HPLC Chlorophyll Processing for the ACT Performance Verification of In Situ Fluorometers

The HPLC method used for chlorophyll *a* analysis followed that of Zapata et al (2000; MEPS 195:29-45) and was conducted by the laboratory of Dr. Welschmeyer at Moss Landing Marine Laboratories (MLML, the West Coast ACT Partner Institution). All pigment samples from Partner sites were collected quantitatively on Whatman 2.5 cm GF/F filters, frozen in liquid N_2 and shipped by overnight courier in liquid N_2 dry shippers to MLML. Samples were removed from liquid N_2, extracted overnight in quantitative volumes (1.8 – 2.0 mL) of N_2-purged 90% acetone (-20 ^{o}C) and ground with a motorized Teflon tissue grinder the following morning (the soak-and-grind method). Samples were kept on ice until loaded on HPLC autosamplers (three identical HPLC systems were used during this project). Replicate test samples supplied by Partner sites showed that extraction by the soak-and-grind method yielded as much as 25% more chl *a* than by routine acetone soaking alone. Prior to grinding, all samples and acetone blanks were spiked with quantitative additions of the internal standard, trans-β-Apo-8′-carotenal (Fluka), which provided quantitative control for volumetric changes in extraction volumes resulting from pipeting imprecision and/or evaporation. Approximately 40 samples were run per HPLC autosampler batch, with three authentic chl *a* standards evenly dispersed through the sample batch. Chl *a* standards were purified from spinach extracts for peak purity and quantified spectrophotometrically at 664 nm using an extinction coefficient of 87.67 L g^{-1} cm^{-1}.

HPLC solvent delivery in binary mode was made using a Varian 9012 pump under the following gradient protocol: 0 min, 100% A; 22 min 60% A, 40% B; 28 min, 5% A, 95% B; 38 min, 5% A, 95% B; 40 min, 100% A; where A solvent was methanol:acetonitrile: 0.25M aqueous pyridine (50:25:25 v:v:v) and solvent B was methanol:acetonitrile:acetone (20:60:20 v:v:v). Linear mixing was used between gradient steps at a constant flow of 1 mL min^{-1}. Samples were injected with a robotic autosampler (Gilson 231XL, cooled to 10 ^{o}C) which was programmed to quantitatively dilute the sample extracts with Milli-Q water (1:2 v:v, Milli-Q:acetone extract) just prior to each injection, thus preventing peak-spreading on early-eluting polar compounds. Quantitative partial-loop injections (250 uL) were made onto a 500 uL sample loop. A 250 mm x 4.6 mm I.D., C-8 column (Varian Microsorb 5 um particle size, 100 angstrom pore size, solid-phase material), with corresponding 30 mm guard column and 0.5 um pore-size steel prefilter was used for compound separations. Absorbance and fluorescence data were collected with in-line detectors (Thermo Separation Products Spectra Focus VIS detector 440 nm and Kratos 950 filter fluorometer). Samples routinely yielded five peaks which were considered to make up the natural 'chl *a*' signal sensed by in situ fluorometry; specifically, parent chl *a* and four derivatives including chlorophyllide *a*, two chl *a* allomers and epimeric chl *a*'. Divinyl chl *a* was not detected in any of the coastal samples analyzed during this project. All peak responses were summed to yield the 'total' chl *a* signal using Peak Simple data acquisition hardware/software. Sample extracts were simultaneously analyzed by routine filter fluorometry (Welschmeyer 1994; L&O 39:1985-1992) as a complement and cross-check for quality control of HPLC samples.