Utilizing Native Fluorescence Detection to Improve icIEF Analysis

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Overview

- Advantages of Fluorescence Detection
- UV to Fluorescence Detection Transition
- Validating a Fluorescence method
- Conclusion

History of icIEF Methods

- Most icIEF methods were developed using the iCE3 and were proven to be equivalent using the Maurice
- Maurice has the added feature of fluorescence detection
  - Since the iCE3 will be discontinued (2029), all Lilly testing labs have a Maurice system and the capability to utilize fluorescence detection
- We are continuously looking for ways to improve icIEF method robustness and fluorescence detection has the potential to do so

iCE280 - 1999

iCE3 - 2012

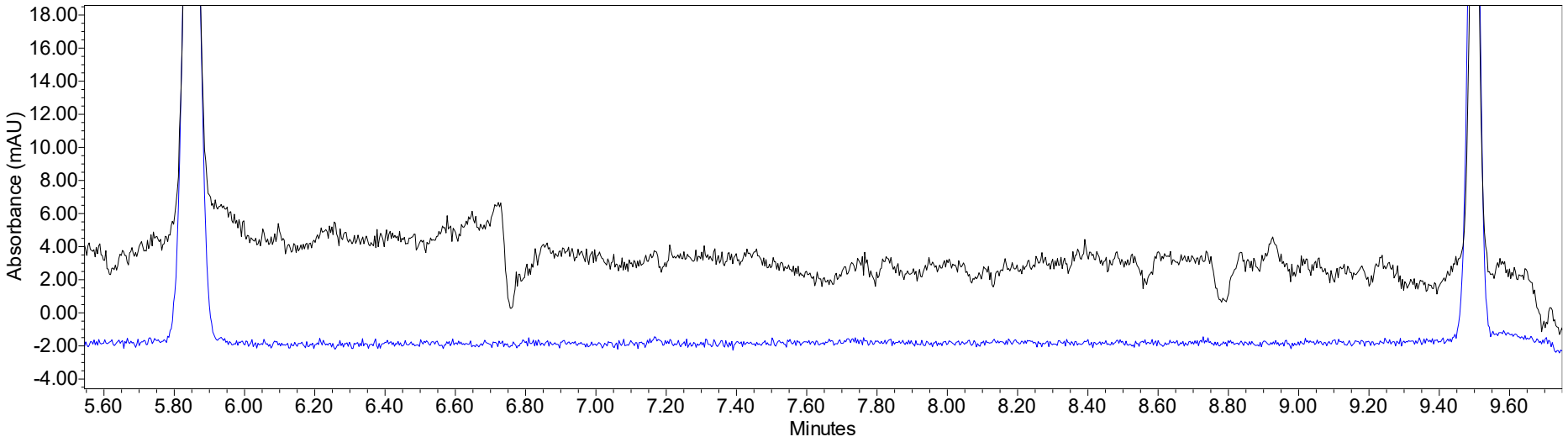
Maurice - 2016

Images provided by Protein Simple

Why Native Fluorescence (FL) Detection?

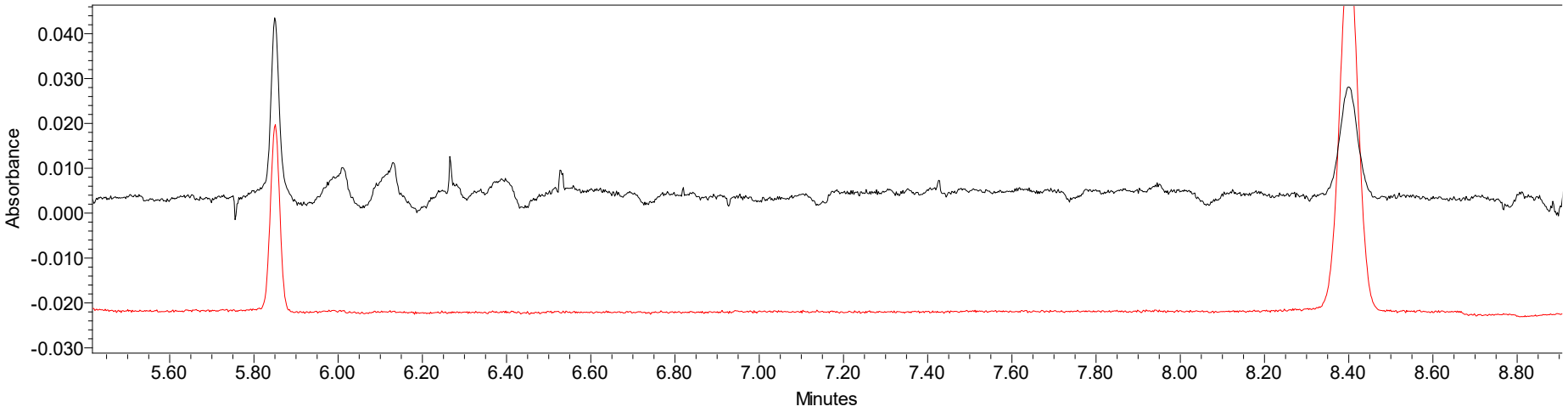
- Native fluorescence detection relies on intrinsic fluorescence emitted by certain amino acids, like tryptophan
- Advantages compared to UV
  - Reduced baseline noise → Improves integration consistency
  - Removes interference from carrier ampholyte dips and air bubbles → Reduces re-runs and need to search for “good” lots of ampholytes
  - Highly sensitive detection → Improved detection of low level variants

Comparison of Baseline: UV vs FL



Pharmalyte 3-10 with know dip around pI 6.7

UV
20 second FL exposure

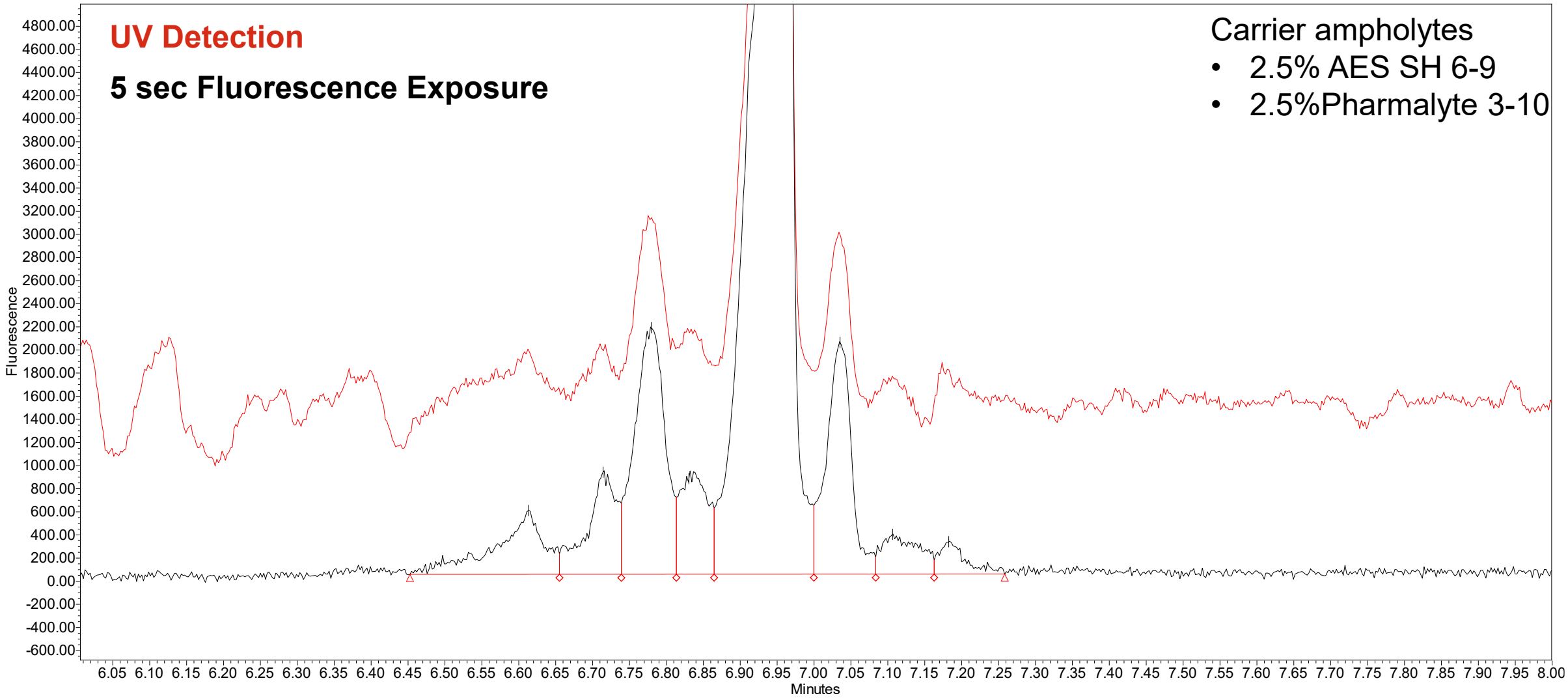


Carrier ampholytes
- 2.5% AES SH 6-9
- 2.5%Pharmalyte 3-10

UV
15 second FL exposure

*Normalized by Y-axis to generate overlay

Improved Integration Strategy



Transitioning a Method to FL Detection

Sample Concentration

- 0.25 mg/mL UV concentration over saturates the detector when using FL detection
- New nominal concentration 0.10-0.15 mg/mL
- Must be assessed for each molecule to find the linearity range of the detector

pI Markers

- Not all iCE3 markers fluoresce and may contain artifact peaks using FL detection
- iCE3 4.65 → Maurice 4.05 or 5.85
- iCE3 9.46 → Maurice 9.50 (highest available is 10.1)

FL Exposure Duration

- Exposure time can be set 1-80 seconds
- Longer exposures produce a higher response
- Ideal exposure is heavily dependent on the molecules structure

Criteria for using Fluorescence Detection

1. Minimal difference between UV and FL numerical results
2. Same number of variants and overall profile appearance
3. Ability to identify a linear range
4. Difference between UV and FL for nominal samples, is comparable to the difference for stressed samples

Assessing a UV method for fluorescence detection

Experiment 1 – Determine Optimal FL Conditions

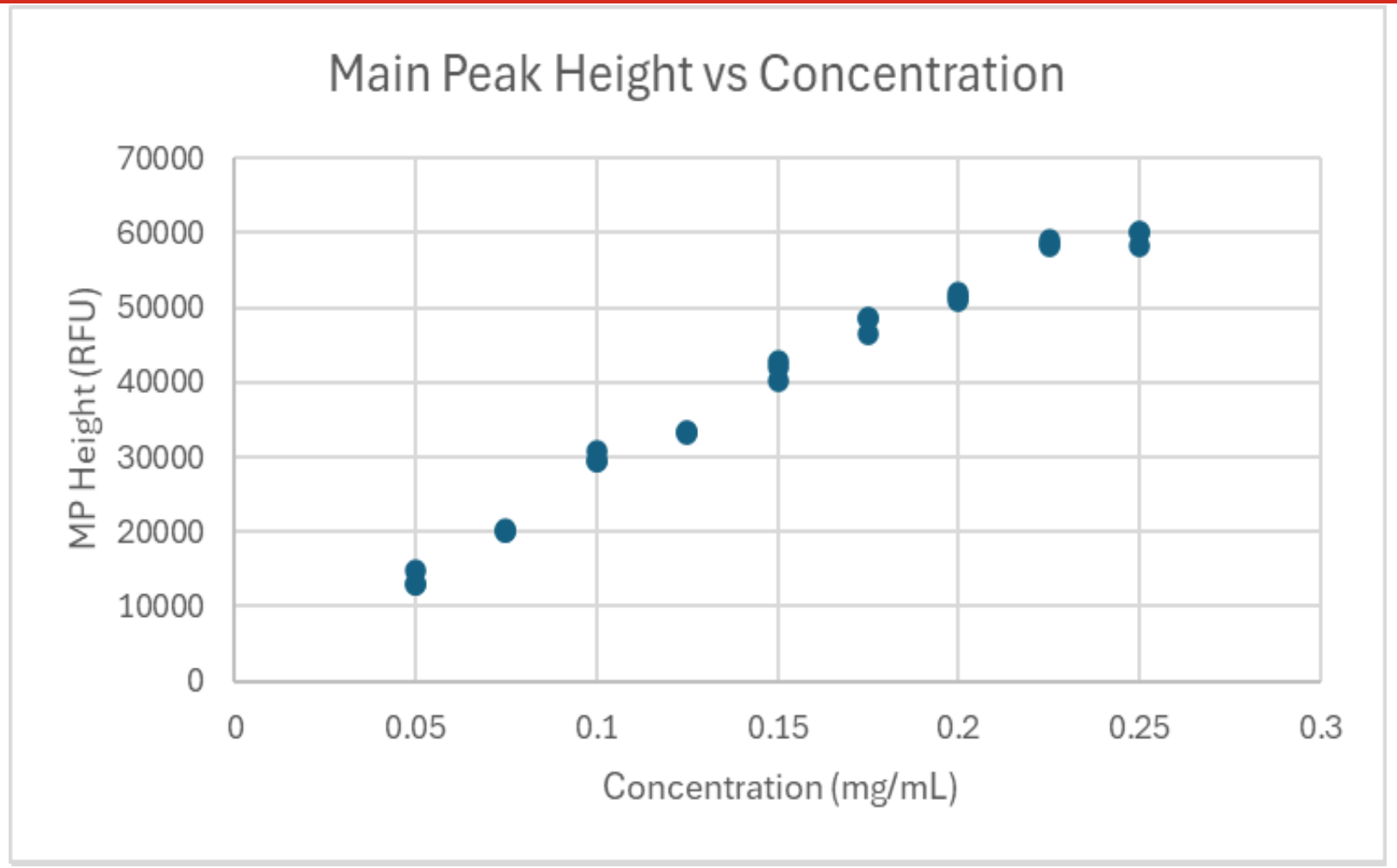
- Evaluate high level linearity using 6 exposures (5-30 sec) to determine a new nominal concentration

Experiment 2 – High and Low Linearity

- Run high and low level linearity using the concentration and exposure +/- 5 sec determined in experiment 1

Experiment 3 – confirm UV and FL comparability

Key Learning: Peak Height



Plotting peak height can be used as a visual to determine the linear range

Assessing a UV method for fluorescence detection

Experiment 1 – Determine Optimal FL Conditions

Experiment 2 – High and Low Linearity

Experiment 3 – confirm UV and FL comparability

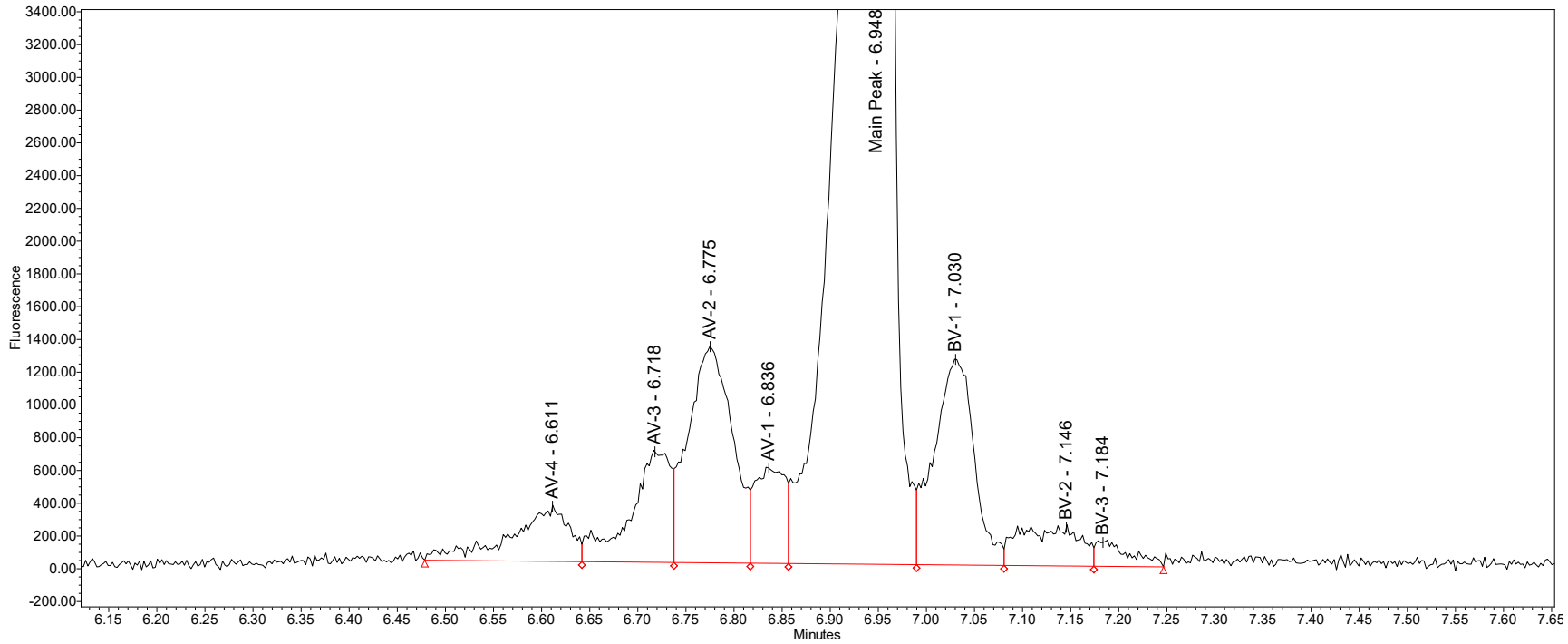
Full FL Linearity Assessment

- Combine day 1 and 2 by running samples to covera wide range (0.0025-0.25 mg/mL)
- Collect 5-30 sec FL – only process potential exposures
- Plot peak height and MP area for each exposure
- Use the graphs to visually determine the linear range
- Check high/low linearity with the data generated

Problem: Experiment 2 failures due to selecting the wrong concentration from experiment 1

Solution: Cover the full range using 0.10-0.15 mg/mL as nominal concentration. Data generated can be used to check high/low level linearity

mAb 1 – Mock Validation



Peak Group	Avg (%)	St Dev (%)	R	R^2	HL/LL slope
MP	71.88	0.82	0.998	0.989	0.91
TAV	18.65	0.77	0.996	0.987	0.87
TBV	9.95	0.34	0.996	0.986	----
Total	----	----	0.998	0.987	0.90

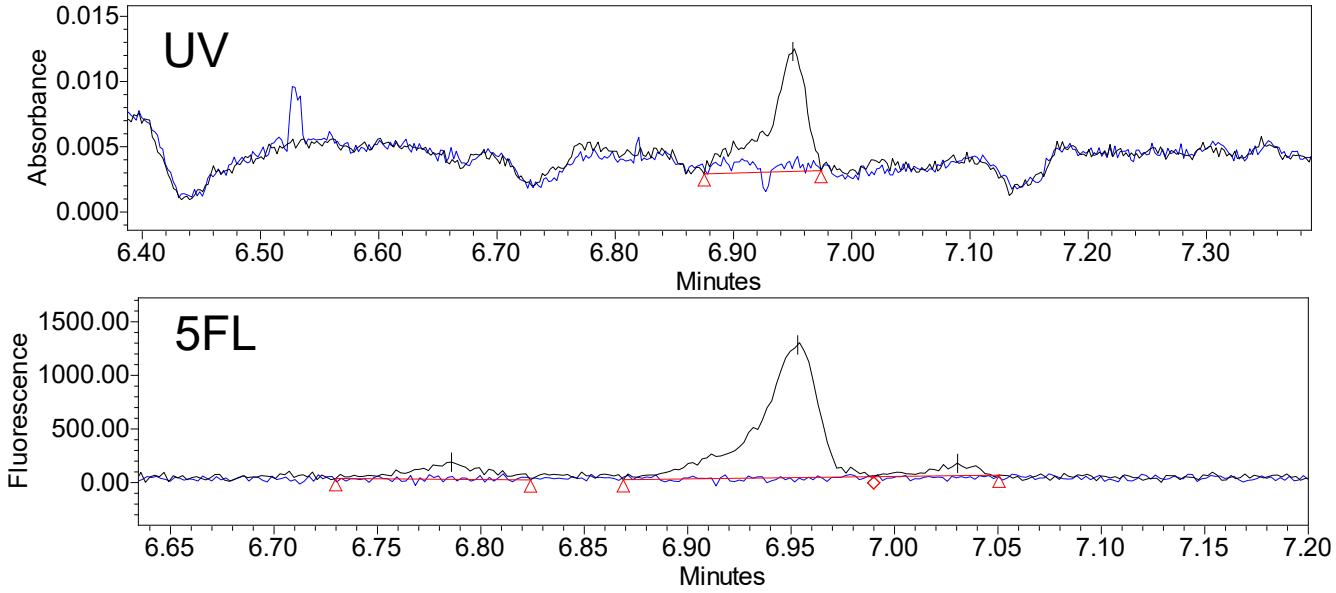
- Carrier Ampholytes
  - Pharmalyte 3-10
  - AES SH 6-9
- AES SH 6-9 has a very noisy baseline – UV detection integration would not be robust due to the noise
- This method was immediately developed for FL detection
- Method passes all validation criteria

Optimal conditions identified: 0.10 mg/mL with 5 second FL exposure

Quantitation and Detection Limit

- Typical QL observed for UV iCE methods is 1-2.5%
- QL expected to improve with FL detection since it reduces interference from air bubbles and carrier ampholytes

Exposure	Conc	Peak	USP S/N	QL (%)	DL (%)
5	0.075	MP	23.0	1.96	0.65
10			50.7	0.97	0.32



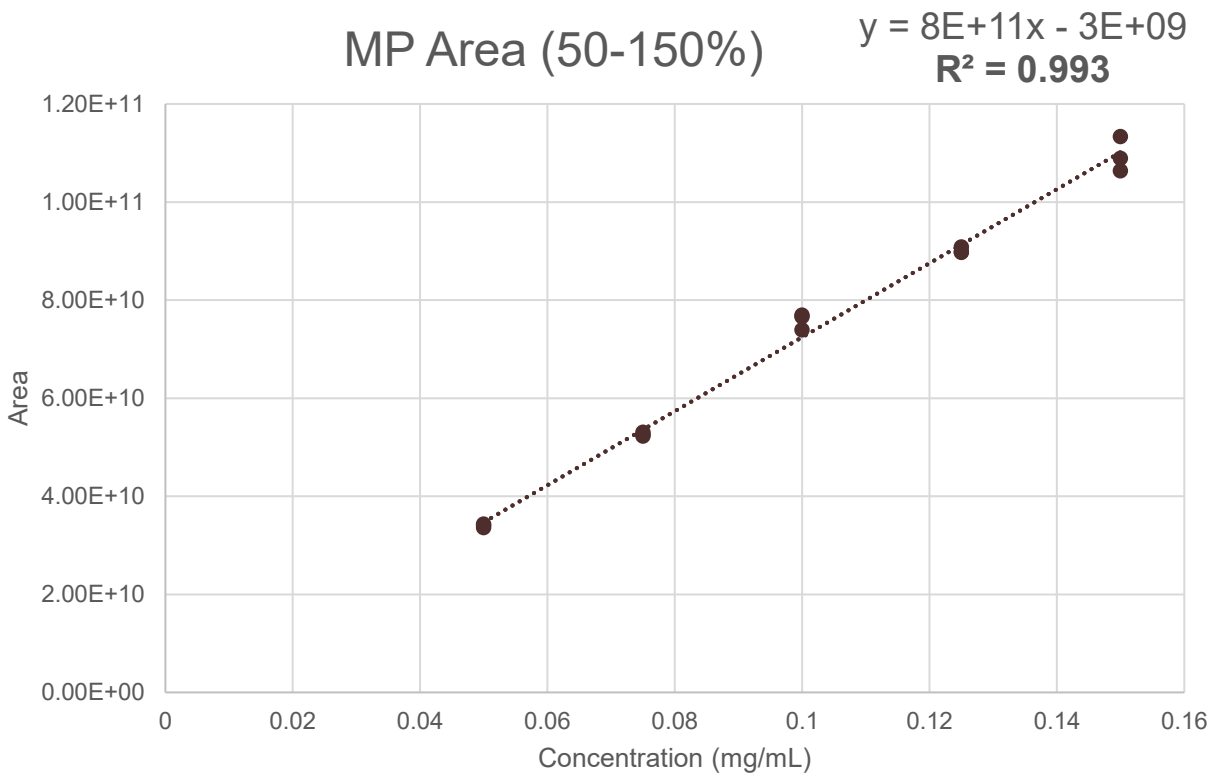
Increase exposure or sample concentration to improve QL

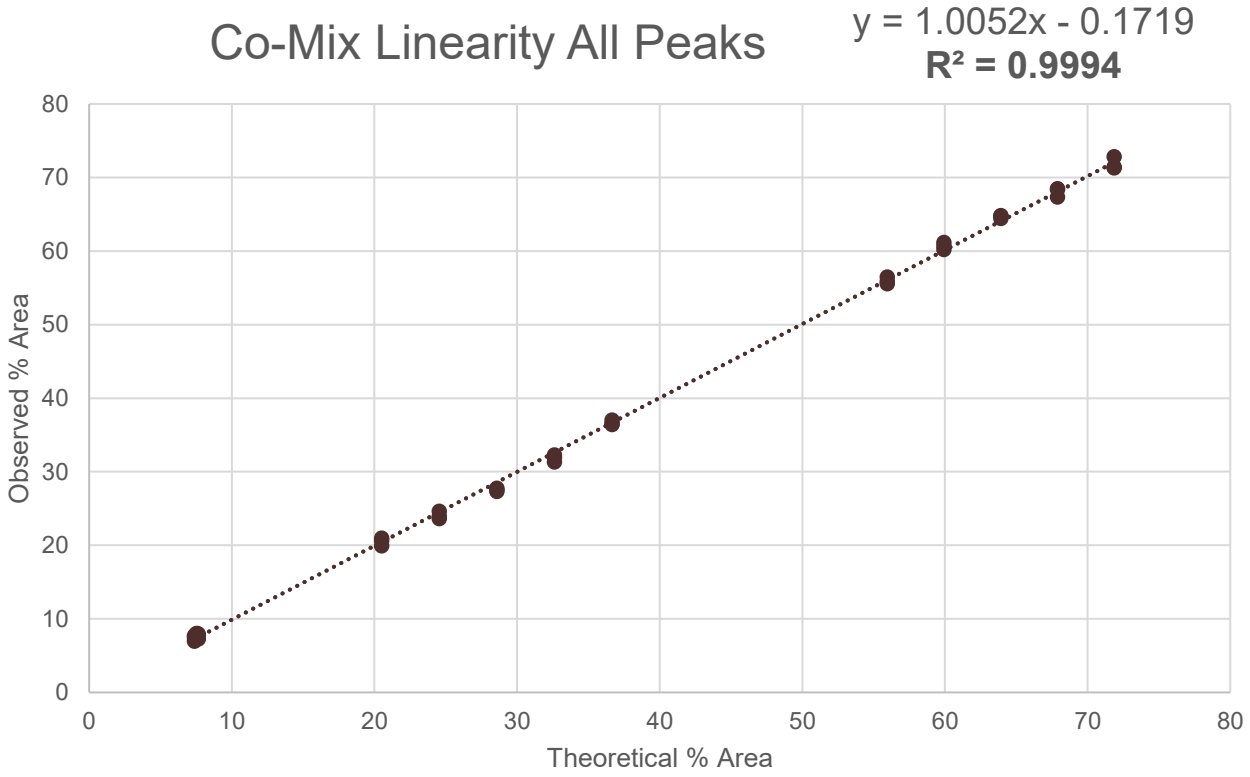
Low and high levels no longer linear when compared to each other

Validating a FL method

Traditional UV Approach	Fluorescence Approach
Linearity by high/low level linearity slope comparison	Linearity by co-mix theoretical vs observed MP, TAV & TBV
Accuracy inferred by low level linearity slope ratio	Accuracy by co-mix MP, TAV and TBV recovery

Re-Evaluating Ideal Conditions





- Decided to increase exposure opposed to concentration – increased 5 to 10 seconds
- Low level is no longer linear compared to high level
- Replacing low level with co-mix to determine accuracy and linearity was successful
  - Co-mix can reduce issues previously seen with low level, such as pipetting errors

Final Conditions: 0.10 mg/mL sample concentration with 10 sec fluorescence exposure
Method was successfully validated

Conclusion

- **FL detection is highly specific to each molecule**
- A different validation approach is needed for FL detection to accommodate the limited linear range of the detector
- FL detection can improve the robustness of our methods and prevent common problems encountered in icIEF analysis
  - Reduce frequency of re-runs due to baseline dips and spikes
  - Does not detect carrier ampholyte interference (Pharmalyte 3-10 baseline dip)
  - Low baseline noise allows for consistent integration of low level variants

Questions?