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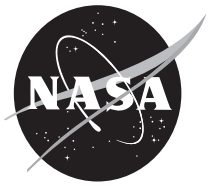

The Fourth SeaWiFS HPLC Analysis Round-Robin Experiment (SeaHARRE-4)

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Chapter 11

The USC Method

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Abstract

The USC method was developed to provide a universal protocol that could be used to analyze freshwater, estuarine, coastal, and ocean habitats, as well as sediment and microbial mat samples. The method employs a unique combination of both monomeric and polymeric C_{18} columns combined with a variable flow binary gradient. This column configuration was originally devised to enhance photopigment separations from sediment samples containing numerous (greater than 150) photopigment and pigment degradation products. The combination of columns provides strong retention and high efficiency (monomeric columns) while selecting for similar compounds with minor differences in molecular structure (polymeric columns). The variable flow binary gradient allows baseline separation of most major pigments including lutein and zeaxanthin, and chlorophyll c_3. Chlorophylls c_1 and c_2 plus divinyl chlorophylls a and b are not completely separated. For HPLC analysis, filters are placed in disposable polypropylene microfuge tubes and lyophilized to remove all water from the filters. The primary advantages of this method are long column life (greater than 2,000 injections), inexpensive and non-hazardous solvents and reagents, no uncertainty regarding the water retained on the filter, and reliability across a range of sample types.

11.1 INTRODUCTION

The HPLC method used at USC for photopigment separations is derived from the Van Heukelem et al. (1992 and 1994) and Pinckney et al. (1996) protocols. Two different reversed-phase C_{18} columns are connected in series. A single monomeric guard column is followed by a monomeric reversed-phase C_{18} column and a polymeric reversed-phase C_{18} column. This column configuration was originally devised to enhance photopigment separations from sediment samples containing numerous (greater than 150) photopigment and pigment degradation products. Monomeric columns provide strong retention and high efficiency, while polymeric columns select for similar compounds with minor differences in molecular structure (Van Heukelem et al. 1992 and Jeffrey et al. 1997b).

In addition to providing for an increase in the number of theoretical plates, the combination of both monomeric and polymeric columns optimizes photopigment separations based on two different molecular properties (coarse and fine structure). This method allows for the baseline separation of most major pigments including lutein and zeaxanthin, as well as chlorophyll c_3. Chlorophylls c_1 and c_2, however, are not completely separated. Divinyl chlorophylls a and b are not completely resolved, but occur as "shoulders" on the monovinyl chlorophylls a and b and can be visually identified in chromatograms.

11.2 EXTRACTION

The SeaHARRE-4 samples were immediately stored in a $-80°C$ freezer upon receipt. For HPLC analysis, filters were placed in disposable polypropylene microfuge tubes (2 mL) and lyophilized ($-50°C$, 0.57 mbar, 12 h; Labconco FreeZone 2.5) to remove all water from the filters. After lyophilization, filters were cut into six equal sections and placed in microfuge tubes. Samples were extracted in 90% acetone (600 µL), and stored at $-20°C$ for 18–20 h. Each sample also received 50 µL of the synthetic carotenoid *trans*-β-apo-8′-carotenal (Sigma-Aldrich, 10810) in 90% acetone as an internal standard using a gastight syringe (Hamilton) and click dispenser (Hamilton, PB600-1). After extraction, the extract was clarified using a 0.45 µm PTFE filter (Gelman Acrodisc). A known volume of the extract (400 µL) was then dispensed into amber glass autosampler vials (2.0 mL) and sealed with PTFE-silicone caps.

11.3 HPLC ANALYSIS

The instrumentation was manufactured by Shimadzu and was part of their Validation and Productivity (VP) series. It consisted of a binary gradient pump (dual LC10-AT and controller SCL-10A), temperature-controlled autosampler (SIL10-A) with a 500 µL injection loop, column

oven (CTO-10AS), and PDA (SPD-M10A with a 200–800 nm range). For the PDA, spectra (380–700 nm) were obtained at 2 s intervals for the duration of each run and photopigment peaks were quantified at 440±4 nm.

Two different reversed-phase C_{18} columns were connected in series. A single monomeric guard column (Rainin Microsorb, 0.46 × 1.5 cm, 3 µm packing) was followed by a monomeric reversed-phase C_{18} column (Varian Microsorb-MV 100-3, 0.46 × 10 cm, 3 µm packing) and a polymeric reversed-phase C_{18} column (Vydac 201TP54, 0.46 × 25 cm, 5 µm packing). The column oven maintained a constant 40°C for the duration of the gradient.

A nonlinear binary gradient, which was adapted from Van Heukelem et al. (1992), was used for pigment separations (Table 38). Solvent A consisted of 80% methanol and 20% ammonium acetate (0.5 M adjusted to pH 7.2), and solvent B was composed of 80% methanol and 20% acetone (Table 38). Solvents were degassed with an in-line degasser (Shimadzu DGU 14A). All solvents were HPLC-grade and chemicals were analytical grade.

Table 38. The gradient used with the USC method. The time is in minutes, the flow rate is in milliliters per minute, and the percentages of solvents A and B are given in the last two columns.

Step	*Time*	*Flow*	A [%]	B [%]
Start	0	0.80	100	0
2	0.5	0.80	50	50
3	35	1.25	0	100
4	36	1.50	0	100
5	37	0.80	0	100
6	38	0.80	100	0
End	50	0.80	100	0

Just prior to the HPLC run, an ion-pairing (IP) solution (1 M ammonium acetate) was added to the vial in a ratio of four parts extract to one part ammonium acetate. Prior work has shown there is negligible pigment degradation within 12 h of adding the IP solution if the sample is placed in a refrigerated autosampler (4.0°C). The IP solution, however, should not be added to the sample if the time until sample analysis is greater than 18 h.

11.4 CALIBRATION

Peaks were identified based on retention time and spectral matches with pigment spectra obtained from DHI standards (Table 39). Peak areas were quantified using Shimadzu `SP1` v7.2.1 software. The PDA was calibrated using a multipoint calibration procedure for a range of injection volumes (25–300 µL) of pigment standards. Regressions were performed using known pigment concentration (y) versus integrated peak area (x), and were of the form $y = mx + b$, where m is the slope and b is the y-intercept.

Table 39. The α values used by the USC method for a variety of pigments as a function of λ. The units for α are liters per gram per centimeter and the units for λ are nanometers. All solvents are at a 100% purity unless indicated otherwise. Not all of the pigments listed were identified and reported for SeaHARRE-4.

Pigment	*Solvent*	λ	α
$\beta\beta$-Car	Acetone	454	250.00
$\beta\varepsilon$-Car	Acetone	448	270.00
$\beta\psi$-Car	Pet Ether	459	318.00
$\varepsilon\varepsilon$-Car	Pet Ether	440	290.00
$\psi\psi$-Car	Acetone	474	344.60
Allo	Acetone	454	250.00
Anth	Ethanol	446	235.00
Asta	Hexane	468	210.00
BChl *a*	Ace./Meth.	771	59.40
But	Acetone	445	147.00
Cantha	Pet Ether	466	220.00
Chl *a*	90% Acetone	664	87.67
Chl *b*	90% Acetone	647	51.36
Chl c_{12}	90% Acetone	631	42.60
Chl c_3	90% Acetone	453	346.00
Chlide *a*	90% Acetone	664	127.00
Chlide *b*	90% Acetone	645	74.07
Croco	Ethanol	443	250.00
Diadchr	Acetone	428	250.00
Diad	Methanol	445	225.00
Diato	Acetone	452	210.00
Dino	Acetone	442	210.00
DVChl *a*	90% Acetone	664	87.67
DVChl *b*	90% Acetone	647	51.36
Echin	Pet Ether	458	215.80
Fuco	Acetone	443	166.00
Gyro	Ethanol	445	262.00
Hex	Acetone	445	142.00
Lut	Ethanol	445	255.00
MgDVP	Methanol	623	58.90
Monado	Diethyl Ether	446	250.00
Myxo	Acetone	478	216.00
Neo	Ethanol	438	227.00
P-457	Acetone	457	164.00
Peri	Acetone	466	134.00
Phide *a*	90% Acetone	667	74.20
Phide *b*	90% Acetone	657	46.37
Phytin *a*	90% Acetone	667	51.20
Phytin *b*	90% Acetone	657	31.80
Pras	Diethyl Ether	446	250.00
Pyrophytin	Diethyl Ether	667	60.29
Siphx	Acetone	445	250.00
Siphn	Ethanol	462	192.00
Vauch	Acetone	444	250.00
Viola	Acetone	442	240.00
Zea	Acetone	452	234.00

All regressions had a coefficient of determination of $r^2 > 0.98$. The slope of the fitted line was used as the response factor for all pigment concentration calculations. The concentrations of pigments for which standards were unavailable were estimated using the ratio method outlined in Jeffrey et al. (1997b).

11.5 VALIDATION

Carotenal blanks (*trans*-β-apo-8′-carotenal in 90% acetone) were run after every 10 samples to verify peak time reproducibility, peak area precision, and instrument performance during the sequence run. Peaks were identified based on retention time and comparison of absorbance spectra with a spectral library derived from pure pigment standards (DHI). Long-term quality control was achieved by analyzing pure standards for chlorophyll *a* and the DHI mix at monthly intervals. Instrument performance was measured and compared with previous measures to determine changes in performance metrics. Volumetric measuring devices were checked weekly.

11.6 DATA PRODUCTS

Pigment concentrations were calculated for each identifiable peak using the following equation:

$$C_{P_i} = \frac{R_I}{V_c} \frac{V_m}{V_f} \frac{\hat{A}_c}{\hat{A}_s} \hat{A}_{P_i} R_{P_i}, \tag{46}$$

where C_{P_i} is the pigment concentration in micrograms per liter; $\hat{A}_{P_i}$ is the pigment peak area; R_{P_i} is the response factor; V_c is the injection volume in microliters; V_m is the total extract volume (volume of added acetone plus volume of internal standard in milliliters); R_I is the ratio of the volume of ion-pairing (IP) solution plus V_m divided by V_m; V_f is the volume of seawater filtered (in liters); $\hat{A}_c$ is the average peak area for carotenal standards; and $\hat{A}_s$ is the peak area of carotenal in the sample.

11.7 CONCLUSIONS

This method has been employed by USC for approximately 15 y to analyze a broad spectrum of sample types from marine and freshwater habitats. The execution of the method is straightforward and involves minimum manipulation of the samples and extracts, is relatively inexpensive, and does not generate hazardous waste products. The primary weakness of the method is the inability to completely separate chlorophylls c_1 and c_2, and divinyl chlorophylls *a* and *b*.