some care must be used when applying LOD or LOQ thresholds to summed pigments. The total chlorophylls, for example, are each the sum of several constituent pigments with TChl *a* usually having the most (e.g., allomers,

epimers, divinyl chlorophyll *a*, and chlorophyllide *a*). If all the constituents are subjected to the LOD or LOQ threshold before the summation is made, the minimum concentration for the total will be artificially increased above a reasonable detectability limit. To prevent this problem, the LOD or LOQ threshold associated with the primary constituent of a summed pigment (e.g., MVChl *a* for TChl *a*) should be assigned to the total; in cases, where two or more constituents contribute rather equally, the largest LOD or LOQ should be used.

A final practical problem with a QA process employing limiting-value thresholds is the unique aspect of divinyl chlorophylls *a* and *b*, which are only present in marine samples if prochlorophytes are present (Goericke and Repeta 1992). In the LOD and LOQ approach to QA, $\left[\text{DVChl } a\right]$, for example, will have a nonzero value, but the geographic location where the sample was taken might strongly suggest—perhaps even to a seemingly high degree of certainty—that prochlorophytes cannot be present and $\left[\text{DVChl } a\right] = 0\,\text{mg}\,\text{m}^{-3}$. Under many circumstances, particularly those involving commercial or scientific analyses not conducted at the institute involved with the field sampling, an HPLC analyst does not know where a sample was collected (during SeaHARRE-2 only *L* and *M* knew the locations of the samples). Given the *blind* nature of most HPLC analyses, it is reasonable for the analyst to use a reporting scheme that always assumes DVChl *a* might be present, which is the perspective adopted here. DVChl *b* is considered similarly, but it is less significant for this study because only *B* separated the monovinyl and divinyl forms of chlorophyll *b*.

The utility of using the described LOD and LOQ approach requires that peak widths, baseline noise, and pigment height responses remain relatively constant over the time set for which the LOD and LOQ values are used. Both purposeful modifications to method parameters and naturally occurring events can affect these aforementioned variables. Changing detector settings (e.g., sample rate, bandwidth, slit width, or reference wavelength characteristics) can affect SNRs and will require a new evaluation of LOD and LOQ limits. Similarly, the natural peak broadening that occurs with column aging has the potential to invalidate LOD and LOQ limits. It is therefore crucial that method changes affecting SNR be accompanied by an evaluation of LOD and LOQ and that method performance criteria that monitor baseline noise and peak width be routinely monitored.

A practical approach to monitoring changes in peak width is to simply monitor the resolution between critical pigment pairs (adjacent pigments whose resolution is adequate for quantitation, but is poorer than other adjacent pigments, as shown in Fig. 5). For example, a new HPLC column with the *H* method is expected to yield resolutions in the range of 1.2–1.4 for the Zea and Lut plus Viola and Hex-fuco critical pairs. With extended column use (which ranges from 500–1,000 injections), however, the peaks broaden and the resolution between these critical pairs degrades. When resolution drops to less than 1.0, the column is removed from service. While this performance metric is useful for ensuring peak resolution is adequate for accurate quantitation, it is also useful to ensure peak widths are constant enough for LOD and LOQ values to remain sufficiently accurate during the same time interval (this would not be true if baseline noise was not also relatively constant over time).

Peak width must also be considered in the context of relative LOD and LOQ values. At HPL, it was shown that relative LOD and LOQ values can be used as an accurate proxy for an observed, or measured, LOD and LOQ value (Table 19). This relationship may not hold true, however, if individual peak widths vary greatly from the *reference* pigments used to compute the relative LOD and LOQ values (in this instance, the reference pigments were Fuco and MVChl *a* and peak widths measured at the half height never varied by more than 30% from the reference peak width). If there are large differences in peak widths, relative LOD and LOQ values may not be an appropriate proxy for the actual LOD and LOQ values, unless they can be matched with a reference pigment of similar peak width. Alternatively, it may be necessary to physically measure the LOD and LOQ values for peaks with aberrant peak widths.

1.4.3.8 An Example QA Procedure

The benefits of applying a QA procedure as part of data analysis and reporting are evaluated here using 46 sets of duplicate filters collected as part of an environmental monitoring program funded by the Connecticut Department of Environmental Protection. The evaluation is based on the precision of pigment concentrations quantitated for duplicate filters according to four increasingly stringent QA procedures, or data reporting scenarios (listed from least to most stringent):

1. Pigments not found in the analysis were reported with zero concentration (this is the null reporting approach used by many analysts),
2. Zero or unspecified (null) concentrations were replaced with a constant value of $0.0005\,\text{mg}\,\text{m}^{-3}$ (this was the procedure used during SeaHARRE-1 and initially proposed for SeaHARRE-2),
3. Concentrations less than the LOD were replaced with the effective LOD, and
4. Concentrations less than the LOQ were replaced with the effective LOQ.

All of the filters were analyzed at HPL for 24 pigments used in this study, which included all of the primary pigments (and the secondary pigments therein). The effective LOD and LOQ values were estimated using (28) and (29) with the following volumetric parameters: $V_c = 150\,\mu\text{L}$, $V_x = V_e + V_w = 3{,}200\,\mu\text{L}$, and $V_f = 500\,\text{mL}$.

The relationship between SNR and precision associated with the analysis of individual pigments in duplicate filters is shown in Fig. 7a. The data fall into three groups as a function of precision: a) the majority of the results have a precision less than 15% (particularly those with an SNR above 1,000; b) a lesser amount of data have elevated CV values between 15–100%, with most of the degradation in precision associated with just a few pigments (Chlide *a*, DVChl *a*, Perid, and But-fuco); and c) there are a relatively small number of data with CV values in excess of 100%, and many of these (but not all) are the same problematic pigments identified in the second grouping.

The data in Fig. 7a also suggest an SNR of 10 is a reasonable threshold for excluding problematic data, although it will not remove all of them. If the threshold is applied, the majority of the remaining data will have a very good average precision, but a few pigments will not—in fact, the poor precision will be mostly independent of SNR. The latter is not a detraction of applying the QA threshold. It is actually an advantage, because the entire process clearly shows which pigments within the combined *H* method and *in situ* sampling protocol are not being adequately quantitated. Note that the problems with Chlide *a* and DVChl *a* will not be so important if they are only used to form TChl *a*, because most of the performance aspects of TChl *a* are set by MVChl *a*, which usually has excellent precision (because it is usually the most abundant pigment).

The average precision (measured as the CV between duplicate filters) for each of the data reporting scenarios is given in Fig. 7b for the primary pigments. Although most of the pigments show an increasing improvement in precision (i.e., a decrease in the CV value) with the application of a more stringent QA procedure, some pigments remain unaffected. The latter correspond to pigments that appear in a sufficiently high (nonzero) concentration they cannot be influenced by an SNR threshold. Considering only those pigments that are influenced, the average precision across these pigments was reduced from 12.3% (null reporting) to 6.6% (application of a LOQ threshold), which represents a 46.5% improvement in precision.

In addition to the analysis of duplicate filters, replicate injections of at least one sample extract were performed with every sequence. This companion QA procedure provides the opportunity for understanding the causes of the poor precision seen with the problematic pigments in Fig. 7a. For example, the average precision with null value reporting for all the primary pigments in duplicate filters was 9.7%, but it was 2.2% for these same pigments in the replicate injections. Average precision for the problematic pigments, Chlide *a*, DVChl *a*, and But-fuco was 33.4, 30.4, and 8.2% for duplicate filters, but was 5.0, 11.4, and 5.2% for replicate injections, respectively.

It is not surprising that Chlide *a* exhibited poor precision, because it is a known artifact of extraction. The poor precision for But-fuco can largely be explained by its low concentration ($0.028\,\mathrm{mg\,m^{-3}}$ on average), but the poor precision for DVChl *a* is unanticipated. HPLC in-line absorbance spectra indicated that a chlorophyll *a* degradation product, not DVChl *a*, was present in these samples at the DVChl *a* retention time. This pigment was typically less than 2% of the main MVChl *a* peak, even though it was frequently at an adequate SNR for quantitation. Consequently, a QA threshold based solely on SNR had little effect on removing these erroneous results.

A continuing concern with any data reporting scheme, particularly one wherein more and more aggressive QA procedures are proposed, is whether or not the average concentrations of the pigments are significantly altered. Fig. 7c presents the average concentration (across all replicate filters) for the primary pigments considered in Fig. 7b as a function of the QA procedure. Most of the pigments exhibit almost no change in average concentration, and only two show a discernible change. The change is a small increase, because the already low values that fail the QA threshold are being replaced with only slightly larger values (in relative terms). Taken together, the Fig. 7 data show the application of a stringent data reporting scheme can significantly improve precision without unduly influencing average pigment concentrations.

1.5 RESULTS

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

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

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

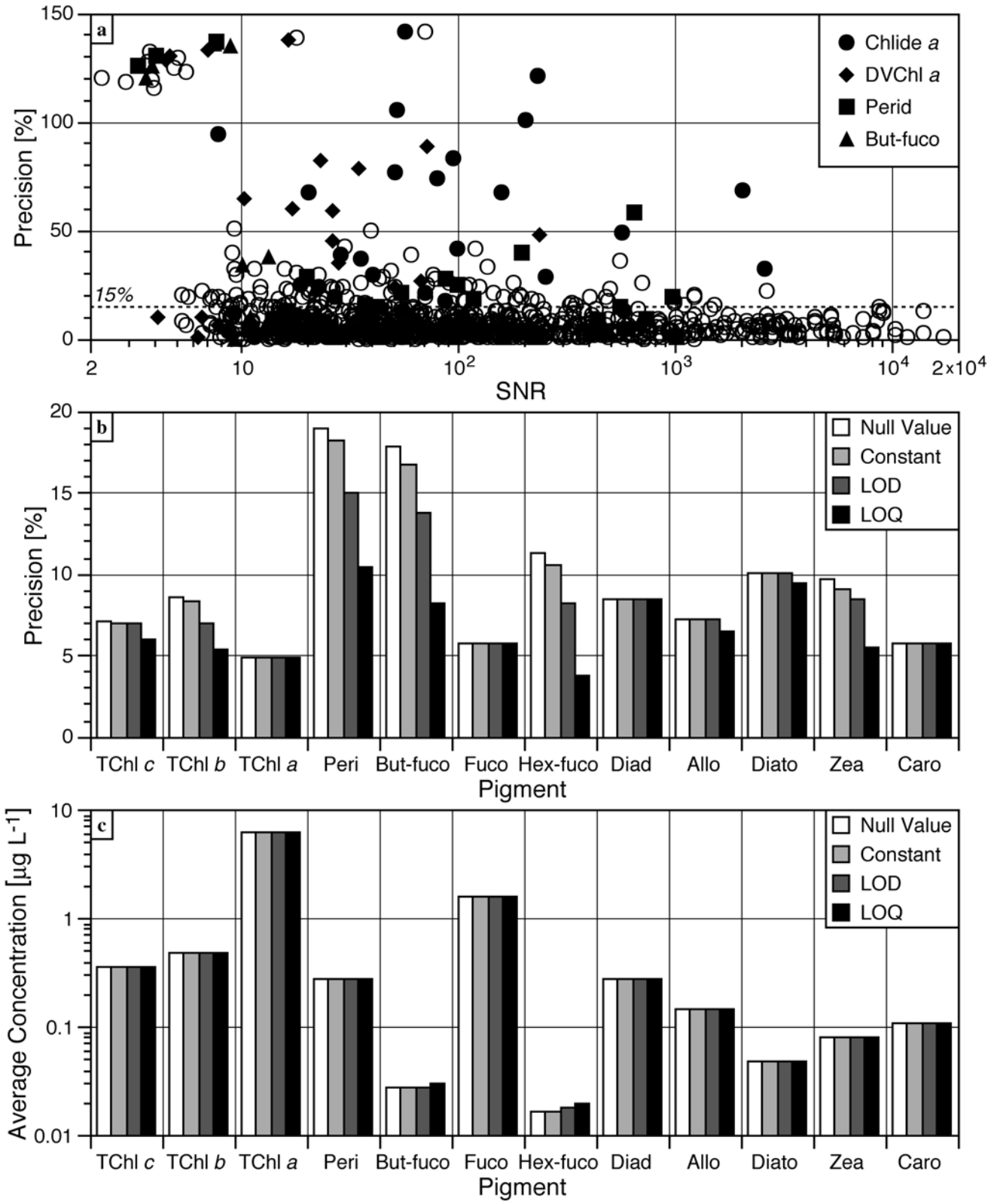

Fig. 7. QA results for the HPL analysis of samples from the Connecticut Department of Environmental Protection (courtesy of J.Y. Li): **a)** precision (estimated from the CV in duplicate filters) as a function of the SNR for each pigment in each sample (the open circles are for all pigments except those denoted by the solid symbols), **b)** average precision (across all filters) for the primary pigments as a function of four QA procedures, and **c)** average concentration for the primary pigments as a function of four QA procedures.

the 12 samples, $|\bar{\psi}|$ (7), and the average precision is given by the average CV values across the 12 samples, $\bar{\xi}$. These are the principal parameters for determining method performance and the uncertainties in the methods.

In anticipation of the statistical results, it is useful to review the most distinguishing aspects of the methods (as compiled principally from the original descriptions by the analysts of the individual methods) in terms of the primary pigments (and the secondary pigments used to create the primary pigments):

- *B* Uses a two-step acidification method, no column temperature control, manual sample injection, no internal standard, single-point calibrations for chlorophyll *a* (with six injections), and Zea coelutes with Lut.
- *C* Uses different absorption coefficients based only on acetone, no mixing of buffer and sample extract during injection, does not quantitate [DVChl *a*], and does not use an internal standard.
- *D* Uses the Latasa et al. (1996) simultaneous equations for determining [DVChl *a*], single-point calibrations for chlorophyll *a* (with four injections), and Fuco coelutes with Phide *a*.
- *H* Uses vitamin E as the internal standard, a high column temperature, separately quantifies Chl c_1 and Chl c_2, a simultaneous equation approach to separately quantify Chl c_1 and Childe *a* (which coelute), and *mix-in-the-loop* injections.
- *L* Uses methanol as the extraction solvent (so the initial mobile phase matches the extraction solvent of the samples), a low flow rate and narrow internal diameter column, no column temperature control, many different absorption coefficients, and [Caro] is computed from [$\beta\beta$-Car] alone.
- *M* Uses unique absorption coefficients for many of the carotenoids, uses a simultaneous equation approach to discriminate Childe *a* from Chl $c_{1+}c_2$, and extraction volume (V_m) varies between samples.
- *P* Not completely known if the samples were degraded from being defrosted, uses single-point calibrations for chlorophyll *a* (with three injections), and [Caro] is computed from [$\beta\beta$-Car] alone.
- *S* Uses the Latasa et al. (1996) simultaneous equations for determining [DVChl *a*], and an estimate of the volume of water on the filter is included in the extraction volume even though an internal standard is used, i.e., $V_x = (V_m + V_w)\hat{A}_{c_1}/\hat{A}_{s_1}$ in the development of (7).

Although not a separate method, it is also useful to remember the *Z* results are from a duplicate set of unequivocally damaged (defrosted) filters analyzed by laboratory *H* and provide a boundary of sorts in evaluating method performance.

1.5.1 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 $\bar{C}^A_{P_i}$ in (19) are the reference values. As noted earlier in Sects. 1.4.1 and 1.4.2, any pigments with significant coelution or specificity problems were not included in producing the overall averages presented in Tables 20 and 21. The level of importance of these types of considerations can be discerned by considering the average and range values for [Zea], which do not include the contributions from *B*, because *B* reported [Zea+Lut]. The average and range for [Zea], had the *B* results been included, would have been slightly larger: $\hat{C}^A_Z = 0.157$ (versus 0.153), $\bar{C}^A_Z = 0.079$ (versus 0.075), and $\check{C}^A_Z = 0.012$ (versus 0.011). Although these are small changes, they ensure an unbiased reference criteria for the uncertainty calculations.

The most important primary pigment, in terms of the field sampling objectives for SeaHARRE-2, is TChl *a* (because of the ocean color prespective). The original plan was to sample the widest range of [TChl *a*] as possible, with an emphasis on eutrophic conditions, that is, the majority of the samples were to be collected such that [TChl *a*] $\geq 1\,\mathrm{mg\,m^{-3}}$. The Table 20 results show a rather extensive and mostly evenly-spaced range in [TChl *a*]. Approximately three decades in concentration were collected (0.345–$25.755\,\mathrm{mg\,m^{-3}}$), and nine of the samples were above $1\,\mathrm{mg\,m^{-3}}$ (samples 7–9 are mesotrophic).

The second most abundant primary pigment in the SeaHARRE-2 samples is Fuco, which spans a range in concentration of 0.040–$12.098\,\mathrm{mg\,m^{-3}}$. TChl *c*, Diadino, and Perid appear in concentrations above $1\,\mathrm{mg\,m^{-3}}$, whereas all the remaining primary pigments are less than $1\,\mathrm{mg\,m^{-3}}$. The pigments with the lowest concentrations are Allo and Diato, although both range across more than three decades of concentration.

Within the secondary and tertiary pigments (Table 21), the most skewed distribution in concentration is associated with DVChl *a*: only two samples had detectable levels (according to those methods that chromatographically separated the monovinyl and divinyl forms of chlorophyll *a*). This means false positives are expected to be a likely problem with many of the DVChl *a* determinations. Chlide *a* is the second most abundant secondary pigment after Chl *a*, but the percent contribution of the sum of [DVChl *a*] and [Chlide *a*] to [TChl *a*] averages 4.5% and is always less than 13%. The remaining secondary and tertiary pigments appear in mostly detectable levels, although there is a persistence of low concentrations associated with mesotrophic samples 7–9 (particularly the latter).

A notable distinction between the primary pigments and the majority of the secondary and tertiary pigments is the latter frequently are at very low quantitation levels.

Table 20. The average primary pigment concentrations (across all eight laboratories) as a function of the batch sample number for the field samples (in units of milligrams per cubic meter). The overall averages for the individual pigments, $\bar{C}^A_{P_i}$, as well as the range in maximum and minimum values, $\hat{C}^A_{P_i}$ and $\check{C}^A_{P_i}$, respectively, are computed across the 12 samples. Exceptions to the general practice of averaging the concentrations from all methods for each sample are given below the overall averages and ranges.

No.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]
1	8.268	0.182	1.851	0.234	0.077	0.241	0.065	0.679	0.042	2.192	1.333	0.086
2	2.958	0.148	0.636	0.097	0.046	0.252	0.046	0.323	0.026	0.436	0.772	0.143
3	4.718	0.056	1.161	0.141	0.026	0.128	0.004	0.619	0.098	1.055	1.227	0.082
4	1.938	0.008	0.378	0.068	0.006	0.049	0.003	0.343	0.098	0.571	0.303	0.092
5	6.055	0.387	1.279	0.178	0.151	0.878	0.163	0.434	0.044	1.941	0.325	0.153
6	25.755	0.509	4.270	0.470	0.080	0.314	0.168	1.550	0.315	12.098	0.456	0.046
7	0.511	0.068	0.096	0.015	0.044	0.190	0.004	0.079	0.009	0.040	0.009	0.011
8	0.833	0.125	0.221	0.016	0.080	0.218	0.013	0.042	0.001	0.149	0.123	0.016
9	0.345	0.028	0.109	0.011	0.078	0.130	0.001	0.019	0.002	0.047	0.006	0.044
10	1.199	0.103	0.291	0.042	0.079	0.399	0.017	0.168	0.033	0.160	0.104	0.078
11	17.403	0.347	2.419	0.378	0.026	0.103	0.078	1.668	0.789	7.981	0.084	0.042
12	3.145	0.171	0.569	0.119	0.124	0.232	0.107	0.311	0.033	0.908	0.125	0.107
$\hat{C}^A_{P_i}$	25.755	0.509	4.270	0.470	0.151	0.878	0.168	1.668	0.789	12.098	1.333	0.153
$\bar{C}^A_{P_i}$	6.094	0.178	1.107	0.147	0.068	0.261	0.056	0.520	0.124	2.298	0.406	0.075
$\check{C}^A_{P_i}$	0.345	0.008	0.096	0.011	0.006	0.049	0.001	0.019	0.001	0.040	0.006	0.011

Notes:

- The [TChl *a*] entries include the [DVChl *a*] values from D and S.
- The [Caro] entries do not include the values from L and P, because they do not include $\beta\varepsilon$-Car.
- The [Zea] entries do not include values from B, because B reported [Zea+Lut].

Table 21. A subset of the average secondary and tertiary pigment concentrations for the field samples presented as a function of the batch sample number, and following the presentation scheme established in Table 20.

No.	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
1	7.700	0.001	0.553	0.011	0.054	0.060	0.177	0.453	0.027	0.036	0.101
2	2.877	0.001	0.078	0.003	0.055	0.058	0.060	0.039	0.041	0.029	0.151
3	4.374	0.001	0.337	0.004	0.003	0.005	0.107	0.188	0.015	0.021	0.089
4	1.836	0.001	0.097	0.003	0.004	0.004	0.024	0.029	0.007	0.005	0.095
5	5.855	0.001	0.190	0.008	0.045	0.079	0.081	0.165	0.053	0.052	0.164
6	25.314	0.001	0.406	0.044	0.782	0.710	0.439	0.973	0.207	0.126	0.090
7	0.503	0.001	0.007	0.004	0.029	0.037	0.001	0.004	0.008	0.016	0.015
8	0.825	0.001	0.006	0.002	0.011	0.016	0.003	0.001	0.015	0.010	0.019
9	0.323	0.038	0.005	0.001	0.002	0.003	0.002	0.001	0.001	0.002	0.046
10	1.184	0.001	0.014	0.004	0.009	0.016	0.003	0.030	0.011	0.010	0.085
11	16.227	0.009	1.137	0.020	0.087	0.108	0.247	0.292	0.021	0.042	0.065
12	2.994	0.001	0.143	0.007	0.062	0.067	0.101	0.184	0.014	0.024	0.119
$\hat{C}^A_{P_i}$	25.314	0.038	1.137	0.044	0.782	0.710	0.439	0.973	0.207	0.126	0.164
$\bar{C}^A_{P_i}$	5.834	0.004	0.248	0.009	0.095	0.097	0.104	0.196	0.035	0.031	0.087
$\check{C}^A_{P_i}$	0.323	0.001	0.005	0.001	0.002	0.003	0.001	0.001	0.001	0.002	0.015

Notes:

- The [Phytin *a*] entries do not include values from C, because they were persistently undetected.
- The [DVChl *a*] entries do not include values from C, because the method did not quantitate it, nor from D and S, because the methods did not chromatographically separate the monovinyl and divinyl forms of chlorophyll *a*.
- The [Phide *a*] entries do not include values from L, because they were persistently undetected.
- The [Viola] entries do not include values from M, because they were persistently undetected.
- The [Neo] entries do not include the values from P, because they were persistently undetected.

Although the individual laboratories did not originally report LOD or LOQ values, concentrations on the order of approximately $0.001\,\mathrm{mg\,m^{-3}}$ are usually associated with being below typical quanitation limits. Note that some of the primary pigments have concentration ranges similar to the usually low values of the tertiary pigments, for example, Allo and Diato.

1.5.2 Method Precision

Method precision for each pigment is estimated by averaging the values computed from the sample replicates across all 12 samples, which is denoted by $\bar{\xi}_{P_i}$. All laboratories analyzed duplicates, except M analyzed triplicates. Table 22 presents the method precision for the primary pigments plus three types of overall precision: a) the average across all primary pigments for each laboratory (Avg.), b) the average values across all methods for each pigment (A), and c) the average across the D, H, M, and S laboratories (A'). The latter are the methods with both an average precision and accuracy for the primary pigments that are less than the average precision and accuracy across all laboratories (the accuracy results are presented in Sect. 1.5.3). Note that within the A' subset are two of the laboratories that participated in SeaHARRE-1, and all four analyzed all of the HPLC laboratory standards (Table 2). The method precision for a subset of the secondary and tertiary pigments, following the presentation scheme established in Table 22, is presented in Table 23.

The method precision results show the superior average precision is associated with $\left[\mathrm{TChl}\,a\right]$, in almost all cases, and there is a general worsening of precision for the other chlorophylls and carotenoids. Individual instances of method precision for specific methods and pigments, show excellent precision is recurringly possible across all pigments (the bold entries in Tables 22 and 23). The precision of these selected instances ranged from 0.2–6.0%, with an overall average of 2.9%. The narrow range of precision excellence, and the fact that it occurs across three different methods, suggests filter inhomogeneity is not a significant component of the variance, and that methodological differences account for the vast majority of the diversity in the precision results.

One of the purposes of this study is to establish realistic performance metrics for HPLC methods. The average results for the primary pigments in Table 22 show a precision level of 7.4% is routinely achievable (even with damaged samples, the Z results), but that 5.4% (A') is attainable (three methods satisfy this criteria). If the best results of the primary pigments are taken to establish an ultimate threshold of the state of the art, perhaps produced by combining specific elements of the methods with excellent precision to create a new heretofore unavailable method, the average threshold is 2.9% (which is rather close to the overall value for laboratory H). The average of the most precise results is 2.9% for the primary pigments and 2.7% for the secondary and tertiary pigments, so a goal of 3% or less emerges as a reasonable metric for a state-of-the-art precision in the analysis of field samples. Note that for both Tables 22 and 23, the best precision for each pigment is usually from the laboratories in the A' subset.

The Z results in Tables 22 and 23, which are from a set of unequivocally defrosted and degraded samples analyzed by HPL (Table 2), provide additional insights. Perhaps the most troubling aspect of the Z results is the average precision for the primary pigments computed from the Z results is 6.7%, which is better than the average precision of all the methods (A=7.4%). In addition, the range in values of precision, with the exception of the results for $\left[\mathrm{TChl}\,c\right]$ and $\left[\mathrm{Diato}\right]$, is relatively narrow. This indicates a good method applied to questionable samples is preferable to a less capable method applied to good samples. It also suggests degraded samples can be a separate and interesting source of information to better understand individual method capabilities. The latter conclusion is only applicable on average (and not for each specific pigment), and differences in precision between Z and H, or Z and A, for the individual pigments establishes other interesting aspects to the general problem.

The $\left[\mathrm{TChl}\,a\right]$ results are worth considering in more detail, because it is the most important pigment in this study, and because improper sample handling is expected to produce a variety of degradation products, which will increase the variance between individual samples (i.e., between filters). The average precision for $\left[\mathrm{TChl}\,a\right]$ is 4.5% for the A results, and 4.4% for Z, which suggests the sample mishandling did not significantly affect overall precision. The $\left[\mathrm{TChl}\,a\right]$ result for H (the laboratory that analyzed the Z samples), however, is 2.6%, so there is a notable degradation in the individual method precision of H as a result of the samples defrosting.

The other pigments within the family of chlorophyll a pigments are expected to be negatively influenced by degradation effects as well (Chlide a, Phide a, and Phytin a). The H result for $\left[\mathrm{Chlide}\,a\right]$ in Table 23 is 8.2%, but the Z result is 24.7%, which is also higher than the A result of 13.6%. The results for $\left[\mathrm{Phide}\,a\right]$ and $\left[\mathrm{Phytin}\,a\right]$ are not as significantly different: they increase from 2.7% for H, to 8.2 and 9.3%, which are only a little larger than the A results of 4.7 and 8.9%, respectively. These results strongly indicate a degradation in chlorophyll for the Z samples, but with a rather small effect on the precision in determining $\left[\mathrm{TChl}\,a\right]$.

The laboratory standards provide an opportunity to explore additional issues with precision, and to confirm some of the conclusions already formulated. The Mix C analysis is the most appropriate, because of all the laboratory standards, Mix C had the most diverse set of pigments and best replicated the complexity of a field sample (Table 6). There are limitations in using Mix C, however: a) laboratories B and P did not analyze it, b) $\left[\mathrm{TChl}\,a\right]$ did not contain $\left[\mathrm{Chlide}\,a\right]$, c) $\left[\mathrm{TChl}\,c\right]$ did not contain $\left[\mathrm{Chl}\,c_1\right]$, and

Table 22. The $\bar{\xi}$ values (coefficients of variation in percent) across all 12 batches of field samples as a function of the method for the primary pigments. The last column presents the (horizontal) average for the primary pigments. Overall averages for all methods and D, H, M, and S are given in the A and A' entries, respectively. The lowest values for each pigment are shown in bold typeface, and the overall averages in slanted typeface. The Z results are not included in the A or A' overall method (vertical) averages.

Lab.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Avg.*
B	7.1	3.9	9.9	17.5	11.0	7.0	6.8	6.4	12.7	8.4	9.0	7.4	8.9
C	7.7	7.7	19.7	13.3	5.4	14.9	15.6	11.3	24.0	11.8	11.9	23.3	13.9
D	6.0	6.8	9.9	9.6	5.7	4.4	8.8	5.7	4.1	4.8	10.2	8.0	7.0
H	2.6	**2.2**	**3.8**	**3.0**	**3.0**	**1.6**	**2.4**	**1.9**	5.2	2.5	3.4	7.0	**3.2**
L	2.8	3.8	4.9	3.7	3.2	3.4	4.6	3.2	**3.6**	**2.3**	**3.2**	**6.0**	3.7
M	3.7	6.2	6.0	3.9	5.4	3.4	10.1	3.1	4.6	3.5	5.4	6.5	5.1
P	3.7	22.6	17.6	4.4	11.8	6.7	15.4	6.9	20.7	6.5	7.7	6.1	10.8
S	**2.2**	4.3	12.9	9.2	4.4	3.9	5.1	2.2	11.9	2.6	8.5	7.0	6.2
A	*4.5*	*7.2*	*10.6*	*9.4*	*6.2*	*5.7*	*8.6*	*5.1*	*10.9*	*5.3*	*7.4*	*8.9*	*7.4*
A′	*3.6*	*4.9*	*8.1*	*6.4*	*4.6*	*3.3*	*6.6*	*3.2*	*6.4*	*3.4*	*6.9*	*7.1*	*5.4*
Z	4.4	3.4	20.3	7.8	4.4	3.0	1.5	6.0	13.6	3.3	6.3	6.4	6.7

Table 23. The $\bar{\xi}$ values (coefficients of variation in percent) across all 12 batches of field samples for a subset of the secondary and tertiary pigments, following the presentation scheme established in Table 22.

Lab.	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
B	7.5	12.5	37.1		5.9	6.4	8.2	6.7	11.4	3.1	7.4
C	7.9		6.3	18.5			0.0†				29.7
D	6.1	13.9	7.8	25.3	4.3	10.3	13.9		5.3	9.4	26.6
H	3.3	**0.2**	8.2	**0.7**	**3.8**	**3.1**	**2.7**	**2.7**	2.1	**1.7**	6.9
L	2.9	0.7	**4.6**	0.8		8.9		0.0†			**6.0**
M	3.9	0.5	21.0	8.7						8.0†	10.9
P	3.8	0.4	10.0	36.9	0.0†	11.5	10.6		**1.8**	11.5	37.4
S	**2.3**	37.9	14.2	8.5					23.4	7.9	11.0
A	*4.7*	*9.5*	*13.6*	*14.2*	*4.7*	*7.2*	*8.9*	*4.7*	*10.6*	*6.9*	*17.0*
A′	*3.9*	0.3‡	*12.8*	*10.8*	*4.0*	*6.7*	*8.3*	*2.7*	*10.3*	*6.7*	*13.9*
Z	4.5	1.1	24.7	4.6	5.3	10.7	9.3	8.2	1.7	9.3	7.9

† Artificially low, because of a persistent nondetection and not included in the A or A' results.

‡ Laboratories D and S are excluded from the average, because they did not chromatographically determine [DVChl *a*].

Table 24. The $\bar{\xi}$ values (coefficients of variation in percent) for Mix C (Table 6) as a function of the method for the primary pigments using the presentation scheme from Table 22. The lowest $\bar{\xi}$ values from the field samples (Table 22) are given in the last row, A^-.

Lab.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Avg.*
C	**0.0**	2.7	5.5	**0.0**	0.0†	5.1	1.2	7.5	2.2	0.0†	9.7	5.3	3.6
D	3.2	1.3	2.0	1.5	0.8	1.7	**0.1**	**0.7**	2.0	0.0‡	0.5	1.3	1.3
H	0.0	**0.5**	2.3	0.5	**0.1**	**0.6**	1.3	2.0	**0.8**	**0.2**	0.3	**0.4**	**0.7**
L	1.4	3.1	0.0†	2.3	1.2	3.5	1.0	1.1	1.2	1.7	**0.1**	1.2	1.6
M	3.5	2.0	**1.7**	2.8	2.1	4.7	5.0	6.1	2.4	3.3	5.0	1.9	3.4
S	2.0	2.9	5.1	3.0	1.4	3.0	1.5	1.4	1.3	2.2	1.6	1.3	2.2
A	*1.7*	*2.1*	*3.3*	*1.7*	*1.1*	*3.1*	*1.7*	*3.1*	*1.6*	*1.9*	*2.9*	*1.9*	*2.2*
A′	*2.2*	*1.7*	*2.8*	*1.9*	*1.1*	*2.5*	*1.9*	*2.5*	*1.6*	*1.4*	*1.9*	*1.2*	*1.9*
A^-	2.2	2.2	3.8	3.0	3.0	1.6	2.4	1.9	3.6	2.3	3.2	6.0	2.9

† Not detected, so not quantitated (and not included in averages).

‡ Coelutes with Phide *a*, so not individually quantitated (and not included in averages).

d) [Caro] did not contain [$\beta\varepsilon$-Car]. Proceeding with these limitations in mind, it is particularly relevant to evaluate the Mix C results within the context of the A' subset.

Table 24 presents the $\bar{\xi}$ values for Mix C. The overall average of the A' subset is 1.9%, which is 3.5% less than the 5.4% A' average precision for the field samples (Table 22). By comparison, the average for the lowest $\bar{\xi}$ values (denoted A^-) from the field sample analysis is 2.9%. These results suggest the variability associated with nonmethodological processes (e.g., filter inhomogeneity, shipping and handling, etc.) is 1.0–3.5%, or approximately 2% (on average). If [TChl a] is taken as a specific example, the difference between the field sample and Mix C $\bar{\xi}$ values for the A' laboratories ranges from 0.2–2.7%, with an average difference of 1.4%. Given the methodological difficulties with many of the carotenoids, and the fact that the quantitation of [TChl a] represents a superior accomplishment, the average difference between the Mix C and field sample $\bar{\xi}$ values for the A' subset is 1.5%. Consequently, it seems appropriate to assess 1.5% of the average variability in HPLC method precision to nonmethodological processes.

1.5.3 Method Accuracy

Method accuracy for each reported pigment product is determined using $|\bar{\psi}|$, the average APD between the reported laboratory values and the average constructed from the values from all the methods. Although the exclusions already noted in determining method precision (Sect. 1.5.1) are relevant to understanding accuracy, most do not actually change the computations. There are exceptions, however, and the two considered here are false positives and false negatives (which are both specificity problems).

A false positive is the most damaging result, in terms of the individual laboratory or overall uncertainty budgets, because of the relative magnitudes of the concentrations in the uncertainty analysis. Using (6) and (7), the basic accuracy computation is formulated as $|\bar{\psi}| = 100|y - x|/x$, where y is the observation (from a particular method) and x is the reference value (the average concentration from all the methods for field samples). In a false positive situation, x is very small (the pigment is not present, so $x \approx 0$), and y is comparatively much larger ($y \gg x$). Using a multiplicative factor, n, approach, $y = nx$, so $|\bar{\psi}| \approx 100(n-1)$. In most false positive cases $n \gg 1$, and $|\bar{\psi}| \gg 100\%$.

In the false negative situation, the magnitude of y in the $|\bar{\psi}| = 100|y - x|/x$ formulation is very small (the pigment was not detected), and x is comparatively larger. The multiplicative formulation becomes $x = ny$, so $|\bar{\psi}| \approx 100|1 - n|/n$. In most false negative cases $n \gg 1$, so $|1 - n| \approx n$, and $|\bar{\psi}| \approx 100\%$ (in fact it is usually a little less than 100%). This near constancy of approximately 100%, makes false negatives rather easy to discern in a set of results.

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

1.5.3.1 Individual Pigments

For SeaHARRE-2 samples, the recurring source for a false positive was for those methods using the Latasa et al. (1996) simultaneous equations for determining [DVChl a] (methods D and S). The impact of this on the uncertainties in [TChl a] are not very significant (Table 25), because the dominant contribution is from [Chl a], but they are very significant when it comes to the uncertainties in [DVChl a] (Table 26). In fact, the uncertainties for D and S in the determination of [DVChl a] are approximately 10,000% or larger (which represents a 100-fold increase and beyond of the estimated concentration with respect to the true (reference) concentration. Note that the accuracy results for P in the determination of [Neo] is 94.3% (Table 26), which is a consequence of a persistent false negative. Not only are these [Neo] results significantly less than the false positives associated with [DVChl a], but the other uncertainties in the determination of [Neo] are sufficiently high so as to make the P result rather ordinary.

Although the anomalies in the accuracy results provide interesting aspects of HPLC uncertainties, the principal interest in this study is the analysis of the primary pigments, and of these, the chlorophylls are particularly important. The overall (A) accuracy in the determination of [TChl a] is 11.4%, although the A' subset is 7.8%. The latter is virtually the same as the inital 7.9% overall value for [TChl a] achieved with the samples from SeaHARRE-1 (Hooker et al. 2000) before additional QA procedures reduced the value to 5.5% (Claustre et al. 2004). There is a notable increase in the uncertainties associated with [TChl b] and [TChl c], which was also seen in the results from SeaHARRE-1. The only method that actually separated chlorophylls c_1 and c_2 was H, so the concept of what constitutes the best proxy for [TChl c] truth requires some discussion (Sect. 1.5.5.2).

Many of the primary carotenoids have uncertainties exceeding the chlorophyll values, with But-fuco, Allo, and Diato being distinguished by uncertainties exceeding 100% (from S, P, and L, respectively). The overall uncertainty for the carotenoids ranges from 18.2–55.1% with an average of 33.0%, which reduces to 10.1–52.5% and 21.8%, respectively, for the A' subset. In comparison, the average accuracy for the SeaHARRE-1 primary pigments is 26.4% (computed from a reanalysis, because the concept of the

Table 25. The $|\bar{\psi}|$ values (average APD in percent) across all 12 batches of field samples as a function of the laboratory method for the primary pigments, and following the presentation scheme established in Table 22.

Lab.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Avg.*
B	19.5	15.4	**11.7**	21.9	66.1	49.1	32.9	28.8	**16.1**	48.5	42.5	43.8	33.0
C	15.6	27.0	53.8	**7.6**	70.8	59.1	89.8	19.5	54.1	54.9	77.8	27.9	46.5
D	7.4	**14.4**	11.7	9.9	37.5	**7.7**	**28.1**	18.6	37.5	7.6	17.4	24.1	18.5
H	**4.4**	18.6	30.5	18.7	**20.9**	8.5	36.9	**5.0**	27.1	**4.2**	**9.4**	**7.0**	**15.9**
L	7.5	23.9	12.1	54.0	43.3	21.5	46.3	32.3	115.9	28.9	65.7	65.5	43.1
M	8.0	39.5	46.3	16.9	48.0	8.8	52.0	7.1	21.4	7.0	23.8	31.9	25.9
P	17.3	21.1	14.2	18.1	26.5	8.8	126.5	24.2	46.8	11.4	27.6	21.3	30.3
S	11.2	18.5	22.0	19.0	103.4	12.2	28.6	9.6	16.1	5.1	19.9	26.5	24.4
A	*11.4*	*22.3*	*25.3*	*20.8*	*52.1*	*22.0*	*55.1*	*18.2*	*41.9*	*20.9*	*35.5*	*31.0*	*29.7*
A′	*7.8*	*22.8*	*27.6*	*16.1*	*52.5*	*9.3*	*36.4*	*10.1*	*25.5*	*5.9*	*17.6*	*22.4*	*21.2*
Z	19.8	21.0	17.6	39.2	36.0	14.5	38.7	35.0	47.5	17.9	11.9	19.0	26.5

Table 26. The $|\bar{\psi}|$ values (average APD in percent) across all 12 batches of field samples for a subset of the secondary and tertiary pigments, and following the presentation scheme established in Table 23.

Lab.	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
B	19.4	38.4	47.6		100.5	96.0	61.7	42.2	86.2	97.1	20.4
C	13.5		73.1	85.5			86.3†				31.2
D	9.4	37,177.0	89.0	85.3	**60.6**	**40.7**	38.7		**68.2**	**32.8**	23.3
H	**6.1**	9.5	123.8	79.1	69.8	48.5	**40.8**	**42.2**	178.1	43.7	**15.5**
L	8.3	**9.3**	76.2	77.4		49.4		84.5†			48.9
M	8.6	11.4	**29.0**	**74.4**						62.1†	36.3
P	19.7	9.8	73.8	96.2	94.3†	42.3	129.0		84.6	56.1	33.1
S	13.3	9,719.6	50.7	125.2					72.0	93.1	20.1
A	*12.3*	*15.7‡*	*70.4*	*89.0*	*77.0*	*58.6*	*67.6*	*42.2*	*97.8*	*64.5*	*27.9*
A′	*9.3*	*10.4‡*	*73.1*	*91.0*	*65.2*	*44.6*	*39.7*	*42.2*	*106.1*	*57.9*	*24.9*
Z	18.6	11.7	48.1	75.7	69.5	51.3	366.0	250.1	131.7	55.0	26.6

† A persistently undetected pigment (Table 23), but the results are included in all averages (unlike precision, accuracy is not misrepresented by specificity problems).

‡ Excluding *D* and *S*, because they did not chromatographically separate monovinyl and divinyl chlorophyll *a*.

Table 27. The $|\bar{\psi}|$ values (average APD in percent) for Mix C as a function of the laboratory method for the primary pigments using the presentation scheme established in Table 22. The uncertainties are computed using the average concentrations of the pigments as the reference in the uncertainty calculation. Laboratory averages are given in the last column; the entries in parentheses do not include the pigments with poor resolution. The lowest $|\bar{\psi}|$ values from the field samples (Table 25) are given in the last row, A^-.

Lab.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Average*
C	7.2	11.6	44.6†	5.3	99.0†	**0.5**	10.2	11.7	52.3†	98.9†	46.6	30.5†	34.9 (13.3)
D	5.8	**3.7**	**8.2**	13.9	14.1	8.8	12.5	20.9	24.7	98.9†	1.7	9.1†	18.5 (11.4)
H	**0.1**	8.3	29.1	16.5	5.1	9.4	**1.1**	**1.2**	14.1	8.6	10.5	7.7	**9.3** (9.3)
L	9.1	53.1	98.6†	45.0	**0.0**	165.1†	22.4	10.1	72.4	17.7	131.1†	20.3†	53.7 (28.7)
M	12.8	25.5	43.8	24.6	33.1	36.2†	1.5	4.7	**13.7**	**1.6**	35.3	6.1	19.9 (18.4)
S	4.9	11.3	64.7	**4.6**	13.9	13.3†	2.9	4.4	19.9	10.7	**0.8**	**1.6**	12.8 (12.7)
A	*6.6*	*18.9*	*48.2*	*18.3*	*27.5*	*38.9*	*8.4*	*8.8*	*32.8*	*39.4*	*37.7*	*12.6*	*24.9* (15.7)
A′	*5.9*	*12.2*	*36.5*	*14.9*	*16.6*	*16.9*	*4.5*	*7.8*	*18.1*	*30.0*	*12.1*	*6.1*	*15.1* (13.0)
A^-	4.4	14.4	11.7	7.6	20.9	7.7	28.1	5.0	16.1	4.2	9.4	7.0	11.4

† Specificity problem (e.g., $R_s < 1$ and a false negative detection).

primary pigment category was not developed until after the exercise).

The lowest uncertainty is recurringly associated with the H method, which has an overall accuracy of 15.9%. Although this establishes an achievable performance metric, the best results from Table 25 (the bold entries) can be used to establish a performance threshold for the current state of the art. In this approach, almost all of the values come from the D and H methods and the average accuracy (A^-) is 11.4%. This theoretical-best value is only 4.5% less than the average result for the H method, which is very nearly within the average precision for the H analysis of field samples (3.2%), and suggests it is an achievable level of accuracy. Despite the close agreement between the A^- and H results, it is still appropriate to independently test whether or not the A^- result is a realistic threshold for defining a state-of-the-art analysis.

A suitable test can be made by considering the uncertainties in determining the Mix C pigments—the only laboratory standard sufficiently complex, in terms of pigment diversity (albeit with artificial concentration levels), to approximate a field sample. To ensure the results are computed as similarly as possible, the Mix C APD values are computed with respect to the average concentrations provided by all the laboratories that analyzed Mix C. If a method had a specificity problem with a particular pigment (e.g., $R_s < 1$ or a false negative detection), the results for that pigment were not included in the reference average. Table 27 presents the uncertainties for Mix C using the edited reference average. The data show the overall uncertainty for all laboratories (A) is 24.9% and 15.1% for the A' subset. The corresponding A and A' values for the field sample analysis (Table 25) are 29.7% and 21.2%, respectively. The difference between the Mix C and field sample uncertainties, 4.8% and 6.1% for A and A', respectively, includes the effects of nonmethodological factors (e.g., sample inhomogeneity), so the level of agreement is actually closer than these values. The excellent agreement of these values suggests Mix C is an appropriate surrogate for a field sample, and the convergence of the A^- and A' results—particularly for the well-resolved pigments—strongly suggests the A^- value is a suitable and achievable threshold to establish the state of the art.

The Mix C results permit an independent exploration of the lower end of the uncertainty budget; the other end is independently accessible through the Z analyses, which also provides some other insights with respect to the methods. As was seen with the analysis of precision derived from the field samples, the accuracy results of the Z samples (Tables 25 and 26) challenge the partitioning of variance between analytical chemistry and sample handling, i.e., the recurring question of, *"Is it the method, or the samples?"* The overall accuracy of the Z primary pigments is 26.5%, which is smaller than the A value of 29.7%, and only a little bit larger than the A' subset value of 21.2%. Although there is a general increase in the uncertainties of the Z pigments with respect to the corresponding H pigments, most of the individual values agree very favorably with the other methods or the overall averages; indeed, on a pigment-by-pigment basis, the Z result is frequently less than at least one of the individual method results.

The most notable aspect of the Z accuracy results is the large increase in uncertainties associated with the TChl a (and Chl a), Phytin a, and Phide a pigments (note the large value for Pras is in keeping with the H result). There is also a significant change in the $\left[\text{Chlide } a\right]$ accuracies, likely because Chlide a is associated with degradation. The investigation of degradation effects in the chlorophyll a pigments is summarized in Table 28. Using the overall average concentrations (A) as a baseline, the H values are usually lower, except $\left[\text{DVChl } a\right]$ is equal and $\left[\text{Chlide } a\right]$ is higher. The Z values show significant shifts with respect to H: $\left[\text{Phytin } a\right]$ and $\left[\text{Phide } a\right]$ both increase, while $\left[\text{Chl } a\right]$ and $\left[\text{Chlide } a\right]$ both decrease, and $\left[\text{DVChl } a\right]$ remains basically the same. These changes are in agreement with the expected composition shifts of $\left[\text{Chl } a\right]$ and $\left[\text{Chlide } a\right]$, which degrade to $\left[\text{Phytin } a\right]$ and $\left[\text{Phide } a\right]$, respectively. It is important to note the values in Table 28 are overall averages, so the RPD values are just to illustrate large-scale changes.

Table 28. The average A (all methods), H, and Z pigment concentrations (milligrams per cubic centimeter) for the chlorophyll a family of pigments across all 12 field samples. The RPD values are computed for Z with H as the reference.

Pigment	A	H	Z	*RPD* [%]
$\left[\text{Chl } a\right]$	5.834	5.435	4.691	−13.7
$\left[\text{DVChl } a\right]$	0.004	0.004	0.003	−23.9
$\left[\text{Chlide } a\right]$	0.248	0.360	0.112	−68.9
$\left[\text{Phytin } a\right]$	0.104	0.089	0.181	103.6
$\left[\text{Phide } a\right]$	0.196	0.179	0.227	26.8
Total	*6.387*	*6.067*	*5.214*	*−14.1*

It is also important to note all the methods have accuracy problems with at least one pigment, which is usually expressed by the average uncertainty approaching or exceeding 100%. The majority of the problems are with tertiary pigments, however, and not primary or secondary pigments (the tertiary pigments are shown underlined):

B Neo, Viola (and Neo+Viola), and Pras;

C Allo, Lut, and Phytin a;

D DVChl a, Chlide a, and Lut;

H Chlide a and Pras;

L Diato and Phide a;

M Viola;

P Allo, Lut, Neo, Phytin a, and Pras; and

S But-fuco, DVChl a, Lut, and Viola.

Part of the burden on the statistical analysis is to show the problems with these pigments are not artificially created by how the proxy for truth is determined.

Table 29 presents the average APD values for the Mix C secondary and tertiary pigments calculated with the known concentrations as the reference. The data show method H is routinely capable of an uncertainty for these pigments on the order of 4% (on average), but excluding $\left[\text{Chl } a\right]$ and $\left[\text{DVChl } a\right]$, the H field sample uncertainties for the corresponding pigments (Table 26) are significantly higher and on the order of 72% (on average). This is more than an order of magnitude increase in uncertainty, and the most likely explanation is a failure in the referencing system (methodological factors not associated with the HPLC analysis have already been shown to be only on the order of a few percent). The uncertainties for $\left[\text{Lut}\right]$, $\left[\text{Neo}\right]$, $\left[\text{Viola}\right]$, $\left[\text{Neo+Vio}\right]$, and $\left[\text{Phide } a\right]$ (the majority of the problematic pigments) in Table 29 are all distinguished by having one or more methods with low uncertaintes and one or more with very high uncertainties. In such a distribution, the average concentration is not close to the true concentration, and those methods with good accuracy are degraded by those with poor accuracy (and vice versa).

If the Table 29 analysis is recomputed using the average pigment concentration as the reference, the average H uncertainty increases from about 4% to 51%. The suggestion here is that a more restrictive set of methods would be a better reference for the tertiary pigments. This is an unanticipated result, because in the most general sense, any pigment that is produced by a validated methodology—and all the methods in SeaHARRE-2 were considered validated by the laboratories using them—should have the same statistical performance properties as any other (within the accepted variance). This concept assumes pigments with severe quantitation problems (e.g., significant coelution with a neighboring pigment) would never be validated and, thus, would not be reported and included in the reported results (except perhaps with well-documented qualification).

1.5.3.2 Pigment Sums

The uncertainties in the determination of the pigment sums for the field samples are presented in Table 30. The lowest overall average values (A) are associated with the chlorophylls and the photosynthetic pigments, $\left[\text{TChl}\right]$ and $\left[\text{PSP}\right]$, plus the total pigments, $\left[\text{TPig}\right]$; the other carotenoids alone are distinguished by higher uncertainties. The average uncertainties for the individual methods are significantly less than the corresponding $\left[\text{PPig}\right]$ values—on average (A) by almost a factor of two, and for the A' subset by more than a factor of three. The lowest uncertainties are defined by S and H.

The overall Z average is very similar to the overall average (A) of the field samples, they differ by only 2.3%, although the A' subset is 11.4% less and H is 13.0% less. In every case, however, there is a method that analyzed unequivocally good samples that has an uncertainty larger than the corresponding Z value. Although this should not be interpreted as support for the concept of trying to make use of degraded filters, it does demonstrate what can be expected with damaged samples (when analyzed by a high-performance method). The most significant change in the Z versus H determinations of the pigment sums occurs for $\left[\text{PPC}\right]$, that is, the photoprotective pigments appear the most vulnerable to sample mishandling.

1.5.3.3 Pigment Ratios and Indices

The uncertainties in the determination of the pigment ratios and indices for the field samples are presented in Table 31. The lowest APD values occur for the $\left[\text{PSP}\right]/\left[\text{TPig}\right]$ ratio which do not exceed 3.0% and are on average 1.5%, although the A' subset is a little better than this. The $\left[\text{PSC}\right]/\left[\text{TCaro}\right]$ and $\left[\text{TChl } a\right]/\left[\text{TPig}\right]$ ratios are also determined with low average APD values (5.2 and 6.5%, which raises the possiblity for these ratios to be used in QA procedures for individual data sets or larger databases (as long as the ratio has a functional relevance). This idea is most applicable if the ratios are consistent across a large range in (predominantly oceanic) trophic conditions, as has already been shown for the $\left[\text{TAcc}\right]/\left[\text{TChl } a\right]$ ratio (Trees et al. 2000). Note that the average APD for the latter is among the highest values for the various ratios considered.

As noted by Claustre et al. (2004), there is a small increase in the APD values of the indices with respect to the ratios, with the $\left[\text{mPF}\right]$ results significantly better than the $\left[\text{pPF}\right]$ and $\left[\text{nPF}\right]$ values. An interesting aspect of the indices is the Z results are not substantially degraded, and in almost every case are superior to many of the results obtained with good samples. The most significant changes in the Z versus H determinations of the pigment ratios occur for $\left[\text{PPC}\right]/\left[\text{TCaro}\right]$ and $\left[\text{PPC}\right]/\left[\text{TPig}\right]$, which change more than 10%, whereas the changes in the other pigment ratios are 3.5% or less.

1.5.4 HPLC versus Spectrophotometric

Filter extracts prepared for HPLC analysis were also analyzed for the spectrophotometric determination of chlorophyll a, $\left[\text{SChl}a\right]$, by laboratories C, D, H, L, and M using the trichromatic equation of Jeffrey and Humphrey (1975). This analysis was possible because all sample extracts were sufficiently concentrated to satisfy the minimum absorbance values suggested for use with this equation (16), as discussed in Appendix F and Appendix G in Jeffrey et al. (1997).

In the initial submission of results, $\left[\text{SChl}a\right]$ was determined with a variety of procedures for estimating the extraction volume (V_x), which was often based on the HPLC internal standard determination. To provide a set of results that were independent of the HPLC analysis, all the $\left[\text{SChl}a\right]$ values were recomputed using extraction volumes

Table 29. The $|\bar{\psi}|$ values (average APD in percent) for Mix C as a function of the laboratory method for a subset of the secondary and tertiary pigments, following the presentation scheme established in Table 23. The uncertainties are computed using the known concentrations of the pigments as the reference in the uncertainty calculation. Pigments with no entries (Chlide *a* and Phytin *a*) were not present in Mix C. The lowest APD values from the field samples (Table 26) are given in the last row, A^{-}.

Lab.	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
C	73.1			60.0	46.0	69.7					10.8
D	52.1	98.2		32.5	13.9	9.8			16.1	4.6	14.2
H	11.7	**4.5**		**2.3**	**2.2**	**0.3**		**9.0**	**1.0**	**3.6**	**1.7**
L	17.0	37.1		55.8		8.4		42.1			30.8
M	27.1	16.0		11.2		20.3			6.7	79.5	14.5
S	**1.3**	10.2		10.3	26.0	11.4			2.3	7.3	1.7
A	*30.4*	*33.2*		*28.7*	*22.0*	*20.0*		*25.5*	*6.5*	*23.8*	*12.3*
A′	*23.1*	*32.2*		*14.1*	*14.0*	*10.5*		*9.0*	*6.5*	*23.8*	*8.0*
A^{-}	6.1	9.3	29.0	74.4	60.6	40.7	40.8	42.2	68.2	32.8	15.5

Table 30. The $|\bar{\psi}|$ values (average APD in percent) across all 12 batches of field samples as a function of the laboratory method for the pigment sums, and following the presentation scheme established in Table 22. The $|\bar{\psi}|$ values of the primary pigments, [PPig], for the field samples (Table 25) are given in the first column for easy comparison. Laboratory averages for the pigment sums are given in the last column.

Lab.	[PPig]	[TChl]	[PPC]	[PSC]	[PSP]	[TCaro]	[TAcc]	[TPig]	[DP]	*Avg.*
B	33.0	15.2	21.4	40.3	22.5	35.2	25.9	22.4	36.5	27.4
C	46.5	21.8	19.9	49.3	29.8	40.6	41.6	28.6	44.3	34.5
D	18.5	6.0	16.8	6.7	5.9	9.1	6.5	6.7	7.3	8.1
H	**15.9**	**4.2**	9.6	4.1	4.1	4.4	5.7	**4.0**	3.7	5.0
L	43.1	6.3	46.3	30.5	10.8	34.8	23.7	14.3	29.4	24.5
M	25.9	14.8	15.5	5.0	9.2	7.1	5.7	6.9	4.9	8.6
P	30.3	13.3	19.4	10.9	12.4	12.7	10.2	12.9	10.5	12.8
S	24.4	5.6	**8.8**	**3.3**	**3.9**	**3.4**	**3.9**	4.3	**2.8**	**4.5**
A	*29.7*	*10.9*	*19.7*	*18.7*	*12.3*	*18.4*	*15.4*	*12.5*	*17.4*	*15.7*
A′	*21.2*	*7.7*	*12.7*	*4.8*	*5.8*	*6.0*	*5.4*	*5.5*	*4.7*	*6.6*
Z	26.5	14.3	33.0	15.7	14.8	20.5	13.8	16.6	15.0	18.0

Table 31. The $|\bar{\psi}|$ values (average APD in percent) across all 12 batches of field samples as a function of the laboratory method for the pigment ratios and indices (following the presentation scheme established in Table 22).

Lab. Code	$\frac{[\text{TAcc}]}{[\text{TChl } a]}$	$\frac{[\text{PSC}]}{[\text{TCaro}]}$	$\frac{[\text{PPC}]}{[\text{TCaro}]}$	$\frac{[\text{TChl}]}{[\text{TCaro}]}$	$\frac{[\text{PPC}]}{[\text{TPig}]}$	$\frac{[\text{PSP}]}{[\text{TPig}]}$	$\frac{[\text{TChl } a]}{[\text{TPig}]}$	*Avg. Ratio*	[pPF]	[nPF]	[mPF]	*Avg. Index*
B	9.0	4.4	11.4	17.8	**3.8**	**0.4**	4.6	7.3	20.1	12.6	10.1	14.3
C	30.6	15.8	39.2	28.6	20.4	2.2	17.5	22.0	54.7	37.0	25.5	39.1
D	**4.4**	4.1	10.1	3.7	10.8	1.3	**2.3**	5.2	14.8	**6.0**	4.6	8.5
H	8.8	3.5	9.0	2.8	10.0	1.1	4.8	5.7	7.3	7.3	**2.3**	**5.6**
L	17.8	2.9	8.1	26.6	28.2	3.0	8.5	13.6	12.3	13.3	8.3	11.3
M	5.3	4.8	12.0	18.8	19.6	2.2	2.4	9.3	21.9	11.3	4.6	12.6
P	11.2	4.0	10.5	7.1	10.9	1.2	5.7	7.2	12.1	28.9	6.6	15.9
S	12.3	**1.8**	**5.4**	**2.2**	4.9	0.5	5.9	**4.7**	**7.0**	24.9	5.3	12.4
A	*12.4*	*5.2*	*13.2*	*13.4*	*13.6*	*1.5*	*6.5*	*9.4*	*18.8*	*17.7*	*8.4*	*15.0*
A′	*7.7*	*3.6*	*9.1*	*6.9*	*11.3*	*1.3*	*3.9*	*6.2*	*12.8*	*12.4*	*4.2*	*9.8*
Z	10.0	7.4	19.5	9.2	20.7	2.2	5.2	10.6	7.3	8.7	3.0	6.3

based on the $V_x = V_e + V_w$ formulation. The $[\text{SChl}a]$ APD values were then determined using the average $[\text{SChl}a]$ value per sample as the reference, except the concentrations of laboratory L for samples 8, 9, and 11 were not included in the reference, because of an anomalous event during the analysis of the extract for sample 8 and insufficient extract volume for samples 9 and 11. Consequently, the APD values for all laboratories except L include all samples and the APD values for L exclude samples 8, 9, and 11.

APD values for $[\text{SChl}a]$ based on recomputed concentrations for the field samples are presented in Table 32. The SChl a uncertainties encompass a narrower range, 3.2–9.0%, than the corresponding HPLC $[\text{TChl}\,a]$ analyses, 4.4–19.5%. The measurement uncertainty for laboratory C greatly improves with the analysis of SChl a (3.7% APD) relative to TChl a (15.6% APD). The overall average APD values for A (5.5%) and A' (5.3%), which in this instance does not include a contribution from laboratory S, are also lower than the corresponding HPLC TChl a values for A (11.4%) and A' (7.8%). Overall precision with field sample extracts analyzed on the spectrophotometer and HPLC were similar (5.3 versus 4.5%, respectively for A). A notable exception was the much improved HPLC TChl a precision of L (2.8%) relative to SChl a precision (7.7%). APD values were frequently lower for Mix E (a chlorophyll a standard analyzed spectrophotometrically) than for Mix D (components of TChl a analyzed individually by HPLC and including Chl a, DVChl a, and Chlide a).

Table 32. A comparison of accuracy ($\bar{\psi}$) and precision ($\bar{\xi}$) for field samples and laboratory standards analyzed for $[\text{SChl}a]$ and $[\text{TChl}\,a]$ (Mix E represents SChl a and Mix D HPLC TChl a). For field samples, uncertainties are computed with respect to the average, and for laboratory standards with respect to the known concentration. Field sample SChl a values were recomputed using $V_x = V_e + V_w$. The field sample CV values describe the precision in the analysis of duplicate filter extracts (CV of SChl a for samples 9 and 11 were unavailable from C, D, and L because of insufficient extract).

Lab.	*Field Samples*				*Mix E*	*Mix D*
Code	$\bar{\psi}_{S_a}$	$\bar{\psi}_{T_a}$	$\bar{\xi}_{S_a}$	$\bar{\xi}_{T_a}$	$\bar{\psi}_{S_a}$	$\bar{\psi}_{T_a}$
B		19.5		7.1		
C	3.7	15.6	5.6	7.7	0.5	8.7
D	3.8	7.4	5.7	6.0	2.2	**0.8**
H	**3.2**	**4.4**	**3.6**	2.6	0.5	1.6
L	7.3	7.5	7.7	2.8	30.3	6.7
M	9.0	8.0	4.0	3.7	**0.3**	3.5
P		17.3		3.7		9.0
S		11.2		**2.2**		2.7
A	*5.5*	*11.4*	*5.3*	*4.5*	*6.8*	*4.7*
A′	*5.3*	*7.8*	*4.4*	*3.6*	*1.0*	*2.1*
Z	11.4	19.8	3.3	4.4		

The lower APD values for $[\text{SChl}a]$ suggest a lower measurement uncertainty relative to HPLC $[\text{TChl}\,a]$, but this should not be taken as an indication that the average $[\text{SChl}a]$ values are necessarily a better proxy for truth than HPLC $[\text{TChl}\,a]$, because $[\text{SChl}a]$ values are elevated by the presence of degradation products (Appendix F in Jeffrey et al. 1997). The utility of using $[\text{SChl}a]$ as a QA check on HPLC $[\text{TChl}\,a]$ is explored by inspection of the $[\text{SChl}a]/[\text{TChl}\,a]$ ratio for laboratories performing both types of analyses. The average ratio across all samples varied among laboratories, but can be separated into three groups: 1.29 for C; 1.14–1.19 for D, H, and M; and 1.03 for L. Laboratories C and L are excluded from further discussion, however, because the HPLC results for laboratory C exhibited abnormally high uncertainties, and the L results were based on methanol extracts, and not acetone, as specified for use with the spectrophotometric equation.

These results were graphed by selecting the x-axis as the $\big([\text{Chlide}\,a]+[\text{Phytin}\,a]+[\text{Phide}\,a]\big)/[\text{TChl}\,a]$ ratio for each sample observed by laboratory H (the only method routinely reporting Phytin a and Phide a) and the y-axis as the overall average $[\text{SChl}a]/[\text{TChl}\,a]$ ratio per sample (for D, H, and M). A linear regression of the data shows the $[\text{SChl}a]/[\text{TChl}\,a]$ ratio is elevated for samples with a greater relative abundance of Chlide a, Phytin a, and Phide a (the correlation coefficient, slope, and y-intercept are $r^2 = 0.636$, $m = 0.729$, $b = 1.089$, respectively).

A more accurate comparison of HPLC and spectrophotometric results can be made by summing the HPLC chlorophyll a pigments that contribute to the SChl a signal in the spectrophotometer, but for which concentrations observed on the HPLC are converted to their molar equivalent response expected in the spectrophotometer (Appendix E in Jeffrey et al. 1997). Such a comparison was performed with the results for laboratory H, and the new HPLC chlorophyll a amounts agreed on average to within 5% of the $[\text{SChl}a]$ values (rather than the 14–19% agreement observed earlier with D, H, and M). Thus, if extracts are sufficiently concentrated and chlorophyll a degradation products are quantified by HPLC, a spectrophotometric evaluation provides a potentially powerful QA check for HPLC analyses. A fluorometric evaluation of $[\text{Chl}\,a]$ is a commonly used QA check in some methods (e.g., the S method), but an advantage of using spectrophotometry as a quality assurance tool is that it is unaffected by uncertainties associated with calibration.

1.5.5 Quality Assurance Procedures

A substantial effort was made during SeaHARRE-2 to understand the importance of parameters that can be exploited as part of a QA process associated with the overall uncertainty budget. The idea was that if these parameters can be isolated and their influence on uncertainties ascertained, then it should be possible to implement a QA procedure that will allow the uncertainty to be controlled or

minimized. Furthermore, it should also be possible to use the parameter in a performance metric approach for validating HPLC methods. The parameters considered here include:

1. Extraction (uncertainties),
2. Separation (specificity),
3. Injection (precision),
4. Degradation (stability),
5. Calibration (accuracy), and
6. Detection (LOD and LOQ).

This is not a complete list, but it includes many primary aspects for minimizing HPLC uncertainties.

1.5.5.1 Extraction

Uncertainties in the extraction volume, V_x (6), can substantially influence accuracy and precision. The formulation of V_x differed between laboratories, as discussed in Sect. 1.3.1 with respect to (7)–(9), and the differences are summarized in Table 8. The consequences of the differences are considered here.

In the original submission of field sample results, laboratory S determined V_x incorrectly (Sect. 1.5), which caused a systematic overestimation (by approximately 5%) in the concentration of all pigments. When the concentrations were recomputed using a correct determination of V_x, the S average APD for $\left[\mathrm{TChl}\,a\right]$ in field samples improved from 11.2% (Table 25) to 6.1%. This correction also changed the accuracy ranking for laboratory S from fifth (according to Table 25) to second, while the average APD of other laboratories for $\left[\mathrm{TChl}\,a\right]$ changed by less than 1%.

Laboratories D, H, L, M, P, and S determined V_x based on an internal standard, which is commonly assumed to improve accuracy and precision. This assumption was evaluated by recalculating $\left[\mathrm{TChl}\,a\right]$ values for these laboratories using $V_x = V_e + V_w$ (for which $V_w = 0.2\,\mathrm{mL}$). The recomputed concentrations were, except for laboratory M, systematically higher than those based on an internal standard and the average increase per laboratory ranged from approximately 1–8%; the laboratory M concentrations decreased on average by 4.6%.

The effects of revised extraction volumes on the average APD for $\left[\mathrm{TChl}\,a\right]$ are summarized in Table 33. The overall average APD (A) was unchanged, but was slightly improved for the A' subset (6.5 versus 6.3%). Increased uncertainties are, therefore, not an *a priori* consequence of the $V_x = V_e + V_w$ formulation; if they were, the high level of accuracy with the revised computations in Table 33 and the spectrophotometric determination of $\left[\mathrm{SChl}a\right]$ in field samples (Table 32) would not be possible. These results also show how the reference concentrations affect uncertainties for laboratories B and C, for which concentrations had not been recomputed and whose APD values changed simply as a function of the reference concentrations.

Table 33. The average laboratory accuracy $\left(\bar{\psi}_{T_a}\right)$ and precision $\left(\bar{\xi}_{T_a}\right)$ for $\left[\mathrm{TChl}\,a\right]$ based on the data as originally submitted and as revised using $V_x = V_e + V_w$. For the original submission, the results for laboratories D, H, L, M, P, and S are based on an internal standard, and the concentrations for S were recomputed to use a correct determination of V_x).

Lab. Code	*Original*		*Revised*	
	$\bar{\psi}_{T_a}$	$\bar{\xi}_{T_a}$	$\bar{\psi}_{T_a}$	$\bar{\xi}_{T_a}$
B	20.4	7.1	17.9	7.1
C	15.0	7.7	16.7	7.7
D	6.9	6.0	6.0	7.3
H	4.1	2.6	4.4	2.8
L	7.8	2.8	9.3	5.2
M	8.7	3.7	5.0	4.5
P	16.7	3.7	16.8	1.5
S	6.1	2.2	9.7	1.9
A	*10.7*	*4.5*	*10.7*	*4.7*
A′	*6.5*	*3.6*	*6.3*	*4.1*

It is not necessarily true that using an internal standard improves precision. Snyder and Kirkland (1979) note that the overall precision associated with two peak area measurements can lead to a degradation in precision, because each peak is a source of imprecision (the two peak area measurements here are represented by $\hat{A}_c$ and $\hat{A}_s$). In most pigment extractions, the effects of evaporation and uncertainties contributed by the water in the filter are generally assumed to be greater than the imprecision associated with peak area measurements. The results presented here showed that the precision of two laboratories (P and S) worsened by using the internal standard.

1.5.5.2 Separation

Round-robin samples may contain a more diverse set of pigments than that for which participating laboratories' methods were developed. Pigments uncommon to a method can interfere with quantitation of pigments routinely analyzed, unless such interferences are evaluated during method development and validation. The pigments each laboratory routinely reports are detailed in Sect. 1.3.2 and Table 4, and with few exceptions, include all primary pigments. Mix C contained 20 pigments (Table 6) and allowed an evaluation of whether primary pigments could be identified and adequately resolved when in the presence of a multitude of interfering pigments, five of which were tertiary and one ancillary. Chromatograms from laboratories analyzing Mix C are shown in Fig. 8. Pigments in Mix C were suspended primarily in acetone (Sect. 1.2.2) to approximate the extraction solvent of the greatest number of laboratories. Concentrations of pigments that elute near each other were adjusted to produce similar peak heights, which facilitated visual inspection and measurements of

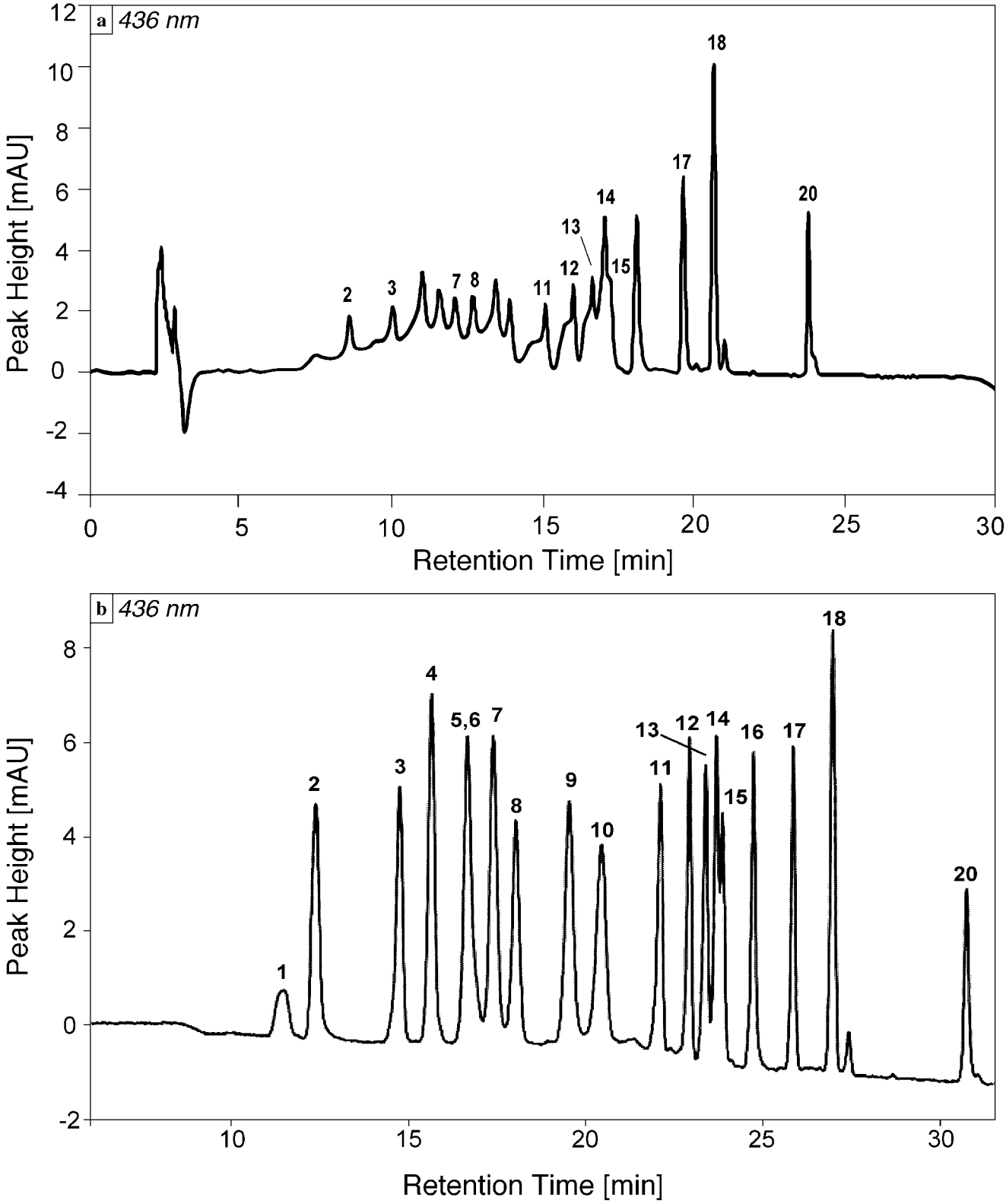

Fig. 8. Chromatograms from the analysis of Mix C for laboratories **a)** *C*, and **b)** *D*. Both chromatograms are from detection at 436 nm. The numbered peaks correspond to the following pigments: 1 Chl c_3, 2 Chl c_2, 3 Perid, 4 But-fuco, 5 Fuco, 6 Phide *a*, 7 Neo, 8 Hex-fuco, 9 Pras, 10 Viola, 11 Diadino, 12 Allo, 13 Diato, 14 Lut, 15 Zea, 16 Cantha, 17 Chl *b*, 18 Chl *a*, 19 DVChl *a*, and 20 $\beta\beta$-Car.

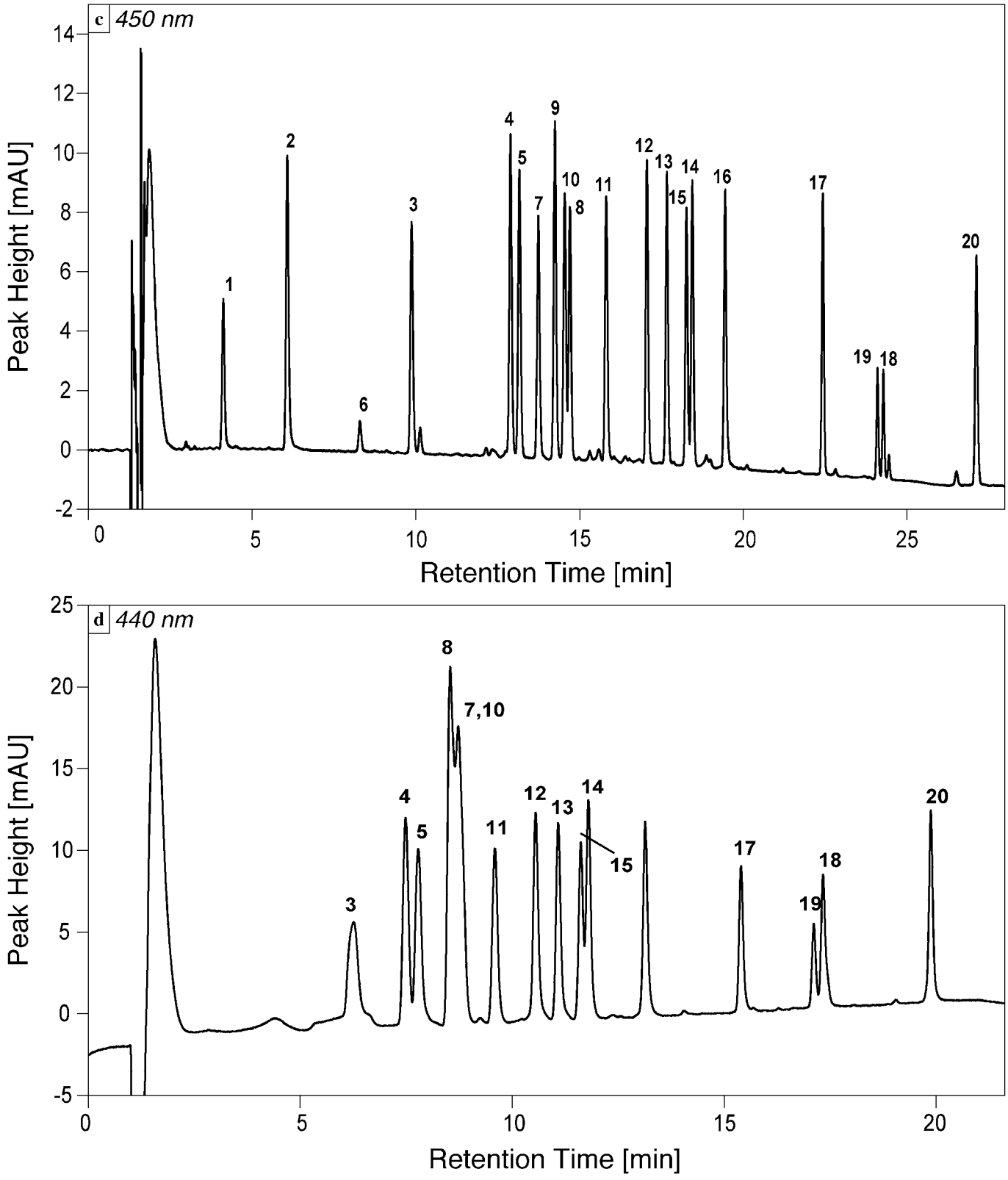

Fig. 8. (cont.) Chromatograms from the analysis of Mix C for laboratories **c)** *H*, and **d)** *L*. The former is for detection at 450 nm, and the latter for 440 nm. The numbered peaks correspond to the following pigments: 1 Chl c_3, 2 Chl c_2, 3 Perid, 4 But-fuco, 5 Fuco, 6 Phide *a*, 7 Neo, 8 Hex-fuco, 9 Pras, 10 Viola, 11 Diadino, 12 Allo, 13 Diato, 14 Lut, 15 Zea, 16 Cantha, 17 Chl *b*, 18 Chl *a*, 19 DVChl *a*, and 20 $\beta\beta$-Car.

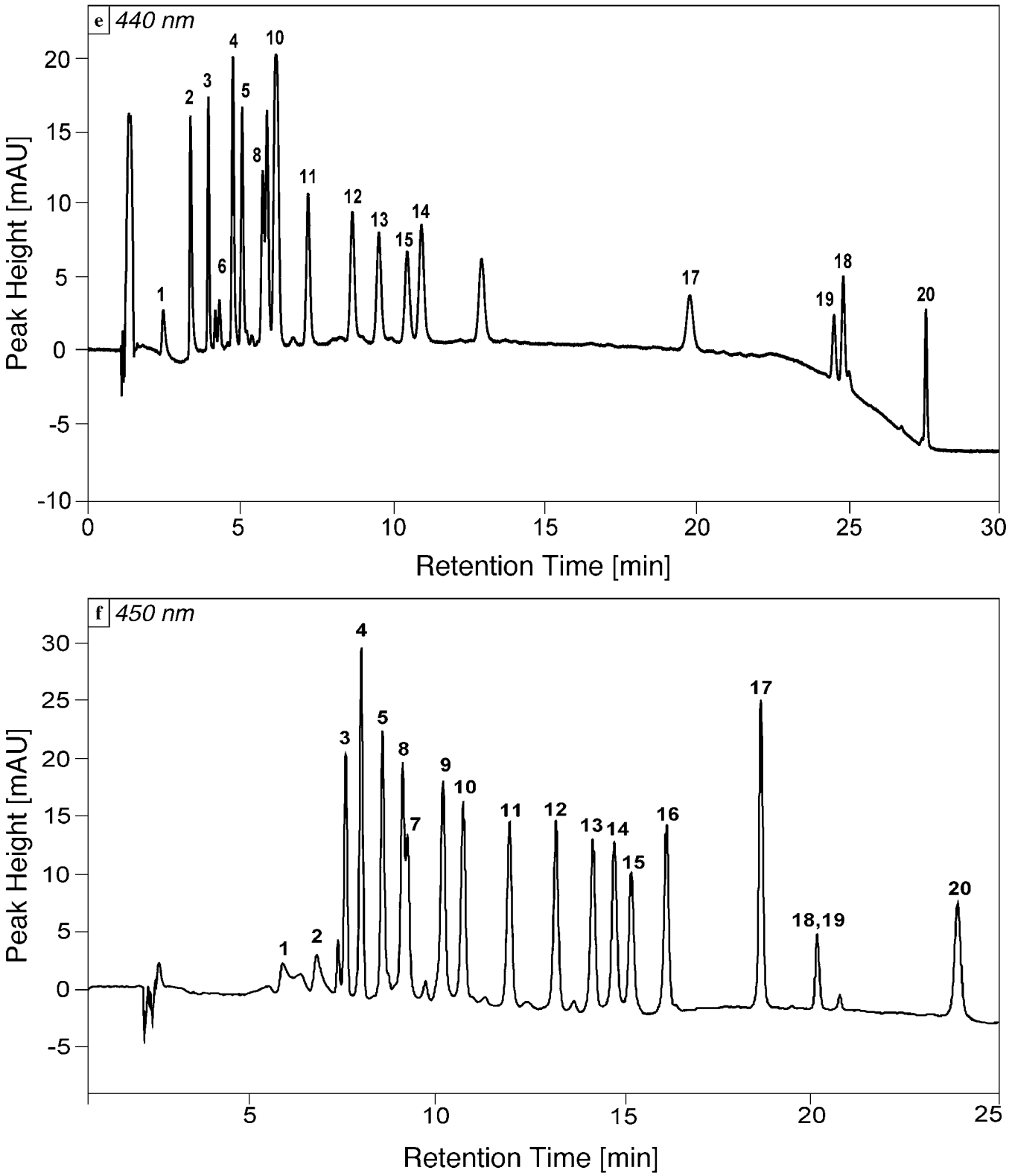

Fig. 8. (cont.) Chromatograms from the analysis of Mix C for laboratories **e)** M, and **f)** S. The former is for detection at 440 nm, and the latter for 450 nm. The numbered peaks correspond to the following pigments: 1 Chl c_3, 2 Chl c_2, 3 Perid, 4 But-fuco, 5 Fuco, 6 Phide a, 7 Neo, 8 Hex-fuco, 9 Pras, 10 Viola, 11 Diadino, 12 Allo, 13 Diato, 14 Lut, 15 Zea, 16 Cantha, 17 Chl b, 18 Chl a, 19 DVChl a, and 20 $\beta\beta$-Car.

peak resolution. These concentrations are not typical of natural samples, so the negative effects of interfering pigments on quantitation of primary pigments is exacerbated.

Laboratories D, H, M, and S (Figs. 8b, 8c, 8e, and 8f, respectively) identified all primary pigments (or components thereof) with the exception that D did not identify DVChl *a*. These laboratories also correctly identified the other pigments in Mix C, except M did not identify Pras, Neo, and Cantha. Based on visual inspection of the M chromatogram (Fig. 8e), the following is speculated: a) Neo coeluted with Viola, b) Pras was misidentified as Cis-fuco, which eluted immediately after but was not fully resolved from Hex-fuco, and c) Cantha eluted freely from adjacent pigments. Such speculations are based in part on previous work by laboratory H with the stationary phase used by M (Van Heukelem and Thomas 2001).

Laboratory L (Fig. 8d) identified all primary pigments (or components thereof) except Chl c_3 and Chl c_2, which were not retained by the stationary phase, presumably because acetone was too strong an injection solvent (with a low polarity index) relative to methanol, for which the method was developed (Table 12). Other pigments not identified by L and C are Phide *a*, Pras, and Cantha. Primary pigments (or components thereof) not identified by C (Fig. 8a) are Chl c_3, But-fuco, Fuco, and DVChl *a* (Viola was also not identified by C). Laboratory C reported atypical baseline behavior when Mix C was analyzed and also evident is peak-shape distortion of many pigments. Peak shape distortion is also present in Fig. 8b for Chl c_3 (D), in Fig. 8d for Peri (L), and in Fig. 8f for Chl c_3 and Chl c_2 (S).

Identification of pigments in Mix C is considered here in the context of their potential for coelution with adjacent pigments. For example, Cantha (peak number 16 in Fig. 8), the pigment that elutes just before Chl *b* in all chromatograms, exhibits baseline resolution ($R_s \geq 1.5$) and therefore does not interfere with quantitation of other pigments, even though most laboratories do not report it. When other pigments in Mix C are unaccounted for, a potential for coelution exists that cannot be completely ascertained. Pigments that were identified in Mix C, and for which an estimate of $R_s < 1.5$ can be determined, are summarized in Table 34. For example, Hex-fuco is not baseline resolved from either Viola, Neo and Viola, Pras, or Neo (H, L, M, and S, respectively); Zea and Lut are unresolved by C, and resolved at levels considered too poor for adequate quantitation ($R_S < 1$) by D and L; and Diato is also unresolved from Lut by C. But-fuco exhibits resolution marginal for quantitation by L and M and the Phide *a* pigment in Mix C coeluted with But-fuco, Fuco and Peri for C, D, and L, respectively. DVChl *a* was not separated from Chl *a* by C, D, and S and for which D and S used a simultaneous equation (Latasa et al. 1996) for quantitation of each component.

Pigments that were not identified, incorrectly identified, or poorly resolved are representative of problems with method specificity, the effects of which are evident with APDs in Table 27 for primary pigments in Mix C. For example, pigments that were not quantified exemplify the false negative scenario and APDs up to 99% are seen in these instances: $\left[\text{TChl } c\right]$ for C and L, $\left[\text{But}\right]$ for C, and $\left[\text{Fuco}\right]$ for C and D (note that D identified Fuco in Mix C (Fig. 8b) but did not quantify it because it coeluted with Phide *a*). Pigments for which $R_s < 1$ usually reflect an APD that is greater than average for that pigment. Specificity problems are most obvious, however, in the laboratory averages, for which results of all pigments are shown alongside results edited to exclude the individual pigment APDs based on false negative values and $R_s < 1$. The average accuracy is remarkably improved for laboratories C and L (by 22 and 25%, respectively), but such exclusions minimally improve results for D, H, M, and S, by a maximum of 7.1% for D. In fact, the small difference in these averages for the A' subset (15.1 versus 13.0) indicates that this subset of laboratories was not severely affected by specificity problems associated with the combination of pigments in Mix C. Inspection of Table 11, however, indicates other pigments, not present in Mix C, that pose other resolution problems for the various methods.

Retention time repeatability and reproducibility are important to the correct identification of pigments. Because Mix C was injected in triplicate, the retention time repeatability over a short time scale was evaluated and the average CV across all identified pigments ranged from 0.02 (H) to 0.16% (S). Chl c_3 and Chl c_2 typically exhibited the poorest retention time repeatability, which averaged 0.24 and 0.13%, respectively, across all laboratories and the best retention time repeatability was often observed for DVChl *a* and Chl *a*, both of which averaged 0.04% across all laboratories.

1.5.5.3 Injection

HPLC analysis precision is estimated using replicate injections on the same day of a sample or standard. The average coefficient of variation for peak area of these injections, $\bar{\xi}_{\text{inj}}$, is referred to here as the injector precision. In its simplest form, injector precision is measured using single-component standards, such as the individual standards from DHI (Mix D) and replicate injections of the internal standard by laboratories that used one. Such precision results are presented in Table 35. Coefficients of variation (percent CV) less than 0.35% are frequent and attained for all pigments evaluated, even though results between pigments and laboratories varied widely (up to and exceeding 10%). The average precision for A is 1.7% and for A', 0.9%. Considered in the context of precision associated with results of a multicomponent mixture (Mix C), where the average CV for A was 2.1% and for A', 1.9%, the analysis of single components implies that complexity of the mixture does not seriously degrade precision. It is unexpected that precision for the internal standard is often

Table 34. The resolution (R_s) between pigments in Mix C. The top panel presents primary pigments or secondary components of a primary pigment sum; the bottom panel contains tertiary and ancillary pigments. If $R_s \geq 1.5$ for a pigment, the cell is empty, and if $R_s < 1.5$, the R_s value and interfering pigment are given. NR indicates no resolution with the interfering pigment, which is listed; NI indicates the pigment was not identified; and MI indicates the pigment was misidentified. Measured values of R_s were not reported by laboratory C and results are based solely on personal communications and visual inspection of chromatograms, in which many pigments exhibited peak fronting and no return to baseline.

Pigment	*C*	*D*	*H*	*L*	*M*	*S*
DVChl *a*	Chl *a* *NR*	Chl *a* *NR*		Chl *a* 1.2		Chl *a* *NR*
Chl *a*	DVChl *a* *NR*	DVChl *a* *NR*		DVChl *a* 1.2		DVChl *a* *NR*
Chl *b*						
Chl c_2				*NI*		
Chl c_3	*NI*			*NI*		
$\beta\beta$-Car						
Allo						
But-fuco	Phide *a* *NR*[1]			Fuco 1.1	Fuco 1.1	
Diadino						
Diato	Lut	Lut 1.2				
Fuco	*NI*	Phide *a* *NR*		But-fuco 1.1	But-fuco 1.1	
Hex-fuco			Viola 1.2	Neo/Viola 0.7	Pras[5] 0.7	Neo[8] ∼0.5
Perid				Phide *a* *NR*		
Zea	Lut	Lut 0.6	Lut 1.4	Lut 0.9		
Cantha	*NI*			*MI*[4]	*MI*[6]	
Lut	Zea/Diato	Diato/Zea[3]	Zea 1.4	Zea 0.9		
Neo				Viola/Hex-fuco	Viola *NR*	Hex-fuco[8] ∼0.5
Phide *a*	But-fuco[1] *NR*	Fuco *NR*		Peri *NR*		
Pras	*NI*			*NI*	*MI*[7]	
Viola	*MI*[2]		Hex-fuco 1.2	Neo/Hex-fuco	Neo *NR*	

Notes:

1 Not reported in the original submission of the results and not resolved.

2 Misidentified as dinoxanthin.

3 The R_s values for Diato/Lut and Lut/Zea are 1.2 and 0.6, respectively.

4 Apparently misidentified as astaxanthin.

5 Hex-fuco was reported as unresolved from *cis*-Fuco which was most likely an incorrect identification of Pras.

6 Misidentified as lycopene.

7 Misidentified as *cis*-Fuco (see note 5 above).

8 Resolution was estimated by visual inspection alone.

Table 35. The injector precision (percent CV) associated with replicate HPLC injections of solutions containing a single component: the individual standards provided as unknowns from Mix D and the internal standard (ISTD) of each laboratory. Laboratories injected the Mix D unknowns in triplicate (except L and S who did duplicate injections). The ISTD precision is associated with replicate measurements of peak area, the $\hat{A}_{c_1}$ term in (7) or $\hat{A}_{c_2}$ in (8) , used by laboratories when they analyzed field samples. Entries in bold face type represent the best precision for each column.

Lab.	[TChl *a*]	[Hex]	[Fuco]	[Peri]	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	*Average*	*ISTD*
C	0.81	†	1.64	1.34	0.62	1.22	1.40	1.17	‡
D	0.91	6.22	3.19	0.92	1.82	1.62	1.03	2.24	1.80
H	**0.22**	0.74	**0.09**	0.36	**0.35**	0.28	0.27	0.33	**0.27**
L	1.08	8.27	12.49	10.39	3.42	1.82	3.20	5.81	3.67
M	0.99	0.37	0.16	1.15	1.21	0.89	0.77	0.79	2.48
P	0.48	2.23	0.91	1.27	1.27	1.66	**0.06**	1.13	11.40
S	0.39	**0.03**	0.35	**0.02**	0.70	**0.20**	0.29	**0.28**	4.57
A	*0.70*	*2.98*	*2.69*	*2.21*	*1.34*	*1.10*	*1.00*	*1.68*	*4.03*
A′	*0.63*	*1.84*	*0.95*	*0.61*	*1.02*	*0.75*	*0.59*	*0.91*	*2.28*

† Sample vial was broken during shipping.

‡ Laboratory C did not use an internal standard.

poor (Table 35) and in all but one instance, exceeds the average for A with Mix D pigments. Peak area precision of the internal standard is especially important because it provides two sources of uncertainty in the calculation equation (7, terms $\hat{A}_c$ and $\hat{A}_s$).

Poor injection precision degrades accuracy with analysis of calibration standards, for which Mix D served as proxy. A possible explanation for poor injection precision can be sought by comparing the volume and composition of the injection solvent with the flow rate and composition of the HPLC solvent at the time of injection. The dissolution solvents in which Mix D pigments are suspended is reviewed in Table 6, gradient conditions are given in Table 10, and a description of the solvent composition, in terms of the polarity index, is given in Table 12. Distorted peak shapes are more likely to occur if the PI of the injection solvent is lower than that of the HPLC solvent when the injection occurs and the effects are exacerbated if a large injection volume is used with a low flow rate; the relationship between injection conditions and peak shape of phytoplankton pigments are evaluated in Zapata and Garrido (1991).

Peak distortion and the accompanying poor precision is most expected with the early eluting pigments in Mix D, Chlide *a*, Peri, Fuco, and Hex-fuco, as is evident in the L results. It is notable, however, that the PI of the sample extract of L is quite different from that of Mix D pigments, and is (appropriately) higher than the HPLC solvent at injection. The poor precision experienced by L for Mix D pigments was not observed with field sample extracts (Table 22). Laboratory D also experienced frequent poor precision (Table 35), but a pipette with poor precision (3.1%) used in the injection process was a contributing factor.

1.5.5.4 Degradation

Typically, a batch of filters is extracted, and HPLC analysis of that batch of sample extracts begins on the same day (Table 3) with the sample extracts residing in a TCAS compartment for 1–30 h during HPLC analysis. The degradation (or stability) of the extracted pigments as a function of temporal evolution, either during analysis or storage, was investigated on a variety of time scales based on the time between extraction and HPLC analysis:

- Short-term, analysis done within 2 days (with the extracts kept in a TCAS compartment and at room temperature);
- Long-term, analysis done within 1 month (with the extracts stored in a freezer); and
- Extended, analysis done after 1 month (with the extracts stored in a freezer).

Although leaving extracts at room temperature or storing them for an excessive amount of time is extreme, there are circumstances that might necessitate analyses well beyond the usually expedient procedures of most methods, e.g., as a result of power or equipment failure. These seemingly anomalous circumstances do occur and are unavoidable, particularly when the samples involved cannot be replaced (i.e., duplicates are not available).

For the purposes of the inquiries into temporal degradation, the initial set of injections in a series of analyses are used as the reference values for the subsequent determination of the RPDs as a function of time. Assuming the initial concentrations are correct, the RPDs give the change or degradation in accuracy as a function of a later time:

$$\psi_{P_i}(t) \;=\; 100\,\frac{C_{P_i}(t) \;-\; C_{P_i}(t_0)}{C_{P_i}(t_0)}, \qquad (30)$$

where t_0 is the initial time and t is a subsequent time, so $t - t_0$ is the elapsed time since the first measurement. All of the temporal degradation analyses were performed by laboratory H, so if a temporal change is persistently of the same sign (positive or negative) and greater than the expected precision of the H method (on average about 3%, and usually less than 7% as shown in Table 22), then a degradation in accuracy is considered to have occurred (remembering that this is not a reflection of method accuracy, but simply a consequence of pigment stability).

To facilitate a compact summary of the stability results, three principal variables are used to describe pigment degradation. These variables provide the average degradation of:

1. All the pigments constituting the total carotenoids, TCaro (i.e., Caro, Allo, But-fuco, Diadino, Diato, Fuco, Hex-fuco, Perid, and Zea);
2. The total chlorophyll pigments, TChl (i.e., TChl *a*, TChl *b*, and TChl *c*); and
3. All the pigments classified as a primary pigment, PPig (i.e., all the pigments in TCaro and TChl).

In some cases individual pigment degradation results are discussed to establish the most important aspects of the group response or because of the singular importance of a particular pigment (e.g., TChl *a*).

1.5.5.4.1 Short-Term Stability

Pigment stability as a function of time (here about 50 h) in the (4°C) TCAS compartment (Table 12), was investigated using replicate field samples from two sites: 10 from the Benguela Current and 10 from the Mediterranean Sea at the *Dynamique des Flux en Méditerranée*† (DYFAMED) site (which are associated with eutrophic and oligotrophic environments, respectively). In addition, two different extractions were performed on all the samples, one using acetone and the other using methanol. The analysis sequence was established by analyzing an initial set of replicate injections of both extracts at a residence time of 2.375 h, and then analyzing seven more replicate

† Translated as "Dynamics of Fluxes in the Mediterranean."

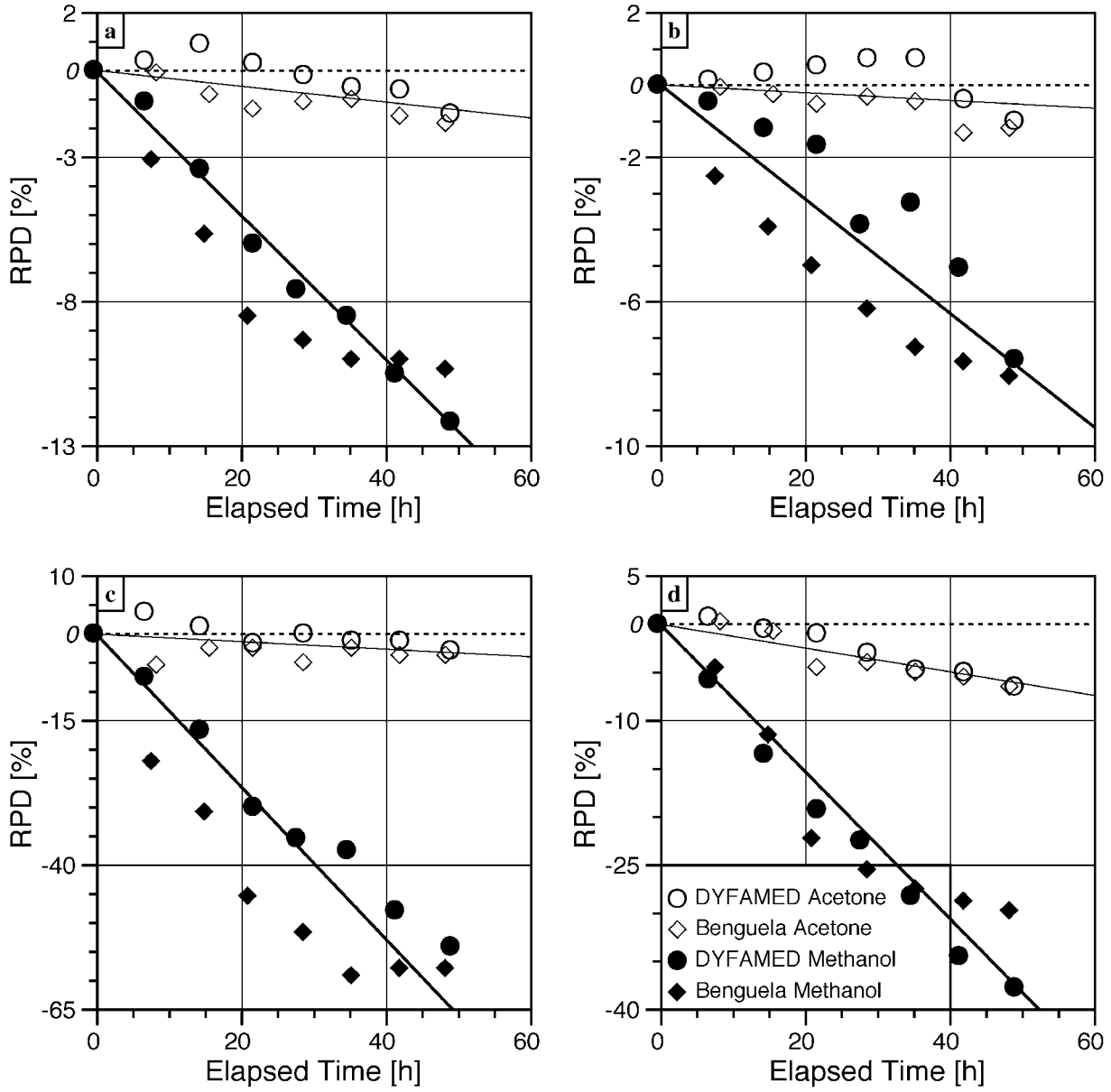

Fig. 9. The change in accuracy as a result of elapsed time in the TCAS compartment determined for **a)** the average degradation of all the PPig pigments, and the individual chlorophylls, **b)** TChla, **c)** TChlb, and **d)** TChlc. Two types of sample extraction procedures are shown, acetone (open symbols) and methanol (solid symbols), for the DYFAMED (circles) and Benguela (diamonds) field sample replicates. The thick and thin solid lines are linear regression fits (forced through zero) for the methanol and acetone data, respectively. The slopes of the fitted lines are as follows:

PPig $-0.7\,\%$/day for acetone and $-6.0\,\%$/day for methanol (remembering that the PPig results are the average for all the individual PPig pigments);

TChl a $-0.3\,\%$/day for acetone and $-3.8\,\%$/day for methanol;

TChl b $-1.6\,\%$/day for acetone and $-31.6\,\%$/day for methanol; and

TChl c $-3.0\,\%$/day for acetone and $-18.3\,\%$/day for methanol.

The dashed line corresponds to an RPD value of 0% (no change with respect to the initial conditions). Although the extent of the x-axis is always the same, note that the y-axis changes from panel to panel.

injections over the next (approximately) 50 h. The final set of samples in the sequence were also a set of replicate injections.

It is useful to first consider the average percent changes in concentrations over the 50 h time period with respect to the initial conditions. Note that the average here corresponds to an analysis time of $t \approx 24$ h in the TCAS compartment. For most methods, analyses occur within 24 h of extraction (Table 3), so the average changes presented here can be considered an upper limit. For the acetone extracts, the average change for the PPig pigments was about −4.1%, which is only marginally within the realm of the degradation threshold (about 3% on average). If the chlorophylls and carotenoids are considered separately, however, the average change for the TChl pigments was −7.2% and the average change for the TCaro pigments was −3.0%. Chlorophylls *b* and *c* emerge as the least stable pigments and the overall changes are comparable to the A' uncertainty in determining $\left[\text{TChl } a\right]$ (Table 25).

The average change in the PPig pigments extracted in methanol was −10.0%, which means they are less stable than the acetone extracts, but this is primarily because of an enhanced instability of the chlorophylls: the average change in the TChl pigments was −34.7%, whereas the average change in the TCaro pigments was only −2.4%. The greater instability of the chlorophylls deserves additional investigation, but it is useful to present this in comparison to the primary pigments, so the derived parameters can be considered within an overall perspective.

Figures 9a–9d present the change in accuracy as a function of the elapsed time in the TCAS compartment for the determination of the average change in the PPig pigments, plus the individual TChl *a*, TChl *b*, and TChl *c* pigments, respectively. The average degradation in accuracy depends strongly on the extraction solvent, with methanol always exhibiting greater sensitivity than acetone. Although the Benguela methanol data exhibit the largest differences, overall there is not much difference between the DYFAMED and Benguela data, which means the results are not strongly dependent on the initial seawater concentration levels.

The strong correlation of the DYFAMED and Benguela data in Fig. 9, plus the well-resolved linear deterioration in accuracy as a function of time, suggests robust degradation rates can be estimated. The acetone degradation rates (per day) always produced a one-day deterioration in accuracy (−0.3 to −3.0%) that was within precision expectations for laboratory *H*, but this is not true for the methanol data. The average degradation rates for the PPig pigments and TChl *a* in methanol yield one-day accuracy deviations within expectations (−6 and −3.8%, respectively), but the TChl *b* and TChl *c* one-day degradations are significantly enhanced (−31.6 and −18.3%, respectively).

The well-resolved linear deterioration of the data in Fig. 9—particularly the acetone extracts—suggests a temporal correction scheme for the concentration of the pigments is worth investigating. Although the data here are well resolved with respect to time, they are not sufficiently diverse as a function of concentration, so a more thorough characterization of the problem as a function of initial concentration and time is needed.

The good stability results for the PPig pigments suggest higher-order averages will be less sensitive to degradation as long as they are composed of a substantial diversity of carotenoids. The average linear regression slopes (forced through zero) for the respective acetone and methanol determinations of the TCaro pigments for the Fig. 9 data, for example, are −0.2 %/day and −0.6 %/day. In contrast, the acetone and methanol average regression slopes for the TChl pigments are −1.6 %/day and −17.9 %/day, respectively, even though the average TChl degradation rates are moderated by the excellent stability of TChl *a*.

The analysis of field samples 9 and 11, mesotrophic and eutrophic, respectively (Table 20), included additional HPLC analyses of acetone sample extracts after 24 h in the TCAS compartment. The analysis of the *H* results for these data provides an opportunity to verify the basic results of the more detailed short-term stability experiment (Fig. 9). For samples 9 and 11, the smallest average degradation occurs for TChl *a* (−1.2%) with larger values for TChl *b* (−1.8%) and TChl *c* (−1.9%), as well as for the average of the pigments comprising PPig (−2.4%). Although there are differences with respect to Fig. 9, the differences are within the precision of method *H*.

There was also an experiment where a set of acetone and methanol extracts were left in a bench drawer at room temperature for approximately two days. Despite this extreme mistreatment, not all of the degradation was severe. The average degradation (over the two-day period) for the TCaro pigments and TChl *a* in acetone was only −1.1 and −3.3%, respectively, which is similar to the two-day degradation values for the TCAS stability experiment. The TCaro and TChl *a* values for the methanol extracts were −5.4 and −26.1%. Although both results are substantially worse than the corresponding acetone values, the low (average) degradation for the TCaro pigments is within the precision range for method *H* and is another strong indicator of the robustness of the carotenoids to temperature effects.

1.5.5.4.2 Long-Term Stability

To investigate the consequences of long-term storage on pigment extract stability, a portion of the large extract volume prepared for the short-term stability experiment was also analyzed with duplicate injections after 7, 14, and 21 days of storage in a −15°C freezer. The average precision (percent CV) for the acetone and methanol extracts across all duplicate injections were 2.3% and 2.1%, respectively, so the concentrations in acetone and methanol were determined to within the expected precision for laboratory *H*.

The average degradation in the TChl pigments as a result of storing the extract in a freezer for 1–3 weeks is

presented in Fig. 10. Although all of the acetone data (open symbols) and the DYFAMED methanol data (solid circles) show a fairly constant degradation with respect to time, the Benguela methanol data (solid diamonds) do not: after an initial large decrease, the degradation rate slows considerably. Nonetheless, the overall degradation of the methanol extracts is significantly more severe than the acetone extracts (by more than a factor of four). The data show that if uncertainties are to be kept to within the average precision of the method (on average about 3% for H), then any long-term storage of the extract is not advisable. Again, if storage is required, the possibility of correcting the concentration of the acetone extracts appears to be worth attempting (after a more thorough characterization as a function of concentration and time).

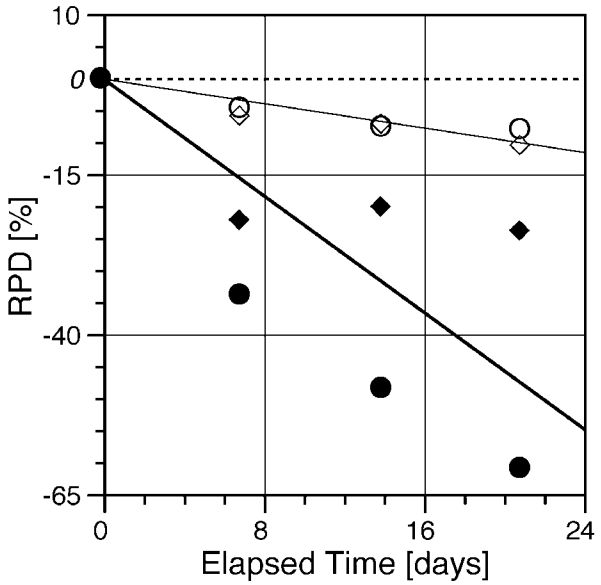

Fig. 10. The average degradation in accuracy for the determination of the extracted TChl pigments as a result of extended freezer storage (following the presentation scheme in Fig. 9). The linear regression slopes (forced through zero) for the acetone and methanol data are −0.5 %/day (thin line) and −2.3 %/day (thick line), respectively.

The TChl results include the very sensitive TChl *b* and TChl *c* pigments. If TChl *a* is considered separately, the degradation rates for acetone and methanol are −0.2 and −0.7 %/day, respectively. A one-week storage of an acetone extract would, therefore, yield only a −1.4% average degradation in the accuracy of [TChl *a*]. The average degradation rates for the carotenoid-dominated pigments comprising PPig and TCaro are −0.3 %/day and −0.2 %/day for acetone, respectively, and −0.7 %/day and −0.2 %/day for methanol, respectively. Again, the low rates for acetone extracts suggest a limited number of days of freezer storage will not result in additional uncertainty greater than the precision of the H method.

Taken together, the long-term freezer results confirm the basic results of the short-term experiments:

1. Pigment extracts in acetone are significantly more stable than extracts in methanol,
2. TChl *a* in acetone is very stable, and
3. The carotenoids are more stable than the chlorophylls (including TChl *a*).

It is important to note the short-term degradations presented in Sect. 1.5.5.4.1 are an intrinsic part of the long-term experiments, but the two are kept separate here to permit individual analyses of the different time scales involved.

1.5.5.4.3 Extended-Term Stability

A five-month (155-day) analysis was also performed on six acetone extracts from the SeaHARRE-2 field samples analyzed by H. The results for this analysis are presented in Table 36, which are based on the following sample distribution: four extracts were from the properly frozen samples and two were from the defrosted Z samples (Table 25). The consistency of the analysis (and the H method) is well demonstrated by the excellent agreement of the CV values between the two data types (including the expected increases in TChl *a* as shown in Table 22). The CV results indicate that both sets of pigments degraded rather uniformly, with TChl *a* and the carotenoids emerging as the most stable pigments.

Table 36. The average percent degradations for the properly frozen and accidently defrosted (during shipping) field samples after the acetone extracts were stored for 155 days in a −15°C freezer. The CV for the average values are given in the following rows and are shown in slanted typeface.

Field Sample Type	PPig [%]	TChl [%]	TCaro [%]	TChl *a* [%]
Frozen (H)	−11.8	−23.7	−7.8	−8.7
(CV)	*2.0*	*3.3*	*1.9*	*1.3*
Defrosted (Z)	−14.2	−26.5	−8.9	−8.0
(CV)	*3.3*	*6.7*	*1.9*	*2.4*

As noted earlier (Sect. 1.5.5.4.2), the longer-term experiments did not include sufficient shorter-term analyses to distinguish the two time scales. If they did, it is very likely the temporal response would in fact be better described by a nonlinear function for some pigments or extraction solvents: an initial rapid decay followed by a slower rate of degradation (and, thus, approximating an exponential curve).

1.5.5.5 Calibration

Laboratories B, L, M, P, and S purchased several calibration standards from DHI and all but S remeasured

Table 37. The R' values of each pigment in Mix A relative to Mix B (from triplicate analyses), for which individual pigment R values were based on the concentration formulated at HPL. To produce each mix, the concentrations of the standards were determined spectrophotometrically using the same ethanol absorption coefficients as DHI (Mix A) or alternatives suggested in Jeffrey (1997) for 100% acetone (Mix B). The concentration of acetone in Mix B was adjusted to 90% before distribution. Underlined results (C and L) have poor precision (the CV of replicate injections of either mix exceeded 9%).

Lab.	[Peri]	[Fuco]	[Hex]	[Diad]	[Allo]	[Zea]	*Average*
C	1.306	1.249	1.075	0.914	1.329	0.923	1.133
D	0.911	0.904	0.791	0.778	0.871	0.848	0.850
H	0.961	0.959	0.840	0.838	0.931	0.921	0.908
L	1.448	0.912	1.011	0.852	0.960	0.932	1.019
M	1.023	1.004	0.887	0.866	0.968	0.958	0.951
S	1.036	1.042	0.907	0.896	1.007	0.912	0.967
A	*1.114*	*1.012*	*0.918*	*0.857*	*1.011*	*0.916*	*0.971*
A′	*0.983*	*0.977*	*0.856*	*0.845*	*0.944*	*0.910*	*0.919*

the concentrations spectrophotometrically (Table 13). The RPD of these measurements with respect to the concentrations supplied by DHI ranged from −32.0 to 17.2%, and the average APD per laboratory ranged from 2.5–19.2% (based on using the same absorption coefficients as DHI). To what degree these RPD values reflect changes in concentration or an uncertainty in the spectrophotometric measurement is not known. Other spectrophotometric interlaboratory calibrations yielded APDs (with respect to known concentrations) typically less than 1% (Mix E results in Table 32) and 1.4% (Van Heukelem et al. 2002) for Chl *a*; for a variety of carotenoids and chlorophylls, the average APD was 3.5%, with infrequent higher APDs not exceeding 11% for some chlorophylls†. In other studies, Chl *a* results (relative to the average consensus) were within ±4%‡ and ±6%, with 10% of results poorer than this (Latasa et al. 1996). Dunne (1999) suggests uncertainties with results of Latasa et al. (1996) are in part a result of low concentrations, for which absorbance was less than 0.18.

Laboratories D, H, P, and S used the same absorption coefficients for the primary pigment carotenoids, but other laboratories used varying absorption coefficients—most notably M and C—for which C used only absorption coefficients in acetone, instead of the more common ethanol values (Table 14). Even absorption coefficients which differ, but use the same solvent, can cause differences approximating 23% (Hex-fuco), 19% (But-fuco), 9% (Diad) and 5–6% (Fuco and Diato), using absorption coefficients of M, relative to those of DHI, as examples. Absorption coefficients for most chlorophyll pigments were more similar among laboratories (Table 15), except for the pigments in [TChl *c*], for which λ was frequently either in the blue (443–453 nm) or red (near 630 nm).

The consequences of using different absorption coefficients (acetone versus ethanol) on quantitation were evaluated by comparing response factors from the HPLC analysis of pigments suspended in ethanol (Mix A), for which concentrations were determined using DHI absorption coefficients in ethanol, relative to the same pigments suspended in 90% acetone (Mix B), for which concentrations were determined with absorption coefficients in 100% acetone (Jeffrey et al. 1997). R was computed for each pigment in Mix A and Mix B for each laboratory based on their reported HPLC peak areas and the known concentrations (as formulated at HPL). The R_{P_i} value in ethanol, relative to the R_{P_i} value in acetone is the relative response factor, R'_{P_i}. Note that if $R'_{P_i} = 1$, each value of R_{P_i} yields the same quantitative result for that pigment, i.e., the response factor in acetone is the same as in ethanol.

A summary of the R' values for each pigment in Mix A relative to Mix B is presented in Table 37. The R' values for the individual pigments range from 0.778–1.042, but most are less than 1 (underlined values are excluded because the precision was poor). The selected ethanol absorption coefficients generally produce lower results than the selected acetone absorption coefficients, and the bias is most pronounced for Diadino and Hex-fuco (0.845 and 0.856, respectively for A'). If absorption coefficients in ethanol for Fuco, Hex-fuco, and Diad are the ones used by M, not DHI, R' approaches 1 more frequently and is 1.025, 1.054, and 0.917, respectively, for the A' subset. In addition, there is a consistent pattern among laboratories, in that D always has the lowest R' value across all pigments and S has the highest (with the exception of Zea).

HPLC calibration accuracy was evaluated with pigments in Mix D, Mix A and Chl *a* in Mix B (Table 2), for which all concentrations were based on the absorption coefficients used by DHI. RPDs (with respect to the known concentrations) are expected to be smaller for laboratories

† Derived from a spectrophotometric round robin led by L. Schlüter (DHI) and reported in an unpublished summary to all participants.

‡ Derived from a spectrophotometric round robin led by R. Ellison (Turner Designs, Inc.) and reported in an uncirculated monograph to all participants.

Table 38. The calibration accuracy of standards analyzed as a single component in Mix D (M_D) or as part of a simple mixture in Mix A (M_A) or Mix B (M_B). Accuracy is described by the RPD with respect to the known concentration for 10 pigments (Sect. 1.2.3): three unique to Mix D (center-left), three unique to Mix A (center), and four common to Mix D and Mix A or Mix B (center-right). For each of the latter pigments, the difference in APD between standard mixtures is $\delta|\psi|$, which is averaged over all four pigments and shown with the average method precision (ξ) for replicate analyses of primary pigments in Mixes A, B, and C. RPD values for M' are based on DHI absorption coefficients, and A'' is the average of D, H, M', and S (overall averages are computed using APD values).

Lab.	C_{T_a}	C_{D_a}	C_{C_a}	C_A	C_{Dd}	C_Z	C_a		C_H		C_F		C_P		*Average*	
Code	M_D	M_D	M_D	M_A	M_A	M_A	M_D	M_B	M_D	M_A	M_D	M_A	M_D	M_A	$\delta\|\psi\|$	ξ
C	8.7	24.6	6.4	−27.4	17.4	18.4	−0.5	0.3		−58.6	−20.7	−63.6	−62.5	−44.6	20.4	7.1
D	−0.8	3.8	0.6	7.1	−3.9	−5.5	−4.7	−7.2	−2.9	−18.7	2.5	1.7	0.7	−3.8	5.6	2.0
H	−1.6	−2.5	−0.5	0.3	0.7	−0.4	−1.6	3.2	−0.3	0.7	0.3	0.9	1.2	−3.1	1.1	0.8
L	6.7	3.1	16.6	12.3	11.1	23.6	3.3	0.7	−13.2	−12.6	6.8	30.0	1.9	−9.6	8.5	2.4
M	3.5	23.7	−19.4	−8.0	−3.4	−11.0	3.4	0.0	26.1	17.9	4.4	3.9	1.5	−0.5	3.3	3.0
M′					*−11.1*				*2.5*	*−4.2*	*−0.5*	*−1.0*				
P	9.0	−0.8	37.3				−1.2		0.5		0.6		−2.6			
S	2.7	6.5	8.9	−0.3	−2.7	−6.8	−3.5	3.6	−7.5	−6.3	−7.1	−6.4	−7.4	−6.6	0.7	3.1
A	*4.7*	*9.3*	*12.8*	*9.2*	*6.5*	*10.9*	*2.6*	*2.5*	*8.4*	*19.1*	*6.1*	*17.7*	*11.1*	*11.4*	*6.6*	*3.1*
A′	*2.1*	*9.1*	*7.4*	*3.9*	*2.7*	*5.9*	*3.3*	*3.5*	*9.2*	*10.9*	*3.6*	*3.2*	*2.7*	*3.5*	*2.7*	*2.2*
A″					*4.6*				*3.3*	*7.5*	*2.6*	*2.5*				

using these same absorption coefficients. Pigments comprising Mix D were provided individually (from DHI) and pigments in Mix A and Mix B (from HPL) were provided in simple mixtures that did not create problems with resolution or detection limits. The pigments in Mix D were more concentrated than the pigments in Mix A and Mix B, by 5–8 fold for carotenoids and 30 fold for Chl *a*.

RPD values for the Mix D individual pigments are provided in Table 38 according to three classifications or sets:

1. Pigments exclusively present in Mix D,
2. Pigments exclusively present in Mix A, and
3. Pigments common to Mix D and another mixture (either Mix A or Mix B).

RPD values to within 1% were observed for each pigment in each mix by at least one laboratory. Many RPDs were in excess of ±15%, especially for laboratory *C*, which, for carotenoids, was related to the use of significantly different absorption coefficients than those used by other laboratories.

To illustrate the effects of different absorption coefficients, four carotenoid results for M are recomputed according to the commonly used absorption coefficients, and the new results are presented as M' in Table 38. In addition, the overall average of D, H, M', and S is given as A''. The M' RPD values for Hex-fuco are the most improved (by approximately 23%) and considering results for all six carotenoids in the table, the average values for A, A', and A'' are now 10.6, 4.9, and 4.2%, respectively. The Chl *a* RPD values are low across all laboratories, mixtures, and subsets (the average APD is never greater than 3.5%) and the TChl *a* RPD values range from −1.6 to 9.0% with an average APD less than 5% in A and A'. DVChl *a* and Chlide *a* were more problematic and RPD values ranged between −19.4 and 37.3% in several instances.

Differences between APD values of individual pigments ($\delta|\psi|$) in Table 38 (center-right), should, converge with the method precision. Such a convergence indicates a) accurate calibration across the range of concentrations provided, and b) low uncertainties in the preparation of standards. Average $\delta|\psi|$ values are presented for each laboratory alongside the average method precision, as described by the average percent CV of primary pigments in replicate injections of Mixes A, B, and C. For all but laboratory *C*, the average $\delta|\psi|$ differs from the method precision by 6% or less and differs on average by 3.5 and 0.5% for A and A', respectively. Consequently, it is routinely possible for uncertainty in the analysis of a single component to approximate the uncertainty for that same pigment when it is found in a simple mixture with no interfering pigments.

Theoretically, uncertainties should be similar whether a pigment is analyzed as a constituent of a simple mixture or as part of a complex mixture, if the method involved is fully validated for the pigments being quantified. Whether or not this was achieved in practice during SeaHARRE-2 was evaluated by comparing the APD of an individual pigment in Mix B (a simple mixture) with its APD in Mix C (a complex mixture). APD values are computed with respect to the known concentrations, and small values of $\delta|\psi|$ describe the potential for accurate quantification, regardless of complexity. Such comparisons are possible because: a) the stock standards used to formulate Mix B were also used to prepare Mix C; b) the absorption coefficients did not vary between mixes; c) the dissolution solvent was primarily the same (Table 6); and d) none of the pigments

Table 39. The effects of analysis complexity on calibration accuracy are described by the change in APD ($\delta|\psi|$) for seven pigments analyzed in Mix B versus their analysis in Mix C, for which many specificity problems occurred (false negative values or chromatographic coelution). The laboratory average $\delta|\psi|$ is shown for all seven pigments and for a subset of pigments (Perid, Fuco, Hex-fuco, and Chl *a*) for comparison with Table 38. The lowest values are shown in bold typeface.

Lab.	[Peri]	[Fuco]	[Hex]	[Diad]	[Allo]	[Zea]	[Chl *a*]	*Ave.* $\delta|\psi|$
C	32.9	44.8†	32.3	3.3	7.5	31.2‡	72.7‡	32.1 (45.7)
D	8.4	92.7†	6.3	7.3	9.6	16.2‡	44.9‡	26.5 (38.1)
H	6.7	**0.6**	**1.1**	**0.1**	1.2	3.3	8.5‡	**3.1** (**4.2**)
L	61.4‡	7.6	91.6‡	0.4	9.4	8.8‡	16.3	27.9 (44.2)
M	**1.2**	1.9	6.0‡	8.0	9.3	3.0	27.1	8.1 (9.0)
S	18.2	2.1	4.0‡	4.5	**0.6**	**1.5**	**2.2**‡	4.7 (6.6)
A	*21.5*	*24.9*	*23.5*	*3.9*	*6.3*	*10.7*	*28.6*	*17.1* (24.6)
A′	*8.6*	*24.3*	*4.3*	*5.0*	*5.2*	*6.0*	*20.7*	*10.6* (14.5)

† False negative. ‡ Chromatographic coelution.

were near detection limits, even though the Mix B carotenoids were 4–6 times more concentrated and Chl *a* was 0.7 times less concentrated.

Stock pigment standards are very rarely completely free of isomers and epimers, which can cause coelution problems and become more problematic as the complexity of the mixture increases. This problem is minimized if the chromatographic purity of the stock pigment standard is high (greater than 95%), as it was for all standards in this presentation except DVChl *a*, for which the chromatographic purity was 80%. Not all laboratory methods were developed to separate and quantify all pigments in Mix C (Tables 4 and 5), but the methods capable of separation are expected to have lower average $\delta|\psi|$ values.

Results are shown in Table 39 for the seven pigments in common to both Mix B and Mix C. The lowest $\delta|\psi|$ value for each pigment is indicated in boldface type, and averages 1.0% across all pigments, which is similar to the best method precision (0.3%) for these pigments in Mix C (Table 24). Considering the overall average results first (A), Allo, Diad, and Zea have the lowest average $\delta|\psi|$ (not greater than 10.7%), while the average $\delta|\psi|$ for the other pigments ranges from 21.5–28.6%. In the A' subset, the average $\delta|\psi|$ for Allo and Diad is relatively stable (within 1% of the A results), but substantial reductions in average $\delta|\psi|$ values (from about 5–19%) are seen for other pigments in A' except Fuco, for which $\delta|\psi|$ remains elevated.

Elevated $\delta|\psi|$ values are largely explained by coelution problems (Table 34 and Fig. 8) and C and D false negatives for quantifying Fuco (Table 27). Allo and Diad exhibited no coelution problems (Table 34), with an exception for H and L (Table 11). High $\delta|\psi|$ values for Chl *a* in Table 39 (the average $\delta|\psi|$ value is 28.6% for A and 20.7% for A') are unanticipated within the context of the average Chl *a* uncertainty in field samples (12.3% Table 26). Unlike field samples, which contained little if any DVChl *a* (Table 21), Mix C contained almost equal proportions of Chl *a* and DVChl *a*. Consequently, Chl *a* accuracy can be severely compromised by coelution with DVChl *a* (C, D, and S) or the DVChl *a* epimer (H, Table 11). It is notable that the simultaneous equation used by S, which had poor accuracy with DVChl *a* in field samples, yielded the lowest Chl *a* $\delta|\psi|$ value in these results (2.2%).

The average change in uncertainty contributed by an increasingly complex analysis is summarized in Table 39 with an average $\delta|\psi|$ across all laboratories of 17.1 and 10.6% for A and A', respectively. Average $\delta|\psi|$ converges with method precision (Table 38) for some laboratories, most notably H, S, and M, suggesting that uncertainty in results for these laboratories is less affected by the complexity of the analysis. The use of a laboratory standard to estimate uncertainties in field samples is potentially biased by the diversity and relative proportions of pigments in the standard mixture, however.

In most cases, calibrations are performed using solitary standards, but they can also be determined from a simple or complex mixture as long as the process is consistent across all analyses and the overall uncertainty converges with method precision. Statistically, calibration is based on single- or multipoint analyses and accuracy should be demonstrated across the entire range of concentrations expected in the field samples. The fitted accuracy of multipoint calibration curves can be determined for each point in the curve by calculating the APD of the fitted amount relative to the known amount, and then computing an $|\bar{\psi}|_{\rm res}$ (Sect.1.4.3.4). These calculations were done for C, H, M, and S based on the x,y data provided by each laboratory, for which x is the Chl *a* amount and y is the peak area (C, H, and M) or height (S). The $|\bar{\psi}|_{\rm res}$ values were 1.3, 1.1, 1.9, and 2.2%, respectively, for C, H, M, and S.

The above-mentioned $|\bar{\psi}|_{\rm res}$ values were computed using calibration points within the range of concentrations suitable for the (eutrophic and mesotrophic) SeaHARRE-2 samples and are not necessarily indicative of what would be attained from standard concentrations suitable for oligotrophic samples. The mode of calibration suggested in

Sect. 1.4.3.4 (and exemplified in Fig. 6) includes standard concentrations near an LOD and LOQ, but these points should only be included in a $|\bar{\psi}|_{\text{res}}$ calculation if they are within the range of concentrations expected in the field samples to be analyzed. The point here is to ensure the $|\bar{\psi}|_{\text{res}}$ value is as representative as possible of the concentration range associated with the field samples.

The measuring devices used to formulate laboratory standards contribute to the uncertainty budget (Table 16). The average percent difference between the delivered volume and the setpoint volume of such devices was 0.35% and ranged from 0.001–2.4%. The average precision was 0.66% and ranged from 0.01–2.85%. In most cases, glass volumetric pipettes and glass syringes (H) performed better than automated pipettes.

1.5.5.6 Detection

A rigorous and properly validated HPLC methodology will involve many elements, which will include, but is not limited to: a) an appropriately implemented reporting scheme with properly defined digits of precision, b) quantitatively established thresholds of detection, c) short- and long-term verification of precision and accuracy, and d) application of the most recent absorption coefficients. None of the SeaHARRE-2 laboratories satisfied all these requirements, although some satisfied one or more of them. The most significant omission was none of the methods properly reported concentrations at, or below, the detection capabilities of the method—indeed most methods did not document their detection limits. The absence of the latter places a rather significant question on the process used here to establish a proxy for truth—which was based on the average reported concentration—when the pigments involved are at low concentrations.

For a sample set derived from eutrophic and mesotrophic waters, the problem of using improperly validated data in a proxy for truth does not just influence the values close to the detection limits of a few pigments, although this is the most severe example. Any unvalidated method will unnecessarily contribute artificial variance to the resulting uncertainty estimate, and this will be unfairly shared by all the other methods. The only way to have a more correct measure of the uncertainties for SeaHARRE-2 is to establish a new averaging procedure for deriving the truth references by excluding those methods that contributed an unwarranted amount of variance to the overall uncertainties.

Improvements to accuracy and precision for the field results are considered here by implementing the following:

1. Application of an LOD and LOQ threshold to all the originally reported data (except for laboratory B, which was unable to make the LOD and LOQ calculations),
2. Correction of any known defects in the reporting scheme, and
3. Reduction of the number of laboratories contributing to the truth reference values by removing those laboratories with significant problems that would probably invalidate their results as a contributor to truth.

The defects that were fixed included: a) D had the misfortune of receiving the `RR2-001m` sample, which took a very long time to filter (Table 1), and the results for that filter were anomalously low, so they were ignored; b) the S results were corrected to remove the use of the wrong formulation for computing the extraction volume; and c) within the subset of methods used to derive the truth reference values, identical absorption coefficients were used to exclude this mostly artificial source of variance.

Within this pursuit of a better proxy for truth, the D, H, M, and S methods, which have already been identified as the A' subset, are used to derive the truth reference values. Methods D, H, and S use the same absorption coefficients for the primary pigments, so the M results need to be corrected to simulate the use of identical absorption coefficients. The reasons for excluding B, C, L, and P (hereafter referred to as the A^+ subset) as contributors to the truth references are as follows:

- The A' subset has a precision and accuracy that is significantly less than the overall (A) average, and that is usually very close to the theoretical best or state-of-the-art result (A^-).
- In terms of average performance, the precision and uncertainty in the individual B, C, L, and P methods is almost always worse than the corresponding Z (defrosted) results.
- The temporal instability of methanol questions the inclusion of the Chl b and Chl c values from the L results in a proxy for truth.
- Laboratories C and L both had significant resolution problems with several of the primary pigments, and they also had difficulties with Mix C (remembering, however, that the L method was not normally prepared for acetone injection, but the calibration standards used by L were in acetone, so this is still a relevant point).
- Although there is no evidence the P samples were significantly degraded, they were unequivocally mistreated, so some question about their efficacy will always be present.
- The B and P methods had extraction solvent concentrations that were high in water content, which made them closer to an 80% acetone solution rather than the recommended 90%, so pigment solubility might be degraded for both methods.
- Laboratories B and P did not participate in all aspects of the round-robin work plan (Table 2), so a full (quantitative) evaluation of these methods is not available.

Table 40. Average uncertainties for a diversity of pigment products subjected to LOD thresholds and organized into four groups: a) individual pigments, b) the pigments within TChl, TCaro, and PPig, c) higher-order products (pigment sums, ratios, and indices), and d) the SChl *a* to TChl *a* ratio. The latter is formed from the SChl *a* and TChl *a* concentration values produced by the applicable individual method (not all methods provided spectrophotometric data). The lowest uncertainties for the individual methods are shown in bold typeface. The average of the B, C, L, and P methods is given by A^+.

Lab.	*Individual Pigments*			*Pigments in*			*Pigment*			$\frac{[\text{SChl } a]}{[\text{TChl } a]}$
Code	[Chl *a*]	[Fuco]	[TChl *a*]	TChl[1]	TCaro[2]	PPig[3]	Sums[4]	Ratios[5]	Indices[6]	
B	19.6	56.7	18.3	17.8	55.4	46.0	32.5	8.7	15.9	
C	13.7	52.4	16.2	34.1	55.0	49.8	32.2	23.5	43.3	17.1
L	19.1	39.4	16.7	19.4	52.4	44.1	30.9	9.3	10.3	20.7
P	19.9	8.2	18.1	16.1	45.5	38.2	11.5	8.2	17.5	
A^+	*18.1*	*39.1*	*17.3*	*21.8*	*52.1*	*44.5*	*26.8*	*12.4*	*21.8*	*18.9*
Z	18.8	13.7	20.5	14.6	25.3	22.7	15.4	9.0	5.1	13.9
D	8.0	**2.8**	6.6	10.6	15.7	14.4	**3.9**	**3.4**	8.3	**3.2**
H	**6.4**	5.7	4.8	**9.1**	**13.6**	**12.5**	5.0	4.7	**3.9**	5.2
M	8.7	6.1	7.0	20.4	21.0	20.8	7.3	7.5	12.4	5.8
S	6.8	4.3	**4.5**	17.7	22.2	21.1	5.3	7.8	10.8	
A'	*7.5*	*4.7*	*5.7*	*14.4*	*18.1*	*17.2*	*5.4*	*5.9*	*8.8*	*4.7*
A	*12.8*	*21.9*	*11.5*	*18.1*	*35.1*	*30.9*	*16.1*	*9.1*	*15.3*	*10.4*
A^-	6.4	2.8	4.5	4.5	12.5	11.0	3.0	3.2	3.9	3.2

The average of the uncertainties in determining:

1. [TChl *a*], [TChl *b*], and [TChl *c*].
2. [Caro], [Allo], [But], [Diad], [Diato], [Fuco], [Hex], [Peri], and [Zea].
3. All the pigments in TChl and TCaro.
4. [TChl], [PPC], [PSC], [PSP], [TCaro], [TAcc], [TPig], and [DP].
5. [TAcc]/[TChl *a*], [PSC]/[TCaro], [PPC]/[TCaro], [TChl]/[TCaro], [PPC]/[TPig], [PSP]/[TPig], and [TChl *a*]/[TPig].
6. [pPF], [nPF], and [mPF].

- Methods B and L used some carotenoid absorption coefficients in alternate solvents, so these pigments cannot be easily reprocessed with respect to the other methods.
- Method C used only acetone absorption coefficients, whereas almost all of the other methods used a mixture of ethanol and acteone absorption coefficients (for the carotenoids and chlorophylls, respectively).

Note that this does not mean there are no problems or deficiencies with the methods comprising the A' subset. For example, D and S did not chromatographically separate DVChl *a*, and S had significant problems with But-fuco. None of these problems substantially invalidate large portions of the data from these methods, however, and all of the difficulties are rather minor compared to the problems listed for the other methods.

It is also important to note that if one of the methods that was excluded from the subset should have in fact been included, this will show up in the uncertainty results: the uncertainties for the incorrectly excluded method will be very close to the A' subset (assuming A' really is a reasonable source for the truth references). Similarly, if the partitioning is correct, the two groups of uncertainties will be demonstrably different—low uncertainties for the A' subset, and much higher uncertainties for the other four A^+ methods.

The application of the aforementioned corrections and LOD thresholds are not expected to substantially influence the average precision of the methods, because the field samples were usually above detection limits, and the use of the $0.0005\,\mathrm{mg\,m^{-3}}$ constant as a limiting value has already mitigated the statistical consequences of null values. Nonetheless, small improvements are expected, because some pigments for some samples are at, or near, detection limits. In addition, a more significant difference is expected between the A^- and A^+ subsets (as already forecast by Table 22), and this is seen: average precision is 5.0 and 8.8%, respectively (which is within 0.5% of what is presented or derived from Table 22). The range in A' precision is 2.5–7.4%, with the highest values associated with the same pigments first identified in Table 22.

A summary of uncertainties for a diverse set of pigment products derived from using the quality-assured A' concentrations as the only contributors to the truth references is presented in Table 40 (a separate table of the individual concentrations based on the LOD analysis is not presented). The Table 40 data show the average uncertainties for the B, C, L, and P subset (A^+) are distinctly different

from the A' subset, and the uncertainties for the former are significantly higher than the uncertainties for the latter for all pigment products—on average, more than 330% higher. The only case where the two subsets are rather similar is for the average uncertainty in the TChl pigments.

The P uncertainties are almost always within the range of the B, C, and L results, and the uncertainties for the A^+ subset are usually greater than the Z results, which suggests the P samples were not significantly degraded (if at all). In fact, because the Z results are usually between the A' and A^+ values, it is unlikely the A^+ higher uncertainties have anything to do with sample transportation or field preparation problems—the enhanced uncertainties are a consequence of the individual laboratory methods.

The original performance perspective for SeaHARRE-2 was derived from the ocean color remote sensing requirements and the results obtained from SeaHARRE-1: an upper uncertainty range of 25% is acceptable, although 15% would (presumably) allow for significant improvements. The A' entries in Table 40 are always less than 25%, and only TCaro and PPig are greater than 15% (and by a small amount that is less than the method precision). In comparison, the A^+ uncertainties are frequently close to, or above, the 25% threshold, and only the uncertainty in determining the pigment ratios is below 15%.

The A^- values in Table 40 establish a theoretical best (or state-of-the-art) capability, but the most intriguing aspect of these data are the recurring convergence of these uncertainties with the overall A' method precision (5.0%). The only lack of convergence occurs (on average) for Chl a, TCaro, and PPig (although the former is rather close), which suggests methodological problems with some of the carotenoids (primarily Allo, But-fuco, Diato, and Zea).

Application of an LOQ, instead of an LOD, threshold further improves average precision by 0.2% for A' and 0.3% for A^+, and almost always improves average uncertainties. The average uncertainties for the A' and A^+ primary pigments, for example, are lowered by 1.2 and 2.3%, respectively; similarly, the average uncertainties for the A' and A^+ pigment indices decrease by 0.5 and 2.1%, respectively. The average uncertainties for the pigment sums and ratios are mostly unchanged as are the $[\text{Chl } a]$, $[\text{Fuco}]$, and $[\text{TChl } a]$ average uncertainties. The largest changes in the primary pigments occur for the carotenoids—the average uncertainties for the TCaro pigments decrease by 1.7 and 2.8% for the A' and A^+ subsets.

The accuracy of the ratio of the spectrophotometric and HPLC determinations of total chlorophyll a in Table 40 further confirms the validity of the A' subset and shows a convergence not only with HPLC procedures, but with spectrophotometric procedures as well. For the D, H, and M methods, the convergence of the $[\text{SChl}a]/[\text{TChl } a]$ ratio is very nearly to within the precision of the individual HPLC methods (the average of which is 4.6%). This does not mean the overall uncertainties in the separate data products across all methods are to within the same level of agreement. The overall determination of $[\text{SChl}a]$ and $[\text{TChl } a]$, for example, is to within an uncertainty of 10.6% (D, H, and M) and 21.1% (C and L), respectively.

The SeaHARRE-1 data set was also analyzed with and without an LOQ quality-assurance process (Claustre et al. 2004). A comparison of the LOQ quality-assured data for the A' and A^+ subsets with the SeaHARRE-1 data is presented in Fig. 11. The comparison shows a decrease in average uncertainty from individual primary pigments, to pigment sums, and then to pigment ratios, followed by a small increase in uncertainty with the pigment indices. In addition, within a pigment category, there is a decrease in average uncertainty with the application of a more stringent QA process. These so-called *functional forms*, establish the advantage of using quality-assured and higher-order data products from different databases—they have the lowest uncertainties.

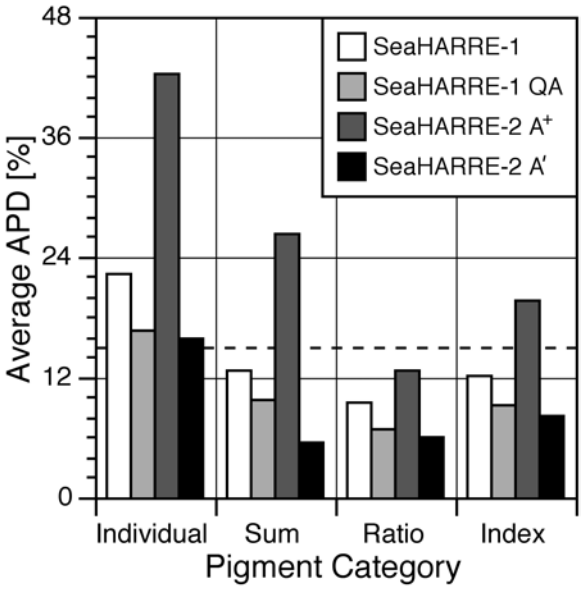

Fig. 11. The average uncertainty (APD) values for SeaHARRE-1 (white bars) versus the LOQ quality-assured data from SeaHARRE-1 (light gray bars) and the SeaHARRE-2 A^+ and A' subsets (dark gray and black bars, respectively). The pigment categories span the individual primary pigments, plus the pigment sums, ratios, and indices. The dashed line delimits the 15% accuracy objective.

1.6 SUMMARY AND DISCUSSION

The diversity of objectives for the SeaHARRE-2 activity, along with the large number of separate analyses involved, makes 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 preceeding sections, and follows the original presentation of the objectives (Sect. 1.1) and much of the organizational scheme used to present the results (Sect. 1.5).

1.6.1 Individual Pigment Uncertainties

The most important individual pigment considered here is TChl *a*, because it is the central pigment in marine bio-optical studies. The average $\left[\text{TChl}\,a\right]$ uncertainty across all laboratories was 10.9%, and the average $\left[\text{TChl}\,a\right]$ uncertainty with the A' subset (laboratories D, H, M, and S) was initially 7.8%. This result is very similar to the 7.9% initial value from SeaHARRE-1 (Hooker et al. 2000), which involved H, L, and M, plus another laboratory that did not participate in SeaHARRE-2. For the SIMBIOS pigment round robin, the average uncertainty in determining $\left[\text{TChl}\,a\right]$ is estimated to be approximately 8.5%† (Van Heukelem et al. 2002).

After the application of a more robust QA process (an LOD threshold followed by an LOQ threshold) and the use of the A' subset as the proxy for truth in the uncertainty calculations, the average uncertainty in $\left[\text{TChl}\,a\right]$ was lowered to 5.9%, for which the average method precision was 3.0%. The application of an LOQ threshold to the SeaHARRE-1 data resulted in a similar uncertainty in $\left[\text{TChl}\,a\right]$ of 5.5% (Claustre et al. 2004). The only other individual pigment to be determined to a like accuracy and precision in the SeaHARRE-2 activity was Fuco (on average 4.7 and 2.7%, respectively). In fact, the average uncertainty for the primary pigments using the quality-assured A' subset was 16.0%. Although this is significantly larger than the $\left[\text{TChl}\,a\right]$ average uncertainty, it is very close to the 15% refinement objective.

1.6.2 Ocean Color Requirements

The remote sensing requirements for the *in situ* determination of $\left[\text{TChl}\,a\right]$ are well satisfied by HPLC analyses. The 25% compliance objective was achieved by all methods, regardless of the truth referencing system, and the 15% refinement objective was achieved by most of the methods. Given that the application of a QA process resulted in an average $\left[\text{TChl}\,a\right]$ uncertainty on the order of 5.5%, an accuracy objective to within 5%—although not routine—seems achievable (Table 40).

Although Chl *a* is currently the primary pigment of interest for ocean color applications, recent inquiries have demonstrated the possibility of deriving pigment indices from satellite observations (Uitz et al. 2005), so the future of remote sensing will most likely include more pigment products than just the chlorophyll *a* concentration. Although no community-wide accuracy objectives exist for any pigments other than Chl *a*, the average uncertainties for the pigment indices are almost always to within 15%. In fact, for the quality-assured A' subset, the pigment indices are determined with an average uncertainty of 8.8% (Table 40).

1.6.3 Higher-Order Variables

The pigment indices are one example of higher-order variables; the other ones considered here are the pigment sums and ratios (Table 5). Regardless of the truth referencing system, the higher-order variables are distinguished by lower average uncertainties than the average uncertainty for the primary (individual) pigments. In all cases—including both SeaHARRE-1 and SeaHARRE-2—the average uncertainties for the pigment sums and ratios are less than the 15% refinement threshold, and with the application of a QA procedure (e.g., use of an LOD or LOQ reporting system), the uncertainties are less than 10% and even to within 6% (Fig. 11). These results confirm the possibility of using higher-order variables to minimize uncertainties in large databases derived from a diverse set of contributors (Claustre et al. 2004).

1.6.4 HPLC versus Spectrophotometric

The overall average uncertainty for (spectrophotometric) $\left[\text{SChl}a\right]$, was 3% lower than for (HPLC) $\left[\text{TChl}\,a\right]$, when results of the five laboratories performing both analyses are considered; the overall precision, however, was similar for each type of analysis (5.3% and 4.5%, respectively). These findings suggest the following:

1. The complexity of HPLC analysis contributes to the uncertainty budget;
2. Low uncertainties with interlaboratory spectrophotometric analyses are attainable; and
3. Uncertainties associated with sample collection and differences in extraction procedures among these laboratories were small.

The convergence of analysis uncertainty (5.5%) with analysis precision (5.3%) for $\left[\text{SChl}a\right]$ reinforces the latter finding, considering that spectrophotometric analyses of chlorophyll *a* standards alone typically contribute uncertainty on the order of 1.4%.

Despite low interlaboratory uncertainty, $\left[\text{SChl}a\right]$ is not necessarily a better estimate of truth than HPLC $\left[\text{TChl}\,a\right]$. The most notable reason is chlorophyll *a* degradation products absorb strongly at 664 nm (Jeffrey et al. 1997), the wavelength used to quantify $\left[\text{SChl}a\right]$. An apparent effect of the presence of degradation products is illustrated by comparing the Z results (the defrosted filters) with the H results (which were undamaged). The sum of $\left[\text{Phytin}\,a\right]$ and $\left[\text{Phide}\,a\right]$ was 5% higher, relative to $\left[\text{TChl}\,a\right]$, in Z results and the $\left[\text{SChl}a\right]/\left[\text{TChl}\,a\right]$ ratio was elevated (1.26 for Z versus 1.16 for H).

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

The Z and H HPLC results converge with their respective $[\mathrm{SChl}a]$ results, however, if HPLC $[\mathrm{TChl}\,a]$ is replaced with a term that represents the sum of all pigment concentrations that contribute to the $[\mathrm{SChl}a]$ value, which include $[\mathrm{Phytin}\,a]$, $[\mathrm{Phide}\,a]$, $[\mathrm{DVChl}\,a]$, $[\mathrm{Chl}\,a]$, and $[\mathrm{Chlide}\,a]$. Before summing, the quantified HPLC weight of these pigments is converted to moles, a correction is made to adjust for differences in their absorptivity relative to chlorophyll a at 664 nm, and the adjusted molar amount of each pigment is converted to its chlorophyll a weight equivalent. With such recomputations, the average $[\mathrm{SChl}a]/[\mathrm{TChl}\,a]$ ratio, 1.05, was the same for both the H and Z modified results, and all ratios were confined to a narrow range, 0.93–1.18. This type of comparison might prove useful as a QA check for accuracy, especially in the absence of intercalibration exercises.

1.6.5 QA Procedures and Parameters

Uncertainties in pigment analyses can be reduced if appropriate QA thresholds are applied to the data before it is supplied to the end user. Such QA thresholds reduce the effects of pigments found either in low concentrations or not found at all (which are routinely reported as a concentration of zero). Two conditions that can contribute significantly to pigment uncertainties are *false positives* and *false negatives*, which frequently occur when pigments are quantified near the method detection limit. The application of a QA threshold that quantifies a detection threshold (LOD) can reduce such uncertainties. A more rigorous approach, with a greater reduction in uncertainty, is the application of a quantitation threshold (LOQ).

Section 1.4.3 describes how to apply an LOD and LOQ, and demonstrates the reduction in uncertainty with results for the analysis of duplicate filters gathered over a one-and-a-half-year time period from coastal waters, where $[\mathrm{TChl}\,a]$ ranged from 0.3–22.5 $\mu\mathrm{g\,L^{-1}}$, pigment diversity was significant, and the resulting complex chromatograms challenging to integrate.

The QA procedures and parameters considered here included extraction (volumetric uncertainties), separation (coelution and resolution), injection (injector precision), degradation (temporal stability), calibration, and detection (application of the LOD and LOQ).

1.6.5.1 Extraction

Prior to SeaHARRE-2, none of the laboratories involved were sharing information on a recurring basis using formulistic communications, that is, a precise lexicon expressed with unique symbols and formulas was not available to document all aspects of an HPLC method. Consequently, some important procedures were described using unnecessarily vague language. The inaccurate determination of extraction volume, for example, is a frequent occurrence. In the SIMBIOS HPLC round robin (Van Heukelem et al. 2002), 25% of the laboratories did not calculate the extraction volume correctly, and this mistake was also made here by laboratory S. In more than one of these cases, the miscalculations were the result of interpretation errors by laboratories trying to implement a particular methodology.

Differences in extraction procedures alone do not always explain differences in the results or uncertainties in the quantitated pigments. The A' subset D, H, M, and S used different extraction procedures (Table 8), but their uncertainties were rather similar (in particular, Table 40). Nonetheless, correlations between extraction problems and higher uncertainties do exist. The two laboratories with extraction solvent concentrations approaching 80% acetone (B and P), for example, had demonstrably higher uncertainties than the A' subset. In addition, the only method to use methanol as an extraction solvent (L), which is less stable than acetone (especially for Chl b and Chl c), had significantly higher uncertainties than the A' subset.

1.6.5.2 Separation

In instances where the effects of poor separation could be quantified, removing the pigments with specificity problems (i.e., reducing the number of the pigments reported) results in lower uncertainties and a convergence of the uncertainties between the overall average (A) and the A' subset (Table 27). Conversely, increasing chromatographic complexity leads to increasing uncertainties. For a common set of pigments (Chl a, Hex-fuco, Fuco, and Perid), which all had resolution problems (Tables 11 and 34), the average increase in APD is 6.6% for A, and 2.7% for A' (Table 38) when going from Mix D (in which pigments were analyzed individually) to Mix A or Mix B, where pigments were part of a simple mixture. The average change in APDs going from Mix B to Mix C (a complex mixture) is 24.6% (A) and 14.5% (A') as derived from Table 39.

1.6.5.3 Injection

The precision of the HPLC analysis method, exclusive of any sample effects, was evaluated with replicate injections of either single-component (Mix D) standards (Table 35) or a multicomponent (Mix C) standard mixture (Table 24). Peak area precision less than 0.5% was frequently observed across all laboratories for various pigments, indicating the injection conditions of all laboratories were able to deliver precise volumes. In most instances, the complexity of the analysis did not strongly affect analysis precision, because the average precision across all laboratories for Mix D pigments was 1.7%, which was slightly lower than the 2.2% average precision for Mix C pigments. Inspection of Mix C chromatograms (Fig. 8), however, reveals the poorest precision experienced by each laboratory was frequently associated with pigments that were only partially resolved ($R_s < 1.0$) or were asymmetrical and

broad in shape. The latter occurred most frequently with early eluting pigments (most notably Chl c_3 and Chl c_2). A contributing cause to peak asymmetry is an incompatibility of injection conditions (e.g., injection volume and injection solvent) with initial separation conditions (e.g., mobile phase composition and flow rate).

HPLC injection conditions should be optimized for all pigments quantified and all injection solvents used. Frequently the solvent used with sample extracts is different from the solvents in which calibration standards are suspended. The wide range in precision with Mix D pigments, 0.02–8.3% for Hex-Fuco and 0.09–12.5% for Fuco (Table 35), indicates injection conditions were frequently not fully optimized for these ethanol injections. The problem of poorly optimized injection conditions is not limited to laboratories in SeaHARRE-2, however. In the SIMBIOS intercalibration exercise, only two of seven laboratories were optimized for peak shapes of chlorophyll *c* pigments†.

1.6.5.4 Degradation

The pigment degradation experiments quantified the sensitivity of pigments (using primarily pigment extracts) to short-, long-, and extended-term temporal deterioration in a 4°C TCAS compartment, a −15°C freezer, and a laboratory drawer. The Z analyses are also applicable to these investigations, because the samples involved were unequivocally degraded (the shipping dewars defrosted and the samples were received at room temperature). Note that the degradation for the Z analysis is for the entire filter sample, and not just the extract.

The average one-day degradations for the stability and Z analyses are presented in Table 41 and confirm the basic conclusions of these inquiries:

1. Short-term degradation rates are the most rapid, which are followed by a slower longer-term decay rate (and taken together approximate an exponential curve);
2. Pigments extracted in acetone are more stable than pigments extracted in methanol, which is consistent with the findings of Latasa et al. (2001) for algal cultures extracted in methanol and acetone;
3. TChl *a* in acetone is very stable;
4. The carotenoids are more stable than the chlorophylls (including TChl *a*); and
5. For data already collected and subjected primarily to short-term sources of deterioration, much of the degradation—particularly for samples extracted in acetone—appears sufficiently linear to suggest a temporal correction scheme for pigment concentrations is worth investigating (although additional experiments as a function of initial concentration and time are needed).

† Although a technical report for the SIMBIOS chlorophyll *a* round robin was produced (Van Heukelem et al. 2002), this information was not part of the published material.

This means any method using an alternative extraction solvent to the ones considered here needs to separately characterize the temporal degradation sensitivity of that solvent and whether other practices, such as storing extracts under an inert gas (argon or nitrogen) or at colder temperatures have important effects.

Table 41. The average one-day degradations for the stability experiments based on the linear regressions (forced through zero) applied to the data. The defrosted dewar entry is based on the Z analysis, so the degradation is for the entire filter sample (and not just the extract), and assumes the samples were defrosting over an 8-day period.

Experiment (and Duration)		PPig [%]	TChl [%]	TCaro [%]	TChl *a* [%]
TCAS† (2 days)	Ace.	−2.6	−8.4	−0.6	−1.7
	Meth.	−10.9	−34.2	−2.7	−13.1
Drawer‡ (2 days)	Ace.	−2.1	−6.8	−0.5	−1.3
	Meth.	−8.9	−27.9	−2.2	−10.6
Def. Dewar (8 days)	Ace.	−2.2	−1.6	−2.4	−2.1
Freezer§ (3 weeks)	Ace.	−0.3	−0.5	−0.2	−0.2
	Meth.	−0.7	−2.3	−0.2	−0.7
Freezer§ (5 months)	Ace.	−0.1	−0.2	−0.1	−0.1

† 4°C ‡ Room temperature (∼22°C) § −15°C

1.6.5.5 Calibration

A theoretical *best* estimate of the accuracy of calibration can be computed using the results from the Mix D analysis and is estimated to be 4.2% $\left(\sqrt{1.7^2 + 1.6^2 + 3.5^2}\right.$, with 1.7% for $\bar{\xi}_{\text{inj}}$, 1.6% for the $|\bar{\psi}|_{\text{res}}$, and 3.5% for the uncertainty in the concentration of the pigment standards$\left.\right)$ and was observed to be 8.9% for A, and 5.2% for the A' subset. Consequently, the A' results very nearly approximate the theoretical accuracy, which confirms a) calibrations can be done a high degree of accuracy, and b) the A' subset is separately distinguishable within the full set of laboratories.

Although the Protocols suggest using the Jeffrey et al. (1997) absorption coefficients, the Protocols also say pigment standards can be purchased from several different sources, which do not always use the Jeffrey et al. (1997) absorption coefficients, so some variance in absorption coefficients is inevitable, even if the Protocols are adhered to. The majority of the laboratories in SeaHARRE-2 used the same absorption coefficients for the chlorophylls and the values involved were in agreement with the Protocols. A preponderance of ethanol absorption coefficients were used for the carotenoids, and is a practical consequence of the fact that pigment standards are not commercially available in acetone. This mismatch was a continuing source

of variance in pigment concentrations, as originally predicted in Jeffrey et al. (1997), for both SeaHARRE-1 and SeaHARRE-2.

1.6.5.6 Detection

The use of an increasingly robust detection-based reporting scheme (null value replacement, LOD threshold, and LOQ threshold) improves method precision and accuracy. As the QA process is made more robust, method precision and accuracy increasingly converge, not only for some of the individual pigments, but most notably for almost all of the higher-order variables. The culmination of the QA process is the identification of the methods capable of providing an adequate proxy for truth—the A' subset.

1.6.6 Validation of Round-Robin Method

There is a close agreement of the overall accuracy results for the field samples and Mix C primary pigments, which agree to within 4.8% and 6.1% for A and A', respectively. In addition, the range of uncertainties, individual method averages, and ranking of the methods from lowest to highest average uncertainty are all very similar. This indicates the two independent approaches for estimating method uncertainties produce comparable results, which is a substantial validation of the round-robin approach, because it shows using a proxy for truth—referencing the accuracy calculations with respect to the overall pigment averages—provides the same basic result as using a laboratory standard.

The best of the field sample results, A^{-}, has an overall accuracy of 11.4%, which is very close to the overall H result of 9.3% for the Mix C analysis. This convergence between a theoretical method (taken almost exclusively from two methods) and an established method is another form of validation, because it demonstrates closure in the statistical description. The latter is particularly important in defining realistic HPLC performance metrics, which is one of the objectives of this study.

Another significant validation of the data analysis approach used here—particularly the ultimate use of the A' subset as the final proxy for truth—is the convergence of the SeaHARRE-1 and SeaHARRE-2 quality-assured uncertainties. Additional evidence is provided by the increasing convergence of uncertainties with method precision as the QA process is made more robust. Ultimately, an inescapable conclusion of the QA results is the D, H, M, and S methods were the only adequate source for truth, and thus, the only properly validated methods.

1.6.7 Performance Metrics

One of the most important SeaHARRE-2 objectives was to establish performance metrics for evaluating HPLC methods used to quantitate marine pigments. Before discussing the various performance categories it is useful to remember the analysis of marine pigment samples requires a so-called *trace analysis* perspective, that is, the analytical resolution is approximately at the 1 ppm ($0.001\,\mathrm{mg\,m^{-3}}$) level. In such an environment, miniscule discrimination becomes ordinary, and the vocabulary used to distinguish the capabilities of the methods quickly becomes divorced from the simple fact that all methods are performing at extraordinary levels. Furthermore, a gradation of capabilities is needed, because the requirements of an investigative problem should be matched to a method with the requisite capability. In the absence of knowing the capabilities of a method, most investigators assume that because marine pigment analysis is potentially a very precise and accurate undertaking, all the investigative requirements of their individual needs will be met—*but this is not true until it has been quantitatively demonstrated.*

The emphasis here is on proposing metrics that are easily reproduced by any practitioner of a marine pigment HPLC method, although some (relatively minor) revisions to the field sampling and laboratory analysis protocols might be required. It is recognized, however, that the hardware and software being used at a particular laboratory might not be capable of running alternative methods beyond a certain performance level. Some institutions are resource limited and have to work with the equipment they have, so they may never be able to produce state-of-the-art results; not because they are not diligent in calibrating and validating their methods, but because the limiting factor is the equipment they have available. Sampling protocols, sample storage, and laboratory procedures are very important, and can all effect the quality of the results if not performed appropriately, but the hardware and software might ultimately limit the capability of the method no matter how well the other procedures are performed.

Four types of performance categories are established based on the overall average results for the primary pigments and total chlorophyll *a*: *routine* (any method should compliant, that is, capable of satisfying the criteria), *semi-quantitative* (most methods should be compliant), *quantitative* (some methods will already be compliant, and others should be compliant with relatively minor changes), and *state-of-the art* (very few methods have the demonstrated capability and only a few others could be compliant).

As already noted, HPLC pigment results are affected by a multitude of procedures, including—but not necessarily limited to—the following:

- The methodology used to collect the sample;
- Sample transportation and storage;
- Pigment extraction procedures;
- System calibration;
- Injection and detection;
- The capabilities of the HPLC hardware; and
- The separation method implemented.

Table 42. The performance metrics for the four categories established for validating the determination of marine pigments using an HPLC method: concentration (average precision and accuracy for TChl *a* and PPig); separation (minimum resolution and average retention time precision); injection precision (the average of an early- and late-eluting pigment standard, e.g., Perid and Chl *a*); and calibration (average residual, $|\bar{\psi}|_{\mathrm{res}}$, for Chl *a* and the precision of the dilution devices, $\bar{\xi}_{\mathrm{cal}}$). The PPig and TChl *a* performance metrics are based on using the analysis of a mixture of laboratory standards (Mix C) and replicate field samples with approximately equal weights applied to each (remembering that uncertainties are assumed to combine in quadrature and that the latter presupposes the inclusion of replicate filter collection during field sampling). The corresponding values for method *H* are given as an example. The overall performance of *H* is considered "state-of-the-art," because the average score of the weights is 3.7, $(4+4+4+3+3+4+4+4+3+4)/10$.

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

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

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

§ The $\lvert\bar{\psi}\rvert_{\mathrm{res}}$ 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, $\lvert\bar{\psi}\rvert_{\mathrm{res}}$ is computed using those calibration points within the range of concentrations expected in the field samples to be analyzed (Sect. 1.5.5.5).

Meticulous attention to detail is required for all of these procedures if consistent and quantitatively accurate pigment concentrations among laboratories are to be attained. To limit uncertainties, all procedural aspects need to be evaluated quantitatively, so they can be properly minimized (especially over time).

A performance evaluation scheme that required every possible parameter to be compared would not be practical, so the approach adopted here is to select a subset of key parameters that are representative of larger groups of procedural parameters. The subset of performance metrics suggested here are presented in Table 42. Precision and accuracy are shown for both TChl *a* and PPig, because of the importance of the former and to promote a continuing effort to improve the quantitation of the problematic pigments (e.g., Allo, But-fuco, Diato, Peri, Zea, Chl *b*, and Chl *c*). Presenting two metrics is not very satisfactory (*a priori*, there is no reason why a preponderance of the primary pigments quantitated by a properly validated and quality-assured method should not be to within the same precision and accuracy as TChl *a*), but it is practical: both of the SeaHARRE activities showed that with or without a quality-assured reporting scheme, the performance in determining $\left[\mathrm{TChl}\,a\right]$ is not usually seen in the other pigments (except Fuco, and to a slightly less degree Diad and Hex-fuco). It should be noted that metrics for TChl *a* and PPig represent the combined results of replicate, *in situ* filters and a mixed standard (in this case Mix C), for which accuracy of the latter is with respect to average concentrations. Combining *in situ* and standards results diminishes the effects of filter inhomogeneity, which could otherwise bias a laboratory's category and score.

The separation (Sects. 1.3.2 and 1.4.3.4), calibration (Sects. 1.3.4 and 1.4.3.4), and injection (Sect. 1.5.5.3) variables in Table 42 are central to understanding the performance of any HPLC method and can be evaluated without participation in an intercalibration exercise. Not all variables are necessarily significantly correlated with lowering uncertainties. For example, the CV of retention time $\left(\bar{\xi}_{t_R}\right)$ varied widely in the A' subset, and apparently not to the detriment of the uncertainty budget. Nonetheless, it is true more often than not, that methods with poor accuracy and precision have high $\bar{\xi}_{t_R}$ values, and none of the poorer performing methods had low $\bar{\xi}_{t_R}$ values.

Table 42 was also constructed with the intent of categorizing overall method performance by evaluating the performance metrics achieved in terms of numerical weights for each. An overall score is produced by summing all the weights and dividing by the number of metrics. If this process is applied to the SeaHARRE-2 laboratories, the only methods to achieve a "quantitative" or "state-of-the-art" score are *D*, *H*, *M*, and *S*, which is a significant verification of using the A' subset as the final source for truth.

The SeaHARRE results show that standardizing some procedures among laboratories improves consistency in results. A particularly relevant example is the use of common absorption coefficients and procedures which a) optimize spectrophotometric accuracy (Clesceri et al. 1998 and Bidigare et al. 2003), b) yield accurate extraction volumes, and c) minimize sample degradation during shipping. It is unclear whether adopting a standardized, or so-called *unified*, HPLC separation method necessarily improves consistency in results among laboratories.

The choice of a separation method should be influenced by analytical requirements and clear documentation showing the method can produce a level of accuracy and precision consistent with intended use of the data. After the SeaHARRE-2 laboratory submissions were analyzed and all groups were provided the opportunity for collaboration at meetings hosted by GSFC and LOV, many analysts considered adopting the H method (Chapt. 5). If a consensus of analysts report future results from the H method, it could be perceived that a unified method has been adopted. It is important to remember, that if this evolution occurs, it is a consequence of the performance metrics achieved (Table 42) and not because a unified method was recommended. The HPLC separation method contributes to, but is not exclusively capable of, producing accurate and precise results.

There are several factors to consider when implementing an HPLC method, whether for the very first time or as a replacement for a method already in use. The most important point is to determine if it is possible to implement the required procedures exactly as documented. Frequently, extensive modifications are made to a published method, and the newly implemented method bears little resemblance to the original citation. Regardless of whether or not changes were made to the foundation method, performance evaluation criteria should be implemented and the resulting performance metrics considered in the context of achievable results (as in Table 42).

The performance metrics in Table 42 can be used to evaluate one method with respect to another, or simply to evaluate the same method at any given point in time. The latter is only meaningful if a time series of observations for the various parameters is first established and is, thus, available for comparison purposes. Performance metrics are used or influence the methodology as exemplified in the follow-on discussion, which uses laboratory H (and the lexicon used by H) for illustrative purposes:

- *Run* describes uninterrupted, repetitive execution of the same HPLC method (on the same instrument and column) during which all injections needed to complete the analysis of extracts from the same batch (all extracted on the same day) and standards used for performance metrics are performed. A batch of samples is extracted on one day and the run is initiated at the completion of sample extraction. If a run is aborted, performance metrics are redone after the run is reinitiated. The same calibration factors are used throughout a run.
- *Sequence* describes continuous, uninterrupted, execution of the same HPLC method and may include several runs. The same HPLC column and calibration factors are used throughout a sequence.
- *Short-term precision* describes the CV of replicate analyses of standards or samples performed during the same run or sequence.
- *Average short-term precision* describes the average of several observations of short-term precision and may be discriminated by run or sequence.
- *Long-term precision* describes the CV associated with the cumulative average of peak area or calibration factors when the same standard is analyzed on several occasions across many runs and/or sequences.
- *Average long-term precision* describes the average of several observations of long-term precision.
- *Calibration reproducibility* describes the CV associated with response factors (or slopes) that are determined from independently formulated standards and includes all aspects of calibration: spectrophotometric determination of pigment concentrations, as well as their subsequent dilution and HPLC analysis.
- *Overall method precision* describes the variation in concentrations of pigments in replicate filters that are extracted on different days (or months), and analyzed on different HPLC sequences and may include different calibration factors. It is, therefore, cumulatively affected by many variables including storage conditions and filter inhomogeneity.

In addition, the following observations are acquired and retained over time for each run:

- *Resolution* (R_s) is determined from a standard mixture containing the critical pairs (either Lut and Zea or Viola and Hex-fuco) and is analyzed near the beginning of each run. The column is replaced if $R_s < 1$ for one of the critical pairs. This standard mixture is also used to visually inspect for peak symmetry of early eluting pigments.
- *Retention time stability* ($\bar{\xi}_{t_R}$) is checked with standard mixtures, which are used to identify the retention times of most pigments quantified and are injected at intervals that bracket every ten sample injections.
- *Short-term precision* ($\bar{\xi}_{\text{inj}}$) is evaluated with duplicate injections of the internal standard (performed at random and immediately after a sample is analyzed) and with replicate injections of a mixed standard (referred to above). If short-term precision is not consistent with average short-term precision, no further analyses are performed until the reason is identified and corrected.

- *Stability* describes the variation in pigment results for a sample extract that has been analyzed at the beginning of a run, and then again at the end of a run. This test evaluates pigment stability with maximum residence time in the TCAS and is done with the first sample extracted in each batch.
- *Calibration repeatability* is monitored via short-term and long-term precision. Pigments with absorption coefficients in ethanol are not combined with pigments using absorption coefficients in acetone.
- *Carryover* is evaluated from chromatograms at 450 and 665 nm from analyses of the internal standard batch solution or acetone.
- *Extraction volume* according to V_m (7) and $V_e + V_w$ (9) are compared to identify aberrant values. These comparisons are possible because practices to minimize evaporation of extraction solvent are implemented.
- *Pigment ratios*, derived from the proportion of chlorophyll *a* allomers, epimers, and degradation products (phaeophytin *a* and phaeophorbide *a*) relative to $\left[\text{Chl } a\right]+\left[\text{DVChl } a\right]$ and $\left[\text{TAcc}\right]/\text{TChla}$, are monitored for each sample.

All quality assurance activities are supervised by experienced HPLC pigment analysts. Performance monitoring conducted more intermittently than on a per-run basis include the following:

- $\bar{\xi}_{\text{cal}}$ is minimized through the use of glass, class-A volumetric pipettes and gas-tight glass syringes in the calibration procedure, because they provide the best accuracy and precision. The measuring device used for V_m in (7) is also calibrated.
- If $|\bar{\psi}|_{\text{res}}$ is suddenly different from the temporal average, perhaps caused by one point, the anomalous point is deleted as long as this yields a new value in keeping with the average; if agreement is not achieved, the calibration is repeated.
- Calibration reproducibility is monitored to establish requirements and the frequency of recalibration. Calibration factors are not changed during a run or sequence.
- Laboratory fortified blanks are extracted and analyzed if there are changes to the extraction procedure.
- Spectrophotometer accuracy is evaluated with interlaboratory comparisons (for chlorophyll *a* only), and is based on neutral density filters traceable to the National Institute of Standards and Technology (NIST) plus comparisons among spectrophotometers at HPL. All standards are analyzed in replicate, CV values are determined, and pigment concentrations recorded, so temporal changes can be observed.
- Alternative analyses in support of HPLC TChl *a* accuracy are sometimes conducted with either fluorometric or spectrophotometric analysis of the sample extract, for which V_x is defined as $V_e + V_w$ (9).
- $|\bar{\psi}_{P_i}|$ describes accuracy with *in situ* samples or a reference mixture containing many pigments. This variable can only be determined and refined by multiple interlaboratory comparisons.
- $\bar{\xi}$ values are determined from the average CV of replicate *in situ* samples, which is performed at a frequency selected by the principal investigator. HPL analysts prefer to analyze replicate filters at a frequency of no less than 1 in 10 samples (or 2 per batch). Nonmethodological factors contribute greatly to differences in $\bar{\xi}$ between projects, and cumulative averages are tabulated according to the principal investigator conducting the field research.

Accuracy and precision with *in situ* samples, shown as $|\bar{\psi}|$, and $\bar{\xi}$ for TChl *a* and PPig in Table 42, are the two most important metrics in the table. They cannot be measured in isolation, but require participation in multiple intercalibration exercises and all other metrics are created to support conclusions regarding them. Recurring round robins are the most appropriate mechanism by which refinements to method accuracy are evaluated. To illustrate, an initial round robin between *H* and another SIMBIOS laboratory yielded average APDs for *in situ* samples of 9% (TChl *a*) and 30% (PPig). Method refinements were implemented by both laboratories and two additional round robins yielded greater accuracy, for which TChl *a* APDs were 4 and 7% and PPig APDs were 20 and 13%. It is worth noting that the latter values for TChl *a* and PPig were from a round robin representing eight oligotrophic and four mesotrophic samples, and each laboratory received solitary (not duplicate filters) from each site. These SIMBIOS round-robin metrics are not exactly comparable to the metrics in Table 42, because they were not modulated by simultaneous evaluation of accuracy associated with analysis of a mixed standard, but they do show performance categories improve with method refinements and that, outside the context of SeaHARRE-2, values assigned to accuracy categories in Table 42 appear appropriate.

Discerning precision effects, free from factors not related to the HPLC method, was possible in SeaHARRE-2, because of the many participants analyzing replicate filters and supporting data from the mixed standards. Consequently, 1.5% of the variability in filter results was identified as coming from nonmethodological factors (Sect. 1.5.2) and the ensuing estimated variability with the *H* method for PPig was 2% (Table 42). The precision for method *H* with duplicate filters typifying samples in Fig. 7 (not SeaHARRE-2 samples), however, is 10.2%. Without participation in intercalibration exercises, non-HPLC method precision is estimated by difference, relative to HPLC performance metrics that can be observed in isolation and are

considered here for $\bar{\xi}_{\rm cal}$ (for the measuring device used for V_m), $\bar{\xi}_{\rm inj}$ for PPig and the internal standard, and a stability term (not in Table 42). Representative values for these terms with method H are 0.4, 1.0, 0.3, and 3.5%, respectively, and the quadrature sum is approximately 4%; thus, approximately 6% of the uncertainty in this illustration is from nonmethodological factors. It is of interest to note that, in SeaHARRE-2, all duplicate filter extracts were analyzed sequentially and would not have been influenced greatly by stability. With the example given here, duplicate filters were analyzed at random within (and across) runs.

1.6.8 Conclusions and Recommendations

The most comprehensive influence of an activity like SeaHARRE is to propose–and quantitatively defend with the results from well-planned experiments—clarifications and revisions to the Protocols as well as the pigment analysis objectives of the marine community. In this way, the state of the art for HPLC analysis is continuously being refined and advanced in a disciplined process. This concept was refined here by introducing the notion of a *theoretical best* HPLC method, wherein the lowest uncertainties or best precision from all the methods were used to define a subset of the results (A^-) which could only be produced by a heretofore unavailable method. Although it might seem fantastical to imagine such a method at this point in time, the idea is to set a threshold of achievability, so the range in performance between the current state of the art and a possible future capability can be described. This necessarily means this report is just a snapshot of the state of the art and it is fully expected—in fact, the greater community requires—that the state of the art will change over time and move incrementally closer to whatever threshold of achievability is chosen.

It is important to remember the perspective adopted here is marine, but more correctly, *oceanic*, i.e., scientific investigations wherein estuarine and riverine influences are usually minor. This is not to say the coastal zone is inapplicable, it is just to clarify that the results and conclusions discussed here may ultimately be invalid as the water depth gets shallower and shallower. Although many of the SeaHARRE lessons—as well as the generalized approach—might still be applicable, it is very likely that a revised set of protocols, QA procedures, and performance metrics will be needed for the shallow water community.

In terms of the original objectives for SeaHARRE-2 and the results achieved, the following are recommended or considered noteworthy (in no particular order of priority, but within the limits of what is considered achievable for the typical analyst and the current state of the art):

1. If linearity in response over the range of concentrations expected for analysis is demonstrated, single-point calibrations are acceptable. Individual calibrations for one chlorophyll and one carotenoid, however, should be made using a dilution series, wherein three concentrations bracket the expected detection limit and three bracket the anticipated concentrations for the samples. The average of the absolute residuals to the fitted line (forced through zero), $|\bar{\psi}|_{\rm res}$, should be retained over time†. The temporal reproducibility of $|\bar{\psi}|_{\rm res}$ is an important quality assurance opportunity that should be exploited.
2. A mixture of PPig standards or an algal source with concentrations appropriate to the trophic conditions associated with the field samples to be analyzed should be produced (or procured) and then analyzed at the start of the field sample analysis and after every 20 samples (with at least one mixture standard analyzed in each *sample set* of field samples, e.g., for each complete analysis of an autosampler compartment). This mixture of pigments is used to monitor retention time and response factor stability. Pigments exhibiting marginal separation should be present in the mixture, so resolution values adequate for quantitation ($R_s \geq 1$) can be demonstrated.
3. Based on pigment stability, acetone is a significantly preferred extraction solvent over methanol. To keep degradation uncertainties to within method precision, all HPLC analyses of acetone extracts should be completed within 24 h of extraction (12 h ensures the uncertainty is to within 50% of method precision). If Chl *b* and Chl *c* are important, HPLC analysis should be completed within 2–3 h of extraction if methanol is used as an extraction solvent, otherwise 12 h is appropriate.
4. Pigment instability during analysis of a batch of samples should be evaluated by placing two vials of the same sample extract in the TCAS when sample analysis is initiated—one of these vials should be the first sample analyzed and the other the last sample analyzed. The time interval between injections describes the maximum residence time for all samples in the sample set.
5. Injector precision should be measured for Perid (or another early-eluting pigment) and Chl *a* (or another late-eluting pigment) using sequential injections of both standards. In addition, the precision of the internal standard should be measured and analyzed over time. This procedure is important for initially demonstrating capability, but over the long term, the precision of the internal standard and the precision of replicate injections of the same sample extract (at the beginning and the end of the run) may be a more useful monitoring metric.

† If an alternative calibration process is used and it does not include the calculation of residuals, then the r^2 and y-intercept values of the fit should be retained.

6. Any volumetric devices used in the execution of the method should be gravimetrically calibrated with the solvent intended for use (usually 100% acetone or 100% methanol), and the CV reported as $\bar{\xi}_{\rm cal}$.
7. The effective LOD and LOQ values for each pigment should be determined (Sect. 1.4.3.7), and either the LOD or the LOQ should be selected as the limiting QA threshold for data reporting purposes, i.e., all concentrations less than the limiting threshold are replaced by the threshold for each reported pigment or pigment product. In addition, all reported concentrations should be with the appropriate number of digits of precision. If the SeaHARRE sampling approach is typical, this will usually be three.
8. The field sampling protocol must respect the desired LOD and LOQ levels established for the QA process, which means the volume of seawater filtered in the field must be sufficient to support the LOD and LOQ objectives. Substituting (24) and (25) into (28) and (29), respectively, and then rearranging the terms yields:

$$V_f \;=\; \max \begin{cases} D_{P_i}^{\rm eff}\left[\dfrac{3}{S_{P_i}(\lambda_d)}\,\dfrac{V_x}{V_c}\right] \\ Q_{P_i}^{\rm eff}\left[\dfrac{10}{S_{P_i}(\lambda_d)}\,\dfrac{V_x}{V_c}\right], \end{cases} \tag{31}$$

   where "max" indicates the maximum value of all the effective LOD and LOQ terms for all the applicable pigments is selected, which means some may be set to zero. For example, if only two pigments are considered and the effective LOD and LOQ for those pigments results in $V_f = 1.5$ and 1.8 L plus $V_f = 5.0$ and 6.0 L, respectively, the final V_f value to be used in the field is 6.0 L. But if the LOQ is not to be considered, the final V_f value to be used in the field is 1.8 L. For proper cruise planning, this means the HPLC analyst supplies estimates for the terms in brackets, and the scientists who will ultimately make use of the data provide the effective LOD and LOQ requirements, and this establishes the volume of seawater that must be filtered.
9. *In situ* sampling should include the collection of quadruplicate samples across the dynamic range in productivity experienced in the field. An appropriate subsampling for replicates is probably one quadruplicate for every 20 *in situ* samples. The extra samples should be used for duplicate analysis at the *home* laboratory with the remaining duplicates sent to another laboratory using a validated method for separate analysis. The intercomparison of the two sets of analyses can be used to establish and monitor the uncertainties in pigment quantitation. Over time, a diversity of laboratories will provide the best estimate of quantitation uncertainties.
10. The performance metrics should be used to initially quantify, and periodically reaffirm, the capabilities of the method. Overall method performance should be a part of all data reporting activities using the labeling and scoring established for the "routine," "semiquantitative," "quantitative," and "state-of-the-art" nomenclature presented in Table 42.
11. The accuracy objective for determining the *in situ* concentration of total chlorophyll *a* in support of ocean color vicarious calibration activities should be changed from the current "routine" value of 25% to a "semiquantitative" value of 15% (with a precision to within 5%). The "semiquantitative" performance category should also be adopted as the minimum QA level for all pigment data being used in validation activities. This means the primary pigments must be determined within an accuracy of 25% and a precision of 8%.
12. The advantage of using higher-order variables (pigment sums, ratios, and indices) to reduce uncertainties in databases, which was first established with the SeaHARRE-1 analysis for predominantly mesotrophic waters (Claustre et al. 2004), is further confirmed with the mostly eutrophic data from SeaHARRE-2. Indeed, the analysis presented here shows how these variables can be used in a QA process, because the superiority of the A' subset as a reference for truth with respect to the A^+ subset was confirmed in part by the significant reduction in uncertainties for the higher-order variables.
13. Although laboratory standards—particularly mixed standards—provide an opportunity to quantify certain aspects of the uncertainties associated with an individual HPLC method, the most comprehensive examination occurs during round robins based on natural samples. Consequently, every laboratory making recurring and substantial contributions to a database in support of calibration and validation activities for ocean color remote sensing should participate in a pigment round robin as frequently as practical.
14. An implicit result of the work presented here was the creation of a detailed vocabulary and symbology to verbally and mathematically describe all aspects of HPLC methodologies in use by the marine community. In the absence of such a unique lexicon, it is unlikely another scientist or analyst can accurately reproduce the results or methods. Indeed, prior to SeaHARRE-2, many participants exposed a variety of misinterpretations in the techniques being employed simply because the language involved was too vague and open to multiple interpretations. Although the word and symbol choices espoused here solved many of the problems associated with documenting SeaHARRE-2, it is just an example, and

the community should use a more detailed revision of the Protocols to discuss and ultimately embrace a more widely-endorsed lexicography.

15. The individual pigments constituting the primary pigments (Table 5) should be adopted as the minimum set of variables for submission to larger marine databases. If this is done, all the higher-order variables discussed in this study (also in Table 5) will be accessible to all database users. Furthermore, a complete temporal understanding of the uncertainties in a significant portion of the databases will be possible.
16. For accurate TChl a concentrations, DVChl a needs to be individually quantitated, whether this is done by a simultaneous equation or chromatographic separation appears to be unimportant, because both approaches produced sufficiently accurate results for $\left[\text{TChl } a\right]$, as evidenced by the fact that the A' subset includes both methodological approaches. The uncertainties in $\left[\text{DVChl } a\right]$ at low concentrations are significantly influenced by the methodological approach, however, primarily because the simultaneous equation leads to the creation of false positives.
17. For the range of concentrations in SeaHARRE-2, the spectrophotometric determinations for chlorophyll a (SChl a) had lower uncertainties—less than 6% on average—than the average HPLC determinations of TChl a (initially 11% and finally about 6% after a rigorous QA process). Because the SChl a analyses were derived from the same filter extracts as TChl a, this strongly implies the nonmethodological sources of uncertainty (e.g., sample collection, transport, and extraction) were very low (in keeping with the 2% value separately estimated in Sect. 1.5.2).
18. Absorption coefficients and solvents used to spectrophotometrically determine the concentrations of pigment standards should be standardized. For six carotenoids within the A' subset, differences averaging approximately 8% (but as much as 15%) were observed when the absorption coefficients in ethanol were compared to those in acetone (Table 37). While it seems sensible to suggest the use of acetone absorption coefficients (because most laboratories extract samples in acetone), it is not currently practical to do so, because many standards currently available in ethanol are not commercially available in acetone.

Chapter 2

The BIO Method

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Abstract

The BIO HPLC method is similar to the method described by Gieskes and Kraay (1989), using a C_{18} HPLC column in combination with a methanol-based, reversed-phase binary gradient system. This method effectively separates the major key pigments in an analysis time of 30 min, but does not permit the separation of mono- and divinyl chlorophylls *a* and *b*. Separation of the divinyl pigments can, however, be achieved by acidifying a second sample, and quantifying the divinyl phaeophytin-like pigments. Acidification of samples with dilute HCL effectively separates divinyl phaeophytin *a* and *b* from phaeophytin *a* and *b*, respectively. Application of the latter procedure to chromatographically separate the divinyl forms requires each sample to be analyzed twice.

2.1 INTRODUCTION

Pigments at BIO were analyzed using a C_{18} column in combination with a methanol-based, reversed-phase binary gradient system. This method does not allow the separation of divinyl chlorophylls *a* and *b* from chlorophyll *a* and *b*, respectively. The latter was achieved by acidifying a second sample with 1 N HCL and quantifying the divinyl phaeophytin-like pigments. The method effectively separates most of the major key pigments except for those that coelute: chlorophyll c_1 and chlorophyll c_2, as well as zeaxanthin and lutein.

The HPLC system includes a fluorescence detector and a diode array detector, but no autosampler (the samples were manually injected) and no temperature control. The samples were stored in a $-80°$C freezer until the HPLC analysis was performed. The pigments were extracted in 95% acetone by mechanical grinding, and clarified by centrifugation.

2.2 EXTRACTION

Immediately prior to analysis, the pigments were extracted by grinding the filter in 1.5 mL 95% acetone in dim light for approximately 1 min, using a glass tube and a motorized grinder fitted with a Teflon pestle. The glass tube was placed on ice midway through grinding to avoid overheating. The extract was then centrifuged for 2 min at 12,000 *g* to clarify the sample, and the supernatant pipetted off.

2.3 HPLC ANALYSIS

The samples were analyzed using a Beckman System Gold HPLC with the following components:

1. A 3 µm Ultrasphere-XL ODS column (70×4.6 mm) with an Ultrasphere-XL ODS guard cartridge (the column temperature is not controlled);
2. A 126 solvent module with binary pump;
3. A 168 UV-VIS diode array detector;
4. A 406 analog interface module;
5. A 157 fluorescence detector; and
6. Data acquisition and analysis software (Beckman System Gold running on a PC with MS-DOS operating system).

A 150 µL subsample of pigment extract was diluted with 300 µL 0.5 M ammonium acetate buffer using a Hamilton microliter syringe, and 250 µL of this solution was manually injected through a 500 µL sample loop into the HPLC system. It has been previously determined that separation of individual pigments can be satisfactorily achieved by using a linear solvent gradient system similar to that used by Gieskes and Kraay (1989). The gradient (Table 43) was run from 100% solvent A to 100% solvent B over a 15 min period, where solvent A was methanol and 0.5 M ammonium acetate (80:20) and solvent B was methanol and ethyl acetate (70:30). The flow rate for the gradient was 1 mL min^{-1} and the column temperature was not controlled.

Table 43. The gradient used with the BIO column. The time is in minutes, and the percentages of solvents A and B are given in the last two columns, respectively.

Step	*Time*	A [%]	B [%]
Start	0	100	0
2	3	100	0
3	18	0	100
4	26	0	100
5	27	100	0
End	28	100	0

Divinyl chlorophyll *a* and divinyl chlorophyll *b* were separated from chlorophyll *a* and chlorophyll *b*, respectively, by acidifying a subsample of the pigment extract. Acidification resulted in divinyl phaeophytin *a* eluting 0.3 min past phaeophytin *a*, and the same was true for divinyl phaeophytin *b* and phaeophytin *b*. To acidify the samples, 10 µL of 1 N HCL was added to 300 µL of the pigment extract and the solution was left in the dark at room temperature for 20 min. Next, 600 µL of 0.5 M ammonium acetate buffer was added to the sample, and 250 µL of this mixture was injected into the HPLC system.

A blank sample (buffer only) was always injected at the beginning of each day. The eluting peaks were monitored at 430 nm using a Beckman 168 UV-VIS diode array detector and a Beckman 157 fluorescence detector fitted with a broad band excitation filter (430–450 nm), a broad band emission filter (585–650 nm) and a red-sensitive extended wavelength photomultiplier tube. The diode array detector collected spectra between 400–550 nm, which were used to verify pigment identification. Peak areas were integrated automatically using the following default parameters:

1. Peak width (0.2);
2. Minimum peak height (0.0005); and
3. Peak threshold (0.002).

Peaks were also visually inspected to confirm correct baselines and optimal integration.

2.4 CALIBRATION

Pigment calibration standards were either purchased from commercial suppliers or isolated from microalgal cultures. Chlorophylls *a* and *b*, and *ββ*-carotene were purchased from Sigma-Aldrich Company (St Louis, Missouri). Divinyl chlorophyll *a*, 19′-butanoyloxyfucoxanthin, zeaxanthin, and 19′-hexanoyloxyfucoxanthin were purchased from DHI Water and Environment (Hørsholm, Denmark). The remaining pigment standards were extracted from microalgal cultures, either grown in the BIO laboratory, or provided by Dr. R. Bidigare. Phaeophytins *a* and *b* and divinyl phaeophytin *a* and *b* were identified using acidified standards. Chlorophyllide *a* was obtained by 50% acetone-water extraction of a filter of material from a *Phaeodactylum tricornutum* culture.

The concentrations of all chlorophyll derivatives are reported as their respective chlorophyll equivalent concentrations. Chlorophyllide *a* was quantified using the chlorophyll *a* calibration factor. Concentrations of all chlorophylls and their derivatives were quantified using the fluorescence chromatogram, except sometimes for chlorophyll *a* peaks that were above the range of the detector. In this case, the corresponding absorption chromatograms were used.

At least six replicate calibrations were run for each standard. The oncentrations of all pigment standards were determined using absorption measurements carried out on a dual-beam Shimadzu 2101 spectrophotometer equipped with a 2 nm bandwidth. The specific absorption coefficients (α) and absorption maxima used for each pigment is given in Table 44. Peak identifications were made through comparison of retention times and diode-array absorbance spectra with the spectra of known pigment standards.

Table 44. The α values used with the BIO method for the various pigment standards as a function of λ. The units for α are liters per gram per centimeter and the units for λ are nanometers.

Pigment	*Solvent*	λ	α
Chl $c_{1+}c_2$	100% Acetone	630.0	38.20
Perid	100% Acetone	468.0	134.00
But-fuco	100% Ethanol	446.0	160.00
Fuco	100% Acetone	446.0	150.70
Hex-fuco	100% Ethanol	445.0	160.00
Cis-neo	100% Ethanol	438.0	227.00
Pras	100% Ethanol	454.0	160.00
Diadino	100% Ethanol	448.0	262.00
Allo	100% Ethanol	454.0	262.00
Zea	100% Ethanol	450.0	254.00
Chl *b*	90% Acetone	646.0	51.40
DVChl *b*	90% Acetone	650.0	51.40
Chl *a*	90% Acetone	664.0	87.67
DVChl *a*	90% Acetone	664.3	87.67
βε-Car	100% Ethanol	446.0	262.00
ββ-Car	100% Ethanol	452.0	262.00

2.5 VALIDATION

Pigment retention times were checked daily by injecting a mixture of pigment standards, and comparing retention times with those of the unknown sample. Pigment identification was also manually verified by comparison of the HPLC in-line diode array detector spectra with that of the pigment standards and published spectra. Response factors for chlorophylls *a* and *b*, and fucoxanthin were computed at the beginning of every cruise (30–100 samples), and those of the other pigments were checked at regular intervals. Response factors for absorption were relatively stable, but those for fluorescence changed with the age of the lamp.

2.6 DATA PRODUCTS

For every injection, a data file and printout was created listing pigment retention times, peak area, peak height, and area percentage. Data were visually inspected to identify the various pigments, and then manually entered into a spreadsheet file, and converted into pigment concentrations. The following formula was used to calculate pigment concentration (C_{P_i}) of each sample (in units of nanograms per liter):

$$C_{P_i} = \frac{V_e + V_w}{V_f} \frac{\hat{A}_{P_i} R_{P_i}}{V_c'/D_b}, \tag{32}$$

where $\hat{A}_{P_i}$ is the peak area of pigment P_i, R_{P_i} is the response factor, D_b is the dilution factor for the volume of buffer plus sample and is computed as the inverse of the proportion of sample in the sample-plus-buffer mixture (i.e., the inverse of the sample volume divided by the sum of the sample volume plus the buffer volume), V_c' is the volume of sample-plus-buffer mixture injected, V_f is the filtration volume in liters, V_e is the volume of extraction solvent in milliliters, and V_w is the volume of water retained by each filter (approximately 0.2 mL). The latter two terms combine to produce an extraction volume ($V_x = V_e + V_w$) of 1.7 mL, and the volume of sample injected onto the HPLC column is $V_c = V_c'/D_b$.

2.7 CONCLUSIONS

The reversed-phase HPLC method with a C_{18} column, and the additional step of acidifying samples, provides good resolution of mono- and divinyl chlorophylls *a* and *b*, and separates most other key pigments in a run time of 30 min (or 60 min if samples are acidified). The method is suitable for a wide range of samples, which only require acidification if the water temperature at which the samples are collected is above about 10°C (Partensky et al. 1999), and if zeaxanthin is present (indicating the possible presence of *Prochlorococcus* sp.).

Chapter 3

The CSIRO Method

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Abstract

The HPLC method used by CSIRO is a modified version of the Wright et al. (1991) method. This method has the capacity to separate 50 different pigments and is used routinely in the CSIRO laboratory to separate approximately 35 different pigments, but does fail to separate monovinyl chlorophyll *a* and chlorophyll *b* from their divinyl forms. CSIRO has continued to use this method, because until recently, it was the recommended method of SIMBIOS (Mueller et al. 2003), and for many years the work of this laboratory has been in temperate waters for which the method works well. More recently, CSIRO has occasionally been working in tropical waters where divinyl chlorophyll *a* is often the predominant form of chlorophyll, and in these cases, the laboratory has started using the Zapata et al. (2000) method. For the SeaHARRE-2 activity, the Wright et al. (1991) method was used exclusively. Samples are extracted over 15–18 h in an acetone solution before analysis by HPLC using a reverse-phase C_{18} column and ternary gradient system with a photodiode array (PDA) detector. The method is regularly validated with the use of external standards and individual pigment calibration. The detection limit of most pigments is within the range of 0.001–0.005 mg m^{-3}. The method has proven to offer a good balance between accuracy of pigment composition and concentration and number of samples analyzed; an important factor in an applications based laboratory.

3.1 INTRODUCTION

The CSIRO method separates pigments on a reversed-phase C_{18} column using a three-solvent gradient system. Using a PDA detector, pigments are verified by the retention time and absorption spectra of each chromatographic peak and quantified by the detector signal at 436 nm. Separation of most pigments is achieved, the exceptions being chlorophyll c_1 and chlorophyll c_2, lutein, and zeaxanthin, plus the monovinyl forms of chlorophyll *a* and chlorophyll *b* from the divinyl forms. Using the PDA detector, the method can establish the presence of the divinyl forms by observation of the spectral shift in the 431 nm peak. Analysis time is 30 min per sample with a further 5 min injection delay to ensure no carry over between sequential samples.

Immediately after collection, field samples are stored in liquid nitrogen until analysis in the laboratory. The SeaHARRE-2 samples were received on 22 January 2003 and were stored in liquid nitrogen until analysis on 15 March 2003.

3.2 EXTRACTION

To extract the pigments, the thawed filters are cut into small pieces and covered with 100% acetone (3 mL) in a 10 mL centrifuge tube (the scissor and forcep blades are cleaned 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) and sonicated once more for 15 min in an ice-water bath in the dark.

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

The centrifuged extracts are then filtered through a 0.2 µm Teflon syringe filter, which has been rinsed with acetone, directly into a 1 mL amber vial. The remaining extract from each sample remains in a centrifuge tube, covered with parafilm, and is stored at −20°C until the HPLC analysis has been successfully completed.

3.3 HPLC ANALYSIS

The CSIRO system is a Waters HPLC comprising a 600 controller, 717 plus refrigerated autosampler, and a 996 PDA detector. The refrigerated autosampler is set to 4°C, but generally runs at 6°C because of room temperature effects. The pigments are separated using a stainless steel 250 × 4.6 mm internal diameter column packed with ODS-2 of 5 µm particle size (SGE). The column is maintained at a temperature of 30°C to reduce the effects of room temperature variation.

The samples and standards are stored in 1 mL amber vials in the autosampler prior to injection. Samples are kept under these conditions for no more than 12 h, and, in general, 200 µL injections are made from a 250 µL loop. After injection, separation of the pigments is achieved using the gradient elution system shown in Table 45 with a flow rate of 1 mL min^{-1}. The analysis time of each sample is 30 min with an additional 5 min injection delay of the next sample to ensure there is no carry over between samples.

Table 45. The three-solvent gradient elution system for the CSIRO method. Solvent A is composed of 80:20 methanol:0.5 M ammonium acetate; solvent B is 90:10 acetonitrile:water; and solvent C is ethyl acetate.

Step	*Time*	A [%]	B [%]	C [%]
Start	0	0	100	0
2	4	0	100	0
3	22	0	0	100
4	24	0	100	0
5	26	100	0	0
End	30	100	0	0

Initial peak integration and identification is performed by the automated features of Waters Millenium 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.

3.4 CALIBRATION

Calibration standards are either purchased, as in the case of chlorophyll *a*, chlorophyll *b*, $\beta\varepsilon$-carotene, and $\beta\beta$-carotene (Sigma), or isolated from algal cultures. The concentration of all standard stock solutions is determined using a GBC 916 UV/VIS dual-beam spectrophotometer with a 2 nm bandwidth. An absorption spectrum of each pigment is recorded between 350–900 nm. The concentration of each pigment is calculated using the absorption coefficient from the literature (Jeffrey et al. 1997) together with the absorption measured at the corresponding wavelength. The absorption coefficients, wavelengths, and solvents used for each pigment are listed in Table 46. The absorbance at the wavelength used is corrected for any absorption measured at 750 nm.

Table 46. The α values used with the CSIRO method for the various pigment standards as a function of λ. The units for α are litres per gram per centimetre and the units for λ are nanometers.

Pigment	*Solvent*	λ	α
Perid	100% Acetone	466.0	134.00
But-fuco	100% Acetone	445.0	147.00
Fuco	100% Acetone	447.0	166.00
Hex-fuco	100% Acetone	445.0	142.00
Neo	100% Acetone	438.0	227.00
Pras	100% Acetone	450.0	250.00
Viola	100% Acetone	442.0	240.00
Diadino	100% Acetone	448.0	223.00
Allo	100% Acetone	454.0	250.00
Diato	100% Acetone	452.0	210.00
Lut	100% Acetone	446.0	255.00
Zea	100% Acetone	454.0	234.00
$\beta\varepsilon$-Car	100% Acetone	448.0	270.00
$\beta\beta$-Car	100% Acetone	454.0	250.00
Chlide *a*	100% Acetone	664.0	127.00
Chl *b*	100% Acetone	646.0	51.36
Chl *a*	100% Acetone	662.5	88.15
Chl c_3	100% Acetone	453.0	334.50
Chl c_2	100% Acetone	628.9	37.20
Phytin *b*	90% Acetone	657.0	31.80
Phytin *a*	90% Acetone	667.0	51.20

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

3.5 VALIDATION

At the beginning of every set of samples analyzed by the HPLC system, a pigment mixture is analyzed to determine if there is any change in the retention time of approximately 30 pigments. A mixture of known concentrations of fucoxanthin, chlorophyll *a*, chlorophyll *b*, and $\beta\beta$-carotene is also analyzed to determine whether or not the HPLC system, including the column, are working appropriately. In the case of the SeaHARRE-2 experiment, where the number of samples analyzed vastly exceeded the normal analysis conditions in the CSIRO laboratory, the mixture of known concentration of standards was analyzed after every tenth injection.

The calibrations of chlorophylls *a* and *b* as well as $\beta\beta$-carotene are done approximately every three months, whereas calibrations of a selection of other pigments are done approximately once a year.

3.6 DATA PRODUCTS

Waters Millenium software creates an electronic file in which each chromatographic peak has its retention time, peak area, and peak height recorded together with an initial pigment identification. Once the chromatograms have been manually checked, the peak areas are transferred to a spreadsheet in which the pigment concentrations are calculated using the appropriate response factor:

$$C_{P_i} = \frac{V_x}{V_f}\,\frac{V_c}{V_s}\,\frac{\hat{A}_{P_i}}{F_{P_i}}\,D_f, \tag{33}$$

where V_x is the final extraction volume (composed of the volume of extraction solvent plus the volume of water retained on the filter); V_f is the volume of sample water filtered; V_c is the volume of sample extract injected onto the column; V_s is the volume of standard injected to determine the inverse response factor, F_{P_i}; $\hat{A}_{P_i}$ is the peak area of pigment P_i; and D_f is a dilution factor (rarely used and only applied if the color of the extract is very dark and it is probable the sample will be outside the linear range of chlorophyll *a*).

Observations over time (in graduated tubes) indicate the extraction volume is rather constant, $V_x = 4.0 \pm 0.1$ mL. Consequently, for each sample extracted, the extraction volume is observed in a graduated tube to see if it falls between these limits. If it is not within 3.9–4.1 mL, the volume observed is recorded for use; otherwise, 4.0 mL is used.

Both V_c and V_s are in the concentration formula (33), because the injected volume of sample can occasionally differ from the injected volume of standard. This is important because F_{P_i} in (33) is not simply the inverse of the response factor (amount injected divided by area) as discussed with (5), F_{P_i} is the inverse of micrograms of pigment per milliliter divided by area. For SeaHARRE-2, V_c was equal to V_s, so (33) can be simplified to $C_{P_i} = (4.0/V_f)(\hat{A}_{P_i}/F_{P_i})D_f$, with V_f given in milliliters.

3.7 CONCLUSIONS

The CSIRO method provides the resolution and quantitation of more than 20 pigments with detection limits ranging from 0.001–0.005 mg m^{-3} within an analysis time of 30 min. One disadvantage of the method is that separation of the monovinyl forms of chlorophyll *a* and chlorophyll *b* cannot be chromatographically separated from their divinyl forms, making the method unsuitable for analysis of samples from tropical waters.

Chapter 4

The DHI Method

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Abstract

The HPLC method used at DHI is a slightly modified version of the method developed by Wright et al. (1991). This method does not separate chlorophyll c_1 and chlorophyll c_2; furthermore, divinyl chlorophylls *a* and *b* are not separated from monovinyl chlorophylls *a* and *b*, respectively. Dichromatic equations were used to separate divinyl chlorophyll *a* from monovinyl chlorophyll *a* (Latasa et al. 1996). The results of the dichromatic equations, however, showed in several instances that divinyl chlorophyll *a* was present although this pigment was not detected by the laboratories that were able to separate the two pigments chromatographically. The dichromatic equation method was apparently giving erroneous results, and should therefore be used with caution. Phaeophorbide *a* was not separated from fucoxanthin, but because these pigments have distinctly different absorption spectra, they could subsequently be separated.

4.1 INTRODUCTION

The HPLC method used at DHI is a slightly modified version of the method developed by Wright et al. (1991). This method is not able to separate chlorophylls c_1 and c_2; furthermore, divinyl chlorophylls *a* and *b* are not separated from monovinyl chlorophylls *a* and *b*, respectively, but they can be separated using dichromatic equations as described in Latasa et al. (1996).

4.2 EXTRACTION

The HPL unknowns and the *in situ* filters were received in dry shippers on 16 and 17 December 2002, respectively, and stored at −80°C until analyzed on 29–31 January 2003. Before analysis by HPLC, the filters were thawed, placed in vials, and 100 µL internal standard (*trans*-β-apo-8′-carotenal), which had to be purified prior to use, and 3 mL 100% acetone were added to the filters. The samples were sonicated on ice for 10 min, and extracted for 24 h at 4°C. The filters and cell debris were filtered from the extract using disposable syringes and 0.2 µm Teflon syringe filters. Subsequently, 1 mL of extract was transferred to HPLC vials and placed in the HPLC cooling rack. Prior to injection, 300 µL of water was added and mixed to the extract in the HPLC vial by the auto injector.

4.3 HPLC ANALYSIS

The DHI HPLC system is a Shimadzu LC-10A/VP HPLC composed of three pumps (LC-10AS), PDA detector (SPD-M10A VP), SCL-10AVP System controller with Class VP software version 5.0, temperature-controlled autosampler (set at 4°C) and column compartments (set at 20°C), and a degasser.

The samples were injected into the HPLC according to the method described by Wright et al. (1991), although the linear gradient was modified slightly in order to obtain good resolution of the pigments (Table 47). Chlorophylls and carotenoids were detected at 436 nm, phaeopigments at 405 nm, while divinyl chlorophylls *a* and *b* were determined using dichromatic equations (Latasa et al. 1996). Peak identities were routinely confirmed by on-line PDA analysis.

Table 47. The gradient used in the DHI method. Solvent A was 80:20 methanol:0.5 M ammonium acetate, pH 7.2; solvent B was 90:10 acetinitrile:water; and solvent C was 100% acetonitrile. The flow rate was 1 mL min^{-1}.

Step	*Time*	A [%]	B [%]	C [%]
Start	0.0	100	0	0
2	1.0	25	75	0
3	2.0	21	69	10
4	12.0	21	69	10
5	13.6	5	60	35
6	23.0	0	31	69
7	28.0	0	31	69
8	31.0	0	40	69
9	33.0	100	0	0
End	36.0	100	0	0

4.4 CALIBRATION

The HPLC system was calibrated with pigment standards from the International Agency for ^{14}C Determination (DHI) in January 2003, before the analysis of the SeaHARRE-2 samples. The concentrations of the calibration standards were determined using a Shimadzu UV-2401-PC dual-beam, monochromator-type spectrophotometer, which is subjected to a regular set of quality control procedures. In addition, selected batch numbers for the standards are controlled for purity and concentration by an independent laboratory. Chl c_1 and Chl c_2 elute together, and were reported as Chl c_1+c_2, using the absorption coefficient of Chl c_2.

The calibrations were made from single-point calibrations repeated four times. The E values for the DHI pigment standards used with the DHI methods are presented in Table 48.

Table 48. The α values used with the DHI method for a variety of pigments as a function of λ. The units for α are liters per gram per centimeter and the units for λ are nanometers.

Pigment	*Solvent*	λ	α
Chl c_3	90% Acetone	452.9	346.00
Chl c_2	90% Acetone	443.8	374.00
Chlide a	90% Acetone	664.0	127.00
Perid	100% Ethanol	472.0	132.50
Phide a	90% Acetone	667.0	74.20
Phytin a	90% Acetone	667.0	51.20
But-fuco	100% Ethanol	446.0	160.00
Fuco	100% Ethanol	449.0	160.00
Neo	100% Ethanol	439.0	224.30
Pras	100% Ethanol	454.0	160.00
Viola	100% Ethanol	443.0	255.00
Hex-fuco	100% Ethanol	447.0	160.00
Diadino	100% Ethanol	446.0	262.00
Allo	100% Ethanol	453.0	262.00
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	664.3	87.67
Chl a	90% Acetone	664.3	87.67
$\beta\varepsilon$-Car	100% Aetone	448.0	270.00
$\beta\beta$-Car	100% Ethanol	453.0	262.00

4.5 VALIDATION

The performance of the HPLC systems was checked every day by injecting a mixture of pigment standards, and controlling that the peak separation was good. This pigment mixture was also used for determining the identity of the peaks by comparing both the retention time and the absorption spectra of the unknown pigments in the samples with the retention time and the absorption spectra of the pigment standards in the mixture.

Injections of a chlorophyll a standard are made four times immediately after running a set of samples and are used routinely to control the accuracy of the response factors.

4.6 DATA PRODUCTS

The peak areas and pigment identities were transferred to a spreadsheet file, and based on the response factors, the pigment concentrations were calculated and corrected for errors during handling by the internal standard (applied in a two-step process, which is denoted by the "2" subscripts):

$$C_{P_i} = \frac{V_s \hat{A}_{c_2}/\hat{A}_{s_2}}{V_f} \frac{\hat{A}_{P_i} R_{P_i}}{V_c}, \tag{34}$$

where V_s is the volume of internal standard added to the extract–sample mixture, $\hat{A}_{c_2}$ is the peak area of the internal standard when it is injected onto the HPLC column prior to its addition to the sample, $\hat{A}_{s_2}$ is the peak area of the internal standard in the sample, V_f is the volume of water filtered to produce the sample, $\hat{A}_{P_i}$ is the peak area of pigment P_i, R_{P_i} is the response factor, and V_c is the volume of sample extract injected onto the column. The formulation in (34) can be presented more compactly†, but this is not done so all the terms are accessible. Dichromatic equations were used to separate divinyl and monovinyl chlorophyll a as described in Latasa et al. (1996).

4.7 CONCLUSIONS

The DHI method was originally developed by Wright et al. (1991) and provides good resolution of most marine pigments. The gradient has to be adjusted to the HPLC and to each new column in order to give good resolution of the peaks, especially Zea and Lut, as well as Neo and Hex-fuco. At DHI, the method has mostly been used for phytoplankton in fresh water, estuaries, and coastal areas, so it has not been necessary to routinely separate the divinyl and monovinyl forms of chlorophyll a for the purpose of detecting prochlorophytes. During SeaHARRE-2, the results of the dichromatic equations used to separate DVChl a from MVChl a in several instances showed that DVChl a was present, although this pigment was not detected by the laboratories which were able to separate the two pigments chromatographically. The dichromatic equation method was apparently giving erroneous results, and should, therefore, be used with caution. The method does not separate phaeophorbide a from Fuco, but because these pigments have different absorption spectra, they are easy to separate by measuring their absorption at different wavelengths.

† Using (1) and (8), (34) reduces to (6), because $\tilde{C}_{P_i} = \hat{A}_{P_i} R_{P_i}$ and $V_x = V_s \hat{A}_{c_2}/\hat{A}_{s_2}$, respectively, that is,
$C_{P_i} = \frac{V_x}{V_f} \frac{\tilde{C}_{P_i}}{V_c}$.

Chapter 5

The HPL Method

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Abstract

The HPL method was developed for use with a variety of water types. Many pigments important to freshwater, estuarine, and oceanic systems are baseline resolved and quantitatively reported, including divinyl and monovinyl chlorophyll *a*. The linear dynamic range for chlorophyll *a* extends approximately from 0.3–700 ng per injection. The method is based on a C_8 HPLC column, a methanol-based reversed-phase gradient solvent system, a simple linear gradient, and an elevated column temperature (60°C). The method can provide quantitative results for up to 25 pigments with qualitative information for additional pigments. Quality assurance measurements are made during sample analysis to confirm method performance is within expectations. Investigations into the uncertainties in the method show the 95% confidence limits were estimated as a) 0.5–3.8% for precision of replicate injections within and across sequences, b) 3.2% for chlorophyll *a* calibration reproducibility, and c) 5.1% for chlorophyll *a* method precision, including filter extraction and analysis.

5.1 INTRODUCTION

The HPLC method used by HPL was developed for a variety of water types, ranging from the coastal environment to the open ocean, with widely varying chlorophyll *a* concentrations. As such, many taxonomically important pigments common to freshwater, estuarine, and oceanic systems are well resolved. Features of this method include:

1. Baseline resolution between the key taxonomically important carotenoids, as well as divinyl and monovinyl chlorophyll *a*, and partial resolution between divinyl and monovinyl chlorophyll *b*;
2. An analysis time for one sample of approximately 30 min; and
3. Adequate sensitivity for the accurate analysis of dilute oceanic samples, e.g., approximately 0.25 ng of chlorophyll *a* yields an SNR of 10 (Sect. 1.4.3.5).

The HPL method uses a C_8 column and a reversed-phase, methanol-based, binary gradient, solvent system. The detector signal at 665 nm is used to quantify chlorophyll *a*, divinyl chlorophyll *a*, chlorophyllide *a*, phaeophorbide *a*, and phaeophytin *a*. All other pigments are quantified from the signal at 450 nm.

Limitations of the HPL method include the coelution of chlorophyllide *a* with chlorophyll c_1, and the coelution of $\beta\varepsilon$-carotene with $\beta\beta$-carotene, the latter of which are not individually quantified. Chlorophyllide *a* as well as chlorophyll c_1 are quantified using a dichromatic equation based on their spectral differences similar to that described in Latasa et al. (1996) for monovinyl and divinyl chlorophyll *a*. Other pigments with a potential for coelution are discussed in Sects. 5.4 and 5.5 and are further detailed in Van Heukelem and Thomas (2001). Subsequent to SeaHARRE-2, unknown pigments in some natural samples were found to elute at retention times of Neo and Myxo.

5.2 EXTRACTION

The SeaHARRE-2 filters were received at HPL on 30 October 2002 and were immediately stored in a −80°C freezer until analysis on 7–8 November 2002. Part of the analysis included samples that were mishandled and unequivocally defrosted during shipment. These analyses were intended to provide insight into the various properties of damaged samples, so they might be identified under circumstances wherein the preservation history of the samples is in doubt.

Each filter was cut into slivers and placed in a heavy-walled, 15 mL conical glass tube; 3 mL of 95% acetone, containing approximately 0.025 $\mu g\,mL^{-1}$ of the internal standard vitamin E acetate (Fluka 95250), was added to the glass tube using a Dispensette Organic™‡ re-pipette set to deliver 3.00 mL and calibrated at 3.078±0.0119 mL.

‡ Dispensette Organic is a registered trademark of BrandTech, Inc. (Essex, Connecticut).

Each tube was then covered with Parafilm™, placed in an ice bath, and kept in the dark until a set of filters (20 or 30) was processed in the same manner. Each filter was then individually disrupted using an ultrasonic probe (Branson Ultrasonics Corp., Model 450) for approximately 30 s with an output of 40 W. Each tube was submerged in a beaker of ice during disruption to prevent heat accumulation.

After each filter was disrupted and the tube was again tightly wrapped in Parafilm, the entire filter set was placed in a freezer (−15°C) for 4 h. Each sample slurry was then well mixed on a vortex mixer, transferred to a 20 mL syringe, and the extract was clarifed by pushing the contents through a Teflon HPLC syringe cartridge filter (Scientific Resources, Inc.) with a 0.45 µm pore size, a 25 mm diameter, and a glass-fiber prefilter. The extract was collected in a 7 mL glass vial. After mixing well, 500 µL of extract was placed in an HPLC vial and put directly into the autosampler compartment. The remaining extract was stored in a freezer (−15°C) until the HPLC analysis was complete.

5.3 HPLC ANALYSIS

The replicate filter extracts were analyzed using a fully automated HP 1100 HPLC system equipped with a quaternary pump, programmable autoinjector, temperature-controlled autosampler, peltier temperature-controlled column oven compartment, PDA detector, and ChemStation software (all from Agilent Technologies, Inc.). Vials of injection buffer, samples, and calibration standards were placed in the autosampler compartment tray (maintained at a constant 5°C) and analyzed over a time period not exceeding 24 h. The sample extract (or standard) was mixed with buffer (90:10, 28 mM aqueous tetrabutyl ammonium acetate, 6.5 pH; methanol) by the autoinjector (within a 900 µL sample loop) immediately prior to injection using an injector program specifically created for use with this method and HPLC injector configuration.

The injector program was optimized to yield peaks that are symmetrical in shape and exhibit consistent peak widths, regardless of elution position. Alternating volumes (in microliters) of buffer and sample, respectively, were drawn into the 900 µL loop as follows: 150–75–75–75–150, after which the entire contents of the sample loop are injected onto the column. This procedure uses 375 µL of buffer and 150 µL of sample extract.

After sample injection, separation was achieved using an Eclipse XDB C_8 HPLC column (part number 963967-906), 4.6 mm×150 mm (diameter by length), manufactured by Agilent Technologies with gradient elution using a linear gradient from 5–95% solvent B in 22 min. Solvent B was methanol, and solvent A was (70:30) methanol:28 mM aqueous tetrabutyl ammonium acetate, 6.5 pH (Table 4).

An isocratic hold on 95% solvent B was necessary from 22–29 min for elution of the last pigment ($\beta\beta$-carotene) at approximately 27 min. After a return to initial conditions (5% solvent B) by 31 min, the column was equilibrated for 5 min prior to the start of the next injection cycle and analysis (Table 49). The flow rate was 1.1 mL min^{-1} and the column temperature was 60±0.8°C†.

Table 49. The gradient used with the HPL column organized by the steps involved in the complete analysis of a sample and the percentages of solvents A and B. All gradient changes are linear.

Step	*Time*	A [%]	B [%]
Start	0	95	5
2	22	5	95
3	29	5	95
4	31	95	5
End	36	95	5

Data from the first injection in an automated sequence of analyses were always disregarded, because retention time and peak area reproducibility were less precise than in subsequent analyses. Pigments were detected at 450 and 665 nm (20 nm bandwidths for both) with the photodiode array detector using a flowcell volume and pathlength of 13 µL and 10 mm, respectively. The signal at 665 nm was used to quantify monovinyl and divinyl chlorophyll *a*, phaeophytin *a*, and phaeophorbide *a*; the signal at 450 nm was used to quantify all other pigments. Peaks were integrated using the automated functions of the ChemStation software, all peaks were visually inspected to verify the automated integrator had drawn peak baselines correctly and in a fashion consistent with the peak integrations of calibration standards. Paper copies and an electronic data file were retained for each analysis.

5.4 CALIBRATION

External calibration standards were either purchased or isolated from naturally occurring sources (Van Heukelem and Thomas 2001) as listed in Table 50. All pigments listed with a bullet (•) symbol in the first column in Table 50 were reported (the mode of quantitation and potential for interference from coeluting pigments are also listed). The pigment standard concentrations were determined using absorption coefficients (Table 51) and absorbance measured with a dual beam, monochromator-type spectrophotometer (Shimadzu Scientific Instruments, Inc., model UV-2401-PC). A spectral bandwidth of 2 nm was used, the sample was corrected for turbidity at 750 nm, and the standards were sufficiently concentrated such that the absolute absorbance of each pigment fell from 0.1–1.0 (in absolute units) for greatest spectrophotometer accuracy (Clesceri et al. 1998). Absorbance accuracy of the spectrophotometer was checked with neutral density filters traceable to NIST as described in Latasa et al. (1999).

† A useful presentation of needed precautions when using elevated column temperatures is provided by Wolcott et al. (2000).

Table 50. The pigments identified by the HPL method, the sources used to identify them, and the in-line visible absorbance spectra from the HPLC DAD (350–700 nm). For the latter, parentheses indicate a spectral shoulder. Peak numbers marked with the bullet symbol (•) are reported for SeaHARRE-2. The source entries indicate the references used to identify pigments (including algal cultures and authentic standards) and the absorbance entries specify the observed in-line HPLC absorbance spectra. The t_R values refer to pigment retention times (in minutes). Quantitation indicates whether the pigment was quantified based on response factors determined from a) a discrete pigment standard whose concentration had been determined spectrophotometrically with absorption coefficients (those listed with α), or b) an alternative mode of quantitation. The SCOR WG 78 abbreviations are listed in Appendix B. The pigment absorption coefficients used are given in Table 51. Pigments without quantitation details are included because they are often poorly resolved from adjacent pigments ($R_s \leq 1.5$) and can, therefore, interfere with accurate quantitation. Resolution values for such pigments are given or listed as NR (not resolved) and identified by their pigment numbers.

No.	*Pigment*	*Source*	*Absorbance* [nm]	t_R	R_s	*Quantitation*
1•	Chl c_3	C, J, L, N, S, T	456, 588, (625)	3.88		Avg. Chl c_1 and Chl c_2 R_f
2	MVChl c_3	J	448, 585, (626)	4.14		
3•	Chl c_2	A–E, H, J, L–O, R–T	446, 584, 634	5.70	NR 3/4	α
4	Mg DVP	G, P	440, 576, 632	5.81	NR 4/a	
a	*Unknown*	K		5.92	NR a/5	
5•	Chl c_1	B, D, E, R	442, 580, 634, 668	6.05	NR 5/6	Simultaneous equation
6•	Chlide a	F, H, N, O	(390), 434, 620, 668	6.06	NR 5/6	Simultaneous equation, part of TChl a
7•	Phide a	DHI†	408, 508, 538, 608, 666	8.05		R_f of Phytin a with a molecular weight correction
8	Phide a-like		408, 508, 538, 608, 666	8.29		Part of Phide a
9	Phide a-like		408, 508, 538, 608, 666	9.19		Part of Phide a
10•	Perid	A, B, M	476	9.32		α
11	Perid isomer	A, B, M	478	9.58		
12	Phide a-like		408, 508, 538, 608, 666	10.60		Part of Phide a
13	Phide a-like		408, 508, 538, 608, 666	10.78		Part of Phide a
b	*Unknown*	P	456, 476	11.37		
14•	But-fuco	DHI†, C, N, S, T	448, 464	12.31		α
c	*Unknown*	G	458	12.68	NR c/15	
15•	Fuco	C, D, E, L, N, R–T	454	12.63	NR c/15	α
16•	Neo	F, G, P, U	414, 438, 466	13.29	NR 16/17	α
17	4k-hex-fuco	J	448, 470	13.31	NR 16/17	
d	*Unknown*	L	446, 468	13.73	NR d/18	
18•	Pras	DHI†, G, P	462	13.74	NR d/18	α
19•	Viola	DHI†, F, G, P, Q, U	418, 442, 470	13.99	1.3 19/20	α
20•	Hex-fuco	DHI†, C, J, L	(430), 452, 480	14.16	1.3 19/20	α
21	Asta	Shrimp carapace, G	480	14.53		
e	*Unknown*	P	466	14.78		
22	Diadchr	M	(410), 428, 456	15.02	NR 22/f	
f	*Unknown*	J	448, 470	15.09	NR f/23	
23	*Unknown*	K	452, 474, 506	15.13	NR 23/24	
24•	Diadino	A–E, J, L–N, R–T	(428), 446, 476	15.23	1.4 24/25	α
25	Dino	A, M	416, 440, 470	15.49		
26	Anth	F	(425), 446, 474	15.99		α
27•	Allo	H, O	(430), 452, 480	16.53		α

Table 50. (cont.) The pigments identified by the HPL method, the sources used to identify them, and the in-line visible absorbance spectra from the HPLC DAD (350–700 nm).

No.	*Pigment*	*Source*	*Absorbance* [nm]	t_R	R_s		*Quantitation*
28	Myxo	DHI†	(450), 474, 506	17.34			α
29•	Diato	C, D, E, M, R, T	(430), 454, 480	17.12	NR	29/30	α
30	Monado	H, O	(422), 444, 472	17.22	NR	29/30	
31•	Zea	C, F, G, I, K, P, Q, V	(430), 452, 478	17.79			α
32•	Lut	F, G, T, V	424, 446, 474	17.98			α
g	*Unknown*	Q	422, 444, 472	18.24	NR	g/h	
h	*Unknown*	G	(408), 428, 454	18.32	NR	g/h	
i	*Unknown*	L, N, S, T	(424), 448, 472	18.84			
33•	Cantha	W	480	19.07			α
j	*Unknown*	Q	422, 444, 472	19.23			
34	Gyroxanthin diester	DHI†, C	(426), 444, 472	19.94			α, based on Diadino α
35	Gyroxanthin diester-like	C	(426), 444, 472	21.00			
36	DVChl *b*	U	478, 608, 654	21.92	0.8	36/37	Part of TChl *b*
37•	Chl *b*	Fluka†, F, G, P	468, 602, 652	22.03	0.8	38/39	α
38	DVChl *b*′	U	480, 608, 658	22.29			
39	Croco	H, O	(428), 446, 476	22.42	NR	39/40	
40	Chl *b*′	Fluka†, F, G, P	470, 602, 652	22.50	NR	39/40	
41	Vitamin E	Fluka†		22.84			Internal standard
42	Chl *a* allomer	Fluka†, A–T	(390), 432, 620, 666	23.30			α, part of TChl *a*
43	Chl *a* allomer	Fluka†, A–T	(390), 432, 620, 666	23.43	NR	43/k	α, part of TChl *a*
k	*Unknown*	R	(464–474)	23.52	NR	k/l	
l	*Unknown*	R	454	23.52	NR	l/44	
44	Phytyl-chl *c*	J, R	460, 588, 636	23.53	NR	l/44	
45•	DVChl *a*	U	(390), 442, 622, 666	23.76			α, part of TChl *a*
m	*Unknown*	L	458, 588, 638	23.91	NR	m/46	
46•	Chl *a*	Fluka†, A–T	(390), 432, 620, 666	23.96	NR	m/46	α, part of TChl *a*
47	DVChl *a*′	U	(386), 440, 622, 666	24.13			α, part of TChl *a*
48	Chl *a*′	Fluka†, A–T	(388), 432, 618, 666	24.33			α, part of TChl *a*
49•	Phytin *a*	Acidified from Chl *a*	408, 508, 538, 608, 666	25.43			α
n	*Unknown*	P	(422), 442, 470	25.58			
50	Phytin *a*′		408, 508, 538, 608, 666	25.62			Part of Phytin *a*
51	$\beta\varepsilon$-Car	Sigma†, G, H, J, L, O, P, S	(422), 446, 474	26.65	NR	42/43	Part of Caro
52•	$\beta\beta$-Car	Fluka†, A–G, I–N, P–U	(430), 452, 476	26.71	NR	42/43	α, part of Caro

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

After the pigment standard concentrations were determined, each standard was injected individually to determine chromatographic purity, peak purity, and the response factor, R. Most pigments listed with absorption coefficients, E (Table 51), were quantitatively combined into one mixture for use as a quality control standard with sample analyses. The response factors for each pigment in this much-diluted mixture were compared to the response factors observed from the highly-concentrated stock standards to confirm single-point calibrations were suitable. Chlorophyll *a*, however, was quantified with a multipoint calibration curve (not forced through zero).

Table 51. The α values (in units of liters per gram per centimeter), used for spectrophotometrically measuring pigment concentrations. Also shown are the solvents and maximum wavelengths (λ_m) specified for use with α values. The units for λ_m are nanometers.

No.	*Pigment*	*Solvent*	λ_m	α	*Ref.*
3	Chl c_2	90% Acetone	631.0	40.40	[1]
5	Chl c_1	90% Acetone	631.0	44.80	[1]
10	Perid	100% Ethanol	472.0	132.50	[2]
14	But-fuco	100% Ethanol	447.0	160.00	[3]
15	Fuco	100% Ethanol	449.0	160.00	[4]
16	Neo	100% Ethanol	438.0	227.00	[5]
18	Pras	100% Ethanol	454.0	160.00	[4]
19	Viola	100% Ethanol	443.0	255.00	[4]
20	Hex-fuco	100% Ethanol	447.0	160.00	[3]
24	Diadino	100% Ethanol	446.0	262.00	[4]
26	Anth	100% Ethanol	446.0	235.00	[1]
27	Allo	100% Ethanol	453.0	262.00	[4]
28	Myxo	100% Acetone	478.0	216.00	[6]
29	Diato	100% Ethanol	449.0	262.00	[4]
31	Zea	100% Ethanol	450.0	254.00	[1]
32	Lut	100% Ethanol	445.0	255.00	[4]
33	Cantha	100% Acetone	474.0	222.90	[7]
34	Gyr diester	100% Ethanol	445.0	262.00†	[4]
37	Chl *b*	90% Acetone	646.8	51.36	[1]
45	DVChl *a*	100% Acetone	663.0	88.15	[1]
46	Chl *a*	90% Acetone	664.3	87.67	[1]
49	Phytin *a*	100% Acetone	665.9	‡	[8]
52	$\beta\beta$-Car	100% Ethanol	453.0	262.00	[9]

† Based on Diadino.
‡ Based on 46,000 molar absorption coefficient.
[1] Jeffrey et al. (1997).
[2] Jeffrey and Haxo (1968).
[3] Vesk and Jeffrey (1987).
[4] Bidigare (1991).
[5] Jeffrey (1972).
[6] Britton (1995).
[7] Latasa et al. (1996).
[8] Watanabe et al. (1984).
[9] Davies (1976).

In all cases, when formulating standard dilutions and mixtures, volumetric class A glassware and gas-tight, calibrated glass syringes were used. Additionally, the concentrations of the stock standards were rechecked on the spectrophotometer prior to the formulation of new calibration standards.

The signal at 665 nm (±10 nm) was used for quantification of Chl *a* and DVChl *a*, because they respond similarly (and strongly) at this wavelength and the linear regression for Chl *a* was used for both Chl *a* species. For determining the contribution of MVChl *a* and DVChl *a* to TChl *a*, the peak areas of all products (including all allomers and epimers) were summed and used in the calculations. When reporting the individual contribution of MVChl *a* and DVChl *a* (when both were present), the following formulas (based on the amount per injection, $\tilde{C}$) were used:

$$\tilde{C}_{D_a} = \frac{\hat{H}_{D_a}}{\hat{H}_a + \hat{H}_{D_a}} \left[\tilde{C}_{D_a} + \tilde{C}_a\right] \tag{34}$$

and

$$\tilde{C}_a = \frac{\hat{H}_a}{\hat{H}_a + \hat{H}_{D_a}} \left[\tilde{C}_{D_a} + \tilde{C}_a\right], \tag{35}$$

where $\hat{H}$ indicates the peak height and the subscript identifies the pigment or pigment association (as per the symbology established in Sect. 1.2).

It should be noted that to the extent that DVChl *a* epimers are present in a sample, the value for Chl *a* will be overestimated and the value for DVChl *a* will be underestimated, because DVChl *a* epimer coelutes with the Chl *a* main peak. In some natural samples, a small peak (usually 2% or less of the Chl *a* peak area) appears at the DVChl *a* retention time. If the absorbance spectra does not match DVChl *a*, but does match Chl *a*, it is summed with Chl *a*. These uncertainties affecting the discrimination between DVChl *a* and Chl *a*, however, do not affect the TChl *a* value.

Pigments not fully resolved and reported as one amount included Chl c_2 and MgDVP, MVChl *b* and DVChl *b*, and $\beta\varepsilon$- and $\beta\beta$-Car. Response factors used for quantification were based on the Chl c_2, MVChl *b*, and $\beta\beta$-Car response factors, respectively.

5.5 VALIDATION

HPL method validation describes pigment identification and elution position, including pigments with a potential for interference, and factors contributing to method imprecision and inaccuracy. Method validation tests were conducted before the method was implemented and are supported and refined by routinely conducting quality control measurements during the analysis of samples.

The pigment elution positions were initially determined from retention times and absorbance spectra of authentic standards (Fluka, Sigma, and DHI), plus known pigments in reference algal cultures recommended by SCOR (Jeffrey and Le Roi 1997). During the analysis of field samples, retention times of pigments in samples are compared

Table 52. A summary of the factors influencing method precision. The number of sequences is given by N_S, the number of calibrations by N_C, and the number of filters by N_F. The entries for the number of columns indicates whether tests were conducted on several columns or a single column. The average results and 95% confidence limits (CL) are expressed as RSD.

Validation Criteria	*Testing Interval*	*Observations*	*Analyte Tested*	*No. of Columns*	*Avg. Result*	*95% CL*
Average Short-Term Precision [1]	Per Sequence	$N_S = 15$	Internal Standard (vitamin E)	Multiple	0.3%	0.5%
Average Short-Term Precision [2]	Per Sequence	$N_S = 5$	12 Carotenoids (in ethanol)	Single	0.7	2.0
Average Short-Term Precision [2]	Per Sequence	$N_S = 7$	$[\text{Chl } a]$, $[\text{Chl } b]$, and $[\text{Chl } c]$ (in acetone)	Single	1.6	3.8
Long-Term Precision [3]	Across All Sequences	$N_S = 6$	$[\text{DVChl } a]$ (in 90% acetone)	Multiple	0.5	1.2
Calibration Reproducibility [4]	Across All Calibrations	$N_C = 21$	$[\text{Chl } a]$ (in 90% acetone)	Multiple	1.5	3.2
Method Precision [5]	Across All Replicates, Sequences, and Calibrations	$N_S = 7$ and $N_C = 7$ ($N_F = 17$)	$[\text{Chl } a]$ (in filter extract)	Multiple	2.1	5.1

[1] Measured using the average RSD per sequence averaged over all sequences.
[2] Measured using the individual pigment RSD values averaged for each sequence. The average RSD per sequence was then averaged across all sequences.
[3] Measured using the average peak area per sequence averaged across all sequences.
[4] Measured using the RSD observed with the average slope.
[5] Measured using the RSD observed with the average $[\text{Chl } a]$ across all filters.

with retention times of pigments in a mixture of standards formulated at HPL and used as a principal quality control procedure. Such mixed standards are analyzed several times per sequence, and the resulting chromatograms are overlaid (on screen) with chromatograms of the samples analyzed during the same sequence.

Pigments quantitated during SeaHARRE-2 are indicated in Table 50 with the bullet symbol (•). Pigments in Table 50 without this symbol, but with quantification details, can also be quantitated. Pigments without quantification details have a potential for interference with quantitation, as do isomers of Diadino, Fuco, and But-fuco (not in Table 50) that coelute with Allo, Diadino, and Hex-fuco, respectively.

HPL method precision is described by the random variation in results for pigments in replicate filters when such filters are extracted on different days, and analyzed on different HPLC sequences with different calibration factors. Method precision is, therefore, cumulatively affected by such factors as short- and long-term instrument precision, calibration reproducibility, filter extraction, and the effects of storage and filter inhomogeneity. The latter contributes to uncertainty, but is not ordinarily within the control of the HPL method. Clarification of terms used at HPL to describe precision is given below and a summary of the validation criteria used to quantify precision is presented in Table 52.

Short-term instrument precision is described by variations in peak area (expressed as RSD) for individual pigments (injected singly or as part of a mixed standard) determined from replicate injections, which may occur sequentially, but usually occur no more frequently than every tenth injection during the same HPLC run or sequence (Sect. 1.6.7 provides run and sequence definitions as used at HPL). The average RSD per pigment per sequence or run is then averaged over several sequences or runs to determine average short-term precision per sequence for that pigment (or pigment category, such as carotenoids, chlorophylls, or internal standard).

Long-term instrument precision describes the random variations in peak area when the same solution of pigments (or pigment) is analyzed during several different HPLC sequences (or runs). Long-term precision, therefore, does not describe precision per sequence (or run), but precision across sequences (or runs).

Variations in extraction volume determinations are described, in part, by the accuracy and precision of the measuring device used to deliver extraction solvent (containing the internal standard) to the sample filter and the stability of the internal standard stock solution, which is analyzed in duplicate each day it is added to samples. The latter is also considered a measure of short-term precision.

Calibration reproducibility is affected by variations associated with the spectrophotometric determination of the

concentration of the stock pigment standard solution, its subsequent dilution for HPLC analysis, the stability of the stock pigment standard during freezer storage, and short-term and long-term instrument precision. Calibration reproducibility describes the variation (expressed as RSD) among slopes observed for different independently-formulated, multilevel chlorophyll *a* calibration standards analyzed on different HPLC sequences and columns.

HPL method precision (Table 52) was evaluated prior to SeaHARRE-2 activities by collecting replicate sample filters (from an algal monoculture), storing the filters at −80°C, extracting and analyzing filters for chlorophyll *a* content at intervals over a 10-month period (these results were evaluated without using an internal standard).

The ability of the HPL method to be accurate was estimated by measuring the recovery from laboratory-fortified blanks (Clesceri et al. 1998). Known amounts of nine pigments were added to three filter blanks, which were then extracted and analyzed as if they were samples (vitamin E was used as an internal standard to estimate extraction volumes). The recovery was calculated following Clesceri et al. (1998), wherein the ratio of the found value to the true value was expressed as a percentage. The true value (the concentration of each pigment before being added to the filter blanks) was quantified by HPLC with the same calibration factors used to measure the found value (the concentration in the filter extract after extraction).

Percent recovery ranged from 92–104% for the pigments that were evaluated: Chl c_2, Perid, Fuco, Diadino, Zea, Lut, Chl *b*, Caro, and Chl *a*. True concentrations of the pigment standards were characteristic of natural samples near a Chl *a* concentration of $0.05\,\mu g\,L^{-1}$ (assuming HPL method extraction and injection volumes and a filtration volume of 2.8 L). Thus, depending on the pigment, the amount injected ranged from 1–7 ng.

The ability to be accurate is affected by the pigment concentration in the filter extract. The MDL and the LOQ were estimated for chlorophyll *a* according to Miller and Miller (2000). MDL and LOQ are 0.8 and 3.0 ng per injection, respectively, and were determined from 25 different chlorophyll *a* curves analyzed over a four-year period. (Sect. 1.4.3 provides an alternate discussion of detection limits.) Reproducibility within 5%, however, is attained when the injected amounts are greater than 10 ng per injection. In the perspective of field sample analysis, 10 ng per injection is equivalent to a field sample of $0.08\,\mu g\,L^{-1}$ (assuming a 2.8 L filtration volume).

5.6 DATA PRODUCTS

A series of formulations are used to calculate micrograms of pigment per liter of seawater beginning with

$$\tilde{C}_{P_i} \;=\; \hat{A}_{P_i}\, R_{P_i}, \tag{37}$$

where $\tilde{C}_{P_i}$ is the amount of pigment injected (in units of nanograms per injection), $\hat{A}_{P_i}$ is the area of the parent peak for pigment P_i, and R_{P_i} is the purity-corrected response factor. Pigment concentration is expressed as

$$C_{P_i} \;=\; \frac{V_x}{V_f}\,\frac{\tilde{C}_{P_i}}{V_c}, \tag{38}$$

where V_x is the extraction volume (in microliters), V_c is the volume (in microliters) of sample injected onto the HPLC column, and V_f is the filtration volume (in milliliters). The procedure for adding internal standard is described in Sect. 1.3.1 as the *one-step approach,* for which large batches (0.5–1.0 L) of extraction solvent (containing the internal standard) are formulated and used until consumed. The internal standard is used to correct V_x for residual water retained on the filter paper (plus any variations in volume caused by evaporation):

$$V_x \;=\; \frac{\hat{A}_{c_1}}{\hat{A}_{s_1}}\,V_m, \tag{39}$$

where $\hat{A}_{c_1}$ is the peak area of the internal standard (within the internal standard batch mixture) when it is injected onto the HPLC column prior to its addition to the sample, $\hat{A}_{s_1}$ is the peak area of the internal standard in the sample, and V_m is the volume of the internal standard batch mixture added to each sample filter (3.078 mL during SeaHARRE-2). Both $\hat{A}_{c_1}$ and $\hat{A}_{s_1}$ are determined during the same HPLC sequence.

5.7 CONCLUSIONS

The HPL method was developed for use with a wide variety of water sample types and, as such, has the ability to separate pigments important to both estuarine and oceanic samples including divinyl and monovinyl chlorophyll *a*. Pigments often abundant in estuarine and freshwater systems (such as Neo, Pras, Viola, Allo, and Lut) are well resolved from each other and from pigments commonly found in oceanic systems (But-fuco, Hex-fuco, Fuco, and Zea). The method is also designed to accommodate samples with a wide range of concentrations, as the linear dynamic range extends nearly to detector saturation and high sample mass does not deleteriously affect peak shape, peak area, or retention time reproducibility.

Validation activities have been conducted to identify and cross reference pigment elution positions across a diversity of algal cultures, however, heretofore unidentified interfering pigments will likely be encountered as more diverse sets of natural samples are analyzed. Method precision is affected by all aspects of sample collection, storage, and analysis, but was found to be within 5.1% (at the 95% CL) for chlorophyll *a* in test filters collected at HPL. Method precision is, however, variable with filtration conditions. Performance metrics and other observations of method performance are recorded with each batch of samples analyzed (Sect. 1.6.7), which are expected to evolve and be refined over time.

Chapter 6

The LOV Method

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Abstract

The LOV method applies a sensitive reversed-phase HPLC technique for the determination of chloropigments and carotenoids within approximately 24 min. The different pigments are detected by a DAD which allows for automatic identification to be carried out on the basis of absorption spectra. Optical densities are monitored at 440 nm (chloropigments and carotenoids) and at 667 nm (chloropigments only). The method provides a good resolution between chlorophyll *a* and divinyl chlorophyll *a*, but uncertainties may arise for the partial separation of chlorophyll *b* and divinyl chlorophyll *b*, and for the resolution of chlorophyll *c* pigments. The problem of coelution between 19′-hexanoyloxyfucoxanthin and prasinoxanthin may also be an issue in coastal Case-2 waters where the latter can be present in more significant concentrations. Effective limits of quantitation for most pigments are low (0.0005 mg m^{-3} for chlorophyll *a* and 0.0008 mg m^{-3} for carotenoids).

6.1 INTRODUCTION

The LOV method applies a sensitive reversed-phase HPLC technique (with a C_8 column) for the determination of most chloropigments (including degradation products) and carotenoids (extracted in methanol) within approximately 24 min. This type of analysis, which separates divinyl chlorophyll *a* from chlorophyll *a* was initially developed for application to open ocean Case-1 waters, especially in oligotrophic areas (effective limits of quantitation of 0.0005 mg m^{-3} for chlorophyll *a*). This method, however, also performs well in Case-2 waters.

6.2 EXTRACTION

The SeaHARRE-2 *in situ* samples (stored and transported in liquid nitrogen) were received on 5 November 2002, the DHI standards on 3 December 2002, and the HPL standards (transported in dry ice) on 17 December 2002. All samples were stored at −80°C until analysis. The filters were extracted and analyzed on 10 February 2003, while the DHI and HPL standards were analyzed on 13 February 2003.

The extraction process involved the following steps:

1. The 25 mm GF/F filter was placed into a 10 mL Falcon™ tube.
2. 3 mL of methanol, including an internal standard (*trans*-β-apo-8′-carotenal), was added to each tube using an Ependorf™ pipette, while making sure that the filter was completely covered.
3. The samples were placed in a −20°C freezer for a minimum of 30 min.
4. The filters were macerated using an ultrasonic probe (Ultrasons-Annemasse), for not more than 10 s (the probe was rinsed with methanol and then wiped between each sample).
5. The tubes were placed back into the freezer for another minimum of 30 min.
6. The sample was clarified using vacuum filtration through a 25 mm GF/C (1.3 µm particle size retention) filter. A glass tube (rinsed with methanol and wiped between each sample) was used to press the sample slurry. The filtrate was collected in a 10 mL Falcon™ tube.
7. The filtrate was stored in a −20°C freezer until HPLC analysis (which occurred within 24 h).

6.3 HPLC ANALYSIS

The LOV HPLC system was composed of the following:

- An Agilent Technologies *ChemStation for LC* software (A.09.03);
- A Thermoquest Autosampler (AS 3000), including a temperature control (set at 4°C), an autoinjector for mixing the sample with the ammonium acetate (1 M) buffer, a 1 mL preparation syringe, and a 1 mL sample syringe;
- A degasser (Agilent Technologies 1100);

- A binary pump (Agilent Technologies 1100); and
- A DAD (Agilent Technologies 1100).

The sample extract and standards were transferred into 2 mL glass vials using Pasteur pipettes (which were disposed of after use). The glass vials were placed in the autosampler at 4°C, and an empty vial was added for mixing each sample with the buffer (1/3 buffer and 2/3 sample extract). The mixed solution in the second vial was then injected onto the column via a 200 µL injection loop. To save time, sample preparation was done in an overlapping mode. Except for the first injection, the mixture in the second vial stood for about 15 min until injection.

Separation was based on segmented gradients between a 70:30 methanol:ammonium acetate 0.5 N mixture and a 100% methanol solution (solvent A and solvent B, respectively) and is similar to the Vidussi et al. (1996) method (Table 53). To increase sensitivity and the resolution of certain peaks, modifications to the latter included the use of a reversed-phase C_8 (Hypersil-MOS 3 µm) column, and a 0.5 mL min^{-1} flow rate. The column was equilibrated for 3 min after returning to initial conditions. The main characteristics of the method are summarized in Tables 8–10 and are also described in Claustre et al. (2004).

Table 53. The gradient used with the LOV column. The time is in minutes, and the percentages of solvents A and B are given in the last two columns.

Step	*Time*	A [%]	B [%]
Start	0	80	20
2	4	50	50
3	18	0	100
End	22	0	100

Measurements were made at 440 nm for carotenoids and chlorophylls, as well as 667 nm for chlorophyll degradation products (chlorophyllide *a* and phaeopigments). The 667 nm signal, which was the most sensitive, was also used for determining extremely low chlorophyll *a* concentrations. For both signals, a reference signal at 750 nm was used to compensate for fluctuations caused by baseline absorbance.

The first step in data processing consisted of the inspection and (when adequate) the modification of the integration and spectral identification of the different peaks using the ChemStation software. The second step consisted of the calculation of concentrations.

6.4 CALIBRATION

A calibration was performed in January 2003 shortly before the analysis of the SeaHARRE-2 samples. The concentrations for eight pigment standards (Perid, But-fuco, Fuco, Hex-fuco, Allo, Zea, Chl *b*, and Chl *a*) provided by DHI International Agency for C^{14} determination (Hørsholm, Denmark) were determined by spectrophotometry using a PerkinElmer Lambda 19, dual-beam spectrophotometer (2 nm slit width, 400–700 nm spectral range, with a correction at 700 nm). The calibration curves were composed of 4–8 points, and the corresponding response factors at 440 and 667 nm were determined by HPLC analysis of each standard solution.

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

a) Knowing the specific absorption coefficients of chlorophyll *a* (or chlorophyll *b*);

b) Accounting for the absorption of chlorophyll *a* and divinyl chlorophyll *a* (or chlorophyll *b* and divinyl chlorophyll *b*) at 440 nm when the spectra of both pigments are normalized at their red maxima; and

c) 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). The absorption coefficients for the LOV standard pigments are listed in Table 54 in the same order as their retention times.

Table 54. The α values used with the LOV method for a variety of pigments as a function of λ. The units for α are liters per gram per centimeter and the units for λ are nanometers.

Pigment	*Solvent*	λ	α
Mg DVP			Chl *c* ¶
Chl c_3	90% Acetone	630.6	42.60 †
Chl c_1+c_2	90% Acetone	630.6	42.60 †
Perid	100% Ethanol	472.0	132.50 ‡
But-fuco	100% Ethanol	446.0	160.00 ‡
Fuco	100% Ethanol	449.0	160.00 ‡
Pras	Diethyl Ether	446.0	250.00 †
Hex-fuco	100% Ethanol	447.0	160.00 ‡
Viola	100% Acetone	442.0	240.00 †
Neo	100% Ethanol	438.0	227.00 †
Diadino	100% Acetone	447.5	223.00 †
Allo	100% Ethanol	453.0	262.00 ‡
Diato	100% Acetone	452.0	210.00 †
Zea	100% Ethanol	450.0	254.00 ‡
Lut	Diethyl Ether	445.0	248.00 †
Chl *b*	90% Acetone	646.8	51.36 ‡
DVChl *b*	90% Acetone	440.0	28.03
DVChl *a*	90% Acetone	440.0	74.20
Chl *a*	90% Acetone	664.3	87.67 ‡
$\beta\varepsilon$-Car	100% Ethanol	440.0	180.00 §
$\beta\beta$-Car	100% Acetone	440.0	180.00 §
Phide *a*	90% Acetone	667.0	74.20 †

¶ Same as chlorophyll *c*
† Jeffrey et al. (1997)
‡ DHI Water and Environment
§ Previous calibration

Absorption coefficients were identified spectrally and then quantified in relation to the peak area (concentrations are given in milligrams per cubic meter). Because Chl c_1 and Chl c_2 coelute, they were first identified spectrally before being quantified then summed. Similarly, because Chl b and DVChl b literally coelute, they were first identified spectrally, then quantified with their respective absorption coefficients, and finally summed. This same principle, based on spectral identification, was also applied to the Neo and Viola critical pair as well as the $\beta\beta$-Car and $\beta\varepsilon$-Car critical pair, which were all quantified according to the spectrally-dominant pigment.

6.5 VALIDATION

Short-term quality control (during a run of samples) was monitored using two injections of methanol (including an internal standard), which were injected at the beginning and end of a run of samples, as well as after every 10 injections. This was done to verify retention time reproducibility, peak area precision, 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 was also regularly monitored during analysis.

The identification of individual pigments was manually verified 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 phytoplankton cultures.

Long-term quality control was temporally monitored using the daily precision of the internal standard, which was also collected for observation of long-term precision of the instrument. The efficiency of the chromatographic column was monitored regularly using the column plate numbers of several major pigments (the ChemStation software can automatically produce this data).

If a problem arose that would prevent one or more samples from being analyzed within 24 h after extraction, the affected extracts were stored under argon or nitrogen and placed in a −80°C freezer until routine analysis was possible.

6.6 DATA PRODUCTS

The *ChemStation for LC* program produces a spreadsheet file for each sample comprising the pigment identification, retention times, peak areas, peak heights, peak widths, and other chromatographic information. This file is used in a Visual Basic™ program to extract the peak areas and names, and then to calculate the internal-standard-corrected concentrations, C (in milligrams per cubic meter) of each pigment P_i as in the following equation:

$$C_{P_i} = \frac{\hat{A}'_{P_i} R_{P_i}}{V_f}, \tag{40}$$

where $\hat{A}'_{P_i}$ is the corrected peak area (mAU s), R_{P_i} is the pigment response factor [mg (mAU s)$^{-1}$], and V_f is the volume of water filtered in units of cubic meters. The $\hat{A}'_{P_i}$ term is computed as

$$\hat{A}'_{P_i} = \frac{\hat{A}_{P_i} \hat{A}_{c_1}}{\hat{A}_{s_1}}, \tag{41}$$

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

6.7 CONCLUSIONS

The LOV method provides a good resolution between Chl a and DVChl a, as well as between Zea and Lut, although uncertainties may arise for the partial separation of Chl b and DVChl b and for the resolution of chlorophyll c pigments (Chl c_1 and Chl c_2). The coelution between Pras and Hex-fuco may also be an issue in coastal Case-2 waters, where the former can be found in more significant concentrations than offshore. The method, adapted for methanol extracts, is sensitive to the injection solvent: for example, standard solutions containing ethanol have caused severe peak fronting, thereby leading to possible uncertainties in the calibration procedures. Accuracy and precision are improved with the use of an internal standard. The effective limits of quantitation for a filtered 2.8 L volume of seawater are estimated to be 0.0005 mg m^{-3} for chlorophyll a and 0.0008 mg m^{-3} for carotenoids.

Chapter 7

The MCM Method

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Abstract

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

7.1 INTRODUCTION

Pigments are analyzed with a reversed-phase HPLC method using a binary solvent system following a linear gradient on a C_8 chromatography column according to Barlow et al. (1997). Baseline separation of mono- and divinyl chlorophyll *a* as well as lutein and zeaxanthin, partial separation of mono- and divinyl chlorophyll *b*, and resolution of other key chemotaxonomic chlorophylls and carotenoids are achieved in an analysis time of approximately 30 min. Chloropylls c_1 and c_2 plus $\beta\varepsilon$-carotene and $\beta\beta$-carotene are not well separated by this technique. Prior to HPLC analysis, all samples are stored either in liquid nitrogen or in a −85°C freezer.

7.2 EXTRACTION

An appropriate amount of *trans*-β-apo-8′-carotenal is dissolved in 0.5 or 1.0 L of 100% acetone, and the absorbance (approximately 0.1) is determined at the blue maximum to estimate the concentration of the *trans*-β-apo-8′-carotenal internal standard in the acetone. The solution is stored at −20°C in the dark, and 2 mL of the acetone and *trans*-β-apo-8′-carotenal mix are added to frozen filter samples (25 mm) in graduated centrifuge tubes. The filter samples contain 0.1–0.2 mL of water after filtration.

The pigments are extracted with the aid of ultrasonication (0.5 min) and soaking for 30 min in the dark. The extract is clarified by centrifugation in a refrigerated centrifuge. Final extract volumes are read from the centrifuge tube graduations, whose graduations have been checked against pipetted solution volumes.

7.3 HPLC ANALYSIS

The sample extracts, which are stored in dark glass vials, are loaded into a Thermo Separations AS3000 autosampler and cooled to 2°C. The autosampler incorporates a column compartment containing a 3 µm Hypersil MOS2 C_8 column plus an autoinjector, and both are heated to 25°C. Prior to injection, the autosampler is programmed to vortex mix 300 µL of extract with 300 µL of 1 M ammonium acetate buffer, after which, 100 µL of the extract-buffer is injected onto the chromatography column. The individual pigments are separated at a flow rate of 1 mL min^{-1} by a linear gradient using a Varian ProStar tertiary pump programmed following the parameters presented in Table 55.

Table 55. The gradient used with the MCM column. The time is in minutes, and the percentages of solvents A and B are given in the last two columns.

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

The column is then reconditioned to original conditions over an additional 7 min. Solvent A consists of 70:30 (v/v) methanol:1 M ammonium acetate, and solvent B is 100% methanol. Pigments are detected by absorbance at 440 nm using a Thermo Separations UV6000 DAD (5 cm×10 µL

flow cell) and any chlorophyll transformation products are simultaneously monitored at 665 nm. Spectral data are collected on every sample from 400–700 nm. Peak areas are initially automatically integrated using instrument default conditions, and then every chromatogram is checked with appropriate peak markers and baselines being manually moved and placed to optimize the integration. The chlorophyllide *a* standard is prepared from a culture of *Dunaliella tertiolecta*, following procedures detailed in Jeffrey et al. (1997).

7.4 CALIBRATION

Triple single-point calibrations are run with all pigment standards, except for chlorophyll *a* and *trans*-β-apo-8′-carotenal where multipoint calibrations are conducted. The *trans*-β-apo-8′-carotenal (Fluka Chemie AG) internal standard is purchased from Sigma-Aldrich Company (St Louis, Missouri). The chlorophyll *a* standard is also purchased from Sigma-Aldrich, and a stock solution is prepared at approximately 1 mg in 100 mL 100% acetone. A 10% working standard is then prepared in 100% acetone from the stock solution.

The following standards (2.5 mL volume) are purchased from DHI Water and Environment Institute (Hørsholm, Denmark):

- Divinyl chlorophyll *a*,
- Chlorophyll *b*,
- Chlorophyll c_2,
- Chlorophyll c_3,
- Peridinin,
- Fucoxanthin,
- 19′-Butanoyloxyfucoxanthin,
- 19′-Hexanoyloxyfucoxanthin,
- Violaxanthin,
- Diadinoxanthin,
- Diatoxanthin,
- Alloxanthin,
- Zeaxanthin,
- Lutein, and
- $\beta\beta$-Carotene.

Chlorophyll standards are shipped in 90% acetone and carotenoid standards in 100% ethanol.

Concentrations of all external standards and the *trans*-β-apo-8′-carotenal internal standard are determined and checked from absorbance measurements, scanning between 400–750 nm, using a monochromatic, double-beam Hitachi U-2000 spectrophotometer with a 2 nm bandwidth. Red and blue wavelength maxima are used for the chlorophylls and carotenoids, respectively, with the absorption coefficients (Table 56) estimated from data given in Jeffrey et al. (1997). Chl c_1 and Chl c_2 are not separated in the MCM method, but diode array spectra show Chl c_2 usually dominates the peak, with smaller amounts of Chl c_1, so $\left[\text{Chl } c_{1+}c_2\right]$ is estimated from the Chl c_2 calibration.

Table 56. The α values used by the MCM method for a variety of pigments as a function of λ. The units for α are liters per gram per centimeter and the units for λ are nanometers.

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

7.5 VALIDATION

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

7.6 DATA PRODUCTS

The data files of the chromatographic results report the pigment identification, retention times, plus peak areas and heights. The reponse factors are computed from the peak areas and pigment standard concentrations, and the relative response factors are calculated by relating individual pigment response factors to the *trans*-β-apo-8′-carotenal response factor. The weight of the *trans*-β-apo-8′-carotenal internal standard added in each 2 mL extrac-

tion volume is known from the concentration of *trans*-β-apo-8′-carotenal in the acetone extract solution and is of the order of 450–500 ng mL^{-1}. The concentration of each pigment, C_{P_i}, within a sample is calculated as:

$$C_{P_i} = \frac{W_s/\hat{A}_{s_1}}{V_f}\,\hat{A}_{P_i}\,R'_{P_i}, \tag{42}$$

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

Because chlorophyllide *a* and chlorophyll c_{1+2} coelute, their individual concentrations are estimated using the following simultaneous equations:

$$\left[\text{Chlide } a\right] = \frac{\hat{A}_1\,R_{y_2} - \hat{A}_2\,R_{y_1}}{R_{x_1}\,R_{y_2} - R_{x_2}\,R_{y_1}} \tag{43}$$

and

$$\left[\text{Chl}c_{1+2}\right] = \frac{\hat{A}_2\,R_{x_1} - \hat{A}_1\,R_{x_2}}{R_{x_1}\,R_{y_2} - R_{x_2}\,R_{y_1}}, \tag{44}$$

where $\hat{A}_1$ and $\hat{A}_2$ are the respective chromatographic areas at 665 nm and 440 nm; R_{x_1} and R_{x_2} are the response factors for chlorophyllide *a* at 665 nm and 440 nm, respectively; and R_{y_1} and R_{y_2} are the response factors for chlorophyll c_{1+2} at 665 nm and 440 nm, respectively.

7.7 CONCLUSIONS

The reversed-phase HPLC method using a C_8 column provides good resolution of mono- and divinyl chlorophylls *a* and *b*, and lutein and zeaxanthin, as well as satisfactory separation of other key pigments within 30 min. The use of *trans*-β-apo-8′-carotenal internal standard improves the accuracy of pigment determinations. Providing a pragmatic balance between good analyte resolution and acceptable sample throughput, the method is suitable for the analysis of a wide range of oceanographic samples.

Chapter 8

The PML Method

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Abstract

The PML method successfully resolves and quantifies over 20 chlorophyll and carotenoid pigments from a variety of water types, ranging from estuaries to oligotrophic oceans. A C_8 column is used in combination with a methanol-based binary solvent system following a linear gradient. The method separates monovinyl and divinyl chlorophylls *a* and *b*, and the carotenoids lutein and zeaxanthin. A DAD is configured at 440 nm to determine chlorophylls and carotenoids and at 667 nm for the determination of chlorophyllide *a* and phaeopigments. The method provides good resolution with a run time of approximately 30 min excluding an additional 15 min to restore the column and solvent gradient prior to the next injection. The internal standard, *trans*-β-apo-8′-carotenal, improves the accuracy of the analysis.

8.1 INTRODUCTION

The PML method, which is based on the method presented by Barlow et al. (1997), was developed to successfully resolve important pigments for a variety of water types, ranging from estuaries to oligotrophic oceans. This method is used predominantly for local field work analyzing samples from both Case-1 and Case-2 waters. The method has also been used to analyze samples from numerous international cruise campaigns, and has performed reliably for many water types.

The separation of mono- and divinyl chlorophylls *a* and *b* plus the carotenoids zeaxanthin and lutein, is achieved over a run time of approximately 30 min. Both $\beta\beta$-Car and $\beta\varepsilon$-Car, as well as chlorophylls c_1 and c_2, are not separated by this method. Use of a DAD configured at 667 nm allows for the determination of chlorophyllide *a* concentration, because at 440 nm it coelutes with chlorophyll c_1+c_2. The use of this method makes it possible to correct the chlorophyll c_1+c_2 concentration. All samples at PML are stored in liquid nitrogen prior to analysis (Mantoura and Llewellyn 1983).

8.2 EXTRACTION

A maximum of 10 samples are extracted at any one time. They are placed into 15 mL centrifuge tubes labeled 1–10 and the corresponding tube and filter identities are documented. A 90% HPLC grade acetone (v:v with Milli-Q water) extraction solvent is used with an amount of *trans*-β-apo-8′-carotenal internal standard similar in concentration to the pigments in the sample. The concentration of the internal standard is determined by spectrophotometric analysis, and then the solution is stored at −20°C. Prior to extraction, this solvent is removed from the freezer and allowed to warm at room temperature for a period of 20 min. This allows for more accurate volumes when pipetting. An Eppendorf™ Multipette plus pipette is then used to add 2 mL of solvent to each centrifuge tube.

Each filter is opened up in the tube using a spatula, which is cleansed with acetone between each sample. The caps are firmly screwed onto the centrifuge tubes to prevent any evaporation. All the tubes are then placed into a small cool box containing crushed ice and covered to keep dark for a period of 30 min. They are then removed one at a time and ultrasonificated using a Sonics Vibra Cell VC130PB with an output of 50 W for 30 s. During this time, the sample tube is held in a beaker of crushed ice to reduce any heating effects. The sample tube is then placed back into the cool box and the probe is wiped down with acetone in preparation for the next sample.

Once all the samples have been ultrasonificated, they are left in the ice for an additional 30 min. After this, the solvent from each sample is transferred to a 1.5 mL centrifuge tube, and centrifuged at 14,000 rpm for a period of 2 min in an Eppendorf microcentrifuge. The centrifuge is not cooled, so the time in the centrifuge is kept to a minimum to prevent any unnecessary heating of the sample. The supernatant from each sample is then transferred into

an amber HPLC vial and stored at −20°C until analysis later the same day.

8.3 HPLC ANALYSIS

Sample extracts are placed into the Thermo Separations AS3000 programmable auto sampler, which is cooled to 0°C. A Hypersil MOS-2 C_8 3 µm column is incorporated into the auto sampler and thermostated to 25°C. The method requires that a clean vial is inserted into the auto sampler after each sample vial. Prior to injection, the auto sampler is programmed to vortex mix 300 µL of extract with 300 µL of 1 M ammonium acetate buffer.

A 100 µL volume of the extract-buffer is injected onto the column. A solvent flow of 1 mL min^{-1} is delivered using a spectraSYSTEM P4000 quaternary pump. A binary solvent method is used composed of 70:30 HPLC grade methanol:1 M ammonium acetate (solvent A) and 100% methanol (solvent B). The linear gradient of these solvents through the column is shown in Table 57.

Table 57. The gradient used with the PML column. The time is in minutes, and the percentages of solvents A and B are given in the last two columns.

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

After the pigments are separated, they are detected using a UV6000LP PDA detector. Detection wavelengths are 440 nm for chlorophylls and carotenoids and 667 nm for chlorophyllide *a* and phaeopigments. Spectral data from 350–800 nm are recorded for each pigment. ChromQuest (version 2.51) software automatically integrates the peaks and identifies the pigments. The chromatograms are then all manually checked for both integration and pigment identification.

8.4 CALIBRATION

The HPLC system was calibrated during January 2003 shortly before the SeaHARRE-2 samples were analyzed. All pigment standards were purchased from the DHI Water and Environment Institute (Hørsholm, Denmark). The standard pigments used during this calibration are shown in Table 58. The table also shows the solvent the pigments were shipped in, the absorption coefficients, and the corresponding wavelengths, all supplied by DHI. Triplicate injections were performed for each pigment standard and an average peak area with corresponding standard deviation was calculated for each.

Table 58. The α values used with the PML method for a variety of pigments as a function of λ. The units for α are liters per gram per centimeter, and the units for λ are nanometers.

Pigment	*Solvent*	λ	α
Chl *a*	90% Acetone	664.3	87.67
DVChl *a*	90% Acetone	664.3	87.67
Phytin *a*	90% Acetone	667.0	51.20
Chl *b*	90% Acetone	646.8	51.36
Chl c_2	90% Acetone	630.9	40.40
Chl c_3	90% Acetone	452.9	346.00
Perid	100% Ethanol	472.0	132.50
But-fuco	100% Ethanol	446.0	160.00
Fuco	100% Ethanol	449.0	160.00
Hex-fuco	100% Ethanol	447.0	160.00
Pras	100% Ethanol	454.0	160.00
Viola	100% Ethanol	443.0	255.00
Neo	100% Ethanol	439.0	224.30
Diadino	100% Ethanol	446.0	262.00
Allo	100% Ethanol	453.0	262.00
Diato	100% Ethanol	449.0	262.00
Zea	100% Ethanol	450.0	254.00
Lut	100% Ethanol	445.0	255.00
$\beta\beta$-Car	100% Ethanol	453.0	262.00

Chlide *a* elutes with Chl c_1+c_2 and cannot be differentiated at the 440 nm detection wavelength. Chl c_1+c_2 has no absorption at 667 nm so this wavelength is used to distinguish Chlide *a*. There is no available standard for Chlide *a*, thus, a response factor calculated from the response factor for Chl *a* at 667 nm is used, while taking into account the differences in molecular weight.

To determine the Chl c_1+c_2 concentration, the absorption due to Chlide *a* is determined at 440 nm and then deducted from the Chl c_1+c_2 peak. Chl c_2 is more common in phytoplankton than Chl c_1. Because Chl c_1 and Chl c_2 coelute (but are distinguishable from their absorption spectra), they are reported as one value using the response factor calculated from the Chl c_2 standard.

The concentrations of all the pigment standards were determined by absorbance measurements. This was carried out on a PerkinElmer Lambda 2 UV/VIS dual beam spectrophotometer, calibrated before use with Helma NIST-traceable absorption and wavelength standards. A bandwidth of 2 nm was set and 1 cm microcuvettes where used. Wavelengths and absorption coefficients used in the determination of pigment concentrations were those shown in Table 58 (as supplied by DHI).

During the calibration of the HPLC, the solvent flow rate was checked by collecting the solvent pumped from the outlet of the DAD into a class A, 10 mL measuring cylinder over a 1 min time period. The stability of the column temperature was checked using a Digitron 2006T digital thermometer. The mean temperature of the column

over 40 sample runs was 25.1°C with a standard deviation of 0.4°C.

8.5 VALIDATION

The first and last three samples of any run are chlorophyll *a* working standards, the concentration of which is calculated spectrally and recalculated regularly. After the first three and before the last three chlorophyll samples, three samples of internal standard are run, the concentration of which is also measured spectrally and checked regularly. This practice allows the reproducibility of the HPLC to be monitored for any drift in the retention times or the peak areas and, hence, the response factors. In addition, because triplicates of chlorophyll and internal standard are run both before and after the samples, it is possible to monitor any drift during the course of the run. As mentioned above, the flow rate of the machine is checked manually and the column temperature is also checked on a regular basis, because temperature change could cause drifting retention times.

Pigment standards from DHI are used within a week after receiving them, and once any vial has been opened, it is used immediately. Samples are always run on the same day they are extracted, and never stay in the auto sampler for a longer period than 24 h before analysis.

8.6 DATA PRODUCTS

The ChromQuest software outputs integrated peak areas and pigment identifications. As already mentioned above, these are checked manually for all samples. Peaks are reintegrated where necessary, and the pigment identifications are checked using both retention times and their absorption spectrum. The peak areas are entered into a spreadsheet along with an average internal standard peak area. This is calculated from the six internal standard injections, three at the beginning and three at the end of each run. The corresponding response factors for each pigment are also entered into the spreadsheet.

The individual pigment concentrations, C_{P_i}, are calculated as (in units of nanograms per liter)

$$C_{P_i} = \frac{\hat{A}_{c_1}}{\hat{A}_{s_1}} \frac{V_m}{V_f} \frac{\hat{A}_{P_i} R_{P_i}}{V_c'/D_b}, \tag{45}$$

where $\hat{A}_{P_i}$ is the peak area of pigment P_i, R_{P_i} is the response factor for the pigment, V_m is the volume of the solvent–standard mixture (in units of milliliters), V_f is the volume of water filtered (in units of liters), $\hat{A}_{c_1}$ is the average peak area of the six internal standard injections, $\hat{A}_{s_1}$ is the peak area of the internal standard in the sample, and D_b is the ammonium acetate buffer dilution (fixed at 2) of the volume of buffer and sample V_c' mixed together. The volume of sample injected onto the HPLC column is $V_c = V_c'/D_b$.

8.7 CONCLUSIONS

PML uses an established method, which has been used to analyze field samples from all over the world with varying pigment concentrations. Cruises include the Atlantic Ocean, the Indian Ocean, the Mediterranean Sea, and the Celtic Sea, with chlorophyll *a* concentrations ranging between 0.03–6.4 mg m^{-3}. Samples range from Case-1 oligotrophic oceans to Case-2 coastal waters. The ability of the method to separate divinyl chlorophylls *a* and *b* from monovinyl chlorophylls *a* and *b*, is essential for oligotrophic ocean work. The method separates the two carotenoids zeaxanthin and lutein and has good separation of a wide range of chlorophylls and carotenoids. The method has a good balance between pigment separation and run time (approximately 30 min). The use of the internal standard has improved the accuracy of the analysis.

Acknowledgments

Alexandre de Menezes is thanked for his assistance with the calculation of the LOD and LOQ values for the PML system.

Chapter 9

The SDSU (CHORS) Method

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Abstract

The CHORS method was developed to provide HPLC phytoplankton pigment analyses for the NASA SIMBIOS program, following the protocols presented in the *Ocean Optics Protocols for Satellite Ocean Color Sensor Validation* (Bidigare et al. 2002). The method (Wright et al. 1991) was designed to support a wide range of pigment concentrations from waters sampled throughout the world ocean, but does not chromatographically separate the divinyl chlorophylls *a* and *b* from their corresponding monovinyl forms. The Latasa et al. (1996) dichromatic equations were used to spectrally resolve divinyl chlorophyll *a* from monovinyl chlorophyll *a*. The method uses a reversed-phase C_{18} column, with a tertiary solvent gradient. In addition, a temperature-controlled autosampler provides continuous sample injection to maintain a quota of analyzing 4,000 samples per year. System calibration is routinely monitored and recorded each month to ensure repeatability and consistency of data products.

9.1 INTRODUCTION

The current focus of HPLC analysis at CHORS is to characterize phytoplankton pigments in the water column collected as part of SIMBIOS field campaigns. The method follows the HPLC protocols established in the *Ocean Optics Protocols for Satellite Ocean Color Sensor Validation* (Bidigare et al. 2002). The chromatographic analyses do not separate monovinyl and divinyl chlorophyll *a*; instead, these pigments are quantified using dichromatic equations at 436 and 450 nm.

Because of the large number of samples processed, approximately 4,000 or more per year, the CHORS HPLC laboratory procedures have been streamlined to accommodate the variety and quantity of water samples collected by the SIMBIOS research community. By using larger HPLC solvent reservoirs, multiple sample tube racks, and running the system 24 h a day, the CHORS laboratory can accommodate 500 samples a month, with the system running continuously over a 3–4 week period.

9.2 EXTRACTION

The SeaHARRE-2 samples arrived at CHORS on 19 November 2002, and were stored in liquid nitrogen pending extraction and analysis. On 5 December 2002, the filters were extracted in 4.0 mL of 100% acetone using a Brinkmann Bottletop Dispenser (with a coefficient of variation of 0.321%). An internal pigment standard, canthaxanthin (which is normally not found in samples), was added to the acetone extract prior to pipetting the 4.0 mL, to correct for any extraction volume changes during sample processing.

After 24 h of extraction in a freezer (maintained at −20°C), the samples were sonicated for 10 s using an ultrasonic microprobe tip at a 60% duty cycle. The samples were then extracted for an additional 24 h (stored in a freezer at −20°C). Glass-fiber particles, generated during filter sonication, were removed from the extract by centrifuging the samples at 5,100 rpm for 4 min and then subsequently filtering the extract using 0.2 µm PTFE in-line filters.

9.3 HPLC ANALYSIS

The equipment used with the CHORS method is as follows:

- ThermoQuest HPLC system with membrane degasser and P4000 quaternary pump;
- AS3000 temperature-controlled autosampler;
- UV6000 PDA detector (scanning 436 and 450 nm);
- FL3000 fluroescence detector; and
- System controller with ChromQuest (v3.0) software.

The HPLC gradient program was the same one specified in the Ocean Optics Protocols for pigment analysis (Bidigare

et al. 2002). Pigments were separated on a reversed-phase, Waters Spherisorb ODS-2 (5 µm) C_{18} column (250 mm long with a 4.6 mm internal diameter), using a three-solvent gradient system and a 1.0 mL min^{-1} flow rate (Table 59). A ThermoQuest P4000 pump and an AS3000 Autosampler (maintained at 4°C) were used for processing all samples. Each sample extract was mixed (605 mL:195 mL, extract:water), and 100 mL of this mixture was injected onto the HPLC column.

Table 59. The tertiary gradient elution system used with the CHORS HPLC method. Solvent A is 80:20 methanol:0.5 M ammonium acetate; solvent B is 90:10 acetonitrile:water; and solvent C is 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	20.0	0	31	69
6	22.0	0	100	0
End	25.0	100	0	0

The separation of the various pigments requires approximately 25 min with the pigment peaks being detected by an absorption detector; a ThermoQuest UV6000 scanning DAD (190–800 nm at 1 nm resolution). Pigments were monitored at 436 and 450 nm, with peak retention time or spectrophotometric recognition used to assign peak identification. In addition, a ThermoQuest FL3000 scanning fluorescence detector was used to detect the various chlorophyll degradation products, which occur at lower concentrations.

Although the absorption peaks for monovinyl and divinyl chlorophyll *a* coelute, each compound absorbs differently at 436 and 450 nm, so it was possible to correct for the divinyl chlorophyll *a* contribution by monitoring changes in the 450/436 ratio as a function of changes in the divinyl percentage (Latasa et al. 1996).

Accuracy for each pigment compound was based on the availability of pigment standards and the selection of pigment-specific absorption coefficients. The standard fluorometric method of Holm-Hansen et al. (1965) was used to calculate chlorophyll and phaeopigment concentrations on an aliquot (100 µL) of the pigment extract. These concentrations were also corrected for extraction volume changes using the canthaxanthin internal standard. The Ocean Optics Protocols (Trees et al. 2002) for fluorometric chlorophyll *a* was also followed.

9.4 CALIBRATION

The HPLC pigment calibration standards were purchased from Sigma-Aldrich and DHI. System calibrations were performed to determine individual standard response factors for each compound. Each pigment response factor was determined using a multipoint calibration. The concentration of the standards were provided by DHI, or verified spectrophotometrically (for chlorophyll *a* and chlorophyll *b*) using published absorption coefficients (Table 60). The coefficient of determination for the chlorophyll *a* calibration curve was $r^2 = 0.999$. The coefficient of variation for the canthaxanthin internal standard average was about 3.8%.

Table 60. The α values used with the CHORS method for a variety of pigments as a function of λ. The units for α are liters per gram per centimeter, and the units for λ are nanometers.

Pigment	*Solvent*	λ	α
Chlide *a*	90% Acetone	664.0	127.00†
Chl c_3	90% Acetone	452.9	346.00†
Chl c_2	90% Acetone	630.9	40.40†
Peri	100% Ethanol	472.0	132.50†
But-fuco	100% Ethanol	446.0	160.00†
Fuco	100% Ethanol	449.0	160.00†
Neo	100% Ethanol	439.0	224.30†
Hex-fuco	100% Ethanol	447.0	160.00†
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†
Chl *b*	90% Acetone	646.6	51.36‡
MVChl *a*	90% Acetone	663.5	87.67‡
DVChl *a*	90% Acetone	664.3	87.67†
Phytin *a*	90% Acetone	667.0	51.20†
$\beta\varepsilon$-Car	100% Acetone	448.0	270.00†
$\beta\beta$-Car	100% Ethanol	453.0	262.00†

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

9.5 VALIDATION

The integration of pigment peaks was performed using ChromQuest (v3.0) software, and manually checked to ensure each peak was properly integrated. Retention times and spectral signatures of the standards were used to verify peak identification. Peak areas were quantified using software-aided integration. Response factors generated during the system calibration were used for final concentration calculations.

9.6 DATA PRODUCTS

Each sample data file included the integrated peak area, which was used with the pigment response factors to com-

pute pigment concentration:

$$C_{P_i} = \frac{V_m}{V_f}\,\frac{V_c}{V_s}\,\frac{\hat{A}_{c_1}}{\hat{A}_{s_1}}\,\hat{A}_{P_i}/F_{P_i}, \tag{46}$$

where V_m is the volume of extract (solvent plus standard) added (in milliliters); V_f is the volume of sea water filtered (in liters); V_c is the volume of sample extract injected onto the column; V_s is the volume of standard injected to determine the inverse response factor, F_{P_i}; $\hat{A}_{c_1}$ is the average peak area of 25 internal standard injections; $\hat{A}_{s_1}$ is the peak area of the internal standard in the sample; and $\hat{A}_{P_i}$ is the peak area of pigment P_i. For the CHORS method, $V_c = V_s$, so (46) can be simplified to:

$$C_{P_i} = \frac{V_m}{V_f}\,\frac{\hat{A}_{c_1}}{\hat{A}_{s_1}}\,\hat{A}_{P_i}/F_{P_i}. \tag{47}$$

The following pigments are reported:

- Chlorophyllide *a*,
- Chlorophyll c_3,
- Chlorophyll c_2,
- Chlorophyll $c_{1+}c_2$,
- Peridinin,
- 19′-Butanoyloxyfucoxanthin,
- Fucoxanthin,
- 19′-Hexanoyloxyfucoxanthin,
- Prasinoxanthin,
- Violaxanthin,
- Diadinoxanthin,
- Alloxanthin,
- Diatoxanthin,
- Lutein,
- Zeaxanthin,
- Chlorophyll *b*,
- Chlorophyll *a*,
- $\beta\varepsilon$-Carotene,
- $\beta\beta$-Carotene,
- Monovinyl chlorophyll *a*,
- Divinyl chlorophyll *a*,
- Total chlorophyll *a*,
- Fluorometric chlorophyll, and
- Fluorometric phaeopigments.

Chlorophyll *a* is computed from the absorbance at 436 nm, assuming no divinyl chlorophyll *a* is present with a single response factor. Total chlorophyll *a* is computed as the sum of monovinyl chlorophyll *a*, divinyl chlorophyll *a*, chlorophyllide *a*, chlorophyll *a* allomer, and chlorophyll *a* epimer. Divinyl chlorophyll *a* is not chromatographically separated; it is computed using the Latasa et al. (1996) simultaneous equations.

9.7 CONCLUSIONS

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

Acknowledgments

The SeaHARRE-2 exercise was sponsored by the SeaWiFS and SIMBIOS Projects along with the individual institutes representing the eight laboratories that participated. SeaHARRE-2 provided unique opportunities to evaluate uncertainties with pigment analysis of both field samples and laboratory standards. An undertaking of this magnitude has not previously been attempted, and the important contributions that have emerged from this work were only possible because of the unselfish efforts of the many participating analysts, each of whom provided extraordinary attention to detail and a willingness to share.

The expertise of the DHI shipping office was instrumental in the successful delivery of many sample sets, and the professional contributions of Bo Guttmann were particularly helpful. The example of how a quality assurance process affects uncertainties (Sect. 1.4.3.9) relied on data collected as part of an environmental monitoring program funded by the Connecticut Department of Environmental Protection, and was made available courtesy of Judy Li.

An important part of the SeaHARRE activity has been the workshops that were hosted by the participating institutes. In the most recent workshop, which was hosted by LOV and dealt with the SeaHARRE-2 data analysis and report, several external groups agreed to participate and send representatives. The voluntary participation of Robert Bidigare, Yves Dandonneau, and Jean-François Berthon was especially appreciated.

Appendices

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

Appendix A

The SeaHARRE-2 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*
DV chl *a*	Divinyl chlorophyll *a*,
DVChl *a*′	Divinyl chlorophyll *a* epimer,
DV chl *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,
Mg DVP	Mg 2,4-divinyl phaeoporphyrin a_5 monomethyl ester,
Monado	Monadoxanthin,
Neo	Neoxanthin,
Perid	Peridinin,
Pras	Prasinoxanthin,
Viola	Violaxanthin,
Zea	Zeaxanthin,
[$\beta\beta$-Car]	$\beta\beta$-Carotene (β-Carotene),
[$\beta\varepsilon$-Car]	$\beta\varepsilon$-Carotene (α-Carotene),

Appendix C

Commercial HPLC Manufacturers and Pigment Suppliers

The commercial HPLC manufacturers and pigment suppliers discussed in this report are presented alphabetically.

Agilent Technologies, Inc.[1]
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Fax: 800–519–6047
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Branson Ultrasonics Corporation
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Net: http://www.bransoncleaning.com

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[1] Formerly the Hewlett-Packard Analytical Division.

[2] Formerly the VKI Water Quality Institute.

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Fax: 414–273–4979
Net: flukausa@sial.com

Hewlett-Packard Company
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Fax: 650–857–5518
Net: http://www.hp.com

Hitachi Instruments, Inc.
3100 North First Street
San Jose, CA 95134
Voice: 800–548–9001
Fax: 408–492–8258
Net: http://www.hii-hitachi.com

Scientific Resources, Inc.
P.O. Box 957297
Duluth, GA 30095–7297
Voice: 800–637–7948
Fax: 770–476–4571

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

ThermoQuest[5]
355 River Oaks Parkway
San Jose, CA 95134–1991
Voice: 408–526–1100
Fax: 408–965–6810
Net: http://www.thermoquest.com

Glossary

%RSD Relative Standard Deviation (expressed in percent)

APD Absolute Percent Difference

BENCAL Benguela Calibration (and Validation)
BIO Bedford Institute of Oceanography (Canada)

CHORS Center for Hydro-Optics and Remote Sensing
CL Confidence Limits
CSIRO Commonwealth Scientific and Industrial Research Organisation (Australia)
CTD Conductivity, Temperature, and Depth
CV Coefficient of Variation

DAD Diode Array Detector
DHI DHI Water and Environment Institute (Denmark)
DP (Total) Diagnostic Pigments
DYFAMED *Dynamique des Flux en Méditerranée* (Dynamics of fluxes in the Mediterranean)

ESA European Space Agency

FRS (South African) Fisheries Research Ship

GSFC Goddard Space Flight Center

HP Hewlett-Packard
HPL Horn Point Laboratory
HPLC High Performance Liquid Chromatography

ID Identification
ISTD Internal Standard

JGOFS Joint Global Ocean Flux Study

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)
MDL Mimimum Detection Limit
MPF Microplankton Proportion Factor

N/A Not Applicable
NASA National Aeronautics and Space Administration
NIST National Institute of Standards and Technology
NPF Nanoplankton Proportion Factor
NR Not Resolved

PDA Photodiode Array
PI Polarity Index
PML Plymouth Marine Laboratory (United Kingdom)
PPC Photoprotective Carotenoids
PPF Picoplankton Proportion Factor
PPig Primary Pigments
PROSOPE *Productivité des Systèmes Océaniques Pélagiques* (Productivity of Pelagic Oceanic Systems)
PSC Photosynthetic Carotenoids
PSP Photosynthetic Pigments

QA Quality Assurance

RPD Relative Percent Difference
RSD Relative Standard Deviation (usually expressed in percent)

S/I Sequence and Injection (number)
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
SeaWiFS Sea-viewing Wide Field-of-view Sensor
SIMBIOS Sensor Intercomparison and Merger for Biological and Interdisciplinary Oceanic Studies
SNR Signal-to-Noise Ratio

TAcc Total Accessory Pigments
TCAS Temperature-Controlled Autosampler
TChl Total Chlorophyll
TPig Total Pigments

[3] Part of Sigma-Aldrich.

[4] Formerly Sigma Chemical.

[5] Formerly Thermo Separation Products.

UNESCO United Nations Educational, Scientific, and Cultural Organization
UPD Unbiased Percent Difference

WG Working Group

Symbols

$a(\lambda)$ The spectral absorption coefficient (12).
A Used to denote the average of all the methods (18) or the spectral absorbance (12), depending on usage.
A' The average across the D, H, M, and S methods.
A^- The set of best results (e.g., lowest uncertainties or precisions) from a group of methods.
$A(\lambda)$ The spectral absorbance (12).
$\tilde{A}(\lambda)$ The corrected spectral absorbance.
$a_{1\%}$ The absorption coefficient in percent weight per volume (usually in units of grams per 100 mL).
$\hat{A}_1$ The chromatographic area at 665 nm.
$\hat{A}_2$ The chromatographic area at 440 nm.
$\hat{A}_c$ The peak area of the internal standard when it is injected onto the HPLC column.
$\hat{A}_{c_1}$ $\hat{A}_c$ determined using a one-step internal standard methodology (7).
$\hat{A}_{c_2}$ $\hat{A}_c$ determined using a two-step internal standard methodology (8).
$a_m(\lambda)$ The molar absorption coefficient.
$\hat{A}_{P_i}$ The area of the parent peak and associated isomers for pigment P_i (1).
$\hat{A}'_{P_i}$ The corrected peak area.
$a_s(\lambda)$ The specific absorption coefficient.
$\hat{A}_s$ The peak area of the internal standard in the sample.
$\hat{A}_{s_1}$ $\hat{A}_s$ determined using a one-step internal standard methodology (7).
$\hat{A}_{s_2}$ $\hat{A}_s$ determined using a two-step internal standard methodology (8).

b The y-intercept of a linear equation (2).
B The BIO method.

C The CSIRO method or the concentration of a pigment (depending on usage).
C_a The concentration of chlorophyll *a*.
C_A The concentration of alloxanthin.
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_{P_i} The concentration of a particular pigment.
$\tilde{C}_{P_i}$ The amount of pigment injected for pigment P_i (1), usually in units of nanograms.
$\hat{C}^A_{P_i}$ The maximum concentration of pigment P_i across all methods.
$\bar{C}^A_{P_i}$ The average concentration of a particular pigment across all methods.
$\check{C}^A_{P_i}$ The minimum concentration of pigment P_i across all methods.
C_{Pr} The concentration of prasinoxanthin.
$\bar{C}_{P_i}$ The average concentration for pigment P_i.
C_{Pb_a} The concentration of phaeophorbide *a*.
C_{Pt_a} The concentration of phaeophytin *a*.
C_{S_a} The spectrophotometric determination of C_a (16), which includes the contribution of all chlorophyll *a*-like pigments.
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_b The dilution factor for the volume of buffer and sample.
D_f A dilution factor.
D_{P_i} The LOD for pigment P_i (24).
D'_{P_i} The relative LOD for pigment P_i (26).
$D^{\text{eff}}_{P_i}$ The effective LOD for pigment P_i (28).

f-ratio The ratio of new to total production.
$f(x)$ A generalized function of the variable x.
F_{P_i} The inverse response factor for pigment P_i (5), i.e., $1/R_{P_i}$.

H The HPL method.
$\hat{H}_{P_i}$ The peak height of pigment P_i.

i An array index.

j An array index.

k An array index.

l An array index.
l_c The thickness of the sample in centimeters (12), i.e., the pathlength of the cuvette being used.
L The LOV method.
L_j The laboratory or method code (17).

m The slope of a (generalized) linear equation (2).
M The MCM method.
M' The MCM method computed with commonly used absorption coefficients.
M_{P_i} The main peak area of pigment P_i.

n A multiplicative factor.
N The number of observations.
N_C The number of columns.
N_L The number of laboratories quantitating a pigment.
N_R The total number of replicates (17): 3 for M and 2 for all other methods.
N_S The number of samples.

P The PML method.
P_i A particular pigment (referenced using index i).
P_I The polarity index (11).

Q_{P_i} The LOQ for pigment P_i (25).
Q'_{P_i} The relative LOQ for pigment P_i (27).
$Q^{\text{eff}}_{P_i}$ The effective LOQ for pigment P_i (29).

r The coefficient of determination for a linear regression.
R The response factor (from a generalized perspective).
R_{P_i} The response factor for pigment P_i (1), usually expressed as the amount of pigment divided by the peak area.
R'_{P_i} The relative response factor for pigment P_i.
R_s The resolution (or separation) between peaks (10).
$\check{R}_s$ The minimum resolution determined from a critical pair for which one of the pigments is a primary pigment.
R_{x_1} The response factor for chlorophyllide a at 665 nm.
R_{x_2} The response factor for chlorophyllide a at 440 nm.
R_{y_1} The response factor for chlorophyll c_1+c_2 at 665 nm.
R_{y_2} The response factor for chlorophyll c_1+c_2 at 440 nm.

S The (SDSU) CHORS method.
S_k The k^{th} station or sample ID (17).
$S_{k,l}$ The station or sample ID set by k, and the replicate number set by l (17).
$S_{P_i}(\lambda_d)$ The slope of the linear regression equation from the calibration of pigment P_i (24).

t Time.
t_0 The initial time.
t_R The retention time.
t_{R_1} The retention time of the first peak (10).
t_{R_2} The retention time of the second peak (10).

V_c The volume of sample extract injected onto the HPLC column (6).
V'_c The volume of sample-plus-buffer mixture injected.
V_e The volume of the extraction solvent (9).
V_f The volume of water filtered in the field to create the sample (6).
V_m The volume of extraction solvent (containing internal standard) added to a filter.
V_s The volume of internal standard (8).
V_x The extraction volume (6).
V_w The volume of water retained on the filter (9), usually assumed to be 0.2 mL for a 25 mm filter.

$\hat{w}_B$ The width of a peak at its baseline.
$\hat{w}_{B_1}$ The width of the first peak at its baseline (10).
$\hat{w}_{B_2}$ The width of the second peak at its baseline (10).
W_m The molecular weight.
W_s The carotenal weight.

x The abscissa.

y The ordinate.
Y A set of questionable samples analyzed by PML.

Z A set of unequivocally defrosted samples analyzed by HPL.

$\alpha(\lambda)$ The specific absorption coefficient expressed in percent weight per volume (e.g., in units of grams per 100 mL).

$\delta|\psi|$ Differences between APD values of individual pigments.

λ The spectral wavelength.
λ_d The detector wavelength (24).

ξ The precision or CV (22); also known as the RSD and %RSD.
$\bar{\xi}$ The average precision (23).
$\bar{\xi}_{\text{cal}}$ The average CV for gravimetric calibration of dilution devices.
$\bar{\xi}_{\text{inj}}$ The injector precision.
$\bar{\xi}_{t_R}$ The CV of retention time.
$\bar{\xi}_{V_x}$ The average CV of the extraction volume.

σ The standard deviation (22).

ψ The UPD or RPD (19).
$|\psi|$ The APD (20).
$|\bar{\psi}|$ The average APD.
ψ_{res} The RPD between the observed and fitted values (i.e., the residuals).
$\bar{\psi}_{\text{res}}$ The average RPD of a linear fit.
$|\bar{\psi}|_{\text{res}}$ The average of the absolute residuals.

References

Barlow, R.G., R.F.C. Mantoura, M.A. Gough, and T.W. Fileman, 1993: Pigment signatures of the phytoplankton composition in the northeastern Atlantic during the 1990 spring bloom. *Deep-Sea Res. II,* **40,** 459–477.

——, D.G. Cummings, and S.W. Gibb, 1997: Improved resolution of mono- and divinyl chlorophylls *a* and *b* and zeaxanthin and lutein in phytoplankton extracts using reverse phase C-8 HPLC. *Mar. Ecol. Prog. Ser.,* **161,** 303–307.

——, H. Sessions, N. Silulwane, H. Engel, S.B. Hooker, J. Aiken, J. Fishwick, V. Vicente, A. Morel, M. Chami, J. Ras, S. Bernard, M. Pfaff, J.W. Brown, and A. Fawcett, 2003: BENCAL Cruise Report. *NASA Tech. Memo. 2003–206892, Vol. 27,* S.B. Hooker and E.R. Firestone, Eds., NASA Goddard Space Flight Center, Greenbelt, Maryland, 64 pp.

Bidigare, R.R., 1991: "Analysis of algal chlorophylls and carotenoids." In: Marine Particles: Analysis and Characterization. *Geophysical Monograph 63,* D.C. Hurd and D.W. Spencer, Eds., American Geophysical Union, Washington, DC, 119–123.

——, R.C. Smith, K.S. Baker, J. Marra, 1987: Oceanic primary production estimates from measurements of spectral irradiances and pigment concentrations. *Global Biogeochem. Cycles 1,* 171–186.

——, and M.E. Ondrusek, 1996: Spatial and temporal variability of phytoplankton pigment distributions in the central equatorial Pacific Ocean. *Deep-Sea Res. II,* **43,** 809–833.

——, and C.C. Trees. 2000. "HPLC Phytoplankton Pigments: Sampling, Laboratory Methods, and Quality Assurance Procedures." In: G.S. Fargion, and J.L. Mueller, Ocean Optics Protocols for Satellite Ocean Color Sensor Validation, Revision 2. *NASA Tech. Memo. 2000–209966,* NASA Goddard Space Flight Center, Greenbelt, Maryland, 154–161.

——, L. Van Heukelem, and C.C. Trees, 2002: "HPLC Phytoplankton Pigments: Sampling, Laboratory Methods, and Quality Assurance Procedures." In: Mueller, J.L., and 39 Coauthors, Ocean Optics Protocols For Satellite Ocean Color Sensor Validation, Revision 3, Volume 2. *NASA Tech. Memo. 2002–210004/Rev3–Vol2,* J.L. Mueller and G.S. Fargion, Eds., NASA Goddard Space Flight Center, Greenbelt, Maryland, 258–268.

——, ——, and ——, 2003: "HPLC Phytoplankton Pigments: Sampling, Laboratory Methods, and Quality Assurance Procedures." In: Mueller, J.L., R.R. Bidigare, C. Trees, J. Dore, D. Karl, and L. Van Heukelem, Ocean Optics Protocols For Satellite Ocean Color Sensor Validation, Revision 4, Volume V: Biogeochemical and Bio-optical Measurements and Data Analysis Protocols. *NASA Tech. Memo. 2002–210004/Rev4–Vol.V,* J.L. Mueller, G.S. Fargion, and C.R. McClain, Eds., NASA Goddard Space Flight Center, Greenbelt, Maryland, 5–14.

Bidlingmeyer, B., 1992: *Practical HPLC Methodology and Applications.* J. Wiley & Sons, New York, 452 pp.

Britton, G., 1995: UV/Visible spectroscopy. In: *Carotenoids, Vol. 1B: Spectroscopy,* G. Britton, S. Liaaen-Jensen, and H. Pfander, Eds., Birkhäuser Verlag, Basel, Switzerland, 13–62.

Chamberlin, W.S., C.R. Booth, D.A. Kiefer, J.H. Morrow, and R.C. Murphy, 1990: Evidence for a simple relationship between natural fluorescence, photosynthesis and chlorophyll in the sea. *Deep-Sea Res. Part A,* **37,** 951–973.

Claustre, H., 1994: The trophic status of various oceanic provinces as revealed by phytoplankton pigment signatures. *Limnol. Oceanogr.,* **39,** 1,206–1,210.

——, F. Fell, K. Oubelkheir, L. Prieur, A. Sciandra, B. Gentili, and M. Babin, 2000: Continuous monitoring of surface optical properties across a geostrophic front: Biogeochemical inferences. *Limnol. Oceanogr.,* **45,** 309–321.

——, S.B. Hooker, L. Van Heukelem, J-F. Berthon, R. Barlow, J. Ras, H. Sessions, C. Targa, C. Thomas, D. van der Linde, and J-C. Marty, 2004: An intercomparison of HPLC phytoplankton pigment methods using *in situ* samples: Application to remote sensing and database activities. *Mar. Chem.,* **85,** 41–61.

Clesceri, L.S., A.E. Greenberg, and A.D. Eaton (Eds.), 1998: Part 10000, Biological examination, Section 10–200 H. In: *Standard Methods for the Examination of Water and Wastewater,* 20th ed. American Public Health Association, American Water Works Association, Water Environment Federation, Baltimore, Maryland, 10-18–10-25.

Davies, B.H., 1976: Carotenoids. In: *Chemistry and Biochemistry of Plant Pigments, Volume 2, 2nd Edition.* T.W. Goodwin, Ed., Academic Press, London, 38–165.

Dunne, R.P., 1999: Spectrophotometric measurement of chlorophyll pigments: A comparison of conventional monochromators and a reverse optic diode array design. *Mar. Chem.,* **66,** 245–251.

Eppley, R.W., and B.J. Peterson, 1979: Particulate organic-matter flux and planktonic new production in the deep ocean. *Nature,* **282,** 677–680.

Gieskes, W.W.C., G.W. Kraay, A. Nontji, D. Setiapermana, and Sutomo, 1988: Monsoonal alternation of a mixed and a layered structure in the phytoplankton of the euphotic zone of the Banda Sea (Indonesia): A mathematical analysis of algal pigment fingerprints. *Neth. J. Sea Res.,* **22,** 123–137.

——, and G.W. Kraay, 1989: Estimating the carbon-specific growth rate of the major algal species groups in eastern Indonesian water by ^{14}C-labeling of taxon-specific carotenoids. *Deep-Sea Res.,* **36,** 1,127–1,139.

Goericke, R., and D.J. Repeta, 1992: The pigments of *Prochlorococcus marinus*: The presence of divinyl chlorophyll *a* and *b* in a marine procaryote. *Limnol. Oceanogr.,* **37,** 425–433.

——, and ——, 1993: Chlorophyll *a* and chlorophyll *b* and divinyl chlorophyll *a* and chlorophyll *b* in the open Subtropical North Atlantic Ocean. *Mar. Ecol. Prog. Series,* **101,** 307–313.

Hoge, F.E., and R.N. Swift, 1981: Airborne simultaneous spectroscopic detection of laser-induced water raman backscatter and fluorescence from chlorophyll *a* and other naturally-occurring pigments. *Appl. Opt.,* **20,** 3,197–3,205.

Holm-Hansen, O., C.J. Lorenzen, R.W. Holmes, and J.D.H. Strickland, 1965: Fluorometric determination of chlorophyll. *J. du Cons. Int'l. pour l'Explor. de la Mer,* **30,** 3–15.

Hooker, S.B., and W.E. Esaias, 1993: An overview of the SeaWiFS project. *Eos, Trans. AGU,* **74,** 241–246.

——, and C.R. McClain, 2000: The calibration and validation of SeaWiFS data. *Prog. Oceanogr.,* **45,** 427–465.

——, H. Claustre, J. Ras, L. Van Heukelem, J-F. Berthon, C. Targa, D. van der Linde, R. Barlow, and H. Sessions, 2000: The First SeaWiFS HPLC Analysis Round-Robin Experiment (SeaHARRE-1). *NASA Tech. Memo. 2000–206892, Vol. 14,* S.B. Hooker and E.R. Firestone, Eds., NASA Goddard Space Flight Center, Greenbelt, Maryland, 42 pp.

Jeffrey, S.W., 1972: Preparation and some properties of crystalline chlorophyll c_1 and chlorophyll c_2 from marine algae. *Biochim. Biophys. Acta.,* **279,** 15–33.

——, 1997: Appendix E, Chlorophyll and carotenoid extinction coefficients. In: *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods*, S.W. Jeffrey, R.F.C. Mantoura, and S.W. Wright, Eds., UNESCO Publishing, Paris, 595–596.

——, and F.T. Haxo, 1968: Photosynthetic pigments of symbiotic dinoflagellates (zooxanthallae) from corals and clams. *Biol. Bull.,* **135,** 149–165.

——, and G.F. Humphrey, 1975: New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1, and c_2 in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanzen,* **167,** 191–194.

——, and J-M. LeRoi, 1997: Simple procedures for growing SCOR reference microalgal cultures. In: *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods.* S.W. Jeffrey, R.F.C. Mantoura, and S.W. Wright, Eds., UNESCO Publishing, Paris, 181–205.

——, R.F.C. Mantoura, and S.W. Wright, 1997: *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods.* UNESCO Publishing, Paris, 661 pp.

——, S.W. Wright, and M. Zapata, 1999: Recent advances in HPLC pigment analysis of phytoplankton. *Mar. Freshwater Res.,* **50,** 879–896.

JGOFS, 1994: Protocols for the Joint Global Ocean Flux Study Core Measurements. Intergovernmental Oceanographic Commission, Scientific Committee on Oceanic Research. *Manual and Guides, UNESCO,* **29,** 91–96.

Kishino, M., M. Takahashi, N. Okami, and S. Ichimura, 1985: Estimation of the spectral absorption coefficients of phytoplankton in the sea. *Bull. Mar. Sci.,* **37,** 634–642.

Latasa, M., R.R. Bidigare, M.E. Ondrusek, M.C. Kennicutt II, 1996: HPLC analysis of algal pigments: A comparison exercise among laboratories and recommendations for improved analytical performance. *Mar. Chem.,* **51,** 315–324.

——, ——, ——, and ——, 1999: On the measurement of pigment concentrations by monochromator and diode-array spectrophotometers. *Mar. Chem.,* **66,** 253–254.

——, K. van Lenning, J.L. Garrido, R. Scharek, M. Estrada, F. Rodríguez, M. Zapata, 2001: Losses of chlorophylls and carotenoids in aqueous acetone and methanol extracts prepared for RPHPLC analysis of pigments. *Chromatographia,* **53,** 385–391.

Li, W.K.W., and A.M. Wood, 1988: Vertical distribution of North Atlantic ultraphytoplankton: Analysis by flow cytometry and epifluorescence microscopy. *Deep-Sea Res.,* **35,** 1,615–1,638.

Lorenzen, C.J., 1966: A method for the continuous measurement of *in vivo* chlorophyll concentration. *Deep-Sea Res.,* **13,** 223–227.

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

Miller, J.N., and J.C. Miller, 2000: Calibration methods in instrumental analysis. In: *Statistics and Chemometrics for Analytical Chemistry,* 4th ed., J.N. Miller and J.C. Miller, Eds., Dorset Press, Dorchester, 107–150.

Morel, A., 1988: Optical modeling of the upper ocean in relation to its biogenous matter content (case 1 waters). *J. Geophys. Res.,* **93,** 10,749–10,768.

Mueller, J.L., and 27 coauthors, 2003: Ocean Optics Protocols for Satellite Ocean Color Sensor Validation: Special Topics in Ocean Optics Protocols and Appendices. *NASA/TM–2003–211621/Rev4–Vol.VI,* J.L. Mueller, G.S. Fargion, and C.R. McClain, Eds., NASA Goddard Space Flight Center, Greenbelt, Maryland, 139 pp.

Neveux, J., and F. Lantoine, 1993: Spectrofluorometric assay of chlorophylls and phaeopigments using the least squares approximation technique. *Deep-Sea Res. I,* **40,** 1,747–1,765.

Olson, R.J., S.L. Frankel, S.W. Chisholm, and H.M. Shapiro, 1983: An inexpensive flow cytometer for the analysis of fluorescence signals in phytoplankton: Chlorophyll and DNA distributions. *J. Exp. Mar. Biol. Ecol.,* **68,** 129–144.

O'Reilly, J.E., S. Maritorena, B.G. Mitchell, D.A. Siegel, K.L. Carder, S.A. Garver, M. Kahru, and C. McClain, 1998: Ocean color chlorophyll algorithms for SeaWiFS. *J. Geophys. Res.,* **103,** 24,937–24,953.

Parsons, T.R., and J.D.H. Strickland, 1963: Discussion of spectrophotometric determination of marine plant pigments, with revised equations for ascertaining chlorophylls and carotenoids. *J. Mar. Res.,* **21,** 155–163.

Partensky, F., W.R. Hess, and D. Vaulot, 1999: *Prochlorococcus*, a marine photosynthetic procaryote of global significance. *Microbiol. Mol. Biol. Rev.,* **63,** 106–127.

Segel, I.H., 1968: *Biochemical Calculations,* 2nd ed. John Wiley and Sons, Inc., New York, 441 pp.

Snyder, L.R., and J.J. Kirkland, 1979: Detectors, Chapt. 4. In: *Introduction to Modern Liquid Chromatography, 2nd Edition.* John Wiley and Sons, Inc., New York, 125–167.

Trees, C.C., D.K. Clark, R.R. Bidigare, and M.E. Ondrusek, 2000: Chlorophyll *a* versus accessory pigment concentrations within the euphotic zone: A ubiquitous relationship? *Limnol. Oceanogr.,* **45,** 1,130–1,143.

——, R.R. Bidigare, D.M. Karl, L. Van Heukelem, and J. Dore, 2002: "Fluorometric Chlorophyll a: Sampling, Laboratory Methods, and Data Analysis Protocols." In: Mueller, J.L., and 39 Coauthors, Ocean Optics Protocols For Satellite Ocean Color Sensor Validation, Revision 3, Volume 2. *NASA/TM–2002–210004/Rev3–Vol2,* J.L. Mueller and G.S. Fargion, Eds., NASA Goddard Space Flight Center, Greenbelt, Maryland, 269–283.

Uitz, J., H. Claustre, A. Morel, and S.B. Hooker, 2005: From surface chlorophyll *a* to phytoplankton community composition in oceanic waters. *J. Geophys. Res.,* (submitted).

Van Heukelem, L., and C. Thomas, 2001: Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. *J. Chromatogr. A.,* **910,** 31–49.

——, and C.S. Thomas, and P.M. Glibert, 2002: Sources of Variability in Chlorophyll Analysis by Fluorometry and High-Performance Liquid Chromatography in a SIMBIOS Inter-calibration Exercise. *NASA Tech. Memo. 2002–211606,* G.S. Fargion and C.R. McClain, Eds., NASA Goddard Space Flight Center, Greenbelt, Maryland, 50 pp.

Vesk, M., and S.W. Jeffrey, 1987: Ultrastructure and pigments of two strains of the picoplanktonic alga *Pelagococcus subviridis* (Chrysophyceae). *J. Phycol.,* **23,** 322–336.

Vidussi, F., H. Claustre, J. Bustillos-Guzman, C. Cailliau, and J.C. Marty, 1996: Determination of chlorophylls and carotenoids of marine phytoplankton: Separation of chlorophyll *a* from divinyl-chlorophyll *a* and zeaxanthin from lutein. *J. Plankton Res.,* **18,** 2,377–2,382.

——, ——, B.B. Manca, A. Luchetta, and J-C. Marty, 2001: Phytoplankton pigment distribution in relation to upper thermocline circulation in the eastern Mediterranean Sea during winter. *J. Geophys. Res.,* **106,** 19,939–19,956.

Watanabe, T., A. Hongu, K. Honda, N. Masataka, M. Konno, and S. Saitoh, 1984: Preparation of chlorophylls and pheophytins by isocratic liquid chromatography. *Anal. Chem.,* **56,** 251–256.

Wolcott, R.G., J.W. Dolan, L.R. Snyder, S.R. Bakalyar, M.A. Arnold, and J.A. Nichols, 2000: Control of column temperature in reversed-phase liquid chromatography. *J. Chrom A,* **869,** 211–230.

Wright, S.W., 1997: Appendix H, Summary of terms and equations used to evaluate HPLC chromatograms. In: *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods*, S.W. Jeffrey, R.F.C. Mantoura, and S.W. Wright, Eds., UNESCO Publishing, Paris, 622–630.

——, S.W. Jeffrey, R.F.C. Mantoura, C.A. Llewellyn, T. Bjornland, D. Repeta, and N. Welschmeyer, 1991: Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Mar. Ecol. Prog. Ser.,* **77,** 183–196.

Yentsch, C.S., and D.W. Menzel, 1963: A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. *Deep-Sea Res.,* **10,** 221–231.

Zapata, M., and J.L. Garrido, 1991: Influence of injection conditions in reversed-phase high-performance liquid chromatography of chlorophylls and carotenoids, *Chromatographia,* **31**, 589–594.

——, F. Rodriguez, and J.L. Garrido, 2000: Separation of chlorophylls and carotenoids from marine phytoplankton: A new HPLC method using a reversed phase C_8 column and pyridine-containing mobile phases, *Mar. Ecol. Prog. Ser.,* **195,** 29–45.

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L. van Heukelem and C.S. Thomas: UMCES Horn Point Laboratory, Cambridge, MD; J. Clastre and J. Ras: LOV Observatoire Océanologique de Villefranche, Villefranche-sur-Mer, France; et al.

14. ABSTRACT
Eight international laboratories specializing in the determination of marine pigment concentrations using high performance liquid chromatography (HPLC) were intercompared using in situ samples and a variety of laboratory standards. The field samples were collected primarily from eutrophic waters, although mesotrophic waters were also sampled to create a dynamic range in chlorophyll concentration spanning approximately two orders of magnitude (0.3–25.8 mgm3). The intercomparisons were used to establish the following: a) the uncertainties in quantitating individual pigments and higher-order variables (sums, ratios, and indices); b) an evaluation of spectrophotometric versus HPLC uncertainties in the determination of total chlorophyll a; and c) the reduction in uncertainties as a result of applying quality assurance (QA) procedures associated with extraction, separation, injection, degradation, detection, calibration, and reporting (particularly limits of detection and quantitation).

15. SUBJECT TERMS
HPLA analysis, SeaWiFS, Round-Robin Experiment, SeaHARRE

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