Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Detecting chlorophylls and carotenoids utilizing a novel chromatographic method designed for HPLC-PDA-MS analyses and application to environmental and cultured algae and cyanobacteria

Zacharias Smith

triggerdog91@gmail.com

USDA ARS https://orcid.org/0000-0002-6347-9325

David E. Berthold

University of Florida

Katherine M. Gaffney

The Ohio State University

H. Dail Laughinghouse

University of Florida

Cathleen J. Hapeman

USDA ARS

Research Article

Keywords: cyanobacteria, HAB, cHAB, pigments, HPLC, remote sensing, carotenoids, chlorophylls, chlorophyll a, pigment analysis, algal bloom, plant pigment

Posted Date: November 4th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11554586/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: The authors declare no competing interests.

Detecting chlorophylls and carotenoids utilizing a novel chromatographic method designed for HPLC-PDA-MS analyses and application to environmental and cultured algae and cyanobacteria

Authors

[Zacharias J. Smith[1], David E. Berthold[2], Katherine M. Gaffney[3], H. Dail Laughinghouse[2], Cathleen J. Hapeman[1]]

Affiliations

1. USDA Agricultural Research Service, Hydrology and Remote Sensing Laboratory, 10300 Baltimore Ave, Beltsville, MD, 20705

2. Agronomy Department, Fort Lauderdale Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, 3205 College Avenue, Davie, FL 33314

3. School of Environment and Natural Resources, College of Food, Agricultural, and Environmental Sciences, Ohio State University, 2021 Coffey Road, Columbus, OH 43210

Corresponding author

Zacharias J. Smith (zach.smith@usda.gov)

Keywords

cyanobacteria, HAB, cHAB, pigments, HPLC, remote sensing, carotenoids, chlorophylls, chlorophyll *a*, pigment analysis, algal bloom, plant pigment

Abstract

Chlorophylls and carotenoid analyses are important in aquaculture and freshwater and marine research as they are used to quantify algal biomass, food and supplement analyses, and to determine ecological diversity. The methods used for these analyses have often been limited to photometric detection, with some methods incorporating chromatographic separation. Mass spectrometric detection offers improved sensitivity, selectivity, and structural information compared to spectroscopy, but requires a full redesign of the separation system as previous chromatographic separations used solvent additives incompatible with mass spectrometry. The separation developed here used a YMC C30 "Carotenoid Column" with a novel mobile phase and elution scheme to separate a suite of algal pigments. Detection can be performed using a photodiode array alone, or in conjunction with mass spectrometry (MS). We report that the carotenoid echinenone (CAS 432-68-8) is actually an isomeric pair, which may have important implications for cyanobacterial photosynthesis and the orange carotenoid protein complex. New chlorophyll analogs, that could be hydroxylated or dehydroxylated derivatives, were detected in *Caulerpa* and

Planktothricoides sp. BLCC-F182. This cyanobacterium produced novel chlorophylls, and it uniquely contained no echinenone or 3-OH echinenone. There was marked diversity in the types of pigments produced in the multiple strains analyzed, and the analyses demonstrated that there is likely to be more to discover in the pigments produced by algae and cyanobacteria. The inclusion of MS added significant value for the analysis of carotenoids and chlorophylls, and novel methodologies such as this may lead the field into important discoveries and advancements.

Introduction

Pigments are found in many plants and animals with a variety of biological functions including: energy capture from the sun, protection from oxidative stress, tissue presentation, coloration, and more. Study of pigmentation is often performed with fluorometric and/or absorbance detectors, while HPLC has regularly been employed to separate and analyze complex mixtures of these analytes. Pigments have often required separation prior to analysis due to the diversity of chemical and stereochemical structures found in nature (Yabuzaki 2017; Taniguchi and Lindsey 2021). This is usually performed with chromatographic techniques, which separate individual components of the mixture and allows for specific identification of each pigment. Chromatographic methodology generally depends on the type of detector chosen, and on whether the method was designed to target chlorophylls, carotenoids, or both.

Chlorophylls and carotenoids are pigments critical to most plants due to their roles in photosynthesis, including being incorporated into photosystems I and II and other accessory structures. Characterization and quantification of chlorophylls and carotenoids has played important roles in studies of food supplies, human and animal nutrition, terrestrial and aquatic remote sensing, and many biological assessment techniques (Mackey et al. 1996; Takaichi 2011; Kuczynska et al. 2015; Aydoğan 2020). In aquatic environments, pigment analyses have been used to broadly assess ecological health and aquatic diversity, understand energy capture on a global scale, and to assess and quantify phytoplankton and hydrophytes.

Combined methods for chlorophylls and carotenoids have primarily focused on photodiode array and/or fluorescence detection, and have been effective at separating complex pigment mixtures with appropriate selectivity and detection limits required for their application (Mantoura and Llewellyn 1983; Garrido and Zapata 1997; Rodríguez et al. 1998; Jeffrey et al. 1999; Lacker et al. 1999; Van Heukelem and Thomas 2001; Gentili and Caretti 2011). Many modern pigment methods still utilize the foundational science first described in the publications of the 1984 Scientific Committee on Oceanic Research (SCOR), which included a method development group documenting a series of techniques for pigment analysis (Jeffrey et al. 1997). While the chromatography and sensitivity in SCOR related methods is excellent, low resolution between analytes could result in the loss of separation from small changes in method

and/or column performance (Zapata et al. 2000). The complex mixtures and many isomeric forms of pigments in natural samples may also introduce unanticipated interferences that inhibit analysis and quantification.

There have been relatively few modifications and/or advancements in analytical approach for pigment analyses that phytoplankton and algal researchers have been able to take advantage of over the last 10 years, with much of the improvement limited to improved HPLC column and detector manufacturing and design, rather than in the core components fundamental to the scientific application and approach (Catlett and Siegel 2018). This is unfortunate because liquid chromatography has been revolutionized by the induction of mass spectrometry, such as electrospray ionization (ESI) (Whitehouse et al. 1985), which has revolutionized numerous fields in the life sciences as it can function as a universal detector, provide important structural information about analytes, offers increased specificity, and at times drastically improved sensitivity compared to photometric methods. However, algal pigment analyses have yet to greatly benefit from this technological advancement as there are a limited number of MS methods for pigments described in the literature.

HPLC/UPLC technology advancement in the last decade, including column technology improvement (e.g. packing, stationary phase chemistry, C30 columns etc.), and increased accessibility to ESI mass spectrometers (MS) have offered researchers new and improved tools for chromatography, but these technologies have not been

102 widely implemented for combined carotenoid/chlorophyll analyses. The SCOR-style
103 pigment methodologies use solvent systems that are largely incompatible with mass
104 spectrometry due to high salt concentrations in the mobile phases, where they are
105 added to introduce ion pairing mechanisms for analyte retention. Because of the
106 importance of these ion pairing salts in analyte retention and selectivity in these
107 chromatographic systems, major chromatographic modifications are required for this
108 method framework to achieve good separation when trying to incorporate MS detection
109 (Jeffrey et al. 1999; Cruz and Casal 2018; Pniewski 2020). Some methods for carotenoids
110 in the literature used MS compatible solvents and/or MS detection, but only a selection
111 of these methods incorporated chlorophylls and therefore would need to be developed
112 further to incorporate chlorophyll analytes (Gentili and Caretti 2011; Rivera et al. 2011;
113 Bijttebier et al. 2014).
114 Even though mass spectrometric detectors offer valuable advantages over
115 photometric detection for pigments, column technology has been the limiting factor for
116 the application. Without ion pairing additives, the selectivity for carotenoid and
117 chlorophyll analytes on C18 HPLC columns can be poor, and even though mass
118 spectrometers allow separation by mass there are isomeric forms, both stereochemical
119 and constitutional, for both the chlorophylls and carotenoids, that require chemical
120 separation prior to identification.

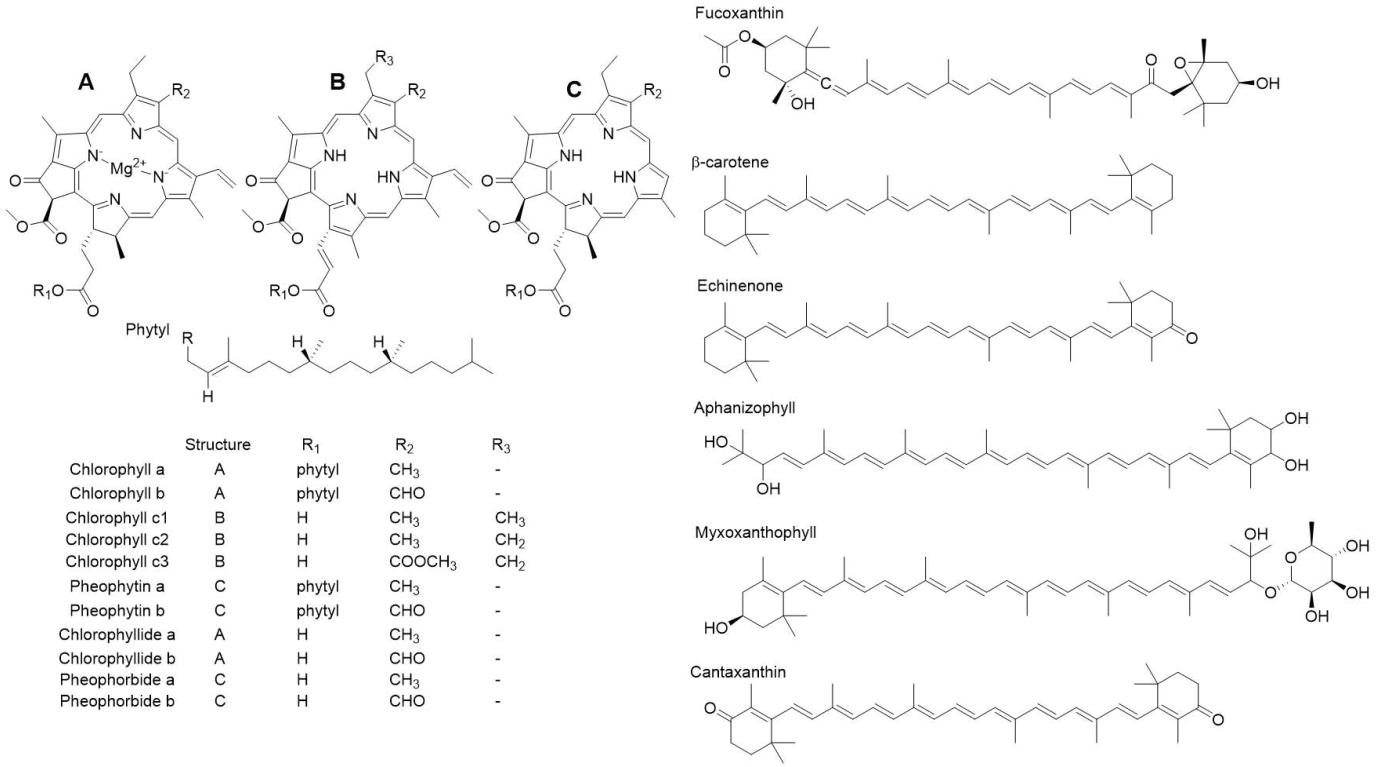
Columns with a C30 stationary phase have been shown to provide increased selectivity between analytes without the need for ion pairing reagents. A specifically engineered phase, The Carotenoid Column™, first described by Sander et al. (Sander et al. 1994), describes the process to design this column for efficiently separating carotenoids; the C30 stationary phase was polymerically bonded to the substrate, and the silica is of intermediate pore size (~20 nm) and is non-endcapped. This design takes advantage of the "three properties…identified as desirable for a column intended for carotenoid separations: enhanced shape recognition of isomers, high absolute retention, and silanol activity" (Sander et al. 1994), of which the importance of these features for carotenoid analyses was determined analytically (Epler et al. 1992; Lesellier et al. 1993). Applications by the column manufacturer, YMC (Kyoto, Japan), demonstrate that this column can also provide excellent separation for chlorophylls in addition to its use with carotenoids (YMC America).

Several methods using MS-based detection or C30 columns have been described for carotenoid and/or chlorophyll analysis, but methodological limitations make them unsuitable for the general chlorophyll/carotenoid analysis that SCOR aimed to provide for oceanographic research. For example, an MS-based method was developed for chlorophylls and carotenoids, but its separation was designed for fruit tree leaves and with short run times, and therefore poorly suited to the highly complex pigment composition of phytoplankton (Wojdyło et al. 2021). A PDA coupled with a quadrupole

time-of-flight MS method for pigment analysis was developed, but its validation only included seven pigments and nine pigments (not including isomers) were identified in a culture of *Dunaliella salina*, which required a relatively difficult extraction procedure (Fu et al. 2012). A separation of various pigments using a C30 column was described, but solvents incorporated tetrabutyl ammonium acetate buffer because the authors were comparing the performance of different columns, and therefore this method could not be utilized in an ESI MS-based detector without modification (Van Heukelem and Thomas 2001). An LC-PDA-MS method using a C30 column was applied to spinach, but the chromatography was designed to separate zeaxanthin, lutein, and their isomers, and was not designed to separate other pigments (Dachtler et al. 2001). An UPLC-MS/MS method was designed for 14 different pigments, but it was operated in targeted mode which would not detect most of the hundreds of pigments known to exist in the environment. The chromatographic separation was limited, with many closely eluting compounds, which required a UPLC system and column to achieve baseline resolution between analytes (Zhang et al. 2016). A MALDI-ToF/ToF method was used to evaluate both pigments and lipids, but it requires relatively uncommon instrumentation, and cannot easily distinguish isomeric forms of pigments unless the isomers produce unique fragmentation pathways (Bianco et al. 2024).

We sought to develop a chromatographic system that could be used to separate the known chlorophylls for which we acquired standards, while also aiming to

chromatographically separate as many carotenoids as possible. We aimed to make this separation methodology applicable to freshwater and marine algal and cyanobacterial pigment analyses with or without an MS detector, as we wanted this method to be usable with only a PDA for detection. It was necessary to redesign the separation so that the method was compatible with mass spectrometric detection, using common and non-toxic solvents and HPLC additives, as this would improve sensitivity, selectivity, and had not yet been demonstrated in a method as comprehensive as the 'standard' photometric HPLC methods regularly employed for phytoplankton. The applicability of our system to phytoplankton is demonstrated by several examples: 1) the effective



	Structure	R_1	R_2	R_3
Chlorophyll a	A	phytyl	CH_3	-
Chlorophyll b	A	phytyl	CHO	-
Chlorophyll c1	B	H	CH_3	CH_3
Chlorophyll c2	B	H	CH_3	CH_2
Chlorophyll c3	B	H	$COOCH_3$	CH_2
Pheophytin a	C	phytyl	CH_3	-
Pheophytin b	C	phytyl	CHO	-
Chlorophyllide a	A	H	CH_3	-
Chlorophyllide b	A	H	CHO	-
Pheophorbide a	C	H	CH_3	-
Pheophorbide b	C	H	CHO	-

Figure 1. Structures of chlorophylls used in this study, and several example carotenoid structures representing early, middle, and late eluting carotenoid compounds.

separation and identification of pigments in a "worst-case" scenario, where a *Caulerpa*

sample containing dozens of pigments, many without commercial reference materials, was evaluated by this system; 2) a comparison of cyanobacterial strains where we investigated the qualitative applications of the methodology for pigment profiling; and 3) a quantitative comparison of the pigment profiles and pigment ratios from *Cladophora* samples collected from Lake Huron and Lake Michigan.

Materials and Methods

Materials

Mass spectrometry grade methanol and ethyl acetate were acquired from Fisher Scientific (Hampton, New Hampshire) and J.T. Baker (Radnor Township, Pennsylvania) respectively. Mass spectrometry grade water was generated inhouse with resistance above 17.8 MΩ·cm. Mass spectrometry grade concentrated ammonium hydroxide was purchased from Supelco (St. Louis, Missouri) and mass spectrometry grade formic acid was purchased from Fisher Scientific (Hampton, New Hampshire).

Authentic certified reference materials were used as standards for the following pigments, delivered as pre-diluted samples in acetone, ethanol, or 90% acetone: alloxanthin (0.848 ppm), aphanizophyll (0.845 ppm), astaxanthin (0.933 ppm), β-cryptoxanthin (1.110 ppm), chlorophylls *a* (1.511 ppm), *b* (1.244 ppm), *c*1 (0.700 ppm), *c*2 (0.819 ppm), *c*3 (0.787 ppm), chlorophyllide *b* (0.688 ppm), diatoxanthin (0.779 ppm), divinyl chlorophylls *a* (0.835 ppm), *b* (0.759 ppm), divinyl Mg-2,4-Divinyl-

phaeoporphyrin a5-monomethylester (MG-DVP) (0.516 ppm), echinenone (total of two isomers determined in this manuscript, 0.923 ppm), lutein (0.933 ppm), myxoxanthophyll (0.922 ppm), neoxanthin (0.720 ppm), peridinin (0.900 ppm), pheophorbide *a* (1.656 ppm), pheophytin *a* (3.345 ppm), and prasinoxanthin (0.980 ppm) were acquired from DHI Group (Hørsholm, Denmark). Standards of cantaxanthin, β-carotene, fucoxanthin, and lycopene were acquired from Sigma Aldritch (St. Louis, Missouri, USA). Linearity was determined on mixtures of 3 or 4 standards, diluted 8× in acetone, measured across five injection volumes of 4, 8, 16, 28, and 40 μL for each mixture.

Pigment extraction

To demonstrate the selectivity of the chromatographic separation, a macrophytic green algal field sample and a unicyanobacterial strain of *Microcystis* sp. BLCC-F108 from the Berthold Laughinghouse Culture Collection (BLCC) (Fort Lauderdale Research and Education Center, University of Florida—IFAS, Davie, Florida, United States) were analyzed. The cyanobacterial strain was cultivated in 2-L Erlenmeyer bioreactors containing 1.8 L of BG-11, aerated using sterile air, and incubated at 25°C under a 12:12h light/dark for four weeks. Cyanobacterial culture was then harvested by centrifugation at 4,700 rpm (Sorvall, X Pro Series). Field samples of the green macroalgae *Caulerpa* (MLP-22-7-3) were collected on March 2022 from Charlotte Harbor

(Matlacha, Florida, USA), manually cleaned to remove debris, stored on dry ice and then held at -80°C prior to lyophilization. Both algal samples were lyophilized, ground into a powder with a mortar and pestle, and stored at -80°C until analysis. Field samples of *Cladophora* were retrieved from sites at Thunder Bay, Lake Huron, and a southwestern portion of Lake Michigan. Other cyanobacterial cultures for the following genera: *Microcystis* BLCC-F159, *Microcystis* BLCC-F209, *Iningainema* BLCC-F205, *Raphidiopsis* BLCC-F278, *Planktothricoides* BLCC-F182, *Scytonema* BLCC-T1, and *Microcoleus* BLCC-T19, and a novel genus BLCC-F213 that has not yet been classified were grown, processed, and extracted in the same way as *Microcystis* BLCC-F108.

Samples were thawed, homogenized by stirring, and aliquots of freeze-dried material 0.05g the cyanobacterial strains, 0.2g for *Caulerpa*, and 0.6g wet weight of *Cladophora* were added to 15-mL falcon tubes and 10 mL of 90% acetone (v/v) was added to each sample, shaken for 15 seconds, sonicated on ice in a sonication bath for 1h and left in the dark for ~16h at 4 °C (US EPA. 1997). Samples were then passed through a 0.2 μm Acrodisk polyvinylidene fluoride (PVDF) syringe filter and analyzed by HPLC-PDA-MS. In early testing, environmental water samples were collected from Perry Lake (Kansas, USA), where 100 mL of water was passed through a glass fiber F filter, and the filtered algal material processed the same as above. All processing was done in the dark or in as low-light conditions as could be managed.

Analysis by LC-PDA-MS

Pigments were separated on a 3μ YMC Carotenoid C30 column (3.0x250 mm) (PN#: CTT00S03-2503WT) equipped with an additional 10-mm, matched-phase guard cartridge (PN#: CT99S03-0103GC). Separation was achieved using a ternary gradient of **A**: methanol with 880 μL and 397 μL of concentrated ammonium hydroxide and formic acid, respectively, diluted to 500 mL; **B:** ethyl acetate; and **C:** water, following the gradient shown in Table 1; all changes were linear (Agilent) or gradient 6 (Waters). The flow rate was 0.5 mL/min and injection volumes for samples were 10 μL while injection volumes for mixtures of standards were 10 – 50 μL. Injection volumes up to 200 μL (90% acetone or other mixes of methanol, ethanol, and acetone) were found to slightly increase peak width but retention times for the majority of compounds remained stable. Development of the chromatographic separation and preliminary validation was achieved using an Agilent (Santa Clara, CA) 1260 BioInert HPLC system with an injection loop of 100 μL, a column compartment maintained at 30°C and a photodiode array equipped with both deuterium and tungsten lamps. Refinement of the methodology and development of the mass spectrometric analysis component of the method was achieved using a Waters (Milford, MA) ACQUITY H-Class UPLC without column temperature control (lab temperatures 15-18 °C during analysis), a 250 μL injection loop and needle, an eλ photodiode array detector with a deuterium lamp and equipped with a high-sensitivity (2400 nL 25 mm path length) flow cell, and a TQ-S

micro triple-quadrupole (MS/MS) detector. All chromatograms in the primary text are from the Waters system LC-PDA-MS system. Separations achieved using the Agilent system are shown in the supplemental and were specifically labeled in the caption where the figure showed chromatograms collected from that HPLC-PDA system.

Table 1. LC gradient used for the separation of chlorophylls and carotenoids. Solvent specifications are described in the text. The switch at 55 minutes to 100% MeOH was to flush check valves and inlet lines of ethyl acetate to make sure that no water-ethyl acetate mixing issues could be introduced into the LC flow path.

Minute	% MeOH:FA:NH_4OH (A)	% EtOAc (B)	% water (C)
0	95	0	5
5	95	0	5
10	70	25	5
15	65	30	5
30	65	30	5
40	25	70	5
46	0	100	0
55	0	100	0
55.01	100	0	0
56	100	0	0
56.01	95	0	5
66	95	0	5

Analyte detection was achieved by a combination of PDA and MS detection. PDA data were collected between 350-700 nm with a scan rate of 1/s and a bandwidth of 1.2 nm. Mass spectrometric detection was achieved with a capillary voltage of 3.0 kV, cone voltage of 40 V, desolvation temperature 350° C, while desolvation and cone gas

flow rates were 650 and 45 L/h respectively. The tandem mass spectrometer (MS/MS) was operated in MS2+ scan mode between 400-950 m/z with a scan time of 0.5s, operated in high-resolution mode which is calibrated to a mass accuracy of 0.5 AMU in Q3. Quantification was performed on extracted ion chromatograms (EICs) of M^+ or $[M+H]^+$ depending on which of these ions was identified for each analytes data processed in centroid mode (Table 2). Several ethyl acetate adducts were identified for the late eluting c-chlorophylls and quantification was performed on a EIC channel of $[M+H]^+$ and $[M+EtAcOH]^+$. Limits of detection (LOD) for the MS were calculated using a minimum detectable peak area of 50,000 area units following Equation 1, response factors measured for each analyte, with the minimum detectable peak area was determined as approximately 5× S/N. Concentration factors are for a standard method of 100 mL of water concentrated onto a filter followed by extraction into 10 mL of extraction solvent.

$$LOD\ \left(\frac{\mu g}{L}\right) = \frac{Std.Conc.\left(\frac{mg}{L}\right) * \frac{Injection\ Volume(\mu L)}{1{,}000{,}000}}{Peak\ Area} * \frac{Minimum\ Detectable\ Area}{\frac{Injection\ Volume\ (\mu L)}{1{,}000}} *$$

$$Concentration\ Factor\ (10) * 1{,}000{,}000$$

Several of the unknown putative chlorophylls in samples were partially purified and characterized. 150 uL of the original *Caulerpa* MLP-22-77-3 extract was injected onto the column and a 3-minute fraction taken that contained the unknown chlorophylls and chlorophyll b. 20 uL of this semi-purified fraction was reinjected with an isocratic composition of 75:20:5 A:B:C at 0.4 mL/min.

Results

1. Notable Separations Achieved by this Method

Chromatographic separation of the ten chlorophylls and a suite of carotenoids was achieved with the gradient separation described in Table 1. Figure 2A shows the separation of a set of chlorophyll and carotenoid standards (Supplemental Text 1). Notable achievements in this methodology were the baseline separations of the c-chlorophylls, *c*1, *c*2, and *c*3, with the separation of these three chlorophylls having presented a considerable challenge over the previous three-decades (Mantoura and Llewellyn 1983; Zapata et al. 1987; Garrido and Zapata 1997; Jeffrey et al. 1999; Van Heukelem and Thomas 2001; Pniewski 2020). The system also separated the chlorophyll *a* and *b* analogs referred to in the literature as chl *a*′ or *b*′ and/or chl *a* or *b* allomer, and the MS analysis confirmed that the molecular weights for these compounds were identical to their parents, supporting that that these are isomeric and/or allomeric chlorophyll derivatives. Analysis of pheophorbide *a* showed significant and unique 'tailing' for the compound in standards and samples, where peak shape was more suggestive of the presence of two closely eluting peaks than 'normal' tailing, meaning it may be possible there are isomeric and abundant forms of this pigment. The same pattern could be seen in some putative pheophorbide *b* peaks that were identified

306 through ‘non-target’ extracted ion chromatograms as there were no available reference
307 materials. Aphanizophyll, diatoxanthin, and myxoxanthophyll are baseline separated in
308 standard analyses (Figure A.1), as well as fucoxanthin and peridinin (Figure A.2), and
309 prasinoxanthin and neoxanthin (Figure A.3). The method also baseline separated lutein
310 and zeaxanthin, a difficult isomeric pair to resolve, where there is considerable interest
311 in these compounds due to their importance in food and nutrition (Figures A.4 and A.5)
312 (Dachtler et al. 2001). Many isomeric forms of carotenoids were separated in natural
313 samples and standards by this method, including multiple isomers of fucoxanthin,
314 echinenone, and β-carotene (Figures 2 and A.6).
315
316 **2. Selection of Solvent System and Separation of the Chlorophylls**
317 The primary goal of our chromatographic system was to separate each
318 chlorophyll analog. The most critical chromatographic components for this separation
319 were the addition and maintenance of 5% water throughout most of the analytical run,

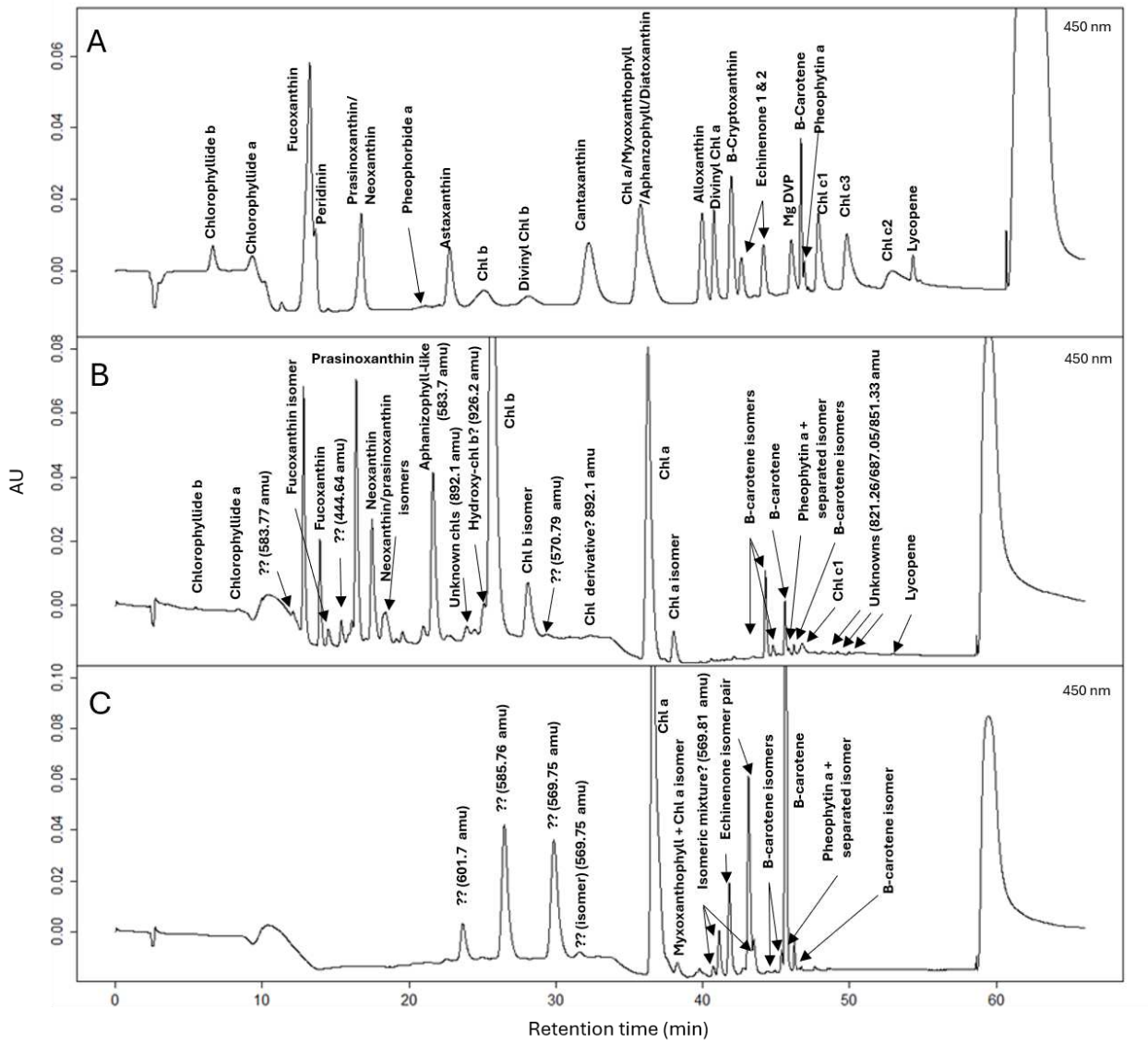


Figure 2. Demonstration of the achieved separation of chlorophylls and carotenoids using the YMC C30 column and novel chromatographic approach. Chromatographic data were collected by photodiode array with an extracted wavelength chromatogram at 450 nm to show chlorophylls and carotenoids. MS data were obtained concurrently with the chromatograms shown and was used for identification. Chromatograms at 665 nm to isolate chlorophylls from carotenoids are shown in Figure 3. (**A**) Pigment standards, (**B**) Chromatogram of an environmental sample, dominated by the genus *Caulerpa*. (**C)** Chromatogram of a cultured strain of *Microcystis* sp. BLCC-F108.

and the isocratic hold of A:B:C at 65:30:5 between 15-30 min. This solvent ratio was where maximum chromatographic selectivity for the *c*-chlorophylls, β-carotene, and pheophytin *a* was identified (Figures 2A, A.7 and A.8).

Changes to the amounts of ethyl acetate during the isocratic hold, or to any other steps of the gradient, which were trialed to increase throughput, led to negative impacts in the c-chlorophyll separation and therefore no further changes to the gradient steps were deemed valuable. Changes to the water percentage were tested but generally caused longer retention time, reduced selectivity and 'smooshed' most of the suite of analytes together, with much worse co-elution for most standards. Therefore, 5% water was deemed the optimum for looking at a broad suite of pigment analytes. A mixture of formic acid and ammonium hydroxide was required to produce good peak shape in the separation, where addition of ammonium hydroxide additive significantly sharpened chlorophyll and carotenoid peak shape in comparison to analyses containing formic acid only (data not shown). Formic acid was required with the non-endcapped stationary phase, as without it major peak broadening became evident. Additional details on the column selection and chemistry, solvent and gradient selection and its impact on the chromatography, and column temperature effects are described in Supplemental Text sections 2, 3 and 4.

3. Separating the Chlorophylls from the Carotenoids

This chromatographic and analytical method was designed to be applicable with or without a mass spectrometric detector. Chlorophyll compounds could be isolated from their carotenoid counterparts by integrating wavelength channels at 660 nm (or

the corresponding 642 λ-max for b-chlorophylls), where carotenoids do not absorb (Figures 3 and A.7). For divinyl chlorophyll *a* and *b*, chlorophyllide *a* and *b*,

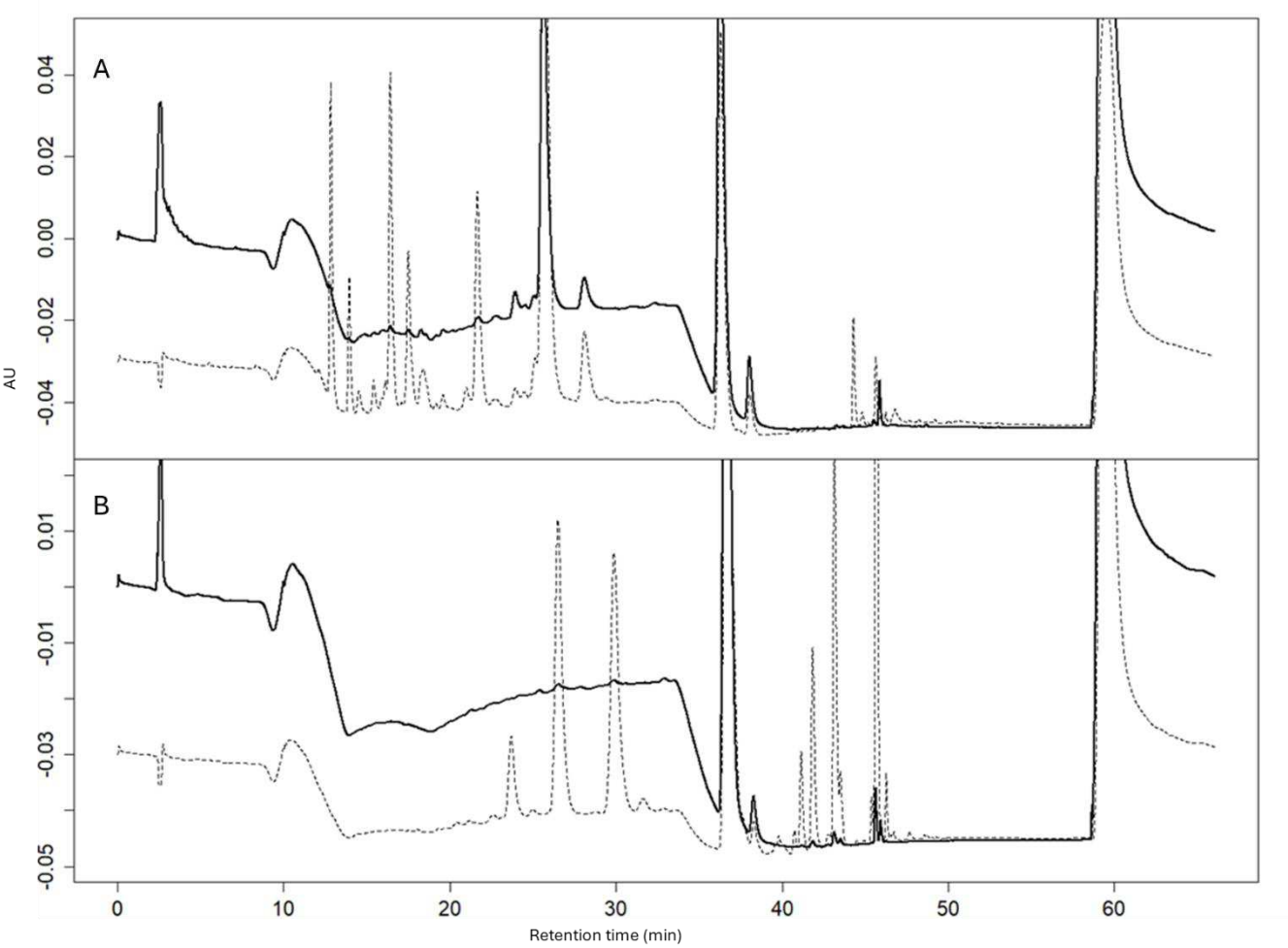


Figure 3. Demonstration of the isolation of chlorophylls from carotenoids using separate extracted wavelength channels from photodiode array data for chlorophylls (665 nm*, thick black lines) and carotenoids (450 nm, dotted line). The wavelength selection can be used to isolate chlorophyll analytes from interferences when compounds were not chromatographically separated. (**A**) Chromatogram of an environmental *Caulerpa* sample, (**C)** Chromatogram of cultured *Microcystis* sp. BLCC-F108 *665 nm was extracted rather than 660 due to the deuterium lamp signal dropout at 660 nm.

pheophorbide *a*, and chlorophyll *a* and *b* there were no interfering pigments at 665 nm (Figures 2 and 3), however, these pigments interfered with some carotenoids at 450 nm.

The chlorophyll *c*'s did not have a strong absorbance in the 600 nm region with the detectors used here, and therefore we aimed to fully separate the chlorophyll *c*'s

from co-eluting carotenoid interferences so that they could be detected and quantified at 450 nm. Specific separation schemes were required to achieve baseline resolution for the three *c*-chlorophylls. Initial development fully separated chlorophyll *c*1, *c*2, and *c*3 from β-carotene, while pheophytin *a* coeluted with these 4 compounds but did not interfere at 450 nm (Figures 2A and A.8). It was notable that our separation had *c*-chlorophylls amongst the latest eluting compounds in the chromatographic separation, in contrast to every other method in the literature that was examined, where capacity factors for the *c*-chlorophyll's were found to be relatively low with, at times, poor selectivity and resolution with each other and with other analytes, where many methods reported on the sum of two or more of these chlorophylls (Jeffrey et al. 1999).

4. Incorporation of Mass Spectrometry

Although the system can be operated for chlorophylls and select carotenoids with only a PDA detector, incorporating mass spectrometry added considerable value by enabling partial structural identification, greater analyte specificity, better identifying of isomeric pigment analogs, separation and quantification of closely or co-eluting compounds with different molecular weights, and significantly improved limits of detection (LOD) (Table 2). Initial analyses of standards supported this; up to 10 isomers were identified in nominally 'pure' carotenoid reference materials, at levels too low for PDA detectors to detect the isomers (Figure A.6). In other cases, similar

structures (e.g. echinenone and 3-hydroxyechinenone) could be distinguished from one another even as they were found to fully coelute. In analyses of complex mixtures of pigments, the majority of EICs were clear of co-eluting contaminants, many of which were not pigments, while the chromatograms rarely contained multiple pigment compounds compared to the PDA traces which naturally showed many pigments in the same wavelength channel.

The sensitivity of the mass spectrometric detector was significantly improved over photometric detection, with limits of detection (LODs) 10-100× lower than those achieved by visible light detection with a PDA detector (Table 2). MS inlet conditions (e.g. needle and cone voltages) did not substantially affect ionization efficiency (e.g. orders of magnitude), and by extension sensitivity, for most compounds. The exception was for the c-chlorophyll's whose responses depended more strongly upon tune conditions, likely due to the carboxylic acid functional group; negative mode ionization may be viable option for the detection of these analytes. No unique tuning parameters were introduced for the chlorophyll *c* compounds in this method, as the sensitivities were acceptable for our purposes using the baseline voltages.

5. Method Performance

Quantitation by this method can be performed well with strong linear relationships for most analytes (Table 2). Only for the c-chlorophylls, with significantly

increased tailing during development, did a notable deviation in linearity appear, where chlorophyll c2 could not be integrated when performing linearity injections due to excessive tailing. Method detection limits for the Agilent PDA-only HPLC system were all below 1 ppb when using a 100 µL injection and assuming a concentration factor of 10 from a lake water sample concentrated onto a filter (Table 2). Detection limits were significantly improved for the MS over the PDA, where 10 µL injections gave MDLs more than low enough for the majority of research questions.

Table 2. Linearity and detection limit information for each of the analytes evaluated in this method. The first two method detection limits (MDLs) represent the MS LOD depending on the injection volume, representing the detection limit of a 100 mL water sample concentrated on a filter and extracted in 10 mL of 90% acetone, assuming 100% recovery of analyte through this standard extraction method. While the MS system was operated with a 10 µL injection, the chromatography does not overly suffer with injection volumes higher than this and therefore we show an LOD at a 100 µL injection volume for comparison. The PDA MDL was determined on the Agilent HPLC system using the same extraction and separation protocol with a 100 µL injection volume.

Pigment Name	Slope	R^2	Target Mass	MDL (10 µL inj. volume) (µg/L)	MDL (100 µL inj. volume) (µg/L)	PDA MDL 100 µL inj. (µg/L)
Alloxanthin	11139.79	0.998	564.7	0.54	0.05	NA
Aphanizophyll	24342.27	0.998	583.7	0.91	0.09	0.25
Astaxanthin	183878.8	1.000	597.7	0.04	0.004	0.21
b-cryptoxanthin	43951.78	1.000	552.7	0.31	0.03	0.26
Beta Carotene	43917.06	0.991	536.7	0.51	0.05	0.1
Cantaxanthin	207418.1	1.000	565.7	0.037	0.004	0.18
Diatoxanthin	17486	1.000	567.7	0.87	0.09	0.12
Echinenone 1	175217	1.000	551.7	0.018	0.002	0.8
Echinenone 2	257401.2	0.999	551.7	0.018	0.002	0.2
Fucoxanthin	351564.7	1.000	659.8	0.027	0.003	0.18
Lutein	26044.24	0.997	568.7 + 551.7	0.25	0.025	0.09
Lycopene	45181.7	0.994	553.7 + 536.7	0.20	0.02	0.17
Myxoxanthophyll	21495.8	0.999	730.9 + 567.7	2.35	0.24	0.28
Neoxanthin	12985.51	0.999	601.8	0.82	0.08	0.13

Peridinin	40436.83	0.999	631.7	0.23	0.02	0.25
Prasinoxanthin	99412.3	1.000	601.8	0.15	0.02	0.11
Chl a	15155.42	0.994	894.1	0.64	0.06	0.59
Chl b	85005.42	0.971	908.1	1.61	0.16	1.0
Chl c1	22665.75	0.991	611.6+699.7	0.53	0.05	0.32
Chl c3	25076.76	0.991	741.7+653.6	1.29	0.13	0.25
Chlorophyllide b	4069.208	0.969	629.6	1.15	0.12	0.25
DV Chl a	9207.111	0.996	892.1	0.95	0.09	0.9
DV Chl b	4285.585	0.992	906.1	1.30	0.13	0.85
Mg DVP	54690.54	0.998	611.6+699.7	0.11	0.01	NA
Pheophorbide a	412553.6	1.000	593.6	0.003	0.0003	0.76
Pheophytin a*	270818	0.989	872.1	0.10	0.01	0.79

406 *Pheophytin a linearity was determined from four points not five.
407

408 Pigments in *Cladophora* samples were quantified using extracted ion
409 chromatograms and evaluated for both precision and quantitative accuracy.
410 Photometric quantitation has been the gold-standard for HPLC pigment analyses for
411 decades, and over the course of the analytical run (~30 hours) standard errors between
412 standards run at the beginning and end of the run were 2% for the PDA detector
413 (Figure A.9). (This average does not include beta-carotene as both the MS and PDA had
414 ~25-30% error suggesting there was an issue with the injection and not the method). By
415 comparison, standard errors for MS analyses were 9%– this was not due to the MS
416 source becoming dirty, as the responses for the second suite of calibration standards
417 had increased rather than decreased over the course of the sample set. The impact of
418 sample matrix effects on analyte response (and quantitative accuracy) were further
419 evaluated by determining the PDA/MS ratios for pheophytin a, beta carotene,
420 chlorophyll a, and fucoxanthin (for the *Cladophora* strains*)* in each sample analyzed.

421 Over the linear range of both detectors this ratio should remain consistent. While there
422 was small deviation at the high and low end of concentrations, the response of both
423 instruments was strongly linear when comparing across sample injections, with R^2 for
424 the four pigments being 0.98, 0.98, 0.97, and 0.98 (Figure A.10). Deviation from linearity
425 occurred at high concentrations in some of the cyanobacterial strains analyzed (e.g., MS
426 response exceeded the linear range), but the MS/PDA response ratios remained mostly
427 linear in the *Cladophora* samples, where pigments were quantified. The implication
428 being that on average the sample matrix was not heavily impacting MS ionization, and
429 by extension quantitation accuracy, across the suite of complex cultured and
430 environmental samples measured in this study.
431
432 **6. Identification and Characterization of Novel Pigments**
433 This study demonstrates for the first time that "echinenone" (CAS 432-68-8), as it
434 has been described in the literature and chemical databases (Schlüter et al. 2006), is
435 actually an epimeric pair, which were identified in cultured cyanobacteria and in
436 *Cladophora* samples that likely contained trace amounts of epiphytic or mixed
437 cyanobacteria. The two primary echinenone isomers were also identified in 'pure'
438 echinenone isolate used for our standard, alongside 6-7+ additional isomers that shared
439 the 551.7 AMU $[M+H]^+$ with echinenone in that same standard (Figure A.6). No samples
440 or standards that have been analyzed in this chromatographic system have ever

441 contained only a single echinenone peak. In some samples there was approximately an
442 equimolar mixture of geometric isomers, however there was marked variability
443 observed between samples/cultures, with the ratio of isomers in the *Microcystis* sp.
444 BLCC-F108 culture (Figure 2C) significantly different from that of the standard (Figure
445 A.6). It was not determined whether the ratios of echinenone could be manipulated
446 during the extraction (e.g. whether temperature change could interconvert between the
447 isomers), but every sample was handled identically throughout extraction and analysis,
448 meaning similar conditions between samples to allow for thermodynamic equilibration.
449 This discovery may have important implications for cyanobacterial photosynthesis due
450 to the presence of echinenone in the orange carotenoid protein (Liguori et al. 2024;
451 Chrupková et al. 2024; Niziński et al. 2025). In addition to echinenone, the molecular ion
452 of 3-OH echinenone was detected in the majority of samples and in our standards co-
453 eluting with echinenone, with this carotenoid derivative also consisting of a pair of
454 isomers rather than as a singular compound.

455 *Caulerpa* and *Planktothricoides* sp. BLCC-F182 both contained putative
456 chlorophylls that may be novel, and are some of the first since the detection and
457 characterization of chlorophyll *f* in 2010 (Chen et al. 2010, 2012). There are several
458 compounds visible in these strains, including what may be a hydroxy- derivative of
459 chlorophyll *b* and a second chlorophyll derivative based on their molecular weights,
460 UV-Vis spectra, and their relative retention times. There are several lines of evidence for

these unknown compounds being chlorophylls: their molecular weights were ~892 (~M-15) and ~926 (~M+18) AMU (referenced against an $[M+H]^+$ of 907.7 for chlorophyll b), heavier than most carotenoids; these compounds shared a peak shape with other chlorophylls and had the distinctive tailing of chlorophylls compared to the gaussian shape of carotenoids (Figure A.11); and the 926 AMU compound eluted very closely to chlorophyll b. The compounds absorbed strongly in the 600 nm range where carotenoids do not absorb (Figure 3), but also had significant differences in their absorbances at 645 and 665, which can be used to help differentiate between chlorophylls a and b (Figure A.12). The putative hydroxychlorophyll b analog had a weaker absorbance at 665, to the point where it was almost indistinguishable from the other co-eluting chlorophyll compounds, but became far more distinct at 645, closer to the λ-max of chlorophyll b. The other unknown chlorophyll at 23.93 remained largely unchanged between both wavelengths. It was not possible to confidently derive a molecular weight or photometric information about the putative chlorophyll visible on the chromatogram at ~24.1 min due to its low abundance.

Mass spectra of the 892 AMU peak showed the parent alongside a series of isotopes (Figure A.13). There were some contaminant molecular weights from 908-913 AMU, but the isotope pattern for chlorophyll b compared to these contaminants and their dissimilarity strongly suggested that this compound was not a chlorophyll b isomer (Figure A.14). Figure A.15 shows the mass spectra of the putative

hydroxychlorophyll b analog; some 908.1 AMU compounds are present in this spectrum because of its co-elution with chlorophyll b, while there are also molecular weights around ~937 AMU in this spectrum, but EICs of this compound did not perfectly align with the PDA spectra and so it is unlikely that these were the correct molecular weights for the unknown.

A partially purified fraction containing the unknown chlorophylls and chlorophyll *b* was obtained, and these compounds were chromatographically separated using a slightly modified isocratic elution to examine the spectra of each individually. The mass spectra of each purified compound are shown in Figures A.16 and A.17, and the PDA spectral traces are shown in Figure A.18. The molecular weights were comparable between the original analysis and the purified extract, while the higher molecular weights in the 926 AMU chlorophyll (those around 937 AMU) were no longer present in the purified fraction. The UV-Vis spectra of the partially purified compounds supports the original observations (Figure A.10): the putative hydroxychlorophyll had a much stronger absorbance around 648 nm, while the 892 AMU compound had a spectrum more closely resembling a chlorophyll *a* analog. These absorbance spectra should not be over-interpreted because the wavelengths around 660 nm are imputed rather than measured due to the deuterium lamp in the detector.

7. Chromatographic Application to Complex Samples

Chromatograms of natural samples in Figure 2 panels B and C and Figure 3 show the resolving power of the designed separation in natural biomass of *Caulerpa* and of *Microcystis* sp. BLCC-F108. Reference materials were unavailable for many of the compounds identified in these samples, but putative identifications, or suspect molecular weights, could be established. Misidentification of non-pigment interferences were mitigated by utilizing the PDA; PDA chromatographic data were overlaid with MS data and EICs were searched for peaks where the retention times were identical between the two channels. For high abundance pigments this was straightforward, and molecular weights were assigned with minimal investigation. Identifying trace pigments was more challenging because some analytes had several plausible molecular weights consistent with retention times. However, many peaks in the EIC's were not perfectly aligned by retention time and therefore many were eliminated as plausible analytes, as they had to have been paired for them to represent the same compound. With a library of known molecular weights, standards, and the chromatographic data, most peaks could be assigned a putative molecular weight or, where possible, a compound name. When pigment concentrations were below the PDA LOD, putative identification was made by MS alone using retention time and molecular mass. The approach was applied to pigments lacking reference materials and to pigments with

519 known molecular weights and well-estimated of retention times (e.g., pheophorbide *b*,
520 chlorophyllide *a* etc.).
521 More than six isomeric forms of some pigments were separated and identified in
522 these samples. These low concentration pigments rarely produced visible spectra strong
523 and precise enough for identification, but they were clearly resolved from background
524 in EICs. Molecular weight data, combined with PDA spectra, confirmed that these
525 peaks were most likely isomers of more abundant and closely eluting pigments. These
526 isomers often co-eluted with other pigment analytes and would have interfered with
527 PDA quantification, but they could be isolated and evaluated independently by the MS.
528 While there were some chlorophylls with co-eluting carotenoid interferences at
529 450 nm, in the 600 nm range the known chlorophylls were separated from carotenoid
530 interferences as carotenoids largely do not absorb above ~510 nm. Each of the
531 chlorophyll analogs for which standards were available was baseline separated, making
532 this method applicable for a system containing only a PDA detector (Figure 3). Some
533 carotenoids were found to co-elute, but the level of interference from this co-elution
534 depended on the mixture of carotenoids in each sample, where many of these
535 compounds may not regularly co-occur.
536

8. Analysis of Cyanobacterial Pigment Profiles

This method was applied towards investigating the pigment profiles of multiple cyanobacterial genera including several strains of *Microcystis*, and one novel genus yet to be described. Nine strains of cyanobacteria were evaluated and several differences in their pigment profiles were identified (Figure 4). Pigments were not quantified in these samples as growth and irradiance conditions can significantly impact pigment ratios, and these cultures were not grown at the same time in the same chamber, nor were they harvested at the same time or in a manner to make sure they were at the same growth stage. The following analyses qualitatively assessed the presence and absence of individual pigment analytes between strains.

Multiple carotenoids were identified across each of the strains of cyanobacteria, but there were several that were present in some strains and notably absent in others (Figure 4). The *Microcystis* sp. BLCC-F159 strain produced two carotenoids with masses of 601.7 and 584.8 AMU, where only the latter was shared with *Microcystis* sp. BLCC-F209. Several carotenoids were found to elute closely to chlorophyll a, including cantaxanthin, the carotenoid at 583.7 AMU ('aphanizophyll-like), and myxoxanthophyll, which were difficult to distinguish chromatographically and photometrically. However, the addition of the MS made differentiation trivial. Only four cyanobacterial strains: *Scytonema* sp. BLCC-T1, Unknown heterocytous sp. BLCC-F213, *Raphidiopsis* sp. BLCC-F278, and *Iningainema* sp. BLCC-F205 contained mixtures of

these three compounds. Myxoxanthophyll has commonly been employed as a pigment marker for the presence of cyanobacteria, but several of these strains, of which the genera can be important producers of cyanotoxins and can form harmful algal blooms, did not produce the carotenoid.

A different ratio of the two echinenone isomers was measured in *Microcystis* sp. BLCC-F209 compared with the other *Microcystis* strains that were analyzed; the two

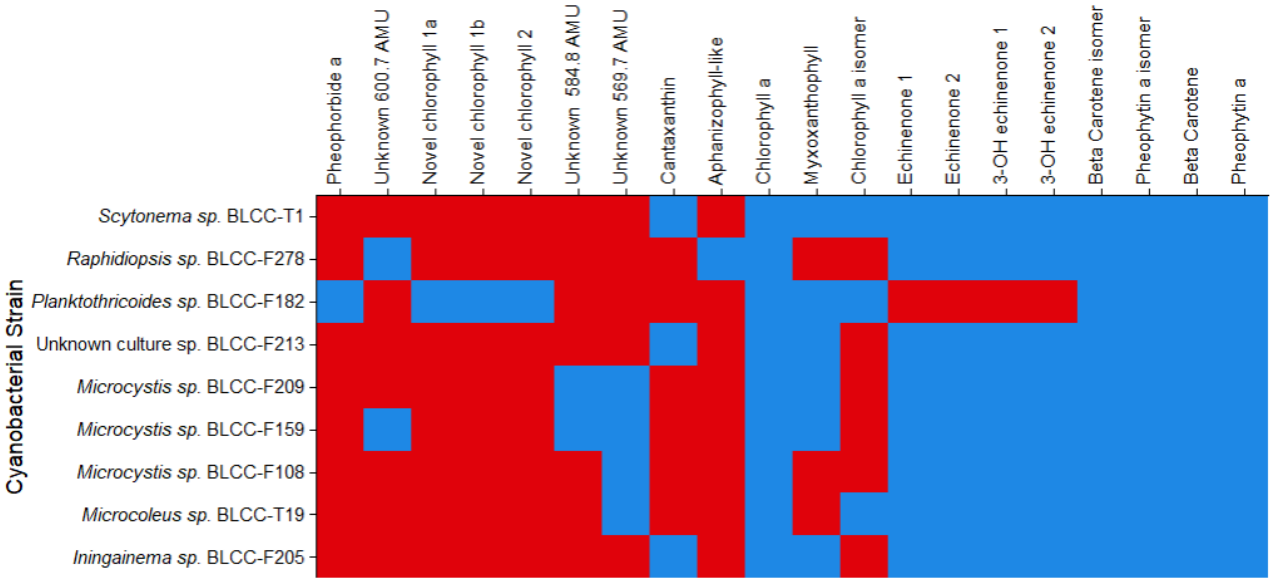


Figure 4. High-abundance pigments present in different cyanobacterial genera and strains. Blue colors represent a compound being present, while red meant the compound was absent. Compounds are ordered based on elution time (early eluting compounds on the left). There were a small number of other trace pigments present in some samples but at abundances too small to reliably identify the parent compound and to compare between samples. Pigments for which there were no standards were reported as unknowns and we report their putative molecular weights – the individual compounds we show were shared between the strains and contained shared molecular weights and retention times. Some pigments not shown in this figure are discussed in the text. PDA chromatograms at 450 nm for each strain are shown as Supplemental Chromatograms.

Microcystis strains BLCC-F108 and BLCC-F159 contained more of isomer 2, and conversely BLCC-F209 contained more of echinenone isomer 1. Unknown heterocytous species BLCC-F213 also contained more of echinenone isomer 1 than isomer 2, but interestingly there may have been a myxoxanthophyll isomer that coeluted at the same

567 retention time as echinenone 1 which impacted the apparent ratio (see supplemental
568 chromatograms). The MS spectral pattern for this particular carotenoid was extremely
569 diagnostic; myxoxanthophyll contains a sugar moiety that fragmented in the ionization
570 source, leading to paired 730.9 and 567.7 AMU peaks in its mass spectra. These two
571 fragments were always present, with the latter being the dominant peak, and the two
572 peaks in the MS spectra were highly suggestive of a myxoxanthophyll isomer being
573 present alongside the parent compound that co-eluted with echinenone 1 in this strain.
574 Chlorophyll isomers (often reported as chl a' or chl allomer in the literature)
575 were identified only in some of the cyanobacterial strains, including *Scytonema* sp.
576 BLCC-T1, *Planktothricoides* sp. BLCC-F182, and *Microcoleus* sp. BLCC-T19. This isomer
577 had a very distinct retention time from that of chlorophyll a, was not found in all
578 samples, and was identified in abundances that make it unlikely to be an artifact of the
579 method and/or extraction. This compound shared the molecular weight and its isotope
580 pattern with chlorophyll a, supporting that it is a chlorophyll a derivative (Walker et al.
581 2003; Steele et al. 2018). The presence of this compound in only some strains warrants
582 further investigation.
583 Several unusual chlorophylls were identified in *Planktothricoides* sp. BLCC-F182,
584 where it was determined that these were likely chlorophyll analogs based on their
585 absorbance spectra at 665 nm and molecular weights. These compounds had high
586 similarity to those we identified in *Caulerpa*, described previously, with many shared

molecular weights and retention times. Notably, pheophorbide a was detected in this strain of *Planktothricoides*, which was not found in any of the other cyanobacterial strains. This strain was also found to not contain echinenone or 3-OH echinenone, which are usually important (and common) accessory pigments in 'normal' cyanobacterial photosynthesis, and which were identified in very high abundance in every other cyanobacterial strain in Figure 4.

9. Evaluation of *Cladophora* Pigment Profiles from the Great Lakes

Algal taxa can vary across ecoregions, and *Cladophora* pigment profiles and concentrations were evaluated in a case study to investigate variability in populations collected from two Great Lakes: Lake Michigan (MWA) and Lake Huron (HTR). There was a diverse but consistent pigment profile for each of the *Cladophora* samples, with the same pigments identified of trace and high concentration analytes in all samples (Figure 5). There was a group of three closely eluting pigments, including chlorophyll b, lutein, and a compound at 583.7 m/z for which no standard was available, but which we putatively identified as possibly being siphonaxanthin following in source fragmentation introducing a loss of –OH (Yoshii et al. 2004; Sugawara et al. 2014). While these two carotenoids co-eluted, we could quantify them separately and without interference using EICs.

606 The presence of fucoxanthin, and the structural isomers prasinoxanthin and
607 neoxanthin were confirmed in these strains, and it was further determined that each of
608 the trace compounds eluting near these latter two analytes were isomeric forms of
609 fucoxanthin through non-target EIC identification of fucoxanthin's m/z. Similarly, co-
610 elution with these trace isomers did not interfere with quantification because each
611 compound could be isolated from contaminants and quantified by MS. Trace quantities
612 of echinenone were identified in each sample that were barely above the LOD for the
613 PDA, but that were identified with ease by MS, suggesting the presence of one or more
614 cyanobacterium associated with the *Cladophora* blooms. The abundance of these
615 echinenone isomers was low enough that PDA detection alone could not identify their
616 presence with certainty.
617 Concentrations of the pigments were generally highly consistent, where ratios of
618 each pigment compound to chlorophyll remained between 1-2 across samples.
619 However, there was significant deviation for some pigment analytes, where Figure 5
620 shows MWA-3 had much higher concentrations of accessory pigments (ratios 4-5) while
621 chlorophylls a and b remained relatively constant. HTR CKD 2 contained much less of
622 the early eluting pigments (pigment/chl a ratios 0-1) and several late eluting pigments
623 relative to the other samples, while several other Lake Huron samples had lower ratios
624 of several individual pigments. HTR CEM6 and CEMI HTR both had lower

625 concentrations of echinenone, suggesting that there may have been a smaller

626 cyanobacterial ensemble associated with these samples.

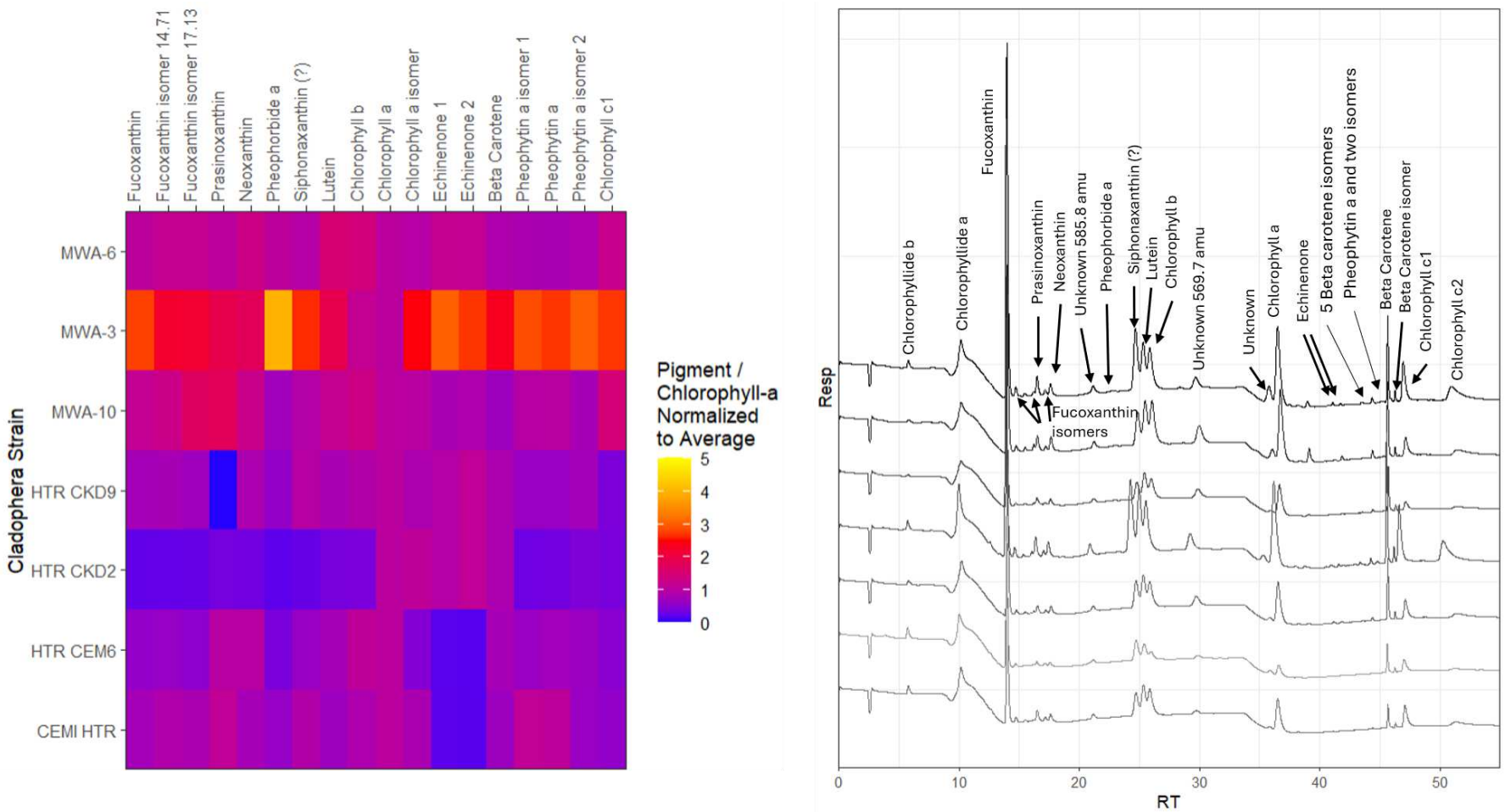


Figure 5. Quantitative evaluation of the pigments identified in *Cladophora* samples. HTR samples were collected from Lake Huron and MWA samples from Lake Michigan. Each pigment was quantified using purchased reference materials. The pigments without reference materials were not included in the left plot, with the exception of the suspected siphonaxanthin analyte because this compound was found in high abundance, which we quantified using standard as the lutein. Comparison of relative concentrations for siphonaxanthin are therefore appropriate, but the true concentrations for this carotenoid reported here could not be determined without a reference standard. Concentrations were determined for each pigment and then taken as a ratio against chlorophyll a. To evaluate changes in pigment ratio between compounds, the average pigment/chl ratio for each compound was determined across all samples, and the concentration for an individual sample normalized against this average. Each individual compound is shown on the right from photodiode array chromatograms at 450 nm where the contribution of each pigment analyte to the total can be seen by the chromatographic peak size. Quantitative data are shown in Figure A.19.

Discussion

A novel chromatographic separation scheme for the combined analysis of chlorophylls and carotenoids is reported here. This methodology was designed from the ground up as the standard chromatographic techniques, with the fundamentals developed decades ago and principally still in use today (Reyna et al. 2017; Latasa et al. 2023), are largely incompatible with mass spectrometric (MS) detection. Through this developmental process the aim was to achieve a highly selective chromatographic separation capable of being implemented on an HPLC system containing only a PDA detector, but that could also be connected into an MS system to incorporate more comprehensive structural analysis for specific compound identification. The method was demonstrated to be able to separate and identify dozens of compounds in highly complex samples, to detect multiple novel chlorophyll analogs in marine and freshwater algae and cyanobacteria, and identify that echinenone is composed of a mixture of isomers that have not been reported to be anything but a single compound in previous research (Schlüter et al. 2006) using this chromatographic methodology.

The separation was designed to use the relatively non-toxic and "green" solvents: methanol and ethyl acetate. Ethyl acetate, which has very low toxicity, is less irritating to the nose, and is rapidly decomposed in the body and in the environment to non-toxic compounds (ethanol and acetic acid). Many methods using the C30 column

stationary phase for separation have used methyl tert-butyl ether (MTBE) as the reversed phase non-polar 'favorable' elution solvent (Van Heukelem and Thomas 2001; Bijttebier et al. 2014). Alongside being an 'unusual' HPLC solvent in the modern era, MTBE is a well-known environmental contaminant and there are some potential human health risks from exposure, making it less than optimal.

Using a C30 phase in this method has substantially changed the retention of pigments that have rarely been examined on this stationary phase (Jeffrey et al. 1999). Historically, the *c*-chlorophylls have been difficult to separate and were often found to elute early with little stationary phase retention. In the separation described here, the *c*-chlorophylls were among the most strongly retained/highest capacity factor analytes, which we hypothesize is due to interactions between free silanol groups on the silica with carboxylic acid moiety of the *c*-chlorophyll's, leading to long retention times from their non-polar character alongside strong dipole-moment interactions. As part of this, it is possible that in this solvent system the carboxylic acid functional groups of these chlorophyll derivatives were protonated through changes in 'pH' (noting that the system is mostly non-aqueous) and/or microenvironments that led to unfavorable charge formation when bound to the highly-nonpolar stationary phase, whereas in previous studies using C18 columns the carboxylic acid may have deprotonated which led to the observed lower capacity factors.

667 The lack of endcapping on the YMC column increased analyte selectivity, but
668 also caused peak tailing for the chlorophyll analogs. Binding of these analytes to free
669 silanol groups is hypothesized to have produced broader peaks relative to their
670 carotenoid counterparts. Carotenoids by comparison have many delocalized electrons
671 and few highly polar or acidic functional groups; London forces dominate their
672 intermolecular interactions, so they would interact only weakly with silanol groups of
673 the stationary phase. In contrast, the chlorophylls *c*'s likely have much stronger dipole
674 moments than the carotenoids and, provided they do not become ionized, they could
675 bind strongly to the silica.
676 Incorporation of MS detection was the crucial advancement in our methodology
677 and several crucial decisions in our approach differed from MS-based methods reported
678 by others. The instrument was operated in scan mode as the effectiveness of the
679 chromatographic separation combined with the many molecular weights meant that the
680 system could remain highly selective even without including fragmentation in multiple
681 reaction monitoring (MRM), meaning the system could then be used for 'non-target'
682 analysis, where we knew at the outset of this study that we would not have standards
683 for many of the pigments that would be detected. While MRM methods have been
684 reported before, such as by Zhang et al., these methods can only detect a very specific
685 set of pigments (Zhang et al. 2016). MRM methods (in triple quadrupole MS/MS
686 systems) can only detect compounds selected *a priori* by the analyst, and generally

require standards for tuning the instrument and to determine the appropriate daughter fragments to use for detection. The pigment standards tested here all ionized well under the same instrumental conditions, meaning that it is likely the majority of pigments are also likely to efficiently ionize and that the system can detect and putatively identify many trace pigments by mass spectrometry, even those where there are no reference materials. The LODs outlined in Table 2 support this, where there were low ppb or parts per trillion detection limits after accounting for relatively minimal (10×) concentration factors.

MS MRM methods are hampered by the fact that many carotenoids undergo in-source fragmentation. The chemical and structural mechanisms for their ionization are non-trivial, with different molecular ions identified depending on the specific analyte. Our MS conditions led to some analytes ionizing to M^+, rather than the more standard $[M+H]^+$ most-commonly observed in electrospray ionization, which was also reported by Bijttebier et al. (Bijttebier et al. 2013). Other carotenoids had either losses or gains of a hydroxy -OH (17 AMU), while others lost or gained H_2O (18 AMU) due to in-source fragmentation/oxidation, which has also been reported previously (Dachtler et al. 2001; Bijttebier et al. 2013). A majority of myxoxanthophyll molecules lost their glycol-functional group, with a resulting molecular weight of 567.7 AMU, alongside correspondingly poor sensitivity when compared to other carotenoids.

There are also many isotopes for chlorophylls, which could be missed by targeted MS methods only looking for a very specific molecular weight. Isotopic peaks for chlorophylls were common due to abundant isotopes of magnesium (21% are ^{25}Mg and ^{26}Mg isotopes) while ^{15}N and ^{13}C isotopes also contributed to the observed spectral patterns. The isotopes present in some analytes interfered with other analyte EIC channels, such as similar molecular weight carotenoids or the divinyl chlorophylls which are 2 AMU lower than the chlorophyll counterparts. Care was necessary to avoid identifying analytes that were not present but were isotopic artifacts. By using MS scan, these instrumental complexities could be accounted for because full scan spectral data was collected for every pigment peak in each chromatogram. This also offered significant advantages for post-hoc analyses, where samples could be reprocessed after-the-fact to look for new pigments, rather than requiring a full reanalysis of the sample using a different or modified acquisition method.

This novel separation and detection methodology was applied to several cyanobacterial and green algal test materials. In several cyanobacterial strains a complex pattern of presence and absence of analytes between strains was observed, where there were several carotenoids present, for which we did not have reference materials. The similarity (or differences) between pigments with similar retention times could be confirmed with high certainty using the MS by comparison of m/z's and isotope patterns for the unknown pigments, which can be difficult to assess with PDA

726 spectra alone. Retention times were very stable, but even small shifts of only a few
727 seconds can make it difficult to ascertain with certainty the similarity between strains
728 when there are many closely eluting compounds. For example, in cyanobacterial strains
729 cantaxanthin (565.7 AMU) was identified in several samples (Figure 4). However, this
730 peak was relatively broad and there were other pigments in this retention time range
731 that could have been mistakenly identified based on retention time and spectral
732 analyses alone but can confidently be reported to not be cantaxanthin as the parent
733 molecular weight was absent. Further analysis confirmed that the closely eluting peaks
734 were three separate compounds, where one of these compounds shared a molecular
735 weight with aphanizophyll, alongside another unknown at 569.7 AMU.
736 While pigment profiles have been evaluated for different marine algal classes
737 (Schlüter et al. 2000) and freshwater organisms including cyanobacteria (Schlüter et al.
738 2006) for application in chemical taxonomy (ChemTAX), these methods have looked for
739 very specific pigments based on shared production amongst a class, where certain
740 pigments are absent from other algal groups and/or classes. Decades of literature have
741 repeatedly demonstrated how complex pigment profiles can be, and our analyses
742 reaffirm this fact. Modern information technology infrastructure and technological
743 advancement in computer science have repeatedly demonstrated the power of machine
744 learning for pattern identification (Mrdjen et al. 2025), which to our knowledge has only
745 been applied recently to integrate a new analytical modeling improvement to

ChemTAX (Hayward et al. 2023). Newer and advanced pattern identification models could take advantage of the complex pigment profiles of different algae for identification.

More advanced input data to these models (from improved analytical methodology) is a limiting factor and advanced measurement methodology could offer more comprehensive chemical identification of phytoplankton. Advanced fingerprinting techniques, common in omics-style analyses, may become more applicable when advanced instrumental spectral data can be incorporated into these models. The chromatographic method designed in this study offered similarly selective chromatographic separation in comparison to the HPLC-PDA methods commonly in use at the time of publication, while adding highly specific structural identification and analyte sensitivity from the addition of MS detection. Pairing selective chromatographic separation with MS detection offers the capability to stringently assess pigment profiles across taxa in ways that have not been available, which could add the advanced data needed to make ChemTAX models more precise.

The *Cladophora* analyses demonstrated the value that MS detection adds to algal analysis, where there may be many closely eluting or co-eluting compounds. Rather than requiring redevelopment of the separation quantify these pigments precisely, quantitation was simplified because MS could fully resolve the co-eluting pigments by mass-to-charge. While chlorophyll *b* can be quantified independently of the two closely

766 co-eluting carotenoids using a longer wavelength, this would not be used to differentiate
767 lutein and siphonaxanthin. Close to a dozen trace pigment isomers that were near or well
768 below LODs for the PDA were also separated and identified by utilizing the MS detector.
769 These isomers sometimes eluted alongside other higher abundance pigments (e.g.
770 prasinoxanthin and neoxanthin) where quantification was important, but could be
771 isolated by mass so that they would not interfere with MS quantitative analyses.

772 Remote sensing has been widely used to monitor large waterbodies and blooms
773 that are difficult or impossible to assess by traditional sampling techniques (Kutser
774 2004; Gower et al. 2005; Wynne et al. 2008, 2010; O'Donnell et al. 2013; Seegers et al.
775 2021; Hou 2022). These measurements often rely on the reflectance from photosystem I
776 and/or II complexes, which can incorporate many pigments, including pheophytin a
777 and various carotenoids (Guskov et al. 2009), which vary between organisms and
778 classes. Some of these techniques require ground-truthing of satellite reflectance data
779 with analytical measurements of pigments to be quantitative (Tomlinson et al. 2016).
780 Fluorescence methods also often use formulae to correct for pheophytin *a*, chlorophyll *b*,
781 and other pigments, but these corrections can be complicated by environmental
782 variability, because their accuracy may change when the mixtures of pigments change
783 in the samples. In addition, the reflectance measurements are made on the intact
784 photosystems, which have different optical properties (absorbance maxima at 680+ nm)
785 than pure chlorophyll a (660 nm), so some studies need to evaluate the whole pigment

786 profiles of an organism or system and understand how accessory pigments impact the
787 optical properties of the photosynthetic apparatus in different organisms. While this
788 study did not pair remote sensed data with HPLC-PDA-MS analysis, there were distinct
789 differences in the pigment profile ratios of samples of *Cladophora*, even within 'similar'
790 populations, which may be worthy of further study coupling HPLC analysis with
791 spectral sensing techniques. The differences between *Cladophora* pigment profiles made
792 it clear that direct measurements of many pigments could be important for calibrating
793 visible light and optical detection techniques, as there was unsurprisingly inherent
794 heterogeneity measured in these photosynthetic organisms. This HPLC-PDA-MS
795 methodology offers a new approach towards quantitative holistic chemical analyses of
796 algal pigments, using either visible light or mass spectrometric detection.

797 There was slightly more analytical imprecision introduced by the MS detector
798 versus the PDA detector (9% standard error versus 2%, respectively). This difference was
799 introduced by the MS, where response in the detector derives from the chemical and
800 physical properties of each analyte, where sensitivity can be principally impacted by the
801 ionization efficiency of the analyte in combination with the matrix surrounding the
802 analyte as it enters the electrospray (or other ionization technique) source. MS systems
803 may therefore require more stringent standardization to deal with variability in the
804 ionization of analytes. For many scientific measurements, the quantitative accuracy
805 achieved here would not impact the results or conclusions. For the data presented in

806 Figure 5, the differences in pigment ratios were orders of magnitude different among
807 strains, so the 9% average error across all of the standards' response factors was
808 inconsequential. However, there is no reason why quantitation cannot be performed
809 using a mixture of PDA and MS data, or by relying on the MS primarily for identification
810 and structural confirmation and the PDA for quantification, in cases where analytical
811 error must be minimized. This method framework was designed to be flexible, allowing
812 modification and adjustment based on the needs of the analyst, including being able to
813 change injection volume, the chromatographic gradient, and HPLC manufacturer while
814 still achieving similar chromatographic results (Supplemental Text 4).

815 *Planktothricoides* sp. BLCC-F182 had a distinctive and unique pigment profile.
816 This strain produced a set of chlorophylls that were not detected in the other strains,
817 and it did not produce echinenone. Structurally similar chlorophyll compounds were
818 also found in the *Caulerpa* analyzed in this study. *Planktothricoides* is a freshwater
819 cyanobacterium, while *Caulerpa* is a marine green alga, making the discovery of similar
820 chlorophylls in both samples intriguing. These compounds were likely not analytical
821 artifacts, because multiple lines of evidence show analytical or processing errors could
822 not have or were unlikely to have produced the observed results (Supplemental Text 5).
823 Given this evidence, these compounds are believed to be present in both samples, but
824 there may have been organisms growing on or with the *Caulerpa* that could have

825 produced these chlorophyll analogs, and that they may not necessarily have been
826 produced by *Caulerpa* itself.
827 These compounds are being isolated and purified at a preparatory scale,
828 allowing for full characterization in additional follow-up studies. Other putative
829 chlorophyll analogs have been identified in other samples (data not shown), but the
830 complex mixture of algae or cyanobacteria present in those samples made it difficult to
831 determine who could have been producing those unusual chlorophylls. It is not a
832 surprising result to find that that there are other chlorophylls in the environment, and
833 the method described here is amongst the first to be able to separate many analogs,
834 detect them with the highly sensitive MS detector, while providing detailed structural
835 information about the unknown compounds to aid in their identification.
836 These measurements describe a limited set of samples; however, many unique
837 features and interesting pigments were identified using this HPLC-PDA-MS
838 methodology. What was reported in this work is only a snapshot of the chlorophyll and
839 carotenoid diversity present in natural systems, and algae are ripe to be explored with
840 new measurement techniques and modern instrumentation. There have been relatively
841 few technological advancements in pigment analytical technique over the last 15 years,
842 where the methods are still fundamentally based on technology and methodology
843 developed in the 1990's and 2000's. There is certainly much more to discover in the field
844 of algal pigments, and the discoveries made during the relatively limited application of

this methodology to freshwater and marine algae and cyanobacteria demonstrate that the field is far from a 'solved' area of research.

Funding

This manuscript was supported by U.S. Department of Agriculture – Agricultural Research Service intramural projects under Natural Resources and Sustainable Agricultural Systems National Programs. This work was partly supported by the Research Capacity Fund (HATCH) project award no. FLA-FTL-006614, from the U.S. Department of Agriculture's National Institute of Food and Agriculture.

Acknowledgements

We would like to thank the U.S. Geological Survey for access to their instrumentation during the initial chromatographic development. We would like to thank Ben Kramer and Mary Anne Evans for providing us with *Cladophora* samples to analyze for this manuscript.

Disclaimer

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or

endorsement by the U.S. government. USDA is an equal-opportunity provider and employer.

Author Contributions

Z.J.S. designed the HPLC methods and performed the analysis, extracted the samples, prepared the first draft of the manuscript and most figures. D.E.B and H.D.L isolated the cyanobacteria strains and collected the green alga used for analysis. K.M.G. developed the code needed to plot Figures 4 and 5 and figured out how to best plot the data. C.J.H. oversaw the instrumentation and provided the funding needed for the pigment standards, columns, LC-MS/MS, and any other materials needed. Z.J.S., D.E.B., and H.D.L. revised and edited the manuscript for submission. All authors reviewed the manuscript prior to submission

Data Availability Statement

All data is fully available in the text and supplemental materials.

Conflicts of Interest

The authors declare no conflicts or competing interests in the completion of this study.

The authors declare no use of artificial or general intelligence in the production of this manuscript.

References

Aydoğan C (2020) Recent advances and applications in LC-HRMS for food and plant natural products: a critical review. Anal Bioanal Chem 412:1973–1991. https://doi.org/10.1007/s00216-019-02328-6

Bianco M, Ventura G, Coniglio D, et al (2024) Development of a New Binary Matrix for the Comprehensive Analysis of Lipids and Pigments in Micro- and Macroalgae Using MALDI-ToF/ToF Mass Spectrometry. International Journal of Molecular Sciences 25:. https://doi.org/10.3390/ijms25115919

Bijttebier S, D'Hondt E, Noten B, et al (2014) Ultra high performance liquid chromatography versus high performance liquid chromatography: Stationary phase selectivity for generic carotenoid screening. Journal of Chromatography A 1332:46–56. https://doi.org/10.1016/j.chroma.2014.01.042

Bijttebier SKA, D'Hondt E, Hermans N, et al (2013) Unravelling ionization and fragmentation pathways of carotenoids using orbitrap technology: a first step towards identification of unknowns: Carotenoid fragmentation by orbitrap technology. J Mass Spectrom 48:740–754. https://doi.org/10.1002/jms.3203

Catlett D, Siegel DA (2018) Phytoplankton Pigment Communities Can be Modeled Using Unique Relationships With Spectral Absorption Signatures in a Dynamic Coastal Environment. JGR Oceans 123:246–264. https://doi.org/10.1002/2017JC013195

Chen M, Li Y, Birch D, Willows RD (2012) A cyanobacterium that contains chlorophyll *f* – a red-absorbing photopigment. FEBS Letters 586:3249–3254. https://doi.org/10.1016/j.febslet.2012.06.045

Chen M, Schliep M, Willows RD, et al (2010) A Red-Shifted Chlorophyll. Science 329:1318–1319. https://doi.org/10.1126/science.1191127

Chrupková P, van Stokkum IHM, Friedrich T, et al (2024) Raman Vibrational Signatures of Excited States of Echinenone in the Orange Carotenoid Protein (OCP) and Implications for its Photoactivation Mechanism. Journal of Molecular Biology 436:168625. https://doi.org/10.1016/j.jmb.2024.168625

Cruz R, Casal S (2018) Direct analysis of vitamin A, vitamin E, carotenoids, chlorophylls and free sterols in animal and vegetable fats in a single normal-phase liquid chromatographic run. Journal of Chromatography A 1565:81–88. https://doi.org/10.1016/j.chroma.2018.06.029

Dachtler M, Glaser T, Kohler K, Albert K (2001) Combined HPLC−MS and HPLC−NMR On-Line Coupling for the Separation and Determination of Lutein and Zeaxanthin Stereoisomers in Spinach and in Retina. Anal Chem 73:667–674. https://doi.org/10.1021/ac000635g

Epler KS, Sander LC, Ziegler RG, et al (1992) Evaluation of reversed-phase liquid chromatographic columns for recovery and selectivity of selected carotenoids. Journal of Chromatography A 595:89–101. https://doi.org/10.1016/0021-9673(92)85149-N

Fu W, Magnúsdóttir M, Brynjólfson S, et al (2012) UPLC-UV-MSE analysis for quantification and identification of major carotenoid and chlorophyll species in algae. Anal Bioanal Chem 404:3145–3154. https://doi.org/10.1007/s00216-012-6434-4

Garrido JL, Zapata M (1997) Reversed-phase high-performance liquid chromatographic separation of mono- and divinyl chlorophyll forms using pyridine-containing mobile phases and a polymeric octadecylsilica column. Chromatographia 44:43–49. https://doi.org/10.1007/BF02466514

Gentili A, Caretti F (2011) Evaluation of a method based on liquid chromatography–diode array detector–tandem mass spectrometry for a rapid and comprehensive characterization of the fat-soluble vitamin and carotenoid profile of selected plant foods. Journal of Chromatography A 1218:684–697. https://doi.org/10.1016/j.chroma.2010.12.001

Gower J, King S, Borstad G, Brown L (2005) Detection of intense plankton blooms using the 709 nm band of the MERIS imaging spectrometer. International Journal of Remote Sensing 26:2005–2012. https://doi.org/10.1080/01431160500075857

Guskov A, Kern J, Gabdulkhakov A, et al (2009) Cyanobacterial photosystem II at 2.9-Å resolution and the role of quinones, lipids, channels and chloride. Nat Struct Mol Biol 16:334–342. https://doi.org/10.1038/nsmb.1559

Hayward A, Pinkerton MH, Gutierrez-Rodriguez A (2023) phytoclass: A pigment-based chemotaxonomic method to determine the biomass of phytoplankton classes. Limnology and Oceanography: Methods 21:220–241. https://doi.org/10.1002/lom3.10541

Hou X (2022) Global mapping reveals increase in lacustrine algal blooms over the past decade. Nature Geoscience 19

Jeffrey SW, Mantoura RFC, Wright SW (1997) Phytoplankton Pigments in Oceanography: Guidelines to Modem Methods, S.W Jeffrey, R.F.C. Mantoura, S.W. Wright (Eds.), Unesco Publishing, Paris (Cedex 15) (1997), 661 pages, F-75732, (ISBN 92-3-103275-5). Urban & Fischer

Jeffrey SW, Wright SW, Zapata M (1999) Recent advances in HPLC pigment analysis of phytoplankton. Mar Freshwater Res. https://doi.org/10.1071/MF99109

Kuczynska P, Jemiola-Rzeminska M, Strzalka K (2015) Photosynthetic Pigments in Diatoms. Marine Drugs 13:5847–5881. https://doi.org/10.3390/md13095847

Kutser T (2004) Quantitative detection of chlorophyll in cyanobacterial blooms by satellite remote sensing. Limnol Oceanogr 49:2179–2189. https://doi.org/10.4319/lo.2004.49.6.2179

Lacker T, Strohschein S, Albert K (1999) Separation and identification of various carotenoids by C30 reversed-phase high-performance liquid chromatography coupled to UV and atmospheric pressure chemical ionization mass spectrometric detection. Journal of Chromatography A 854:37–44. https://doi.org/10.1016/S0021-9673(99)00584-1

Latasa M, Rodríguez F, Agustí S, Estrada M (2023) Distribution patterns of phytoplankton groups along isoirradiance layers in oligotrophic tropical and subtropical oceans. Progress in Oceanography 217:103098. https://doi.org/10.1016/j.pocean.2023.103098

Lesellier E, Tehapla A, Krstulovic AM (1993) Use of carotenoids in the characterization of octadecylsilane bonded columns and mechanism of retention of carotenoids on monomeric and polymeric stationary phases. Journal of Chromatography A 645:29–39. https://doi.org/10.1016/0021-9673(93)80615-F

Liguori N, van Stokkum IHM, Muzzopappa F, et al (2024) The Orange Carotenoid Protein Triggers Cyanobacterial Photoprotection by Quenching Bilins via a Structural Switch of Its Carotenoid. J Am Chem Soc 146:21913–21921. https://doi.org/10.1021/jacs.4c06695

Mackey M, Mackey D, Higgins H, Wright S (1996) CHEMTAX - a program for estimating class abundances from chemical markers:application to HPLC measurements of phytoplankton. Mar Ecol Prog Ser 144:265–283. https://doi.org/10.3354/meps144265

Mantoura RFC, Llewellyn CA (1983) The rapid determination of algal chlorophyll and carotenoid pigments and their breakdown products in natural waters by reverse-phase high-performance liquid chromatography. Analytica Chimica Acta 151:297–314. https://doi.org/10.1016/S0003-2670(00)80092-6

Mrdjen I, Smith ZJ, Webster AM, et al (2025) A novel artificial intelligence–powered cell counting tool coupled with digital microscopy for rapid field-assessment of harmful cyanobacterial blooms. Limnology and Oceanography: Methods. https://doi.org/10.1002/lom3.10725

Niziński S, Hartmann E, Shoeman RL, et al (2025) Two-Photon-Driven Photoprotection Mechanism in Echinenone-Functionalized Orange Carotenoid Protein. J Am Chem Soc 147:4100–4110. https://doi.org/10.1021/jacs.4c13341

O'Donnell DM, Effler SW, Strait CM, et al (2013) Remote sensing reflectance in the Great Lakes: In situ measurements, closure analyses, and a forward model. Journal of Great Lakes Research 39:137–150. https://doi.org/10.1016/j.jglr.2013.06.016

Pniewski F (2020) HPLC separation of cyanobacterial and algal photosynthetic pigments. Biologia 75:223–233. https://doi.org/10.2478/s11756-019-00407-8

Reyna NE, Hardison AK, Liu Z (2017) Influence of Major Storm Events on the Quantity and Composition of Particulate Organic Matter and the Phytoplankton Community in a Subtropical Estuary, Texas. Front Mar Sci 4:. https://doi.org/10.3389/fmars.2017.00043

Rivera S, Vilaró F, Canela R (2011) Determination of carotenoids by liquid chromatography/mass spectrometry: effect of several dopants. Anal Bioanal Chem 400:1339–1346. https://doi.org/10.1007/s00216-011-4825-6

1008 Rodríguez F, Zapata M, Garrido JL (1998) High performance liquid chromatographic
1009 separation of chlorophyllc forms from marine phytoplankton on octylsilica
1010 bonded phases. Chromatographia 48:677–680. https://doi.org/10.1007/BF02467598

1011 Sander LC, Sharpless KEpler, Craft NE, Wise SA (1994) Development of Engineered
1012 Stationary Phases for the Separation of Carotenoid Isomers. Anal Chem 66:1667–
1013 1674. https://doi.org/10.1021/ac00082a012

1014 Schlüter L, Lauridsen TL, Krogh G, Jørgensen T (2006) Identification and quantification
1015 of phytoplankton groups in lakes using new pigment ratios – a comparison
1016 between pigment analysis by HPLC and microscopy. Freshwater Biology
1017 51:1474–1485. https://doi.org/10.1111/j.1365-2427.2006.01582.x

1018 Schlüter L, Møhlenberg F, Havskum H, Larsen S (2000) The use of phytoplankton
1019 pigments for identifying and quantifying phytoplankton groups in coastal
1020 areas:testing the influence of light and nutrients on pigment/chlorophyll a ratios.
1021 Mar Ecol Prog Ser 192:49–63. https://doi.org/10.3354/meps192049

1022 Seegers BN, Werdell PJ, Vandermeulen RA, et al (2021) Satellites for long-term
1023 monitoring of inland U.S. lakes: The MERIS time series and application for
1024 chlorophyll-a. Remote Sensing of Environment 266:112685.
1025 https://doi.org/10.1016/j.rse.2021.112685

1026 Steele DJ, Kimmance SA, Franklin DJ, Airs RL (2018) Occurrence of chlorophyll
1027 allomers during virus-induced mortality and population decline in the
1028 ubiquitous picoeukaryote *Ostreococcus tauri*: Chlorophyll allomers in *Ostreococcus*
1029 *tauri*. Environ Microbiol 20:588–601. https://doi.org/10.1111/1462-2920.13980

1030 Sugawara T, Ganesan P, Li Z, et al (2014) Siphonaxanthin, a Green Algal Carotenoid, as
1031 a Novel Functional Compound. Mar Drugs 12:3660–3668.
1032 https://doi.org/10.3390/md12063660

1033 Takaichi S (2011) Carotenoids in Algae: Distributions, Biosyntheses and Functions.
1034 Marine Drugs 9:1101–1118. https://doi.org/10.3390/md9061101

1035 Taniguchi M, Lindsey JS (2021) Absorption and Fluorescence Spectral Database of
1036 Chlorophylls and Analogues. Photochem Photobiol 97:136–165.
1037 https://doi.org/10.1111/php.13319

1038 Tomlinson MC, Stumpf RP, Wynne TT, et al (2016) Relating chlorophyll from
1039 cyanobacteria-dominated inland waters to a MERIS bloom index. Remote
1040 Sensing Letters 7:141–149. https://doi.org/10.1080/2150704X.2015.1117155

US EPA. (1997) *Method 445: In Vitro Determination of Chlorophyll a and Pheophytin a in Marine and Freshwater Algae by Fluorescence*; US EPA: Washington, DC, USA, 1997; pp. 1-22.

Van Heukelem L, Thomas CS (2001) Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. Journal of Chromatography A 910:31–49. https://doi.org/10.1016/S0378-4347(00)00603-4

Walker JS, Jie C, Keely BJ (2003) Identification of diastereomeric chlorophyll allomers by atmospheric pressure chemical ionisation liquid chromatography/tandem mass spectrometry. Rapid Comm Mass Spectrometry 17:1125–1131. https://doi.org/10.1002/rcm.1015

Whitehouse CM, Dreyer RN, Yamashita Masamichi, Fenn JB (1985) Electrospray interface for liquid chromatographs and mass spectrometers. Anal Chem 57:675–679. https://doi.org/10.1021/ac00280a023

Wojdyło A, Nowicka P, Tkacz K, Turkiewicz IP (2021) Fruit tree leaves as unconventional and valuable source of chlorophyll and carotenoid compounds determined by liquid chromatography-photodiode-quadrupole/time of flight-electrospray ionization-mass spectrometry (LC-PDA-qTof-ESI-MS). Food Chemistry 349:129156. https://doi.org/10.1016/j.foodchem.2021.129156

Wynne TT, Stumpf RP, Tomlinson MC, et al (2008) Relating spectral shape to cyanobacterial blooms in the Laurentian Great Lakes. International Journal of Remote Sensing 29:3665–3672. https://doi.org/10.1080/01431160802007640

Wynne TT, Stumpf RP, Tomlinson MC, Dyble J (2010) Characterizing a cyanobacterial bloom in Western Lake Erie using satellite imagery and meteorological data. Limnol Oceanogr 55:2025–2036. https://doi.org/10.4319/lo.2010.55.5.2025

Yabuzaki J (2017) Carotenoids Database: structures, chemical fingerprints and distribution among organisms. Database 2017:bax004. https://doi.org/10.1093/database/bax004

YMC America YMC C30 Analytical Phases pg. 64. http://file.yizimg.com/989/20058231105875392697.pdf. Accessed 27 Sept 2022

Yoshii Y, Hanyuda T, Wakana I, et al (2004) Carotenoid Compositions of Cladophora Balls (aegagropila Linnaei) and Some Members of the Cladophorales (ulvophyceae, Chlorophyta): Their Taxonomic and Evolutionary Implication.

Journal of Phycology 40:1170–1177. https://doi.org/10.1111/j.1529-8817.2004.03210.x

Zapata M, Ayala AM, Franco JM (1987) Separation of Chlorophylls and Their Degradation Products in Marine Phytoplankton by Reversed-Phase High-Performance Liquid Chromatography. 23:6

Zapata M, Rodríguez F, Garrido J (2000) Separation of chlorophylls and carotenoids from marine phytoplankton:a new HPLC method using a reversed phase C8 column and pyridine-containing mobile phases. Mar Ecol Prog Ser 195:29–45. https://doi.org/10.3354/meps195029

Zhang J, Liu D, Cao P, et al (2016) A highly sensitive method for analyzing marker phytoplankton pigments: Ultra-high-performance liquid chromatography-tandem triple quadrupole mass spectrometry. Limnology and Oceanography: Methods 14:623–636. https://doi.org/10.1002/lom3.10117

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformation102226Submitted.docx