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This report presents the results of marine and freshwater scientific work carried out by Marine Scotland Science.

The Development & Validation of a Method for Quantification of Pigments of Interest in Marine Waters using High Performance Liquid Chromatography (HPLC) with Diode array Detection (DAD)

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Executive Summary

1. Diode array detectors (DAD) are widely used for the analysis of complex phytoplankton pigments in marine waters. A spectrum of each peak in the sample is collected, and when compared with that of a reference standard, the presence of a particular pigment can be confirmed or refuted, resulting in an increased confidence in pigment identification over a conventional UV detector. A method was developed and validated using high performance liquid chromatography (HPLC) with a DAD with the aim of replacing the pigment HPLC method using a UV detector.

2. A C8 column was used as these have been shown to separate monovinyl and divinyl forms of chlorophyll *a* and lutien and zeaxanthin. This was not possible using a C18 column.

3. Vitamin E actetate was added to the extraction solvent and used as the internal standard.

4. All pigments of interest (19 – butanoyloxyfucoxanthin, 19-hexanoyloxyfucoxanthin, alloxanthin, alphacarotene, antheraxanthin, beta-carotene, chlorophyll *a*, chlorophyll *b*, chlorophyll c_2, chlorophyll c_3, chlorophyllide *a*, diadinoxanthin, diatoxanthin, divinyl chlorophyll *a*, fucoxanthin, gyroxanthin-diester, lutein, neoxanthin, peridinin, prasinoxanthin, violaxanthin and zeaxanthin) were quantified. Correlation coefficients of >0.99 were obtained for each pigment when a quadratic polynomial curve was drawn.

5. The limit of detection (LoD) for all pigments was calculated either based on a low matrix sample or the lowest calibration standard and ranged from 0.001 µg/l for lutein and zeaxanthin to 0.21 µg/l for chlorophyll *a*.

6. Replicate analysis of a mixed pigment standard, and marine water samples met the target limit for reproducibility (coefficient of variation less than 25%) given in the UK Clean Safe Seas Environmental Monitoring Programme (CSEMP) Green Book for chlorophyll *a* and chlorophyll *b* and the majority of the pigments. When the target limit was not met, this was due to the small amounts present in these samples, and for chlorophyllide *a* this was a result of the complexity of the chromatographic peak.

7. QUASIMEME samples were used to determine bias / recovery and this was calculated to be 98% for chlorophyll *a* and, 87% for chlorophyll *b*, meeting the criteria set by the UK National Marine Chemistry Advisory Group (% recovery should be 70 – 110%). Bias / recovery could not be readily determined for the other pigments as these are not within the scope of the QUASIMEME Laboratory Performance Studies. Spiked samples would have had to have been prepared for the other pigments and the cost was prohibitive.

Introduction

The objective of the OSPAR eutrophication strategy is to combat eutrophication within the OSPAR maritime area, in order to achieve and maintain a healthy marine environment where anthropogenic eutrophication does not occur. The strategy builds on long standing commitments from contracting parties to achieve a substantial reduction of nutrient inputs at source where pollution is likely to be caused. Progress in reducing nutrient inputs is regularly assessed by OSPAR based on national implementation reporting by the contracting parties. OSPARs assessment work is supported by monitoring under the Eutrophication Monitoring Programme as part of the OSPAR Co-ordinated Environmental Monitoring Programme (CEMP).

Parts of the OSPAR maritime area are also covered by the requirements of the EU Water Framework Directive (WFD) (European Commission, 2016) and the Marine Strategy Framework Directive (MSFD) (HM Government, 2012), and there are strong links between all three;

- Parties contracted to OSPAR must assess and classify the eutrophication status of their waters as being either “a non-problem area” or “a problem area” (OSPAR, 2013).

- The WFD was introduced in 2000. The purpose of the directive was for member states to assess the ecological status of coastal and transitional waters from a wide variety of human pressures including nutrient input by assigning them as High, Good, Moderate, Poor or Bad, with the aim that. all water bodies must have achieved at least Good status by 2015. Achieving the objectives of the WFD was a challenge with 47% of EU surface waters not meeting good ecological status in 2015 (Voulvoulis *et al*., 2017) Member states that did not meet the objectives in 2015 are required to achieve all WFD environmental objectives by the end of the second and third management cycles, which extend from 2015 to 2021 and 2021 to 2027 respectively.

- The MSFD has eleven qualitative descriptors that are used to determine Good Environmental Status. Descriptor 5 deals with eutrophication and the aim of this is "human-induced eutrophication is minimised, especially adverse effects thereof, such as the loss in biodiversity, ecosystem degradation, harmful algal blooms and oxygen deficiency in bottom waters". The overall aim of the MSFD is to achieve Good Environmental Status across Europe's marine environment by 2020.

The MSFD criteria for monitoring and assessment of eutrophication status have recently been revised (Official Journal of the European Union, 2017). There are now eight criteria elements:

1. Nutrients in the water column;

2. Chlorophyll a in the water column;

3. Harmful algal blooms in the water column;

4. Transparency of the water column;

5. Dissolved oxygen in the bottom of the water column;

6. Opportunistic macroalgae of benthic habitats;

7. Macrophyte communities of benthic habitats;

8. Macrofaunal communities of benthic habitats.

Criteria 1, 2 and 5 are primary criteria with the remaining being secondary. However, criteria 5 can be substituted with criteria 8. Primary criteria must be used, but beyond coastal waters, the use of secondary criteria will be agreed upon at regional or sub regional level.

Eutrophication occurs when waters are enriched by nutrients, especially compounds of nitrogen and/or phosphorus, causing an accelerated growth of algae and higher forms of plant life to produce an undesirable disturbance to the balance of organisms present in the water and to the quality of the water concerned. In UK waters, eutrophication problems are restricted to small estuaries, embayments and coastal waters where water circulation is restricted and conditions are favourable (OSPAR, 2008).

Chlorophyll is the biological compound which plants and algae use to produce food from sunlight in a process known as photosynthesis. Phytoplankton are small single celled organisms at the base of the marine food web. Most contain chlorophyll *a* which they use to harvest light energy from the sun. Accelerated growth and changes in phytoplankton species composition and an increase in harmful algal blooms are just two indicators of eutrophication and potential changes to the environment as a consequence of climate change. *Phaeocystis* and *Noctiluca* are examples of nuisance bloom forming species (OSPAR, 2008). Dinoflagellates become dominant in Scottish waters in the summer after the growing season when the thermocline builds up. Some dinoflagellate species are potentially toxic and their presence could indicate eutrophication. *Gymnodinium mikimotoi* and *Alexandrium spp*. are examples of such species.

Although the determination of chlorophyll *a* in marine waters is considered a routine measurement, a high degree of variability can be observed depending on the methodology used. The many different chlorophyll compounds (chlorophyll *a*,*b*,c_1,c_2,c_3 plus at least 8 other chlorophyll *c* subtypes, divinyl chlorophyll *a* and divinyl chlorophyll *b*) (Zapata *et al.*, 2006) have different and overlapping absorption and emission/excitation spectra (Aminot & Rey, 2001). Traditionally, samples collected to estimate phytoplankton biomass were analysed by either photometric or fluorometric techniques (Baretta-Bekker *et al.*, 2015). The trichromatic photometric method determines chlorophylls *a*, *b* and *c* in the absence of degradation products. Chlorophyll degradation products, such as pheophytin *a* and pheophorbide *a*, may be present in relatively high concentrations in natural samples which will result in an overestimation of

chlorophyll *a* as the absorption characteristics are similar. Methods to correct for pheopigment interference were developed for both photometric and flurorometric techniques. The samples are treated with weak acid to convert chlorophyll *a* to pheophytin *a*. The calculation of pheopigments assumes that all of this pigment is pheophytin *a*, which is probably not the case. The presence of chlorophylls *b* and *c* can significantly interfere with chlorophyll *a* measurements depending on the amounts present. The acidification method is no longer recommended because it is both time consuming and the results are questionable (OSPAR, 2012). Although it is widely recognised that there are a number of issues with the spectrophotometric and fluorometric protocols they are still used to estimate phytoplankton biomass in marine samples by many international monitoring programmes and time series data.

There is also confusion over the use of the term 'chlorophyll *a*' for estimation of phytoplankton biomass as neither the photometric or fluorometric methods can measure chlorophyll *a* exclusively due to the presence of degradation products. When using these methods the term 'chlorophylls' should be used rather than chlorophyll *a* (Baretta-Bekker *et al*, 2015). The only way to measure chlorophyll *a* exclusively is to use a separation technique such as high performance liquid chromatography (HPLC). Automated HPLC methods for the routine determination of phytoplankton chlorophylls, degradation products and carotenoids were first developed in the 1980s and have continually been improved upon (Jeffrey *et al.*, 1997, Smith *et al.*, 2007). These methods can now separate, identify and quantify over 50 chlorophylls, cartenoids, their derivatives and isomers from marine phytoplankton. HPLC data provides valuable information about the contribution of different functional groups to the biomass of the phytoplankton community. Whilst fucoxanthin is a useful indicator of diatoms, some phytoplankton groups contain unique pigment signatures which facilitate their identification e.g. thecate dinoflagellates and the pigment peridinin (Zapata *et al*., 2012) and the dinoflagellate genus *Karenia* and the pigment gyroxanthin (Brand *et al*., 2012). A reliable interpretation of the pigment HPLC data should be supported by microscopic verification.

Detectors

Four types of detectors are commonly used in the HPLC analysis of pigments (Jeffrey *et al*., 1997);

I. Fixed wavelength absorbance detector;
II. Variable wavelength detectors;

III. Full-spectrum detectors;
IV. Fluorescence detectors.

Fixed wavelength detectors are simple, cheap and robust. Mercury lamps produce discrete emission lines at 435.8 nm and 404.7 nm which are ideal for chlorophylls, carotenoids and pheopigments respectively. Variable-wavelength detectors use a continuous spectrum lamp with wavelength dispersal from a diffraction grating monochromator to produce light of a narrow bandwidth. They are more versatile but less sensitive than fixed-wavelength detectors. They can be programmed to change wavelength during a run, but this is not practical for pigment analysis due to the close proximity of peaks with different absorption wavelengths in the chromatogram. Diode array detectors are the most common type of full-spectrum detector. These are extremely useful for pigment analysis in that they allow the full spectrum of a peak to be collected without stopping the flow. Approximately 20 spectra can be collected across a peak, allowing checks of spectral identity and peak purity on both the upslope and downslope of the peak. These are strongly recommended for the detailed analysis of complex pigment mixtures. Fluorescence detectors are one to two orders of magnitude more sensitive than absorbance detectors for chlorophylls but cannot detect carotenoids. Fluorescence detectors are often used in sequence with an absorbance detector, as the data produced are complimentary. The fluorescence detector is positioned after the absorbance detector as the larger fluorescence flow cell causes more peak spreading than the absorbance cell and the fluorescence chromatogram is less sensitive to the small amount of peak spreading introduced by the absorbance detector.

As a result of the increasing need to identify a wider range of pigments and monitor changes in phytoplankton species composition, Marine Scotland Science (MSS) developed a HPLC method in 2009 (Smith *et al*., 2010). The method utilised a variable-wavelength ultra-violet (UV) detector as this was what was available within MSS at the time. Detection using the UV detector was made primarily on the basis of retention time and analyst experience. However, when pigment concentrations were low, identification by retention time alone was difficult due to the close proximity with other pigments. The MSS HPLC-UV method was also limited in that it could not resolve the pigments lutein and zeaxanthin, and 19-hexanoyloxyfucoxanthin and prasinoxanthin. Resolution of these pigments is necessary for the use of CHEMTAX software. CHEMTAX software (Mackey *et al*., 1996) is a matrix factorisation program which can be used to estimate plankton class abundances from concentrations of marker pigments determined by

HPLC analysis. The user constructs a matrix of marker pigment: chl a ratios for each algal class known to be present in the samples. Multiple CHEMTAX analyses are performed to improve initial pigment ratio values and give a good estimation of the biomass.

This report will describe the development and validation of an HPLC method with diode array detection to quantify 19 – butanoyloxyfucoxanthin, 19-hexanoyloxyfucoxanthin, alloxanthin, alphacarotene, antheraxanthin, beta-carotene, chlorophyll *a*, chlorophyll *b*, chlorophyll c_2, chlorophyll c_3, chlorophyllide *a*, diadinoxanthin, diatoxanthin, divinyl chlorophyll *a*, fucoxanthin, gyroxanthin-diester, lutein, neoxanthin, peridinin, prasinoxanthin, violaxanthin and zeaxanthin. Chlorophyllide *a* and divinyl chlorophyll *a* were added to the 2009 suite of pigments as these give a more accurate chlorophyll *a* concentration when reported as total chlorophyll *a* (total chlorophyll *a* is the sum of chlorophyllide *a* + divinyl chlorophyll *a* + chlorophyll *a*). This work was based on the HPLC method described by Van Heukelem and Thomas (2001) which resolves the marker pigments required for CHEMTAX application, and the methods reported in the fifth Sea-viewing Wide Field-of-view Sensor (SeaWiFS) HPLC analysis Round-Robin Experiment (SeaHARRE-5) (NASA Technical Memorandum, 2012). SeaHARRE brings together international laboratories specialising in the determination of marine pigment concentrations using HPLC.

Experimental

Sampling

MSS have established 'The Scottish Coastal Observatory' (Bresnan *et al*., 2016), consisting of a number of sites around the Scottish coast where long term monitoring of different combinations of temperature, salinity, nutrients, carbonate chemistry, pigments, algal toxins and plankton occurs. Monitoring of chlorophyll, using the fluorometric method, has taken place at the Stonehaven monitoring site on the east coast of Scotland (56º 57.80N, 02º 06.20W) since 1997 and the Loch Ewe monitoring site, on the west coast (57º 50.14'N, 05º 36.61'W) since in 2002 (Figure 1).

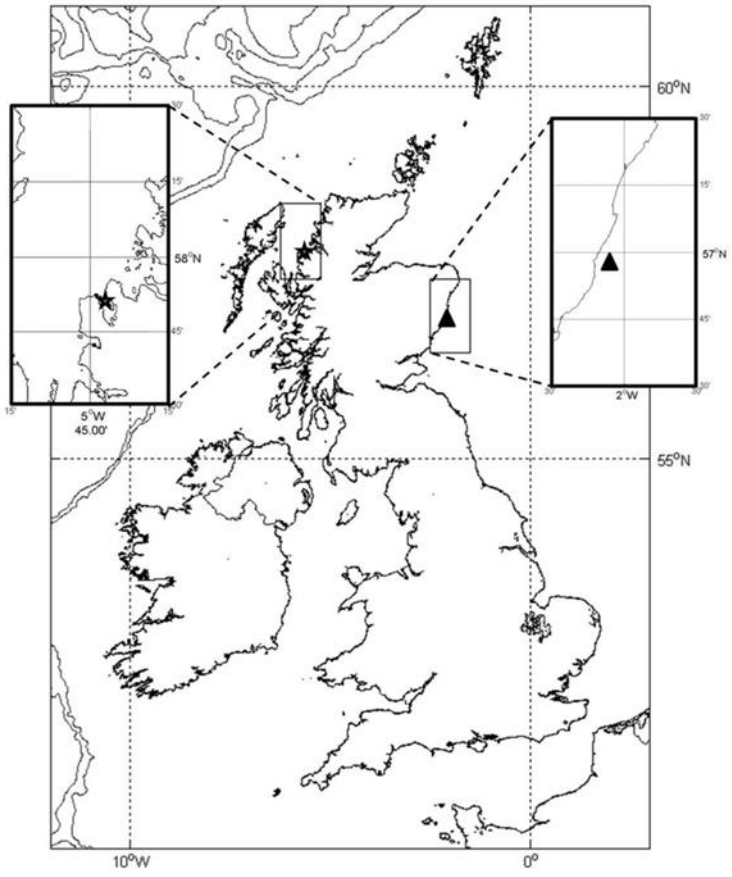


Figure 1: Location of Stonehaven (▲) and Loch Ewe (★) monitoring sites.

For this work, only seawater collected from the Stonehaven monitoring site was used to prepare the samples used for development and validation of the HPLC-DAD method,

due to its close proximity to the laboratory. Water samples collected from Loch Ewe demonstrate similar pigment profiles to those collected from Stonehaven (Smith *et al*., unpublished observations) and, therefore, collection of validation samples at Loch Ewe are not required.

A 10 m integrated Lund tube sampler was deployed to collect water samples for chlorophyll analysis. The contents of the tube were then emptied into an insulated carboy which was protected from light. These were then filtered on return to the laboratory within four hours of collection as algal populations change quickly due to photodecomposition. Prior to sub-sampling the insulated carboy containing the sample was gently agitated to re-suspend any large cells that may have settled. Two litres of sample were filtered through a Whatman GF/F glass fibre filter paper (47 mm diameter), under a low vacuum to avoid damaging the cells. The filter paper was removed from the fritted base using tweezers, avoiding disturbing the filtrate, and folded once (algae inside). The folded filter paper was blotted gently with tissue to remove excess moisture and placed in a pre-numbered centrifuge tube. The centrifuge tubes containing the samples were stored in a cryogenic freezer until analysis.

Reagents

Acetone, methanol and water were all HPLC grade and purchased from Rathburn Chemicals Ltd., Walkerburn, Scotland, UK. Tetrabutyl ammonium acetate (TbAA) buffer was prepared by diluting 0.4 M HPLC grade tetrabutyl ammonium hydroxide, with water and pH adjustment with glacial acetic acid. Both 0.4 M HPLC grade tetrabutyl ammonium hydroxide and glacial acetic acid were purchased from Fisher Scientific UK Ltd., Loughborough, UK. Magnesium carbonate ($MgCO_3$) and vitamin E acetate were purchased from Sigma-Aldrich Company Ltd., Dorset, UK.

The solvent systems prepared for the HPLC gradient were:

- Solvent A, Methanol : 28 mM TbAA pH 6.5 buffer (70 : 30 v/v)
- Solvent B, Methanol (100%)
- Solvent C, Acetone (100%)

The extraction solvent was prepared by diluting vitamin E acetate in 90% buffered acetone (~25 mg/l). 90% buffered acetone was used as the extraction solvent; this was

prepared by mixing the powder free solution from a saturated magnesium carbonate solution with acetone. Vitamin E acetate was used as the internal standard.

Tetrabutyl ammonium acetate was used as the ion-pairing reagent.

Extraction of the Samples

The pigments were extracted from the phytoplankton on the filter paper by adding extraction solvent (5 ml) to a 15 ml polypropylene centrifuge tube containing the filter paper and then sonicating for one minute using a Sanyo Soniprep 150 Ultrasonic Disintegrator fitted with an exponential microprobe, at setting 15 amplitude microns. To avoid heating of the solvent and degradation of the pigments, the centrifuge tube was placed in a beaker of ice during this process. The samples were then placed in a laboratory refrigerator to soak for between 16 to 30 hours. After at least one hour of soaking, the extraction using the Sanyo Soniprep 150 Ultrasonic Disintegrator was repeated, before being returned to the refrigerator to complete the soaking period. The supernatant was clarified using a 0.45 µm PTFE syringe filter. An aliquot of the filtered extract was transferred to an amber HPLC autosampler vial. These were placed in the refrigerated autosampler at 4°C for no more than 24 hours prior to injection. Any remaining filtered extract was stored in a cryovial in a cryogenic freezer.

Pigment Analysis by HPLC with a Diode Array Detector

A Thermo Fisher Scientific Surveyor Plus HPLC with diode array detection and a PC with ChromQuest software version 5.0 was used. The Surveyor Plus Autosampler was fitted with an automated sample preparation facility and a refrigerated sample tray. An automated sample preparation facility is required for pigment HPLC, as losses of hydrophobic pigments occur within one hour if the extracts are prebuffered. Pigment extracts should also be kept cold while awaiting injection (Jeffrey *et al*., 1997).

An Agilent Zorbax Eclipse XDB C-8 column, 150 mm long x 4.6 mm internal diameter was used. This column utilises the technologies of Extra-Dense Bonding (XDB) of organo-silane ligands and double endcapping to protect the ultra-pure silica support from dissolution of mobile phases of intermediate pH. The special Zorbax silica support is designed to reduce or eliminate strong adsorption of highly polar compounds and has a 5 µm particle size. The column is suitable for operation at 60°C as used in the Van Heukelem and Thomas method (2001). The standards and sample extracts were mixed

with ion-pairing reagent immediately before injection [710 µl buffer: 290 µl sample (this is discussed further in the method development section of the report)]. The identification and integration of pigment peaks occurred at three different wavelengths; chlorophyllide *a*, chlorophyll *a* and divinyl chlorophyll *a* were determined at 665 nm, the internal standard was determined at 222 nm and all other pigments were determined at 450 nm (all had a bandwidth of 11 nm and a scan rate of 1.0 Hz). Absorbance spectra were collected between 300-700 nm, with a bandwidth of 1 nm and a scan rate of 10 Hz. The flow cell volume was 10 µl and the path length was 5 cm.

Calibration and Quality Control

Chlorophyll *a* from *Anacystis nidulans* algae, chlorophyll *b* from spinach and vitamin E acetate were purchased from Sigma-Aldrich Company Ltd., Dorset, UK. Vitamin E acetate was used as the internal standard (further information is given below). Other pigment standards were purchased from DHI Lab Products, Hoersholm, Denmark.

Solutions of chlorophyll *a* and chlorophyll *b* were prepared by diluting the solid compounds with 90% buffered acetone. The concentration of chlorophyll *a* and chlorophyll *b* in these solutions was determined using the trichromatic spectroscopic method (Aminot and Rey, 2001). An ultraviolet (UV)/visible spectrophotometer (Ultraspec 3300 pro, Amersham Pharmacia Biotech) was used to make the measurements. A series of diluted standard solutions were then prepared from the stock standard solutions covering the range 20 µg/l to 2000 µg/l chlorophyll *a* and 5 µg/l to 500 µg/ l chlorophyll *b.* Vitamin E acetate in 90% buffered acetone (1g/l) was added to all of the diluted standard solutions prior to making up to volume so that the final concentration of vitamin E acetate was 25 mg/l.

Standard solutions were prepared using the DHI standards for all other pigments covering the range 10 µg/l to 1000 µg/l chlorophyll c_3, chlorophyll c_2, chlorophyllide *a*, peridinin, 19-butanoyloxyfucoxanthin, fucoxanthin, prasinoxanthin, 19-hexanoyloxyfucoxanthin. diadinoxanthin, divinyl chlorophyll *a* and the carotenes; and 5 µg/l to 500 µg/l neoxanthin, violaxanthin, antheraxanthin, alloxanthin, diatoxanthin, zeaxanthin, lutein and gyroxanthin-diester. Vitamin E acetate in 90% buffered acetone (1g/l) was added to all of the diluted standard solutions prior to making up to volume so that the final concentration of vitamin E acetate was 25 mg/l. The spectrophotometrically determined concentrations provided by DHI were used to compute the response factors for each pigment.

Pigments are extremely light and temperature sensitive. Therefore, standard solutions were prepared in amber volumetric flasks or volumetric flasks wrapped in aluminium foil. The standards were prepared in a temperature controlled laboratory (20°C ± 3°C) with subdued lighting. The standards were measured on the same day as preparation.

Vitamin E acetate was used as the internal standard to correct for errors which may have arisen from sample handling and HPLC analysis since standard/sample and internal standard are treated identically. The peak area of vitamin E acetate was determined both when it was injected onto the HPLC column prior to adding to the standard and in the standard. The corrected pigment peak area was then calculated as follows;

$$\textbf{Corrected pigment peak area} = \left(\frac{A_c}{A_s}\right) x\, A_{pi}$$

Using Microsoft Excel a quadratic polynomial curve was drawn where the regression was forced through zero, for each pigment. The concentration of each pigment in the samples was calculated using Microsoft Excel.

Spectral libraries were created by saving the scans of each pigment for all of the diluted standard solutions to assist in accurate identification. Further information on the use of spectral libraries is given below.

Antheraxanthin standard (50 µl of a 0.812 mg/l solution containing 0.041 µg antheraxanthin) and gyroxanthin – diester standard (50 µl of a 0.675 mg/l solution containing 0.034 µg gyroxanthin-diester) were added to mixed pigment standard (650 µl of a solution containing 3.93 mg/l total chlorophyll *a*). This new mixed pigment standard was injected onto the chromatograph each day and the retention times for the individual pigments in the mixed pigment standard were entered into the peak/group table.

A laboratory reference material (LRM) was prepared by diluting Phyto Feast Live (Supplier: Varicon Aqua Solutions Ltd., Malvern, Worcestershire, UK) with seawater collected from a depth of approximately 45 m from a site on the east coast, 3 km offshore from the town of Stonehaven (56º57.8'N, 02º06.2'W). Phyto Feast Live contains a blend of the most important marine microalgae; *Pavlova*, *Isochrysis*, *Thalassiosira*, *Tetraselmis* and *Nannochloropsis*. A series of filter papers were prepared by filtering a known volume of diluted Phyto Feast Live through a Whatman GF/F filter paper. The filter papers were preserved by 'flash freezing' in liquid nitrogen

and stored in a cryogenic freezer. An LRM was extracted and analysed with every HPLC run, and the concentration of individual pigments determined. The data obtained from the LRM was plotted on Shewhart control charts with warning and action limits drawn at ± 2 x and ± 3 x the standard deviation of the mean.

Results and Discussion

Method Development

The MSS HPLC method with UV detection (MSS HPLC-UV) (Smith *et al*., 2010) is limited in that it could not resolve the pigments lutein and zeaxanthin, and 19-hexanoyloxyfucoxanthin and prasinoxanthin. This method utilised a C18 polymeric column and a tertiary gradient and was based on the work of Wright *et al.* (1991) and Kraay *et al.* (1992). At the 2014 QUASIMEME Workshop on Algal Pigments and Nutrients the use of a C8 column was recommended as this can separate monovinyl and divinyl analogues of chlorophyll *a* in the open ocean and also lutein and zeaxanthin. There are two widely accepted methods for HPLC pigment analyses which utilise C8 columns; the Van Heukelem and Thomas method (2001) uses a methanol-based gradient solvent system and the Zapata method (2000) which uses pyridine – containing mobile phases. The Zapata method was discounted due to the health and safety issues associated with the use of pyridine, and, therefore, the method developed was based on the Van Heukelem and Thomas method.

Pre-Treatment Method

For pigment analysis, sample extracts require to be pre-buffered immediately prior to injection. This ensures that even the most polar pigments are adsorbed as a narrow zone on top of the HPLC column. (Jeffrey *et al*., 1997). The Van Heukelem and Thomas method utilises an autosampler tray temperature of 4°C and a mixing in the loop pre-treatment method. Whilst developing the MSS HPLC-UV method it was found that the reaction between the ion-pairing reagent and the sample extracts was temperature dependant and had to be carried out at ambient temperature to ensure adequate mixing (Smith *et al*., 2010). This was thought to be a result of the ion-pairing reagent and the sample extract being held within different areas of the autosampler and, therefore, at different temperatures. Better mixing should be achieved when the ion-pairing reagent is held in the autosampler sample rack with the samples. A standard containing ~ 1000 µg/l chlorophyll *a* and ~250 µg/l chlorophyll *b* was prepared. The

concentrations were not accurately determined as the aim of the experiment was to establish if good mixing was achieved. Using the MSS HPLC-UV method conditions (Smith *et al*., 2010) a pre-treatment method was prepared where vials containing ion-pairing reagent and standard were held in alternating positions in the autosampler sample rack. The autosampler tray temperature was set at 4 °C. The peak areas for chlorophyll *a* and *b* are given in Table 1.

Table 1

Peak area data for chlorophyll *a* and chlorophyll *b* standard solution when the ion-pairing reagent is held in the autosampler rack at 4°C.

Replicate	Chl *a* Peak Area	Chl *b* Peak Area
1	526,813	257,178
2	460,060	226,058
3	607,249	293,239
4	402,185	202,132
5	448,780	220,801
6	437,209	216,896
Mean	**480,383**	**236,051**
Stdev	**74,352**	**33,367**
%CV	**15.5**	**14.1**

The % CV for both chlorophyll *a* and chlorophyll *b* were below the reproducibility target limit of 25% given in The Clean Seas Environment Monitoring Programme, Green Book (2012) demonstrating that adequate mixing is achieved at 4°C when the ion-pairing reagent is held in vials in the autosampler rack rather than in the reagent vessel within the autosampler.

Laboratories participating in the fifth SeaWiFS HPLC analysis round-robin experiment (NASA Technical Memorandum, 2012) use a mixing in the loop pre-treatment method where the autosampler is programmed to draw successive aliquots of sample extract and buffer into the sample loop prior to injection. This was tested at MSS with various different combinations of sample loop, syringe size, volumes of buffer and sample, injection volumes, syringe speed. In all cases poor chromatography was obtained (see Figure 2) due to poor mixing.

The best chromatography (see Figure 3) and reproducibility was achieved when the autosampler was fitted with a 1000 µl sample loop, a 2.5 ml syringe and adopting a mixing in the vial method (see Table 2). Here the autosampler was programmed to draw an aliquot of buffer and an aliquot of sample into a clean HPLC vial, mix and immediately inject 500 µl onto the column. It is important to note that the injection mode was set to a 'partial loop injection' rather than a 'full loop injection'. In 'full loop injection' the amount of sample required is more than 3 times the injection volume which would exceed the capacity of the HPLC vial with the volumes used in this method.

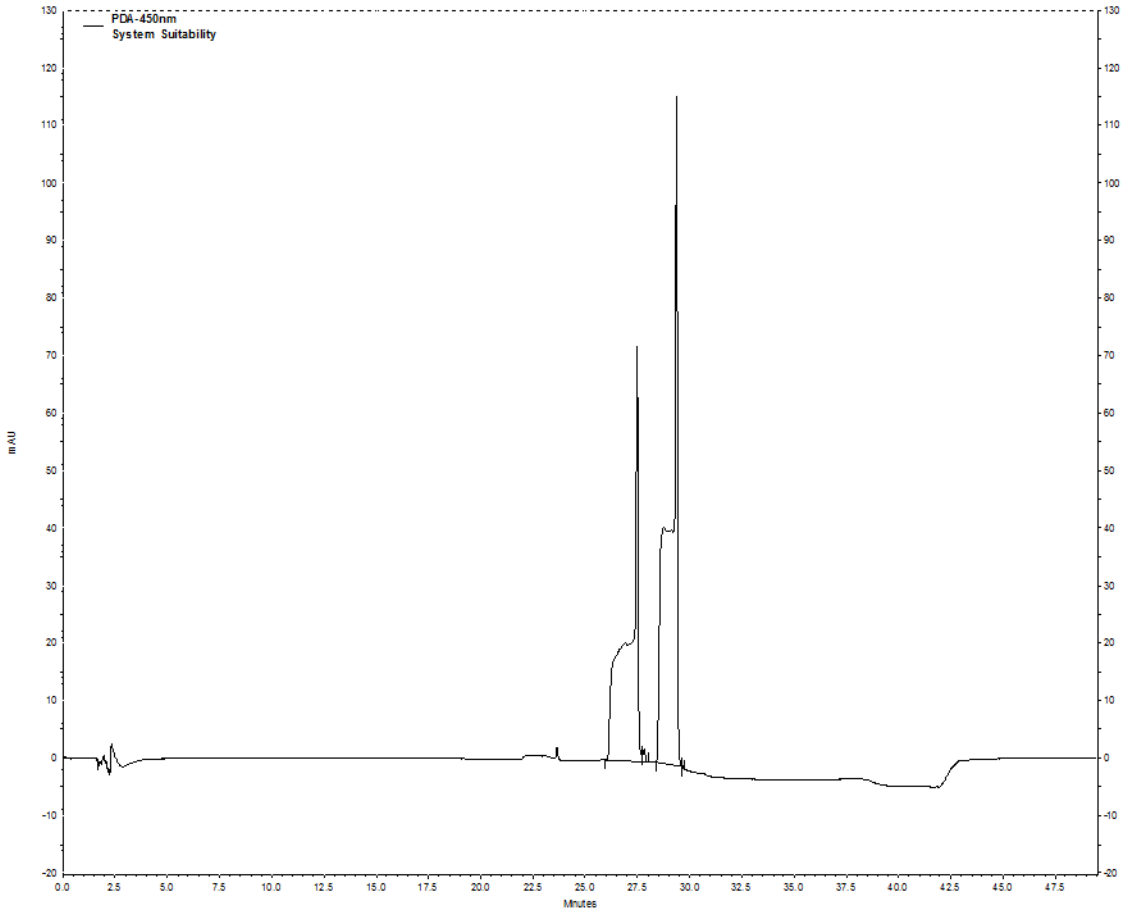


Figure 2: Chromatogram of chlorophylls *a* and *b* using a mixing in the loop pre-treatment method.

Table 2

Mixing in the vial pre-treatment method.

Draw from sample	Draw 710 µl from current + 2 at 150 µl/s, 3 µl bubble volume and 2 mm needle height
Draw from sample	Draw 290 µl from current + 1 at 150 µl/s, 3 µl bubble volume and 2 mm needle height
Deposit liquid in sample	Deposit 1006 µl to current at 250 µl/s & 2 mm needle height
Mix at sample	Mix 900 µl in current at 100 µl/s and 250 µl/s for 10 cycles, 0.5 mm needle height
Wash needle	Wash needle at bottle with 100 µl

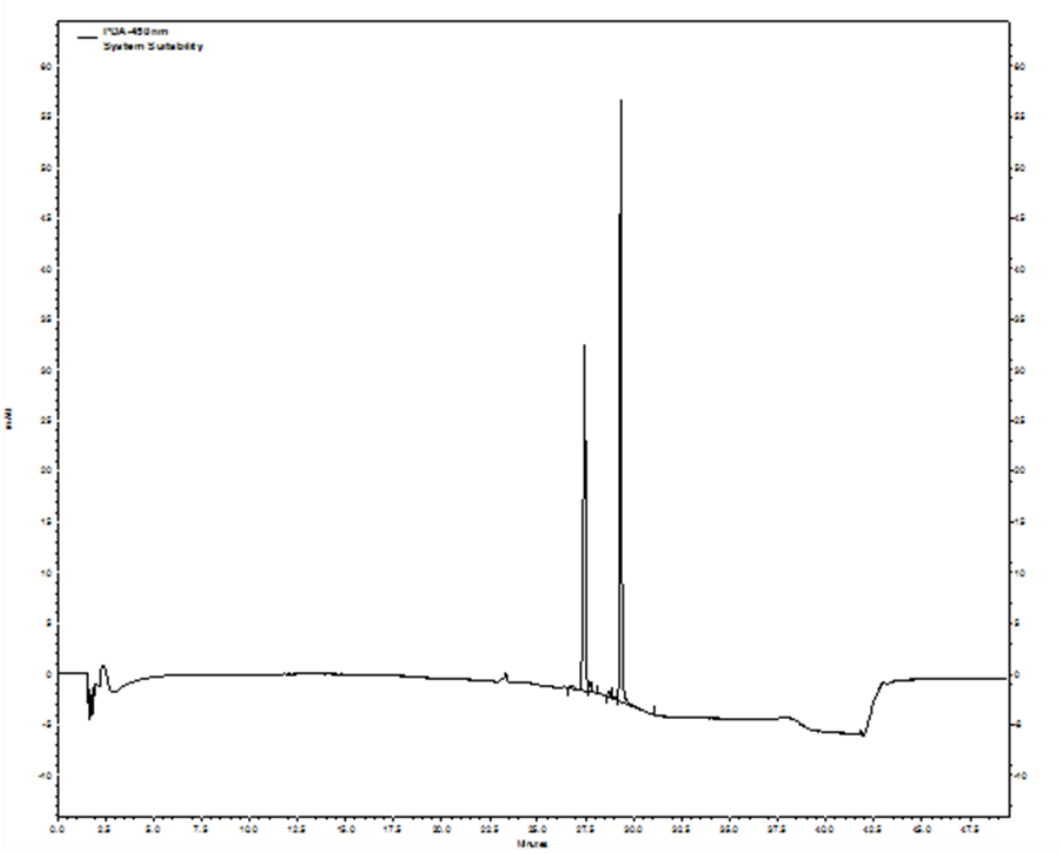


Figure 3: Chromatogram of chlorophylls *a* and *b* using a mixing in the vial pre-treatment method.

HPLC Mobile Phase and Gradient

Initially the mobile phase and gradient reported by DHI was used (NASA Technical Memorandum, 2012). DHI holds a Danish Accreditation and Metrology Fund (DANAK) accreditation for carrying out accredited measurements of pigment concentration in aquatic environments in accordance with International Organisation for Standardisation (ISO) 17025. This gradient is given in Table 3.

Table 3

DHI Gradient system where solvent A is methanol: 28mM TbAA pH 6.5 buffer (70 : 30 v/v) and solvent B is methanol (100%). The flow rate is 1.1 ml/min.

Time (minutes)	% A	% B
0	95	5
5	95	5
27	5	95
34	5	95
35	0	100
38	0	100
39.5	95	5

Initially each pigment was analysed individually by HPLC-DAD to determine the retention times using the pre-treatment method given in Table 2 and the HPLC gradient in Table 3. Each pigment standard solution was diluted with extraction solvent to give a pigment concentration of ~100 µg/l pigment. Gaussian shaped peaks were not observed for chlorophylls c_3 and c_2. Peak splitting of the chlorophyll c_2 peak was observed (Figures 4a and 4b).

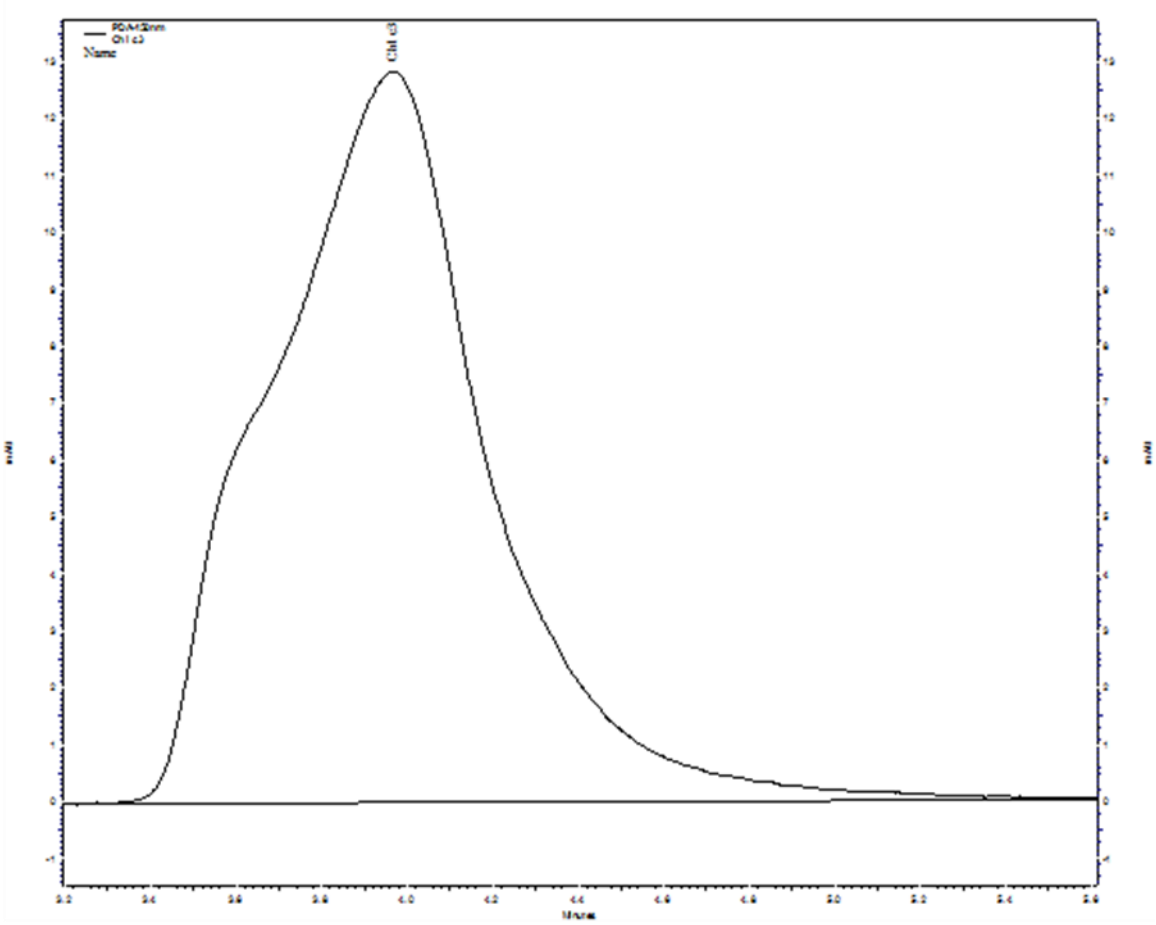


Figure 4a: Chromatogram of chlorophyll c_3 using the DHI gradient given in Table 3.

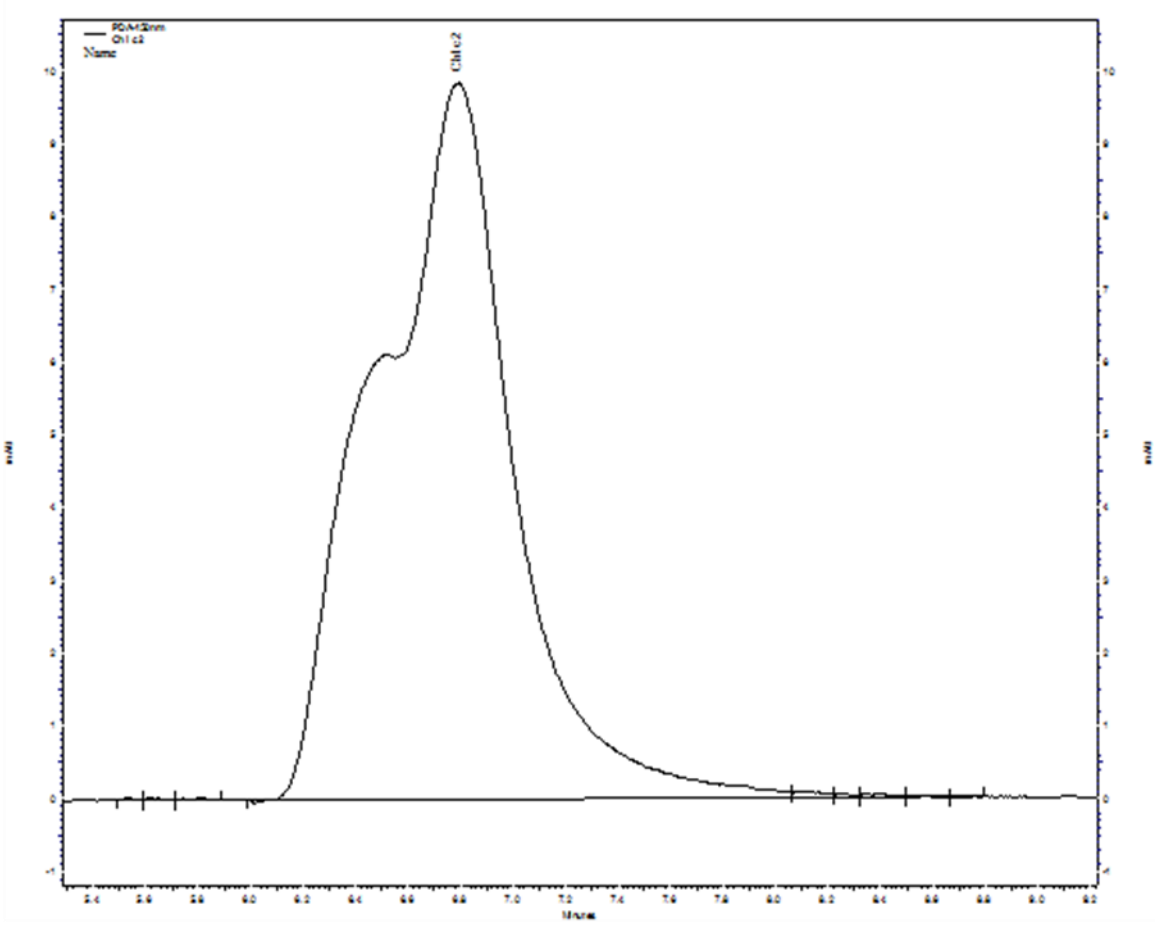


Figure 4b: Chromatogram of chlorophyll c_2 using the DHI gradient given in Table 3.

When the five minute hold time was removed the chromatography was improved for chlorophyll c_2 (Figure 5). The chlorophyll c_3 peak shape is still not ideal but the use of the spectral library will assist in confirming the identity of this pigment.

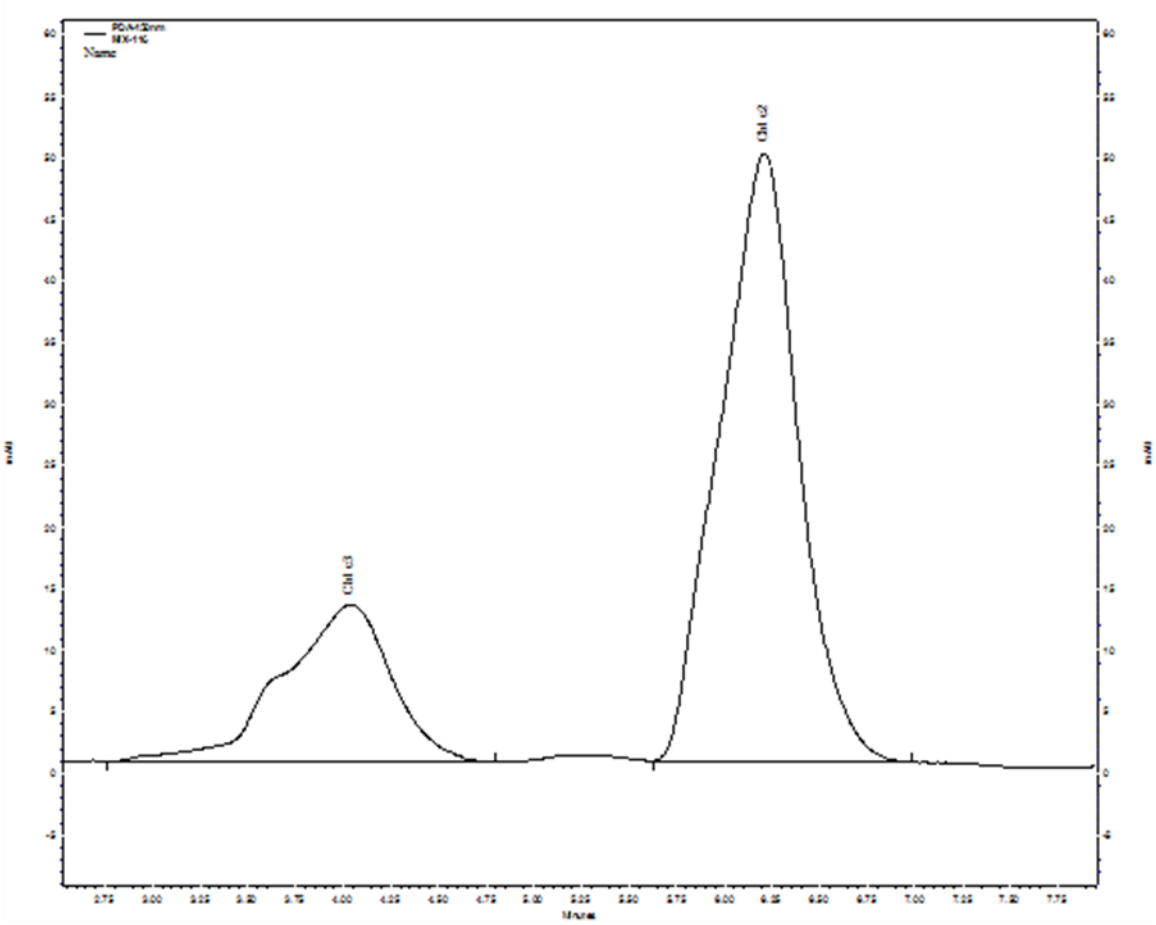


Figure 5: Chromatogram of chlorophylls c_3 and c_2 in DHI Mixed Pigment -116 using the gradient given in Table 4.

Carryover

Method blanks were analysed with each batch of samples to check for contamination of the extraction solvent and/or carryover between injections. Depending on where in the analytical run, the method blank was placed, carryover was observed at both 450 nm and 665 nm (Figures 6a and 6b).

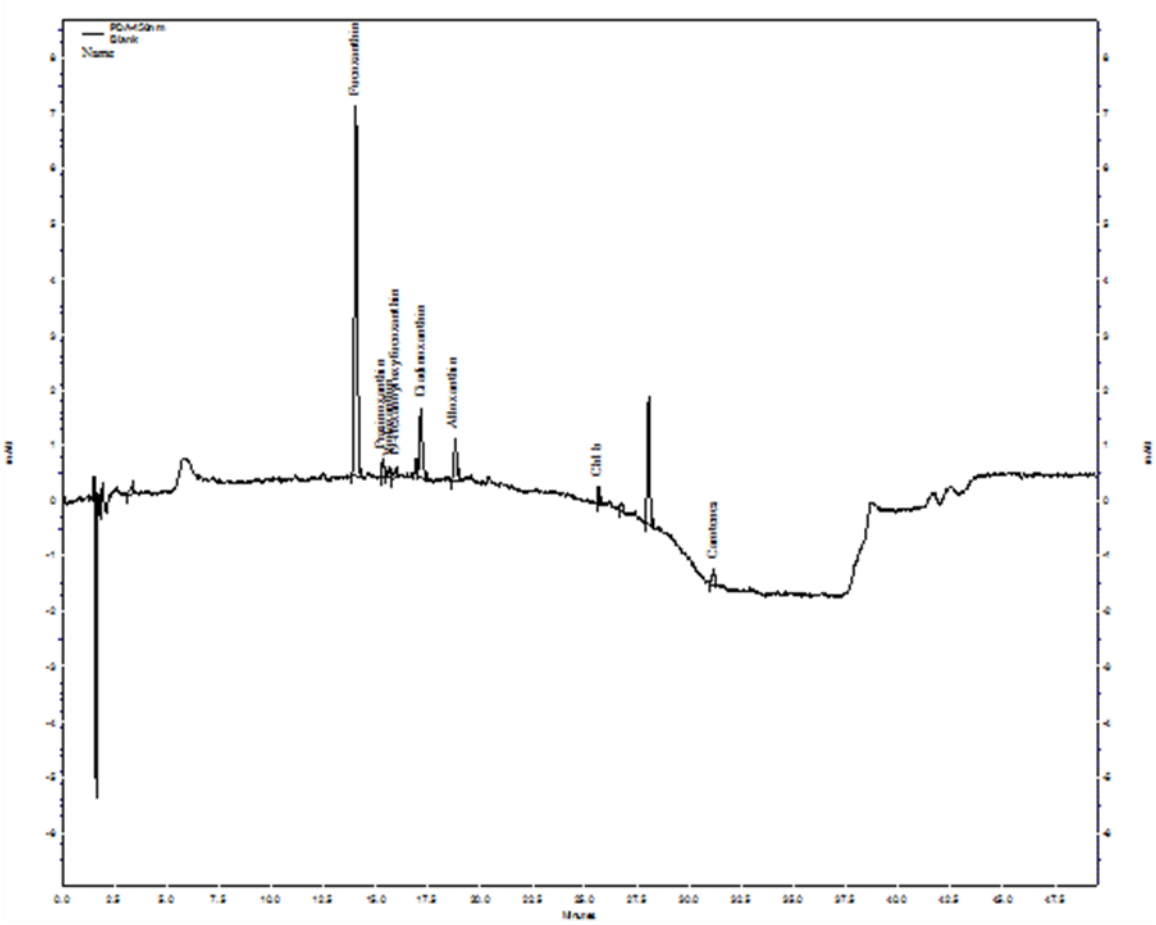


Figure 6a: Chromatogram of a procedural blank showing carryover at 450 nm when analysed immediately after a high concentration sample.

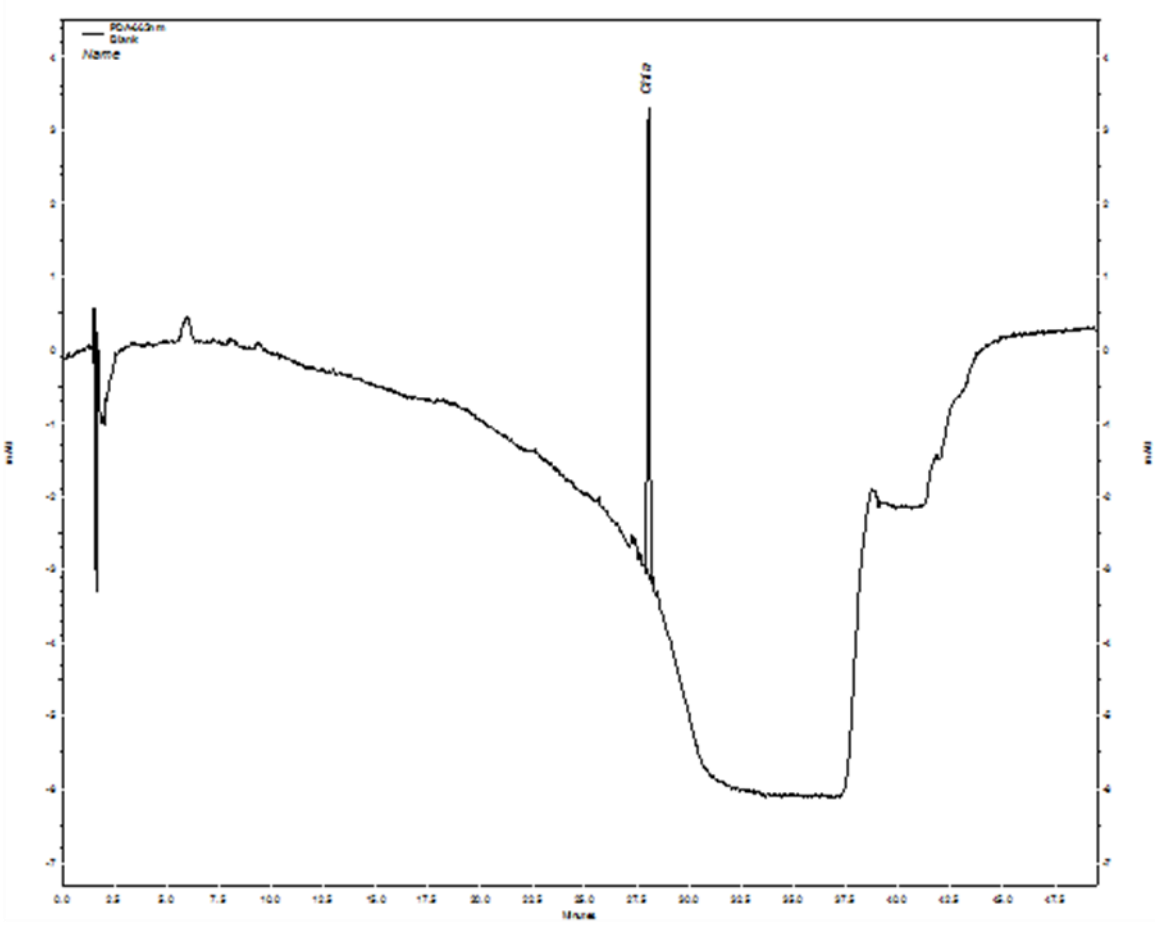


Figure 6b: Chromatogram of procedural blank showing carryover at 665 nm when analysed immediately after a high concentration sample.

Some of the methods reported in the fifth SeaWiFS HPLC analysis round-robin experiment (NASA Technical Memorandum, 2012) include an acetone rinse in the gradient to alleviate carryover. Although this was found to help, carryover was still present and was only eradicated when the volume of methanol used to flush the needle and the syringe was increased after each injection to 6 ml. The new gradient is given in table 4 and the new pre-treatment method given in Table 5.

Table 4

Gradient system where solvent A is methanol : 28 mM TbAA pH 6.5 buffer (70 : 30 v/v), solvent B is methanol (100%) and solvent C is acetone (100%). The flow rate is 1.1 ml/min.

Time (minutes)	% A	% B	% C
0	95	5	0
27	5	95	0
34	5	95	0
35	5	65	30
40	5	65	30
41.5	95	5	0
51.5	95	5	0

Table 5

Mixing in the vial pre-treatment method with extra flush.

Draw from sample	Draw 710 µl from current + 2 at 150 µl/s, 3 µl bubble volume and 2 mm needle height
Draw from sample	Draw 290 µl from current + 1 at 150 µl/s, 3 µl bubble volume and 2 mm needle height
Deposit liquid in sample	Deposit 1006 µl to current at 250 µl/s & 2 mm needle height
Mix at sample	Mix 900 µl in current at 100 µl/s and 250 µl/s for 10 cycles, 0.5 mm needle height
Flush to waste	Flush 2500 µl to bottle at 250 µl/s
Wash needle	Wash needle at bottle with 100 µl

Injection of a standard containing all of the pigments of interest was made using these chromatographic conditions and the retention times are in Table 6. All of the pigments are resolved with the exception of alpha - carotene and beta-carotene which co-elute. However, as these as not key marker pigments for CHEMTAX (Mackey *et al.,* 1996) analysis this was not thought to be an issue.

Table 6

Detection wavelengths and retention times of pigments of interest in mixed pigment standard.

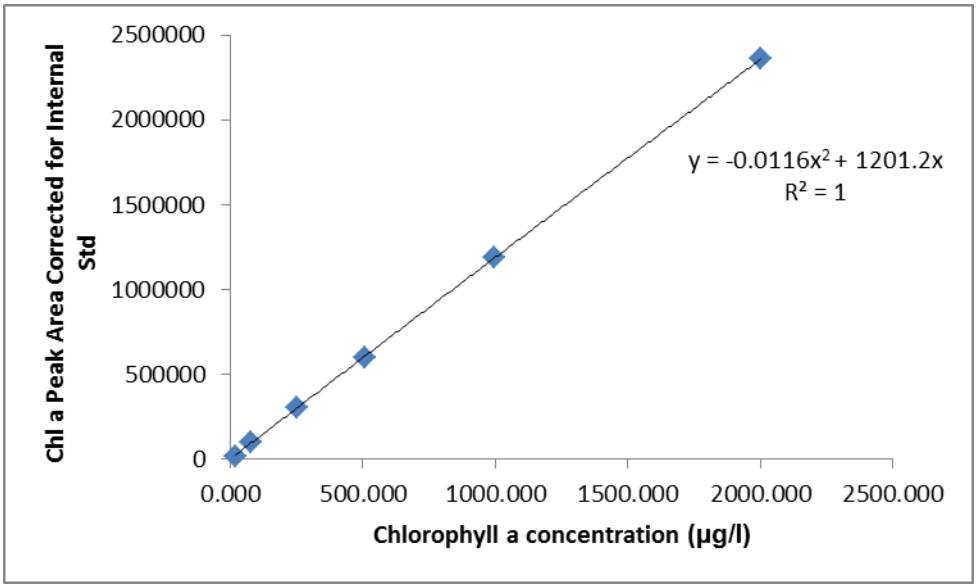
Pigment	Detection Wavelength (nm)	Retention time (minutes)
Chlorophyll c_3	450	3.39
Chlorophyll c_2	450	5.41
Chlorophyllide *a*	665	6.03
Peridinin	450	9.80
19-Butanoyloxyfucoxanthin	450	13.42
Fucoxanthin	450	13.74
Neoxanthin	450	14.29
Prasinoxanthin	450	15.03
Violaxanthin	450	15.31
19-Hexanoyloxyfucoxanthin	450	15.62
Diadinoxanthin	450	16.86
Antheraxanthin	450	17.72
Alloxanthin	450	18.43
Diatoxanthin	450	19.27
Zeaxanthin	450	20.08
Lutein	450	20.31
Gyroxanthin-diester	450	22.99
Chlorophyll *b*	450	25.41
Vitamin E acetate (internal std)	222	26.49
Divinyl chlorophyll *a*	665	27.57
Chlorophyll *a*	665	27.81
Carotenes	450	30.84

Use of an Internal Standard

As discussed above a ‘partial loop injection’ mode was used which is not as reproducible as a ‘full loop injection’ meaning that there is a need to use an internal standard to account for errors during the pre-treatment and injection steps. Recent guidelines (Roy *et al*., 2011) recommend that the extraction solvent and internal standard are combined together in a mixture prior to adding to the sample. A small volume of this mixture is added to the tube containing the filter paper and the pigments are extracted from the phytoplankton, clarified and injected onto the chromatograph.

Vitamin E acetate was used as the internal standard and was prepared at a concentration that is sufficiently high so that baseline drift does not interfere with accurate peak determinations. The wavelength at 222 nm is used exclusively to quantify vitamin E acetate (NASA Technical Memorandum, 2012). Chlorophyllide *a*, chlorophyll *a* and divinyl chlorophyll *a* were determined at 665 nm and all other pigments were determined at 450 nm.

Standard solutions containing chlorophyll *a* and chlorophyll *b* were prepared in 10 ml volumetric flasks, at concentrations of ~ 2000, 1000, 500, 250, 100 and 20 µg/l chlorophyll *a* and 500, 250, 100, 50, 20 and 5 µg/l chlorophyll *b* (standards 1 to 6 respectively). These solutions were used to establish linearity. A standard solution containing approximately 1800 µg/l chlorophyll *a* and 450 µg/l chlorophyll *b* (90% standard) was prepared to establish repeatability. All solutions also contained ~25 mg/l vitamin E acetate. Extraction solvent blanks were also injected onto the chromatograph throughout the run. Quadratic polynomial calibration curves were drawn and shown in Figure 7 and the repeatability results given in Table 7.



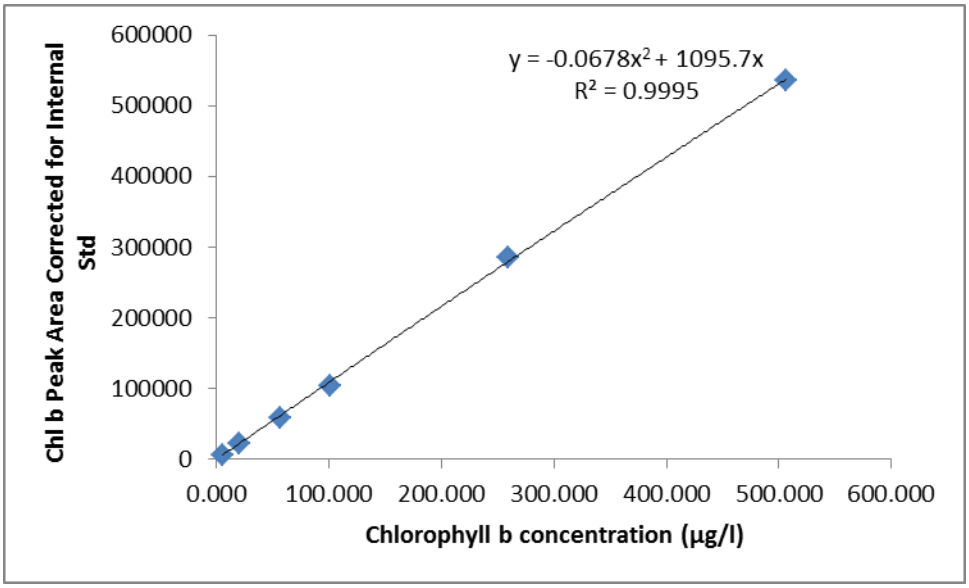


Figure 7: Quadratic polynomial curves for chlorophyll *a* (upper figure) and chlorophyll *b* (lower figure) when the internal standard is added to the 90% buffered acetone.

Table 7

Repeatability when vitamin E acetate is added to 90 % buffered acetone and used as an internal standard solution.

Replicate	Chl a Conc (µg/l)	Chl b Conc (µg/l)
1	1927	464
2	2069	497
3	2005	488
4	1819	428
5	1818	451
6	1966	481
Mean	**1955**	**469**
Stdev	**108**	**31**
%CV	**5.5**	**6.6**

The results demonstrate that vitamin E acetate can be used as an internal standard when it is added to the 90% buffered acetone, and then using this mixture as the extraction solvent.

Spectral Library

The DAD can confirm the identity of a pigment by searching the spectral library and the presence of co-eluting pigments can be assessed by evaluating the peak purity (similarity). In order to obtain a good spectral match, the spectral library must consist of spectra of pigment standards run at similar concentrations to that in the samples. The search can be performed on several spectral libraries simultaneously. Spectral libraries were set up for the pigments at all calibration levels to assist in accurate identification. The similarity of a peak's spectrum with that of the designated reference spectrum in the spectral library is used in addition to retention time as the basis for peak identification. The similarity is quantified through the calculation of a similarity index for each pigment at each calibration level. The similarity indices are used to generate a hit list of the ten best matching entries. A perfect match would have a similarity index of one but for conformational matches a similarity of at least 0.8 is required.

Method Validation

The following were investigated:

- Linearity;
- Method limit of detection;
- Standard and sample reproducibility (between batch);
- Bias/Recovery.

Linearity

A series of diluted standard solutions were prepared covering the range 20 µg/l to 2000 µg/l chlorophyll *a*; 10 µg/l to 1000 µg/l chlorophyll c_3, chlorophyll c_2, chlorophyllide *a*, peridinin, 19-butanoyloxyfucoxanthin, fucoxanthin, prasinoxanthin, 19-hexanoyloxyfucoxanthin, diadinoxanthin, divinyl chlorophyll *a* and the carotenes; and 5 µg/l to 500 µg/ l chlorophyll *b*, neoxanthin, violaxanthin, antheraxanthin, alloxanthin, diatoxanthin, zeaxanthin, lutein and gyroxanthin-diester. All solutions also contained ~25 mg/l vitamin E acetate. The peak areas of the pigments in the standard solutions were corrected for internal standard. For each pigment a second order polynomial curve was drawn using Microsoft Excel where the regression was forced through zero. The correlation coefficients were found to be >0.99 for all pigments.

Method Limit of Detection

Seawater collected from the Stonehaven (56º57.8'N, 02º06.2'W) monitoring site was used to prepare the low matrix samples. The seawater samples were collected in 2 x 10 litre carboys, which were protected from light by a black jacket, using a 10 m integrated tube sampler (Lund sampler). The seawater for the low matrix samples was collected at the end of January 2015 when pigment concentrations are low due to limited amount of light and low temperatures. The seawater was filtered within two hours of return to the laboratory. Prior to sub sampling, the 10 litre carboys were gently agitated to re-suspend any large cells that may have settled. Two litre subsamples were filtered through a Whatman GF/F 47 mm filter paper, using a low vacuum of approximately 0.5 atm to avoid damaging the cells. The filter paper was removed from the fritted base using tweezers, placed on a piece of aluminium foil, folded and placed inside a labelled Nunc cryovial. The samples were frozen immediately at -80ºC. The samples were extracted as described in the experimental section on each of eight days. The results are given in Table 8. The LoD was calculated by multiplying the standard

deviation of the eight replicates by 4.65 (Water Research Centre, UK, 1989). The pigments chlorophyll c_3, 19-hexanoyloxyfucoxanthin, antheraxanthin and gyroxanthin-diester were not detected in the low matrix samples. The LoD for these pigments was calculated using the concentration of the lowest calibration standard. The validation of the additional pigments, chlorophyllide *a* and divinyl chlorophyll *a* was carried out during summer 2016, when pigment levels are high, so again the LoD for these pigments was calculated using the concentration of the lowest calibration standard. The Clean Seas Environment Monitoring Programme, Green Book (2012) states a target for LoD of 0.1 µg/l for chlorophyll *a*; target LoDs are not given for any of the other pigments.

The calculated LoD for chlorophyll *a* was 0.21 µg/l, which is higher than the target. This was due to the chlorophyll *a* concentration of the seawater and the precision of the method. The chlorophyll *a* concentrations in January are at their lowest in the annual cycle, and it is not possible to collect samples with an even lower concentration. The LoD was accepted as this was based on a real sample. The LoD set by the CSEMP is not based on an HPLC method and is at the time of writing is due for review.

Table 8

Concentration data of the pigments of interest in a seawater sample collected during January 2015, to establish the method limit of detection (LOD). The LOD was calculated as 4.65 x Stdev

* The LOD for these pigments was calculated using the concentration of the lowest calibration standard.

Replicate	Conc Chl c3* (µg/l)	Conc Chl c2 (µg/l)	Conc Chlida* (µg/l)	Conc Peridinin (µg/l)	Conc 19- But (µg/l)	Conc Fucoxanthin (µg/l)	Conc Neoxanthin (µg/l)	Conc Prasinoxanthin (µg/l)	Conc Violaxanthin (µg/l)	Conc 19-Hex* (µg/l)	Conc Diadinoxanthin (µg/l)
1	-	0.04		0.01	0.003	0.16	0.004	0.01	0.002	-	0.01
2	-	0.01		0.01	0.003	0.08	0.003	0.01	0.001	-	0.01
3	-	0.01		0.01	0.003	0.08	0.003	0.01	0.003	-	0.01
4	-	0.01		0.01	0.002	0.09	-	-	0.001	-	0.01
5	-	0.02		0.01	0.003	0.09	0.004	0.01	0.002	-	0.01
6	-	0.01		0.01	0.003	0.09	0.003	0.01	0.002	0.01	0.01
7	-	0.01		0.01	0.004	0.10	0.002	-	0.001	-	0.01
8	-	0.02		0.01	0.003	0.08	0.003	0.01	0.002	-	0.01
Mean		**0.02**		**0.01**	**0.003**	**0.10**	**0.003**	**0.01**	**0.002**	**0.01**	**0.01**
Stdev		**0.01**		**0.002**	**0.0004**	**0.03**	**0.001**	**0.001**	**0.001**		**0.002**
%CV		**55.3**		**22.5**	**12.4**	**26.3**	**24.1**	**13.3**	**35.3**		**25.2**
LOD	***0.03***	***0.04***	***0.03***	***0.01***	***0.002***	***0.12***	***0.003***	***0.01***	***0.003***	***0.04***	***0.01***

Replicate	Conc Antheraxanthin* (µg/l)	Conc Alloxanthin (µg/l)	Conc Diatoxanthin (µg/l)	Conc Zeaxanthin (µg/l)	Conc Lutein (µg/l)	Conc Gyroxanthin diester* (µg/l)	Conc Chl b (µg/l)	Conc DVA* (µg/l)	Conc Chl a (µg/l)	Conc carotenes (µg/l)
1	-	0.01	0.005	0.002	0.001	-	0.02		0.25	0.006
2	-	0.01	0.001	0.001	-	-	0.02		0.16	0.004
3	-	0.01	-	0.002	0.001	-	0.02		0.15	0.005
4	-	0.01	-	-	-	-	0.01		0.15	0.005
5	-	0.01	0.001	-	-	-	0.02		0.18	0.004
6	-	0.01	0.002	0.002	0.002	-	0.03		0.27	0.007
7	-	0.01	-	-	-	-	0.02		0.17	0.005
8	-	0.01	0.001	-	-	-	0.02		0.17	-
Mean		**0.01**	**0.002**	**0.002**	**0.001**		**0.02**		**0.19**	**0.01**
Stdev		**0.001**	**0.002**	**0.0003**	**0.0003**		**0.004**		**0.05**	**0.001**
%CV		**17.8**	**72.7**	**18.0**	**23.3**		**20.9**		**24.3**	**19.6**
LOD	***0.01***	***0.01***	***0.01***	***0.001***	***0.001***	***0.01***	***0.02***	***0.04***	***0.21***	***0.005***

Standard and Sample Reproducibility

DHI Water & Environment, Denmark supply mixed pigment standard solutions with an accompanying total chlorophyll *a* concentration. The total chlorophyll *a* concentration is determined by spectrophotometer using the trichromatic equation (Jeffrey *et al*., 1997). The concentration includes all chlorophylls absorbing at 665 nm (chlorophyll *a* + divinyl chlorophyll *a* + chlorophyllide *a*). The concentration is not accurate and the mixed pigment standard solutions cannot be used for calibration, but they can be used for identifying peaks, determining elution order and documenting the precision of the HPLC. Two different types are available. PPS-MIX-1 is suitable for analysing common pigments present in phytoplankton samples. The total chlorophyll *a* concentration is between 3-6 µg/ml. PPS-MIX-2 is especially suitable for analysing samples with pigments present at concentrations near the detection limit. The total chlorophyll *a* concentration is between 0.1-0.2 µg/ml. PPS-MIX-1 and PPS-MIX-2 contained all of the pigments of interest with the exception of antheraxanthin and gyroxanthin-diester. Solutions of PPS-MIX-1 and PPS-MIX-2 containing antheraxanthin and gyroxanthin diester were prepared in 2 ml volumetric flasks. All solutions also contained ~25 mg/l vitamin E acetate. These solutions were known as 'High Std' and 'Low Std'.

The calculated concentrations of chlorophyll *a* and chlorophyll *b* in the 'High Std' were out with the calibration range (0 - 1996.5 µg/l chlorophyll *a* and 0 – 505.2 µg/l chlorophyll *b*). A dilution of the 'High Std' was made so that chlorophyll *a* and chlorophyll *b* were within the calibration range. Each of these solutions were analysed per day for seven days.

Samples were prepared using seawater collected from Stonehaven monitoring site in June 2015 and in July 2016 for chlorophyllide *a* and divinyl chlorophyll *a* validation. Pigment concentrations are higher in the summer months when light and temperature are more favourable for growth. These were prepared in the same manner as the method limit of detection samples. One sample was extracted and analysed per day for seven days.

A laboratory reference material (LRM) was prepared as outlined in the Calibration and Quality Control section. One LRM was extracted and analysed per day for seven days.

The mean, standard deviation and CV% is given in Tables 9a to 9e.

Table 9a

Concentration data of the pigments of interest in PPS-MIX-1 to establish reproducibility of a high standard. Batch MIX-119 spiked with CHLIDA-126 was used for chlorophyllide *a* and divinyl chlorophyll *a*. Batch MIX-116 was used for the other pigments.

Replicate	Conc Chl c3 (µg/l)	Conc Chl c2 (µg/l)	Conc Chlida (µg/l)	Conc Peridinin (µg/l)	Conc 19-But (µg/l)	Conc Fuco (µg/l)	Conc Neoxanthin (µg/l)	Conc Prasinoxanthin (µg/l)	Conc Violaxanthin (µg/l)	Conc 19-Hex (µg/l)	Conc Diadinoxanthin (µg/l)
1	86.6	211.6	90.3	197.8	58.5	162.4	178.5	198.8	162.5	178.4	94.9
2	86.2	232.0	100.3	195.2	58.0	182.9	174.4	197.7	160.5	177.8	95.0
3	133.7	279.4	70.4	250.5	70.1	219.1	200.7	243.1	200.0	218.3	114.4
4	93.9	237.1	86.9	231.2	67.1	192.3	193.6	230.3	186.6	208.5	105.7
5	101.1	241.7	98.8	229.9	65.6	185.2	186.4	224.5	184.0	202.1	104.4
6	84.9	218.9	24.6	208.3	60.9	168.8	164.9	206.5	167.5	186.2	98.2
7	127.7	287.8	89.6	270.2	72.1	198.8	208.2	246.2	213.6	217.6	116.0
8			42.6								
Mean	**102.0**	**244.1**	**75.4**	**226.2**	**64.6**	**187.1**	**186.7**	**221.0**	**182.1**	**198.4**	**104.1**
Stdev	**20.4**	**29.0**	**27.8**	**27.9**	**5.6**	**19.0**	**15.3**	**20.3**	**20.0**	**17.6**	**8.7**
%CV	**20.0**	**11.9**	**36.8**	**12.3**	**8.6**	**10.1**	**8.2**	**9.2**	**11.0**	**8.9**	**8.3**

Replicate	Conc Antheraxanthin (µg/l)	Conc Alloxanthin (µg/l)	Conc Diatoxanthin (µg/l)	Conc Zeaxanthin (µg/l)	Conc Lutein (µg/l)	Conc Gyroxanthin diester (µg/l)	Conc Chl b (µg/l)	Conc DVA (µg/l)	Conc Chl a (µg/l)	Conc carotenes (µg/l)
1	62.4	83.6	68.7	64.3	137.3	41.6	895.0	466.3	1820.6	33.6
2	61.3	82.9	71.5	64.3	138.9	41.0	897.6	510.0	1823.7	34.4
3	68.4	103.3	94.7	77.6	172.1	52.2	1142.4	407.4	2488.5	43.0
4	64.5	96.0	89.2	73.0	164.4	42.8	987.4	472.6	1996.1	35.8
5	63.5	95.2	77.7	74.3	163.2	43.8	1018.3	559.2	2111.5	39.0
6	63.1	87.4	82.5	67.3	147.3	39.2	931.7	536.4	1891.4	34.2
7	74.1	106.7	99.6	82.7	194.0	50.9	1101.1	470.4	2453.0	45.5
8								532.6		
Mean	**65.3**	**93.6**	**83.4**	**71.9**	**159.6**	**44.5**	**996.2**	**494.4**	**2083.6**	**37.9**
Stdev	**4.5**	**9.3**	**11.6**	**7.0**	**20.2**	**5.0**	**97.5**	**49.4**	**283.6**	**4.7**
%CV	**6.8**	**10.0**	**13.9**	**9.7**	**12.7**	**11.3**	**9.8**	**10.0**	**13.6**	**12.5**

Table 9b

Concentration data of the pigments of interest in diluted PPS-MIX-1 (MIX-116), to establish reproducibility of a high standard.

Replicate	Conc Chl c3 (µg/l)	Conc Chl c2 (µg/l)	Conc Peridinin (µg/l)	Conc 19- But (µg/l)	Conc Fucoxanthin (µg/l)	Conc Neoxanthin (µg/l)	Conc Prasinoxanthin (µg/l)	Conc Violaxanthin (µg/l)	Conc 19-Hex (µg/l)	Conc Diadinoxanthin (µg/l)
1	25.3	69.8	77.0	22.3	62.2	54.8	74.3	58.2	67.8	35.0
2	29.0	78.5	86.1	24.5	67.1	60.4	81.0	63.7	73.7	38.9
3	22.5	69.9	74.8	22.2	77.6	53.1	78.7	56.7	65.9	35.5
4	22.9	62.9	70.8	21.6	58.7	50.9	76.0	55.4	64.0	33.7
5	21.2	61.6	70.5	20.8	56.9	52.0	71.5	52.2	63.1	32.4
6	24.0	63.7	74.5	20.7	56.8	50.3	67.4	52.5	62.2	31.8
7	23.9	67.5	76.3	21.7	58.1	50.6	69.8	53.9	63.6	32.9
Mean	**24.1**	**67.7**	**75.7**	**22.0**	**62.5**	**53.2**	**74.1**	**56.1**	**65.8**	**34.3**
Stdev	**2.5**	**5.8**	**5.2**	**1.3**	**7.6**	**3.6**	**4.9**	**4.0**	**4.0**	**2.4**
%CV	**10.4**	**8.5**	**6.9**	**5.9**	**12.2**	**6.7**	**6.6**	**7.1**	**6.0**	**7.1**

Replicate	Conc Antheraxanthin (µg/l)	Conc Alloxanthin (µg/l)	Conc Diatoxanthin (µg/l)	Conc Zeaxanthin (µg/l)	Conc Lutein (µg/l)	Conc Gyroxanthin diester (µg/l)	Conc Chl b (µg/l)	Conc Chl a (µg/l)	Conc carotenes (µg/l)
1	18.6	28.8	27.1	23.7	51.5	14.1	345.9	716.6	11.1
2	22.5	33.7	31.2	27.2	55.5	16.3	398.8	872.5	14.3
3	18.8	30.4	27.2	24.3	49.9	13.5	347.7	702.2	10.7
4	18.9	28.0	25.6	23.1	47.4	12.9	322.0	651.4	9.6
5	19.8	27.8	25.4	19.9	45.3	12.7	319.3	669.3	10.0
6	16.9	27.0	24.3	22.8	46.4	14.1	341.3	759.7	12.0
7	17.9	28.3	26.3	23.9	46.3	13.6	338.7	719.4	11.1
Mean	**19.1**	**29.1**	**26.7**	**23.6**	**48.9**	**13.9**	**344.8**	**727.3**	**11.3**
Stdev	**1.8**	**2.3**	**2.2**	**2.1**	**3.6**	**1.2**	**26.3**	**73.1**	**1.6**
%CV	**9.3**	**7.8**	**8.2**	**9.1**	**7.4**	**8.7**	**7.6**	**10.1**	**13.8**

Table 9c

Concentration data of the pigments of interest in diluted PPS-MIX-2 (MIX-103), to establish reproducibility of a low standard.

Replicate	Conc Chl c3 (µg/l)	Conc Chl c2 (µg/l)	Conc Chlida (µg/l)	Conc Peridinin (µg/l)	Conc 19- But (µg/l)	Conc Fucoxanthin (µg/l)	Conc Neoxanthin (µg/l)	Conc Prasinoxanthin (µg/l)	Conc Violaxanthin (µg/l)	Conc 19-Hex (µg/l)	Conc Diadinoxanthin (µg/l)
1	7.2	8.1	10.3	18.4	11.4	15.7	14.2	5.9	11.5	4.3	5.9
2	6.7	10.0	8.5	20.7	12.4	16.4	14.2	6.7	12.4	4.9	6.3
3	8.4	9.5	8.8	19.7	11.8	15.3	13.6	6.3	11.6	4.5	5.4
4	9.2	13.4	7.9	20.0	12.0	21.1	13.9	6.3	11.6	4.5	5.8
5	13.0	7.9	8.5	20.9	12.3	16.1	14.2	6.8	12.3	5.0	6.1
6	9.1	12.1	7.3	22.8	13.1	18.6	15.8	9.2	13.5	6.9	7.6
7	8.6	7.8	6.9	19.0	11.6	14.8	12.9	6.2	11.1	4.2	5.5
8											
Mean	**8.9**	**9.8**	**8.3**	**20.2**	**12.1**	**16.9**	**14.1**	**6.8**	**12.0**	**4.9**	**6.1**
Stdev	**2.0**	**2.2**	**1.1**	**1.4**	**0.6**	**2.2**	**0.9**	**1.1**	**0.8**	**0.9**	**0.7**
%CV	**23.0**	**22.1**	**13.4**	**7.1**	**4.6**	**13.2**	**6.4**	**16.5**	**6.6**	**19.2**	**12.3**

Replicate	Conc Antheraxanthin (µg/l)	Conc Alloxanthin (µg/l)	Conc Diatoxanthin (µg/l)	Conc Zeaxanthin (µg/l)	Conc Lutein (µg/l)	Conc Gyroxanthin diester (µg/l)	Conc Chl b (µg/l)	Conc DVA (µg/l)	Conc Chl a (µg/l)	Conc carotenes (µg/l)
1	3.2	12.0	5.3	11.9	6.0	3.8	25.1	40.4	84.6	1.6
2	3.7	13.0	4.6	13.1	7.0	4.9	29.5	32.5	94.5	1.9
3	3.5	12.2	5.2	12.3	6.7	4.7	28.2	34.1	88.1	1.9
4	3.3	12.5	6.5	12.3	7.4	4.6	36.4	26.9	111.3	1.8
5	3.4	12.8	5.5	13.1	7.1	4.5	32.7	38.3	96.8	1.4
6	4.1	13.6	6.7	13.3	8.3	4.6	38.2	30.7	119.9	1.9
7	3.1	11.7	5.2	12.2	6.2	3.9	25.5	29.0	90.2	1.7
Mean	**3.5**	**12.5**	**5.6**	**12.6**	**7.0**	**4.4**	**30.8**	**33.1**	**97.9**	**1.8**
Stdev	**0.3**	**0.6**	**0.8**	**0.5**	**0.8**	**0.4**	**5.1**	**4.9**	**13.0**	**0.2**
%CV	**9.4**	**5.1**	**13.7**	**4.3**	**11.2**	**9.0**	**16.7**	**14.7**	**13.2**	**10.2**

Table 9d

Concentration data of the pigments of interest in a seawater sample collected in June 2015, to establish reproducibility of a sample. Seawater sample collected in July 2016 was used to determine the concentration of chlorophyllide *a* and divinyl chlorophyll *a*.

Replicate	Conc Chl c3 (µg/l)	Conc Chl c2 (µg/l)	Conc Chlida (µg/l)	Conc Peridinin (µg/l)	Conc 19- But (µg/l)	Conc Fucoxanthin (µg/l)	Conc Neoxanthin (µg/l)	Conc Prasinoxanthin (µg/l)	Conc Violaxanthin (µg/l)	Conc 19-Hex (µg/l)	Conc Diadinoxanthin (µg/l)
1	-	0.30	0.15	0.02	0.00	0.67	0.01	0.01	0.01	0.02	0.13
2	-	-	0.11	0.03	0.00	1.08	0.02	-	0.02	0.03	0.20
3	-	0.38	0.11	0.03	0.00	0.79	0.01	-	0.01	0.02	0.14
4	-	0.39	0.06	0.03	-	0.81	0.02	-	0.01	-	0.15
5	-	0.35	0.14	0.02	-	0.77	0.01	-	0.01	-	0.14
6	-	0.50	0.17	0.03	0.00	1.07	0.02	-	0.02	-	0.21
7	-	0.36	0.15	0.02	0.00	0.72	0.02	-	0.01	-	0.14
8			0.04								
Mean		**0.38**	**0.12**	**0.03**	**0.00**	**0.85**	**0.02**		**0.01**	**0.02**	**0.16**
Stdev		**0.06**	**0.05**	**0.01**	**0.00**	**0.16**	**0.00**		**0.00**	**0.00**	**0.03**
%CV		**17.1**	**39.0**	**19.8**	**37.2**	**19.5**	**13.2**		**15.2**	**20.2**	**20.5**

Replicate	Conc Antheraxanthin (µg/l)	Conc Alloxanthin (µg/l)	Conc Diatoxanthin (µg/l)	Conc Zeaxanthin (µg/l)	Conc Lutein (µg/l)	Conc Gyroxanthin diester (µg/l)	Conc Chl b (µg/l)	Conc DVA (µg/l)	Conc Chl a (µg/l)	Conc carotenes (µg/l)
1	-	0.03	-	-	-	-	0.08	-	1.30	0.03
2	0.01	0.04	-	-	-	-	0.09	-	1.72	0.04
3	0.00	0.03	-	-	-	-	0.08	-	1.30	0.02
4	0.00	0.03	-	-	-	-	0.07	-	1.16	0.03
5	0.00	0.03	-	-	-	-	0.11	-	1.63	0.03
6	0.00	0.04	-	-	-	-	0.11	-	2.00	0.04
7	0.00	0.03	0.01	0.00	0.00	-	0.07	-	1.12	0.02
Mean	**0.00**	**0.03**					**0.09**		**1.46**	**0.03**
Stdev	**0.00**	**0.00**					**0.02**		**0.33**	**0.01**
%CV	**57.9**	**14.4**					**19.3**		**22.4**	**26.2**

Table 9e

Concentration data of the pigments of interest in a seawater sample collected in a LRM, to establish reproducibility of a sample.

Replicate	Conc Chl c3 (µg/l)	Conc Chl c2 (µg/l)	Conc Peridinin (µg/l)	Conc 19- But (µg/l)	Conc Fucoxanthin (µg/l)	Conc Neoxanthin (µg/l)	Conc Prasinoxanthin (µg/l)	Conc Violaxanthin (µg/l)	Conc 19-Hex (µg/l)	Conc Diadinoxanthin (µg/l)
1	0.01	1.28	-	0.04	3.24	0.20	0.05	0.02	0.03	-
2	0.02	1.39	-	0.05	3.48	0.23	0.07	0.03	0.05	-
3	0.01	1.32	-	0.05	3.65	0.21	0.07	0.03	0.05	-
4	0.01	1.60	-	0.05	3.60	0.21	0.07	0.03	0.06	-
5	0.01	1.57	-	0.06	3.69	0.23	0.08	0.03	0.06	-
6	0.01	1.45	-	0.06	3.80	0.22	0.07	0.03	0.06	-
7	0.01	1.72	-	0.06	3.97	0.25	0.08	0.05	-	-
Mean	**0.01**	**1.48**		**0.05**	**3.63**	**0.22**	**0.07**	**0.03**	**0.05**	
Stdev	**0.004**	**0.16**		**0.01**	**0.23**	**0.02**	**0.01**	**0.01**	**0.01**	
%CV	**36.1**	**10.8**		**11.4**	**6.4**	**7.3**	**15.9**	**27.7**	**23.2**	

Replicate	Conc Antheraxanthin (µg/l)	Conc Alloxanthin (µg/l)	Conc Diatoxanthin (µg/l)	Conc Zeaxanthin (µg/l)	Conc Lutein (µg/l)	Conc Gyroxanthin diester (µg/l)	Conc Chl b (µg/l)	Conc Chl a (µg/l)	Conc carotenes (µg/l)
1	0.04	0.02	0.55	0.09	0.43	-	1.22	2.57	0.28
2	0.05	0.03	0.59	0.11	0.45	-	1.38	3.10	0.30
3	0.04	0.03	0.67	0.13	0.50	-	1.44	3.04	0.38
4	0.04	0.03	0.65	0.12	0.50	-	1.49	3.09	0.37
5	0.04	0.03	0.68	0.13	0.52	-	1.57	3.65	0.31
6	0.04	0.04	0.72	0.14	0.53	-	1.84	4.95	0.49
7	0.05	0.04	0.74	0.16	0.51	-	1.69	3.66	0.27
Mean	**0.04**	**0.03**	**0.66**	**0.12**	**0.49**		**1.52**	**3.44**	**0.34**
Stdev	**0.01**	**0.01**	**0.07**	**0.02**	**0.04**		**0.20**	**0.77**	**0.08**
%CV	**13.9**	**23.7**	**10.3**	**16.5**	**8.0**		**13.3**	**22.4**	**22.2**

Most of the pigments , including chlorophyll *a* and chlorophyll *b,* meet The Clean Seas Environment Monitoring Programme, Green Book (2012) target for precision of 25%. The exceptions are chlorophyllide *a* in the high standard (Table 9a), chlorophyllide *a*, 19 – butanoyloxyfucoxanthin, antheraxanthin and carotenes in the seawater sample (Table 9d), chlorophyll c_3 and violaxathin in the LRM (Table 9e). With the exception of chlorophyllide *a*, the variation is a result of the small amounts present in the samples. A Gaussian shaped peak was not obtained for chlorophyllide *a*, but a more complex peak containing chlorophyllide *a* and chlorophyllide *a* allomers. This was consistent with the certificate of analysis for the chlorophyllide *a* standard (see Appendix 1). The chlorophyllide *a* peak was spilt into its various components and each peak checked for a spectral match by comparing the absorption spectra with those held in the spectral libraries.

Bias/Recovery

Seven samples from QUASIMEME round 2014.2 were purchased for the determination of bias/recovery. QUASIMEME only provide assigned values for chlorophyll *a* and chlorophyll *b*. The other pigments are out with the scope of the QUASIMEME Laboratory Performance Studies. The samples were extracted and analysed as described in the experimental section on each of seven days and the % recovery calculated for chlorophyll *a* and chlorophyll *b*. The results are given in Table 10.

Table 10

Bias data for QUASIMEME Sample 2014-2.

Replicate	Chl a conc (µg/l) M3150	Assigned value Chl a	% Recovery Chl a	z-score Chl a
1	3.819	3.989	95.7	-0.3
2	3.587	3.989	89.9	-0.8
3	2.881	3.989	72.2	-2.1
4	3.921	3.989	98.3	-0.1
5	4.106	3.989	102.9	0.2
6	4.883	3.989	122.4	1.7
7	4.030	3.989	101.0	0.1
Mean	**3.9**		**97.5**	**-0.2**
Stdev	**0.6**		**15.1**	**1.1**
%CV	**15.5**		**15.5**	

Replicate	Chl b conc (µg/l) M3150	Assigned value Chl b	% Recovery Chl b	z-score Chl b
1	0.129	0.142	90.7	-0.6
2	0.107	0.142	75.1	-1.6
3	0.096	0.142	67.4	-2.0
4	0.126	0.142	89.0	-0.7
5	0.141	0.142	99.6	0.0
6	0.162	0.142	114.0	0.9
7	0.106	0.142	74.9	-1.6
Mean	**0.124**		**87.2**	**-0.8**
Stdev	**0.023**		**16.2**	**1.0**
%CV	**18.6**		**18.6**	

The % recovery was calculated to be 97.5% for chlorophyll *a* and 87.2% for chlorophyll *b*. This meets the limits of 70 -110% as set by the National Marine Chemistry Advisory Group (The National Marine Chemistry Advisory Group is a subgroup of UK Clean and Safe Seas Evidence Group (CSSEG) to cover quality assurance and quality control needs). The % CV for chlorophyll *a* was 15.5%, and % CV for chlorophyll *b* was 18.6%. Z scores were calculated, and most were found to be satisfactory; $-2 < z < 2$, with one chlorophyll *a* z score being questionable; $-3 < z < -2$. Bias/recovery could not be readily determined for the other pigments as there is no certified reference material available and external quality assurance

samples only have data for chlorophylls *a*, *b* and *c*, it would be possible to prepare spiked samples containing the pigments, however the cost is prohibitive.

Comparison between MSS HPLC Methods

This new method has many advantages over the HPLC-UV method developed at MSS for pigment analysis;

- Use of the diode array detector greatly facilitates the identification of pigments by comparing the spectrum of the pigment with that of a known standard. Using a UV detector identification is based on retention time alone.

- The pigments lutein and zeaxanthin, and 19-hexanoyloxyfucoxanthin and prasinoxanthin are resolved using a C8 column.

- A pre-treatment method has been developed where the samples and the buffer can be held in the autosampler tray at 4°C.

- Vitamin E acetate has been successfully used as an internal standard when it is combined with the extraction solvent. This allows for errors to be accounted for during the pre-treatment and injection steps.

- All of the pigments of interest are quantified. With the HPLC-UV method only chlorophylls *a* and *b* were quantified.

Conclusions

1. A high performance liquid chromatography (HPLC) method utilising a C8 column and a diode array detector has been developed for pigment analysis at MSS.

2. The diode array detector was used to confirm or refute the presence of a particular pigment by comparing the spectrum in the sample with that of a known standard.

3. Twenty two pigments have been identified and quantified using this method. The identification of all pigments has been accredited. Only the quantification of chlorophyll *a* and chlorophyll *b* have been accredited as the bias/recovery could not be determined for the other pigments as no certified reference materials or external proficiency testing is currently available.

4. Correlation coefficients of >0.99 were obtained for all pigments, when plotting corrected peak area versus concentration for all pigments and applying a quadratic polynomial curve.

5. The limit of detection (LoD) for all pigments was calculated either based on a low matrix sample or the lowest calibration standard. The LoD was calculated to be 0.21 µg/l for chlorophyll *a* and 0.02 µg/l for chlorophyll *b.*

6. The analytical precision was assessed through replicate analysis of pigment standards and natural samples. For the majority of pigments the %CV was less than 25%, meeting the target for reproducibility.

7. The % recovery for chlorophyll a was 98%, with a %CV of 16, and for chlorophyll b was 87%, with a CV% of 19 meeting the criteria of 70-110% recovery.

8. The method has been converted to a standard operating procedure (SOP) and is given in Appendix 2. The method is now used routinely for analysis of pigment samples collected from Scottish Coastal Observatory monitoring sites.

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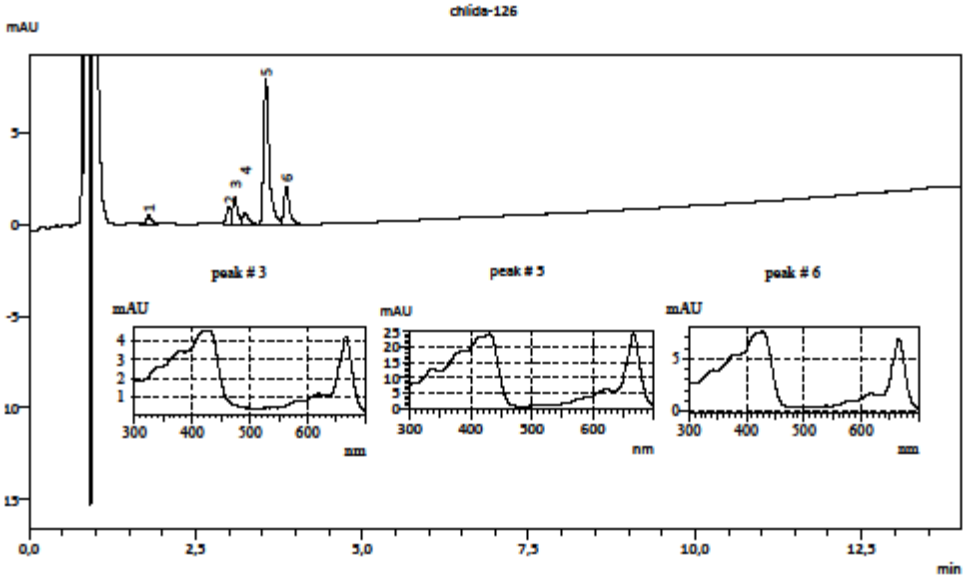
Appendix 1

Certificate of Analysis for Chlorophyllide *a* Standard

CERTIFICATE OF ANALYSIS

Content:	**Plant pigment in 90% acetone**
Name of pigment:	**Chlorophylide a**
Batch No.:	**chlida-126**
Concentration:	**0.867 mg/L**
Standard deviation:	**0.002, n=3**
Absorption coefficient:	**127 L/g/cm at 664 nm**
Volume:	**2.5 mL**
Extracted from:	**Bacillariophyceae**
Measured by:	**mea**
Approved by:	**lsc**

The concentration has been determined from absorbance readings on spectrophotometer (Shimadzu UV-2410 PC). The spectrophotometer is quality controlled using standard quality control procedures (control of wave length accuracy and absorbance control with secondary spectrometric calibration standards and with potassium dichromate (certified reference materials from NIST), control of stray light, noice, etc.). Furthermore, on randomly selected batch numbers an independent laboratory controls both the concentration and the purity of the pigment standards.



PDA Ch1 430nm

Peak#	Ret. Time	Area%	Name
1	1,79	3,32	
2	3,00	5,33	
3	3,09	11,30	chlid a
4	3,25	5,66	
5	3,56	58,93	chlida-126
6	3,86	15,26	chlid a

Method: Van Heukelem & Thomas (2001, J. Chroma. A, 910: 31-49), applied to Shimadzu UHPLC. Column: Eclipse Zorbax RRHD Eclipse Plus C8 (2.1*100 mm, 1.8 µm) injection volume: 100 µl, 28.5% standard: 71.5% TBAA, mix in the loop.

Stored frozen, below -20 °C, in the sealed vial, the pigment is stable for at least three years. The standards should, however, be used immediately after breaking the seal.

In case you need further information or have any questions regarding our products, please feel free to contact:

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Appendix 2

Procedures

1. Introduction and Scope

This SOP describes the preparation of standards for pigment analysis using HPLC with photo diode array detection (M 3150).

2. Principle of Method

To prepare standards for pigment analysis using HPLC with photo diode array detection (M 3150). Vitamin E acetate is used as an internal standard. This is used to correct for errors which may occur as a result of sample handling (e.g. evaporation of solvent) and HPLC analysis (e.g. errors in injection volume).

3. Reference Materials – Not relevant

4. Reagents

- 19 – Butanoyloxyfucoxanthin (Supplier: DHI Water and Environment, Denmark)
- 19- Hexanoyloxyfucoxanthin (Supplier: DHI Water and Environment, Denmark)
- Alloxanthin (Supplier: DHI Water and Environment, Denmark)
- Alpha-carotene (Supplier: DHI Water and Environment, Denmark)
- Antheraxanthin (Supplier: DHI Water and Environment, Denmark)
- Beta-carotene (Supplier: DHI Water and Environment, Denmark)
- Chlorophyll *a* from *Anacystis nidulans* algae (Supplier: Sigma Catalogue number: C6144)
- Chlorophyll *b* from Spinach (Supplier: Sigma Catalogue number: C5878)
- Chlorophyll c_2 (Supplier: DHI Water and Environment, Denmark)
- Chlorophyll c_3 (Supplier: DHI Water and Environment, Denmark)
- Chlorophyllide *a* (Supplier: DHI Water and Environment, Denmark)
- Diadinoxanthin (Supplier: DHI Water and Environment, Denmark)
- Diatoxanthin (Supplier: DHI Water and Environment, Denmark)
- Divinyl chlorophyll *a* (Supplier: DHI Water and Environment, Denmark)
- Fucoxanthin (Supplier: DHI Water and Environment, Denmark)
- Gyroxanthin-diester (Supplier: DHI Water and Environment, Denmark)
- Lutein (Supplier: DHI Water and Environment, Denmark)
- Neoxanthin (Supplier: DHI Water and Environment, Denmark)
- Peridinin (Supplier: DHI Water and Environment, Denmark)
- Prasinoxanthin (Supplier: DHI Water and Environment, Denmark)
- Violaxanthin (Supplier: DHI Water and Environment, Denmark)
- Zeaxanthin (Supplier: DHI Water and Environment, Denmark)
- 90% buffered acetone (see M 3150, section 9.1.9, for preparation details)
- Internal standard solution (see M 3150, section 9.1.10, for preparation details)

5. Equipment

- 1 cm fluorescence cell (Supplier: Fisher Scientific Cat no; CXA-145-155Y)
- Amber volumetric flasks with glass stoppers; 10 ml and 100 ml
- Beakers; various sizes
- Calibrated pipettes; various sizes
- Disposable gloves
- Disposable measuring cups
- Duran bottles; various sizes
- Pasteur pipettes
- Tissues
- Volumetric flasks with glass stoppers; 2 ml
- UV Spectrophotometer (EN1262) – **This instrument requires to be serviced annually in order to check and confirm the traceability of wavelength accuracy and absorbance accuracy.**

6. Environmental control

All photosynthetic pigments are light and temperature sensitive. Laboratory work must be performed in subdued lighting and with the minimum of delay.

Chlorophyll *a* from *Anacystis nidulans* algae, chlorophyll *b* from spinach and vitamin E acetate are held in a laboratory freezer. DHI pigment standards are held in a cryofreezer.

Prepared standards are held in a cryofreezer until use.

Fridge, freezer and cryogenic freezer temperatures are set and monitored as in SOP 280.

7. Interferences – Not relevant

8. Sampling and Sample Preparation – Not relevant

9. Analytical Procedure

9.1 Refer to M 3150 section 9.1.9 for preparation details of 90% buffered acetone. Refer to M 3150 section 9.1.10 for preparation details of the internal standard.

9.2 Preparation of Chlorophyll a and Chlorophyll b Standard Solutions

9.2.1 Chlorophyll *a* from *Anacystis nidulans* algae and chlorophyll *b* from spinach are shipped in amber glass ampoules. The manufacturer states that these have a shelf life of 1 year from the date of delivery. These standards should be stored frozen at -20^{o}C in the dark.

9.2.2 Allow the standards to come to room temperature; this will take at least 30 minutes. Transfer the entire contents of each ampoule (approximately 1 mg) into individual 100 ml amber volumetric flasks. This can be achieved by filling the ampoule with 90% buffered acetone using a Pasteur pipette. Using a clean Pasteur pipette,

transfer the 'green' solution to the 100 ml amber volumetric flask. Repeat this process until the solution turns clear (i.e. is no longer green in colour). Wrap the flasks in aluminium foil. Dilute to volume with 90% buffered acetone and label the flasks chl *a* SSS and chl *b* SSS. These solutions are stable for at least 6 months when stored in a light and airtight container in a freezer. Record the batch number of the chlorophyll *a* from Anacystis nidulans algae and the chlorophyll *b* from spinach in the Chlorophyll HPLC laboratory book.

9.2.3 If 1 mg of standard was diluted to 100 ml then the concentration of chlorophyll *a* and chlorophyll *b* in the SSSs would be 10000 µg/l. The actual concentration of the chlorophyll *a* and chlorophyll *b* stock standard solutions is determined spectrophotometrically using a multiwavelength spectrophotometer and the trichromatic equations described by Aminot & Rey (2001).

9.2.4 Switch on the spectrophotometer. The spectrophotometer will initialise and then do a self-calibration. A message will appear that the self-calibration has passed. Press <enter>. If the self-calibration fails then consult the user manual. Allow the spectrophotometer to warm up for at least 15 minutes before making any measurements.

9.2.5 Ensure that the eight position cell changer has been fitted. Instructions for doing this are given on page 35 of the User Manual for the instrument.

9.2.6 Fill a 1 cm cell with 90% buffered acetone. Insert into position 1.

9.2.7 Fill a 1 cm cell with the chl *a* SSS. Insert into position 2.

9.2.8 Fill a 1 cm cell with the chl *b* SSS. Insert into position 3.

9.2.9 Press <enter> for basic mode and <enter> for absorbance.

9.2.10 Set the wavelength to 630nm using the keypad.

9.2.11 Ensure that cell one is selected. The cell position can be changed by pressing the required number on the keypad. Press <set ref> to zero the instrument.

9.2.12 The cell changer will automatically move to position two and display the result for the 90% buffered acetone reference.

9.2.13 Press <run> to measure the absorbance of the chl *a* SSS. Record the value in the Chlorophyll HPLC laboratory book. The cell changer will automatically move to position three.

9.2.14 Press <run> to measure the absorbance of the chl *b* SSS. Record the value in the Chlorophyll HPLC laboratory book. The cell changer will automatically move to position four.

9.2.15 Repeat steps 9.2.9 to 9.2.14 for wavelengths of 647nm, 664nm and 750nm. Press the <mode> button to change the wavelength.

9.2.16 Calculate the concentration of chlorophyll *a* and chlorophyll *b* in the stock standard solutions using the trichromatic equations described by Aminot & Rey (2001) and B 662

9.2.17 Prepare standard solutions containing known concentrations of chlorophyll *a* and chlorophyll *b* in 10 ml amber volumetric flasks as outlined in the table below.

Standard solution	Nominal Chlorophyll a calibration solution (µl)	Nominal Chlorophyll b calibration solution (µl)	Volume of Internal Standard Solution (µl)	Final Volume (ml)	Nominal Chlorophyll a Concentration (µg/l)	Nominal Chlorophyll b Concentration (µg/l)
CHLAB_1*	2000 Stock	500 Stock		10	2000	500
CHLAB_1	2000 Stock	500 Stock	250	10	2000	500
CHLAB_2	1000 Stock	250 Stock	250	10	1000	250
CHLAB_3	500 Stock	100 Stock	250	10	500	100
CHLAB_4	250 Stock	50 Stock	250	10	250	50
CHLAB_5	400 1*		250	10	80	20
CHLAB_6	200 1*		250	10	40	10
CHLAB_7	100 1*		250	10	20	5

9.2.18 B 662 will automatically calculate the volume of chl *a* and chl *b* SSS to be pipetted into standard solution 1* to give the required concentration. Pipette these volumes into the flask and make up to the 10 ml mark with 90% buffered acetone.

9.2.19 B 662 will automatically calculate the volume of chl *a* and chl *b* SSS to be pipetted into standard solutions 1 to 4, and the volume of standard solution 1* to be pipetted into standard solutions 5 to 7 to give the required concentrations. Pipette these volumes into the flasks. Then pipette 250 µl internal standard solution into each flask and make up to the 10 ml mark with 90% buffered acetone.

9.2.20 Record the actual volume of chl *a* and chl *b* SSSs and standard solution 1* pipetted into the 10 ml amber volumetric flasks on B 662 . The actual concentration of the standard solutions will then be calculated automatically.

9.3 Preparation of Pigment Standard Solutions Using DHI Pigment Standards

9.3.1 Chlorophylls c_3 and c_2, Chlorophyllide *a*, Peridinin, 19-Butanoyloxyfucoxanthin, Fucoxanthin, Prasinoxanthin, 19-Hexanoyloxyfucoxanthin, Diadinoxanthin and Divinyl chlorophyll *a*

9.3.1.1 DHI pigment standard solutions are supplied in vials containing 2.5 ml of individual pigments at a concentration of ~1mg/l. To prepare standard solutions of the

pigments listed above two vials are required for each pigment. These must be from the same batch. The batch number and the actual concentration are given on both the vial and the certificate of analysis. Record the batch number and concentration on B 662

9.3.1.2 Prepare standard solutions containing known concentrations of chlorophyll c_3, chlorophyll c_2, chlorophyllide *a*, peridinin, 19-butanoyloxyfucoxanthin, fucoxanthin, prasinoxanthin, 19-hexanoyloxyfucoxanthin, diadinoxanthin and divinyl chlorophyll *a* in 2 ml volumetric flasks as outlined in the table below.

Standard solution	Nominal Pigment calibration solution (µl)	Volume of Internal Standard Solution (µl)	Final Volume (ml)	Nominal Pigment Concentration (µg/l)
1	1950 Stock	50	2	1000
2	1000 Stock	50	2	500
3	500 Stock	50	2	250
3*	500 Stock	0	2	250
4	250 Stock	50	2	100
5	400 3*	50	2	50
6	200 3*	50	2	25
7	100 3*	50	2	10

9.3.1.3 Pipette these volumes into a 2 ml volumetric flask and make up to the 2 ml mark with 90% buffered acetone. **Note – standard solution 1 is made up to volume with that particular pigment calibration solution.**

9.3.1.4 B 662 will automatically calculate the actual concentration of the pigments in the standard solutions.

9.3.2 Neoxanthin, Violaxanthin, Antheraxanthin, Alloxanthin, Diatoxanthin, Zeaxanthin, Lutein, Gyroxanthin-diester, Alpha & Beta Carotenes

9.3.2.1 DHI pigment standard solutions are supplied in vials containing 2.5 ml of individual pigments at a concentration of ~1mg/l. To prepare standard solutions of the pigments listed above one vial is required for each pigment. The batch number and the actual concentration are given on both the vial and the certificate of analysis. Record the batch number and concentration on B 662.

9.3.2.2 These standard solutions are prepared by mixing two of the pigments together as outlined in the tables below.

Standard	Pigments
A	Neoxanthin + Diatoxanthin
B	Violaxanthin + Alloxanthin
C	Antheraxanthin, + Lutein
D	Zeaxanthin + Gyroxanthin-diester
E	Alpha-carotene + beta-carotene

Standard solution	Nominal 1st Pigment calibration solution (µl)	Nominal 2nd Pigment calibration solution (µl)	Volume of Internal Standard Solution (µl)	Final Volume (ml)	Nominal Pigment Concentration (µg/l)
1	900 Stock 1	900 Stock 2	50	2	450
2	500 Stock 1	500 Stock 2	50	2	250
3	250 Stock 1	250 Stock 2	50	2	125
3*	250 Stock 1	250 Stock 2	0	2	125
4	125 Stock 1	125 Stock 2	50	2	62.5
5	400 3*		50	2	25
6	200 3*		50	2	12.5
7	100 3*		50	2	6.25

9.3.2.3 Pipette these volumes into a 2 ml volumetric flask and make up to the 2 ml mark with 90% buffered acetone.

9.3.1.4 B 662 will automatically calculate the actual concentration of the pigments in the standard solutions.

9.4 HPLC Analysis

9.4.1 Run the standards on the HPLC as M 3150, section 9.6.

9.4.2 Integrate the chromatograms as M 3150, section 9.7.

9.4.3 Export the peak areas to B 663 as M 3150, section 9.9 and correct for internal standard.

9.4.4 Copy the corrected peak areas for each pigment into the appropriate worksheet on B 663. A graph of pigment concentration versus corrected pigment peak area is automatically drawn. A second order polynomial trend line is drawn where the intercept is forced through zero. The equation of the line and the correlation coefficient (r^2) is shown on the graph. The value for r^2 must be 0.99 or greater. If it is not then evaluate the curve for any obvious outliers and discuss with technical management.

9.4.5 Once satisfactory calibration curves have been obtained for all pigments, analyse seven LRM's in accordance with M 3150 . Plot the pigment concentrations on the

control chart and check that they meet the rules outlined in SOP 1380. If any pigment does not meet the rules then discuss with Technical Management.

9.5 Creating the Spectral Library

9.5.1 If satisfactory calibration curves have been obtained then the individual spectra are saved and the spectral library created. Pigments in samples are identified by retention time and comparison with absorption spectrum from the photo-diode array detector with those of pigment standards held in the spectral library.

9.5.2 Open the appropriate data file by selecting C:\ ChromQuest \ Chlorophyll \ PDA \ Data \ Month Year and clicking on the data file.

9.5.3 From the Instrument window menu select Window \ PDA Scan-Mixed View or Views \ PDA Display \ Mixed View from the Navigation Pane. In the Chromatogram View move the cursor to the apex of the peak of interest. In the Spectrum View move the cursor to the wavelength of interest (665 nm for chlorophyll *a* and 450 nm for all other pigments). Right click in the Spectrum View and select Utilities \ Save Trace to save the spectrum to a file with an .spc extension for inclusion in the Spectral Library. Save the trace by selecting C:\ ChromQuest \ Chlorophyll \PDA \ Data \ Spectra\ Calib Month_Year. Save the spectrum as Peak Name_Calibration Level _Date; e.g. Lutein_Std 2_Aug 15. Repeat for all spectra to be saved.

9.5.4 Once all the spectrum files have been saved the Spectral Library can be created. Select File \ Spectral Library \ New to display the Library Definition dialogue box. Click in the Spectrum File cell of row 1 to display the Open dialogue box. Double click the chlorophyll a Std 1 .spc file in the list box by choosing C:\ ChromQuest \ Chlorophyll \ PDA \ Data \ Spectra\ Calib Month_Year. The .spc file name is entered into that cell and the associated spectrum is simultaneously displayed. Repeat this for the std 1.spc files for all of the other pigments. Select File \ Library \ Save As \ C:\ ChromQuest \ Chlorophyll \ PDA \ Data \ Spectra\ Calib Month_Year\M3150_Level1_Calib Month_Year.lib.

9.5.5 Repeat 9.5.4 for calibration levels 2 to 7.

9.5.6 Open the M 3150 master method by selecting C:\ ChromQuest \ Chlorophyll \ PDA \ Methods \ Master methods \ VHT Method \ M 3150_Master.met.

9.5.7 From the Navigation Pane select Method \ PDA Options. On the Library page enter the spectral libraries to be searched that were saved in steps 9.5.4 and 9.5.5 by clicking the file button. Select the Enabled check box for each library. Save the method as the calibration month_year by selecting File \ Method \ Save As \ C:\ ChromQuest \ Chlorophyll \ PDA \ Methods \ Master methods \ VHT Method \ M 3150_Calib Month_Year.met

9.5.8 Ensure the method created in 9.5.7 is open. From the Instrument window menu bar, click on Peak / Group tables. On the Spectrum column, enter the spectrum collected from Standard 3 for each pigment of interest by selecting C:\ ChromQuest \ Chlorophyll \ PDA \ Data \ Spectra\ Calib Month_Year \ Pigment_Std 3_Calib date.spc. Save the method as 9.5.7.

10 Calculation of Results

10.1 Calculation of concentration of chlorophyll *a* and chlorophyll *b* stock standard solutions using the Trichromatic equations.

$$Chlorophyll\ a\ (\mu g/l) = \left(\frac{11.85(E_{664} - E_{750}) - 1.54(E_{647} - E_{750}) - 0.08(E_{630} - E_{750})}{l} \right) \times 1000$$

$$Chlorophyll\ b\ (\mu g/l) = \left(\frac{-5.43(E_{664} - E_{750}) + 21.03(E_{647} - E_{750}) - 2.66(E_{630} - E_{750})}{l} \right) \times 1000$$

Where:

- E_{630} is the absorbance reading measured at 630nm
- E_{647} is the absorbance reading measured at 647nm
- E_{664} is the absorbance reading measured at 664nm
- E_{750} is the absorbance reading measured at 750nm
- l is the path length of the cuvette in centimetres

10.2 Calculation of the concentration of chlorophyll *a* and chlorophyll *b* in the diluted standard solutions

$$Vol\ Pigment\ SS\ required\ (ml) = \left(\frac{Conc\ Pigment\ working\ solution\ required\ (\mu g/l)}{Conc\ Pigment\ SS\ prepared\ (\mu g/l)} \right) \times Vol\ \ flask\ (ml)$$

$$Conc\ Pigment\ diluted\ \ solution\ (\mu g/l) = \left(\frac{Vol\ Pigment\ SS\ pipetted\ (ml)}{Vol\ \ flask\ (ml)} \right) \times ConcPigment\ SS\ (\mu g/l)$$

10.3 Calculation of the concentration of pigments in the diluted standard solutions

$$Conc\ Pigment\ diluted\ \ solution\ (\mu g/l) = \left(\frac{Vol\ Pigment\ SS\ \ pipetted\ \ (ml)}{Vol\ \ flask\ (ml)}\right) \times ConcPigment\ SS(\mu g/l)$$

10.4 Calculation of the pigment peak area corrected for internal standard

$$Corrected\ Pigment\ Peak\ Area\ \ = \left(\frac{Average\ peak\ area\ of\ IS\ in\ IS\ blanks}{Peak\ area\ IS\ in\ pigment\ std}\right) \times Peak\ area\ of\ pigment$$

Where:

- IS is internal standard.

11. Method Validation – Not relevant

12. Reports

Data files saved onto the hard drive of the instrument PC (EN379) are copied to Sose0014f \ data \ UKAS-Archive \ Clean and Safe \ Water chemistry \ Chlorophyll_HPLC along with the associated method and sequence files.

B 662 and B 663 are filed on G \ Data \ Clean and Safe Seas \ Chemdat \ Water Chemistry \ Chlorophyll \ HPLC \ PDA Calibrations \ Month_Year

13. Safety

Refer to RAI154.

14. Literature References

Aminot, A. & Rey, F., 2001, 'Chlorophyll a: Determination by spectroscopic methods', *ICES Techniques in Marine Environmental Sciences*, 30, (reference only).

Jeffrey, S.W., Mantoura, R.F.C. & Wright, S.W., 1997, Phytoplankton Pigments in Oceanography, UNESCO Publishing, (reference only).

Roy, S., Llewellyn, C.A., Egeland, E.S. & Johnsen, G., 2011, Phytoplankton Pigments- Characterization, Chemotaxonomy and Applications in Oceanography, Cambridge University Press, (reference only).

The Fifth SeaWiFS HPLC Analysis Round-Robin Experiment (SeaHARRE-5), NASA Technical Memorandum 2012-217503 (reference only).

Surveyor LC Pump Plus Hardware Manual, 60053-97120, Revision B, September 2006 (controlled)

Surveyor Autosampler Plus Hardware Manual, 60053-9106, Revision D, March 2008 (controlled)

Surveyor PDA Plus Detector Hardware Manual, 60053-97107, Revision E, January 2009 (controlled)

Surveyor Plus Getting Started with ChromQuest 5.0 Tutorial, 60053-97125, Revision A, March 2008 (controlled)

ChromQuest 5.0 Chromatography Data System Reference Guide, CHROM 97253, Revision A, March 2008 (controlled)

Surveyor Plus Manual Set, 60053-64200, Revision B

Ultraspec 3300 pro UV/Visible Spectrophotometer User Manual (controlled)

15. Uncertainty of Measurement – Not relevant

1. Introduction and scope

This method describes the procedure to be followed for the identification and quantitative determination of chlorophyll *a,* chlorophyll *b*, 19-butanoyloxyfucoxanthin, 19- hexanoyloxyfucoxanthin, alloxanthin, alpha-carotene, antheraxanthin, beta- carotene, chlorophyll $c_{2,,}$ chlorophyll $c_{3,,}$ chlorophyllide *a*, diadinoxanthin, diatoxanthin, divinyl chlorophyll *a*, fucoxanthin, gyroxanthin-diester**,** lutein, neoxanthin**,** peridinin, prasinoxanthin, violaxanthin and zeaxanthin in marine phytoplankton using HPLC with diode array detection.

This method is accredited for the quantification of chlorophyll *a* and chlorophyll *b* only. The other pigments are accredited for identification only, with the exception of chlorophyllide *a* and divinyl chlorophyll *a*. These two pigments have been recently added to the method scope and although validated, the data has still to be assessed by UKAS. Quantified data will be reported for all of the pigments, and with the exception of chlorophyll *a* and chlorophyll *b*, must be used for information purposes only.

The data will be reported internally and used in conjunction with the transmission electron microscope data produced by the Phytoplankton Ecology Group to characterise phytoplankton and provide valuable information about the contribution of different functional groups to the biomass of the phytoplankton community at the Stonehaven and Loch Ewe long term monitoring sites.

2. Principle of the method

It is widely reported that the fluorometric acidification technique is inaccurate when chlorophyll *b* and/or chlorophyll *c* are present in the sample, and that the only way to accurately assess all chlorophylls in the presence of degradation products is to use a separation technique such as HPLC.

Chlorophyll containing phytoplankton in a measured volume of seawater is concentrated by filtering at low vacuum through a glass fibre filter paper. The pigments are extracted from the phytoplankton by sonication in a known volume of an acetone solution containing vitamin E acetate (vitamin E acetate is used as the internal standard solution), followed by a soaking period of between 16 and 30 hours. An aliquot of the supernatant is then filtered through a 0.45 µm syringe filter and then analysed by HPLC. The HPLC method is based on the Van Heukelem and Thomas method which uses a simple aqueous methanol to methanol gradient at 60 ºC with tetrabutyl ammonium acetate as the ion-pairing reagent and a highly efficient monomeric C8 column. An acetone rinse is added towards the end of the gradient to alleviate carryover. The high efficiency of the column is enhanced by operation at an elevated temperature which reduces the resistance to mass transfer between phases, thus producing very sharp peaks. Monomeric C8 columns have shown special selectivity towards compounds with subtle differences in polarity such as mono and divinyl chlorophylls and lutein and zeaxanthin. The identification of pigment peaks occur at three different wavelengths; chlorophyll *a,* chlorophyllide *a* and divinyl chlorophyll *a* are determined at 665nm, the internal standard is determined at 222 nm and the remaining pigments are determined at 450 nm.

Pigments are identified by retention time and the absorption spectrum from the photo-diode array detector are compared with those of pigment standards held in the spectral library using ChromQuest Chromatography Data System version 5.0.

The HPLC is calibrated using chlorophyll *a* from *Anacystis nidulans* algae and chlorophyll *b* from spinach. The concentration of these standard solutions are determined using the trichromatic equations for chlorophylls *a* and *b* in 90% buffered acetone (Aminot & Rey 2001). Other pigment standard solutions were purchased from DHI Water and Environment which are provided with accompanying certificates of analysis. See SOP 3060 for details.

3. Reference materials

The LRM is prepared as directed in SOP 3030 and is used to maintain Shewhart control charts (for current LRM refer to B040). An LRM sample is analysed with every HPLC run, and the concentration of the individual pigments is calculated in the same manner as the samples, based on 1 litre of seawater filtered.

4. Reagents

- Vitamin E acetate (Supplier: Sigma)
- Acetone (Supplier: Rathburn Grade: HPLC or equivalent)
- Acetic acid, glacial (Supplier : Fisher Scientific Grade: AnalR or equivalent)
- Magnesium carbonate (Supplier: Sigma Catalogue number: M7179-500G or equivalent)
- Methanol (Supplier: Rathburn Grade: HPLC or equivalent)
- Tetrabutyl ammonium hydroxide 0.4M (Supplier: Fisher Scientific Grade: HPLC or equivalent)
- Water (Supplier: Rathburn Grade: HPLC or equivalent)
- See SOP 3060 for details of pigment standards

5. Equipment

- 0.45um PTFE syringe filters (supplier: Fisher; Cat No; FDP-465-005A)
- 1 litre conical flask
- 15 ml centrifuge tubes, polypropylene (Supplier VWR Cat no; 525-0150)
- 5 ml disposable syringes
- 9 mm screw caps with PTFE seal and amber vials (Supplier: Fisher Scientific; Part Numbers : 11581434 & 11573690)
- Agilent Zorbax Eclipse XDB-C8 Column 4.6 x 150 mm (Part Number: 993967-906). Pump 95% solvent A : 5% solvent B through new columns for 2 hours before use followed by injecting at least 7 system suitability standards until a stable response has been achieved. See sections 9.1.12 and 9.5.
- Amber Duran bottles; 1000 ml
- Amber volumetric flasks with glass stoppers; 10 ml and 100 ml
- Beakers; various sizes
- Black box or centrifuge rack wrapped in aluminium foil for transporting and storing samples in
- Calibrated timer
- Calibrated bottle top dispenser

- Calibrated pipettes; various sizes
- Calibrated top pan balance (2 decimal places).
- Disposable gloves
- Disposable measuring cups
- Duran bottles; various sizes
- Glass rod
- Laboratory refrigerator
- Magnetic stirring bar
- Magnetic stirring plate
- Measuring cylinders; various sizes
- pH meter (EN2013)
- Pasteur pipettes
- Thermo Fisher Scientific Surveyor Plus HPLC comprising of;
    - Surveyor Autosampler Plus (EN1603)
    - Surveyor LC Pump Plus (EN1602)
    - Surveyor PDA Plus Detector (EN1963)
    - PC with ChromQuest software version 5.0 (EN379)
- Tissues
- Tweezers
- Ultrasonic probe (EN1627)
- Whatman GF/F filter papers, 47mm.

6. Environmental control

All photosynthetic pigments are light and temperature sensitive. Laboratory work must be performed in subdued lighting and with the minimum of delay.

Chlorophyll *a* from *Anacystis nidulans* algae, chlorophyll *b* from spinach and vitamin E acetate are held in a laboratory freezer. DHI pigment standards are held in a cryofreezer.

The extraction solvent is held in a laboratory freezer at the end of the working day.

The autosampler rack is set to 4 °C for this method so that the standards and samples are kept cold whilst awaiting injection.

Any remaining standards and sample extracts are held in a cryofreezer. A portion of the extraction solvent must also be held in the cryofreezer. If a sample has to be re-run on the HPLC, the extraction solvent used to extract that particular sample must be run with it to determine the average peak area of the internal standard in the extraction solvent, which is required for the calculations.

Fridge, freezer and cryogenic freezer temperatures are set and monitored as in SOP 280.

7. Interferences

Not applicable.

8. Sampling and sample preparation

The sampling and sample preparation is carried out by the client. Gloves must be worn when carrying out the sampling and these shall be supplied.

8.1 Collect the water sample using a water sampling bottle closed by messenger or electronic command at the sampling depth, by pump sampling from a specific depth or by using a depth integrating hose sampler. Most often water samplers are deployed alongside a conductivity/temperature/depth (CTD) sampler and electronic sensors for chlorophyll fluorescence etc.

8.2 Gently decant the seawater into a clean polyethylene bottle (typically 1, 2, 5 or 10 litres), preferably keeping the sample in the dark or at least out of sunshine and chilled in dim light.

8.3 Samples collected aboard research vessels are filtered on board, whereas samples collected from inshore sites are filtered upon return to the laboratory.

8.4 A known volume of the seawater sample should be filtered within 4 hours of collection as algal populations change quickly. If there is a delay in filtering the sample then it should be stored chilled and in the dark and filtered as soon as possible.

8.5 Sampled volume should usually be standardised by reference to previous data or local considerations. Prior to filtration, gently agitate the bottle containing the sample, to resuspend any large cells that may have settled.

8.6 The sample is filtered through a Whatman GF/F filter paper (glass fibre, nominal porosity 0.7 µm), with a gentle vacuum, generally not more than 0.5 atm to avoid cell damage and loss of material through the filter. Sufficient seawater has been filtered when there is colour visible to the naked eye on the filter paper. In the event that the filtration slows strongly and is terminated before completing the standard volume, ensure that the actual volume filtered is recorded on B 614.

8.7 Remove the filter paper from the fritted base using tweezers, avoiding disturbance of the filtered material and fold once (algae inside). Blot with tissue paper to remove excess moisture and place in a numbered centrifuge tube. The centrifuge tubes containing the samples are protected from light and frozen immediately. The centrifuge tubes containing the samples can be stored frozen at -20 ^{o}C for 1 month, or up to 1 year in a cryogenic freezer.

8.8 Make a record of the sample number and the volume filtered on B 614.

8.9 Samples are logged into the laboratory according to LIMS CS010, LIMS CS020, LIMS CS060, LIMS CS070 and SOP 60.

9. Analytical procedure

9.1 Preparation of reagents

Note: other volumes can be prepared from that stated below by adjusting the volumes accordingly. The manufacturer and batch number of all solvents and reagents used should be recorded on B 660, along with the balance EN number, weights and volumes used.

9.1.1 Preparation of 28 mM TbAA pH 6.5 buffer

9.1.1.1 Calibrate the pH meter as SOP 3050.

9.1.1.2 Using a 100 ml measuring cylinder add 70 ± 1 ml 0.4 M tetrabutyl ammonium hydroxide and with a 1000 ml measuring cylinder add 500 ± 10 ml water to a 1000 ml beaker. Place a magnetic stirring bar into the beaker and stir on a magnetic stirring plate.

9.1.1.3 Adjust the pH of the solution with glacial acetic acid until the pH is 6.5 ± 0.05 pH units. Note : this requires between 1 and 2 ml of glacial acetic acid.

9.1.1.4 Transfer the TbAA solution to a 1000 ml measuring cylinder. Rinse the beaker at least twice with HPLC grade water bringing the solution up to the 1000 ml mark.

9.1.1.5 Transfer the solution back into the 1000 ml beaker, add a magnetic stirring bar and stir on a magnetic stirring plate.

9.1.1.6 Mix the solution thoroughly and check that the pH is still within 6.5 ± 0.05 pH units.

9.1.1.7 Transfer to an amber reagent bottle. Label the bottle with the following information:

- Contents
- Analyst initials
- Preparation date
- Expiry date (1 month from the date of preparation)

9.1.2 Preparation of solvent system A; methanol : 28 mM TbAA pH 6.5 buffer (70 : 30 v/v)

9.1.2.1 Using a 1000 ml measuring cylinder add 300 ml ± 10 ml 28 mM TbAA pH 6.5 buffer to a 1000 ml Duran bottle. Using a 1000 ml measuring cylinder add 700 ml ± 10 ml methanol to the Duran bottle. Cap and swirl vigorously several times to mix thoroughly. Label the bottle with the following information:

- The contents of the bottle
- Analyst initials
- Preparation date
- Expiry date (1 week from the date of preparation)
-

9.1.3 Preparation of solvent system B; 100% methanol

9.1.3.1 Add approximately 1000 ml methanol to a 1000 ml Duran bottle. Label the bottle with the following information:

- The contents of the bottle
- Analyst initials
- Preparation date
- Expiry date (1 week from the date of preparation)

9.1.4 Preparation of solvent system C; 100% acetone

9.1.4.1 Add approximately 300 ml acetone to a 500 ml Duran bottle. Label the bottle with the following information:

- The contents of the bottle
- Analyst initials
- Preparation date
- Expiry date (1 week from the date of preparation)

9.1.5 Preparation of solvent system D; methanol : water (70 : 30 v/v)

9.1.5.1 Using a 250 ml measuring cylinder add 210 ml ± 2 ml methanol to a 500 ml Duran bottle. Using a 100 ml measuring cylinder add 90 ml ± 1 ml water to the Duran bottle. Cap and swirl vigorously several times to mix thoroughly. Label the bottle with the following information:

- The contents of the bottle
- Analyst initials
- Preparation date
- Expiry date (1 week from the date of preparation)

9.1.6 Preparation of wash bottle solvent; 100% methanol

9.1.6.1 Add approximately 500 ml methanol to a 500 ml Duran bottle. Label the bottle with the following information:

- The contents of the bottle

- Analyst initials
- Preparation date
- Expiry date (1 week from the date of preparation)

9.1.6 Sonicate solvent systems A, B, C and D and the wash solvent for 20 minutes in an ultrasonic bath.

9.1.7 Preparation of the injection buffer

9.1.7.1 Pipette 10 ml methanol into a 100 ml volumetric flask and make up to the 100.0 ml mark with 28 mM TbAA pH 6.5 buffer. Label the flask with the following information:

- The contents of the flask
- Analyst initials
- Preparation date
- Expiry date (1 week from the date of preparation)

9.1.8 Preparation of saturated magnesium carbonate solution

9.1.8.1 Weigh 10 g ± 0.1 g magnesium carbonate into a 1000 ml conical flask. Add 1000 ml water and mix thoroughly. The solution is allowed to settle for a minimum of 24 hours. **Note only the clear 'powder free' solution is used during subsequent steps.** This can be easily done by decanting the 'powder free' solution into a 1000 ml reagent bottle. Label the bottle with the following information:

- The contents of the bottle
- Analyst initials
- Preparation date
- Expiry date (1 month from the date of preparation)

9.1.9 Preparation of 90% buffered acetone

9.1.9.1 Measure 100 ml ± 1 ml of 'powder free' saturated magnesium carbonate solution into the 1000 ml measuring cylinder. Transfer to a reagent bottle. Measure 900 ml ± 10 ml of acetone into the 1000 ml measuring cylinder and transfer to the reagent bottle. Mix thoroughly. Label the bottle with the following information:

- The contents of the bottle
- Analyst initials
- Preparation date

- Expiry date (1 week from the date of preparation)

9.1.10 Preparation of internal standard solution

9.1.10.1 Allow the vitamin E acetate to come to room temperature; this will take at least 30 minutes. Transfer the entire contents of the ampoule (approximately 100 mg) into a 100 ml amber volumetric flask. This can be achieved by filling the ampoule with 90% buffered acetone using a Pasteur pipette. Vitamin E acetate is a dense liquid. Using a clean Pasteur pipette, transfer the dense liquid to the 100 ml volumetric flask. Repeat this process until all of the vitamin E acetate has been transferred. Dilute to volume with 90% buffered acetone, wrap in aluminium foil and label the flask with the following information:

- M 3150 Internal Standard
- Analyst initials
- Preparation date
- Expiry date (1 month from the date of preparation)

9.1.10.2 When a new batch of internal standard solution has been prepared, the extraction solvent is prepared (step 9.1.11) and **SYSTEM SUITABILITY CHECK** (step 9.5) **PERFORMED PRIOR TO EXTRACTING ANY SAMPLES** to demonstrate the suitability of the solution. **THIS MUST BE RUN ALONGSIDE AN EXTRACTION SOLVENT PREPARED FROM THE BATCH OF INTERNAL STANDARD SOLUTION WHICH IS BEING REPLACED**. Note that a new batch of internal standard solution has been prepared in the comments box on the HPLC DA System Suitability Control Chart in Quality Analyst.

9.1.11 Preparation of extraction solvent

9.1.11.1 Using a calibrated pipette add 5.0 ml internal standard solution, (step 9.1.10), into a 100 ml measuring cylinder. Make up to 100 ml ± 1 ml with 90% buffered acetone (step 9.1.9) Transfer to an amber reagent bottle. Add a further 100 ml ± 1 ml 90% buffered acetone into the cylinder and transfer to the amber reagent bottle. Mix thoroughly. Label the bottle with the following information:

- The contents of the bottle
- Analyst initials
- Preparation date
- Expiry date (1 week from the date of preparation)

9.1.11.2 The extraction solvent is held in a laboratory freezer after use. When next used it must be brought to room temperature; this will take at least 30 minutes.

9.1.12 Preparation of system suitability solution

9.1.12.1 Prepare internal standard solution as 9.1.10 above.

9.1.12.2 Prepare standard solution CHLAB_2 as directed in SOP 3060 section 9.2 using the worksheet labelled system suitability in B 664 to calculate the volumes of chlorophyll *a* and chlorophyll *b* stock standard solutions to be pipetted to prepare the system suitability solution. The system suitability solution also contains 250 µl of internal standard solution.

9.2 Preparation of the HPLC

9.2.1 If the HPLC has had mobile phase pumping at a low flow rate then equilibrate the column for at least 30 minutes as described in step 9.2.13. If the HPLC system is being started up then proceed to step 9.2.2.

9.2.2 Switch on the pump, autosampler, PDA and computer.

9.2.3 Open up ChromQuest by double clicking on the desktop icon. Double click on the PDA icon in the main menu window. The last method which was used will be loaded.

9.2.4 Connect solvent bottle A to solvent line A, solvent bottle B to solvent line B, solvent bottle C to solvent line C, solvent bottle D to solvent line D and the wash solvent to the wash line.

9.2.5 After changing the solvents the system will have air in the solvent lines. To remove air from the solvent lines the pump is purged as section 9.2.6. To remove air from the syringe, flush it as section 9.2.10.

9.2.6 The purge command draws eluent from the solvent reservoir bottle at a rate of approximately 10 ml / min. To purge the solvent lines, open the drain valve by turning it 180º anti-clockwise to the purge position. The word DRAIN on the knob appears upside down. From the Instrument window menu bar, click Control \ Instrument Status. Click the Surveyor LC Pump tab and click Diagnostics. The Diagnostics operation page appears. Enter a purge time of 5 minutes and select solvent valve A. Click start to purge solvent line A. If there is still air in the solvent line then purge for a longer period of time.

9.2.7 Repeat for solvent lines B, C and D, by selecting the appropriate solvent valve.

9.2.8 Once all the solvent lines have been purged of air, close the drain valve by gently turning it clockwise as far as it will go. Click close to exit the Diagnostics dialog box.

9.2.9 Check that the pump is pumping solvent through at 1.1 ml/min. To do this attach a length of pump tubing to the top of the column and place a beaker underneath. Select File \ Method \ Open \ LocalDiskC \ ChromQuest \ Chlorophyll \ PDA \ Methods \ Master Methods \ VHT Method \ Pump Check A_B. Then click Control \ Download Method from the Instrument window menu bar. Collect the solvent in a beaker for 10 minutes. After 10 minutes, set a calibrated timer to 10 minutes and collect the solvent in a 25 ml measuring cylinder. After 10 minutes record the volume

of solvent collected on the HPLC System Suitability Shewhart control chart. The volume should be 11.0 ± 0.5 ml. If it is not consult the Technical Manager for guidance. Repeat the process but select File \ Method \ Open \ LocalDiskC \ ChromQuest Chlorophyll \ PDA \ Methods \ Master Methods VHT Method \ Pump Check B_C. Record the volumes collected on the HPLC DA System Suitability Control Chart in Quality Analyst.

9.2.10 To flush the syringe, click Control \ Instrument Status from the Instrument window menu bar, and then click the Surveyor AS tab. Click Diagnostics. Click the Direct Controls tab. In the Direct Commands list, select the Flush Syringe command. In the Parameters area, select Bottle from the reservoir list, enter a volume of 6000 µl and a flush speed of 100 µl/sec. Click Submit to execute the command. Repeat this until there are no air bubbles present in the syringe. Click Done to exit Diagnostics when finished.

9.2.11 Allow the detector lamp to warm up for at least 1 hour prior to making any injections. The lamps will be turned on when you download the method. To do this click File \ Method \ Open \ LocalDiskC \ ChromQuest \ Chlorophyll \ Methods \ PDA \ Master Methods \ VHT Method and select M3150_Master method_Calib Month_Year. Save this method as a new method in order to adjust retention times, sample information etc. This is done by selecting File \ Method \ SaveAs \ LocalDiskC \ChromQuest \Chlorophyll \ PDA \ Methods \ Methods Month Year. Save the method as M3150_date. Then click Control \ Download Method from the Instrument window menu bar.

9.2.12 Equilibrate the column with solvent A (95%) and solvent B (5%) for at least 2 hours prior to making any injections. Check that the pump is pumping at 1.1 ml /min, that the column pressure is stabilised and the tray and oven temperatures are at the correct settings by clicking Control \ Instrument Status from the Instrument window menu bar and selecting the appropriate tab.

9.3 Preparation of Standard Solutions

The instrument is calibrated when the column is changed. To prepare the calibration standard solutions see SOP 3060.

9.4 Extraction of the pigments

9.4.1 Prepare a method blank by inserting a clean Whatman GF/F filter paper, 47mm into a clean 15 ml centrifuge tube. The LRM is also placed into a clean 15 ml centrifuge tube.

9.4.2 Using a calibrated pipette or a calibrated bottle top dispenser add 5 ml of extraction solvent to the numbered centrifuge tube containing the filtered samples (**a maximum of 10 samples are extracted per batch**), method blank and LRM. Mix thoroughly by inversion. If necessary use a glass rod to ensure that the filter paper is completely submerged in the extraction solvent.

9.4.3 Place the centrifuge tube in a beaker of ice. Sonicate for 1 minute using the Sanyo Soniprep 150 Cell Disruptor, at setting 15 amplitude microns. After sonication,

remove the centrifuge tube from the beaker of ice and mix thoroughly by inversion. If necessary use a glass rod to ensure that the filter paper is completely submerged in the extraction solvent. Transfer the centrifuge tube to the black box or centrifuge rack wrapped in aluminium foil. Clean the probe of the Sanyo Soniprep 150 Cell Disruptor by rinsing with 90% buffered acetone before sonicating any further samples.

9.4.4 Once all of the samples have been sonicated, return the black box or centrifuge rack wrapped in aluminium foil containing the samples to the refrigerator to soak for between 16 to 30 hours. After at least 1 hour of soaking, mix thoroughly by inversion and repeat step 9.4.3. Return the black box or centrifuge rack wrapped in aluminium foil containing the samples to the refrigerator to complete the soaking period.

9.4.5 After the soaking period the supernatant is filtered through a 0.45 µm PTFE syringe filter. This is achieved by emptying the supernatant from the centrifuge tube into a clean disposable measuring cup. Draw the supernatant into a clean disposable 5ml syringe. Attach a 0.45 µm PTFE syringe filter to the end of the syringe and carefully filter the supernatant into a second clean disposable measuring cup. Dilute the filtered extract with extraction solvent if required.

9.4.6 Transfer approximately 0.5 ml of the filtered extract or diluted filtered extract to an amber HPLC autosampler vial. Each sample requires three vials; one containing the filtered extract or diluted filtered extract, an empty vial in which the pre-treatment procedure will occur and one vial containing approximately 1 ml of injection buffer. The vials are placed in the autosampler rack as follows; empty vial for pretreatment, filtered extract, injection buffer. The samples can be stored in the refrigerated autosampler for up to 18 hours before injection. Keep the remainder of the filtered extract in the cryofreezer in case of repeat.

9.4.7 Note the times that the extracts were placed in and removed from the fridge in the Chlorophyll HPLC laboratory book or directly into the **Results** tab for the LIMS batch (refer to LIMS CS070 for further details). Also make a note of any samples that were diluted and the dilution factors.

9.5 System Suitability Check

9.5.1 Prior to running the samples a system suitability check is performed to ensure that the HPLC system is performing satisfactorily. This is done by injecting 500 µl of system suitability solution (prepared in section 9.1.12) onto the chromatograph. To do this click the **Single Run** button from the toolbar or click Control \ Single Run. In the Run Information box, enter System Suitability as the Sample ID, select the method saved in 9.2.11, enter C:\ ChromQuest \ Chlorophyll \ PDA \ Data \ Month Year in the Data Path box and System Suitability_Date in the Data File box. In the Autosampler box, check the Pretreatment program box and select C:\ ChromQuest \ Chlorophyll \ PDA \ Pretreatment \ PDA Pretreatment_M3150.ape, enter the vial number and an injection volume of 500 µl. Place in the autosampler in position 2, along with an empty vial for pretreatment in position 1 and a vial containing approximately 1 ml of injection buffer in position 3. Leave all other settings at the default. The vial containing the extraction solvent is left in the autosampler for at least

30 minutes prior to injection to allow the temperature to stabilise. Click the clock next to Begin run and enter the time as appropriate and click OK. Click Submit. Complete the Chlorophyll HPLC injection notebook.

9.5.2 Once the data has been acquired, check that the peaks have been correctly integrated. To do this open the data file by clicking File \ Data \ Open and select the appropriate file. Ensure that the method saved in 9.2.11 is selected. To do this click File \ Method \ Open and select the appropriate method.

9.5.3 The internal standard chromatogram at 222 nm will open. Adjust the integration as required using the Integration Events toolbar. This is described in more detail in the 'Getting Started with ChromQuest 5.0 Tutorial'. The adjustment can either be 'Insert into Integration Events table' or 'Insert into Manual Integration Fixes table'. 'Insert into Integration Events table' will make this adjustment to all chromatograms run using this method after they have been reprocessed, whereas 'Insert into Manual Integration Fixes table' will only make the adjustment to the particular chromatogram which is being reintegrated. Click Analyze Now. This adds the integration event to the appropriate table and analyses the chromatogram. Save any changes to the method by selecting File \ Method \ Save.

9.5.4 To review the chlorophyll *a* chromatogram at 665 nm, select Window \ Cascade and click on the PDA 665 nm window. Adjust the integration as required as described in section 9.5.3. The method saved in 9.2.11 is set to detect the pigments based on retention time with spectral confirmation of the spectrum made on standard 3 (SOP 3060). If chlorophyll *a* has not been detected, and there is a peak present, verify the identity of the peak by comparing its spectrum with those held in the spectral libraries. To do this open the PDA Scan – Mixed View, either by selecting Window \ PDA Scan – Mixed View from the Instrument window menu or Views \ PDA Display \ Mixed View from the Navigation Pane. In the Chromatogram view move the cursor to the apex of the peak of interest. In the Spectrum view move the cursor to 665 nm. Click Actions \ Search Library. Assess the results of the spectral library search. For a conformational match the similarity must be 0.9 or greater. If in doubt consult the Technical Manager. If a match for chlorophyll *a* has been reported, change the spectrum from standard 3 to the spectrum of the standard which a match has been made. To do this select the Peak \ Group Table button or by clicking Method \ Peak \ group Table. In the Spectrum box, click on the green diamond, then select File \ Method \ Open \ Local Disk C \ ChromQuest \ Chlorophyll \ PDA \ Data \ Spectra \ Calib Month_Year \ Chla_ Std_No of spectral match.spc. Save the method as a new revision of the method saved in 9.2.11, i.e. M3150_date_version 1. Click Analyze now.

9.5.5 Repeat 9.5.4 for the chlorophyll *b* chromatogram at 450 nm. In the Spectrum view move the cursor to 450 nm.

9.5.6 Record the peak areas for internal standard, chlorophyll *a* and chlorophyll *b* on the HPLC DA System Suitability Control Chart in Quality Analyst. The peak area can be obtained directly from the chromatogram or by selecting Reports/View/External Standard. Enter the concentration of chlorophyll *a* and chlorophyll *b* in the system

suitability solution on the HPLC DA System Suitability Control Chart in Quality Analys as calculated using worksheet labelled system suitability in B 664. The response for chlorophyll *a* and chlorophyll *b* will be calculated. If the values for the internal standard peak area, the chlorophyll *a* response and the chlorophyll *b* response fall within the limits of the charts proceed with running the samples as described below. If the system suitability check is not satisfactory try re-running the check and if not satisfactory discuss with the Technical Manager.

9.6 Checking retention times and setting up the peak table

9.6.1 Check the certificate of analysis for the current batch of DHI mixed pigment to ensure that all of the pigments of interest are present. If they are not then these must be added. Pipette 500 µl DHI mixed pigment solution into an amber HPLC vial and 50 µl of any of the remaining individual pigments. Record the preparation details in the Chlorophyll HPLC laboratory book. Place in the autosampler in position 2, along with an empty vial for pretreatment in position 1 and a vial containing approximately 1 ml of injection buffer in position 3. The remaining pigments can be kept frozen in the cryofreezer for future use.

9.6.2 Inject 500 µl of this mixture onto the chromatograph. To do this click the **Single Run** button from the toolbar or click Control\Single Run. In the Run Information box, enter Mixed Pigments as the Sample ID, select the method saved in 9.2.11, enter C:\ ChromQuest \ Chlorophyll \ PDA \ Data \ Month Year in the Data Path box and Mixed Pigments_Date in the Data File box. In the Autosampler box, check the Pretreatment program box and select C:\ ChromQuest \ Chlorophyll \ PDA \ Pretreatment \ PDA Pretreatment_M3150.ape, enter the vial number and an injection volume of 500 µl. Leave all other settings at the default. The vials containing the mixed pigments and the injection buffer are left in the autosampler for at least 30 minutes prior to injection to allow the temperature to stabilise. Click the clock next to Begin run and enter the time as appropriate and click OK. Click Submit. Complete the Chlorophyll HPLC injection notebook.

9.6.3 Once the data has been acquired, check that the peaks have been correctly identified. To do this open the data file by clicking File \ Data \ Open and select the appropriate file. Ensure that the method saved in 9.2.11 is selected. The identity of a peak can be confirmed by comparing the spectrum of the pigment with that held in the spectral library. From the Instrument window menu select Window \ PDA Scan-Mixed View or Views \ PDA Display \ Mixed View from the Navigation Pane. In the Chromatogram view move the cursor to the peak of interest. In the Spectrum view move the cursor to the wavelength of interest. Click Actions \ Search Library. Assess the results of the spectral library search. The similarity should be 0.9 or greater. If in doubt consult the Technical Manager. In the Chlorophyll HPLC laboratory book make a note of the retention time for each pigment. Refer to the Mixed Pigment chromatogram in the appendix of this document. Select the Peak \ Group Table button or by clicking Method \ Peak \ Group Table. Enter the new retention times. Save the changes to the method by clicking File \ Method \ Save. Click the Analyse button or select Analysis \ Analyse to reintegrate the chromatogram.

9.6.4 Adjust the integration as required using the Integration Events toolbar. This is described in more detail in the 'Getting Started with ChromQuest 5.0 Tutorial'. The adjustment can either be 'Insert into Integration Events table' or 'Insert into Manual Integration Fixes table'. 'Insert into Integration Events table' will make this adjustment to all chromatograms run using this method after they have been reprocessed, whereas 'Insert into Manual Integration Fixes table' will only make the adjustment to the particular chromatogram which is being reintegrated. So in this instance choose 'Insert into Integration Events table'. Click Analyze Now. This adds the integration event to the appropriate table and analyses the chromatogram. Save the changes to the method by clicking File\Method\Save.

9.7 Running the Samples

9.7.1 Use the Sequence Wizard to set up the sequence. A typical sequence would contain a method blank, an LRM, 10 samples and a minimum of 3 extraction solvent (internal standard) blanks. **Note – the total run time should not exceed 18 hours.** The first extraction solvent (internal standard blank) blank is run at the beginning of the sequence and the others are placed randomly throughout the run. Click File \ Sequence \ Sequence Wizard. Select the method set up in 9.2.11. In the Data File Type box check For Acquisition. Leave the Amount Values box at the default setting. Click Next.

9.7.2 Leave the Sample ID box blank. In Data path select C:\ ChromQuest \ Chlorophyll \ PDA \ Data \ Month Year. Leave the Data File box blank. Enter the number of unknown runs in the sequence. Click Next.

9.7.3 Enter the first unknown vial, for example B;1 and increment by 3. Note avoid using racks A and E as additional strain will be put on the needle tubing during the pre-treatment procedure. Enter an autosampler injection volume of 500 µl. In the Pretreatment program file box select C: \ ChromQuest \ Chlorophyll \ PDA \ Pretreatment \ PDA Pretreatment_M3150.ape. Click Next.

9.7.4 Leave everything at the default settings in the Calibration page. Click Next. Leave everything in the Reports page blank and Click Finish.

9.7.6 At the end of the sequence, add a low flow (see 9.7.7) or a column wash and shutdown (see 9.7.8) at end of run.

9.7.7 If the instrument is to be used again during the working week, the pump is kept running at a reduced flow rate. In the next empty row of the sequence table, click in the method box, click on the green diamond, then select File \ Method \ Open \ LocalDiskC \ ChromQuest \ Chlorophyll \ PDA \ Methods \ Master Methods \ VHT Method \ M3150_lowflow.met \ Open. Save the sequence by selecting File \ Sequence \ SaveAs \ LocalDiskC \ChromQuest \ Chlorophyll \ PDA \ Sequence \ Month Year and name the file by the date then click Save.

9.7.8 If the instrument will not be used again during the working week, then it is flushed out with solvent system D followed by methanol (solvent system B) to remove all traces of buffer and then closed down. The last two samples will be methanol. Enter the vial

number, an injection volume of 500 µl, leave the Pretreatment box blank, enter 'Methanol Wash' as the Sample ID. In the method box, click on the green diamond, then select File \ Method \ Open \ Local Disk C \ ChromQuest \ Chlorophyll \ PDA \ Methods \ Master Methods \ VHT Method \ Solvent D_No buffer column wash.met \ Open. Repeat for the next row but in the method box, click on the green diamond, then select File \ Method \ Open \ Local Disk C \ ChromQuest \ Chlorophyll \ PDA \ Methods \ MasterMethods \ VHT Method \Methanol Column Wash \Open. In the next row, click the blue arrow in the Run Type column. In the Sample Run Type(s) dialog box, select the Shutdown check box, and click OK. In the Method column of the sequence table, click the green diamond, then select File \ Method \ Open \ Local Disk C \ ChromQuest \ Chlorophyll \ PDA \ Master Methods \ VHT Method \ M3150_shutdown method.

9.7.9 Enter the sample ID and the filename for each row. The format for filename is sampleID_date. Leave all other boxes in the sequence table at their default settings

9.7.10 Save the sequence table by choosing File \ Sequence \ Save As \ Local Disk C \ ChromQuest \ Chlorophyll \ PDA \ Sequence \ Month Year and name the file by the date then click Save.

9.7.11 Load the vials into the sample tray in the autosampler and close the door. From the online Instrument window toolbar, click the Sequence Run button. The Sequence Run dialog box appears.

9.7.12 Select the sequence to be run, by selecting Local Disk C \ ChromQuest \ Chlorophyll \ PDA \ Sequence \ Month Year \ and the file date saved in 9.7.10.

9.7.13 Select the appropriate run range, usually 'All'.

9.7.14 The samples must be placed in the autosampler for 30 minutes to come to the same temperature as the autosampler tray prior to injection. If necessary, click on the clock next to Begin Run and delay for 30 minutes. Click Start.

9.8 Reviewing and Reprocessing the Data

9.8.1 Review each chromatogram in turn by selecting File \ Data \ Open \ Local Disk C \ ChromQuest \ Chlorophyll \ PDA \ Data \ Month Year \ Sample ID_date. Ensure the method saved in 9.2.11 is open.

9.8.2 The internal standard chromatogram at 222 nm will open. Adjust the integration as required using the Integration Events toolbar. This is described in more detail in the 'Getting Started with ChromQuest 5.0 Tutorial'. The adjustment can either be 'Insert into Integration Events table' or 'Insert into Manual Integration Fixes table'. 'Insert into Integration Events table' will make this adjustment to all chromatograms run using this method after they have been reprocessed, whereas 'Insert into Manual Integration Fixes table' will only make the adjustment to the particular chromatogram which is being reintegrated. Click Analyze Now. This adds the integration event to the appropriate table and analyses the chromatogram. Save any changes to the method by selecting File \ Method \ Save.

9.8.3 To review the chromatogram at 665 nm, select Window \ Cascade and click on the PDA 665 nm window. Review the chlorophyll *a* peak and adjust the integration as required as described in section 9.8.2. The method saved in 9.2.11 is set to detect the pigments based on retention time with spectral confirmation of the spectrum made on standard 3 (SOP 3060). If chlorophyll *a* has not been detected, and there is a peak present, verify the identity of the peak by comparing its spectrum with those held in the spectral libraries. To do this open the PDA Scan – Mixed View, either by selecting Window \ PDA Scan – Mixed View from the Instrument window menu or Views \ PDA Display \ Mixed View from the Navigation Pane. In the Chromatogram view move the cursor to the apex of the peak of interest. In the Spectrum view move the cursor to 665 nm. Click Actions \ Search Library. Assess the results of the spectral library search. For a conformational match the similarity must be 0.9 or greater. If in doubt consult the Technical Manager. If a match has been reported, change the spectrum from standard 3 to the spectrum of the standard which a match has been made. To do this select the Peak \ Group Table button or by clicking Method \ Peak \ group Table. In the Spectrum box, click on the green diamond, then select File \ Method \ Open \ Local Disk C \ ChromQuest \ Chlorophyll \ PDA \ Data \ Spectra \ Calib Month_Year \ Chla_ Std_No of spectral match.spc. Save the method as a new revision of the method saved in 9.2.11, i.e. M3150_date_version 1. Click Analyze now. Repeat for the chlorophyllide *a* and divinyl chlorophyll *a* at 665 nm.

9.8.4 Repeat 9.8.3 for the 450 nm chromatogram. In the Spectrum view move the cursor to 450 nm.

9.8.5 Repeat 9.8.3 and 9.8.4 for the remaining samples in the batch.

9.8.6 If it is found that the same change needs to be more than one chromatogram these can be reprocessed as a batch rather than adjusting each one individually. Save the integration events to the method by selecting File \ Method \ Save. Select Sequence \ Process or click the Sequence Process button. The Process Sequence dialog box appears. Select the sequence to be processed by selecting Local Disk C \ ChromQuest \ Chlorophyll \ Sequence \ Month Year and the date saved in 9.7.10. Select the range of samples to be reprocessed and click Start to begin. Review each chromatogram in turn as 9.8.3 to 9.8.4.

9.8.7 If it is found that different changes need to be made to different chromatograms then the method for each sample integrated will have a new revision of the method saved in 9.2.11, i.e. M3150_date_version 2, M3150_date_version 3, M3150_date_version 4 etc. Record which method has been used to integrate which sample in the Chlorophyll HPLC Laboratory Notebook or directly into the notes section of the **Results** tab for the LIMS batch (refer to LIMS CS070 for further details).

9.9 Method Blanks

9.9.1 Assess the method blank. Typically there are no Gaussian shaped peaks for any of the pigments of interest, but if there are or there is any doubt, consult the Technical Manager for guidance.

9.10 Exporting the Data

9.10.1 M 3150 has been set up so that the area data is exported automatically to the hard drive of the HPLC PC C\HPLC Exported Files. Three AREA files are created; 222 nm, 450 nm and 665 nm. Copy these files onto a pen drive.

9.10.2 Open B 664 from Workbench. Remove the protection by selecting Tools / Protection / Unprotect Workbook. Click on the sheet labelled **Sheet 2**. Select File / Open / My Computer / G: / Data / IT Data Files / LIMS / Prod / CS / Batches / Water Chemistry / Chlorophyll / Export from LIMS and select the appropriate csv file (LIMS CS070)

9.10.3 Right click on the worksheet tab in the csv file and select **Move or Copy**. Change the **To Book** selection to B 664 and click OK. Save the workbook as Microsoft Office Excel Workbook with exactly the same name as the LIMS batch tab in the Import to LIMS folder.

9.10.4 The HPLC data can then be exported to Micosoft Excel. This is done by selecting File \ Open \ USB DISK CD (F:) and double clicking the required file. Then select Delimited \ Next \ Comma \ Next \ Finish. Copy the 222 nm data into the 222 nm Exported Area sheet on B 664 as appropriate. Repeat for the 450 nm data and the 665 nm data.

9.10.5 Delete any of the data which is not required e.g. the columns titled;

- Time
- File Name
- Method Name
- User Name
- Volume
- Autosampler Program

A row of data is created for each sample every time an update has been made to the integration. Therefore it is the **LAST ROW** of data for each particular sample that **IS REQUIRED**. Any previous rows can be deleted. **For pigments which have not been detected, enter a zero for peak area.**

9.10.6 Once the data has been edited the extraction solvent blank data is copied into the Extraction Solvent sheet. The peak areas for the extraction solvent blank are copied to the HPLC DA System Suitability Control Chart in Quality Analyst and must fall within the limits. Remove any obvious outliers. The %CV is calculated. This should be <10 %. If it is not discuss with the Technical Manager.

9.10.7 Copy the date analysed, field ID and area data for each pigment and internal standard in the sample into the Calculations sheet. Enter the UKAS/LIMS IDs, the volume of seawater filtered, the extract volume and the dilution factor

9.10.8 The calibration curves for each pigment are saved on B 662 and saved on G\ Data \ Clean and Safe Seas \ Chemdat \ Water Chemistry \ Chlorophyll \ HPLC \ PDA

Calibrations \ Month_Year. Enter the values for a and b from the calibration graph for each pigment into the cells on the Calculations sheet of B 664. The concentration of pigments in the samples will then be calculated automatically. Copy and paste the calculated values from the Calculations worksheet onto the LIMS worksheet in the Workbook. Save the changes and import into LIMS as LIMS CS070.

10. Calculation of Results

10.1 Calculation of pigment concentration from the calibration graph

A second order polynomial graph is drawn for each pigment as SOP 3060. The regression is forced through zero and the equation of the line is:

$$y = ax^2 + bx$$

Where:

- y is the peak area
- x is pigment concentration (µg/l)

To determine x the equation is:

$$x = \frac{-b + \sqrt{(b^2 + 4ay)}}{2a}$$

10.2 Calculation of pigment concentration in the samples

$$Cpi = \left(\frac{Vx}{Vf}\right) \times \left(\frac{Area\ IS\ x}{Area\ IS\ sam}\right) \times (Conc\ pigment\ from\ calibration\ graph\ (x)) \times DF$$

Where:

- Cpi is the pigment concentration (µg/l)
- Vx is the extraction volume (l)
- Vf is the volume of sample filtered (l)
- Area ISx is the average peak area of the internal standard in the extraction solvent
- Area IS sam is the peak are of the internal standard in the sample
- DF is the dilution factor

11. Method Validation

Validation raw data is maintained under G \ Services \ Quality Management \ Method Validation \ Chlorophyll by HPLC \ M3150_PDA.

Summary method performance information is maintained under B 045.

12. Reports

Reports are issued using Buisness Objects Web Intelligence according to LIMS CS090.

Data files, method files and sequence files saved on the hard drive of the instrument PC (EN379) are copied to Sose 0014f \ data \ UKAS-Archive \ Clean and Safe \ Water Chemistry \ Chlorophyll_HPLC

13. Safety

Refer to MEA083

14. Literature References

Jeffrey, S.W., Mantoura, R.F.C. & Wright, S.W., 1997, Phytoplankton Pigments in Oceanography, UNESCO Publishing, (reference only).

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Surveyor LC Pump Plus Hardware Manual, 60053-97120, Revision B, September 2006 (controlled)

Surveyor Autosampler Plus Hardware Manual, 60053-9106, Revision D, March 2008 (controlled)

Surveyor PDA Plus Detector Hardware Manual, 60053-97107, Revision E, January 2009 (controlled)

Surveyor Plus Getting Started with ChromQuest 5.0 Tutorial, 60053-97125, Revision A, March 2008 (controlled)

ChromQuest 5.0 Chromatography Data System Reference Guide, CHROM 97253, Revision A, March 2008 (controlled)

Surveyor Plus Manual Set, 60053-64200, Revision B

15. Uncertainity of Measurement

Uncertainty values are maintained under B 045.

Sources of uncertainty:

- **Sampling:** Samples are analysed and results reported on the samples as received – outwith uncertainty calculations.
- **Sub-sampling:** The filter is the whole sample, not applicable.
- **Storage conditions:** Samples are stored frozen in the dark. Samples are not stored for longer than 1 month at -20ºC and 12 months in a cryofreezer – negligible contribution to uncertainty.
- **Reagent purity:** All reagents used are HPLC grade quality – uncertainty accounted for in validation data.
- **Instrument effects:**
- **Weight:** Tolerance of balances used to prepare reagents is generally <1%. 2 decimal places used, this is sufficient for accuracy required. Uncertainty accounted for in validation data.
- **Volume:** Pipettes, used to prepare calibration standards and dilute samples are calibrated to <1%. Dispenser, used to dispense the extraction solvent, is calibrated to <2% - uncertainty accounted for in validation data.
- **Time:** Timers used to time sonication process are calibrated against the NIST clock to an accuracy of ±1 second – uncertainty accounted for in validation data.
- **Spectrophotometer:** This instrument is serviced annually in order to check and confirm the traceability of wavelength accuracy and absorbance accuracy. Minimal contribution to uncertainty.
- **HPLC:** The HPLC is flushed with methanol / water and methanol at the end of each working week to ensure that all traces of buffer are removed and to keep the instrument in good working order. Minimal contribution to uncertainty.
- **Computational effects:** Concentrations are calculated by Microsoft Excel spreadsheet. Manual checks of calculations have been carried out and found to be acceptable – negligible contribution to uncertainty.
- **Environmental conditions:** Contamination is minimised by the use of a

dedicated laboratory and equipment – uncertainty accounted for in validation data.

- **Operator effects:** All measurement methods are described in fully documented standard operating procedures to limit inconsistencies between operators. Only trained personnel may perform method unsupervised. Variations between operators are accounted for by control chart data. Uncertainty accounted for in validation data.
- **Random effects:** These will be accounted for by validation and control chart data.

Appendix

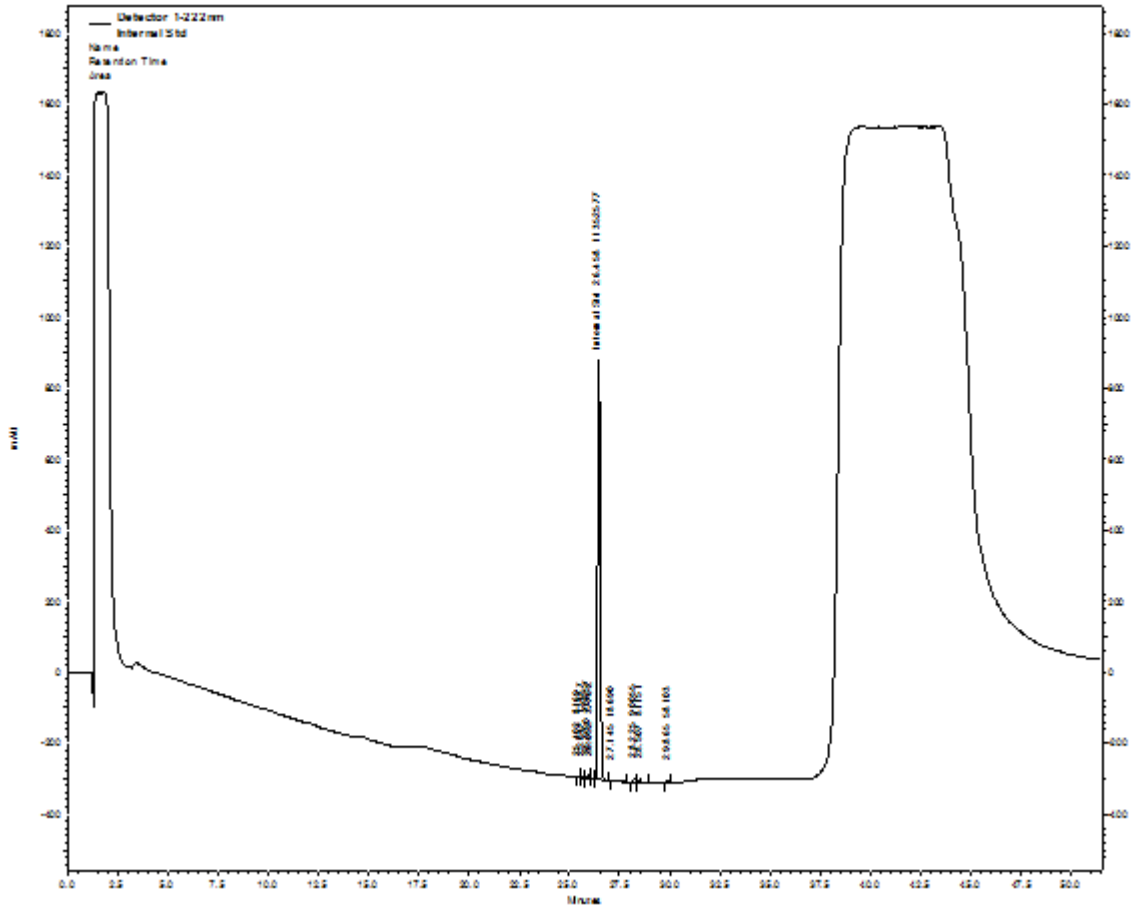
HPLC Conditions where solvent A is methanol : 28mM TbAA pH 6.5 buffer (70 : 30 v/v), solvent B is methanol (100%) and solvent C is acetone (100%).

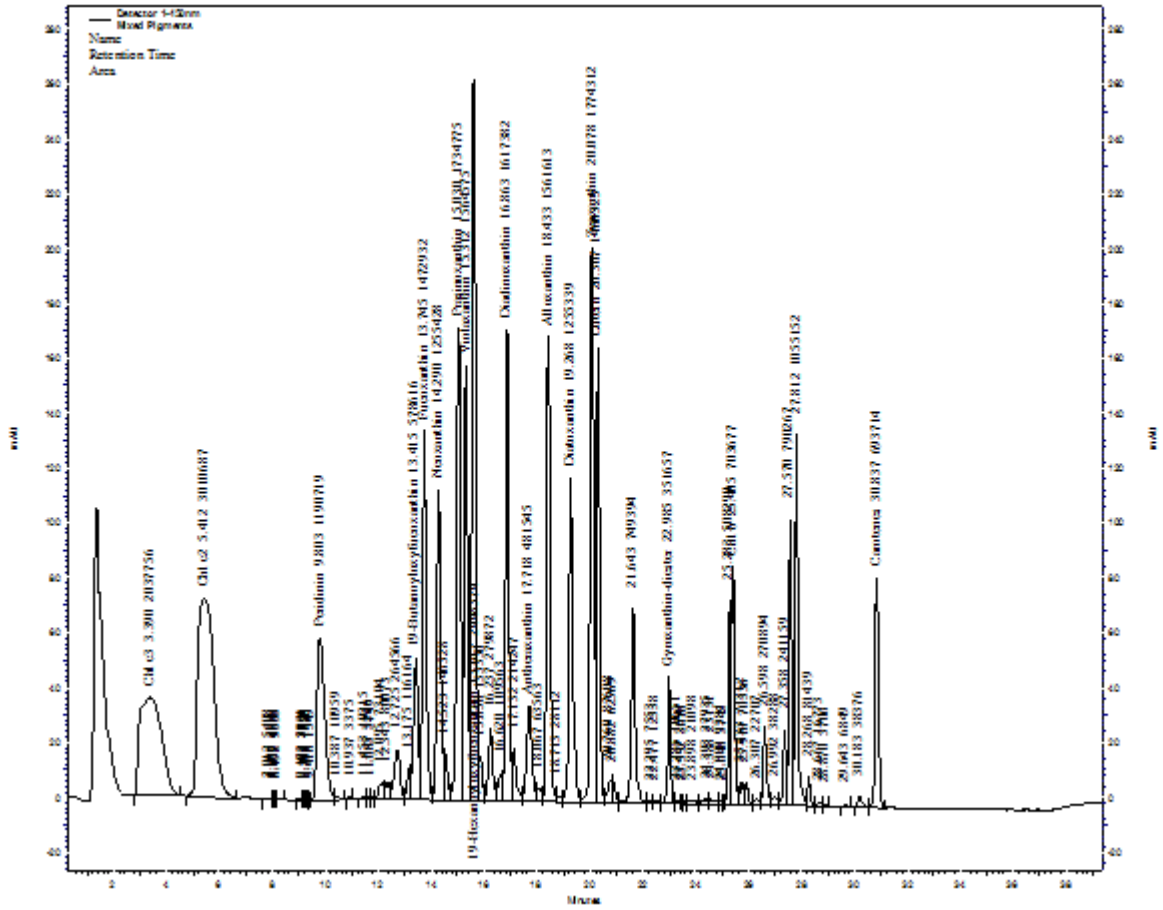
Time (minutes)	% A	% B	% C
0	95	5	0
27	5	95	0
34	5	95	0
35	5	65	30
40	5	65	30
41.5	95	5	0
51.5	95	5	0

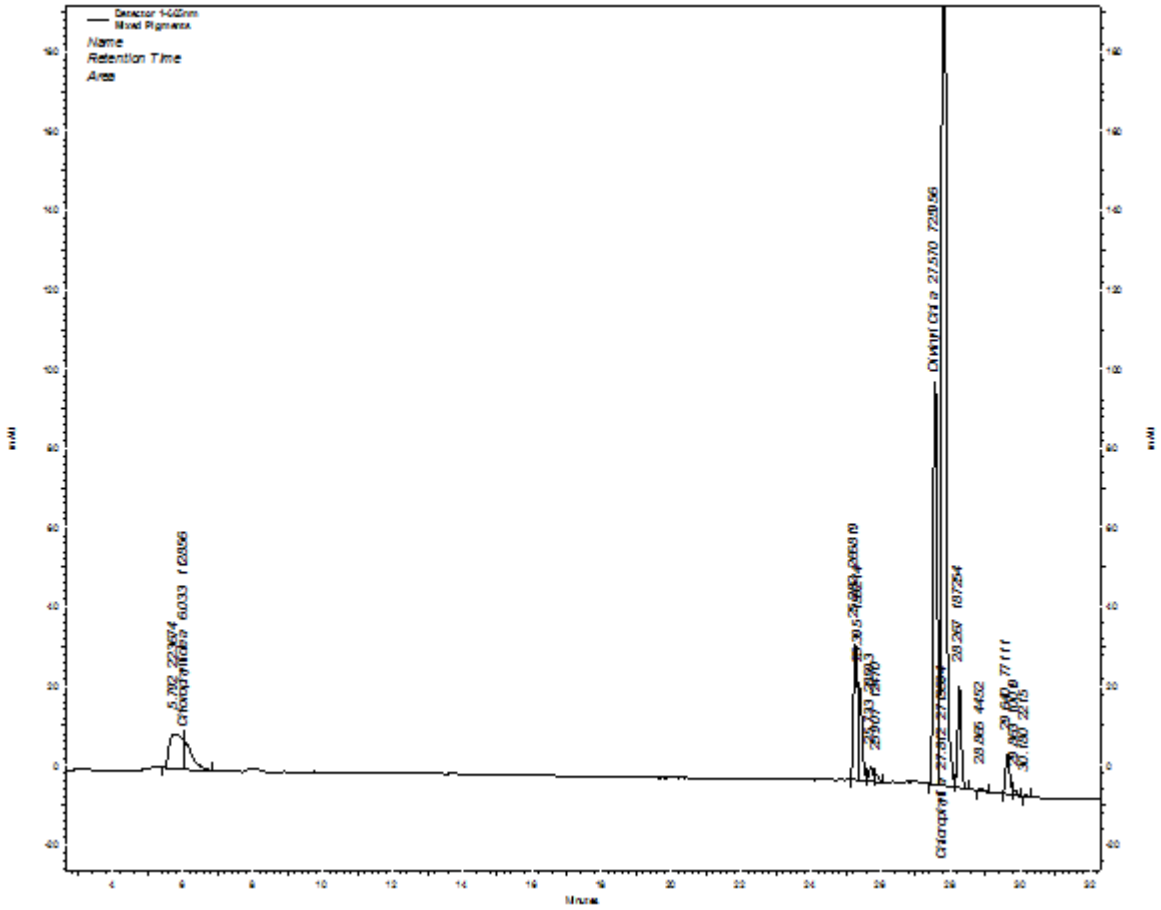
Flow rate:	1.1 ml /min
Detection wavelengths:	222 nm (internal standard), 665 nm (chlorophyllide a, divinyl chlorophyll a, chlorophyll a), 450 nm (all other pigments)
Wavelength range:	300 to 700 nm
Similarity threshold:	0.800
Tray temperature:	4 ºC
Column oven temperature:	60 ºC
Injection mode:	partial loop
Injection volume:	500 µl
Needle height for injection:	2 mm from bottom

Pretreatment Method

Draw from sample	Draw 710 µl from current + 2 at 150 µl/s, 3 µl bubble vol and 2 mm needle ht
Draw from sample	Draw 290 µl from current + 1 at 150 µl/s, 3 µl bubble vol and 2 mm needle ht
Deposit liquid in sample	Deposit 1006 µl to current at 250 µl/s & 2 mm needle ht
Mix at sample	Mix 900 µl in current at 100 µl/s and 250 µl/s for 10 cycles, 0.5 mm needle height
Flush to waste	Flush 2500 µl to bottle at 250 µl/s
Wash needle	Wash needle at bottle with 100 µl


Detector 1-222nm
Internal Std
Name
Retention Time
Area
mAU
Minutes


Detector 1-440nm
Mixed Pigments
Name
Retention Time
Area
Chl c3 3.800 2037756
Chl c2 5.412 3010687
Peridinin 9.803 1190219
Diadinoxanthin 16.863 1617382
Alloxanthin 18.433 1561613
Diatoxanthin 19.268 1253339
Gyroxanthin-diester 22.985 351657
Canthaxanthin 30.837 693704
Minutes


Detector 1-440nm
Mixed Pigments
Name
Retention Time
Area
mAU
Minutes

Detection wavelengths and retention times of pigments of interest in mixed pigment standard.

Pigment	Detection Wavelength (nm)	Retention time (minutes)
Chlorophyll c3	450	3.39
Chlorophyll c2	450	5.41
Chlorophyllide a	665	6.03
Peridinin	450	9.80
19-Butanoyloxyfucoxanthin	450	13.42
Fucoxanthin	450	13.75
Neoxanthin	450	14.29
Prasinoxanthin	450	15.03
Violaxanthin	450	15.31
19-Hexanoyloxyfucoxanthin	450	15.62
Diadinoxanthin	450	16.86
Antheraxanthin	450	17.72
Alloxanthin	450	18.43
Diatoxanthin	450	19.27
Zeaxanthin	450	20.08
Lutein	450	20.31
Gyroxanthin-diester	450	22.99
Chlorophyll b	450	25.41
Vitamin E acetate (internal std)	222	26.49
Divinyl chlorophyll a	665	27.58
Chlorophyll a	665	27.81
Carotenes	450	30.84