www.csiro.au

The CSIRO Method

- The SeaHARRE - 5 Samples

Lesley Clementson

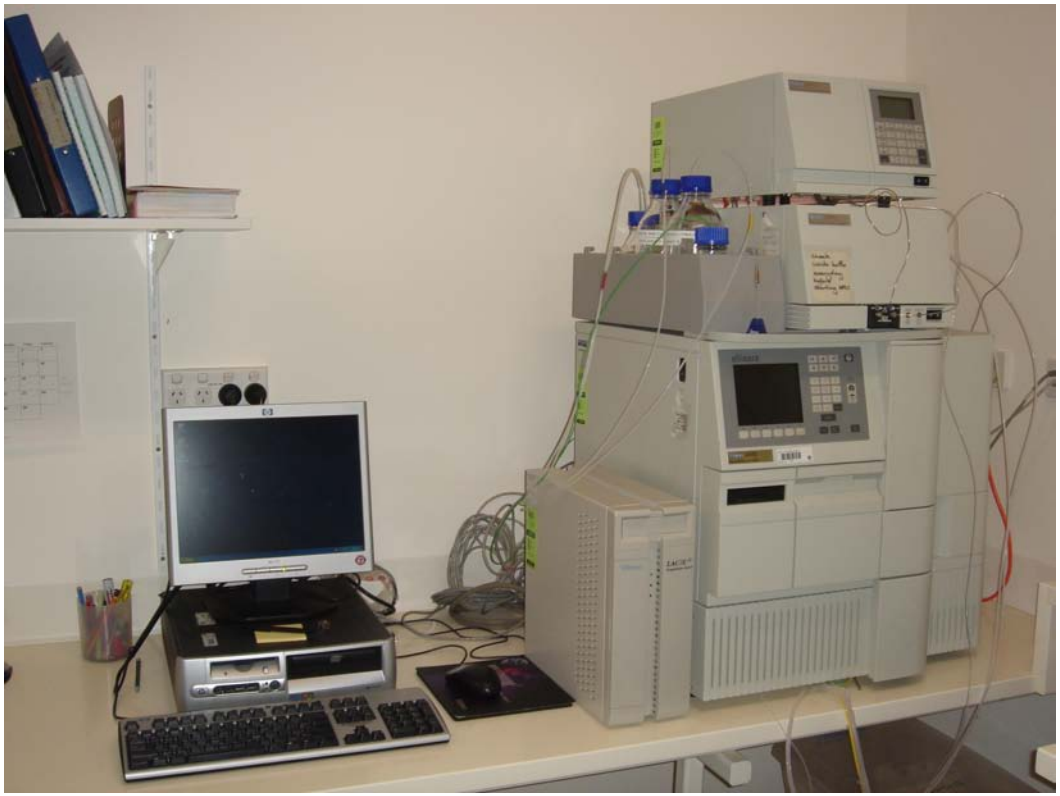
CSIRO Marine and Atmospheric Research, Hobart, Australia

SeaHARRE- 5 Meeting, Hobart, April 2010

CSIRO

HPLC hardware

- Waters - Alliance high performance liquid chromatography system, comprising a 2695XE separations module with column heater and refrigerated autosampler and a 2996 photo-diode array detector.



Method

- Based on Van Heukelem, L., Thomas, C. (2001)

  Computer assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. J. Chromatogr. A., 910, 31-49.

- Zorbax Eclipse XDB-C8 stainless steel 150 mm x 4.6 mm ID column with 3.5 µm particle size (Agilent Technologies)

- Binary gradient elution procedure with flow rate of 1.1 mL per minute

  solvent A is 70:30 28 mM tetrabutyl ammonium acetate, pH 6.5 : methanol

  solvent B is 100% methanol.

Method continued

Time	% solvent A	% solvent B
	95	5
11	45	55
15	45	55
22	5	95
29	5	95
31	95	5

After 31 minutes there is a 5 minute injection delay where 95% A and 5% B run at 1.1 mL per minute.

Column temperature 55°C

Autosampler 4°C

Applications

Analyse 1000 – 2000+ during an average year for multiple projects

- Remote sensing/ocean colour validation
- Biological oceanography
- Biofuels from microalgae

Samples come from different water types and sample types

- Water column phytoplankton – open ocean, coastal, estuarine and riverine from the tropics to Antarctica
- Sea-ice algae
- Microphytobenthos (MPB)
- Seagrass and macroalgae
- Microalgal cultures

Sample handling – SH5 samples

Australian samples collected 10 November – 04 December 2008

USA samples were received 09 February 2009

USA samples were transferred to liquid N_2 dewars in the laboratory

Samples were extracted in groups of 12

A – D	26 May 2009
E – H	27 May 2009
I – L	28 May 2009
AA – AD	29 May 2009
AE – AH	02 June 2009
AI – AM	03 June 2009

There were no delays between extraction and analysis

Sample handling – SH5 samples

A second set of samples (USA and Australian) was sent and were received 09 February 2010

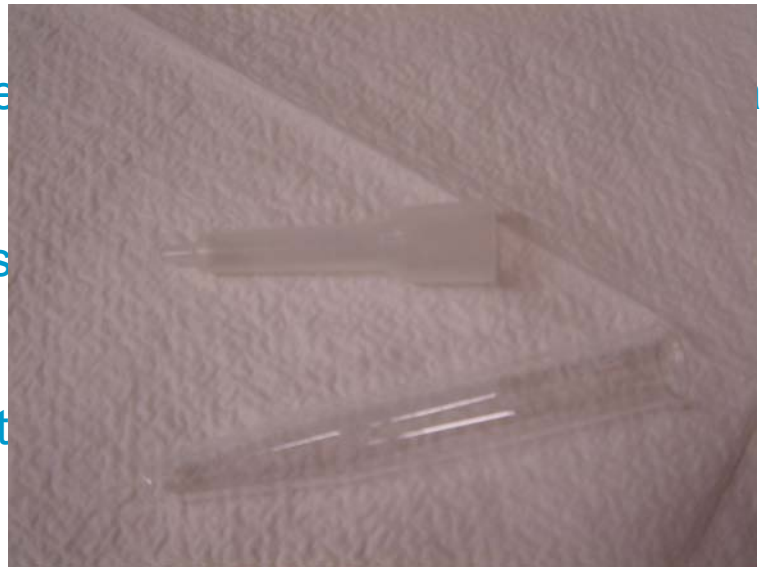
Samples were transferred to liquid N_2 dewars in the laboratory

Samples were extracted in groups of 12

A – D	29 March 2010
E – H	30 March 2010
I – L	yet to be analysed
AA – AD	31 March 2010
AE – AH	yet to be analysed
AI – AM	yet to be analysed

A set of samples collected from Storm Bay 01 April 2010

Extraction procedures



Filters were cut into small pieces and cove
10 ml centrifuge tube.

The samples were vortexed for about 30 s
ice-water bath for 15 minutes in the dark.

The samples were then kept in the dark at

After this time 200 μL water was added to
mixture was 90:10 acetone:water (vol:vol) and sonicated once more in an ice-water bath for 15 minutes.

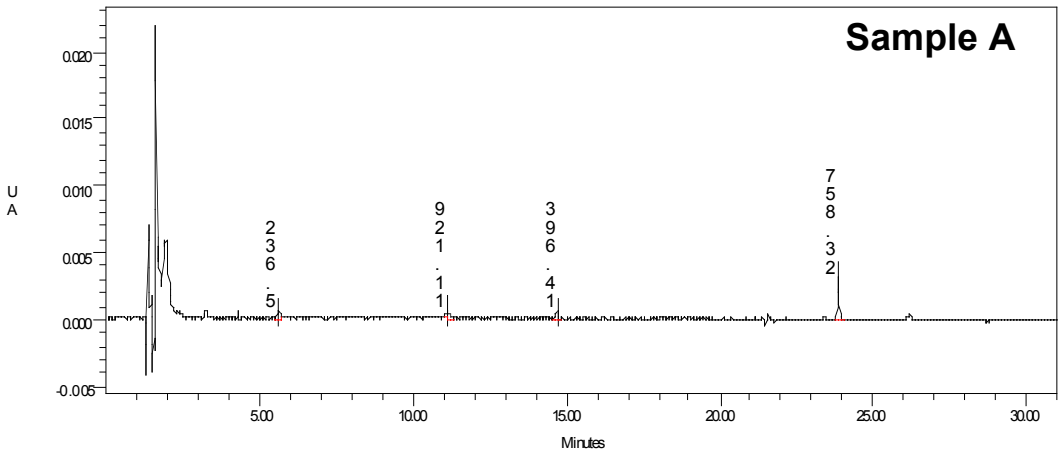
The extracts were quantitatively transferred to a clean centrifuge tube and column, including 2 x 0.5 mL rinses and centrifuged to remove the filter paper.

The volume of the final extract was recorded and the extract then filtered through a 0.2 μm membrane filter (Whatman, anatope) into a 2 mL amber vial prior to analysis by HPLC

Extraction procedures continued

In SeaHARRE -3 and -4 the CSIRO method was part of the reference group.

So what went wrong in the SH5 experiment?



SH5 results – USA set 1

	Total chlorophyll-a			
	C Sample	D Sample	H Sample	L Sample
A	0.373	0.692	0.742	0.738
B	0.772	1.406	1.506	1.537
C	0.516	0.906	0.945	0.947
D	0.967	1.644	1.817	1.738
E	0.906	1.784	1.978	1.862
F	0.810	1.475	1.655	1.590
G	2.481	3.860	4.234	3.706
H	1.219	2.397	2.467	2.324
I	1.522	3.481	3.810	3.456
J	2.619	5.124	5.620	5.193
K	2.569	5.192	5.497	5.075
L	1.238	2.055	2.314	2.178

USA Results – extraction volume

	C results		Chl-a results		
Filter code	2 mL extraction	3 mL extraction	D results	H results	L results
A	0.405	0.721	0.679	0.728	0.729
B	0.733	1.308	1.369	1.465	1.510
C	0.512	0.812	0.885	0.919	0.933
D	0.898	1.659	1.527	1.694	1.640
E	0.840	1.644	1.631	1.815	1.732
F	0.752	1.037	1.475	1.540	1.518
G	1.966	2.739	3.860	4.108	3.632
H	1.035	1.471	2.397	2.156	2.147

SH5 results – Aust set 1

	Total chlorophyll-a			
	C Sample	D Sample	H Sample	L Sample
AA	3.766	3.780	4.806	4.330
AB	10.356	12.067	12.999	10.792
AC	8.666	8.528	9.536	8.338
AD	1.352	1.435	1.549	1.477
AE	7.263	7.397	8.611	7.509
AF	0.394	1.250	1.581	1.126
AG		0.973	1.088	1.098
AH	5.147	5.011	5.822	4.997
AI	1.112	1.437	1.696	1.534
AJ	2.025	2.497	2.876	2.444
AK	6.240	9.472	9.642	8.944
AM	3.295	3.986	4.505	3.695

Aust Results – extraction volume

	C results		Chl-a results		
Filter code	2 mL extraction	3 mL extraction	D results	H results	L results
AA	4.022	4.328	3.780	4.616	4.257
AB	11.093	11.288	12.067	12.847	10.675
AC	9.262	8.735	8.528	9.353	8.250
AD	1.283	1.283	1.435	1.447	1.441

Extraction volume

Filter code	2 mL extraction	3 mL extraction
2a		0.448
2b		0.458
2c		0.435
2d	0.381	
2e	0.376	
2f	0.386	
3a		0.334
3b		0.320
3c		0.317
3d	0.296	
3e	0.282	
3f	NR	

Acetone : water ratio

Filtered 100 mL of Milli-q water through each of 6 25 mm GF filters

255, 257, 214, 220, 224, 214 mg - average = 230.6 mg

Acetone:water (3 mL extraction) = 88:12

Acetone:water (2 mL extraction) = 86:14

Acetone : water ratio

	2 mL extraction		3 mL extraction	
	Acetone (mL)	Water (mL)	Acetone (mL)	Water (mL)
Acetone ext.	2.0		3.0	
Water add		0.133		0.200
Water in filter		0.231		0.231
Rinse	0.900	0.100	0.900	0.100
TOTAL	2.900	0.464	3.900	0.531

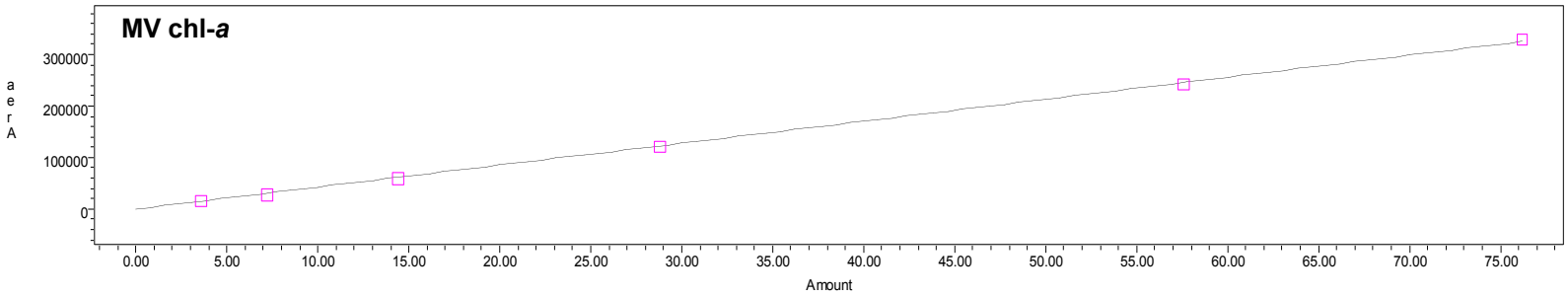
Quality Assurance variables

What parameters are monitored over time as quality assurance variables?

Multipoint calibrations

all standards approximately 5-6 standards in the range 2 – 100 ng/inj.

Exceptions are MV chl-*a* and MV chl- *b* (2 – 400 ng/inj)



One was done for each pigment in May 2010

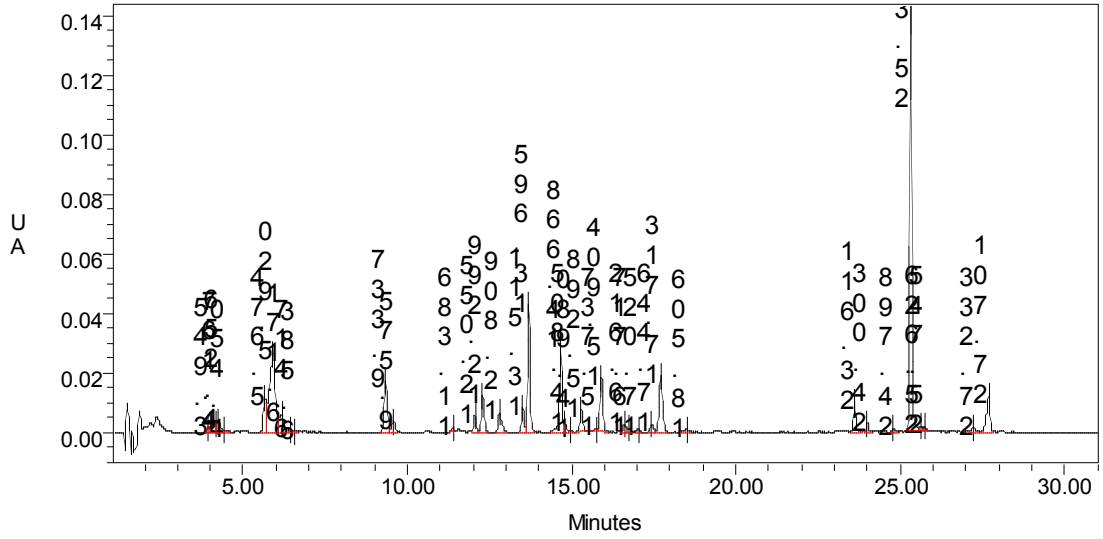
Response factor for chl-a was 3620 – Oct 2004; 3925 – Mar 2007 and 3750 – May 2010

Response factor for chl-b was 2000 – Oct 2004; 2128 – Mar 2007 and 2020 – May 2010

Quality Assurance variables - continued

Culture mix – qualitative only

5 SCOR cultures (mixed) injected with each analysis run.

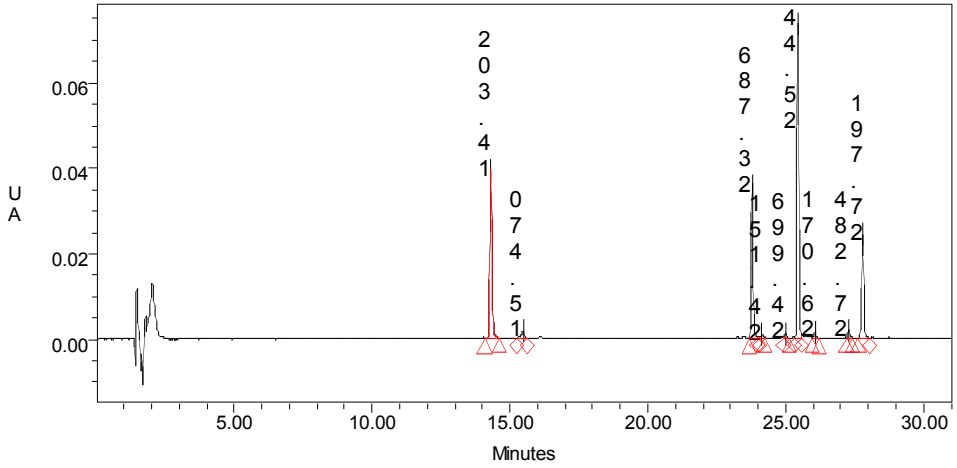


Provides quality assurance on the retention times of the pigments throughout the entire chromatogram. – cf the mixed standard.

Quality Assurance variables - continued

Single point calibrations

4 standards (mixed) injected with each analysis run.

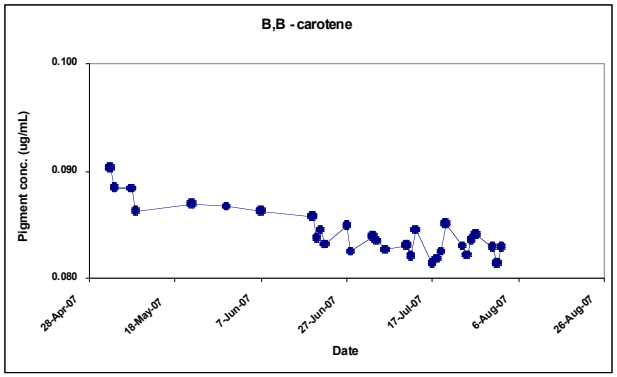


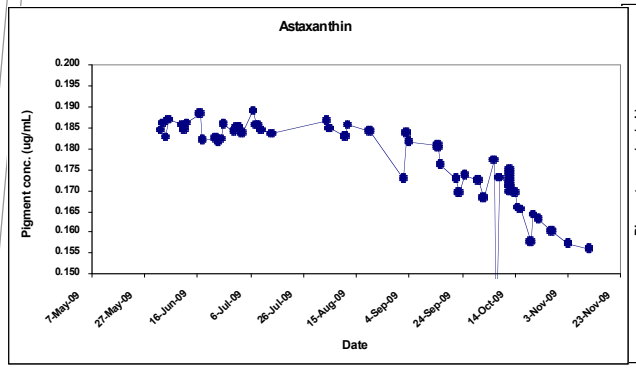
Astaxanthin

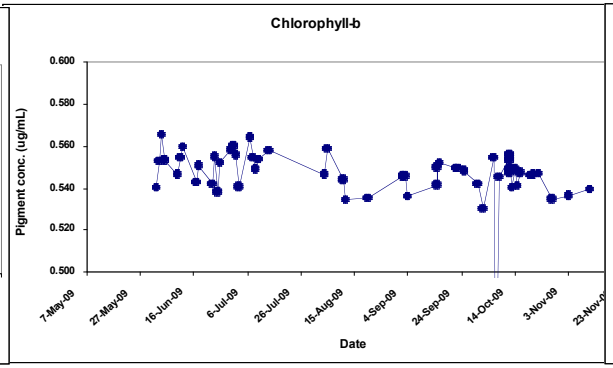
MV Chl-*b*

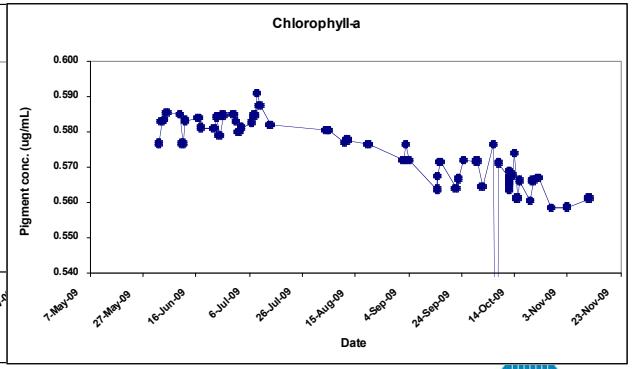
MV Chl-*a*

β,β-carotene









CSIRO

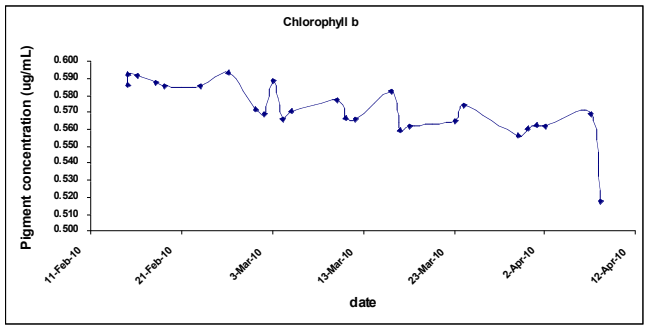
Conclusions

Is the problem due to

The change in the extraction volume

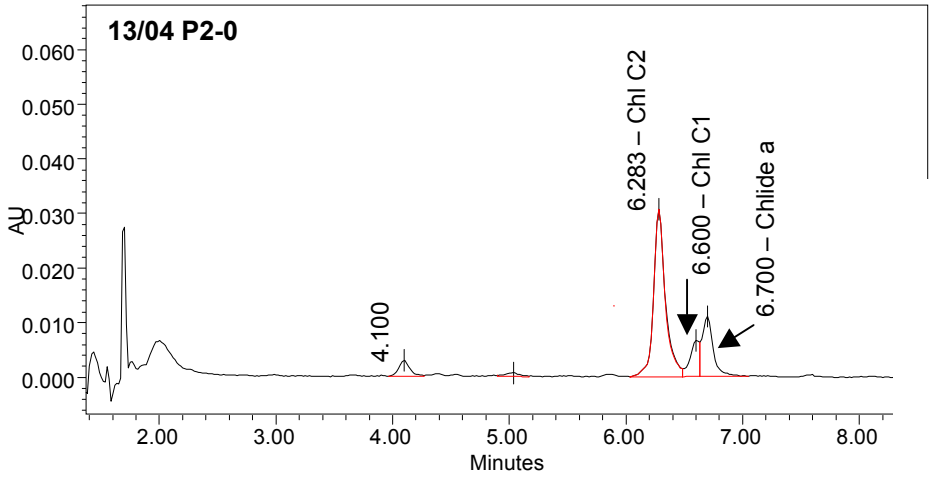
The slight change in the acetone to water ratio of the final extraction solution

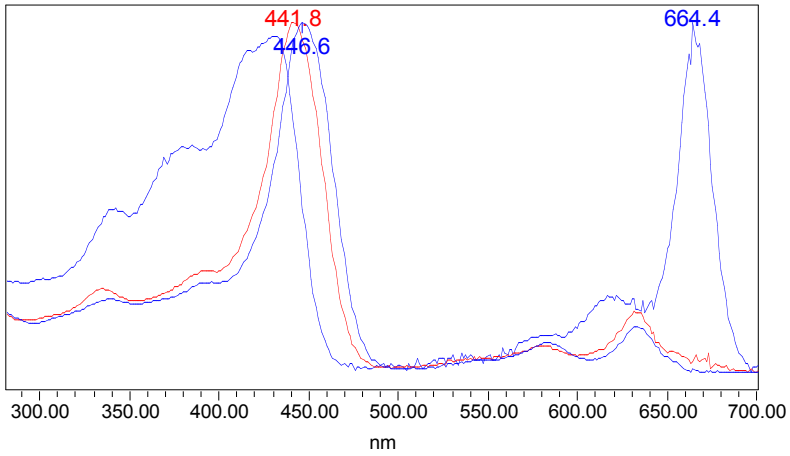
Why is there a difference between the USA and the Australian samples?

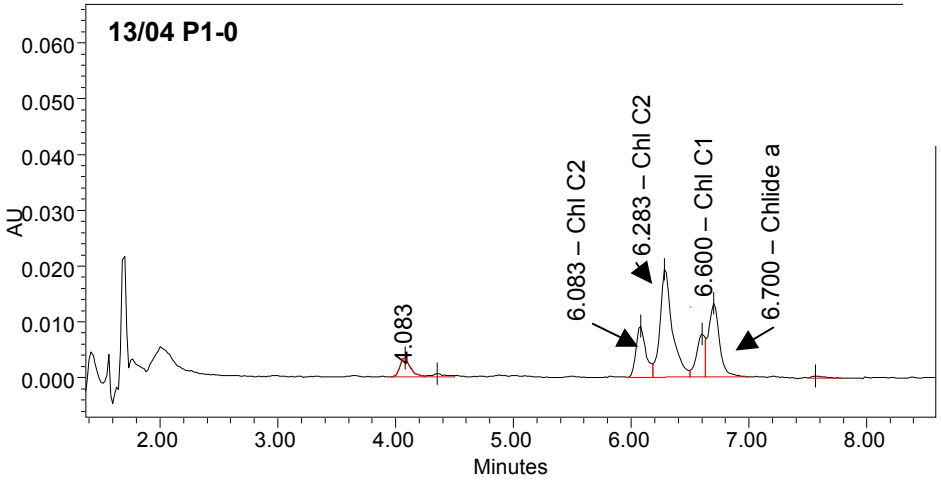

Chlorophyll b
Pigment concentration (ug/mL)
0.600
0.590
0.580
0.570
0.560
0.550
0.540
0.530
0.520
0.510
0.500
11-Feb-10
21-Feb-10
3-Mar-10
13-Mar-10
23-Mar-10
2-Apr-10
12-Apr-10
date

Data Reporting – confidence scale continued

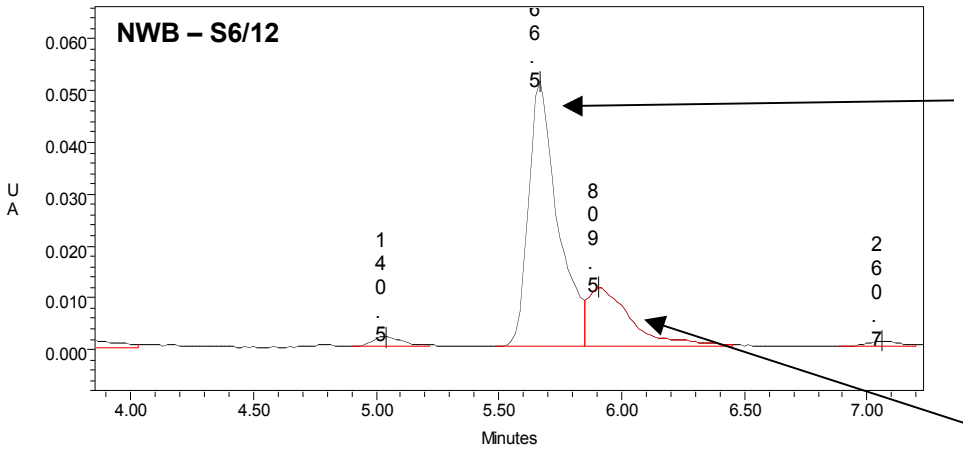
Chlorophyll C2, Chlorophyll C1, Chlorophyllide a 0 – 2 Why?

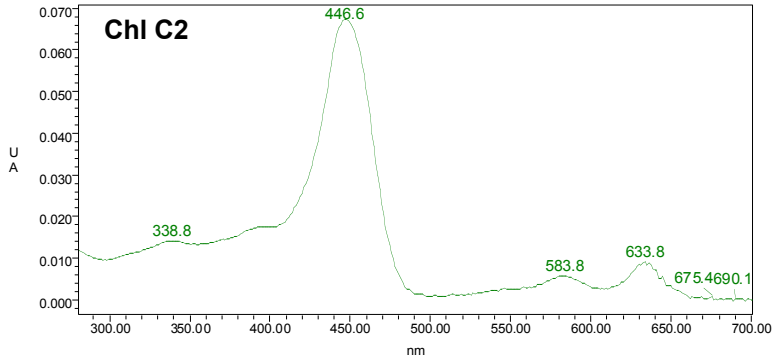


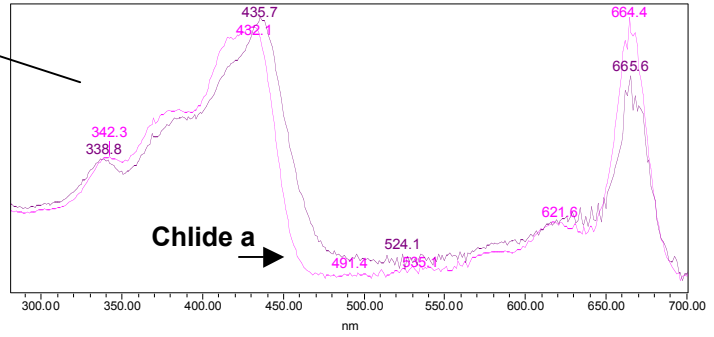


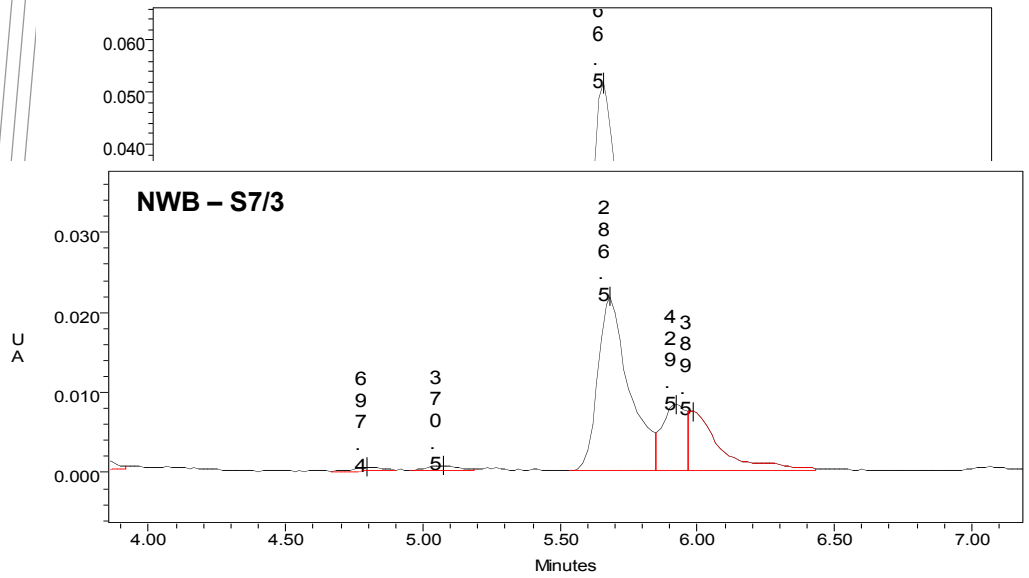


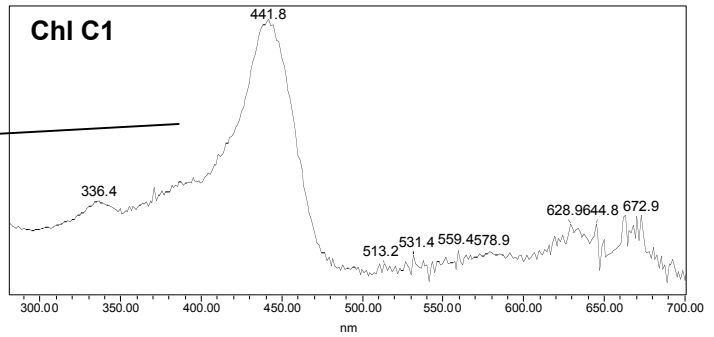
Data Reporting – confidence scale continued





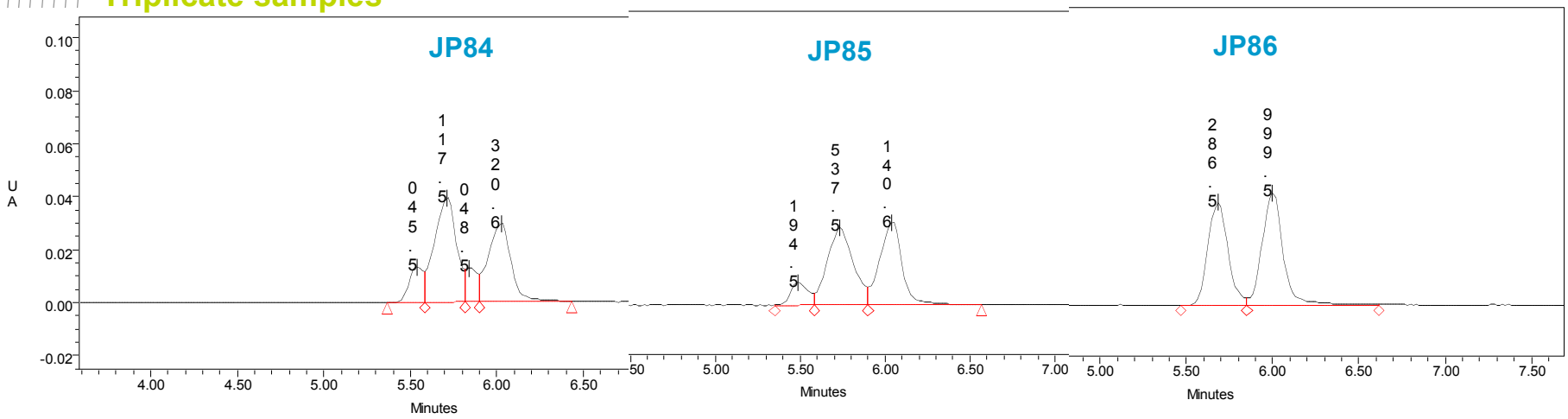






Data Reporting – confidence scale continued

Triplicate samples

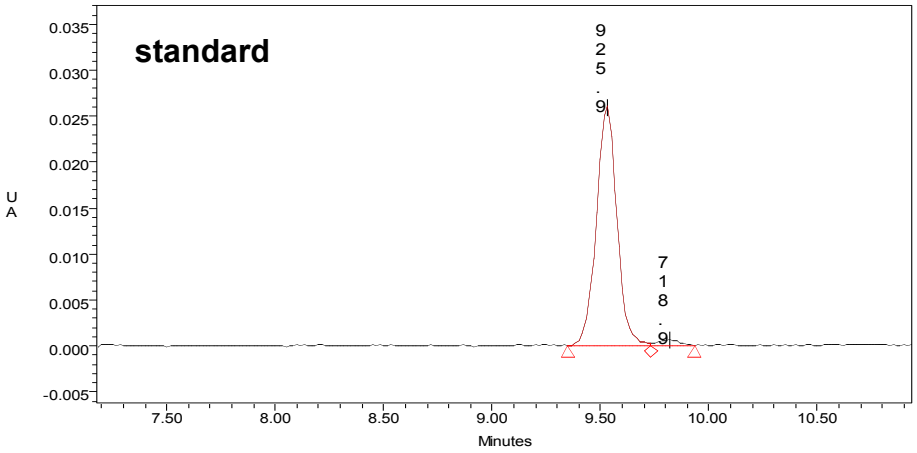


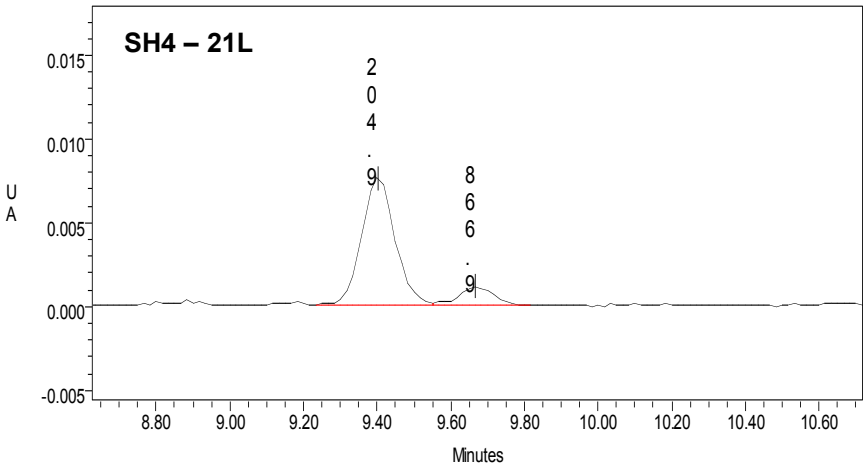
In SH-4 samples, B - G there was an additional peak that seemed to be neither Chl C2 or Chl C1. After analysing a SCOR culture, known to contain MgDVP, I decided that this additonal peak was MgDVP. However lacking Chl C1 and MgDVP standards, the concentration of these pigments was determined using the Chl C2 response factor.

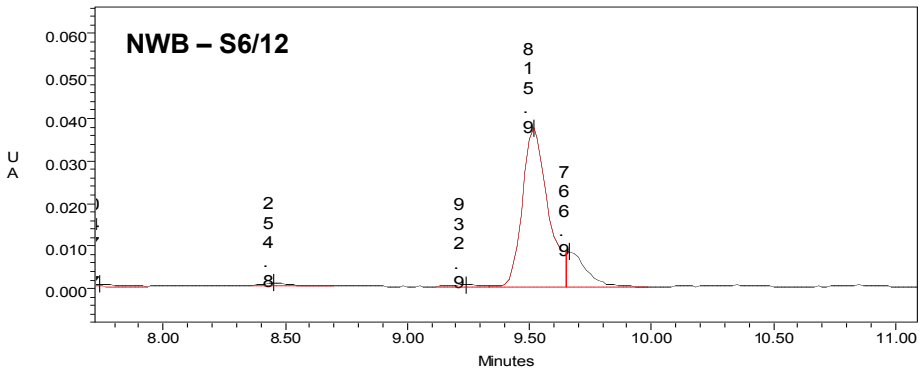
To answer a later question, the close elution of these peaks is probably the greatest limitation of my analyses – in samples.

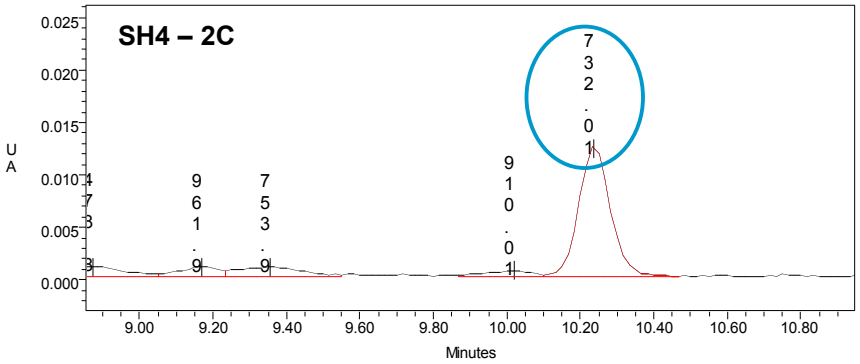
Data Reporting – confidence scale continued

Peridinin, 0 – 1



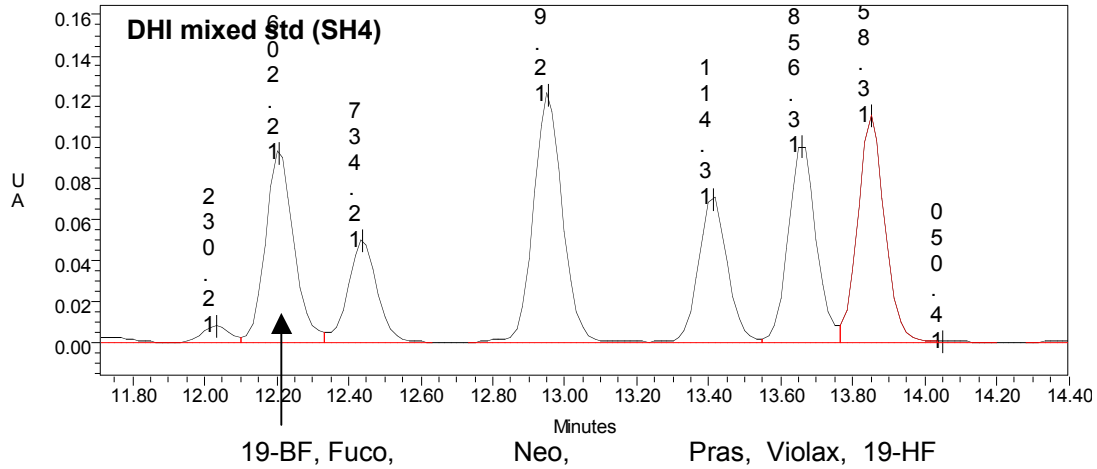


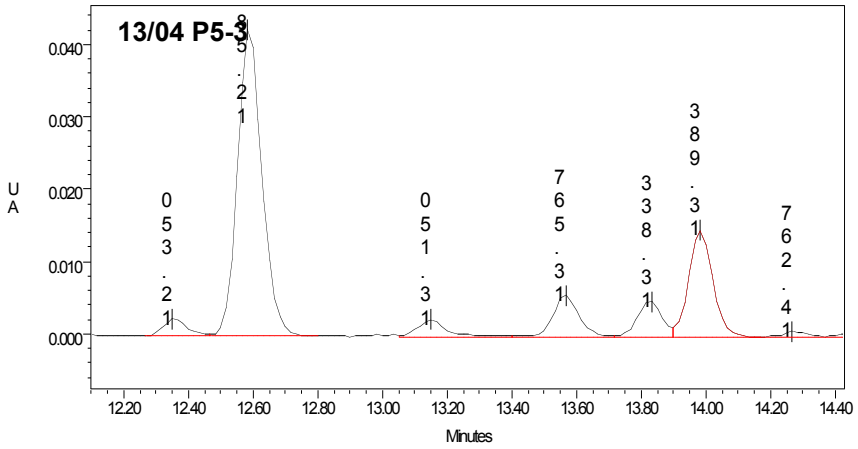


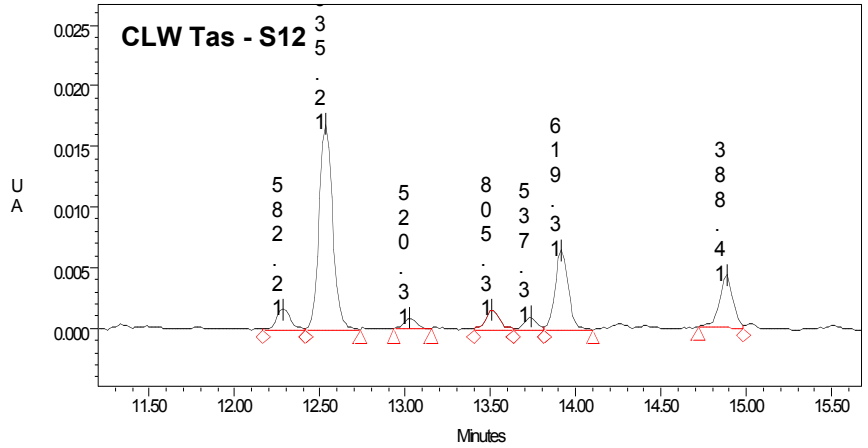


Data Reporting – confidence scale continued

Violaxanthin and 19- Hexanoyloxyfucoxanthin

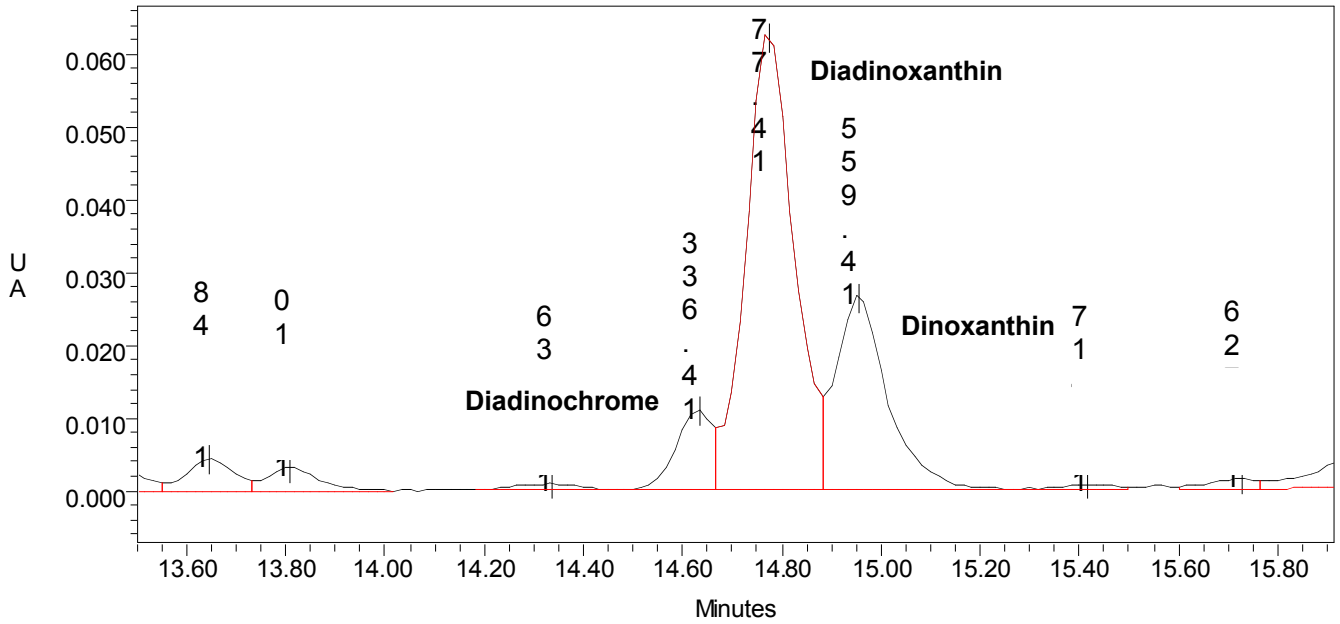






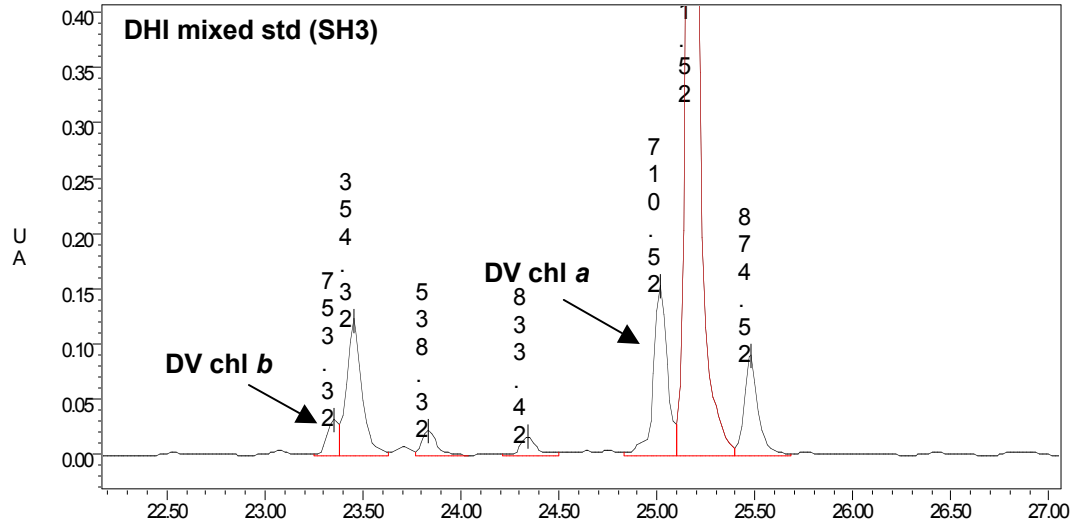
Data Reporting – confidence scale continued

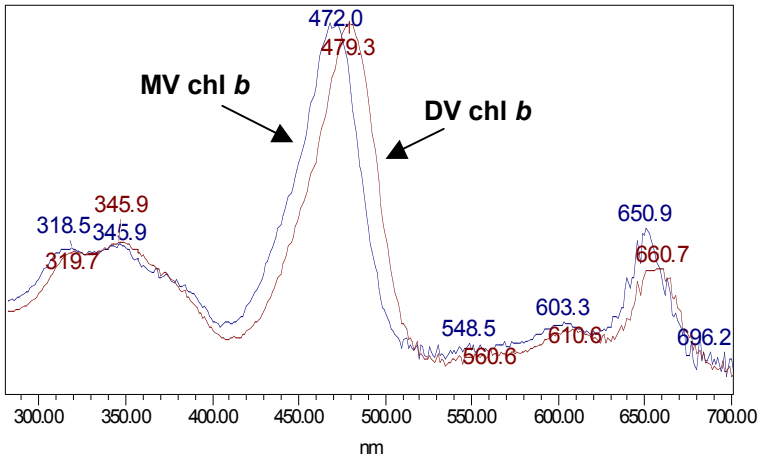
Diadinochrome, Diadinoxanthin, Dinoxanthin

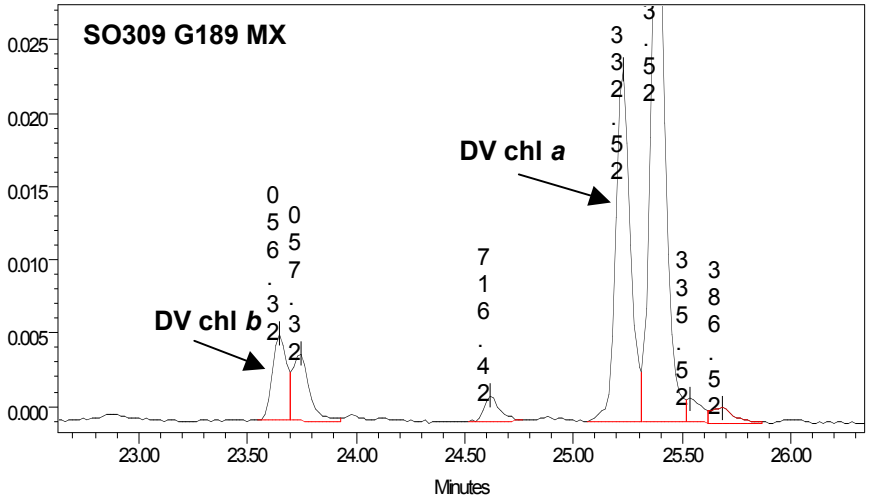


Data Reporting – confidence scale continued

DV and MV chlorophylls *a* and *b*

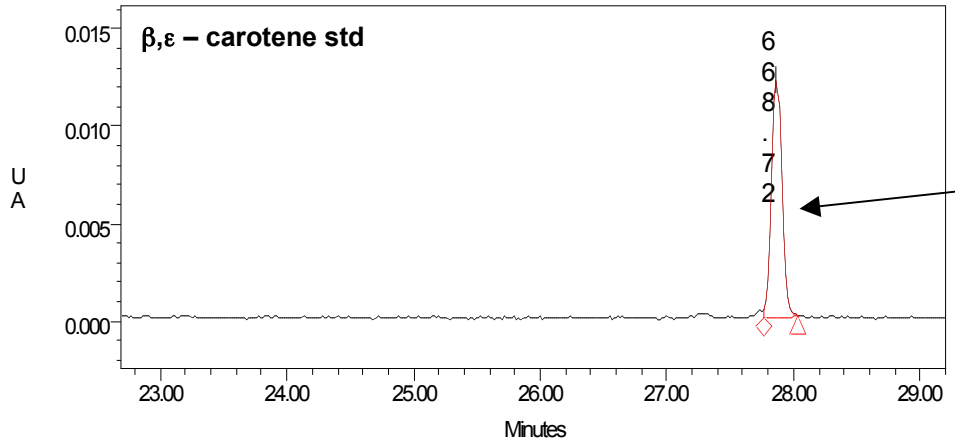


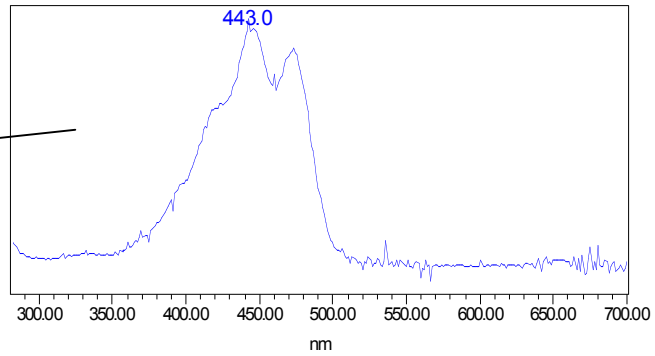


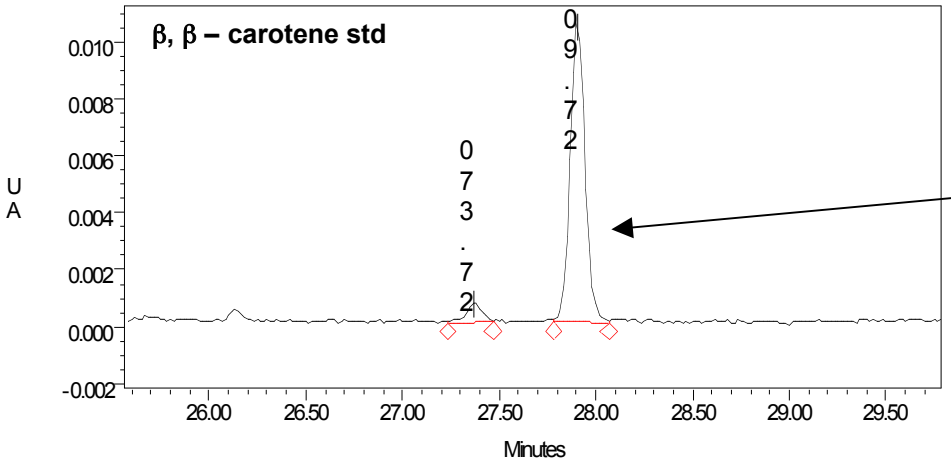


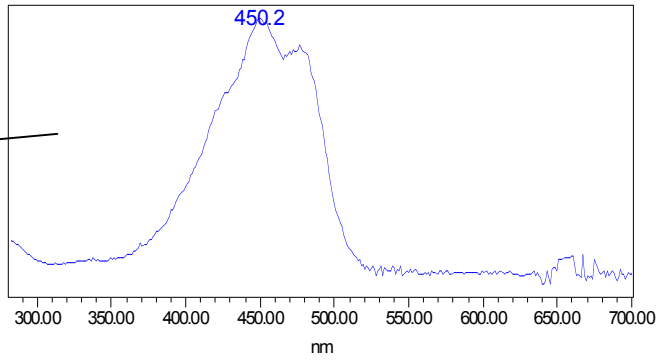
Data Reporting – confidence scale continued

β,ε – carotene, β,β- carotene

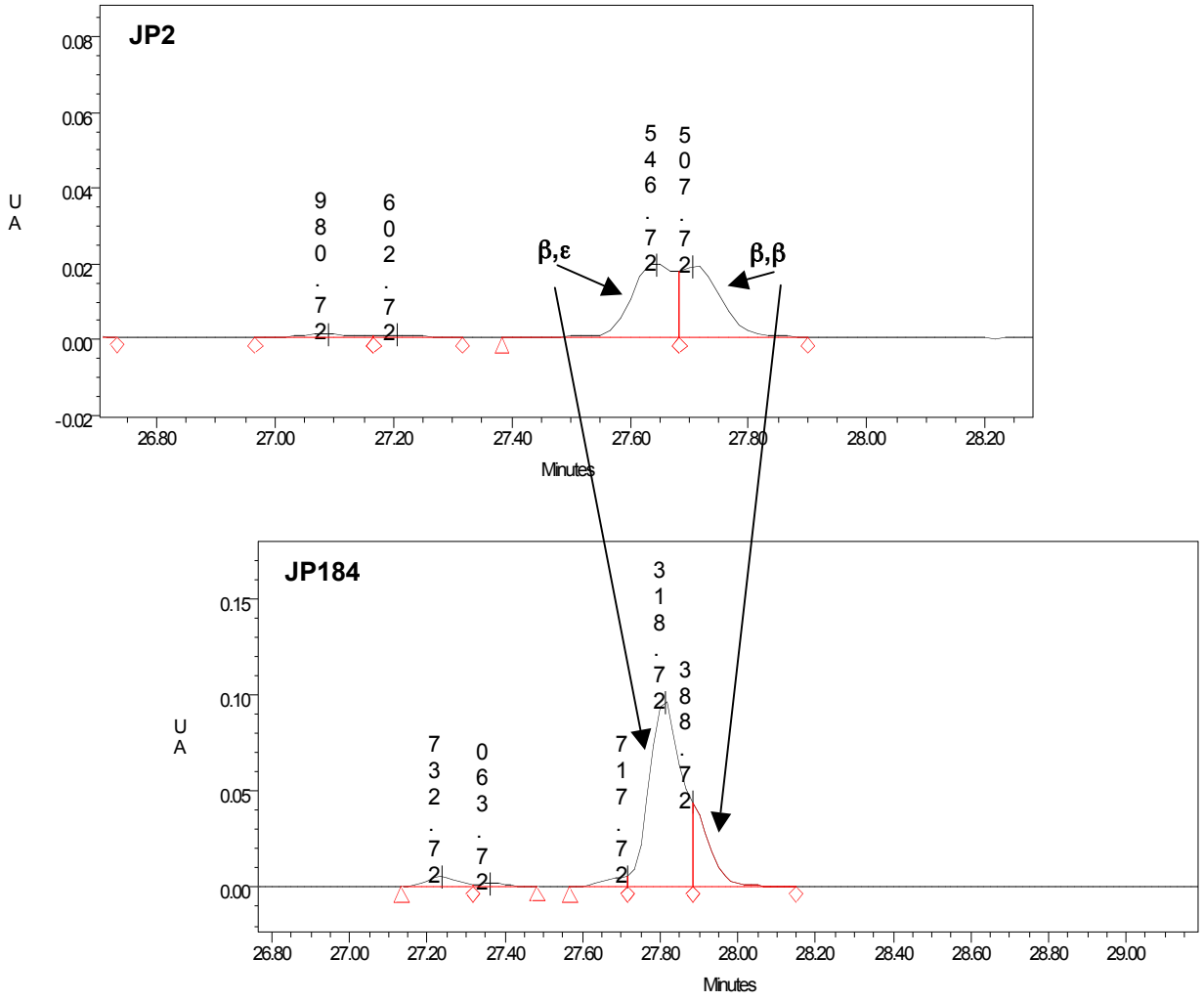




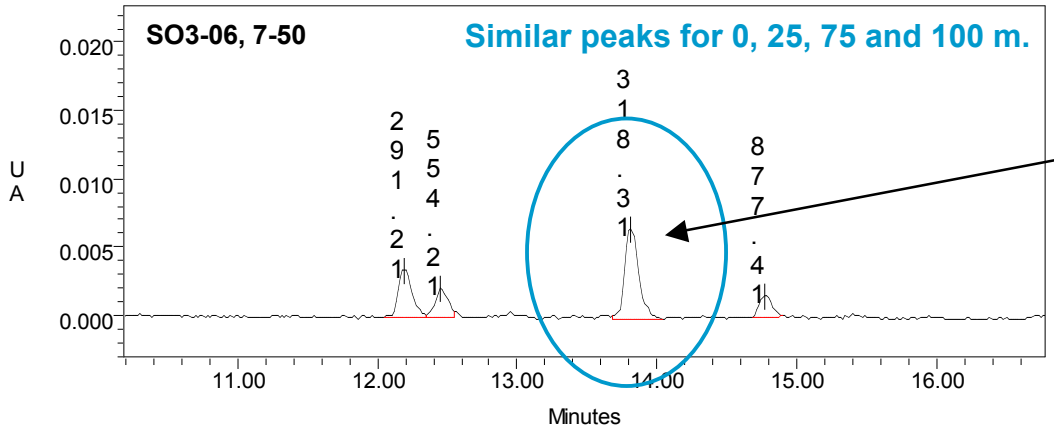


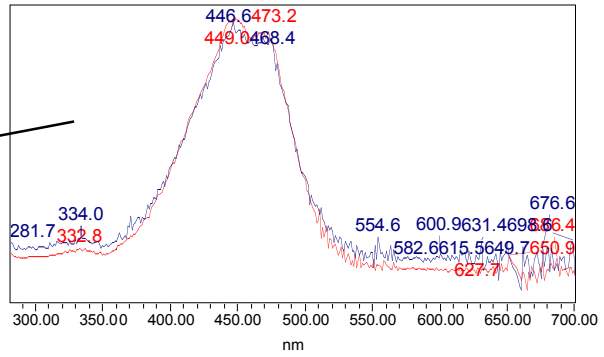


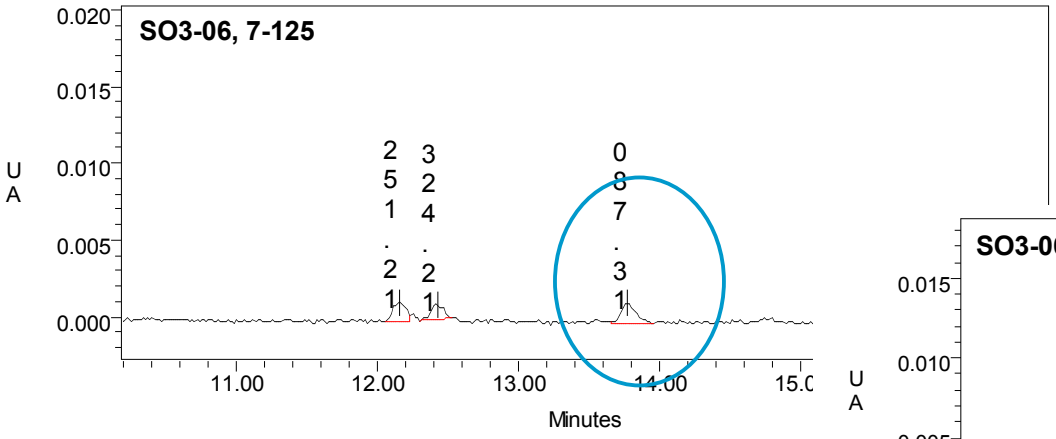
Data Reporting – confidence scale continued

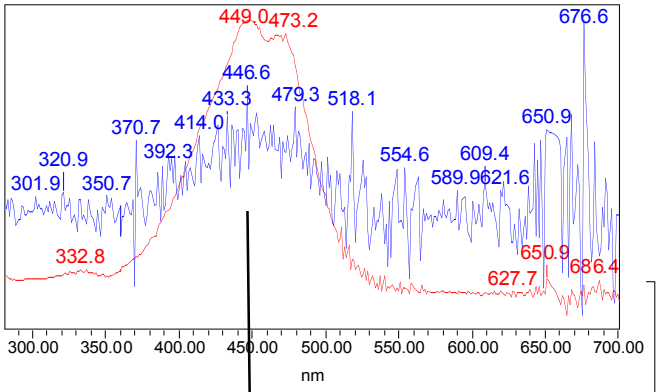


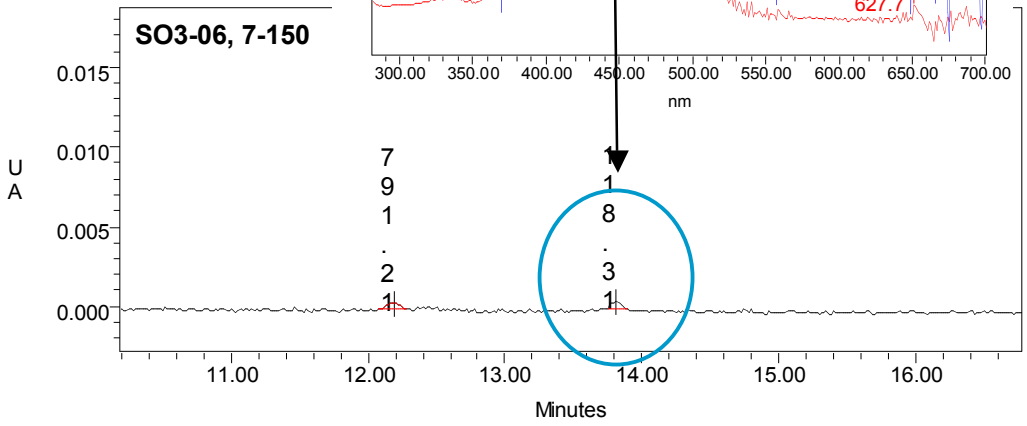
Data Reporting – small peaks







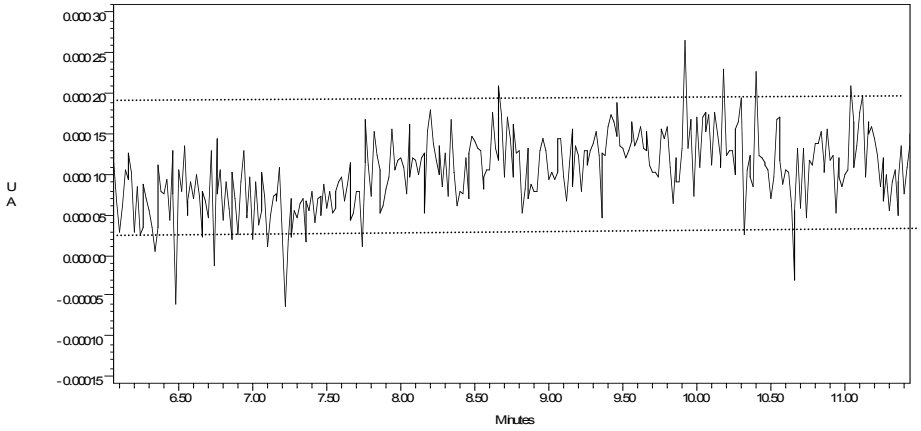




How is the choice made between reporting and not reporting a small peak?

Data Reporting – small peaks continued

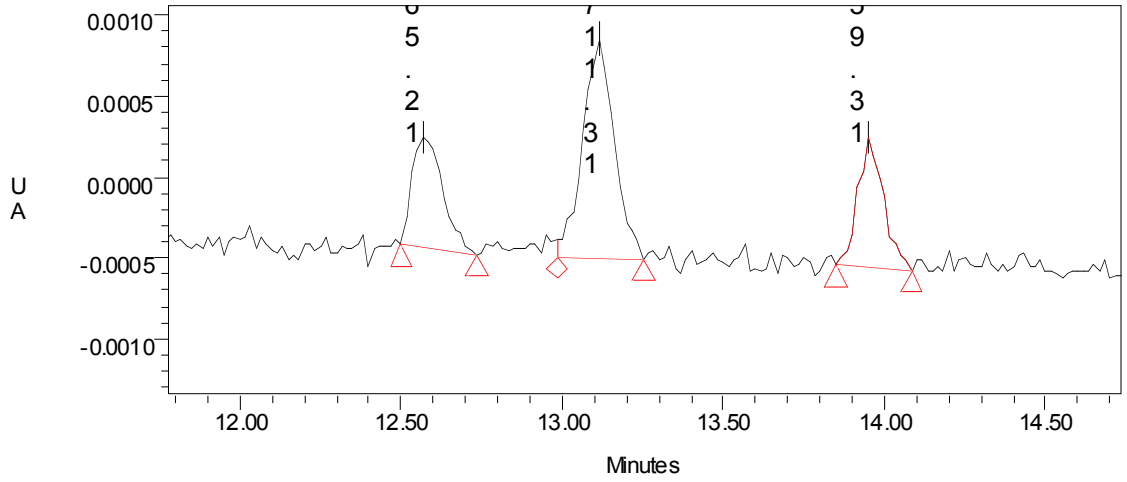
How do you quantitate small peaks?



Baseline noise:

Range 0.00002 - 0.00018 AU

= 0.00016 AU



Fuco = 0.59 ng/inj

Neo = 0.69 ng/inj

19-HF = 0.53 ng/inj

Data Reporting – small peaks continued

How is the choice made on the number of digits reported?

For Fucoxanthin (reference λ = 436 nm)

baseline = 0.00016

peak ht = 0.0007

S:N = 4.375

Limit of detection based on a S:N = 3

Therefore a S:N = 3 is equivalent to 0.405 ng/inj

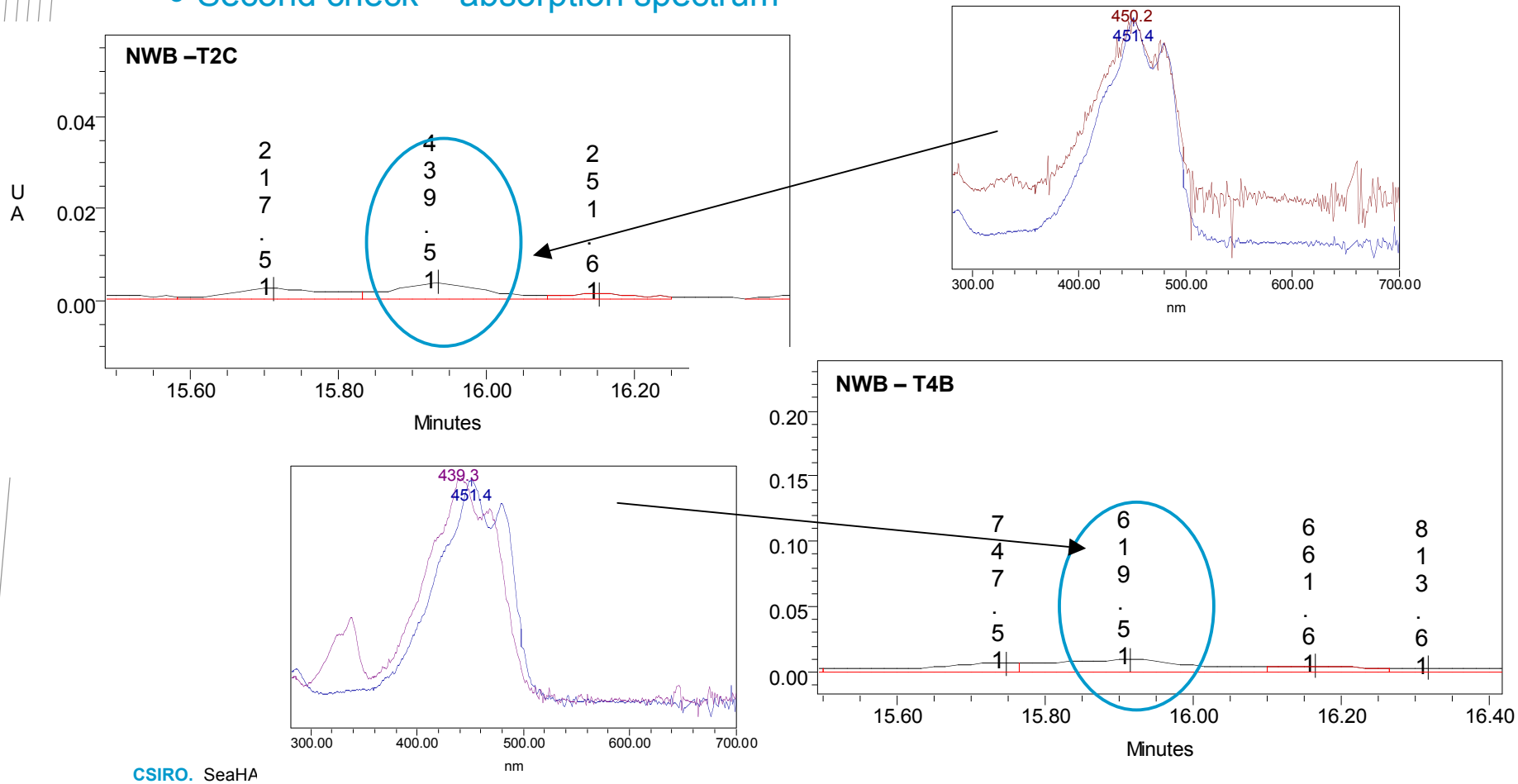
Injection volume = 150 μL

LOD equivalent to 0.003 μg/mL

Data Reporting – absorption spectra

How does the absorption spectrum affect peak identification?

- First check - retention time
- Second check - absorption spectrum



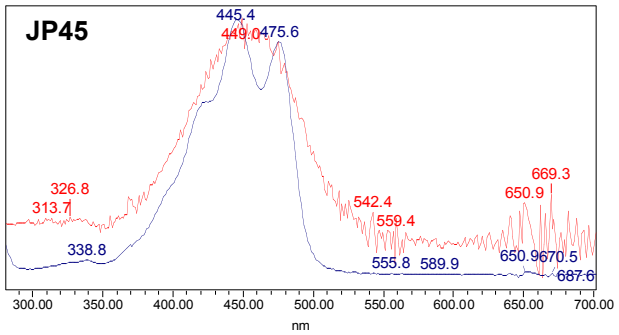
Data Reporting – absorption spectra continued

What is your criteria for rejecting a peak based on peak spectra?

- Small peak in a single sample (not part of a set of samples) without any absorption spectrum.

  ie: not in a depth profile where concentration of a pigment declines.

- Any peak where the absorption spectrum does not match the absorption spectrum of the standard pigment.



Diadinoxanthin – same retention time, different spectrum

Data Reporting – pigments not found

How do you report pigments that are not found in a particular sample, but for which you typically quantify?

Options: bdl – below detection limit

flag of some description – zero, one, blank

value of LOD or LOQ

We use zero!

Data Reporting – standard calibration

Do you report pigments for which you do not use a discrete calibration standard? Which pigments and how do you quantify the pigment concentration?

Report on 32 pigments of which only five do not have an associated standard calibration.

- Chlorophyll C1
- MgDVP
- Diadinochrome
- Dinoxanthin
- DV Chlorophyll b

All of these pigments do not have standards readily available. Because of close elution with other pigments, they would be difficult to purify

Chl C1, MgDVP – use Chl C2 RF

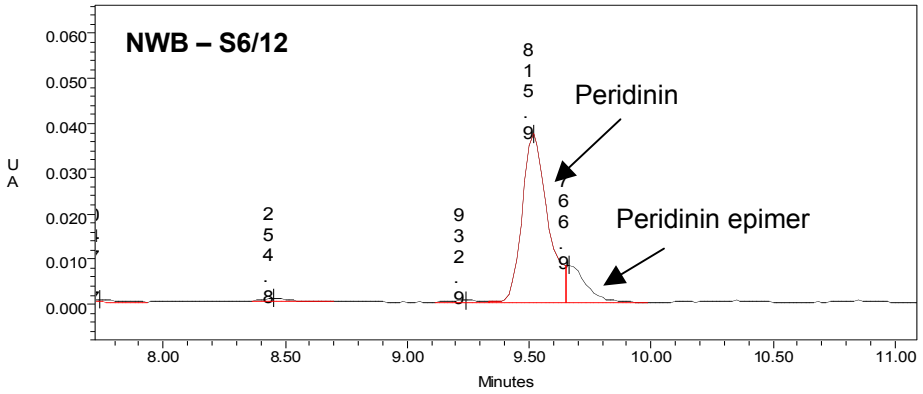
Diadinoch – use Diadinox RF

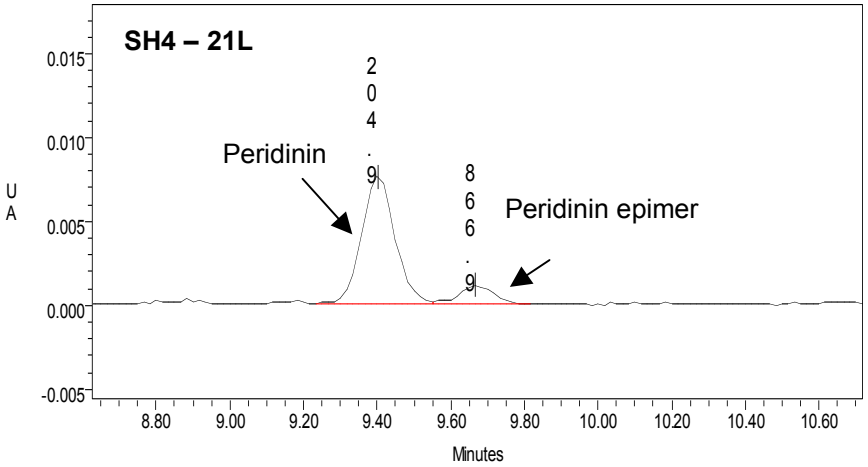
Dinox – use violax RF

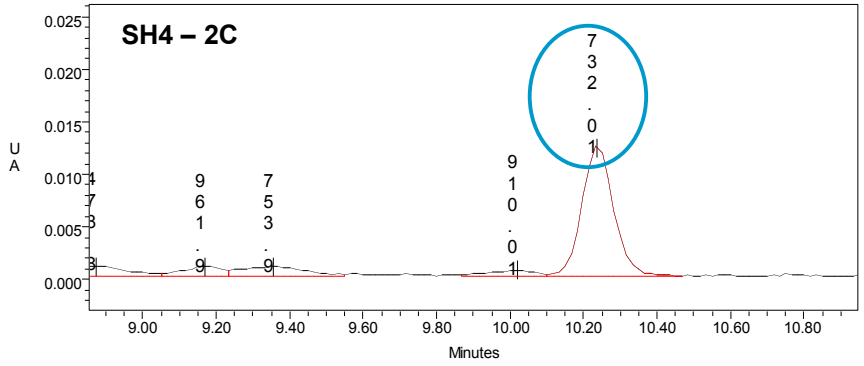
DV Chl b – use MV Chl b RF

Epimers

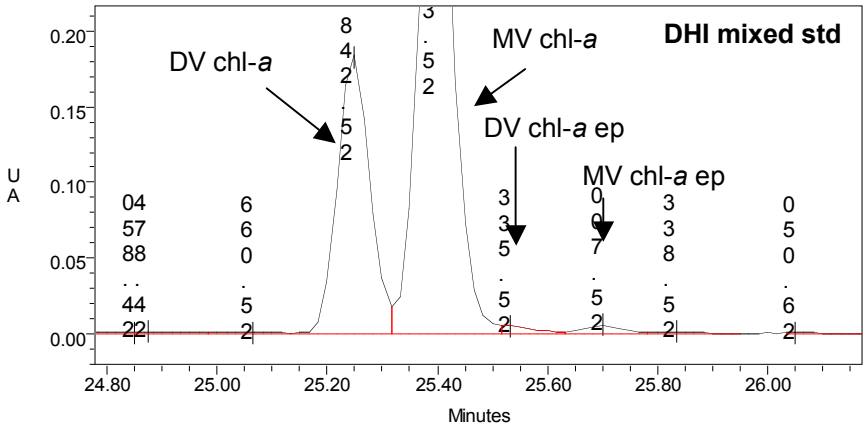
How are epimers dealt with and are they included in the value reported?

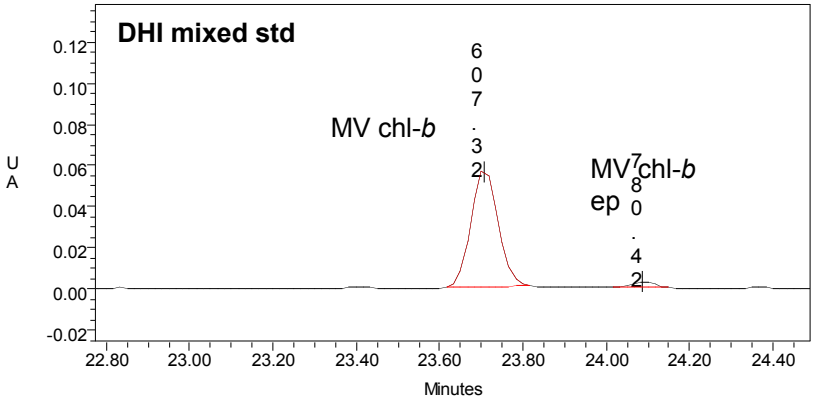


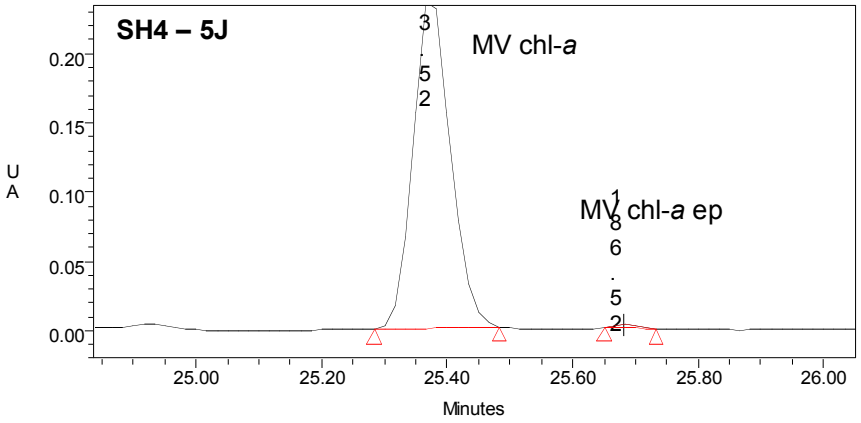




Epimers - continued







Epimers are added into the final concentration as are allomers for chl-*a*.

Isomers are not added in, but treated as individual pigments

Sample Procedures – logging and storage

What are the procedures when samples are received for analysis?

Samples are transferred from the dry shipping dewar or dry ice to a shallow box containing ice.

The samples are sorted and put into cryo-boxes for storage in a liquid nitrogen dewar.

Sample description, cryobox number etc are written into the “dewar log” book

External samples are written into the “sample received” book;

Number of samples received from who, by who etc

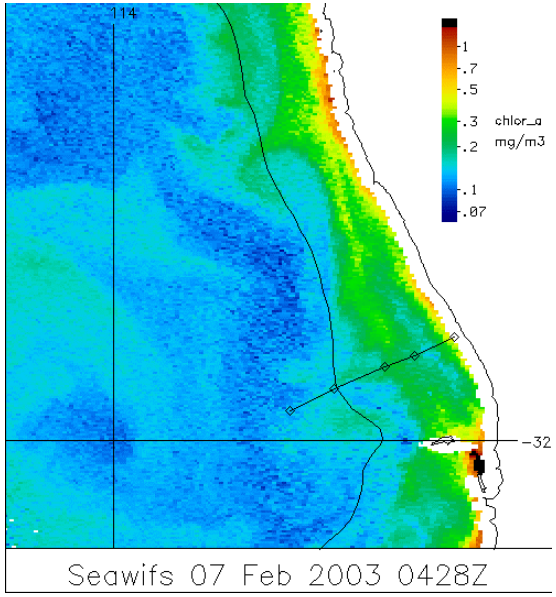
Performance metrics

Sample type

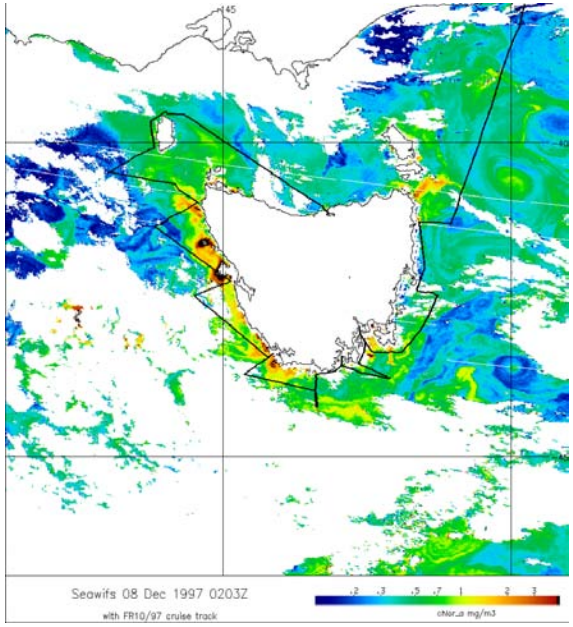
Are coastal or estuarine samples part of routine analyses?

Are coastal samples more influenced by oceanic or estuarine systems?

We do analyse coastal and estuarine samples and they can be influenced by both oceanic and estuarine systems.

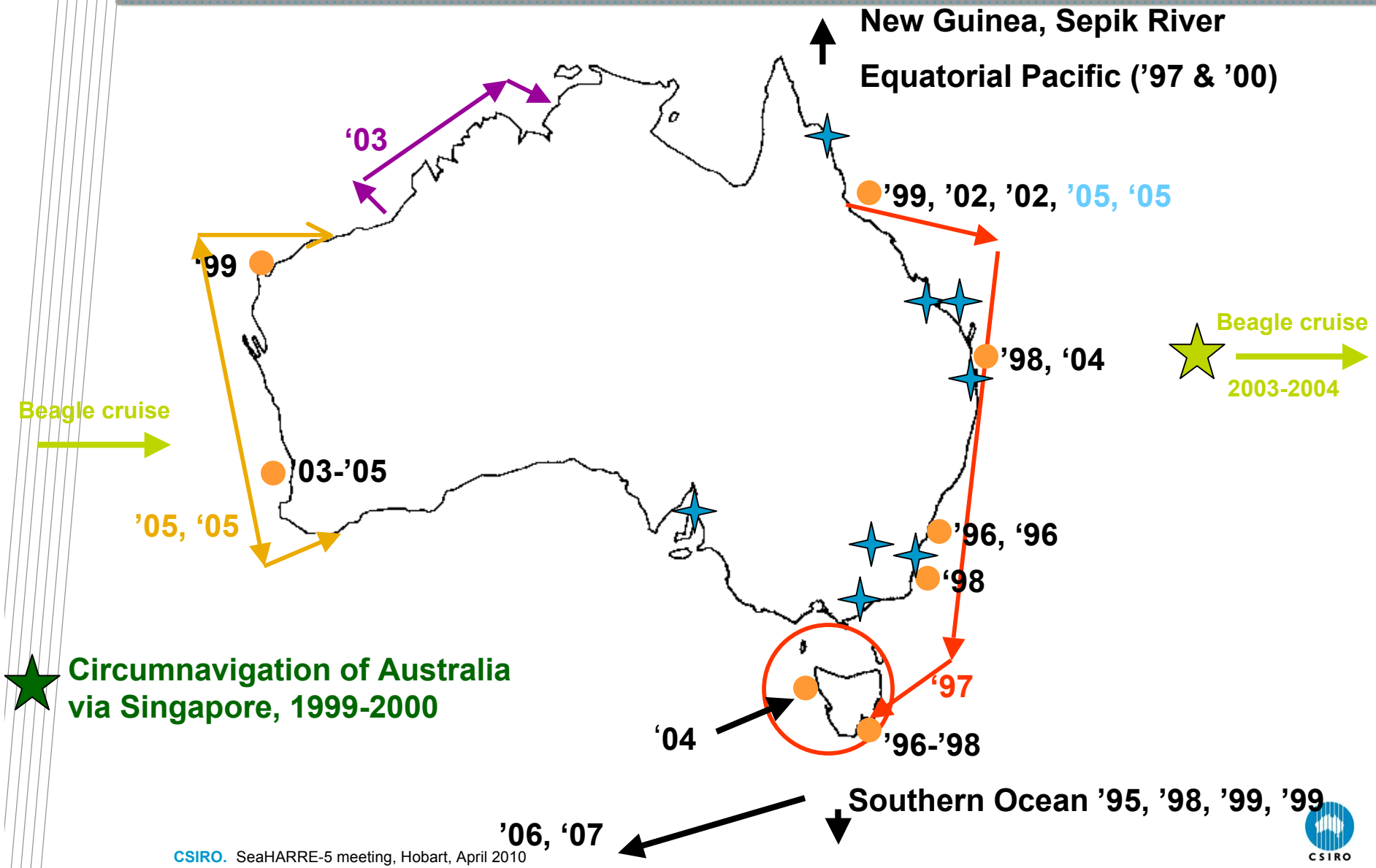


Coastal SW Western Australia
- oceanic influence



West coast Tasmania
- estuarine influence

Sample type


New Guinea, Sepik River
Equatorial Pacific ('97 & '00)
'03
'99, '02, '02, '05, '05
'99
'98, '04
Beagle cruise
2003-2004
Beagle cruise
'03-'05
'05, '05
'96, '96
'98
'97
'04
'96-'98
Circumnavigation of Australia
via Singapore, 1999-2000
Southern Ocean '95, '98, '99, '99
'06, '07

Method Limitations

What is the biggest limitation of your analyses?

Extinction Coefficients

How do you choose absorption coefficients for determining concentrations of pigment standards?

From the literature

"Phytoplankton pigments in Oceanography"

Jeffrey, Mantoura and Wright (Eds)

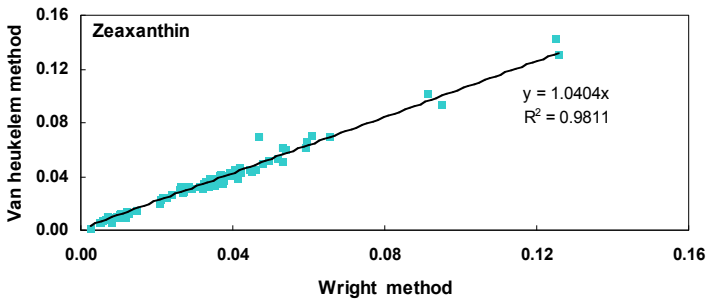
Method validation

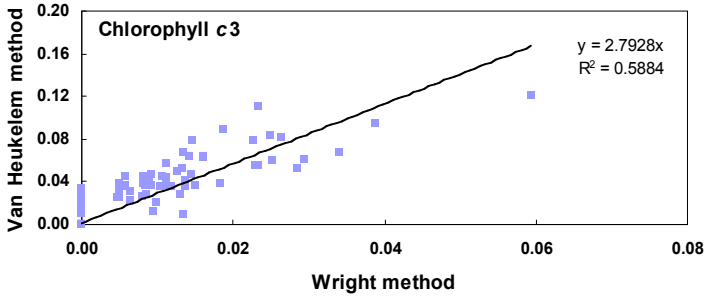
For the SH-2 samples we were using the Wright et al. (1991) method.

At around the time of the last meeting in Villefranch, we were looking at upgrading our HPLC system and changing our method to one that could separate the DV and MV forms of chl-*a*.

As a result of the Villefranche meeting, we decided to try the Van Heukelem and Thomas method

Before we changed instruments and methods, we ran the two systems with the two methods simultaneously for approx 100 samples.





Method validation

	R^2 value
• Chlorophyll C3	0.58
• Chlorophyll C2	0.79
• Peridinin	not detected
• 19- Butanoyloxyfucoxanthin	0.95
• Fucoxanthin	0.90
• 19- Hexanoyloxyfucoxanthin	0.96
• Diadinoxanthin	0.92
• Zeaxanthin	0.98
• carotenes	0.96
• MV Chl-*a* (incl Dv)	0.98
• MV Chl-*b* (incl DV)	0.97

Low R^2 values for Chls C3 and C2 are primarily due to the close elution of the chl C's in the Wright et al. method.

CSIRO

Archival of Results

Are your results routinely archived in a database?

Usually a 2 – 3 year time period for project use, then results are linked (as a .csv and .xls files) to a meta data file in the CSIRO Marine and Atmospheric Research (CMAR) datacentre ???? – MarLIN.

Web address

www.csiro

Public access to all meta data files and some data links.

Often put into a project database, but has not generally been put into global databases.

SH-3 and SH-4 Results

Concentration Calculations

Determine the response factor (RF) from a multipoint calibration.

$$[P_a] = (PA_a / RF_a) \times (Ve_a / Vf_a) \times (1/Vinj) \times DF$$

where
- P_a = pigment a
- PA_a = peak area for pigment a
- RF_a = response factor for pigment a
- Ve_a = volume of final extract in μL
- Vf_a = volume of sample filtered in mL
- Vinj = volume of sample injected in μL
- DF = dilution factor

Sample Extraction and Preparation

Cut filter into 3-4 small pieces - in a 10 ml centrifuge tube → Add 3mL acetone

Vortex for 30 secs, cover with parafilm, sonicate for 15 mins and rest overnight at 4°C → Add 0.2 mL H_20, sonicate for 15 mins

Transfer filter pieces and solvent to Biorad column in a clean centrifuge tube, rinse old tube 2 x 0.5 mL 90:10 acetone:H_20 → Record final volume of extract; Filter extract through a 0.2 μm syringe filter into an amber HPLC vial.

At all times when samples are not being handled, they are kept at 4°C.

CSIRO Marine and Atmospheric Research
Lesley Clementson
Hobart site

Phone: +61 3 6232 5337
Email: Lesley.Clementson@csiro.au
Web: www.csiro.au

www.csiro.au

Thank you

Contact Us
Phone: 1300 363 400 or +61 3 9545 2176
Email: enquiries@csiro.au Web: www.csiro.au

CSIRO