HPLC Results, Lisbon, Portugal

Vanda Brotas, Rafael Mendes, Paulo Cartaxana

Center of Oceanography
University of Lisbon

From the Mud to the Southern Ocean

- Microphytobenthos
- Coastal and estuarine phytoplankton
- Oceanic phytoplankton (Portugal, Antarctica, Patagonia)

General Details

- Number of samples analyzed per year: ca. 2000.
- Samples are analyzed for multiple investigators.
- Samples come from many different water types (lagoons, estuaries, open ocean) and sediment.
- Data only for our research group, do not provide for external customers.
- We analyze primarily field samples, but we also have microalgal cultures.
- Results used for research, scientific projects, PhDs and Masters, and monitoring programs.

Chromatographic Separation

Chromatographic separation with Supelcosyl **C18**:

- monomeric octadecylsilica
- dimensions 250 x 4.6 mm
- pore size 100 A
- particle size 5 µm
- surface area 170 m^2 g^{-1}
- carbon load 11%

Chromatographic separation with Simmetry **C8**:

- monomeric octylsilica
- dimensions150 x 4.6 mm
- pore size 100 A
- particle size 3.5 µm
- surface area 337 m^2 g^{-1}
- carbon load 12.27%

Room temperature

Elution Gradients

C18

A = methanol:water (85:15 v/v)*

B = acetonitrile:water (90:10 v/v)

C = ethyl acetate

* buffered with 0.5M ammonium acetate

0 min, A 60%, B 40%
2 min, B 100%
7 min, B 80%, C 20%
17 min, B 50%, C 50%
21 min, B 30%, C 70%
28.5 min, B 30%, C 70%
29.5 min, B 100%
30.5 min, A 60%, B 40%
35 min, A 60%, B 40%

with a flow rate of 0.6 mL min^{-1}, and duration of 35 min

Kraay et al, 1992, J Phycol

C8

A = methanol:acetonitrile:aqueous pyridine solution (50:25:25 v/v/v)

B = methanol:acetonitrile:acetone (20:60:20 v/v/v)

0 min, A 100%
20 min, A 60%
26 min, A 5%
38 min, A 5%
40 min, A 100%

with a flow rate of 1 mL min^{-1}, and duration of 40 min

INJECTION: volume of solvent that flows through the column to re-equilibrate the column to initial conditions before a sample is injected: ca 6 ml

Zapata et al, 2000. MEPS

Advantages and Disadvantages

- C18
  - Cheaper, faster sample throughput, higher sensitivity.
  - Does not separate Chls *c* and DVChla/Chla.
- C8
  - Separates Chls *c* and DVChl
  - Lower sensitivity.

Suggestion: C8 method with lower flow rate??

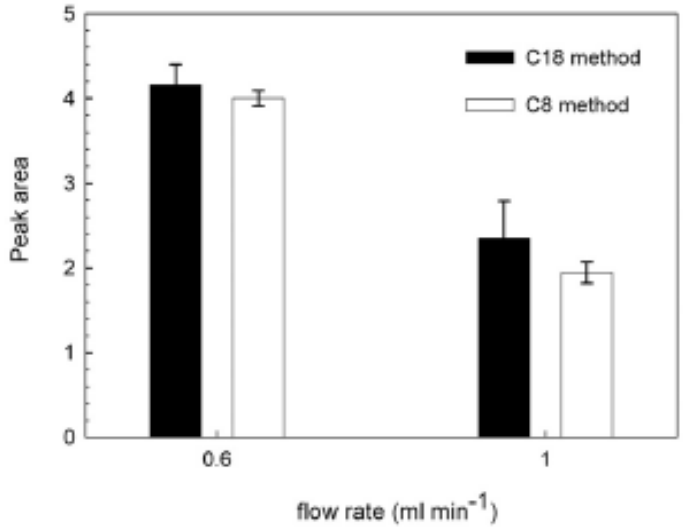


Fig. 7. Influence of flow rate on fucoxanthin standard (0.342 mg L^{-1}) peak area for the C_{18} and C_8 method (mean ± standard deviation, $n = 3$).

Mendes et al, 2007, L&O Methods

Detection

- Photodiode Array (Shimadzu SPD-M10ADvp).

- Fluorescence Detector (Shimadzu RF-10AXL); Ex. 430 nm; Em. 670 nm.

-We visually inspect the integration of all peaks in the chromatograms, and corrections are made, if necessary, with Class VP/LC Solution integration tools.

- Peak acceptance/rejection criteria are basically the ones provided by SeaHarre5.

Calibration

- Sigma (Chl *a*, *b*, and β-carotene) and DHI standards (all others).
- Calibration is done at or close to maximum absorption wavelength, ex: Chl *a* 430 nm, Fuco 448nm, Zea, 454nm.
- No internal standard used.
- We don't quantify pigments for which we don't have standards.
- We use multi-point calibration.
- Our calibration response factors are quite stable.
- We use the concentrations provided by DHI and re-measure spectrophotometrically Sigma standards.
- We dilute standards. Calibrate micropipetes regularly.

Quantification

- Usually, isomers are added to pigment area (ex: Chla). Sum of Chla plus closest peak of epimer and allomere.
- Quantification always by area
- We use the same integration settings for all chromatograms. We determine a min peak area, the first 6 min are not quantifiable: integration off 0-6 min.
- In order to quantify pigments: Minimum width 0.01, Threshold 1500 (area),
- We don't quantify by peak height.

❖Other Stan's questions:

❖What volume of water do you estimate is in the filter? – not addressed yet

❖Do you use the same injector program with samples and standards? yes

SeaHarre Samples

Sample handling: Samples were put in -80º C after arrival. Analysis performed Between February 16th and March 4th.

Extraction:

- 2.5 ml of 95% cold-buffered methanol (2% ammonium acetate).

- 30 s sonication bath (50W).

- 30 min at -20ºC in the dark.

- centrifuge 4000 rpm ,15min, at 4ºC

- filtration prior to injection (0.2 µm)

Injection: Immediately after extraction (maximum of 10 min. at refrigerated autoinjector, -4ºC); injection volume 100 µl.

Chromatographic Separation: C8 Method

Identification of problems

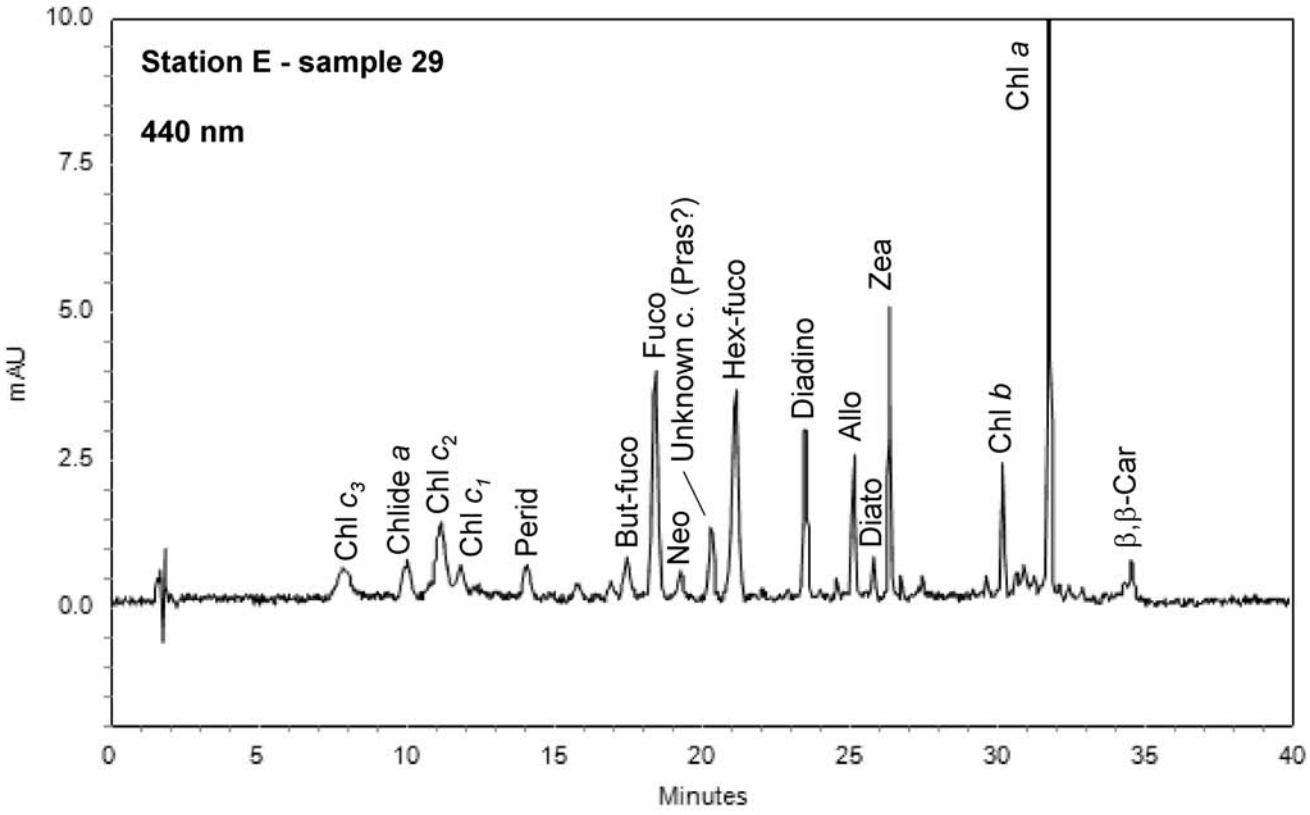
Extraction as a decisive step:

•Manual grinding is operator dependent.

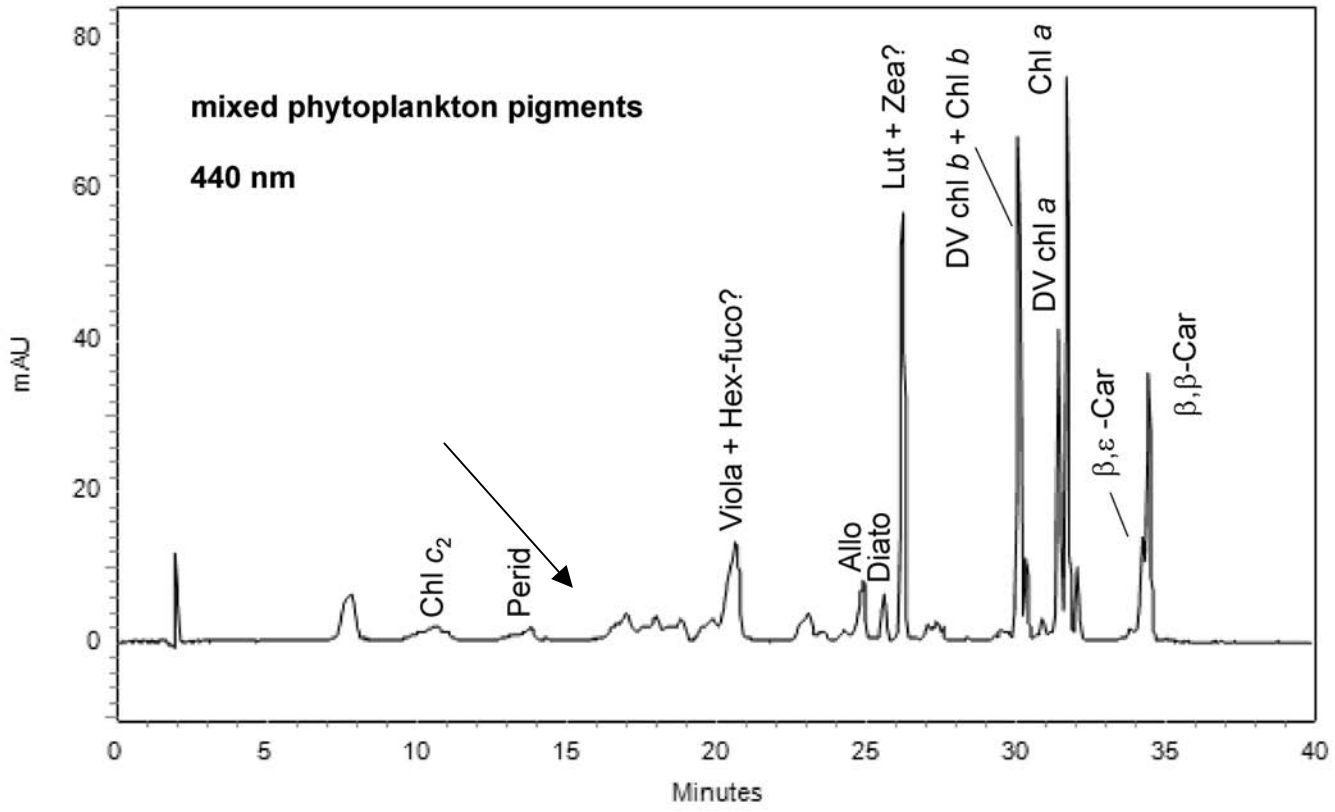
•Extraction solvent volume: Equilibrium between extraction efficiency and sample dilution

Previous underestimation due to inadequate extraction solvent volume in Estuarine phytoplankton samples

Chromatogram: example



Our Problematic chromatogram



In the past, we had problems with early eluting peaks of standards in acetone

Possible explanation:

- Distortion of early eluting peaks, when acetone is used as injection solvent with mobile phases based on methanol (Zapata & Garrido, 1991).

- Different viscosities of sample solvent and mobile phases? (Castells & Castells, 1998).

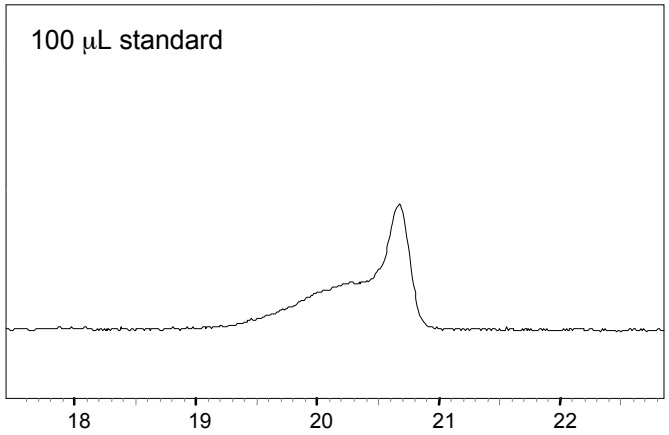
A = methanol:acetonitrile:aqueous pyridine solution

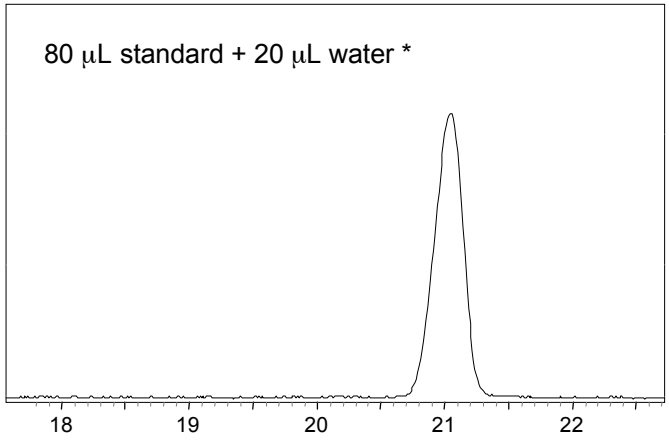
B = methanol:acetonitrile:acetone

0 min – A 100%
20 min, A 60%

When A concentration dominates the gradient, we have problems with standard mix.

Effect of water addition on peak shape

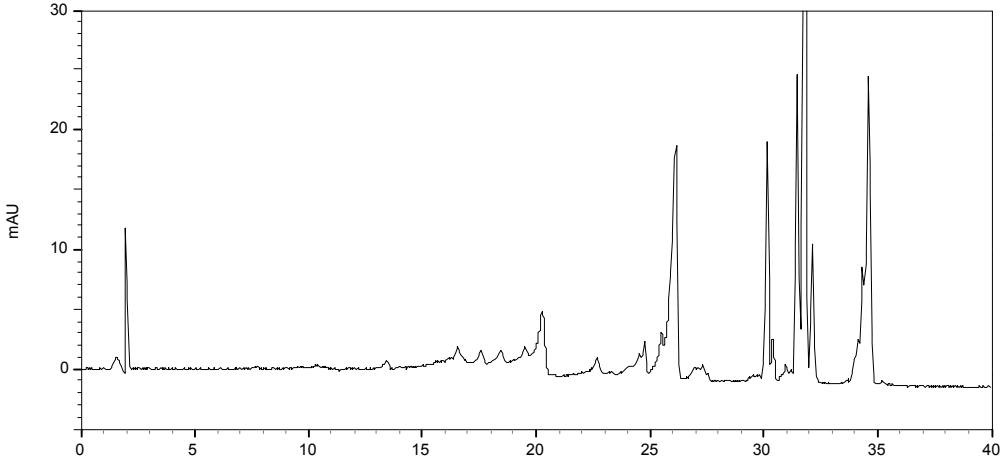




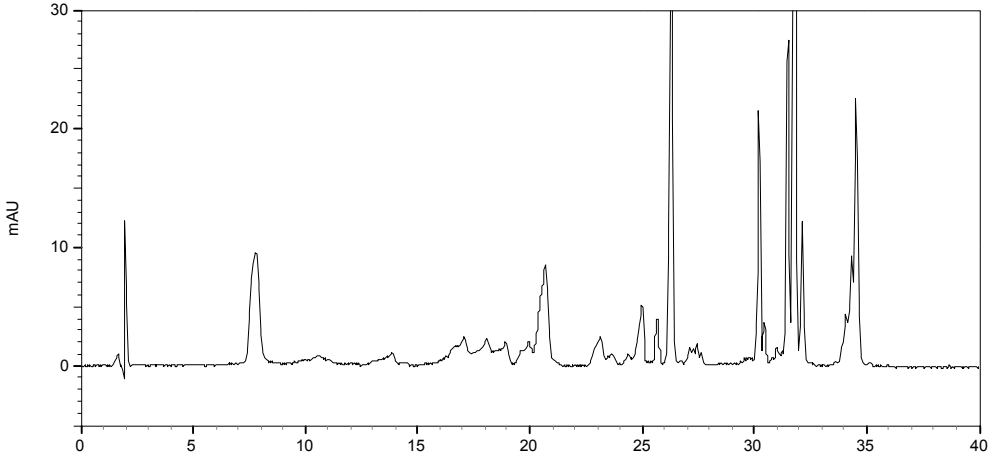
Hexanoyloxyfucoxanthin

*** Our usual calibration conditions**

Effect of water addition on pigment mix

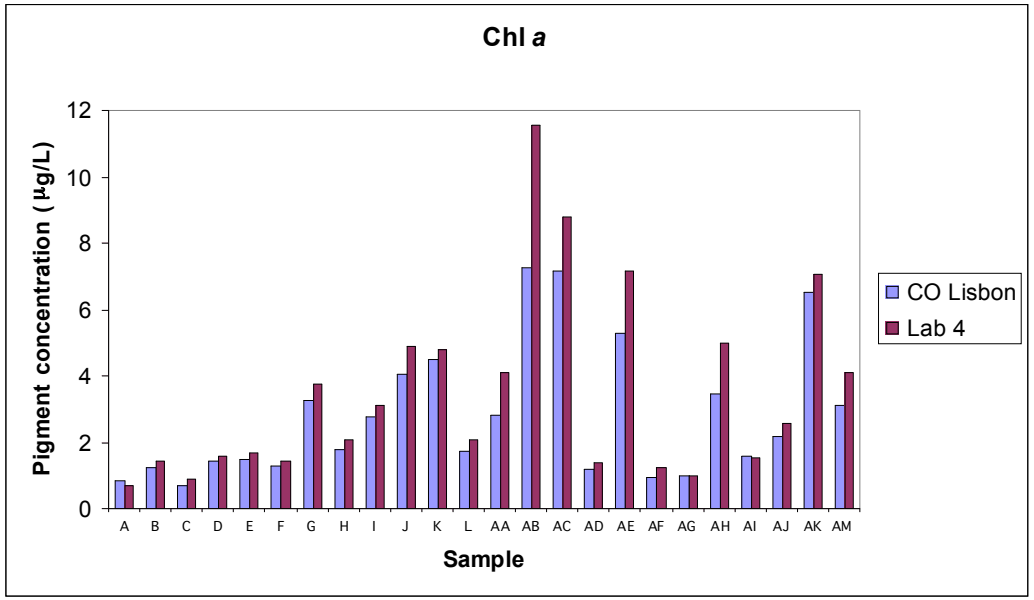


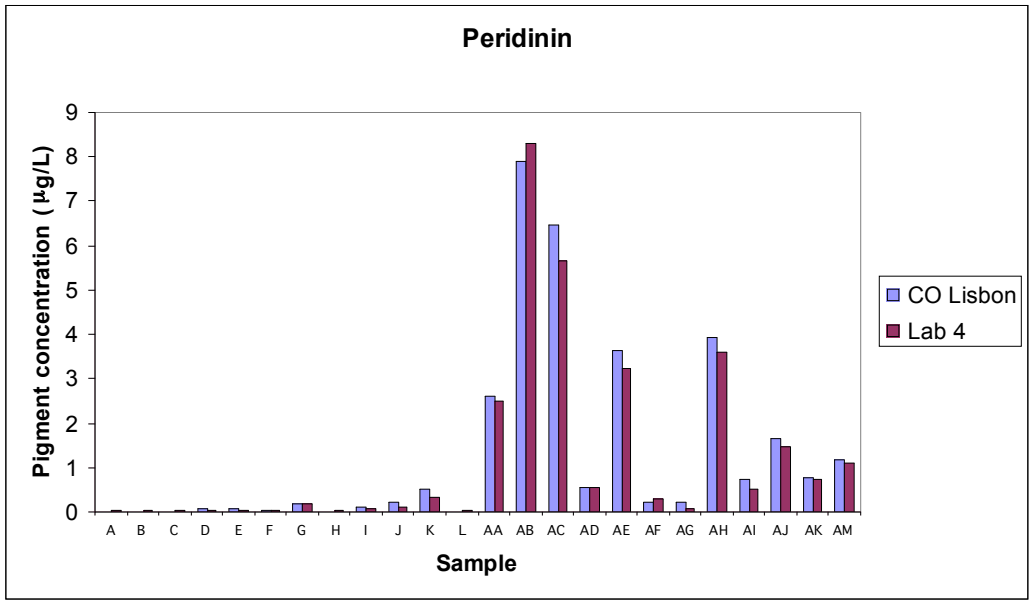
100 μL mixed standard



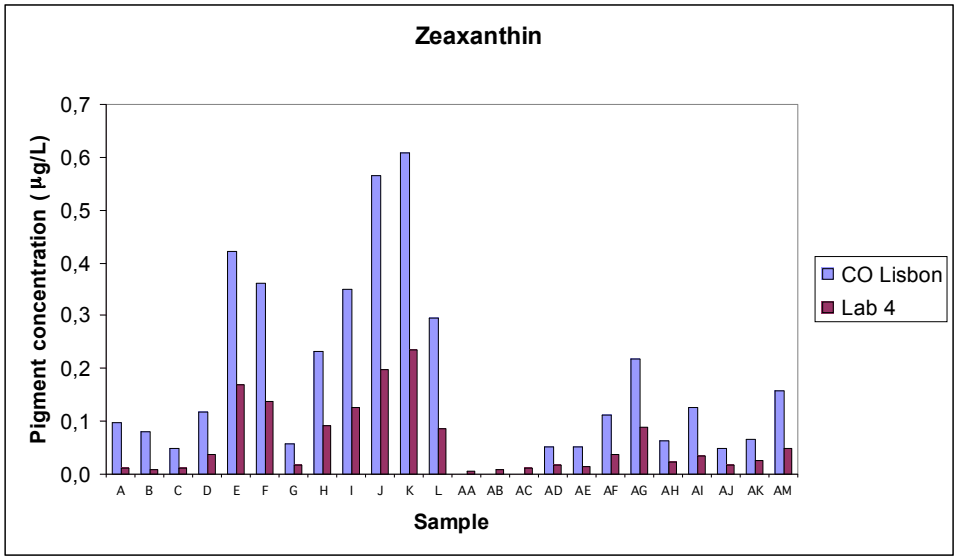
90 μL mixed standard + 10 μL water

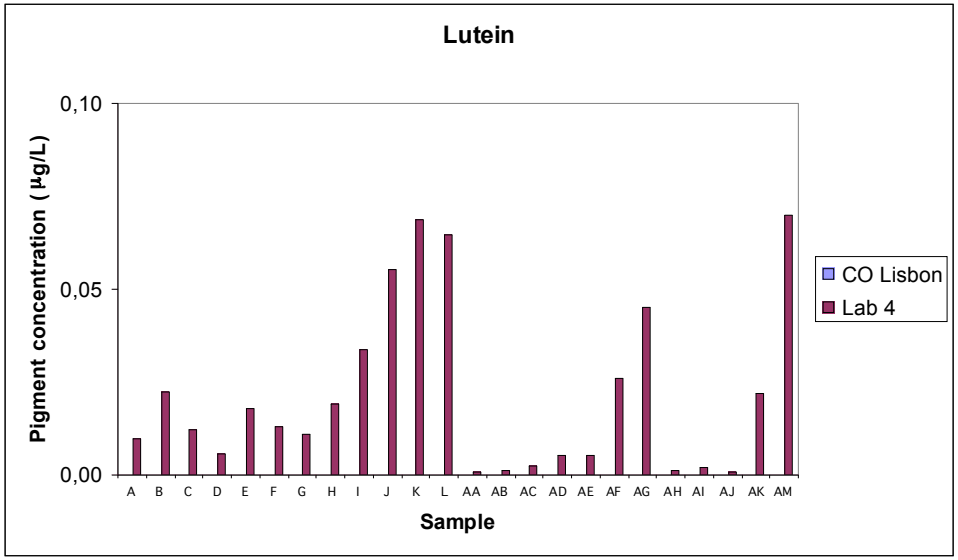
Comparison of Pigment Results





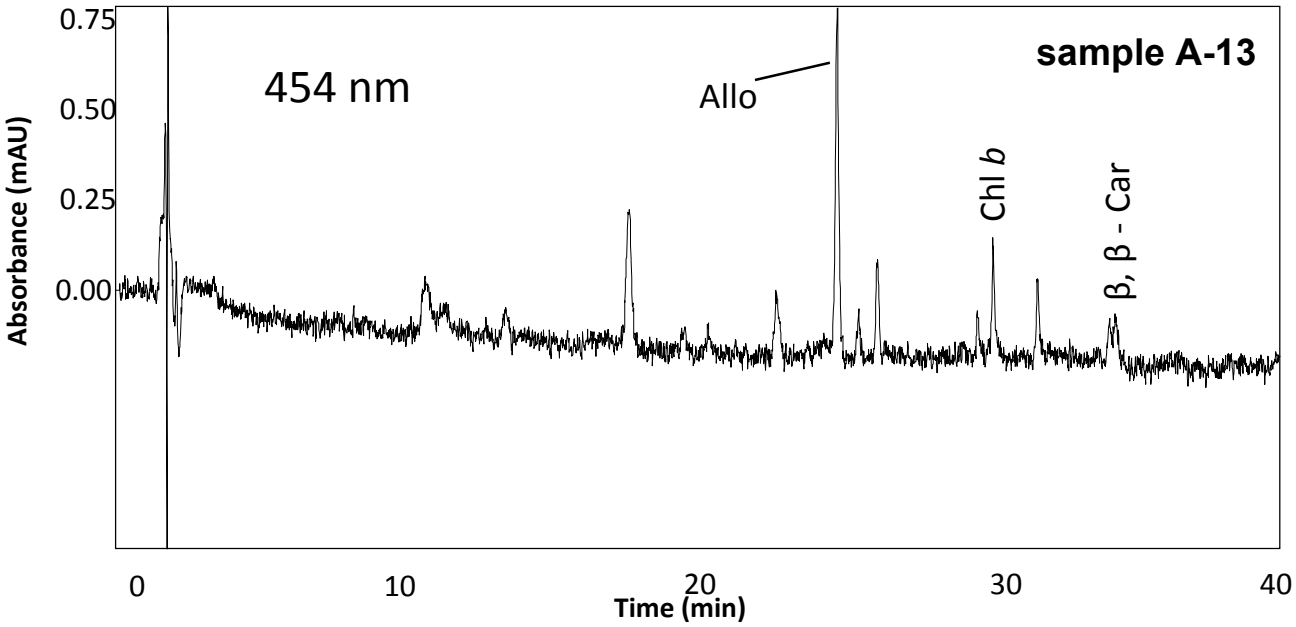
Co-elution of Zeaxanthin and Lutein





Signal to Noise: SNR's

❑ Alloxanthin, Chlorophyll *b* and β, β - Carotene



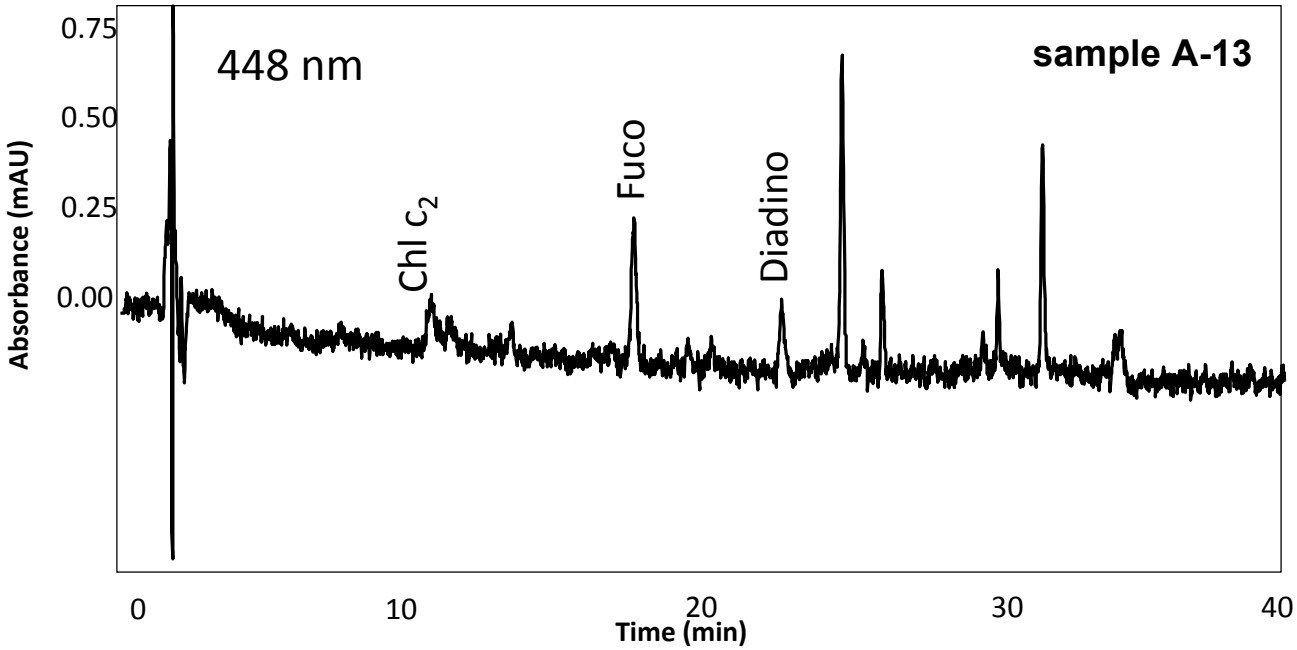
Alloxanthin
0.81 ng
SNR = 25
>LOD (0.09 ng)
>LOQ (0.31 ng)

Chlorophyll *b*
± 0.40 ng
SNR = 9
>LOD (0.17 ng)
< LOQ (0.56 ng)

β, β-Carotene
SNR = 4
Partially co-eluted with β,ε-Carotene

SNR's

❑ Chlorophyll c_2,Fucoxanthin and Diadinoxanthin



Chlorophyll c_2
SNR = 3
<LOD (0.16 ng)
<LOQ (0.53 ng)

Fucoxanthin
0.86 ng
SNR = 12
>LOD (0.21 ng)
>LOQ (0.70 ng)

Diadinoxanthin
± 0.32 ng
SNR = 7
>LOD (0.14 ng)
<LOQ (0.46 ng)

Ideas for improving results

- Standardize extraction procedures