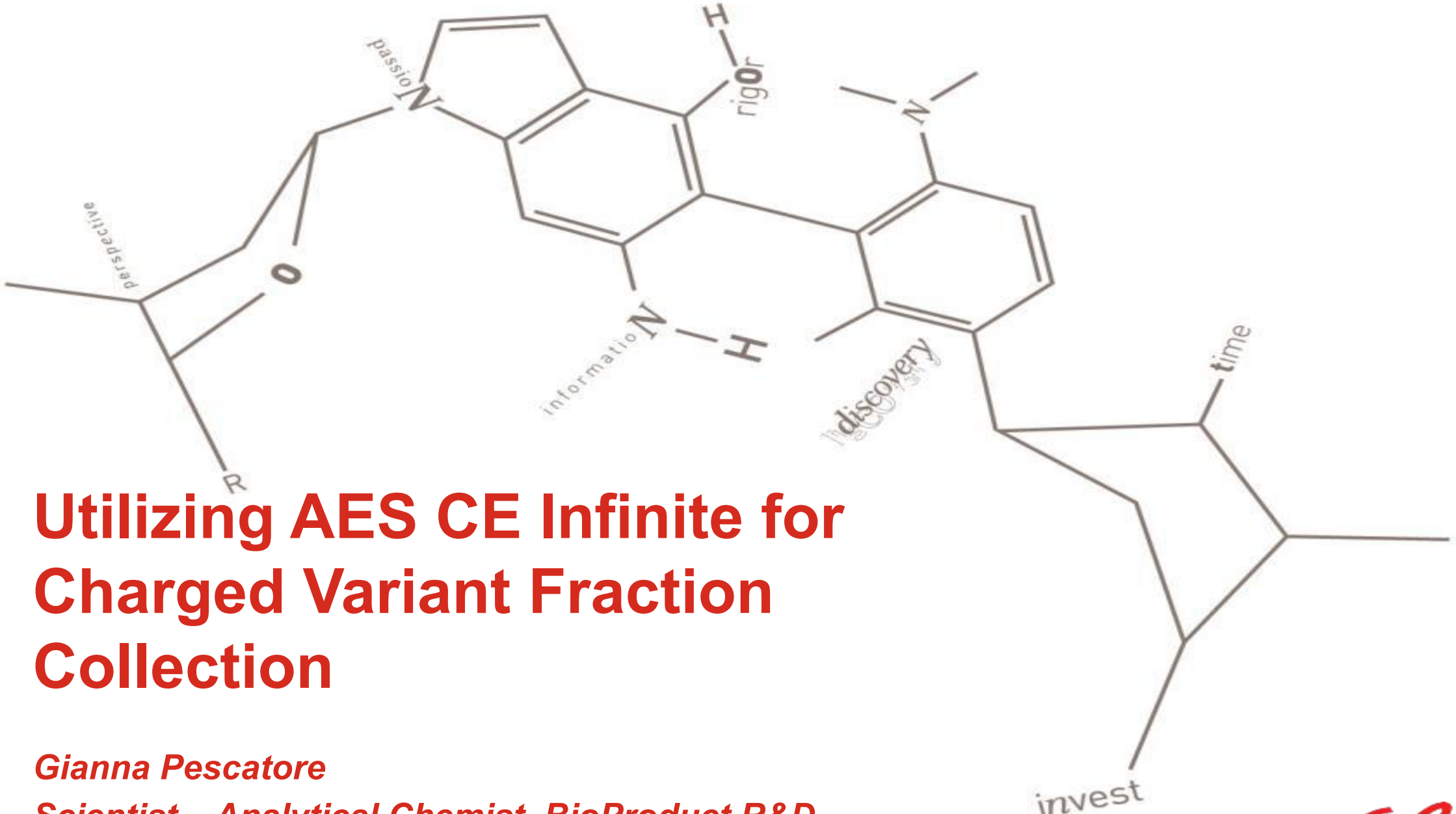


Utilizing AES CE Infinite for Charged Variant Fraction Collection

Gianna Pescatore

Scientist – Analytical Chemist, BioProduct R&D

Lilly

Uses of icIEF fraction collection

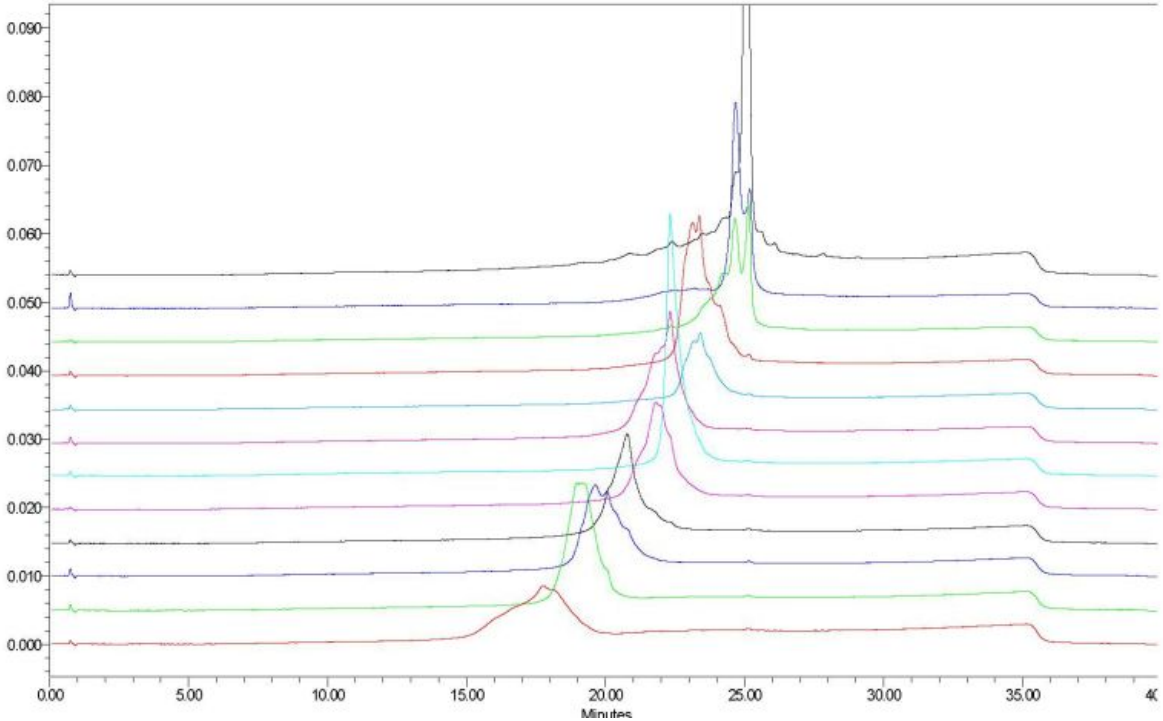
- Charged variant peak ID and potency testing is used to support BLA submissions
- Identification of post translational modifications in forced degradation sample
- Evaluate manufacturing process consistency
- Fractions can be tested using the molecules other analytical methods to determine how each variant is detected by the overall analytical control strategy

CEX vs icIEF Collection

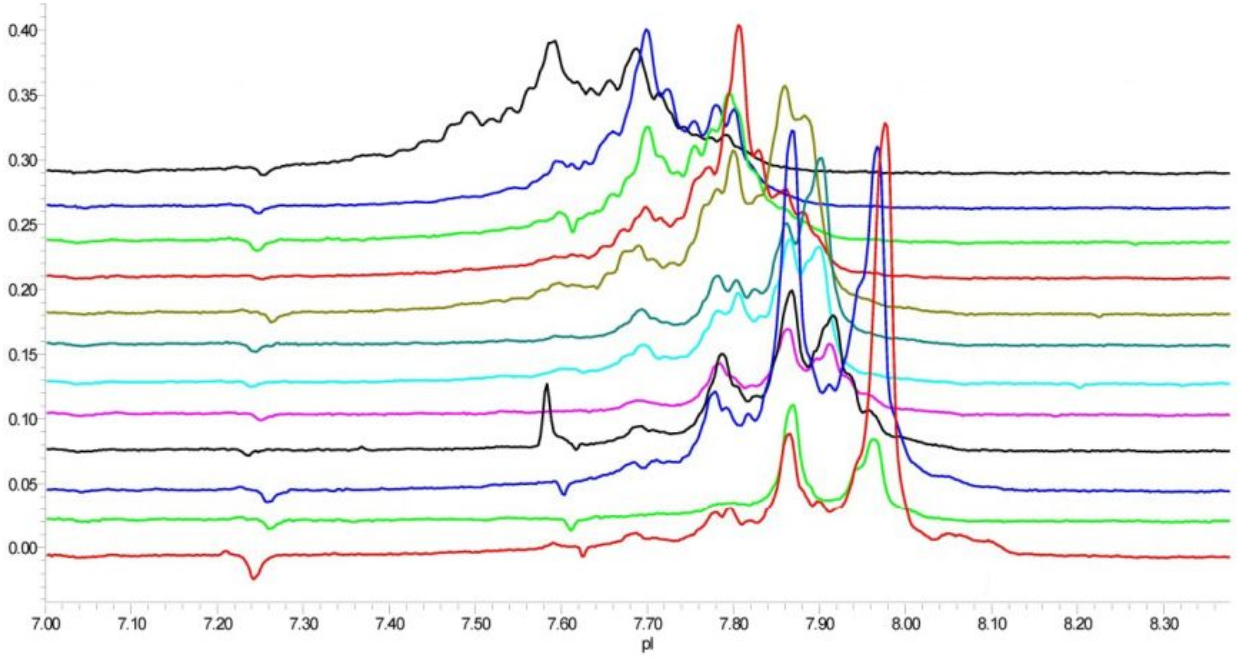
- Historically, CEX has been used to collect charged variant fractions
- Advantages of CEX
  - Ability to inject a substantially higher sample volume and concentration
    - CEX – 50 mg/mL 500 uL injection
    - icIEF – 4 mg/mL 35 uL injection
  - Shorter collection period
- Disadvantages of CEX
  - Difficult to verify icIEF identity due to the different selectivity between the separations
  - CEX fractions that contain a single variant when confirming with CEX, can have multiple peaks when tested using icIEF

CEX Fraction Confirmation: CEX vs iclEF

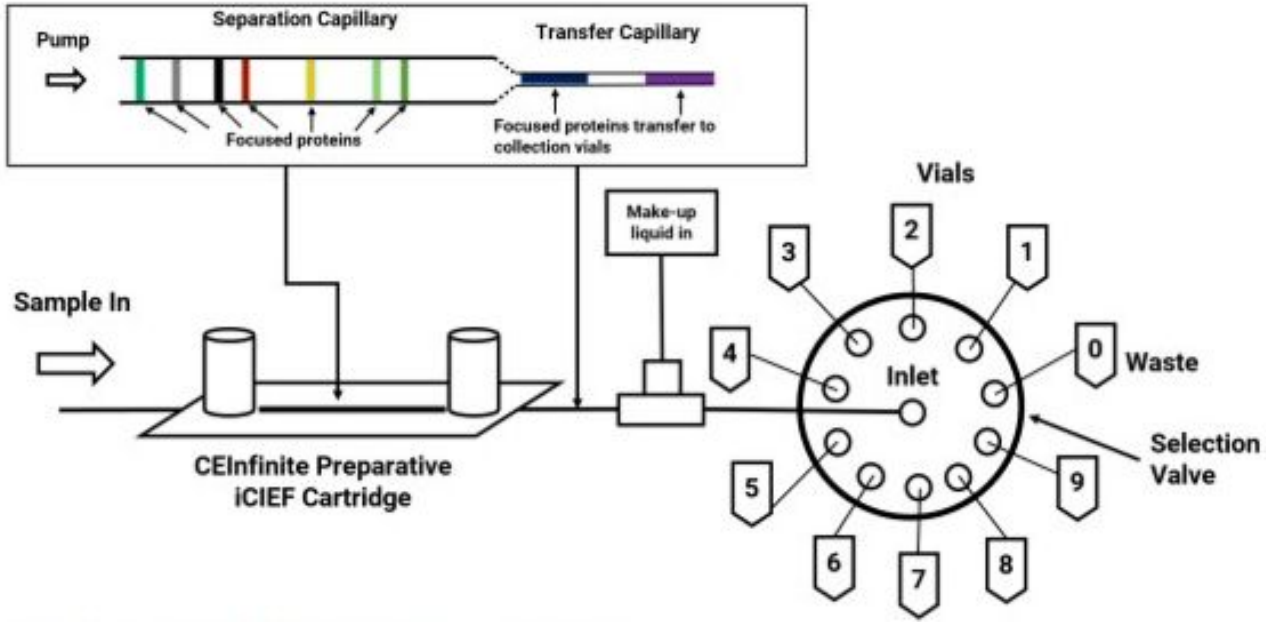
CEX



iclEF



AES CE Infinite Capabilities



- Quick method development
  - Fractions can be verified from ~ 3 injections
- Automated collection based on an absorbance trigger point
- Collects 9 different fraction segments
- 36 fraction collection injections can be done in a single setup
  - Ideal instrument for collecting fractions to be used for peptide mapping and potency testing
- 35-50 minute run time per injection (varies per method)
- Reverse polarity function allows acidic variants to elute first to obtain pure fractions
- Can be directly connected to a mass spectrometer to allow for direct icIEF-MS

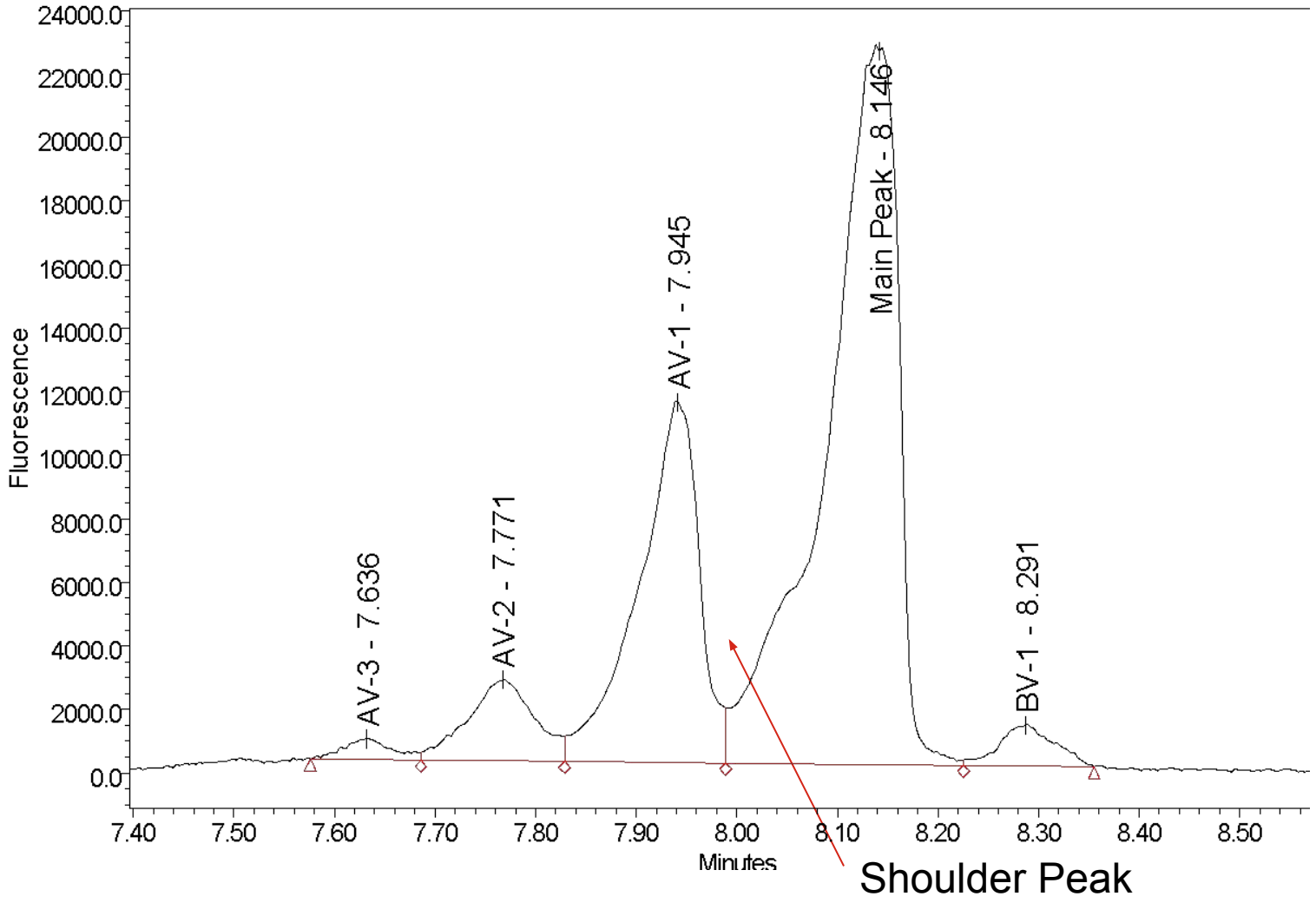
Fraction Collection Workflow

1. Develop a method that provides sufficient resolution on the CE infinite (1-3 Days)
2. Determine fraction collection parameters (1-3 Days)
3. Collect 3 injections worth of material to ensure collection prediction is consistent (~3 hours)
4. Prepare collected fractions for verification using the molecules icIEF method (~1-2 hours)
5. Run prepared fraction samples to confirm peak ID (~2-4 hours)

Molecule 1: Background

- IgG1 Monoclonal Antibody
- Material used was stressed at 40C for 4 weeks
- Peak summary
  - AV3 – 1.3%
  - AV2 – 6.6%
  - AV1 – 26.4%
  - MP – 63.1%
  - BV1 – 2.7%

- Identified problem: Integration strategy of the shoulder peak on the acidic side of the MP (10-15% of MP)
  - Is it stability indicating?
  - Can it continue to be integrated with the MP?



Sample Components

- **Carrier ampholytes** – 5% of the final sample
- **Solubilizer** – Urea or solubilizer of choice
- **Anodic/Cathodic spacers** – Can be used to adjust the pixel location of the MP to increase/decrease run time
- **Sample concentration** – Start at 1-2 mg/mL and assess target concentration based on current
  - Current > 100 uA damages the cartridge
  - Highest successful concentration – 4 mg/mL
- Water is added to achieve a final sample volume of 250 uL, yields 6 injections (Each injection is 35 uL)
- No methyl cellulose – Cartridges are coated with an acrylamide derivative which allows a higher sample concentration to be achieved

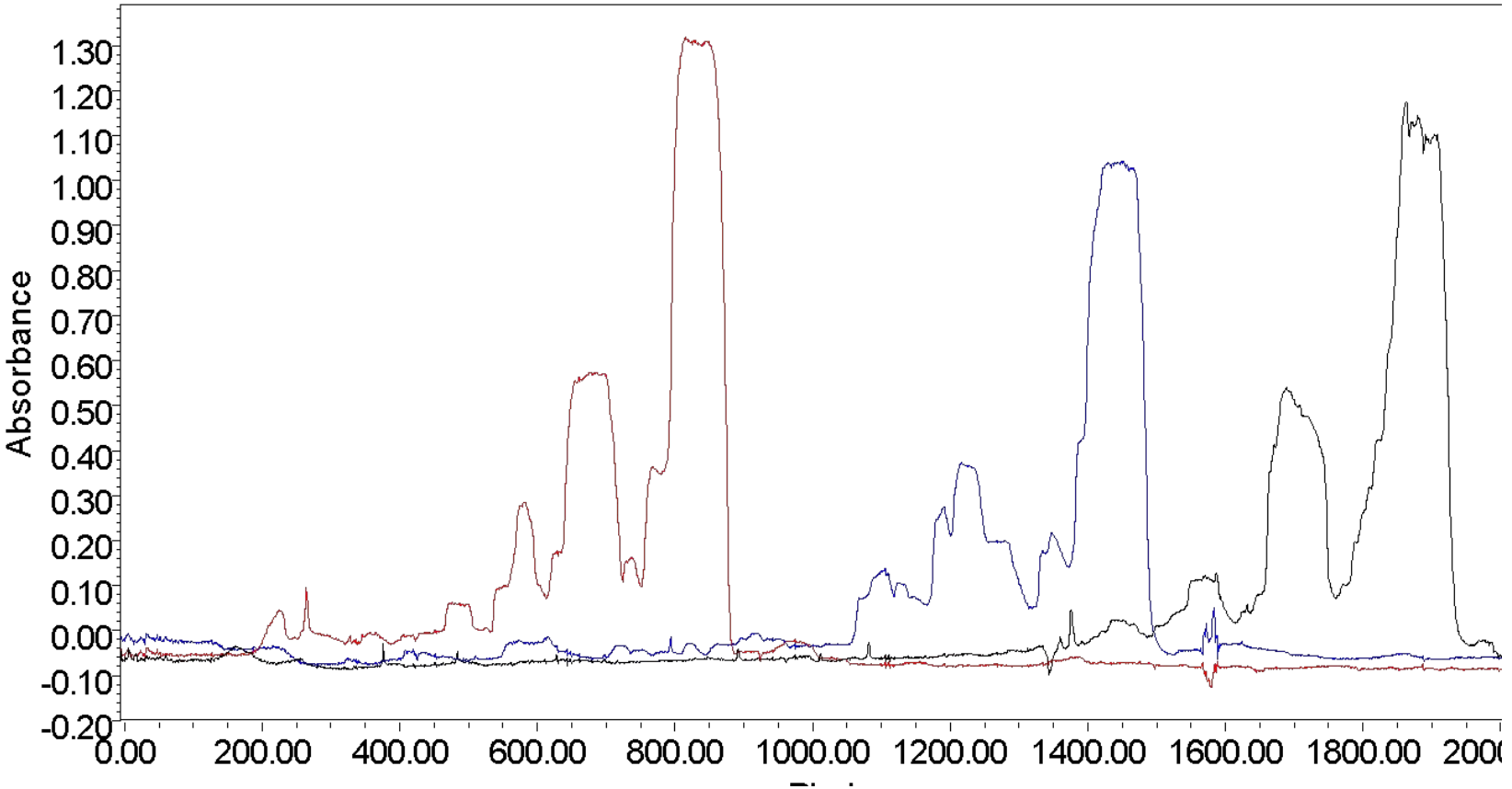
Carrier Ampholyte Screening

1. 5% AES SH 6-9
2. 5% AES UH 7-9
3. 5% AES UH 7-10
4. 3% AES SH 6-9 & AES UH 7-10
5. 5% AES SH 6-9 + 10 mM Arginine
6. 5% AES SH 6-9 + 5 mM Arginine
7. 1% Pharmalyte 5-8, 2% AES HR 7-9 & 2% AES HR 8.5-9.5
8. 1% Pharmalyte 5-8, 2% AES SH 6-9 & 2% AES HR 8.5-9.5
9. 1% AES SH 4-8, 2% AES SH 6-9 & 2% AES HR 8.5-9.5
10. 1% Pharmalyte 5-8, 2% AES SH 6-9 & 2% AES HR 7-9

Experiment 1 – Single Ampholyte (1-3)
Experiment 2 – Cathodic Spacer (4-6)
Experiment 3 – 3 Ampholyte Combinations (7-10)

Experiment 1 - Single Ampholyte Analysis

- Evaluating single carrier ampholytes alone is the first step in method development
- Using one narrow range ampholyte can allow for increased separation
  - However, these typically require longer focusing times which may not be ideal for bulk collection
- It helps establish which carrier ampholyte type and ideal pI range for the final fraction method

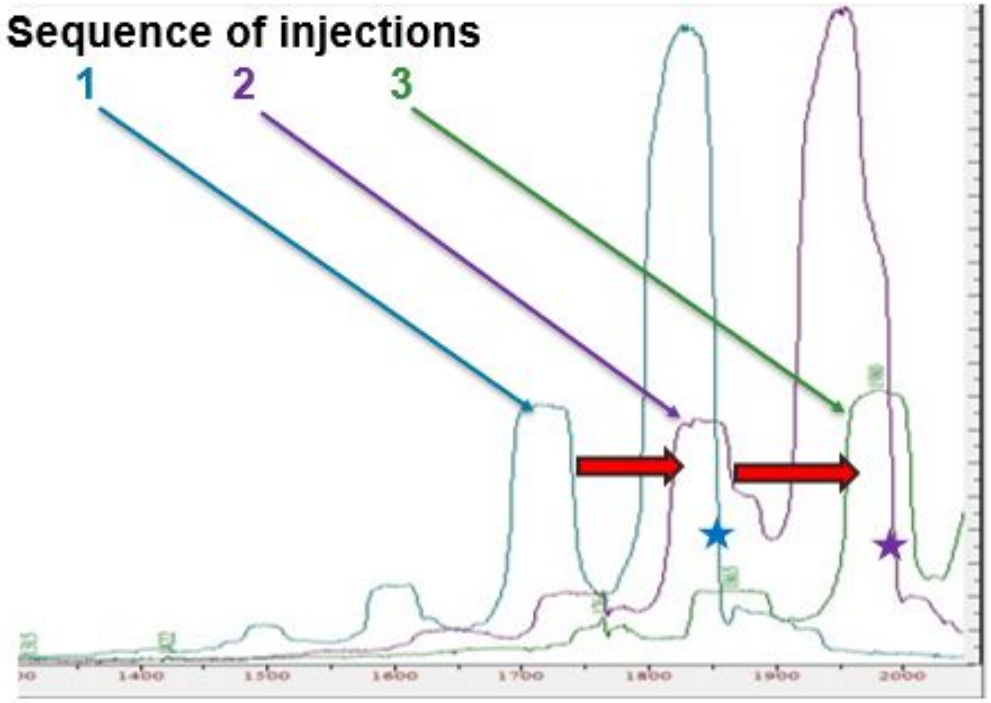


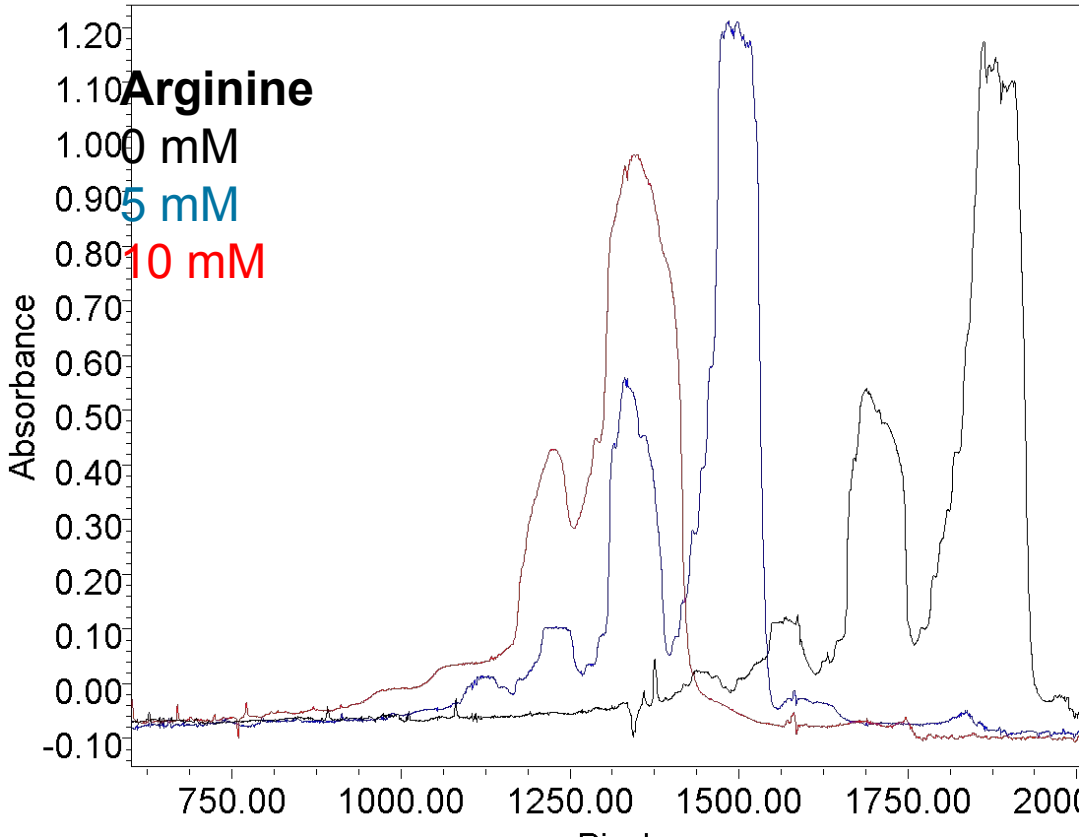
1. 5% AES SH 6-9 (16 min Focus)
2. 5% AES UH 7-9 (20 min Focus)
3. 5% AES UH 7-10 (20 min Focus)

Experiment 2 - Cathodic and Andodic Spacers

- Anodic and Cathodic spacers can be used to adjust the pixel location of the profile.
  - Cathodic spacers (arginine) shifts the profile to the acidic side which reduces risks of micro air bubbles shifting the trigger point out of the detection window
  - Anodic spacers (IDA) shifts the profile to the basic side and reduces mobilization time

Air bubble shift





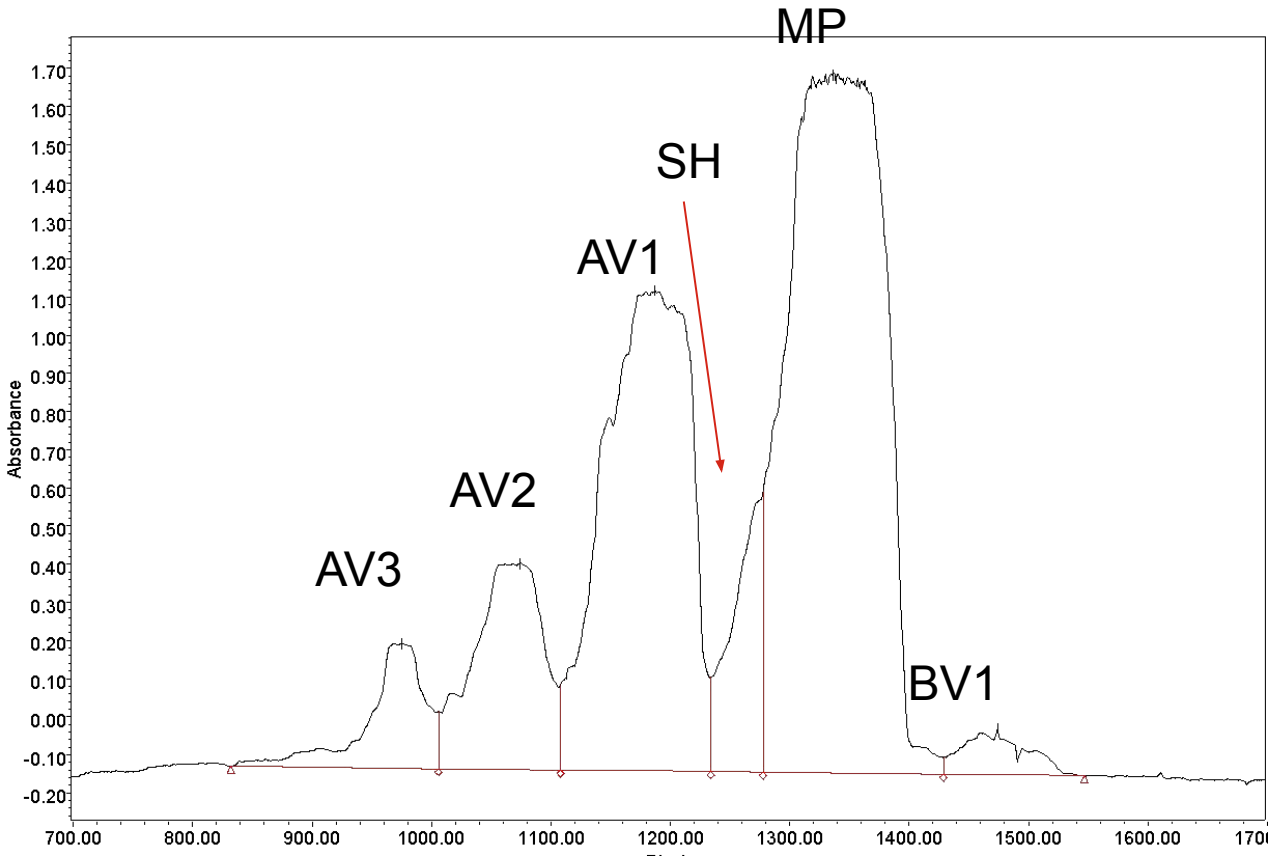
Experiment 3 – 3 Ampholyte combinations

- A combination of 3 carrier ampholytes has shown to be the best approach for 3 different molecules
- AES SH 6-9 had sufficient resolution and ideal run time in experiment 1
  - Chosen as the “base” carrier ampholyte in the final mix
- Pharmalyte 5-8 was added to maintain the acidic side of the gradient to shorten runtime
- AES HR 7-9 was used to add a higher concentration of the ampholytes in the molecules pI range to improve separation

Final Conditions

Component	Stock Conc	Units	Target	Required Volume (µL)
Pharma 5-8	100	(%)	1	2.5
AES 6-9 SH	100	(%)	2	5.0
AES 7-9 HR	100	(%)	2	5.0
Urea	8	Molar	3.5	109.4
Sample	9.4	mg/mL	4	106.4
Water	--	--	--	21.7
250 uL yields six injections (35 uL each)			Total Volume (µL)	250

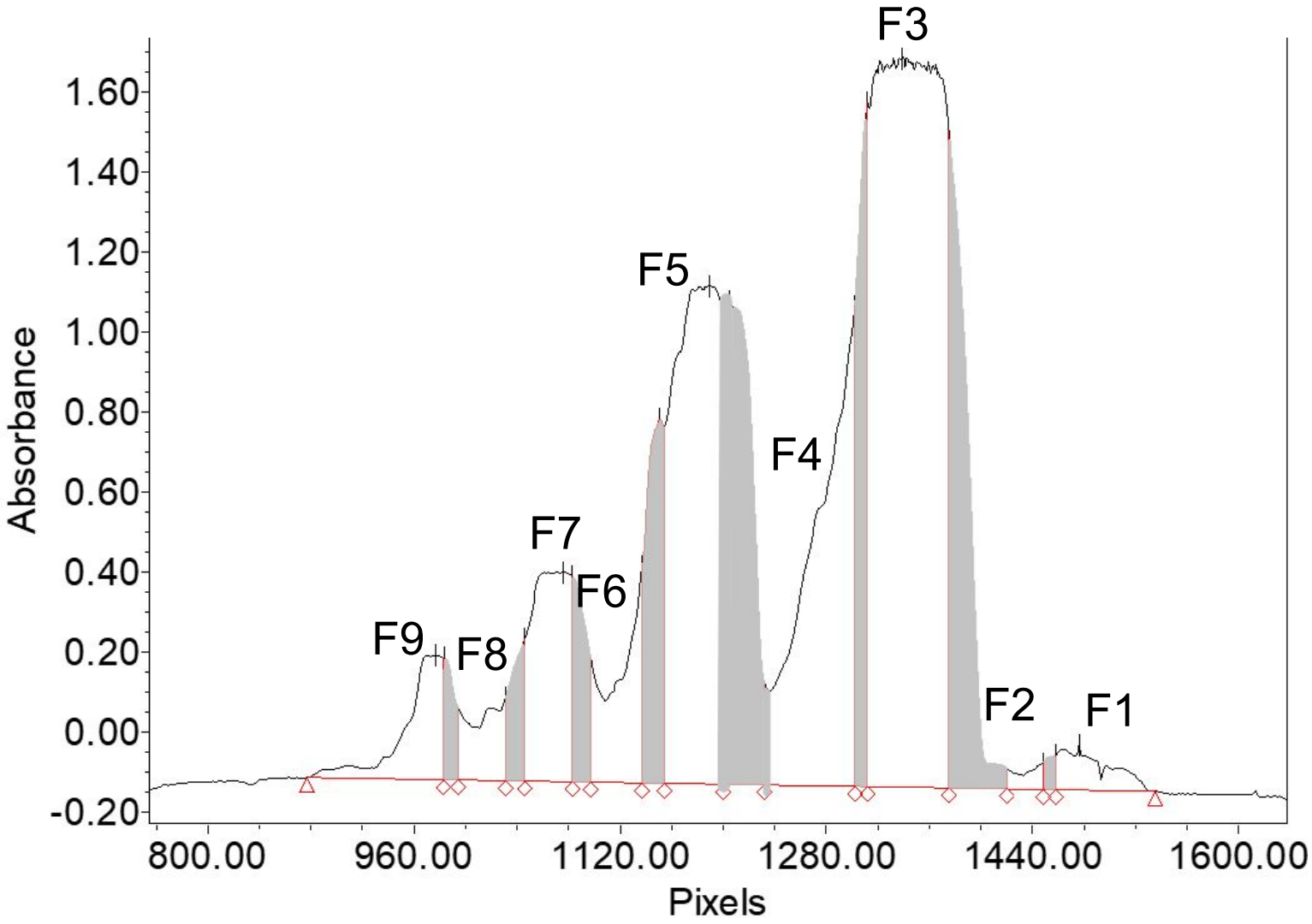
Parameter	Setting
Focusing Period 1	1 minute, 1000 volts
Focusing Period 2	1 minute, 2000 volts
Focusing Period 3	13 minutes, 3000 volts
Mobilization Phase	19 minutes, 3000 volts
Mobilization flow rate	0.16 uL/min



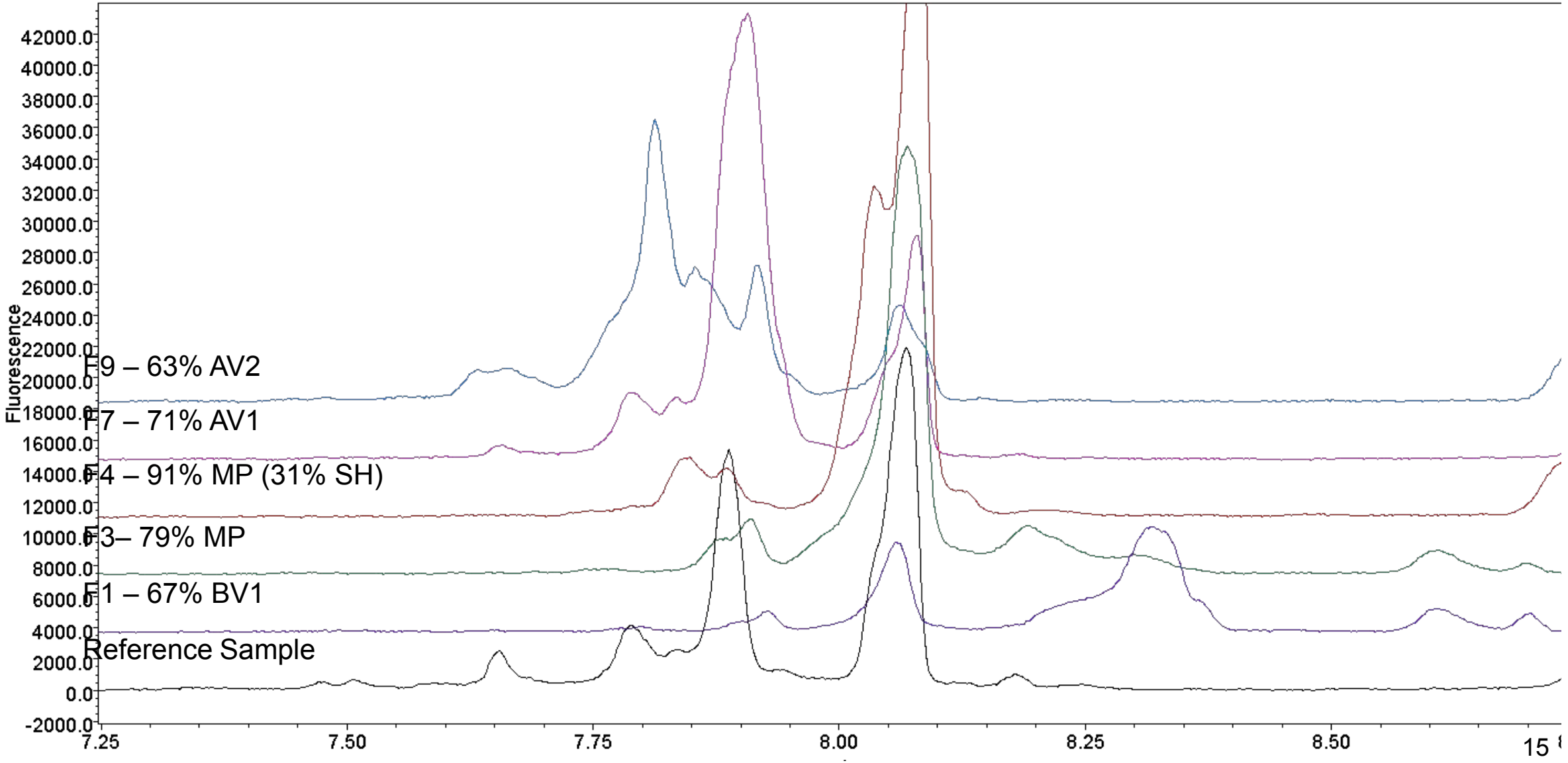
Fraction Collection Settings and Prediction

Parameter	Setting
Focusing Period 1	1 minute, 1000 volts
Focusing Period 2	1 minute, 2000 volts
Focusing Period 3	13 minutes, 3000 volts
Mobilization Phase	19 minutes, 3000 volts
Mobilization flow rate	0.16 uL/min
Absorbance trigger	0.10 AU (2020 pixels)
Auto collection	-150 seconds

Fraction	Collection Time (sec)	Interval Post Collection (sec)
1	55	1
2	35	40
3	45	1
4	45	45
5	35	10
6	35	15
7	35	15
8	35	20
9	45	---



Fraction confirmation



Collected Material

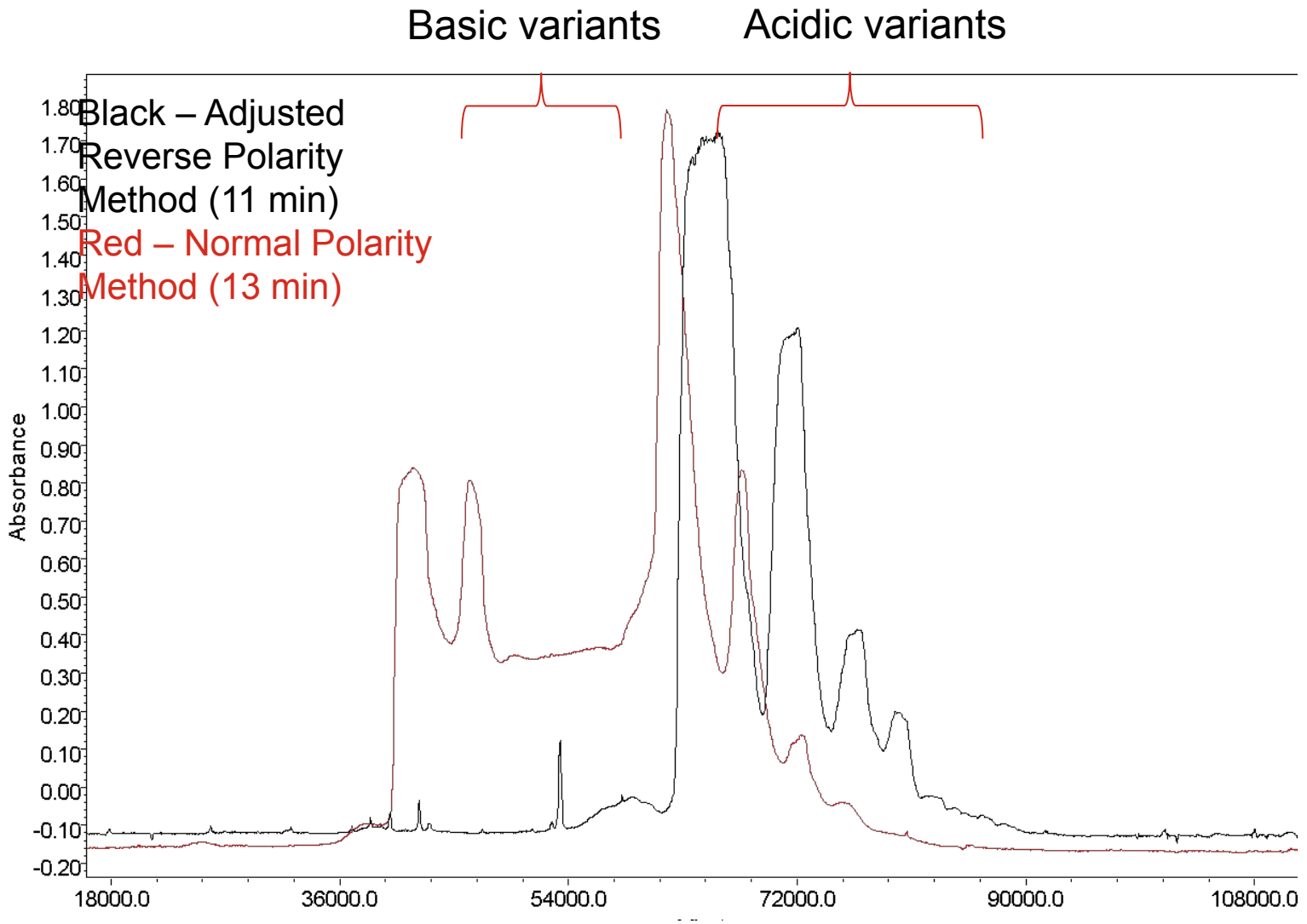
- Fractions collected over a course of 4 days, totaling 114 injections
- Fractions were buffer exchanged using centrifugal concentrators to remove additives and increase concentration
- DropSense was used to obtain the concentration for each fraction
- Verified identity and purity by analyzing the fractions using the molecules analytical icIEF method

Fraction	Variant	Purity (%)	Concentration (mg/mL)	Vol collected (uL)
1	BV1	66.7	0.02	55
2	MP	52.1	0.02	65
3	MP	79.0	0.03	60
4	MP (SH)	90.8 (SH 30.8)	0.18	127
5	MP (SH)	81.0 (SH 22.5)	0.66	140
6	AV1	51.5	0.34	130
7	AV1	71.4	0.36	115
8	AV1	58.8	0.19	107
9	AV2	62.9	0.1	145

Note: volume collected is remaining material after concentration and icIEF analysis

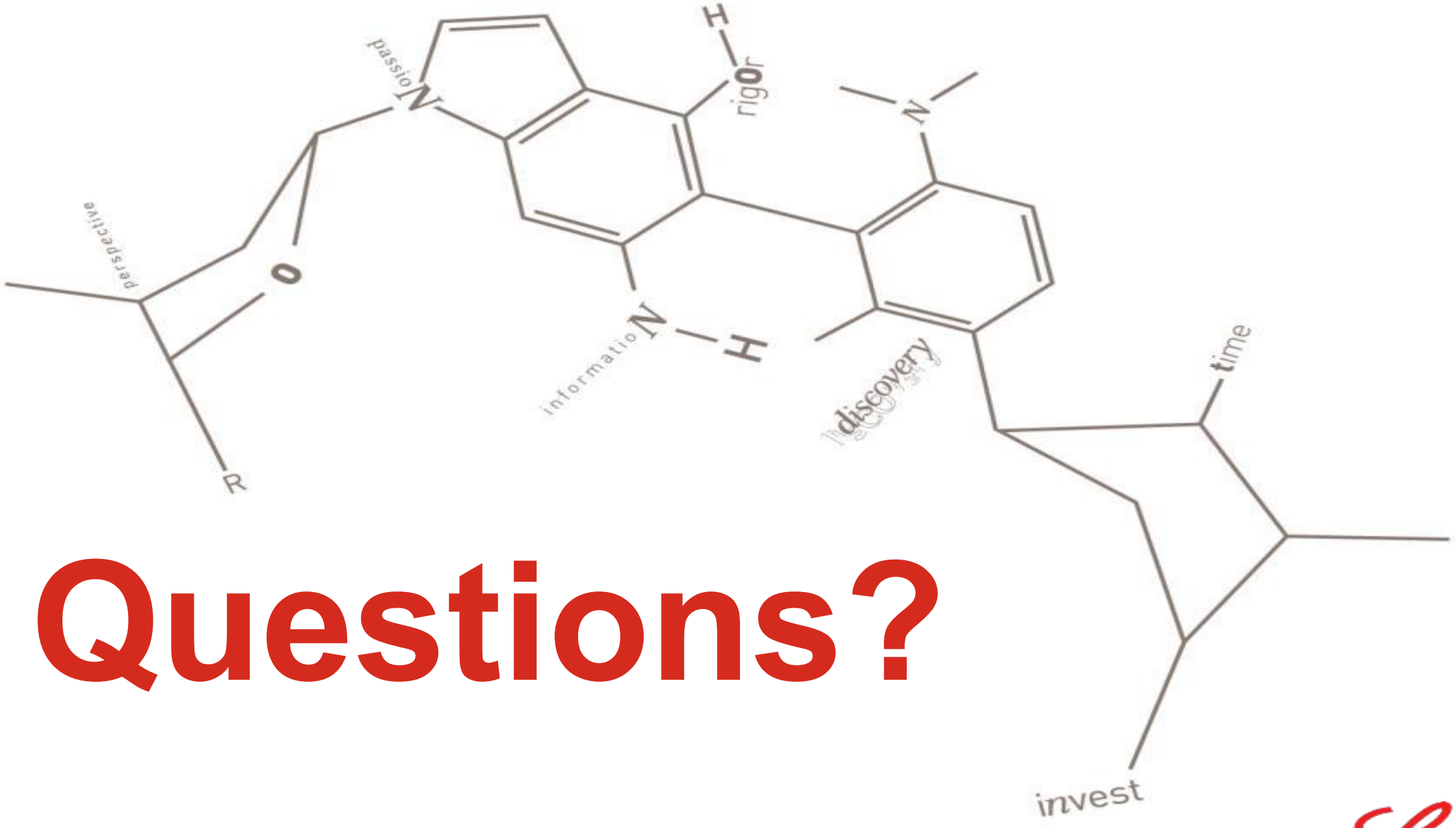
Transition to Reverse polarity (1 Day)

- Allows acidic variants to elute first
  - Increases purity of acidic variants
  - AV3 could not be collected >60% purity using normal polarity – blending of AV2
- For this molecule the ampholytes were adjusted for reverse polarity
  - 1% pharmalyte 5-8
  - 2% AES HR 7-9
  - **2% AES HR 8-10.5 (AES SH 6-9)**
- Typically a longer run time – working against electroosmotic flow
  - Mobilization flow is roughly double of what is used for normal polarity



Conclusion

- The full method development process can be completed in 1-2 weeks
- After 4 days of collection, enough material was collected to support ID testing of MP, AV1 and potentially the shoulder peak
  - BV1 (2.7%) would take ~ 3 Months to collect enough material for ID
  - Bioassay requests ~150 ug of material for potency
    - MP and AV1 potency material can be collected in 1-2 months
- This projects collection was put on pause, but reverse polarity showed potential for collecting the lower level acidic variants
  - The transition of a normal to reverse polarity method can be completed in a day



Questions?

Lilly