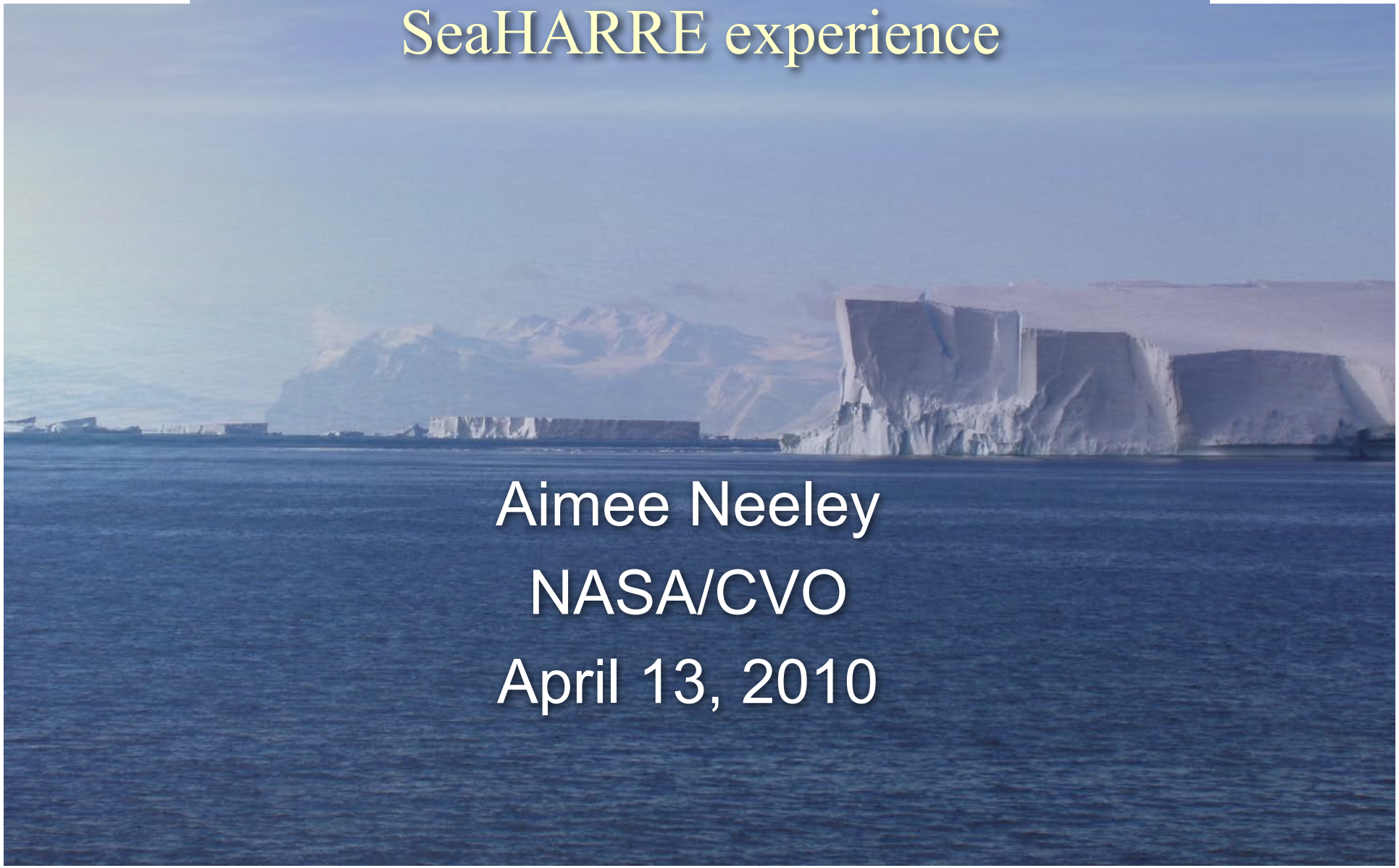
Tales from an HPLC analyst: My first SeaHARRE experience



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NASA/CVO
April 13, 2010

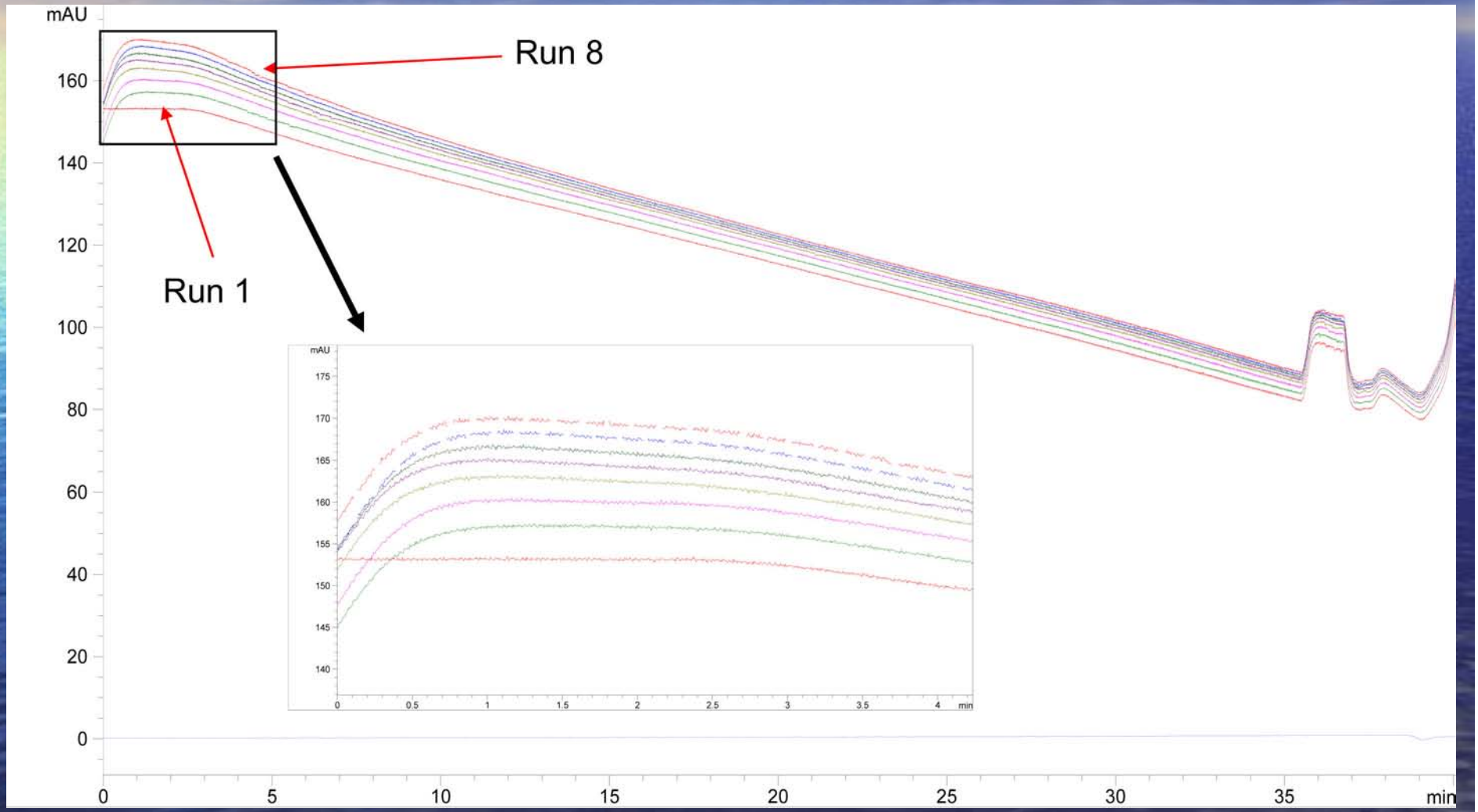
Summary of things to come…

- Original system configuration of an Agilent 1200 series RRLC System
- Approaches to troubleshooting
- Final system configuration for SeaHARRE
- Calibration and analysis procedures
- Integration problems I encountered
- Lessons learned and improvements to be made

The plan and how it was foiled!

- Agilent 1200 Series Rapid Resolution LC System
  - Shorter columns plus lower flow rates: faster method, better resolution, solvent savings
  - Binary Pump SL (up to 600 bar)
  - Microvacuum degasser (internal volume 1 ml/channel)
  - Temperature Controlled Autosampler (200 µL metering head)
  - Thermostatted column compartment
  - Diode Array Detector SL (80Hz rate)
    - Standard Flow Cell-10 mm nominal path lengths (9.80±0.07mm actual), 13 µL cell volume

VHT Method - Modified for an Agilent 1200 series system

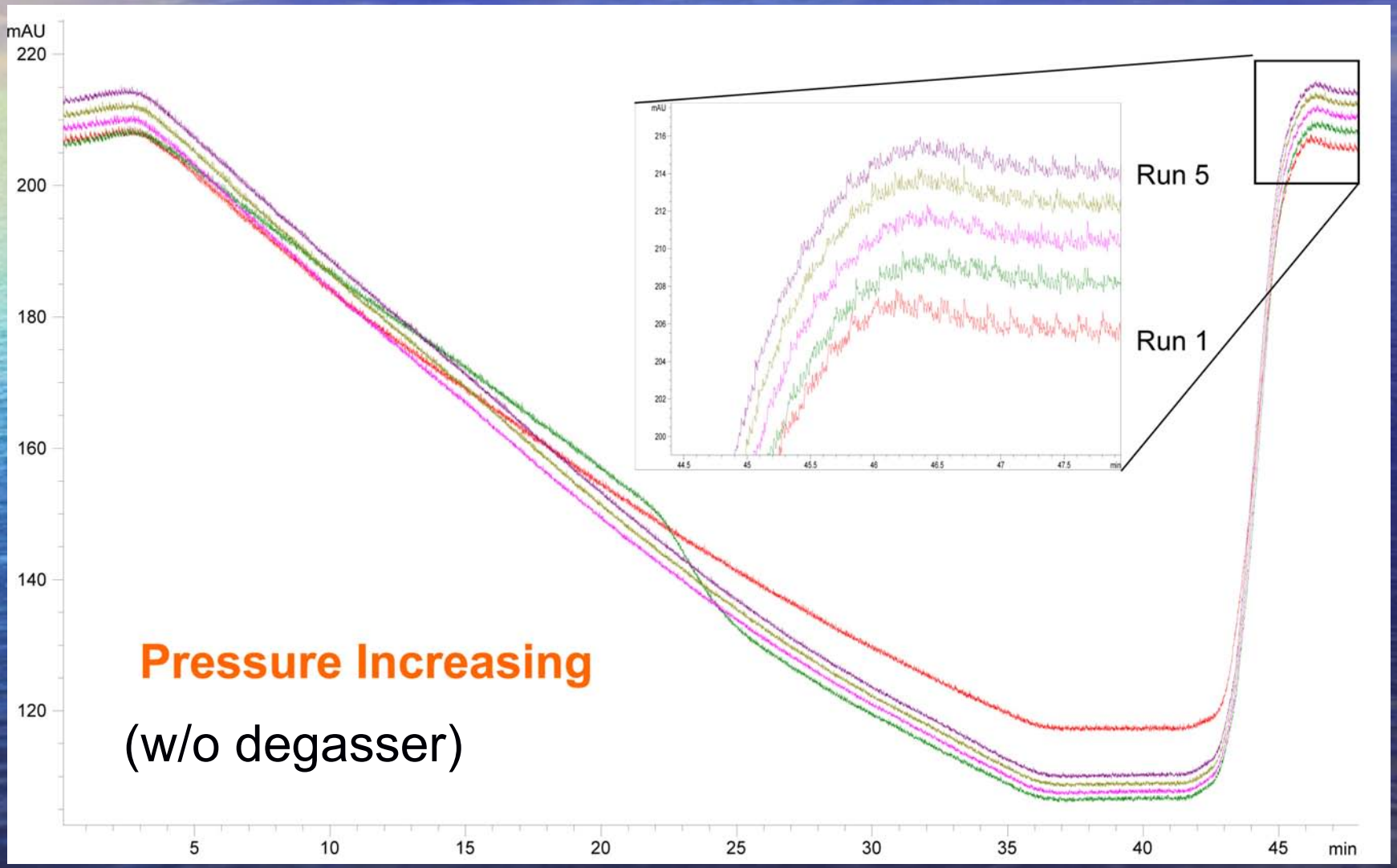


VHT Method - Modified for an Agilent 1200 series system

- Initially thought problem originated in mixing chamber but problem occurred with 100% Solvent A
- Multiple communications conducted with Agilent
  - Original response:Pressure will be higher with binary pump
  - Agilent field tech disconnected degasser and ran blanks but still saw pressure increase (next slide)
  - Situation was ‘escalated’ within Agilent
- Compressibility compensation
- Guard column and filter frit: precipitates forming on column?

VHT Method - Modified for an Agilent 1200 series system

Pwesha Problems!!!

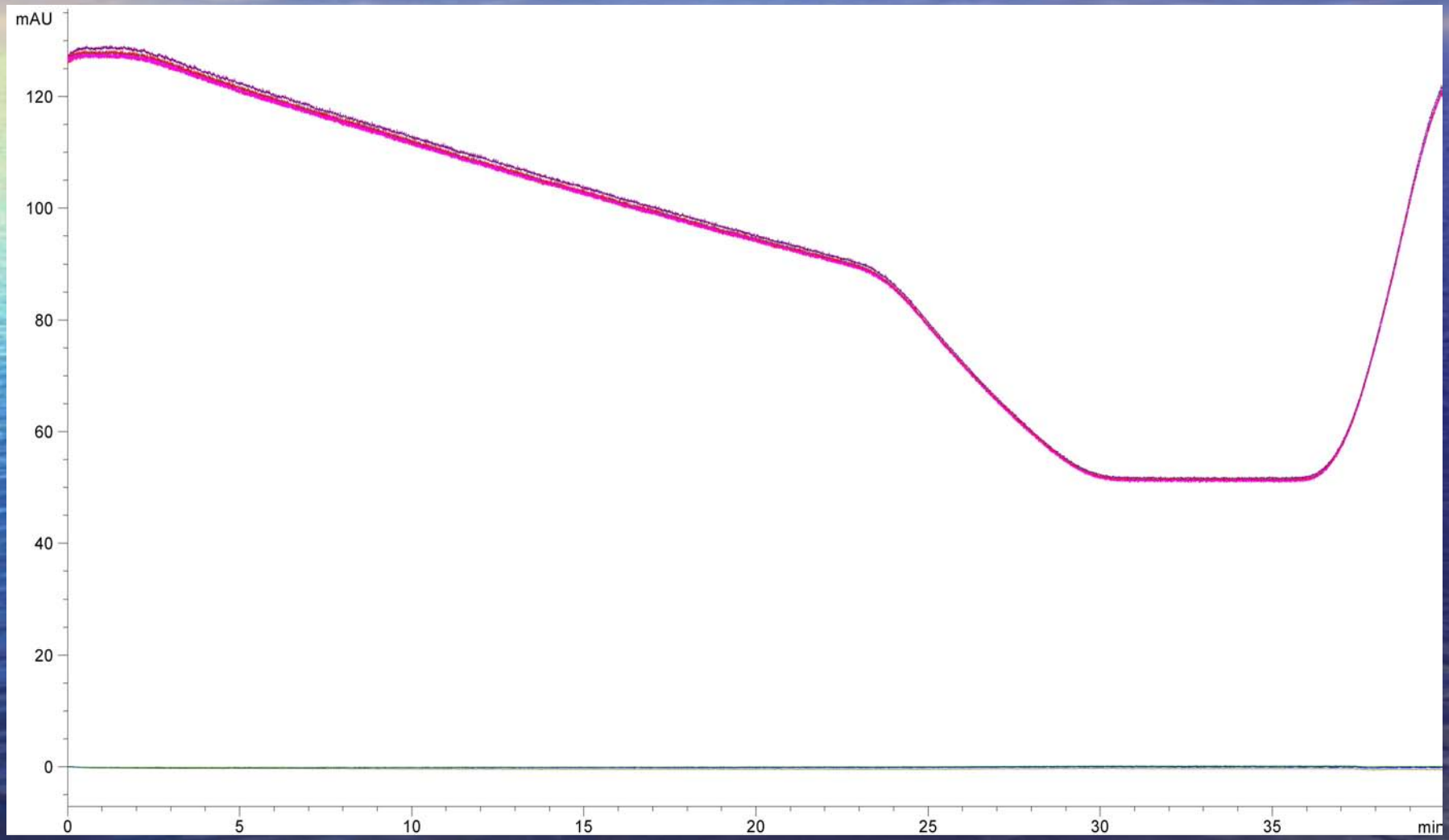


VHT Method - Modified for an Agilent 1200 series system

- Compliance test conducted on all hardware: Passed
- Ran injections of Reserpine (mobile phase 70% ammonium formate, 30% water): no pressure problems
- Run another method that doesn't use ammonium acetate: Zapata method (next slide)

VHT Method - Modified for an Agilent 1200 series system

Zapata Method over 3 days:Pressure is stable



VHT Method - Modified for an Agilent 1200 series system

Final Hardware Configuration

System components:

- Quaternary Pump and Degasser
- Temperature Controlled Autosampler (900 µL metering head)
- Thermostatted column compartment
- Diode Array Detector SL (80Hz rate)
  - Standard Flow Cell-10 mm nominal path lengths (9.80±0.07mm actual), 13 µL cell volume
- Fluorescence detector (hasn't really been used yet)

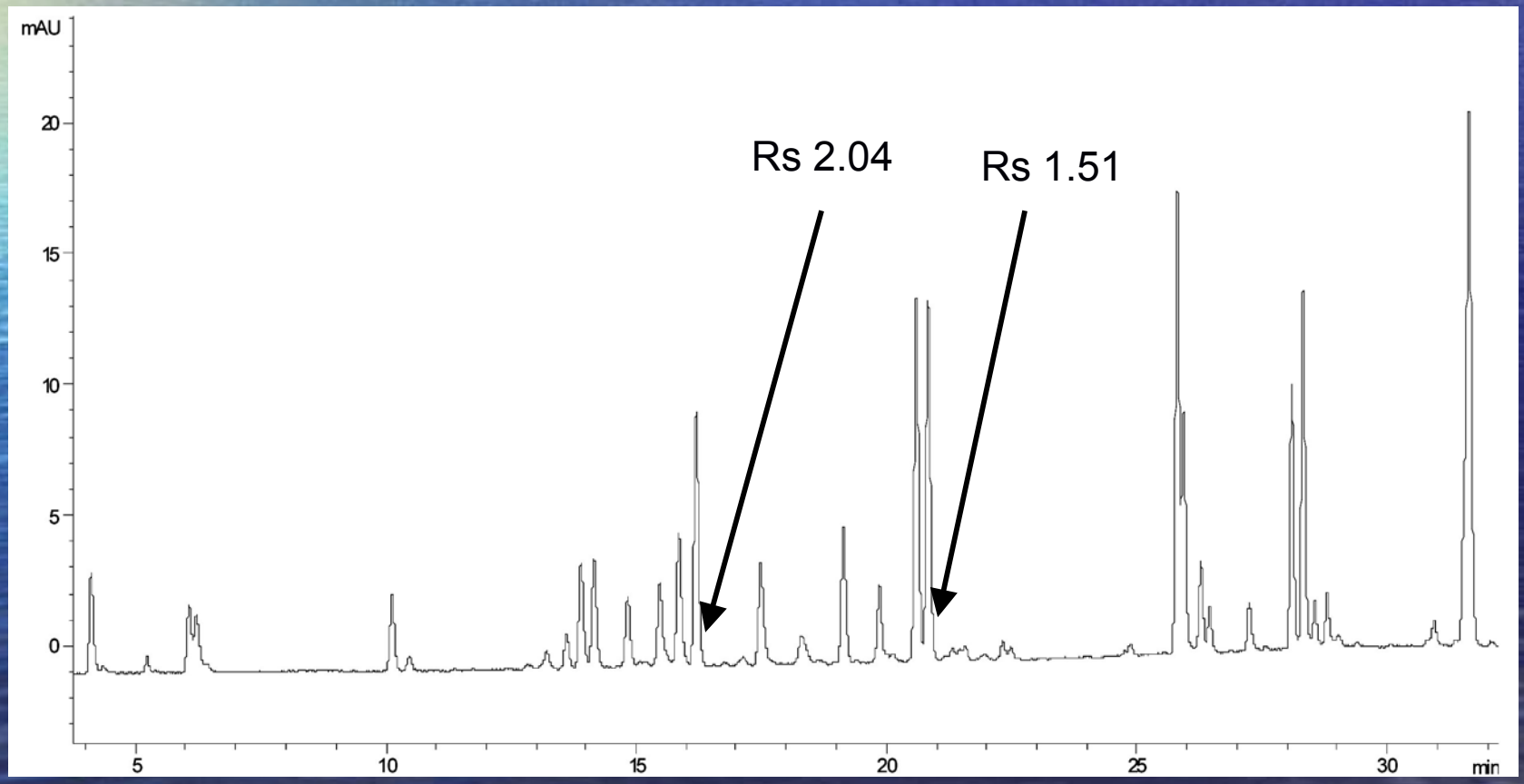
VHT Method - Modified for an Agilent 1200 series system

- Column: Eclipse XDB C8 3.5 µm, 4.6 x150mm
- Solvents: 70% MeOH/30% 28nM TBAA (pH 6.5), Methanol, Acetone
- Gradient:
  - 0-27 minutes 95% A, 5% B to 5% A, 95% B
  - 29.50 minutes 5% A, 95% B
  - 29.75-30.85 minutes 30% A, 65% B, 5% C (Acetone)
  - 31.10 minutes 95% A, 5% B
  - 34.10 Stop
  - Flow rate 1.100 ml/min

CVO gradient is 5 minutes longer than HPL.
Achieved better separation of critical pairs (Rs 1.50).

VHT Method - Modified for an Agilent 1200 series system

Standard Mix 105 during SeaHARRE: 100 µl, 5-fold dilution



VHT Method - Modified for an Agilent 1200 series system

TCAS was allowed to equilibrate to 4C for ≥1 hr

Injector Program:

- Draw 175 µl from buffer
- Draw 75 µl from sample
- Needle wash
- Draw 75 µl from buffer
- Draw 75 µl from sample
- Needle Wash
- Draw 175 µl from buffer
- Draw speed = 130 µl/min and Eject speed = 250 µl/min

Added 25 µL of buffer to the beginning and end of injector program as compared to VHT method.

VHT Method - Modified for an Agilent 1200 series system

Extraction Procedure (same as used by HPL):

- 100 µl water + 2.5 ml 100% acetone/Vitamin E added to Polypropylene tubes
- Covered with Parafilm and placed in freezer for 30 minutes
- Add filters and place back in freezer for approx. 1 hour
- Sonicated on ice for 25-30 seconds (or until filter is 'macerated'). Samples returned to cooler (on ice)
  - Pulse on: 1 second
  - Pulse off: 0.5 second
  - Amplitude: 25%
- Samples returned to freezer for 3-6 hours
- Remove samples from freezer and filter through 0.45 µm PTFE filter and plastic syringe
- Place in scintillation vials with foil-lined caps
- Place in freezer or immediately analyze

VHT Method - Modified for an Agilent 1200 series system

- Method Particulars
  - Column temperature was set at 60°C
  - TCAS was set at 4°C
  - Wavelengths:
    - 665nm (Bw 20)
    - 450nm (Bw 20)
    - 474nm (Bw 10)
    - 222nm (Bw 10)
  - Peak width/data rate: >0.03 min/10Hz
  - Response Time: 0.5 sec
  - Slit width: 2 nm

VHT Method - Modified for an Agilent 1200 series system

- Chl a Calibration
  - System was calibrated with Spinach Chl *a* from Sigma (C5753)
  - Solid was dissolved in 90% acetone overnight (in a drawer) for dissolution
  - Was read on the spectrophotometer on the following day

DHI Standards for Calibration:

Chl c_3	(450nm)	Viol	(450nm)
Chl c_2	(450nm)	Hex-fuco	(450nm)
MgDVP	(450nm)	Diadino	(450nm)
Chlide *a*	(665nm)	Allo	(450nm)
Phide *a*	(665nm)	Diato	(450nm)
Peri	(450nm)	Zea	(450nm)
But-fuco	(450nm)	Lut	(450nm)
Fuco	(450nm)	Chl *b*	(450,474nm)
Neo	(450nm)	Phytin *a*	(665nm)
Pras	(450nm)	a caro	(450nm)
		B caro	(450nm)

VHT Method - Modified for an Agilent 1200 series system

Calibration Procedures

- Performed multipoint calibrations for all DHI Standards (5 points; 5-200ng/inj)
- Standards were diluted using 90% acetone
- Dropped one point if needed to improve residuals
- Acceptance or rejection of calibration was based on 2% threshold for residuals. Calibration was repeated if necessary.
- Average residuals were 2.0 - 2.5% or less except for Neo and Phytin *a* (I had to drop 2 points to get average residuals below 2%)
- Y-intercept was forced through zero for al curves. Y-intercept was converted to concentration. Threshold was 0.005 µg/ml. All met except phide a, Zeax Phytin a.
- ABS(Y-int)*(Rf)
- Percent purity was calculated for each standard and concentrations were adjusted accordingly

VHT Method - Modified for an Agilent 1200 series system

SeaHARRE Analysis QA/QC Procedures

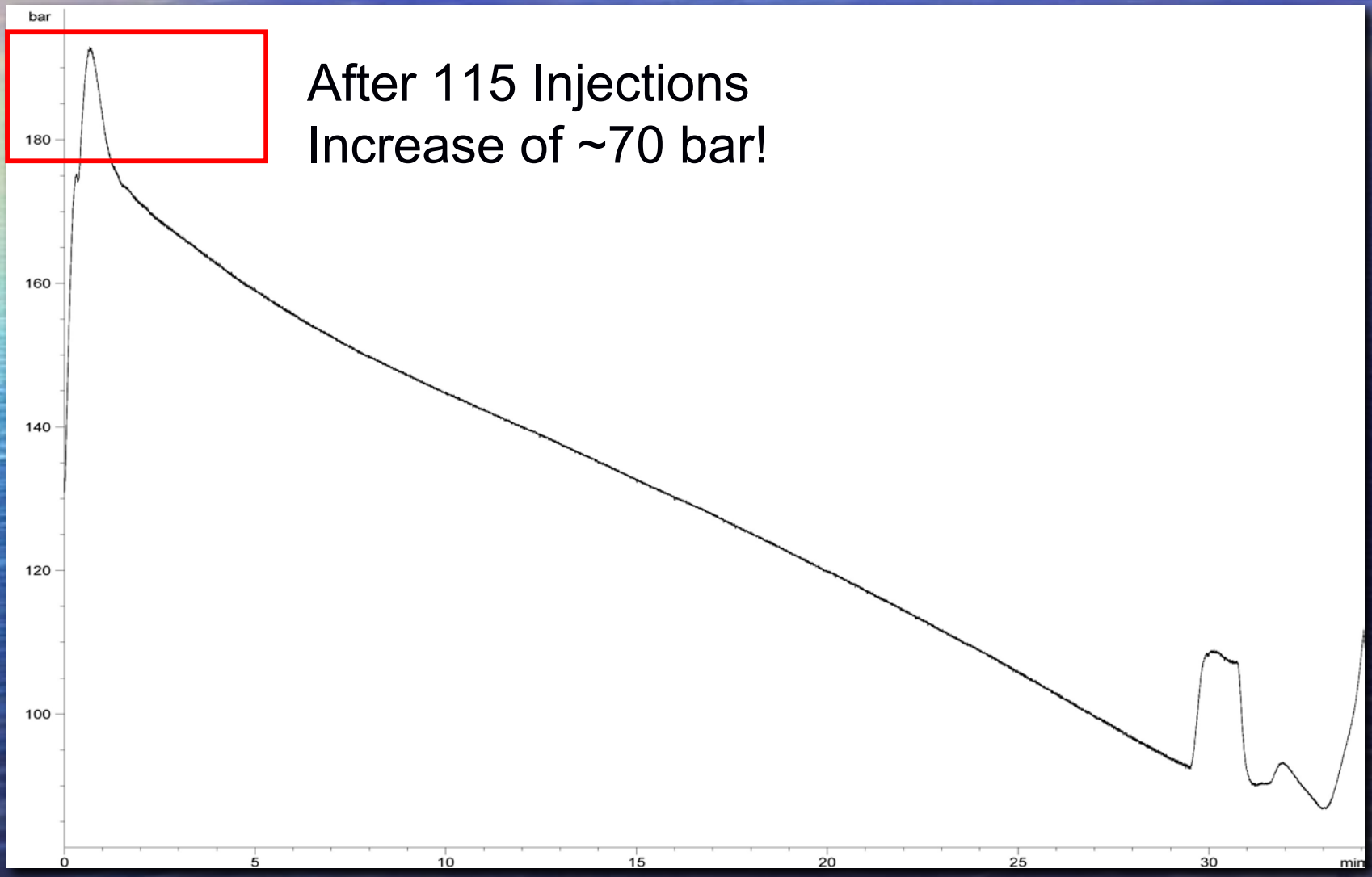
- DHI Chl *a*, Vitamin E and Standard 105 mix were interspersed between every 6 samples (alternated between Std 105 mix and Chl *a*)
- Vitamin E precision: 0.474%
- DHI Chl *a* precision: 1.039%
- Poor precision of Standard 105 mix caused by dilution

VHT Method - Modified for an Agilent 1200 series system

- TChl *a*
  - Precision U.S.: 3.7%
  - Precision AUS: 8.0%
- PPig Precision
  - US: 5.272%
  - AUS: 7.00%

- Separation
  - Average R*s* Zea/Lut (from samples): 1.51
  - Ret time precision Chl *a*: 0.036%
  - Ret time precision Chl c_3: 0.755%
  - Ret time precision B caro: 0.094%
- Injection Precision
  - Chl *a:* 1.040%
  - Peri (from std mix): 3.341%
  - Vit E: 0.474%
- Calibration
  - Chl a (with all 5 points): 1.732%
  - Without lowest point (2.458 ng): 0.792%

VHT Method - Modified for an Agilent 1200 series system

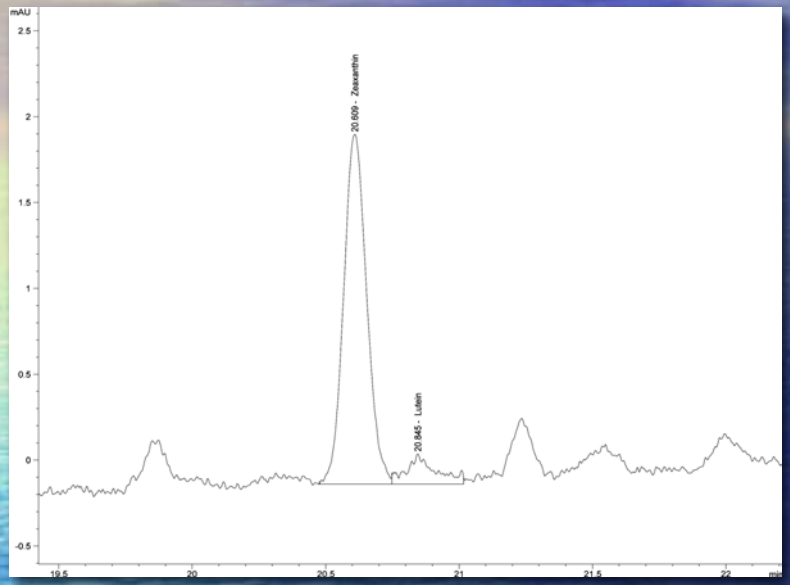


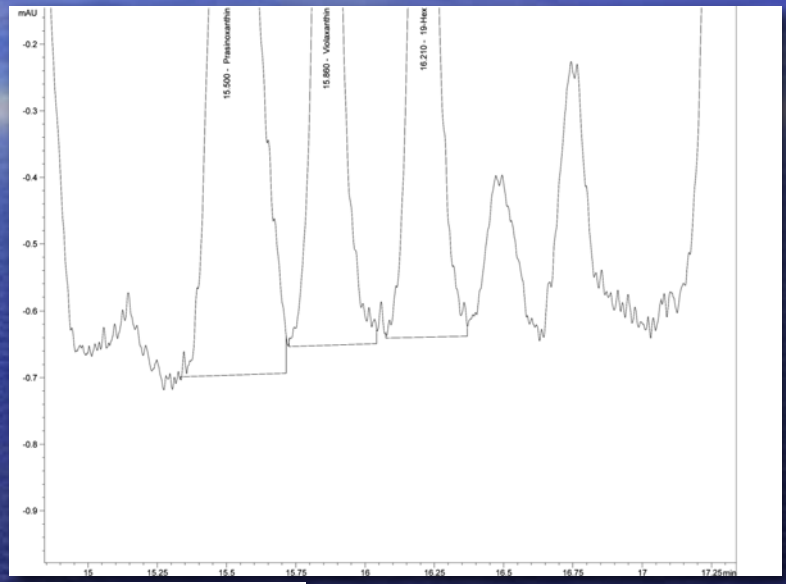
VHT Method - Modified for an Agilent 1200 series system

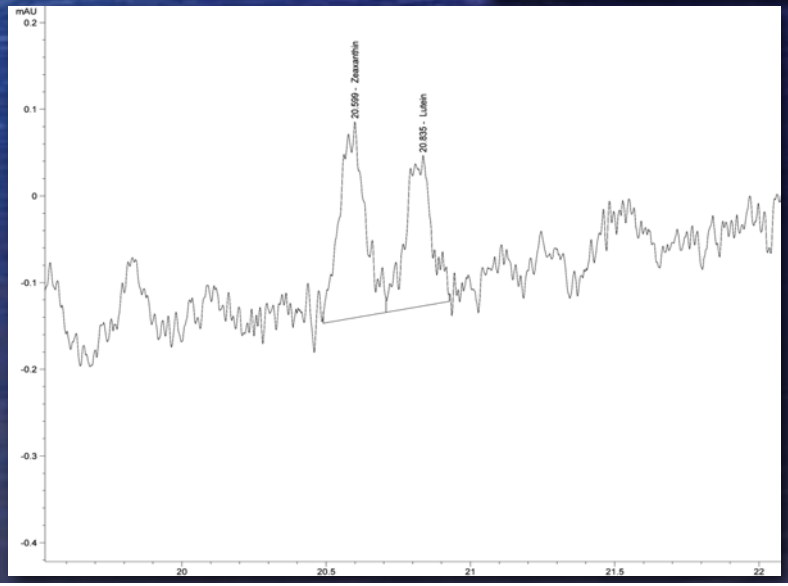
- Reinstate RRLC system configuration
  - Binary pump and micro degasser
  - 100 µl metering head
  - Semi-micro flow cell
  - Remove solvent mixer to decrease delay volume
  - Shorter columns (100mm, 1.8 um particle size)
  - Smaller diameter tubing (decrease delay volume)
- Method Translation/development
  - Method translator on Agilent website
  - Use DryLab software for further method development
  - Test other columns
  - Use method compatible with mass spectrometry analysis
  - Consider extract volume and injection volume

VHT Method - Modified for an Agilent 1200 series system

Integrations Questions

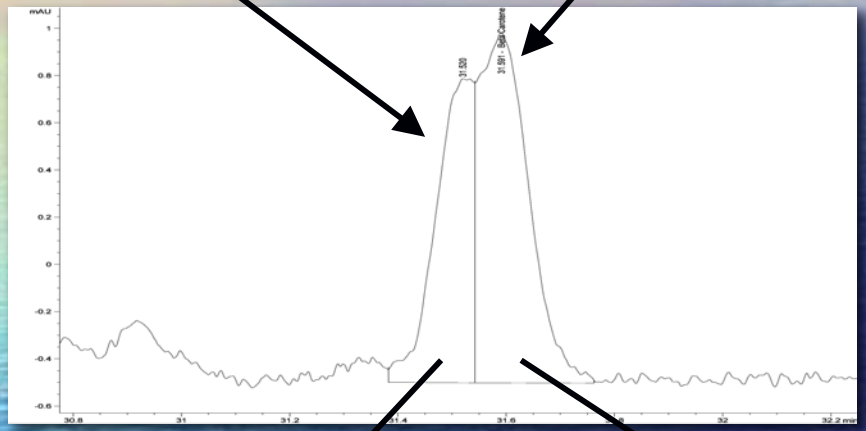


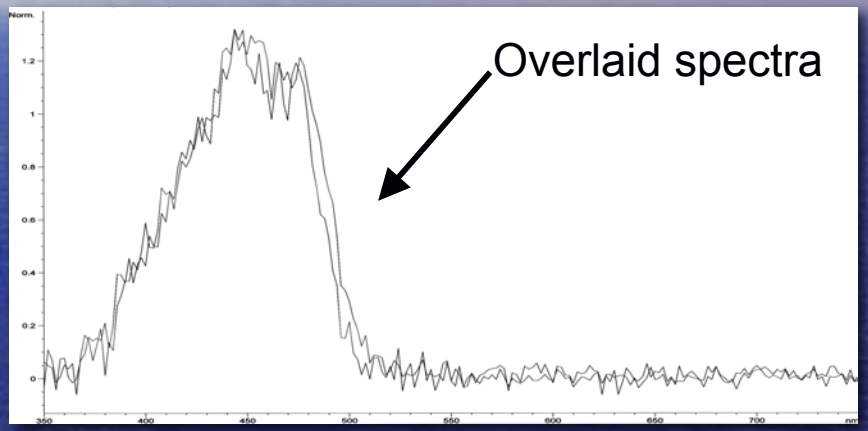


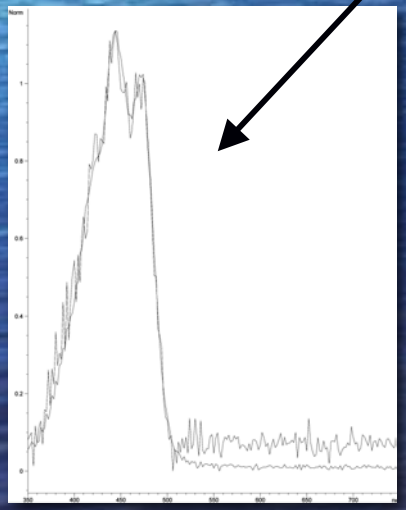


VHT Method - Modified for an Agilent 1200 series system

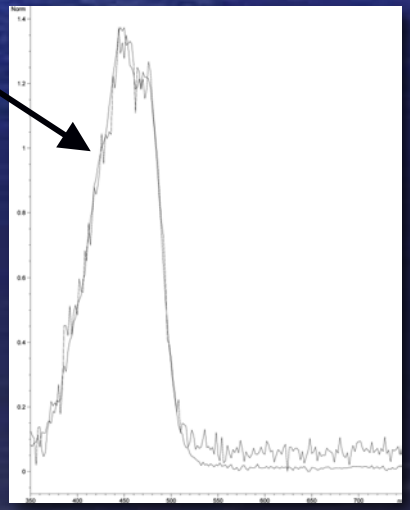
Alpha Carotene and Beta Carotene: Why I identified a carotene in sample set B







Alpha Carotene



Beta Carotene

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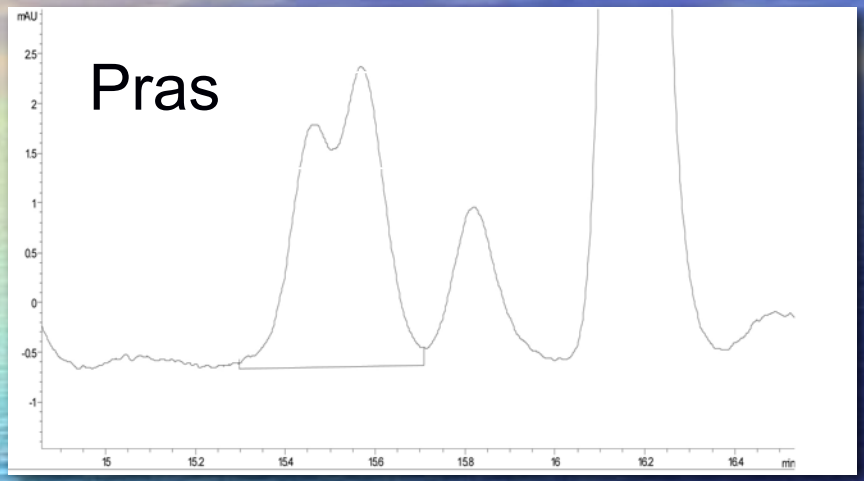
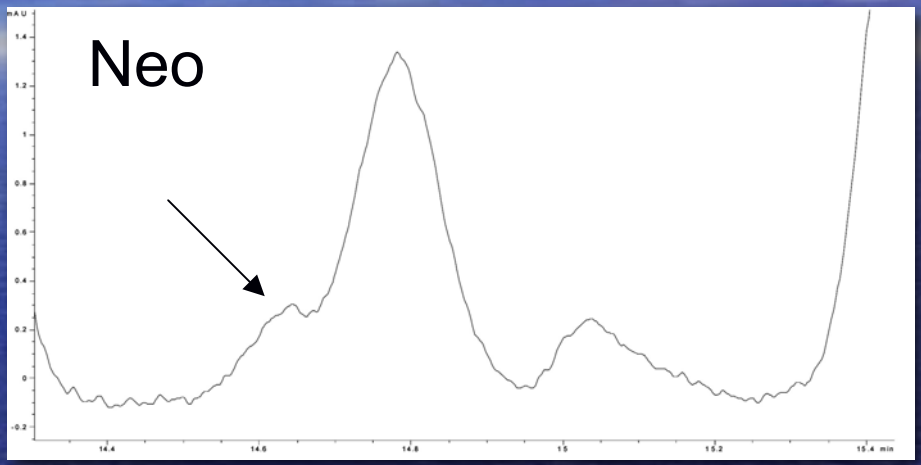
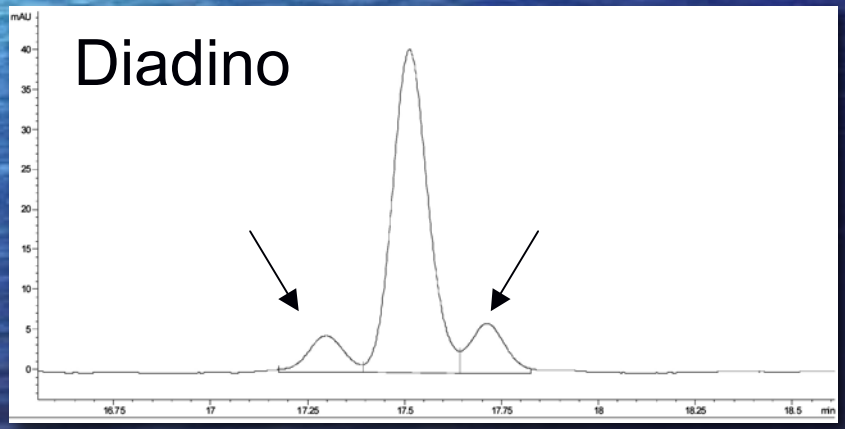
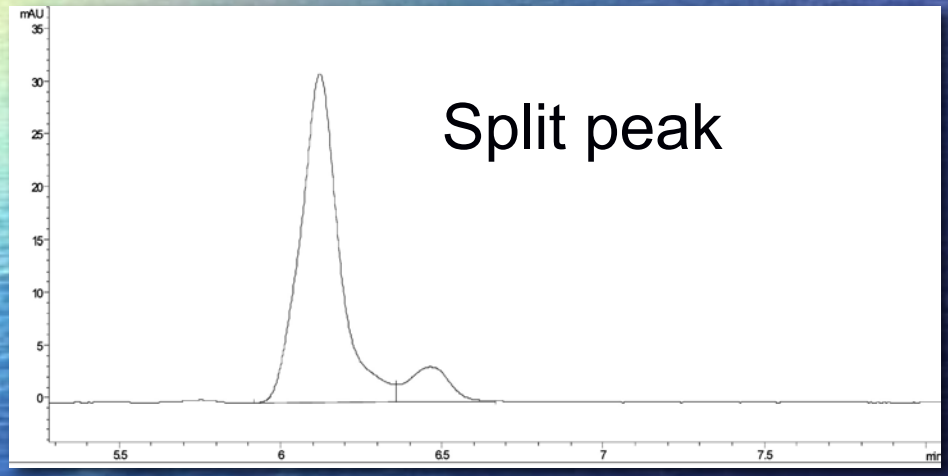


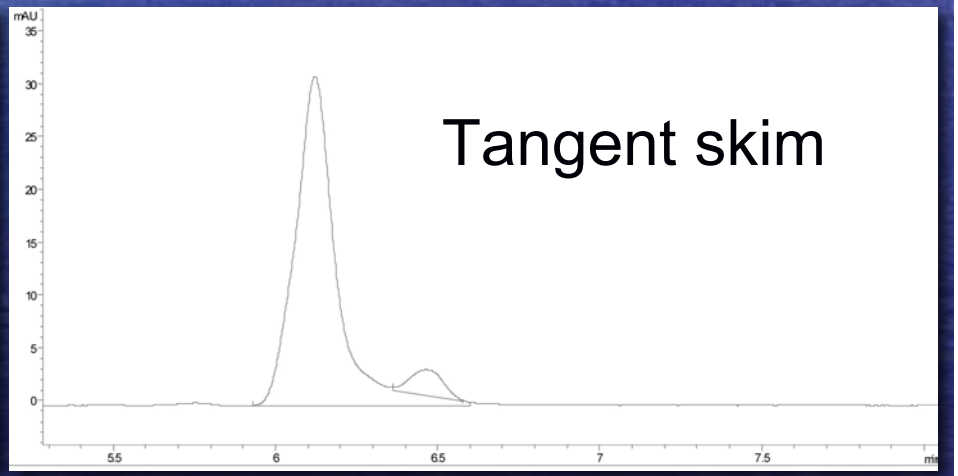




VHT Method - Modified for an Agilent 1200 series system

Chlorophyll c_2 and Chlorophyllide





VHT Method - Modified for an Agilent 1200 series system

October 1, 2009

Integration Problems

By John W. Dolan

John W. Dolan

I recently received an e-mail inquiry from a reader, along with the two chromatograms shown in Figure 1. Although not explicitly stated in the e-mail, it was clear that a debate was raging about how to best integrate this group of peaks. Proper integration procedures is a topic that comes up with surprising regularity, so I would like to look at some aspects of integration in this month's "LC Troubleshooting."

The Best Approach

In Figure 1, it is possible to distinguish three peaks. Peak 1 is just a shoulder on the front of peak 2, whereas peaks 2 and 3 are distinct peaks. So the question is how to best integrate this set of three peaks to get results that are the most accurate — that is, most closely reflect the true area under the peaks. In Figure 1a, a valley-to-valley integration method is used. On the one hand, it may look like this is a good approach, but it misses peak 1 altogether. And, although the integrated area (above the drawn baseline) clearly belongs to peak 2 or peak 3, there is a gross under-integration of the two peaks. That is because all of the area beneath the integration line is ignored.

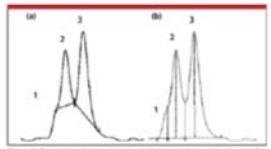
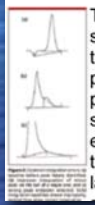


Figure 1

The correct way to integrate a group of peaks like this is to draw a perpendicular line from the valley between the peaks to the baseline extended between the normal baseline before and after the group of peaks, as seen in Figure 1b. For peak 1, it takes a bit of imagination to pick the correct point to drop the valley, and as we'll see in a minute, this is probably not appropriate anyway. For peaks 2 and 3, the process is simple. First draw a baseline connecting the real baseline before and after the peak group. Then draw a perpendicular line from the valley between each peak pair to the baseline.



The errors involved in the perpendicular drop method are as follows: If the peaks are approximately the same size, and tailing or fronting is ignored, the amount of the peak tail from the first peak (peak 2 in the present case) hiding under the second peak (peak 3) should be about the same as the amount of peak front from the second peak hiding under the first. If this is the case, the errors should cancel and peak areas should be fairly accurate. If the second peak fronts significantly or if the first peak has a strong tail, the weighting will be distorted, with corresponding errors. If the peak ratio is large — for example, 20:1 — the larger peak will be little affected by the minor contribution of the smaller peak, but the smaller peak will have excess area contributed by the major peak. In this case, the accuracy for the larger peak should be much better than for the minor peak. When the resolution between the peaks is

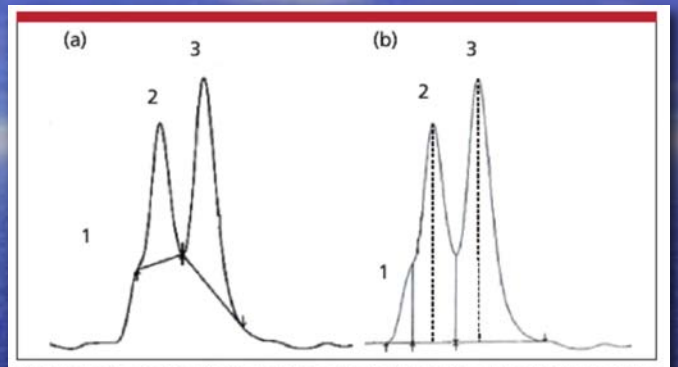


Figure 1: Two options for integrating partially resolved peaks: (a) valley-to-valley integration; (b) perpendicular drop to baseline. Dashed lines in (b) show peak height measurement.

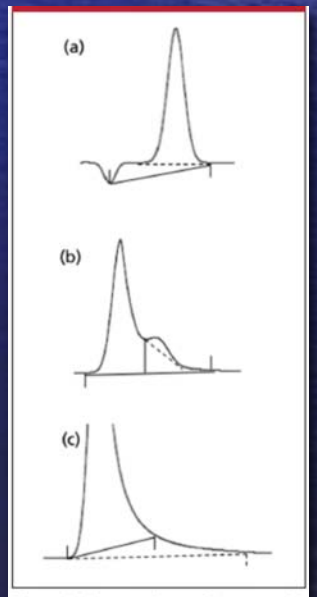


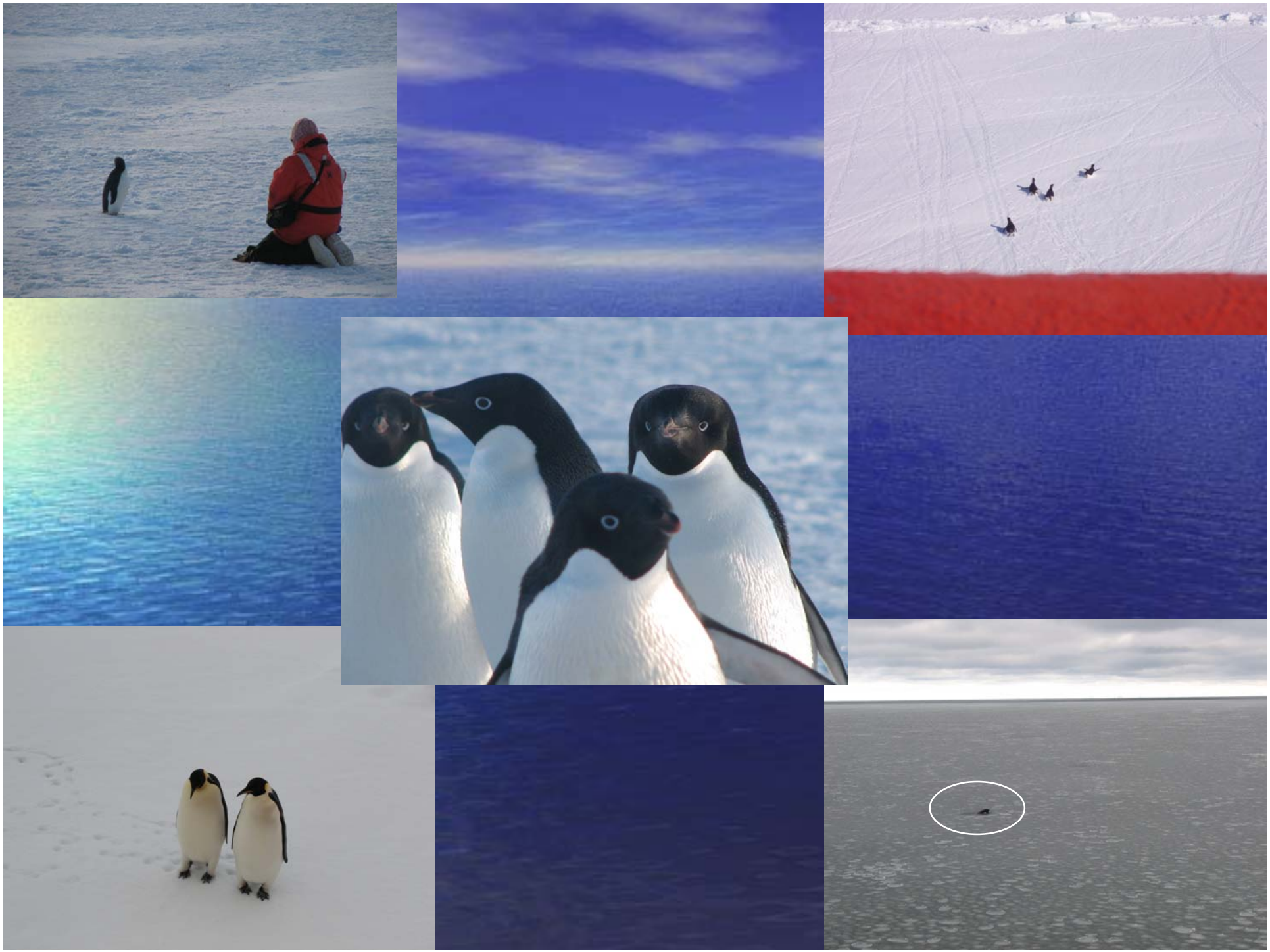
Figure 2: Common integration errors: (a) baseline before peak falsely identified; (b) improper integration of minor peak on the tail of a major one; and (c) wrong peak endpoint selected. Solid integration baselines drawn improperly; dashed lines show correct integration.

Lessons learned and improvements for the future

- Better organization during chromatogram analysis and everything else
- Measure accuracy and precision of diluting devices (used a pipette instead of syringe for standard dilutions)
- Awareness of performance metrics made all the difference!
- Determine best procedures for integrating, accepting and rejecting noisy peaks (low concentration)
- Determine LOD and LOQ for instrument

I still have much to learn!!!

Obligatory Penguin Video



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