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Department of Chemical and Biomedical Engineering,
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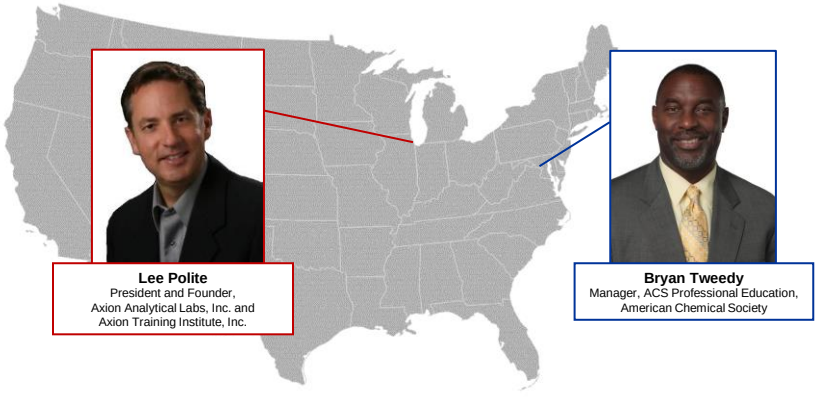

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President and Founder,
Axion Analytical Labs, Inc. and
Axion Training Institute, Inc.

Bryan Tweedy
Manager, ACS Professional Education,
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President and Founder
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HPLC: The World's Most Powerful Separation Tool

- HPLC works by separating complex mixtures into pure compounds
- Why do we separate?
- **We separate in order to:**
  - Identify – What is present in the sample
  - Quantify – How much is present in the sample
  - Purify – Isolate a compound from the sample
- But step one is always to separate!
- Most people expect HPLC separations to be really complicated, but there are only 3 parameters that affect the separation!
- **And here's the best part**: YOU are in charge of those 3 parameters, so YOU are in charge of the separation.
- **So let's take a closer look** at these 3 parameters and how to set them properly.

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HPLC Master Resolution Equation

$$R_s = \left(\frac{k}{1+k}\right) x \left(\frac{\alpha - 1}{\alpha}\right) x \frac{\sqrt{N}}{4}$$

Resolution	Capacity / Retention Factor	Selectivity	Efficiency ("Peak Skinniness")
R>1.50	1 < k < 5	α > 1.2	Avg ~ 10,000 Max ~ 28,000
	$k = (t_r - t_0)/t_0$	$\alpha = k_B/k_A$	$N = 5.545 \times \left(\frac{t_r}{W_h}\right)^2$
	Weaken Mobile Phase ↑%H2O by 10% Increases k 2-3 fold	Function: Mobile Phase Stationary Phase pH, Temp, buffer, additive, etc.	↑ Column Length (Lc) ↓ Particle Diameter (D_p) Optimize Flow Rate (μ) Min. Extra Col. Volume

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Audience Survey Question

ANSWER THE QUESTION ON BLUE SCREEN IN ONE MOMENT

What is the definition of good resolution? It should be greater than or equal to:

- 0.50
- 0.70
- 1.00
- 1.50
- Any of these values

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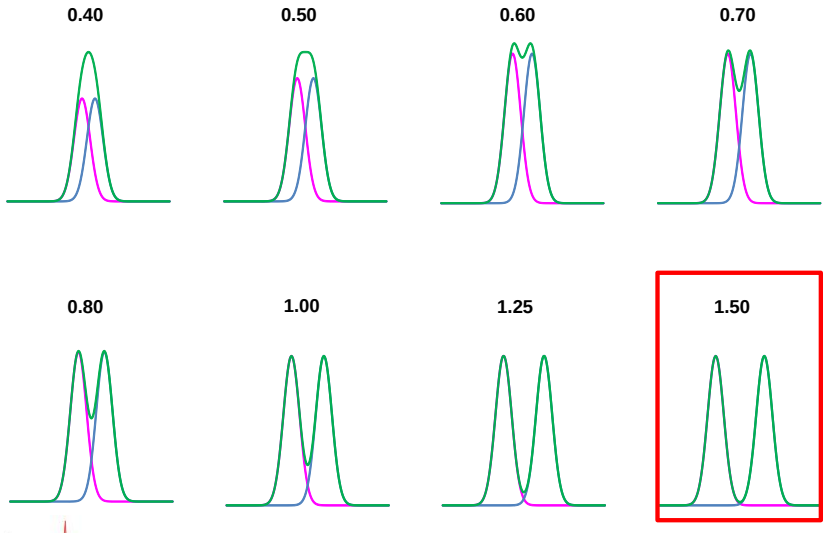
Different Resolution Values



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Method Development Step 1: Maximize Efficiency

$$R_s = \left(\frac{k}{1+k}\right) \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \frac{\sqrt{N}}{4}$$

Capacity **Selectivity** **Efficiency**

- Start with the highest efficiency column that you can buy.
- Try a 15 cm with 3.5 um particles (~20,000 plates) or
- 10 cm with 1.8 um particles (~28,000 plates) – Requires high pressure
- Note: During method optimization, we may opt for a shorter column.
- Column length is proportional to the efficiency.

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Method Development Step 2: Find the Correct Selectivity

$$R_s = \left(\frac{k}{1+k}\right) \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \frac{\sqrt{N}}{4}$$

Capacity | **Selectivity** | **Efficiency**

- Choose reversed phase because…
- Approximately 80% of all HPLC separations are carried out in the reversed phase mode!
- Acetonitrile or methanol blended with water on a good C18 column

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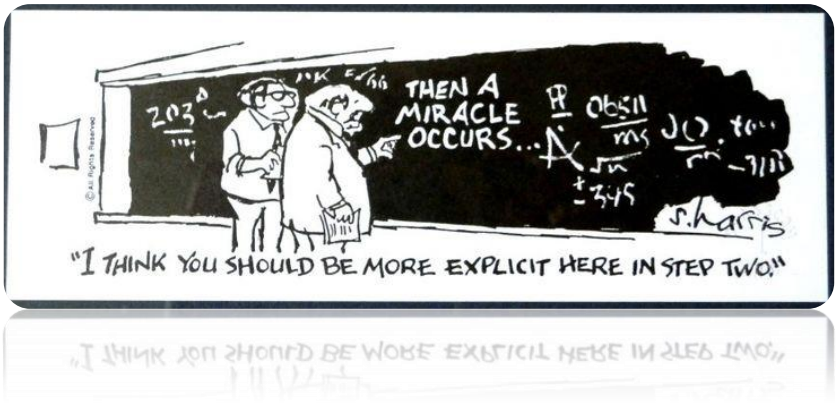

Choose Reversed Phase Because…
It Just seems to work for most applications!

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Audience Survey Question

ANSWER THE QUESTION ON BLUE SCREEN IN ONE MOMENT

Why do we usually choose reversed phase?
(multiple answers may exist)

- The mechanism seems to work for most separations
- It allows us to analyze polar compounds
- The solvents are less hazardous
- To impress my friends at the next cocktail party

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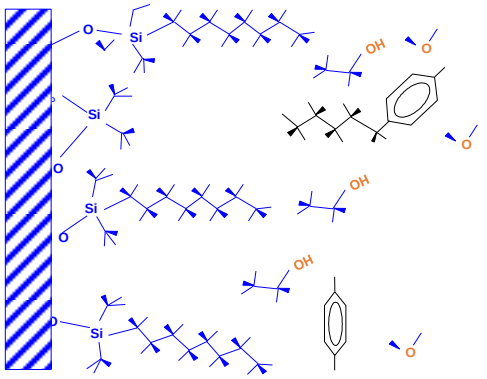
Reversed-Phase Mechanism

- **The analytes partition between the non-polar stationary phase and the polar mobile phase.**
- **Relative affinity means there are two dimensions to the separation.**
- **Reversed phase is especially sensitive to minor differences in hydrophobicity.**
- **The addition or subtraction of just about any group leads to hydrophobicity changes: methyl, hydroxyl, amino, carbonyl, acid, etc.**



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When to Choose Reversed Phase

- **Neutral, polar and nonpolar compounds with a molecular weight less than ~2000**
- **Homologous series**
- **Organic acids and bases**
- **Proteins and peptides**

More Challenging to do by reversed phase

- **Extremely polar compounds**
- **Extremely non-polar compounds**

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Method Development Step 3: Optimize Capacity Factor

$$R_s = \left(\frac{k}{1+k}\right) \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \frac{\sqrt{N}}{4}$$

Capacity Factor | **Selectivity** | **Efficiency**

- How do you find the correct mobile phase strength?
- Try all of the strengths... and see where you peaks elute!
- Scouting Run: Gradient from weakest to strongest mobile phase
- Listen to your sample. The peaks will elute at their desired %B
- There are 3 simple rules for finding the correct mobile phase

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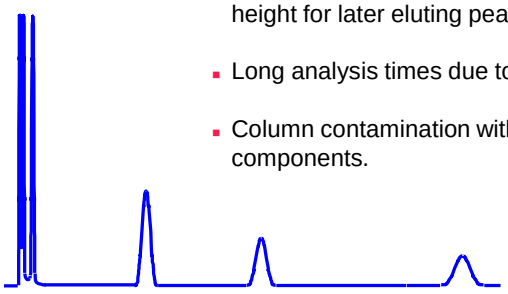
Isocratic Elution Example

Isocratic 50/50 Water/Methanol



- Poor resolution of early eluting peaks.
- Increase in peak width and decrease in peak height for later eluting peaks.
- Long analysis times due to a wide range in k'.
- Column contamination with strongly retained components.

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Audience Survey Question

ANSWER THE QUESTION ON BLUE SCREEN IN ONE MOMENT

The first two peaks are coming off together near the void volume (low capacity factor). What should we do to the mobile phase in order to improve the separation?

- Make the mobile phase stronger
- Make the mobile phase weaker
- Slow down the flow rate
- Change the detector lamp

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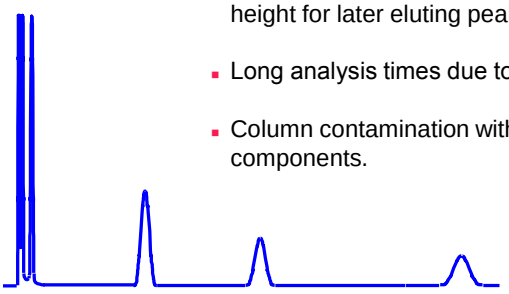
Isocratic Elution Example

Isocratic 50/50 Water/Methanol



- Poor resolution of early eluting peaks.
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- Long analysis times due to a wide range in k'.
- Column contamination with strongly retained components.

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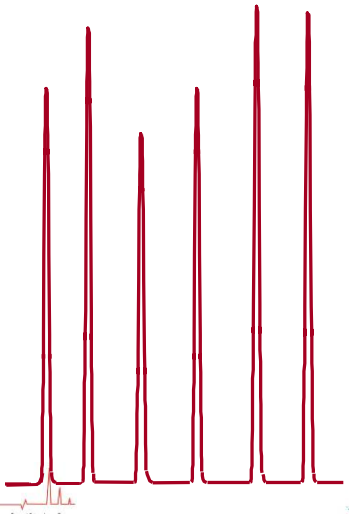
Gradient Elution

Gradient from 10-100% Methanol



Gradient Elution - Mobile phase composition is changed (strengthened) during the separation.

Advantages

- Improved *overall* resolution
- Increased detection
- Ability to separate complex samples
- Shorter analysis times
- Decrease in column deterioration due to strongly retained components

Other Uses

- Column Cleaning
- Scouting run in method development

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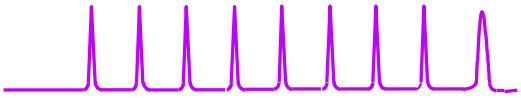
3 Simple Gradient Rules



10% Organic

100% Organic

3 Important Rules for Setting Gradient Parameters

1. Initial Composition – Must be weak enough to give the first peak a k' of at least 1.
2. Final Composition – Must be strong enough to elute the last peak from the column.
3. Gradient Steepness – The shallower the gradient, the more resolution, but longer time.

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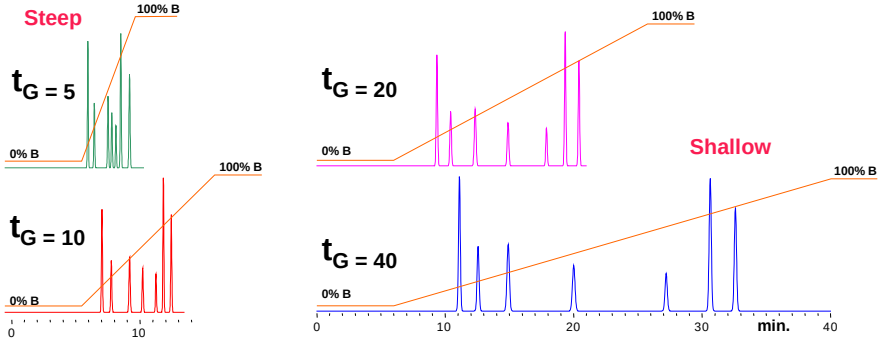
Select Gradient Steepness



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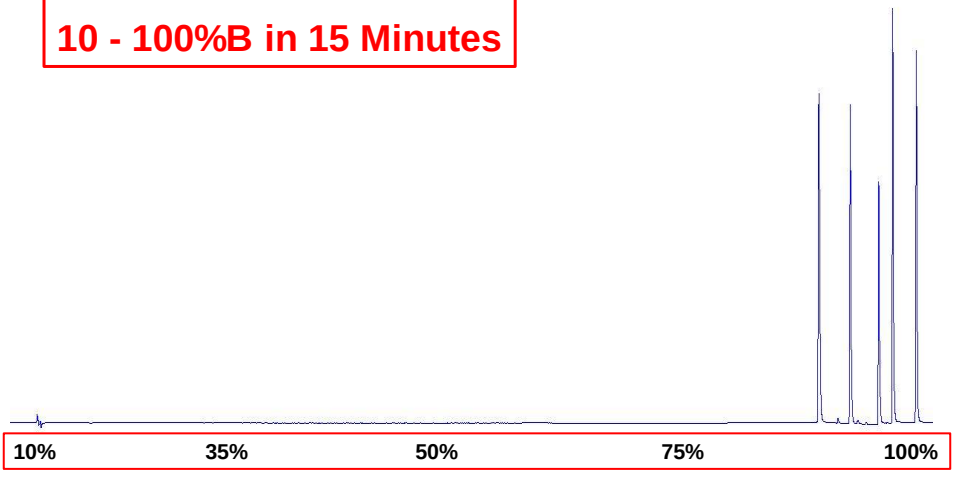
Unknown Sample #1
Gradient Scouting Run

10 - 100%B in 15 Minutes



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%B (Acetonitrile or Methanol)

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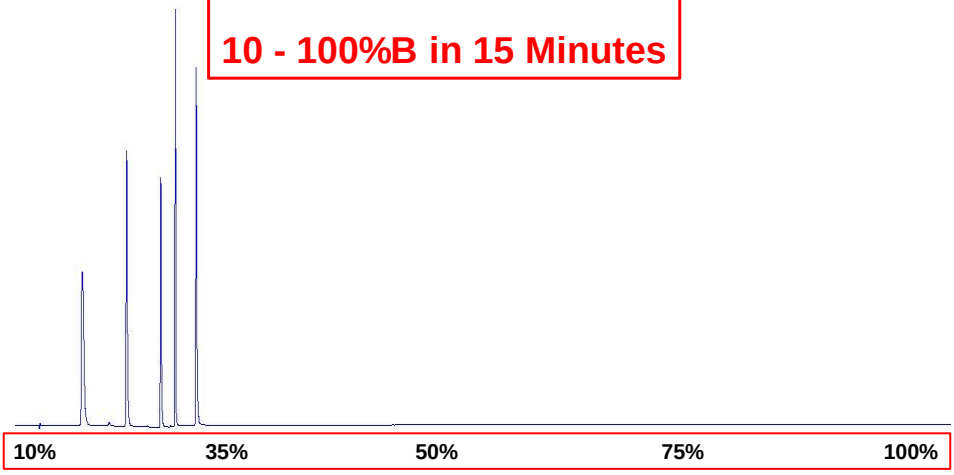
Unknown Sample #2
Gradient Scouting Run

10 - 100%B in 15 Minutes



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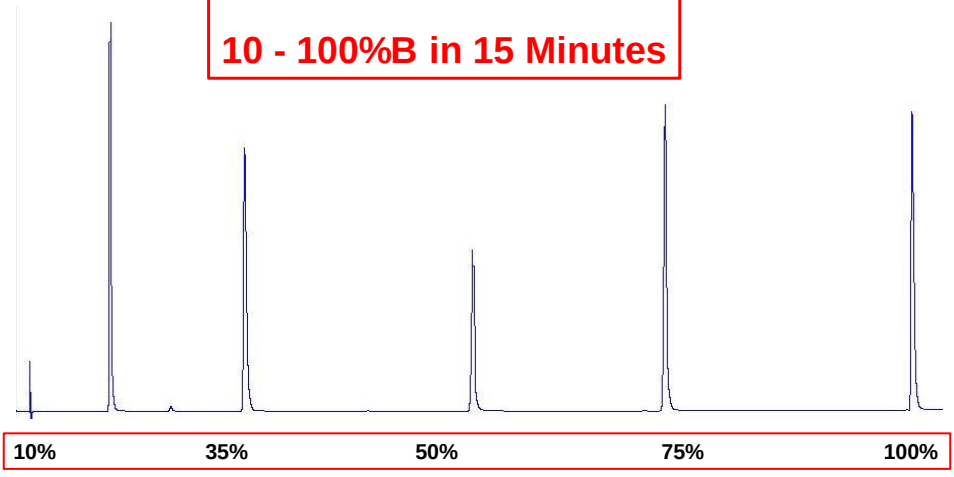
%B (Acetonitrile or Methanol)

32

Unknown Sample #3
Gradient Scouting Run

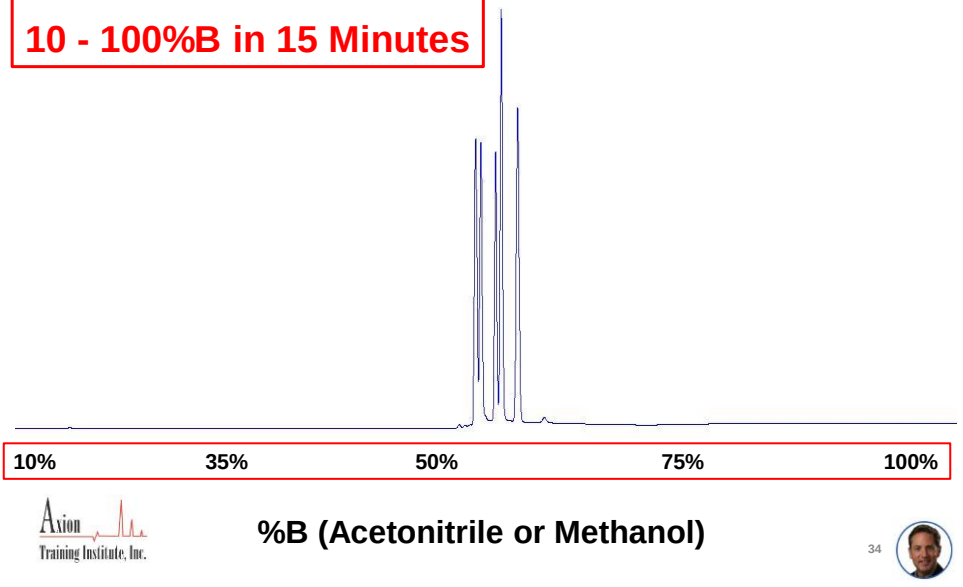
10 - 100%B in 15 Minutes



%B (Acetonitrile or Methanol)

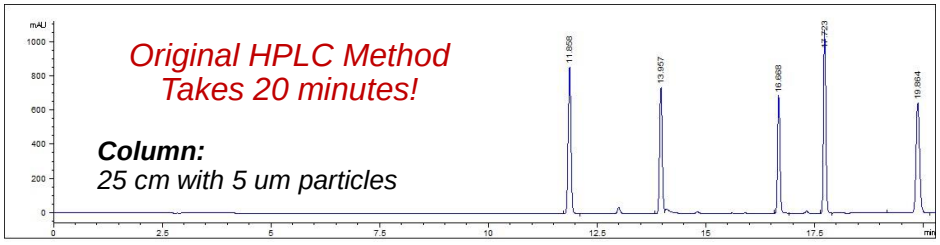
Unknown Sample #4
Gradient Scouting Run

10 - 100%B in 15 Minutes



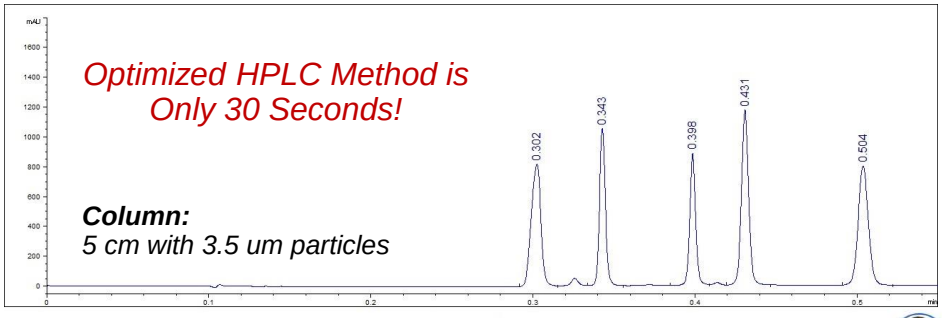
%B (Acetonitrile or Methanol)

After Method Development, Use the Resolution Equation to Cut Analysis Time



Original HPLC Method Takes 20 minutes!

Column:
25 cm with 5 um particles



Optimized HPLC Method is Only 30 Seconds!

Column:
5 cm with 3.5 um particles

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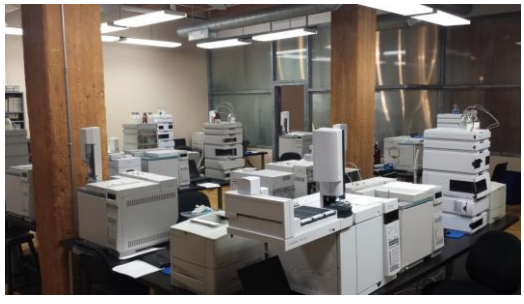

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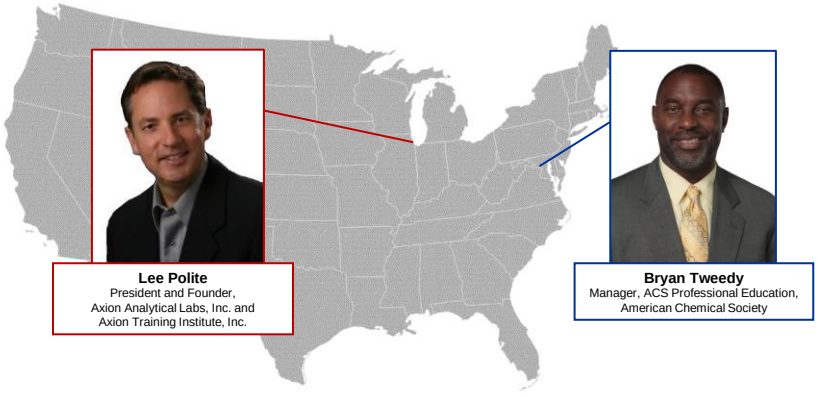

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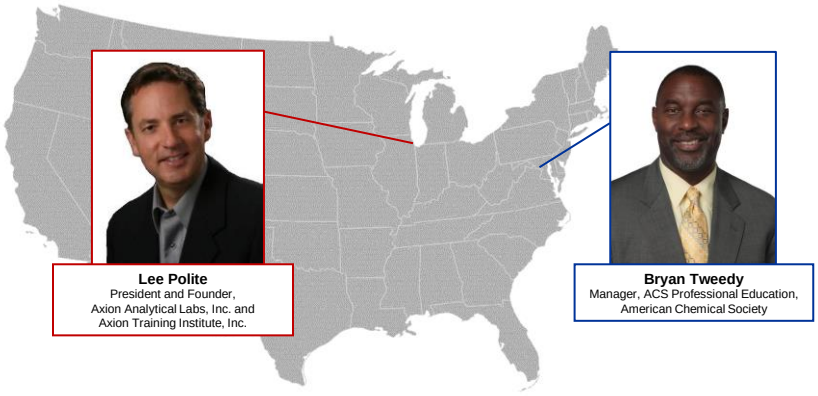

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Fan of the Week

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Department of Chemical and Biomedical Engineering,
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