The Nonlinearity of the Thermo UV6000 Detector, which Affects Both the CHORS C_8 and C_{18} Methods

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HPLC Experiments at CHORS Using HPL Calibration Practices

Laboratory experiments were designed to explore why calibration reproducibility for the CHORS C_8 method was frequently poor and why injector precision was substandard to what had previously been achieved by CHORS during SeaHARRE-2 with a C_{18} method (Wright et al. 1991). Many calibration factors produced on the C_8 method at CHORS were grossly inaccurate because of the following:

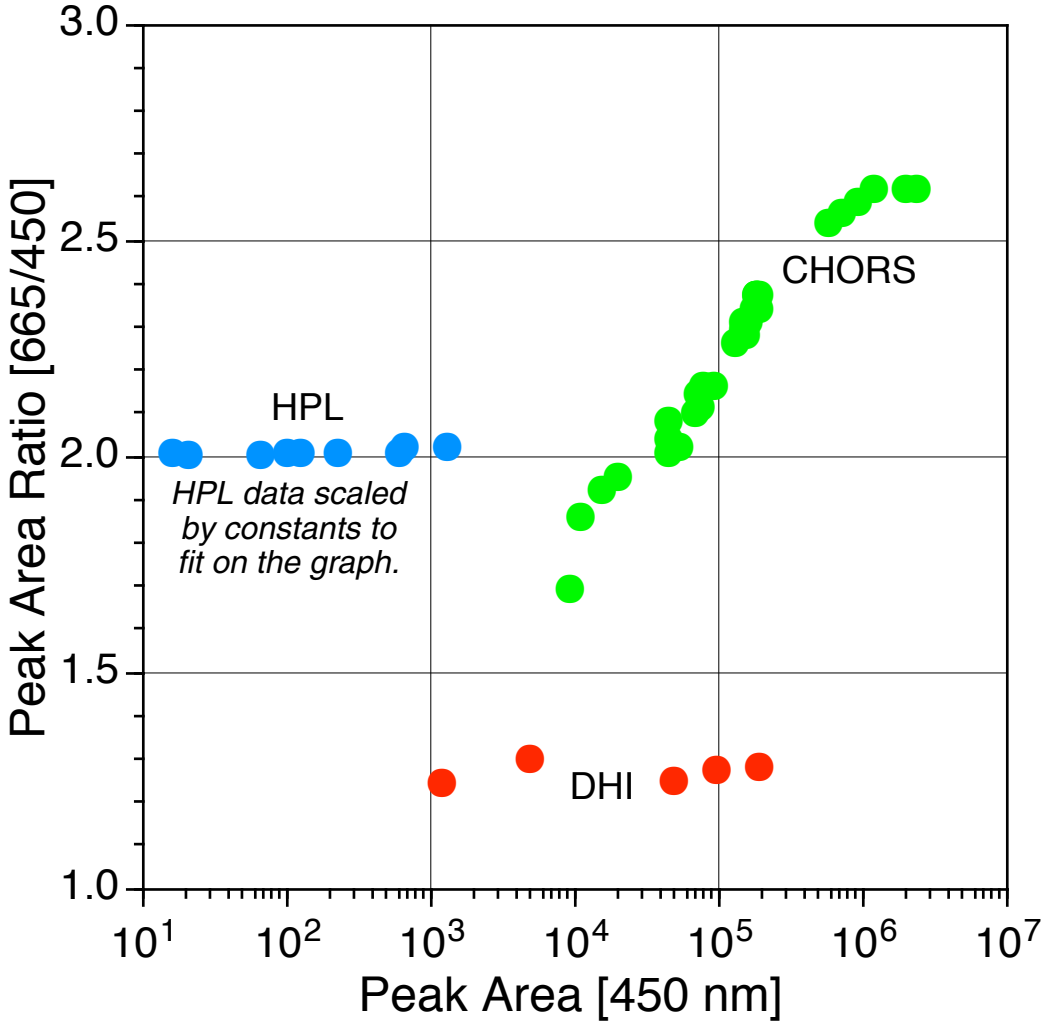
- The calibration curves were *not* linear—the percent residuals were very large and increased as concentration decreased),
- The range of concentrations expected in samples was *not* fully described by the calibration standards (e.g., the linear dynamic range was *not* identified),
- Calibration points were deleted based on visual inspection without supporting evidence based on statistical analyses (such as, percent residuals with respect to the fit), and,
- At times, a theoretical calibration was put into effect whereby a physical calibration with Chl *b* was performed and then all other calibration factors were computed based on previous observations of the relationship between Chl *b* calibration factors and those of other pigments.

The latter was *not* considered valid, because it was never demonstrated that Chl *b* calibration factors and those of all other pigments were stable, accurate, or reproducible.

The Red-to-Blue (664 nm-to-450 nm) Detector Ratio

The CHORS detector acquires data at 436, 450, and 664 nm (the HPL detector is set at 665 and 450 nm). The red-to-blue ratio for Chl *a* calibration standards changes as a function of the 450 nm signal at CHORS, but the same ratio is constant at HPL (Agilent) and DHI (Shimadzu) for the same C_8 method used by CHORS. This has also been confirmed by CSIRO (Waters). The 436/450 ratio at CHORS is closer to being constant, but is still not linear. The significance of this result was explored by consulting with chromatography experts.

This line of inquiry was considered speculative, but it was too curious an observation to leave uninvestigated.



C_8 and C_{18} Detection Differences and the Importance of the Red-to-Blue Nonlinearity

Pigment	C_8 PDA	C_{18} PDA
Chl ***c*$_3$**	450	450
Chl ***c*$_2$**	450	450
Chlide *a*	450/664	664
Phide *a*	664	664
Perid	450	450
But	450	450
Fuco	450	450
Neo	450	450
Pras	450	450
Viola	450	450
Hex	450	450
Diadino	450	450
Allo	450	450
Diato	450	450
Zea	450	450
Lut	450	450
MVChl *b*	450	450
DVChl *a*	664	450/436
MVChl *a*	664	450/436
Phytin *a*	664	664
α-Caro	450	450
β-Caro	450	450

The C_8 and C_{18} methods used very similar calibration procedures and a common set of photodiode array (PDA) wavelengths for detecting carotenoids (yellow), but there were differences for detecting the chlorophyll (green) pigments. For the most important pigments (boldfaced pigment names and blue or red PDA entries)—which directly or indirectly comprise the so-called *primary pigments* (PPIG)—the differences mostly affect TChl *a*, because the final concentration of this pigment is the sum of MVChl *a*, DVChl *a*, and Chlide *a*, and one or more of these constituents are detected at a red wavelength (664 nm).

For the C_8 method, all of the TChl *a* constituents are detected with a red wavelength, so the importance of the red-to-blue nonlinearity is expected to have a maximum effect. In comparison, the quantitation of TChl *a* with the C_{18} method involves predominately blue wavelengths; the exception is Chlide *a*, but it is usually the smallest contributor to TChl *a*, so the red-to-blue nonlinearity is expected to have a minimum effect.

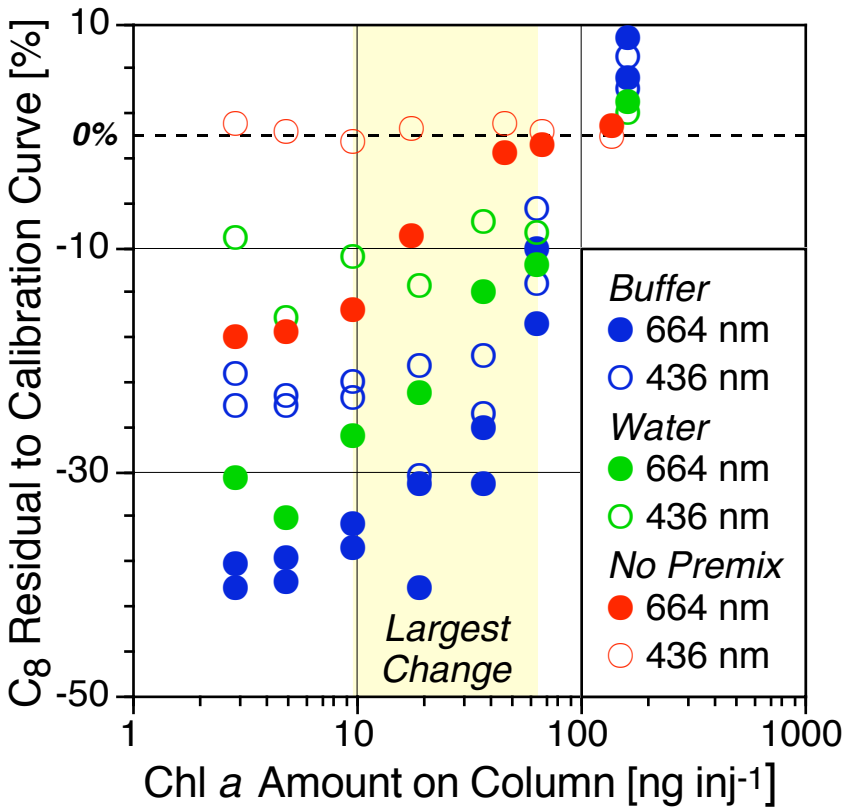
Unresolved Issues Identified During the Investigations of CHORS C_8 Quantitation Problems

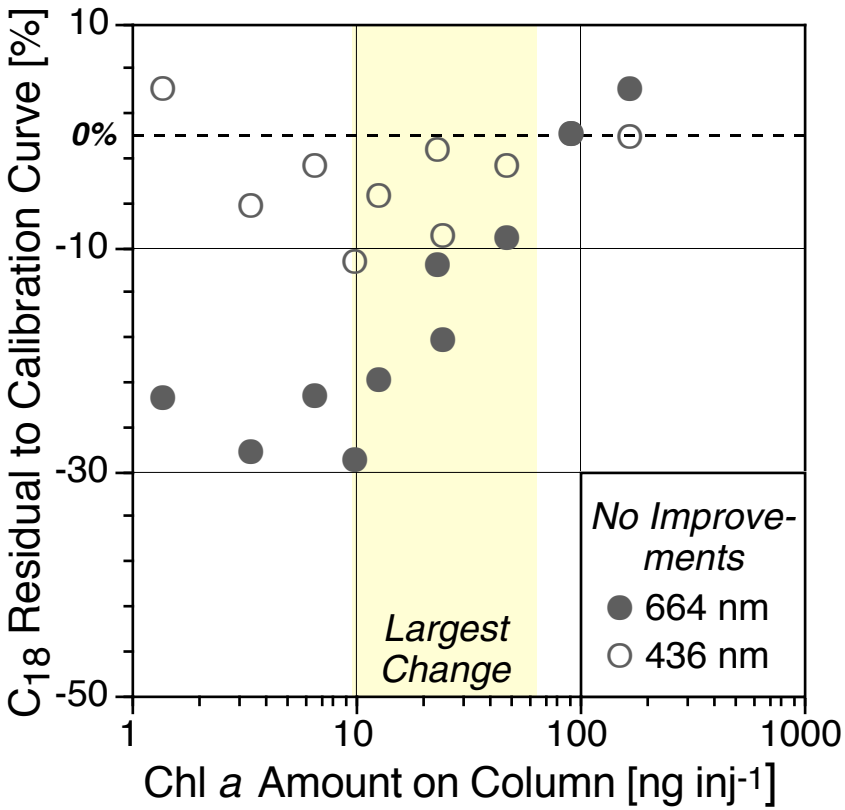
The primary unresolved issues from the first investigation of CHORS C_8 quantitation problems was the nonlinearity of the calibrations and the red-to-blue PDA ratio (for those pigments using 664 nm in the calibration process).

- The C_8 Chl *a* calibration is at 664 nm, whereas the carotenoids and most of the other chlorophylls are at 450 nm; *all the calibrations are nonlinear* as exhibited by an anomalously low response for the more diluted samples, but the Chl *a* calibration is the most nonlinear.
- For the 664 nm calibrations, *no explanation as to why the red-to-blue ratio is not constant was found, but it is correlated with the nonlinearities in the calibrations, so it is assumed to be connected to the calibration nonlinearity*.
- The hypothesized source of calibration nonlinearity was the use of glassware that was not deactivated (i.e., not silanized), but the use of silanized HPLC vials did not reduce the nonlinearity. *The current hypothesis is the Thermo injector program does not permit combining in the loop before injection onto the column, and the requirement to mix in the vial is producing chemical reactions that are reducing the amount of pigment injected onto the column or increasing the amount of pigment retained on the column—both effects result in lower pigment quantitations and nonlinear calibrations. In addition, it is very likely the detector response in the red domain is not correct.*

The Nonlinearity of the CHORS Detector for Both the C_8 and C_{18} Methods

First injection of duplicates used to reduce variance.

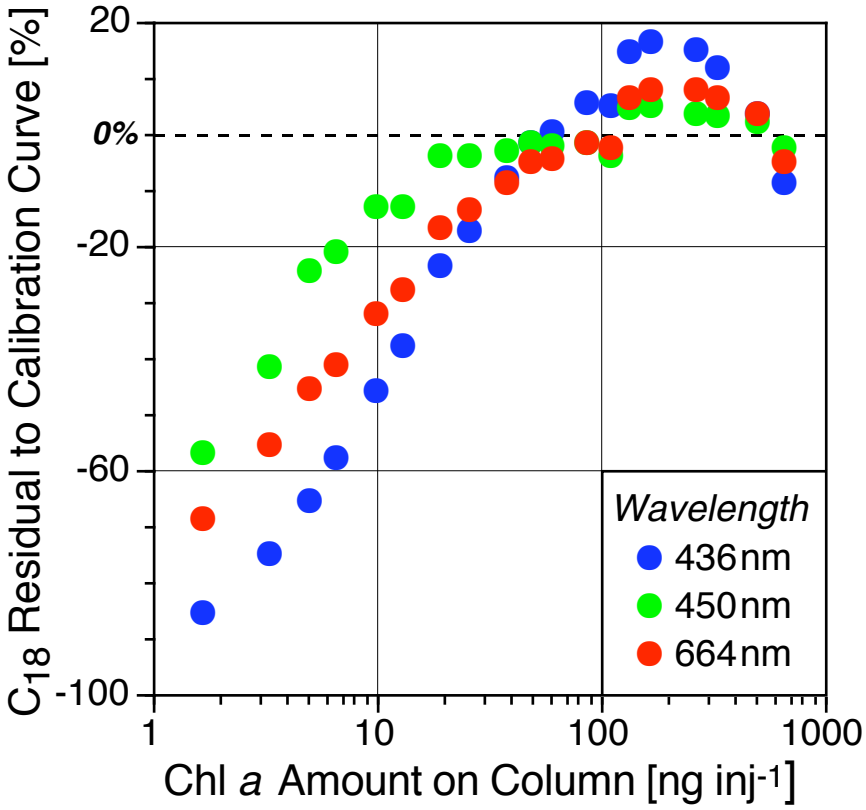


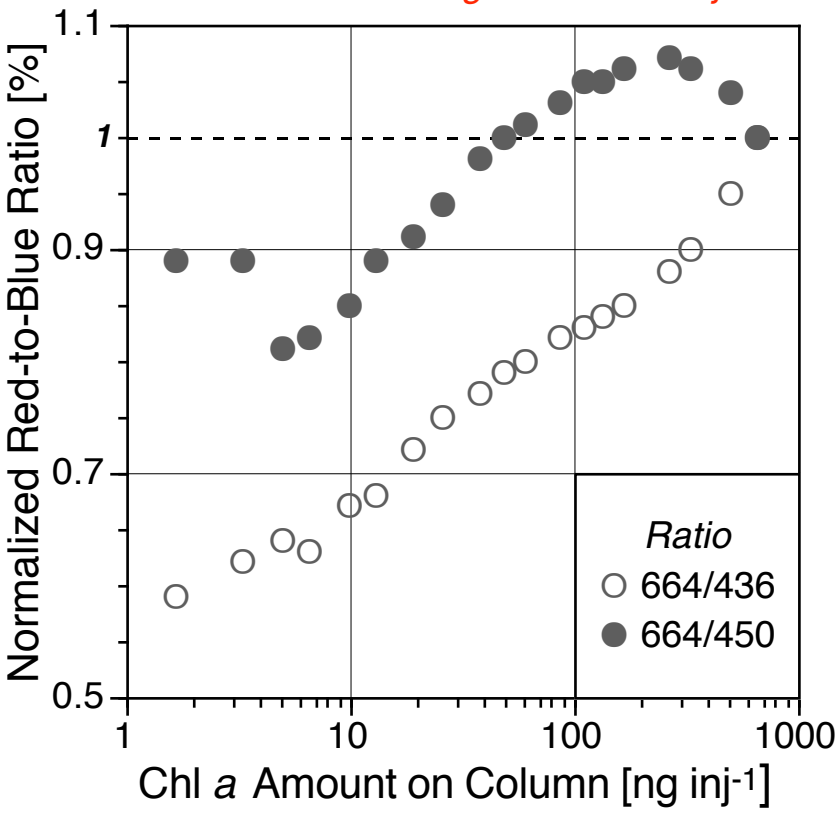


The nonlinearity in CHORS C_8 calibrations are reduced if the buffer is replaced by water, and is completely removed at 436 nm if there is no premixing (left plot). The nonlinearity of the C_{18} method (no methodological changes to improve precision applied), is almost the same as the C_8 *water-only* results. The area of largest change in the nonlinearity is also the same for both methods (yellow region).

The Nonlinearity of the CHORS Detector Reinvestigated Using a New C_{18} Calibration

Normalization based on largest amount injected.

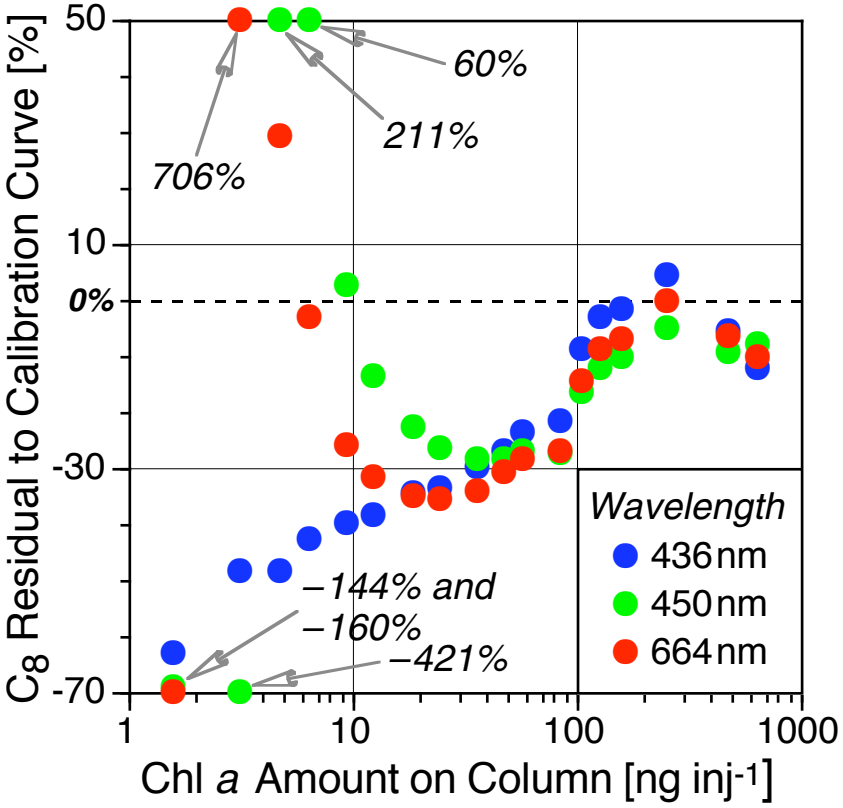


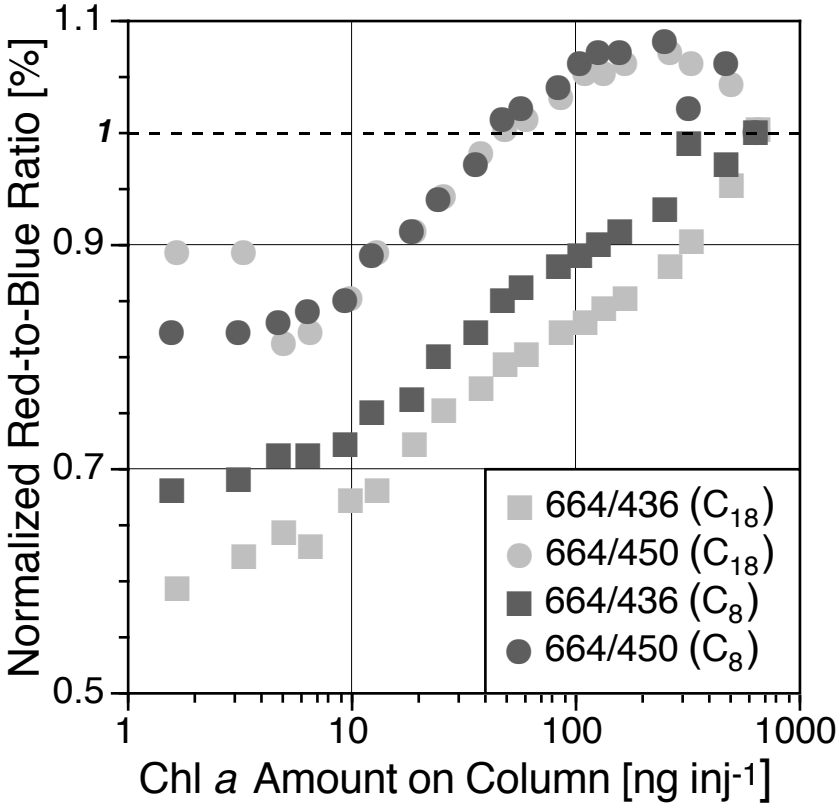


A CHORS calibration is based on immediately forcing through zero, rather than first confirming a negligible *y*-intercept (and average fit residuals to within 2%) before forcing through zero. If the most recent CHORS calibration is not forced through zero, the residuals show a much stronger nonlinearity—*with 436 the largest*—and they are more similar in shape and amplitude. A normalized presentation shows the 664/436 ratio has the most easily characterized functional form.

The Nonlinearity of the CHORS Detector Reinvestigated Using a New C_8 Calibration

Normalization based on largest amount injected.





If the most recent CHORS C_8 calibration is not forced through zero, the residuals show a strong nonlinearity—*as was seen with the most recent C_{18} calibration*. They are similar in shape and amplitude until the effects of the large (and negative) *y*-intercept are encountered (where the fit crosses the *x*-axis). The normalized presentation shows the red-to-blue ratios for the two calibrations are similar, and once again the 664/436 ratio has the most easily characterized functional form.

CHORS Pigment Calibrations Explained Using A C_8 Example

200507aDHI_V2.1.xls (SeaHARRE-3)

	MVChla 664nm		MVChl a	
	Concen.	Area	ng/inj	% Range
1	12.6	7287	0.92	0.3
2	31.1	16418	2.26	0.8
3	60.8	43318	4.41	1.6
4	89.1	53042	6.46	2.4
5	116.1	74190	8.42	3.1
6	212.8	119653	15.43	5.7
	MVChla 1/RF	631.7		

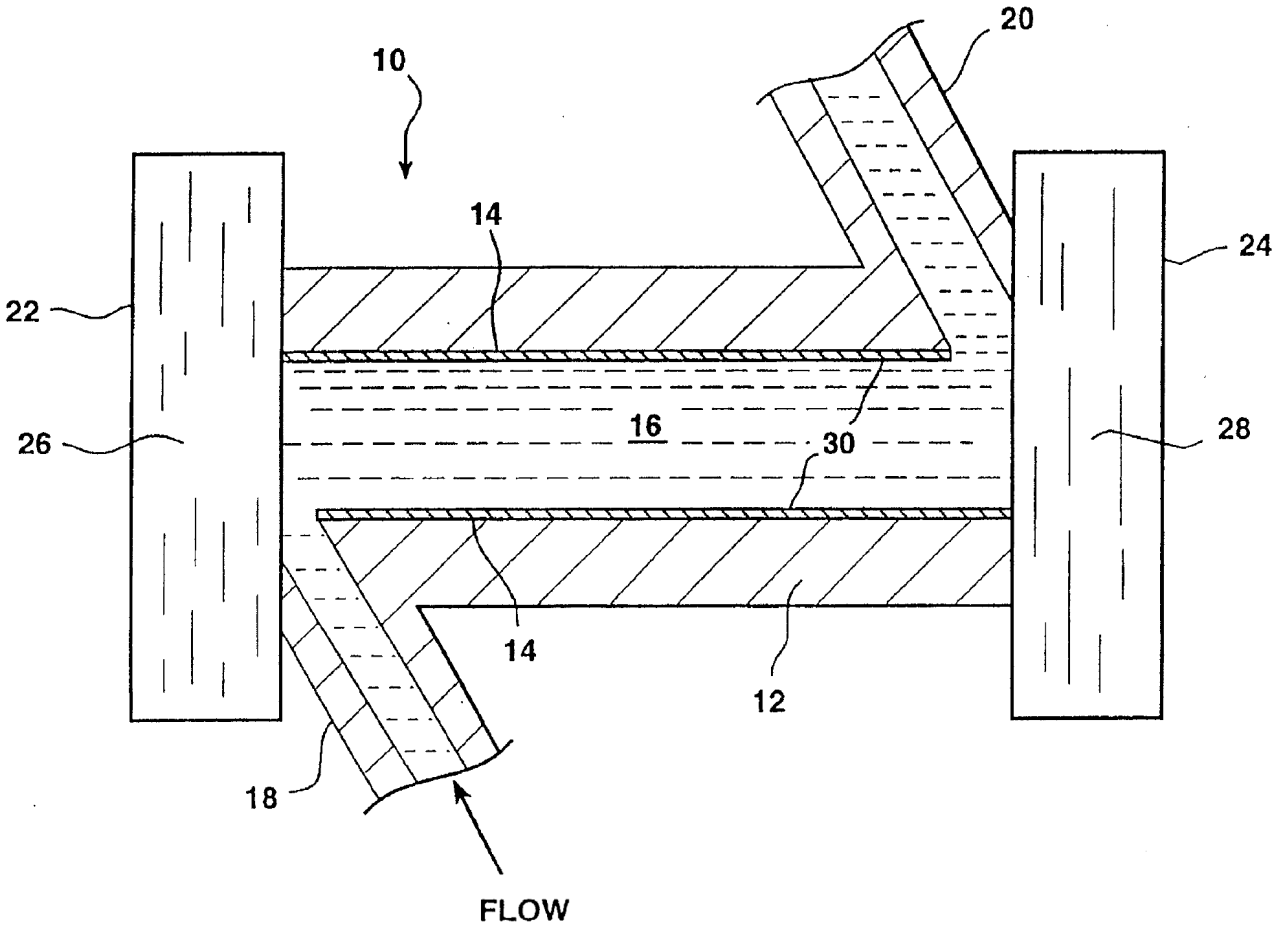
54% Difference

200412b HPLC Calib.xls

	MVChla 664nm		MVChl a	
	Concen.	Area	ng/inj	% Range
3	3.2	1252	0.23	0.1
4	8.0	5892	0.58	0.2
5	16.0	13177	1.16	0.4
6	23.9	22964	1.74	0.6
7	31.9	25242	2.31	0.9
8	79.8	77089	5.79	2.1
9	159.6	153442	11.57	4.3
10	239.4	249383	17.36	6.4
11	319.3	300164	23.15	8.6
	MVChla 1/RF	973.0		

Calibrations are usually done only after the linear dynamic range of the system has been determined, which in fact never occurred. Typically, a calibration begins close to the limit of detection to the top of the working range of the anticipated analyses (which must be within the linear dynamic range). For the CHORS analyses, wherein worldwide samples were expected, a calibration for MVChl *a* should span 2–270 ng (i.e., 1–100% of the working range). Typically, CHORS calibrations spanned a very small concentration range, which were also too low in magnitude (yellow highlight). In some cases, this problem was exasperated by the arbitrary removal of one or more data points (orange highlight)—ostensibly in an effort to produce more consistent results—which still yielded grossly inadequate inverse response factors (737.7 average value).

The Thermo UV6000 Flow Cell



The flow cell (14) of the Thermo Separations UV6000 (US patent 5,608,517) uses a thin polymer (30), Teflon AF, to pipe light down the flow cell using total internal reflection between a source aperture (26) and a detector aperture (28). The light pipe design provides greater sensitivity, because a longer flow cell can be used.

The Nonlinearity of the CHORS Detector is Part of the Design of the Thermo UV6000 DAD

The optimal response for this flow cell is designed to be 190–300nm, and observed nonlinearity can be caused by two problems (US patent 6,281,975B): a) light can be piped inside the cell wall so it never sees the sample, but is still detected by the detector, and b) light is reflected back into the liquid flow path, but still spends some time in the cell wall not interacting with the sample.

This does not explain why the absorbance ratio between the blue and red regions of the detector are not constant, because total reflectance does not depend on wavelength. European patent 1,478,913C also describes the issue of stray light from reflectance in the cell wall: *linearity is improved by using carbon-doped Teflon AF to make the polymer opaque, and the characteristics of the polymer used in the UV6000 makes the material more opaque at 200 nm than at 600 nm*. This would cause the response at the lower wavelengths to be more linear than at the higher wavelengths, because less light is able to be piped through the flow cell wall.

Expert Evaluation of the Thermo UV6000 Detector (by Ron Farnbach)

All HPLC systems have to contend with stray light effects in the flow cell wall, and proprietary optics and other design elements are used to minimize this problem. The use of a light pipe accentuates this problem, and the Thermo design does not deal with it adequately *except in a very narrow spectral range which is not used for the quantitation of marine pigments*.

- There is no refractive index correction, but if one is implemented using the ChromQuest software, the nonlinearity is reduced slightly and the baseline is improved.
- A 1% stray light problem can have a 20% effect on absorbance.
- The CHORS system is best suited for detecting trace quantities of analytes and not for the linear range required for the analysis of marine pigments.
- The detector is more linear in the UV range, which is where most HPLC work (explosives, environmental, and pharmaceutical) is performed; *the detector has marginal to poor linearity over the range used for the analysis of marine pigments*.
- Although the CHORS results should be considered invalidated, because the methodology used requires a linear response and the system was not able to provide such a response, nonlinear calibrations are not an unknown aspect of HPLC methods—it should be possible to correct the calibrations and data, as long as the calibrations are extensive enough to describe the nonlinearity.

A Summary of CHORS C_{18} Quantitation Uncertainties

CHORS participated in several HPLC intercomparisons, all of which included participation by HPL. Two of these were part of SIMBIOS round robins (SB-1 and SB-2), and three others were part of SeaHARRE activities (SH-2, SH-3, and SH-4). The results of these intercomparisons can be used to establish the uncertainties in CHORS pigment quantitations, although the SIMBIOS estimates are only with respect to the HPL results and not to a wider international community.

Data	TChl *a*	TChl *b*	TChl *c*	Caro	But	Hex	Allo	Diad	Diato	Fuco	Peri	Zea	PPig
SB-1	20	54	29	92	22488	4618	22	26	30	12	28	22	2287
SB-2	9	30	42	21	32176	2569	32	17	26	16	37	24	2917
SH-2	5	13	35	19	57	7	28	11	25	4	17	31	21
SH-3	17	17	11	57	23	20	20	8	182	32	41	18	37
SH-4	34	24	32	67	2750	3068	5	14	31	8	284	55	531

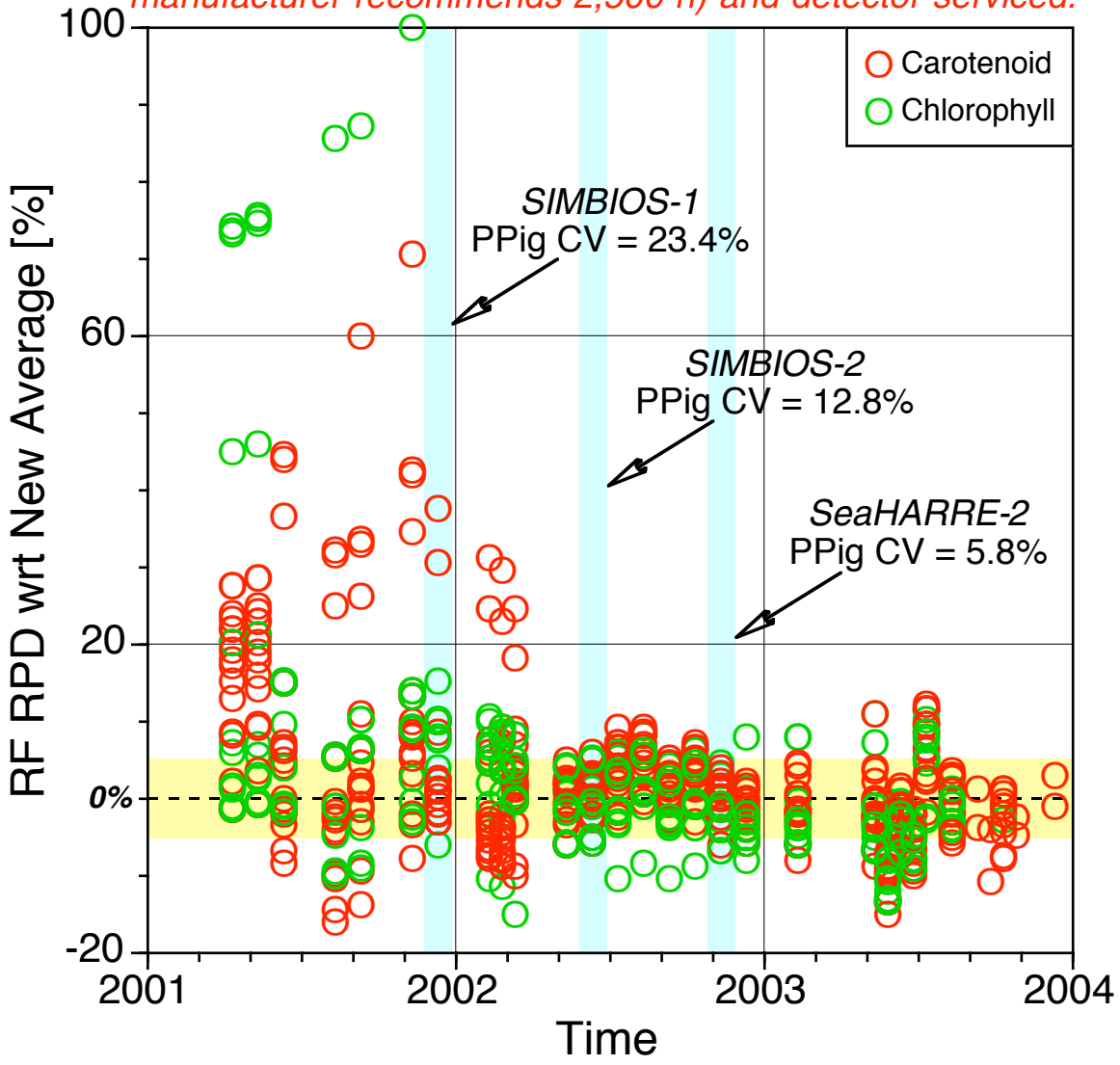
A QA laboratory will quantitate TChl a to within 15% and the PPig average will be to within 25%.

The intercomparisons show the only time period when the average CHORS C_{18} quantitations were to within calibration and validation requirements was during SeaHARRE-2, although some individual pigments always satisfied this requirement (removing But and Hex for SB-1, SB-2, and SH-4 reduces the PPIG uncertainty to 33.6, 25.5, and 55.4%, respectively). The temporal patterns in the uncertainties correspond with the variance in calibrations and the largest uncertainties are seen in the carotenoids, which also have the greatest range in calibration variances.

The Temporal Stability of the CHORS C_{18} HPLC System Prior to the C_8 Method: RFs of All Pigments

The CHORS C_{18} RFs show a large variance for the first year of analysis, but frequent consistency to within ±5% (the yellow band) and most to within ±10%, thereafter. The RPD value is referenced with respect to a *new* average RF computed by excluding the anomalous values in the early data (up to March 2002). Both Phide *a* and Phytin *a* did not yield RPDs within the limits of the plot. In addition, Chl c_2 and Chl c_3 had several RPDs exceeding 100%, so these data points are absent. The data also show the Sea-HARRE-2 results coincide with a time period of good RF consistency and have the best precision (PPig CV).

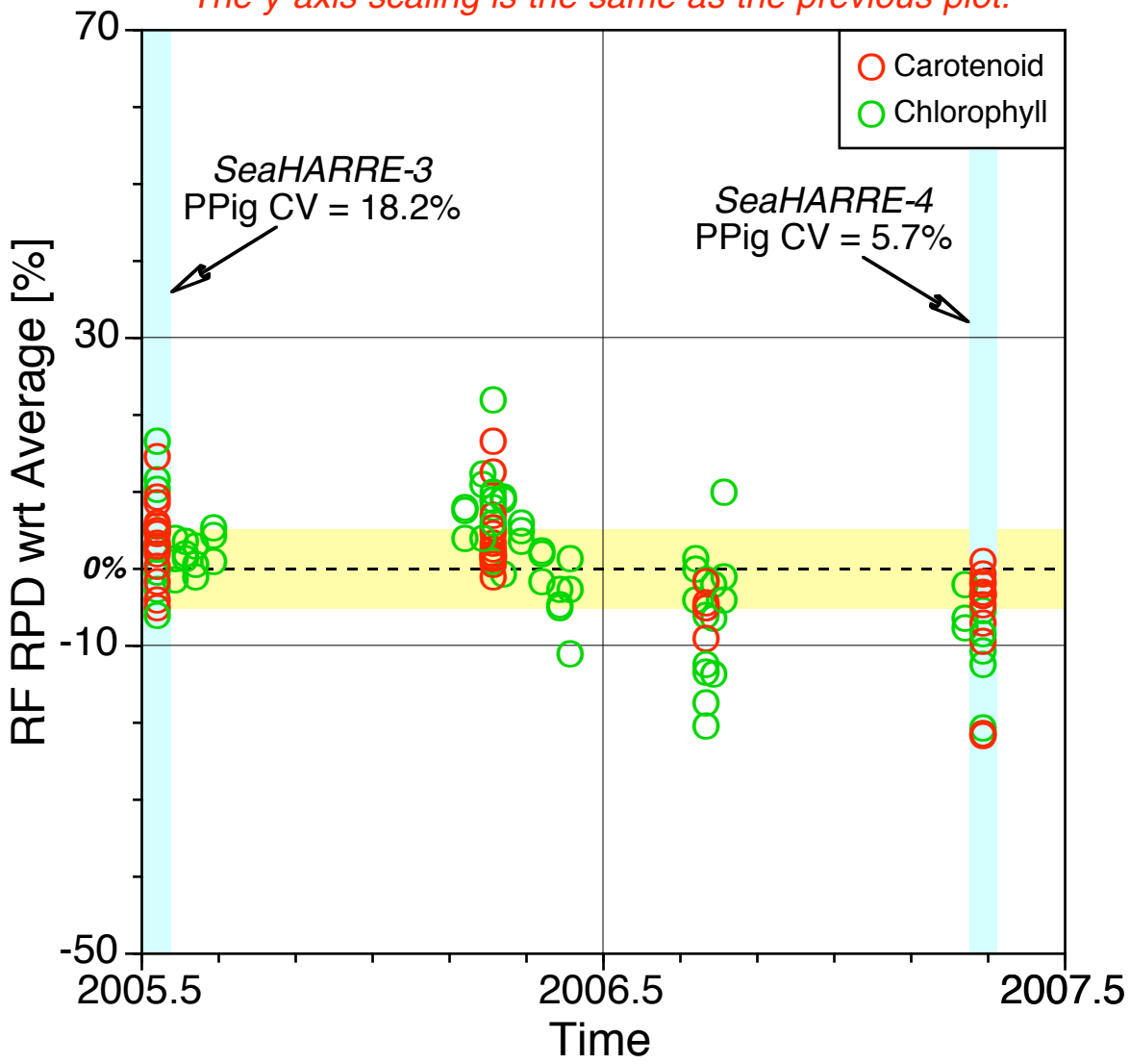
Detector lamp changed in March 2002 with over 5,000 h (the manufacturer recommends 2,500 h) and detector serviced.



The Temporal Stability of the CHORS C_{18} HPLC System After the C_8 Method: RFs of All Pigments

After the C_{18} method was re-implemented, the RFs show a large variance, but none of the extreme values seen earlier. Although, there is frequent consistency for the carotenoids to within ±5% (the yellow band), the range in RPDs is rather large and routinely exceeds ±10%. The SeaHARRE-3 results, for example, show a larger range in RPDs than was seen for SeaHARRE-2, and probably explains why the carotenoid results for SeaHARRE-3 are not as good as the SeaHARRE-2 results. Note also the poor precision for SeaHARRE-3, but the good precision for SeaHARRE-4.

The y-axis scaling is the same as the previous plot.



Another CHORS Quantitation Problem Found in Both the C_{18} and C_8 Calibration and Field Data

2007 Calibration		*SIMBIOS-1*		*SIMBIOS-2*		*SeaHARRE-3*		*SeaHARRE-4*	
Sample	MVChl *a*	Sample	But	Sample	Fuco	Sample	Peri	Sample	Zea
Test-025	1232	SB1-T05	0.048	SB2-003	0.006	SH3-9a	0.024	SH4-D08	0.024
Test-026	1229	SB1-T09	0.024	SB2-008	0.006	SH3-9b	0.002	SH4-D23	0.024
Test-027	1785	SB1-T12	0.048	SB2-012	0.012	SH3-9c	0.002	SH4-D35	0.017
Average	1415	Average	0.040	Average	0.008	Average	0.009	Average	0.021
StanDev	320	StanDev	0.014	StanDev	0.003	StanDev	0.012	StanDev	0.004
CoeffVar	22.6	CoeffVar	34.0	CoeffVar	42.9	CoeffVar	133.2	CoeffVar	18.4

These data are all from the C_{18} method, but the same type of problem is found in the C_8 data.

An unexpected aspect of the most recent inquiries into the response of the CHORS system is the occurrence of large outliers during triplicate injections of a calibration standard. These anomalies have been seen in both the most recent C_8 and C_{18} calibrations. The question arises whether or not this was a feature of past intercomparisons, because if it was, this is an aspect of the system that cannot be explained by the nonlinear response of the detector, and it would probably be impossible to detect in the analysis of natural samples (which are rarely done in triplicate). For those intercomparisons involving triplicates (SeaHARRE-2 was based on duplicates), the appearance of outliers is always found and it varies in magnitude. Furthermore, it is found in pigments whose detection and quantitation has been found to be similar to Chl *a* (e.g., Fuco).

Correcting all the Data Involved is Going to be a Significant Undertaking

Year(s)	Method	NASA	Others
98-00	C_{18}		2,642
2001	SIMBIOS C_{18}	1,819	
2002	SIMBIOS C_{18}	3,986	
2003	SIMBIOS C_{18}	3,421	
2004	MODIS C_8	2,151	168
2005	MODIS C_8	4,965	792
2006	MODIS C_8	512	
2006	C_{18}		2,347
2007	C_8		667
2007	C_{18}		318
	Total	16,854	6,934

The total number of samples analyzed by CHORS for NASA or of interest to NASA is very large: about 17–20,000. Given all the sources of variance in the CHORS results and the fact that the correction scheme may very well require the inspection or manipulation of the chromatograms involved, it seems sensible to first determine how many corrections in the calibration and quantitation process can be applied. The size of the task will require a prioritization of the samples and a verification of the data archive, after which, the basic correction scheme can be executed:

Minutes	Time Period
480	Per day
2,400	Per week
9,600	Per month
62,400	Per 6 months
124,800	Per year

Samples	Per Year
12,480	10 mins each
15,600	8 mins each

- Determine the nonlinear functional response.
- Plot all prior calibrations with respect to the new functional description to see how well they fit the nonlinearity.
- Query the PIs to find out what pigments they need.
- Recalibrate the requisite calibration data.
- Establish QA procedures (another analyst also quantitates a subset of chromatograms which are intercompared).
- Requantitate the chromatograms.