HPLC Pigments – General Notes

Written 03/08 WKoz; Updated 09/08 WKoz

From each depth sampled, 0.5-3 L of seawater is filtered, at < 12 psi vacuum, under dim light conditions (no direct overhead or outside light), onto 25 mm Whatman glass fiber filter (pore size nominally 0.7 µ; hereafter referred to as "GF/F"), and stored in liquid nitrogen until analysis. Samples are extracted and injected as soon as possible after collection (minimum of 24 hours in liquid nitrogen); for years other than 2000, time between filtration and analysis is typically less than one month and no more than three months. Due to instrumentation problems, samples from January 2000 were transported (in liquid nitrogen) to Scripps Institution of Oceanography and analyzed within thirteen months of collection.

Filters are extracted in 90% HPLC grade acetone and either (a) manually crushed with a clean Teflon pestle, stored at -80°C for 24 hours, and pre-filtered through another GF/F before injection (1995-2001) or (b) ultrasonicated while held in a -20°C bench-top cooler for 10 seconds, stored at -80°C for 24 hours and pre-filtered through a 1 µ syringe tip glass fiber filter (2003-2005) or 0.45 µ Whatman nylon Puradisk filter (2006 onward) before injection. Testing confirmed that the changes in pre-injection filter type affected only pre-column and column longevity, not separation or retention times.

For samples between 1995 and 1999, pigments were separated using a Hitachi system (D-6500 in 1995; D-7000 from 1996 to 1999) with L-4250 fixed wavelength (440 nm) and L-4500 diode array (DAD, scanning 350-650 nm) detectors. HPLC grade mobile phase eluents followed the method of Wright et al. (1991):

Solvent A: 80:20 methanol : 0.5 M aqueous ammonium acetate (pH 7.2)
Solvent B: 90:10 acetonitrile : water
Solvent C: 100% ethyl acetate

The analytical gradient was modified from Wright et al. (1991) to reduce spreading of the early eluting, highly polar chlorophyll c3, and is listed in Table 1. Solvents were degassed by sparging with 99% pure Helium gas, and separations were performed on a Waters Resolve C18, 300 mmx3.9 µ, 5 µ column. Hitachi's ConcertChrome software package was used for system control and data collection, and peaks were quantified at 440 nm on the DAD.

Samples collected in January 2000 were, and any samples analyzed at SIO after August 2008 will be analyzed on a Waters 600 controlled system with a Thermo Separation Products (TSP) AS3000 sampler, a TSP Spectra 1000 variable wavelength detector (VWD) for peak quantification and a Waters 470 Scanning fluorescence detector for peak identification when applicable (excitation at 440 nm, emission at 665 nm). Data iss collected using the Waters Millennium 32 software package, and peaks quantified at 440 nm on the VWD.

Samples collected between 2001 and January 2008, were separated using an Agilent Technologies (Hewlett-Packard) 1100 Series HPLC system, equipped with G1314A variable wavelength (fixed at 440 nm), G1315A diode array (scanning 330-800 nm) and G1321A fluorescence (440 nm excitation, 650 nm emission) detectors. Solvents were degassed using a vacuum degasser and column temperature was maintained at 25°C with a G1316A column thermostat. Agilent Technologies ChemStation for LC 3D software was used for system control and data collection and peaks were quantified at 440 nm on the VWD.

From 2000 onward (both Waters and Agilent Technologies systems), HPLC grade mobile phase eluents followed the method of Zapata et al. (2000):

Solvent A: 50:25:25 methanol : acetonitrile : 0.25 M aqueous pyridine
Solvent B: 20:60:20 methanol: acetonitrile : acetone

The analytical gradient is modified slightly from Zapata et al. (2000) to allow adequate separation of the mid-chromatogram range xanthophylls, and is listed in Table 1. Separations with this method are performed on Waters Symmetry C8, 150 mm x 4.6 µ, 3.5 µ columns, and samples are injected as either (a) a 2:1 sample:water mixture in 2000 or (b) a 5:4 sample:water mixture from 2001 onward.

In 1995 and 1996, chlorophylls a and b and alpha and beta carotene were quantified using measured spectral absorbance and published extinction coefficients; all other standards were isolated and quantified by Moss Landing Marine Laboratory and all were injected as external standards to determine signal response and retention times. From 1997 on, commercially produced plant pigment standards (Sigma Chemical for chlorophylls a and b and alpha and beta carotene until 2000; DHI (formerly VKI), Denmark (http://www.c14.dhi.dk/PhytoplanktonPigmentStandards.htm) for all others) were used for system calibration. Four to six point response curves are built

using standard peak areas measured at 440 nm, and daily injections of chlorophyll a (chl_a), as well as random single-point injections of all other pigments, are made to confirm system stability and monitor column degradation throughout the duration of sample processing. Sample peaks are identified based on retention times and confirmed spectrally using the diode array and fluorescence detectors, and pigment concentration was calculated by regression in Excel. Pigments quantified, average retention times, and extinction coefficients used for determining standard concentrations are listed in Table 2.

Table 1. HPLC Gradient Protocols

HPLC System	Time (min)	% Solvent A	% Solvent B	% Solvent C
Hitachi	0	100	0	0
	0.5	0	100	0
	7	0	75	25
	17	0	30	70
	24	0	20	80
	26	0	100	0
	29	100	0	0
	35	100	0	0
Waters†/Agilent*	0	100	0	n/a
	18	60	40	n/a
	22	0	100	n/a
	35	0	100	n/a
	38	100	0	n/a
	40*	100	0	n/a
	42*	100	0	n/a
	45†	100	0	n/a

† * A modification of the Zapata et al., 2000 method is used for both the Waters and Agilent systems; minor adjustments were made based on system configuration to minimize total run time while allowing adequate post-run equilibration.

Table 2. HPLC Pigment Information

Pigment	Abbreviation	Retention Time Wright	Retention Time Zapata	Extinction Coefficient (L g^{-1} cm^{-1})	Wavelength (nm)
chlorophyll c3	chl_c3	6.2	7.7		
chlorophyll c2†‡	chl_c2	7.1	10.8		
chlorophyllide a	chlide-a	n/a	10.9		
peridinin‡	per	9.1	13.2		
19' butanoyloxyfucoxanthin†‡	but	9.3	17.7		
fucoxanthin†‡	fuc	10.1	18.6		
19' hexanoyloxyfucoxanthin†‡	hex	10.4	21.2		
neoxanthin	neox	10.8	19.1		
prasinoxanthin	pras	11.3	19.9		
diadinoxanthin‡	dd	12.9	23.1		
alloxanthin†‡	allox	13.8	24.2		
diatoxanthin‡	dt	14.4	24.6		
lutein‡	lut	14.8	25.0*	255	448
zeaxanthin‡	zeax	15.1	24.9*	234	454
chlorophyll b†‡	chl_b	18.2	20.6	51.36	646
violaxanthin	viol	n/a	20.9		
crocoxanthin	croc	n/a	27.4		
echinenone	ech	n/a	28.1		
chlorophyll a†	chl_a	19.3	29.2	87.67	664
α,α - carotene‡	alpha	21.8	31.3	280	444
β,ε - carotene‡	beta	21.9	31.4	259.2	453

Pigments typically quantified in the Vernet Lab, average retention time (RT) for each method (Wright et al., 1991 used from 1995 to1999, Zapata et al., 2000 used from 2000 onward), and for those pigments mixed from powdered standards, extinction coefficients (α) applied and application wavelengths used. Additional comments: * Note elution order change between lutein and zeaxanthin with Zapata et al., 2000 method.
† Denotes pigments used in CHEMTAX input ratios for composition determination.
‡ Denotes pigments used in assemblages analysis.