

# AN EFFECTIVE METHOD DEVELOPMENT STRATEGY USING FUSED-CORE® COLUMNS

**Thomas J Waeghe**<sup>1</sup>, Stephanie Schuster<sup>2</sup>, and Conner McHale<sup>2</sup>

<sup>1</sup>MAC-MOD Analytical, Inc.  
Chadds Ford, PA 19317

<sup>2</sup>Advanced Materials Technology, Inc.  
Wilmington, DE 19810



# ABSTRACT

**The development of a stability-indicating method (related substances method) can be among the most challenging activities in support of pharmaceutical or other UHPLC method development for complex samples. The objective of such work is to develop a robust and rugged separation of all of the impurities and degradants from a drug substance or formulation, or the analytes from a multi-component mixture. To discover that your stability-indicating method does not separate all of the known and unknown impurities at a later stage can seriously affect product registration.**

**A sensible approach for LC separation development is to screen various selectivity parameters up front, when careful selection of the best combination of stationary phase, organic modifier, mobile phase pH, temperature and other parameters can be made.**

**In this presentation we will show examples of how such a method development strategy can be applied using Fused-Core stationary phase selectivities with samples such as a degraded pharmaceutical active ingredient and a double-blind-prepared mixture of acidic, basic and neutral pharmaceuticals.**

# OUTLINE

- **Factors That Affect Selectivity in RPLC**
  - Relative impact of various parameters
- **Review of method development strategies**
  - Expected or actual sample complexity
  - Method performance requirements
    - Assay method vs. impurity profile/related substances method
  - What should performance criteria be?
- **Screening approach example**
  - Blind sample, unknown number of components
  - Gradient screening: stationary phase, organic modifier, pH
- **Degraded pharmaceutical sample**
  - Screening results for phases and organic modifier at single pH
- **Summary**

# WHICH FACTORS<sup>1</sup> AFFECT SELECTIVITY MOST ?

MOST  
Influence

## Isocratic Separations

- Column Stationary Phase
- Organic modifier
- Mobile phase pH  
(for ionised analytes only)
- % Organic modifier
- Column temperature
- Buffer choice
- Buffer concentration
- Additive concentration



LEAST  
Influence

## Gradient Separations

- All parameters for isocratic
- Gradient steepness
- $k^*$  (that is,  $t_G$ ,  $F$ ,  $\Delta\Phi$ ,  $V_M$ ,  $MW$ )

$$k^* = \frac{85 \times t_G \times F}{\Delta\Phi \times V_M \times S}$$

- Instrument delay volume

# POWER OF CHANGING MULTIPLE PARAMETERS

## Relative Impact of Different Changes in RPLC Parameters on Selectivity<sup>1</sup>

| Selectivity Parameter   | Change in Parameter                                     | Maximum $ \delta \log \alpha $ |
|-------------------------|---|--------------------------------|
| pH                      | 5 pH units  | 0.70                           |
| Organic modifier choice | CH <sub>3</sub> CN $\leftrightarrow$ CH <sub>3</sub> OH | 0.20                           |
| Gradient time           | 10-fold   | 0.20                           |
| Orthogonal column       | $\Delta F_s \sim 65$                                    | 0.19                           |
| % Organic modifier      | 10% (v/v)   | 0.08                           |
| Column temperature      | 20 °C   | 0.07                           |
| Buffer concentration    | 2-fold  | 0.02                           |

Change of only 0.10 needed to go from co-elution to baseline resolution

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

For  $\Delta R_s = 1.5$ ,  $N = 10,000$  and  $k \geq 1$

$(\alpha - 1) = 0.12$  and  $\alpha = 1.12$

$\log \alpha = 0.05$  and Snyder proposed  $|\delta \log \alpha|_{\text{avg}} \geq 0.10$

For a change in both column phase and organic modifier, the expected change is magnified

$$|\delta \log \alpha|_{\text{avg}} = [(0.20)^2 + (0.19)^2]^{0.5} = 0.28$$

Use of different column phases, organic modifiers, pHs, and temperatures can be powerful in changing  $\alpha$  and  $R_s$

<sup>1</sup> Adapted from Snyder et al., "Orthogonal" separations for reversed-phase liquid chromatography, Journal of Chromatography A, 1101 (2006) 122–135

# HALO PHASES FOR REVERSED-PHASE HPLC AND UHPLC

| Packing Description | Bonded Phase                                | Types of Interactions  |
|---------------------|---|--|
| <b>C18</b>          | C18<br>(dimethyloctadecylsilane)            | <ul style="list-style-type: none"> <li>• Hydrophobic</li> </ul>  |
| <b>C8</b>           | C8<br>(dimethyloctylsilane)                 | <ul style="list-style-type: none"> <li>• Hydrophobic</li> </ul>  |
| <b>Phenyl-Hexyl</b> | Phenyl-Hexyl<br>(dimethylphenylhexylsilane) | <ul style="list-style-type: none"> <li>• Hydrophobic</li> <li>• <math>\pi - \pi</math></li> </ul>  |
| <b>ES-CN</b>        | ES-CN<br>(di-isopropylcyanopropylsilane)    | <ul style="list-style-type: none"> <li>• Hydrophobic</li> <li>• Dipole-dipole</li> </ul>   |
| <b>PFP</b>          | PFP<br>(pentafluorophenylpropylsilane)      | <ul style="list-style-type: none"> <li>• Hydrophobic</li> <li>• <math>\pi - \pi</math></li> <li>• Dipole-dipole</li> <li>• Hydrogen bonding</li> </ul> |
| <b>RP-Amide</b>     | C16 Amide                                   | <ul style="list-style-type: none"> <li>• Hydrophobic</li> <li>• Hydrogen bonding</li> </ul>  |
| <b>AQ-C18</b>       | proprietary                                 | <ul style="list-style-type: none"> <li>• Hydrophobic</li> </ul>  |



# HALO PHASES USED IN THIS WORK

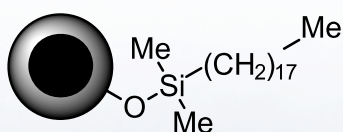
## HYDROPHOBIC SUBTRACTION MODEL PARAMETERS<sup>1</sup>

$$\log k = \eta'H - \sigma'S^* + \beta'A + \alpha'B + \kappa'C + \log k_{EB}$$

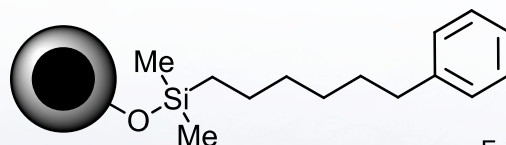
Dissimilarity vs. C18

| $F_s$ | Name                     | H    | $S^*$ | A     | B     | C (pH 2.8) | C (pH 7.0) | EB retention factor | USP type | Phase type |
|-------|--------------------------|------|-------|-------|-------|------------|------------|---------------------|----------|------------|
| 0.00  | <b>HALO C18</b>          | 1.10 | 0.04  | 0.00  | -0.05 | 0.05       | 0.04       | 6.10                | L1       | C18        |
| 17.35 | <b>HALO Phenyl-Hexyl</b> | 0.78 | -0.09 | -0.23 | 0.00  | 0.10       | 0.45       | 3.50                | L11      | Phenyl     |
| 22.78 | <b>HALO ES-CN</b>        | 0.57 | -0.11 | -0.34 | 0.02  | 0.13       | 1.15       | 1.88                | L10      | CN         |
| 52.83 | <b>HALO RP-Amide</b>     | 0.85 | 0.08  | -0.38 | 0.19  | -0.41      | 0.31       | 4.60                | L60      | EP         |
| 94.45 | <b>HALO PFP</b>          | 0.70 | -0.12 | -0.07 | -0.06 | 1.17       | 0.97       | 2.30                | L43      | F          |

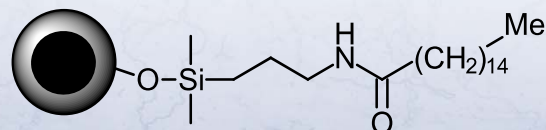
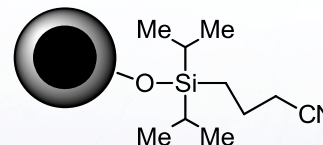
**HALO C18**



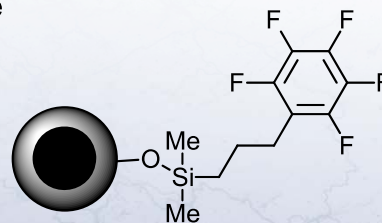
**HALO Phenyl-Hexyl**



**HALO ES-CN**



**HALO RP-Amide**



**HALO PFP**

<sup>1</sup> [www.hplccolumns.org](http://www.hplccolumns.org)

# APPROACH DICTATED BY SAMPLE COMPLEXITY

## Simple

- 1 stationary phase
- 1 organic modifier
- 1 pH
- Use broad gradient range
- Assess need for isocratic vs. gradient

$$\frac{\Delta t_R}{t_G} \leq 0.25, \text{ then isocratic}$$

$$\frac{\Delta t_R}{t_G} \geq 0.40, \text{ then gradient}$$

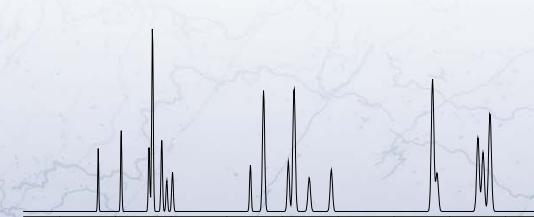
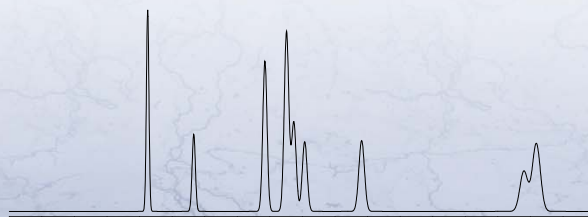
- Isocratic?
  - Identify isocratic conditions for evaluation
  - Compare %Bs at, above and below suspected or predicted conditions
- Gradient?
  - Optimize gradient slope and range
  - Assess impact of temperature/optimize

## Moderate Complexity

- 2 or more stationary phases
- 2 organic modifiers and blend
- 1 or more pHs
- Use broad gradient range to screen phases, organic modifiers, pHs
- Compare results based on peak performance

## Very Complex

- 2 or more stationary phases
- 2 organic modifiers and blend
- 2 or more pHs
- Use broad gradient range to screen phases, organic modifiers, pHs
- Compare results based on peak performance





# HOW SHOULD SCREENING RESULTS BE EVALUATED OR GRADED?

## Performance Objectives

- Best overall peak shape
- Highest # of peaks observed
- Highest limiting resolution
- Best overall average resolution
- Highest likelihood for improvement or optimization

## “Must Not” Haves

- Poor peak shape
- Significant peak bunching

# CONTRIVED COMPLEX, BLINDLY-PREPARED MIXTURE

## 13-20 COMPOUNDS: ACIDS, BASES AND NEUTRALS

### Strategy

- Screened four HALO phases
  - C18
  - Phenyl-Hexyl
  - ES-CN
  - RP-Amide
- Different organic modifiers
  - CH<sub>3</sub>CN, CH<sub>3</sub>OH
- Different pHs with LC-MS compatible buffers
  - pH 2.8, 3.8 (NH<sub>4</sub>COOH)
  - 4.8 and 5.8 (NH<sub>4</sub>OAc)
- Identify one or more possible combinations for further improvement/optimization

Columns: 3 x 50 mm, 2.7 µm

Flow Rate: 0.6 mL/min

Temperature: 30 °C

Gradient: 2–90% organic/buffer

Gradient Time: 10 min

Initial Hold: 1 min

Agilent 1200 binary 600 bar system

- Delay volume: 0.74 mL (from DryLab runs)
- Hold 1 min at %B initial x 0.6 mL/min = 0.6 mL
- Effective delay volume: 1.34 mL

|      |           |  |              |      |     |
|------|-----------|--|--------------|------|-----|
| Time | %B        |  | Phases       | 4    |     |
| 0    | 2         |  | Modifiers    | 2    |     |
| 1    | 2         |  | pHs          | 4    |     |
| 11   | 90        |  | # injections | 2    |     |
| 12   | 90        |  | Total Runs   | 64   |     |
| 12.5 | 2         |  |              |      |     |
| 5    | Post Time |  |              |      |     |
| 17.5 | min       |  | Total Time   | 1120 | min |
|      |           |  | Total hrs    | 18.7 | hr  |

# HOW SHOULD EXPERIMENTAL RESULTS BE EVALUATED OR GRADED?

## 1<sup>st</sup> Approach

|  |   |
|--|---|
| <b>C18</b><br>CH <sub>3</sub> CN<br>pH 2.8   | <b>Phenyl-Hexyl</b><br>CH <sub>3</sub> CN<br>pH 2.8 |
| <b>ES-CN</b><br>CH <sub>3</sub> CN<br>pH 2.8 | <b>RP-Amide</b><br>CH <sub>3</sub> CN<br>pH 2.8     |

And so on for CH<sub>3</sub>OH and other pHs

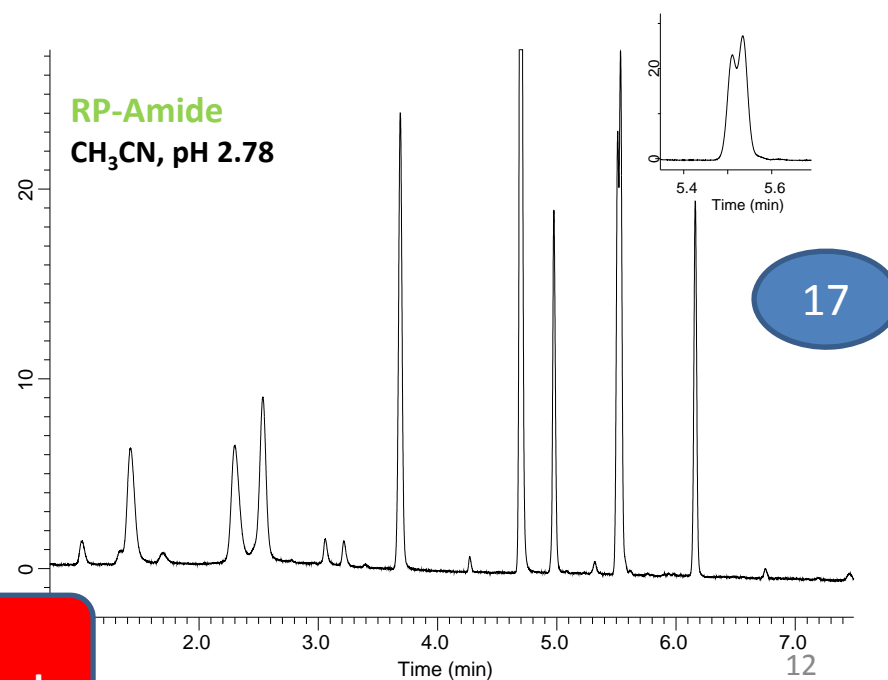
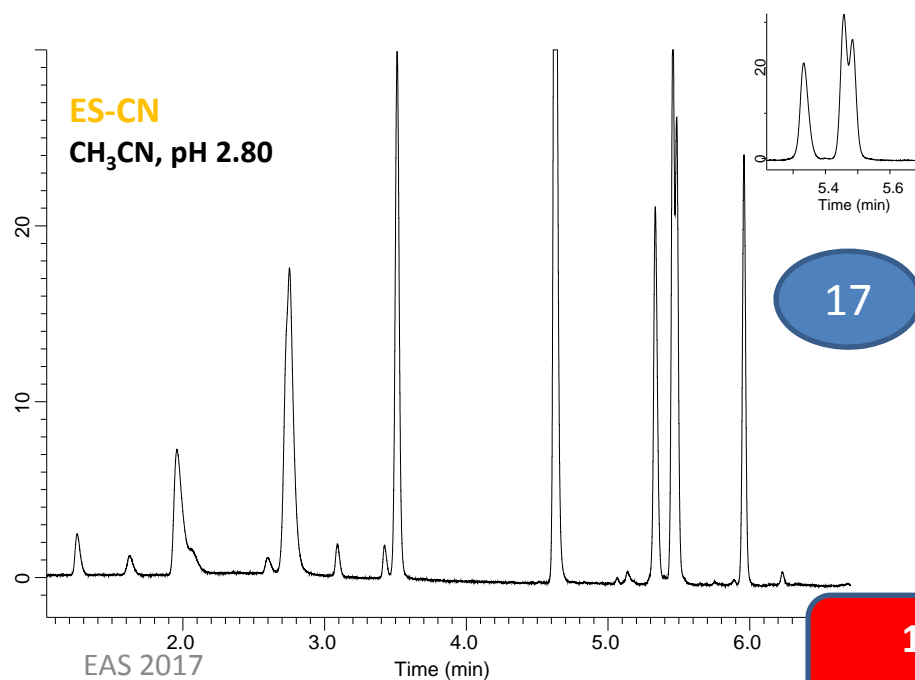
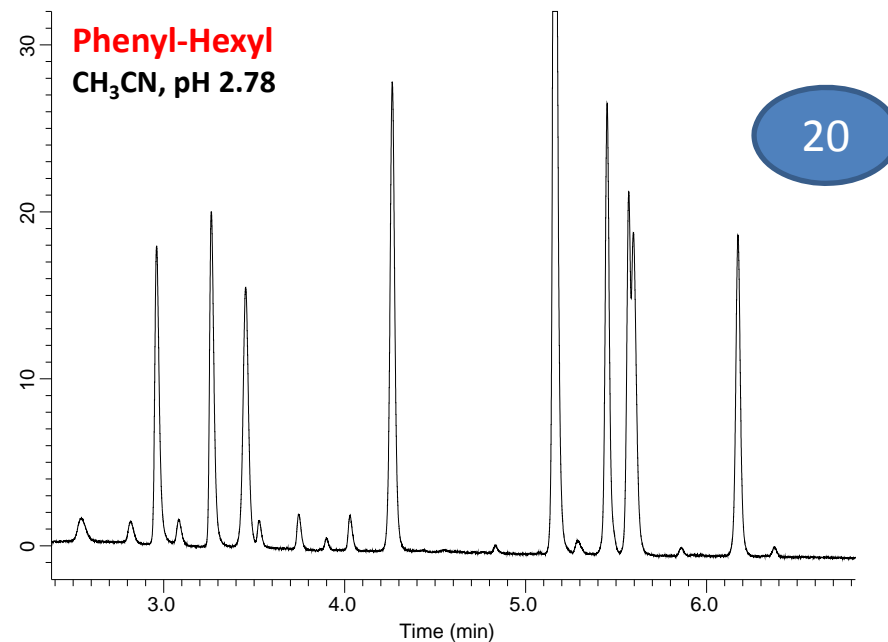
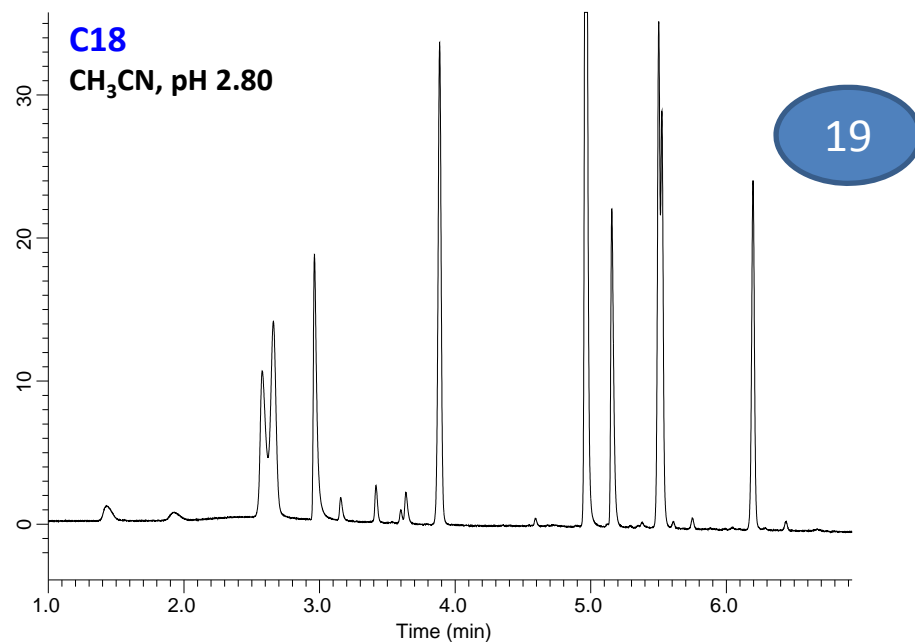
**Compare different phases with each modifier at the same pH**

## 2nd Approach

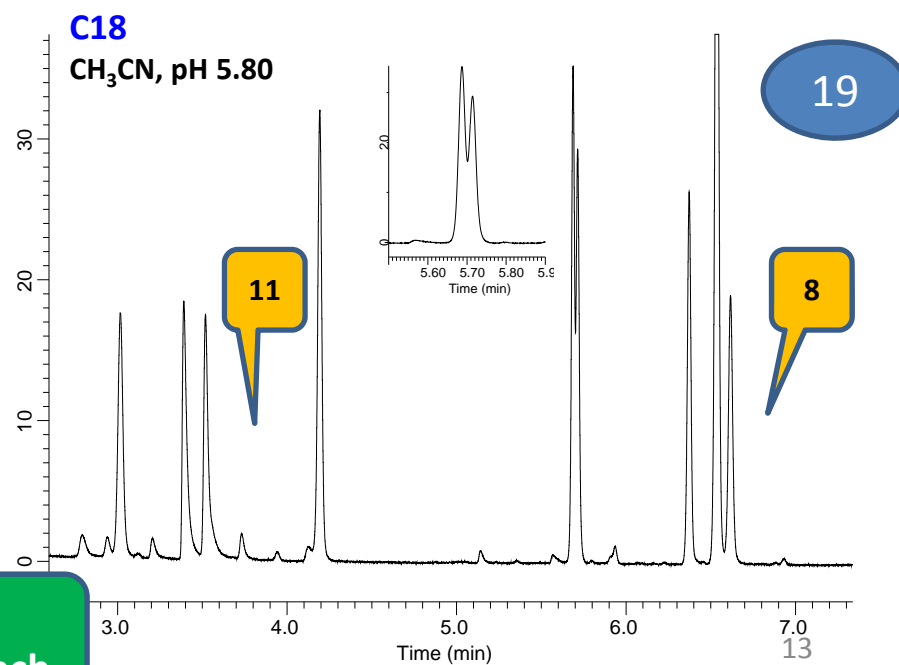
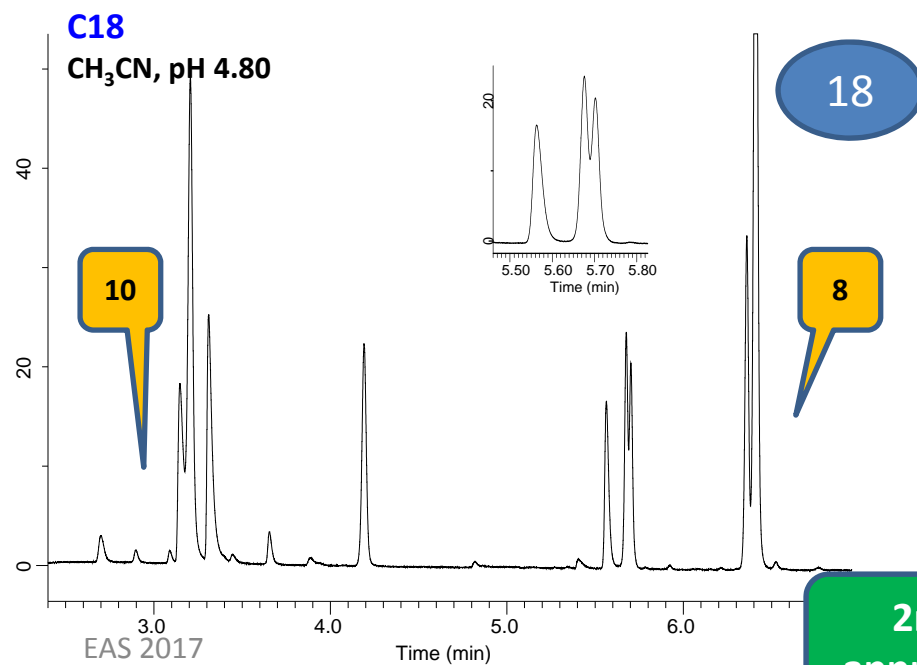
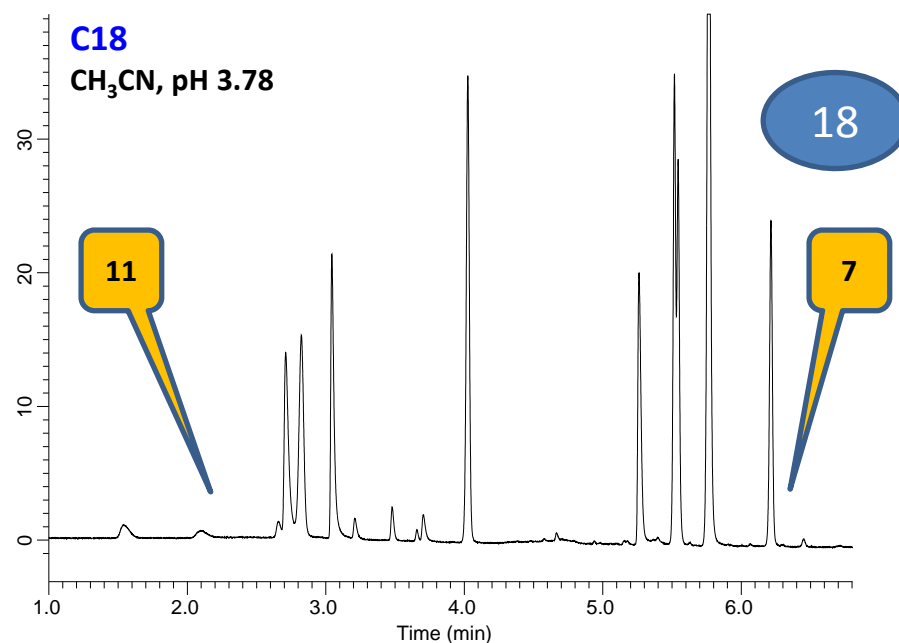
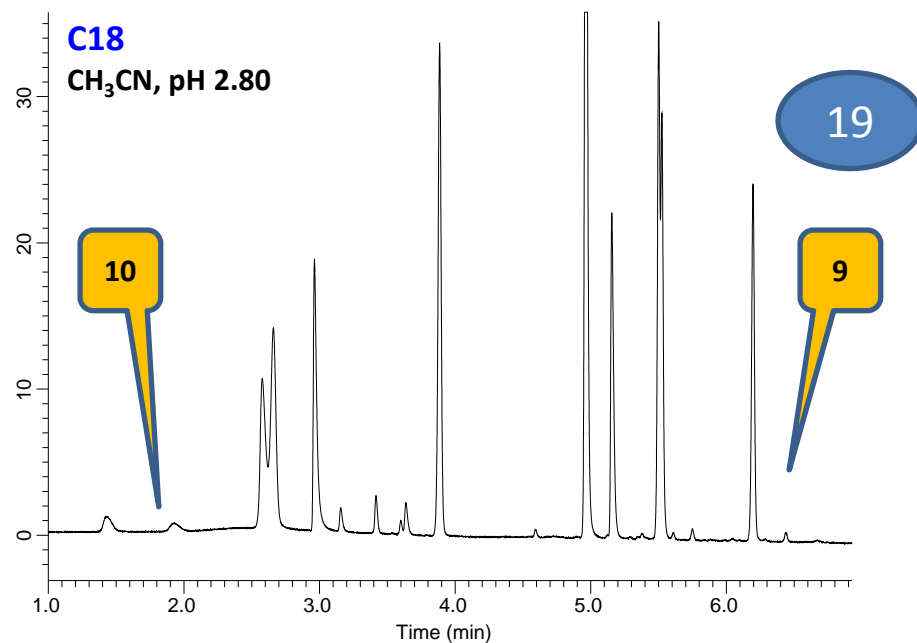
|  |  |
|--|--|
| <b>C18</b><br>CH <sub>3</sub> CN<br>pH 2.8 | <b>C18</b><br>CH <sub>3</sub> CN<br>pH 3.8 |
| <b>C18</b><br>CH <sub>3</sub> CN<br>pH 4.8 | <b>C18</b><br>CH <sub>3</sub> CN<br>pH 5.8 |

And so on for CH<sub>3</sub>OH and other phases

**Compare different pHs for same phase with each modifier separately**

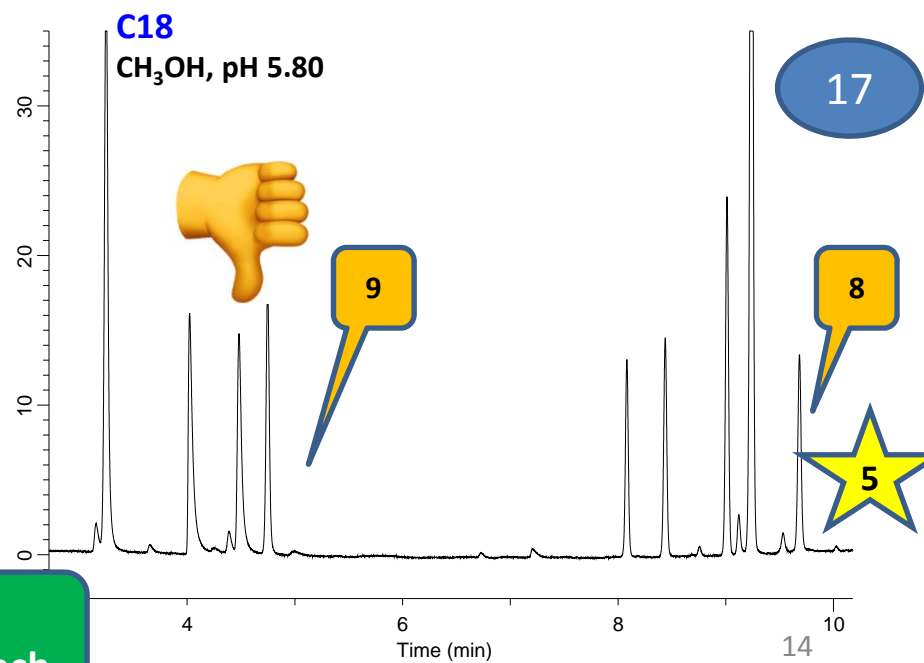
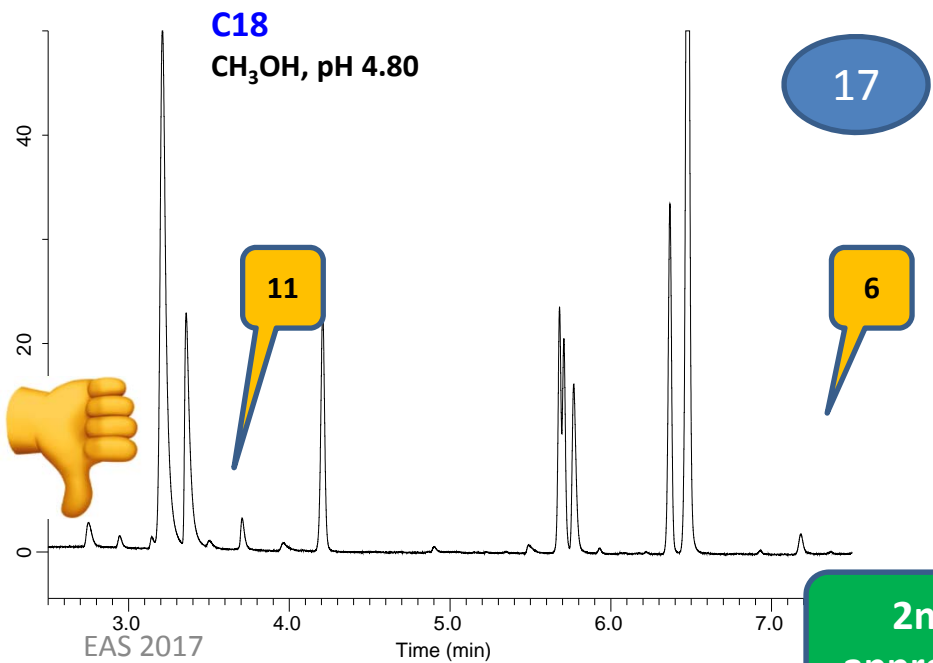
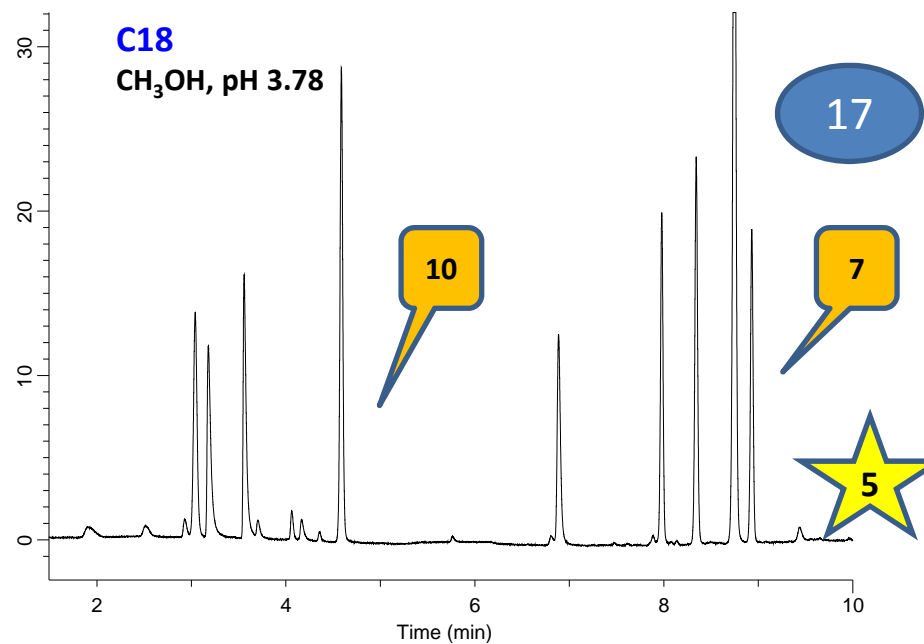
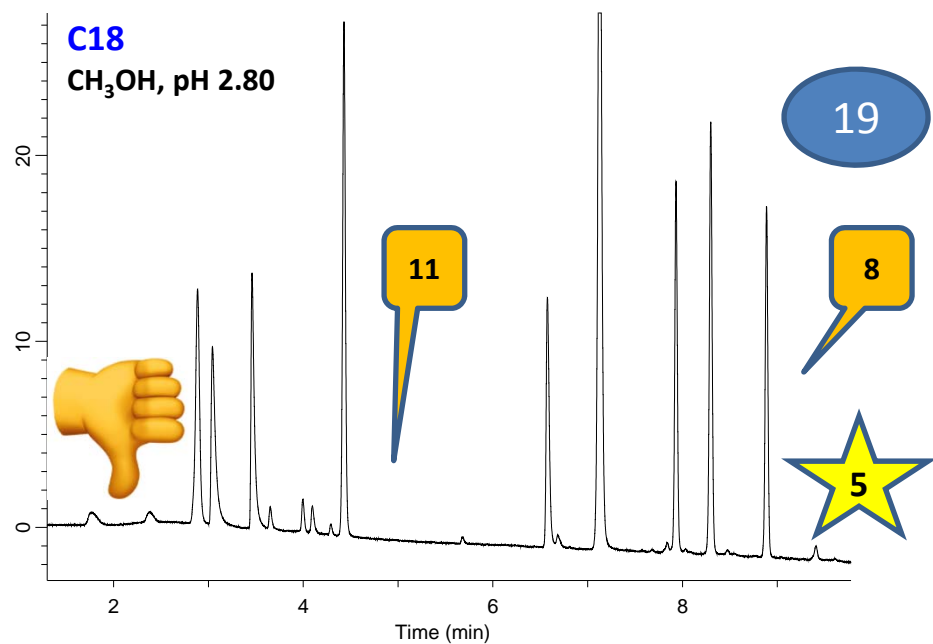


1st  
approach

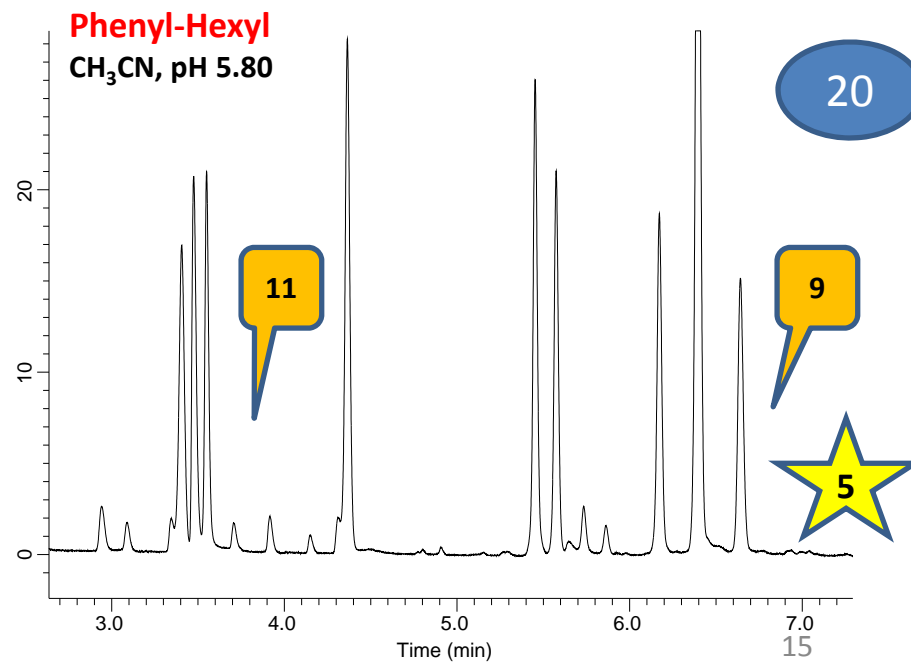
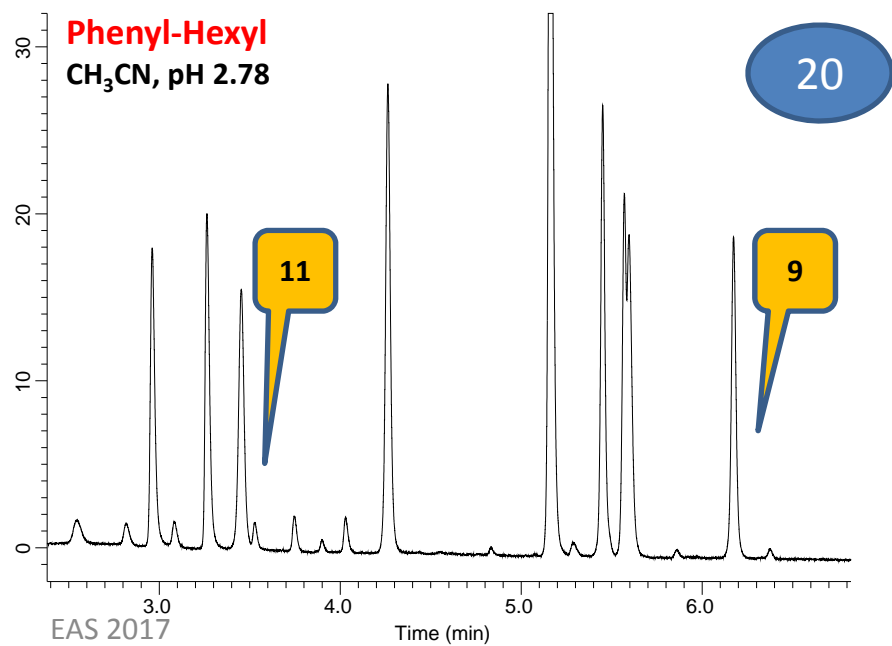
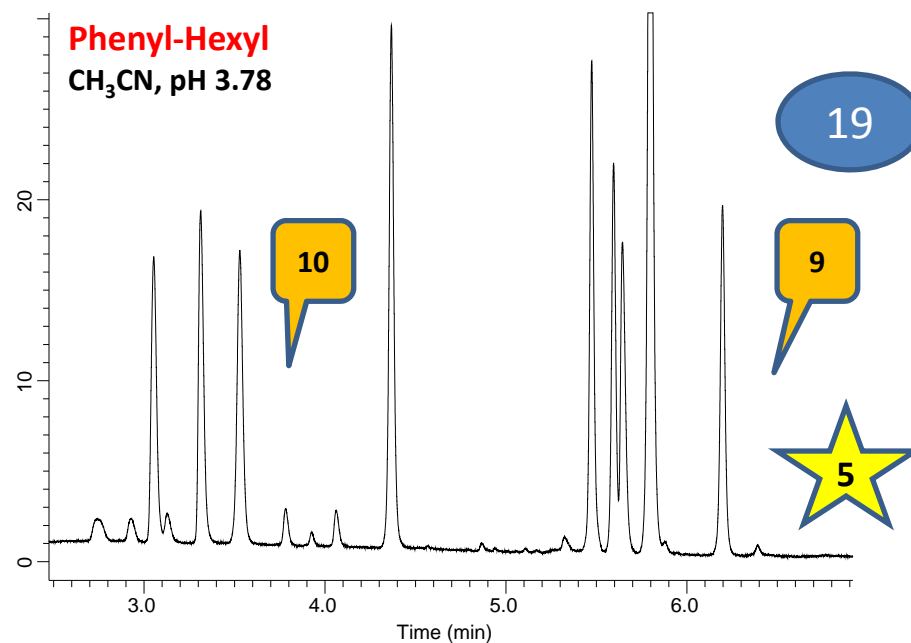
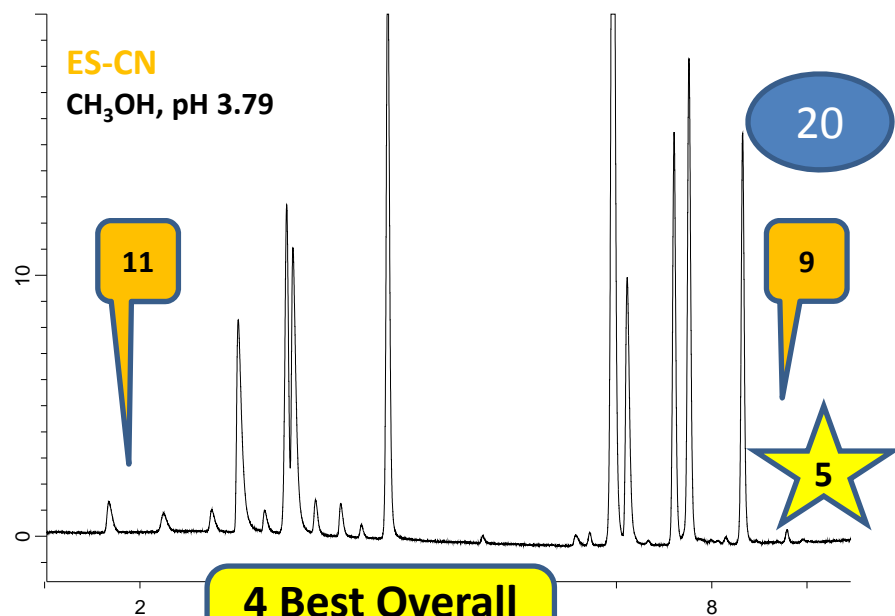


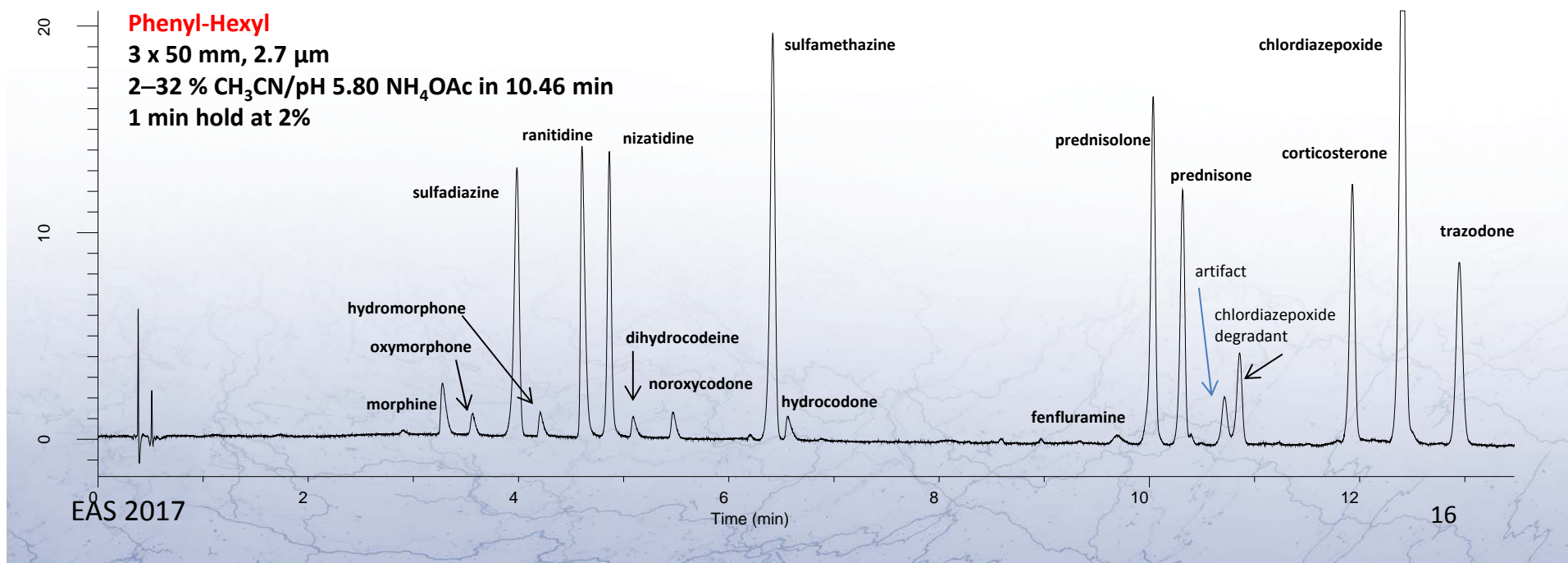
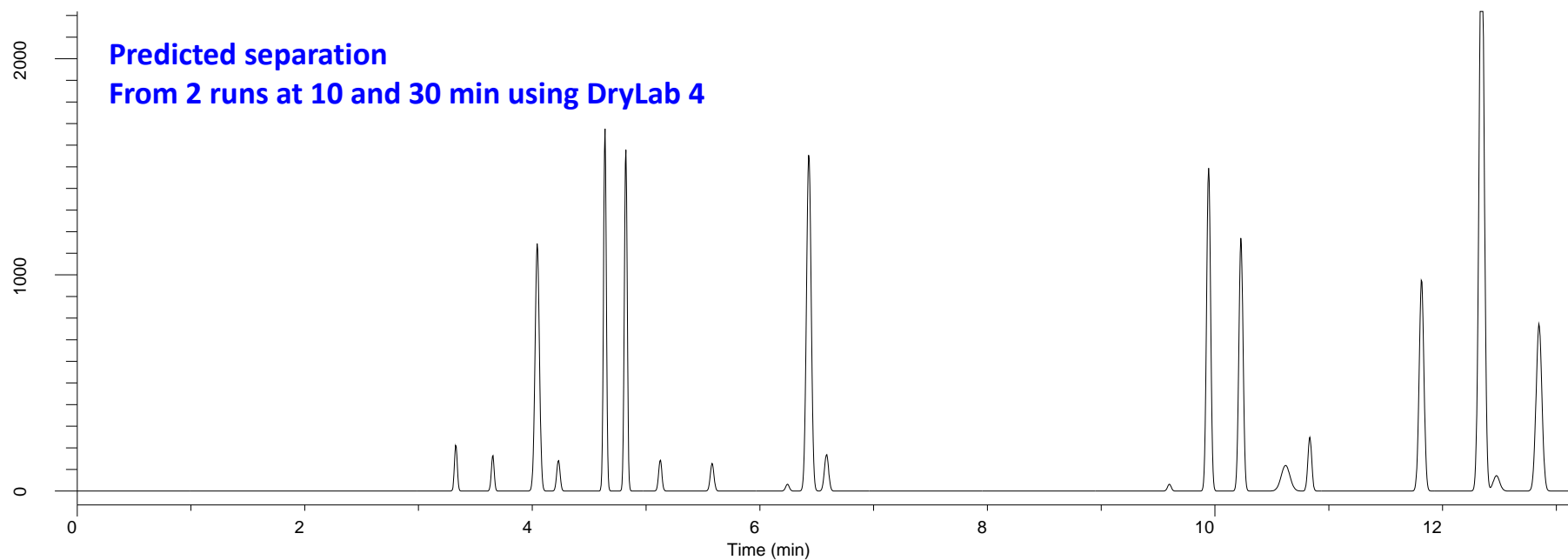
2nd  
approach





2nd  
approach



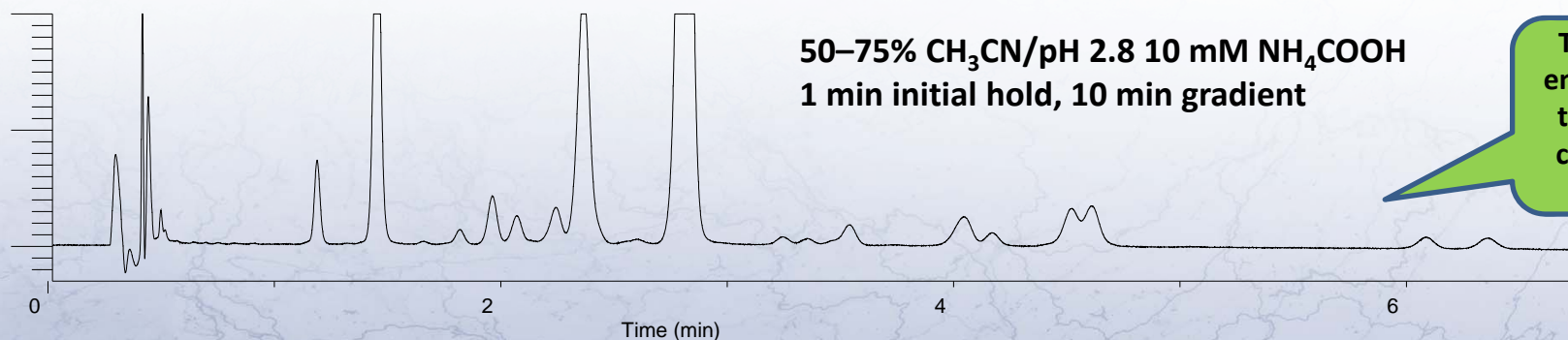
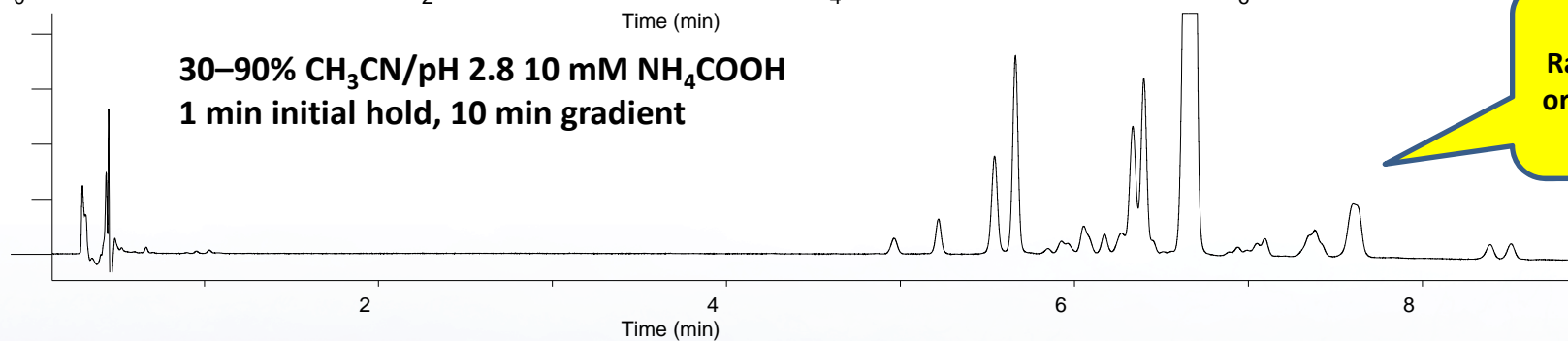
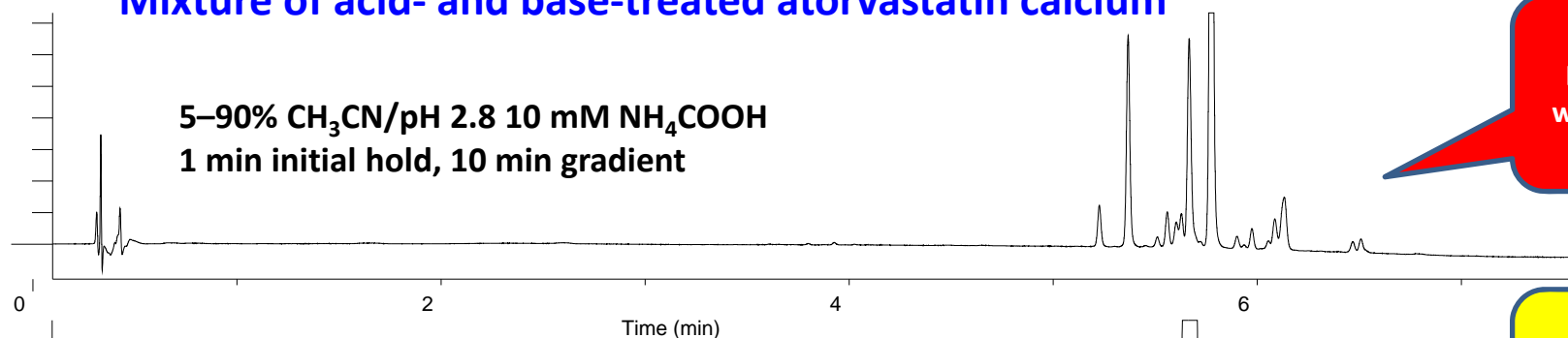


# APPLICATION OF MULTIPLE PHASES FOR STABILITY INDICATING METHOD DEVELOPMENT

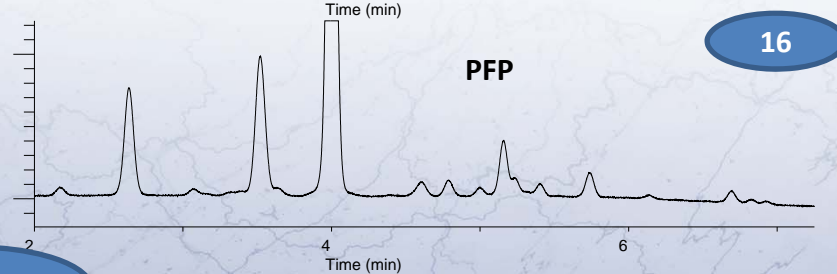
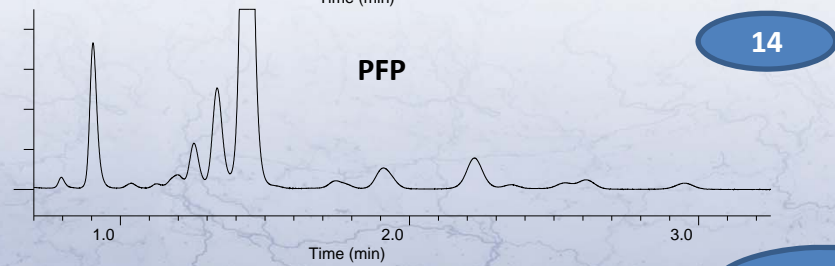
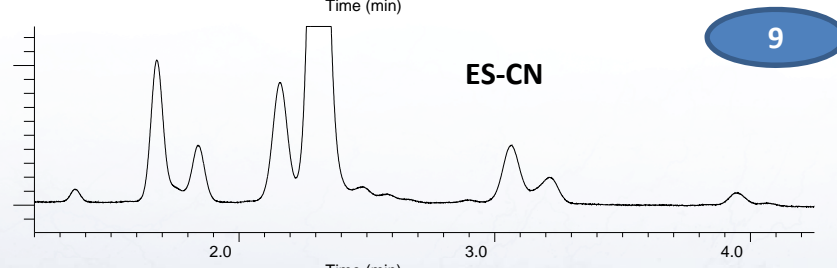
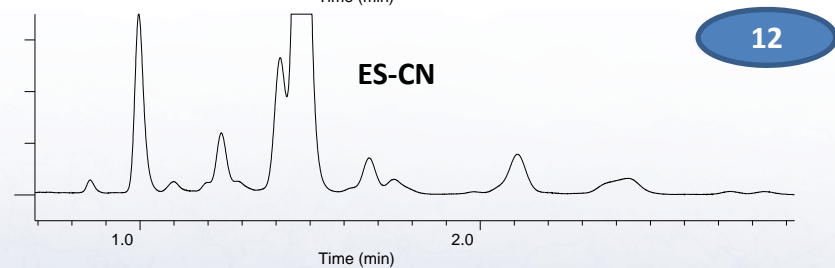
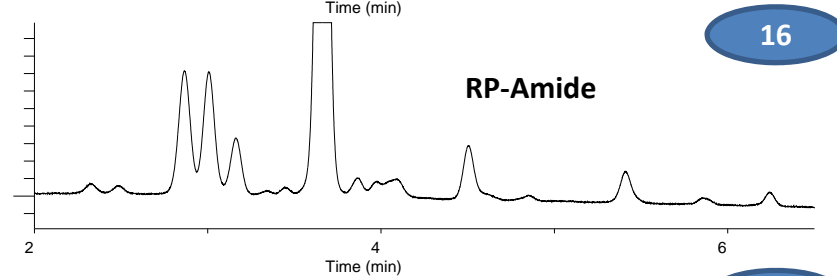
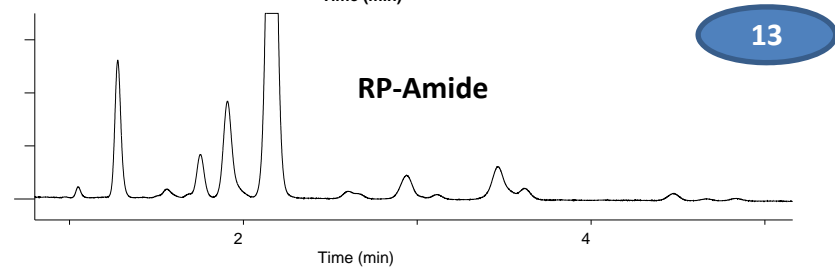
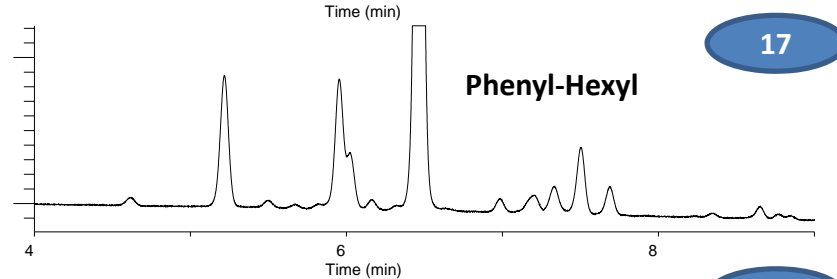
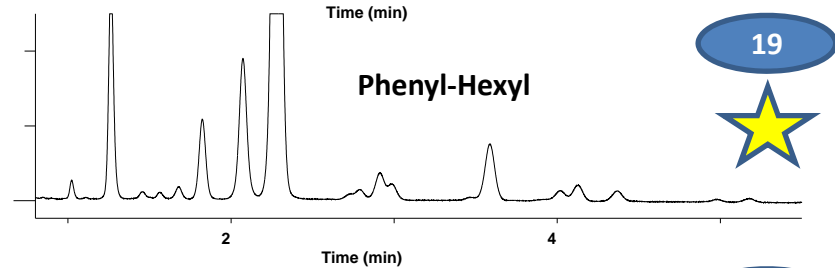
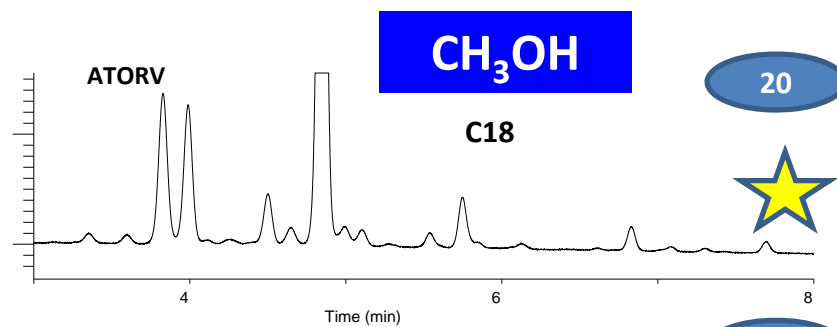
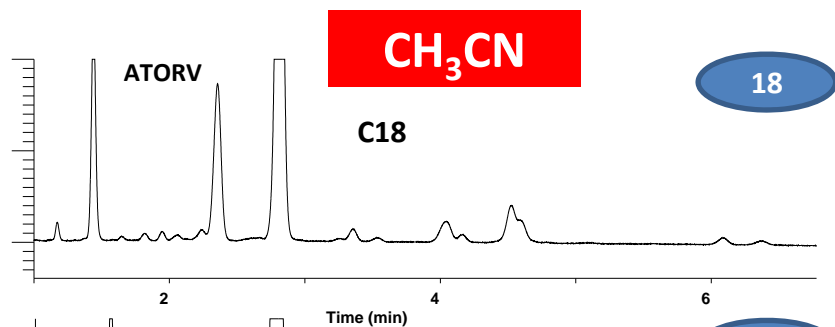
- Atorvastatin Calcium  
10 mg active/310 mg tablet
- Generate HCl-degraded and NaOH-degraded samples
- Pool acid- and base-treated samples together
- Compared five different HALO phases using both CH<sub>3</sub>CN and CH<sub>3</sub>OH at one pH (2.8, ammonium formate)
- Compared results and identified best option(s) for further development and optimization
- Again, used 3 x 50 mm, 2.7 µm HALO column geometry
- Initially screened C18 column using broad gradient with CH<sub>3</sub>CN
- Fine tuned to narrower ranges
- Compared all phases using narrower range using both CH<sub>3</sub>CN and CH<sub>3</sub>OH

# A BROAD RANGE GRADIENT MAY NOT BE AS USEFUL WHEN SCREENING MORE COMPLEX SAMPLES

## Mixture of acid- and base-treated atorvastatin calcium

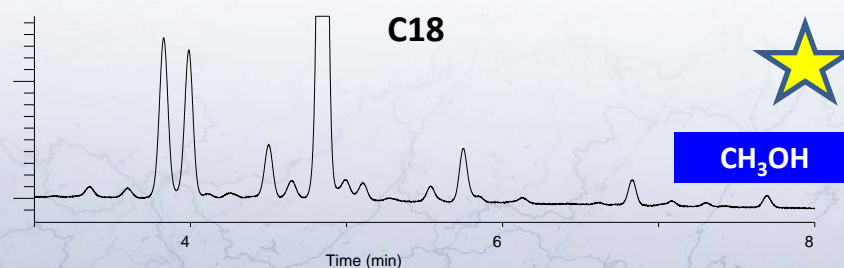
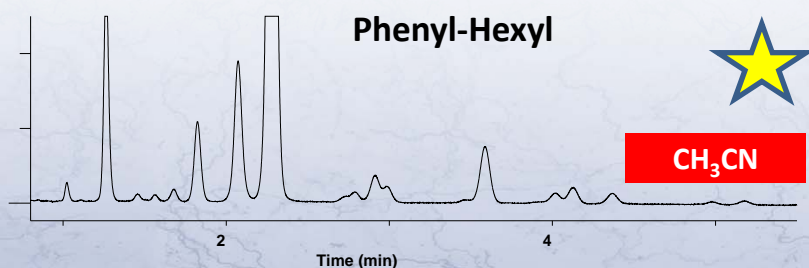






# HOW DO YOU CHOOSE WHICH COMBINATION TO DEVELOP AND OPTIMIZE FURTHER?

- Compare chromatogram for number of peaks observed
- Compare shapes for all detected peaks
- Select phase/modifier combination(s)
  - # peaks separated
  - minimum critical  $R_s$  for peak pair
  - shortest analysis time
  - most peaks with acceptable USP  $T_f$
- If no clear winning combination, carry out several gradients having differing slopes
  - For example, 50–75% in 10 minutes and 25 minutes for C18 and Phenyl-Hexyl
  - Assess whether either combination stands out vs. criteria
- Compare separation on longer column with higher efficiency



# SUMMARY AND CONCLUSIONS

- Use of different column selectivities, with different organic modifiers and pHs, can be an effective approach for ensuring:
  - **all sample components can be “seen” and,**
  - **acceptable combination(s) of column/modifier/pH can be found**
- For moderately complex and very complex samples, it can be effective to screen different stationary phase types, organic modifiers and pHs to identify a promising combination for further refinement or optimization
  - **Related substance methods**
  - **Multiple active ingredient drug products (OTCs)**
  - **Impurity profiles**
  - **Forensic analyses**
  - **Environmental samples**
- Short, efficient, narrow-ID Fused-Core columns allow faster screening of various combinations of conditions and faster answers to (U)HPLC challenges

# KEY FOR GRADING CHROMATOGRAMS



Means that latter 5 larger peaks are separated to a reasonable degree prior to optimization



Signifies the # of peaks in the 1<sup>st</sup> or 2<sup>nd</sup> half of the separation



Signifies the total # of peaks in the separation

Remind symbols  
verbally

# METHOD DEVELOPMENT: TIME CONSIDERATIONS

**Columns: 3 x 50 mm, 2.7 µm**

**Flow Rate: 0.6 mL/min**

**Temperature: 30 °C**

**Initial Gradient: 5–90% organic/buffer**

**Gradient Time: 10 min**

**Initial Hold: 0 initial, later 1 min**

**Agilent 1200 binary 600 bar system**

**Delay volume: 0.74 mL (from DryLab runs)**

**Hold 1 min at %B initial x 0.6 mL/min = 0.6 mL**

**Effective delay volume: 1.34 mL**

|      |           |  |              |      |     |
|------|-----------|--|--------------|------|-----|
| Time | %B        |  | Phases       | 4    |     |
| 0    | 2         |  | Modifiers    | 2    |     |
| 1    | 2         |  | pHs          | 4    |     |
| 11   | 90        |  | # injections | 2    |     |
| 12   | 90        |  | Total Runs   | 64   |     |
| 12.5 | 2         |  |              |      |     |
| 5    | Post Time |  |              |      |     |
|      |           |  |              |      |     |
| 17.5 | min       |  | Total Time   | 1120 | min |
|      |           |  | Total hrs    | 18.7 | hr  |

## Column Treatment and Handling (no col. switching valve)

1. 5 min: Flush column with 50:50 organic/buffer
2. 5–7 min: Flush column with 90:10 organic/buffer
3. 5 – 7 min: Equilibrate column with 2:90 organic/buffer
4. 2 replicate gradient runs
5. End of use: Flush with 50:50 organic/water, 7 min
6. End of day: Flush with 100% organic (CH<sub>3</sub>CN), 7 min



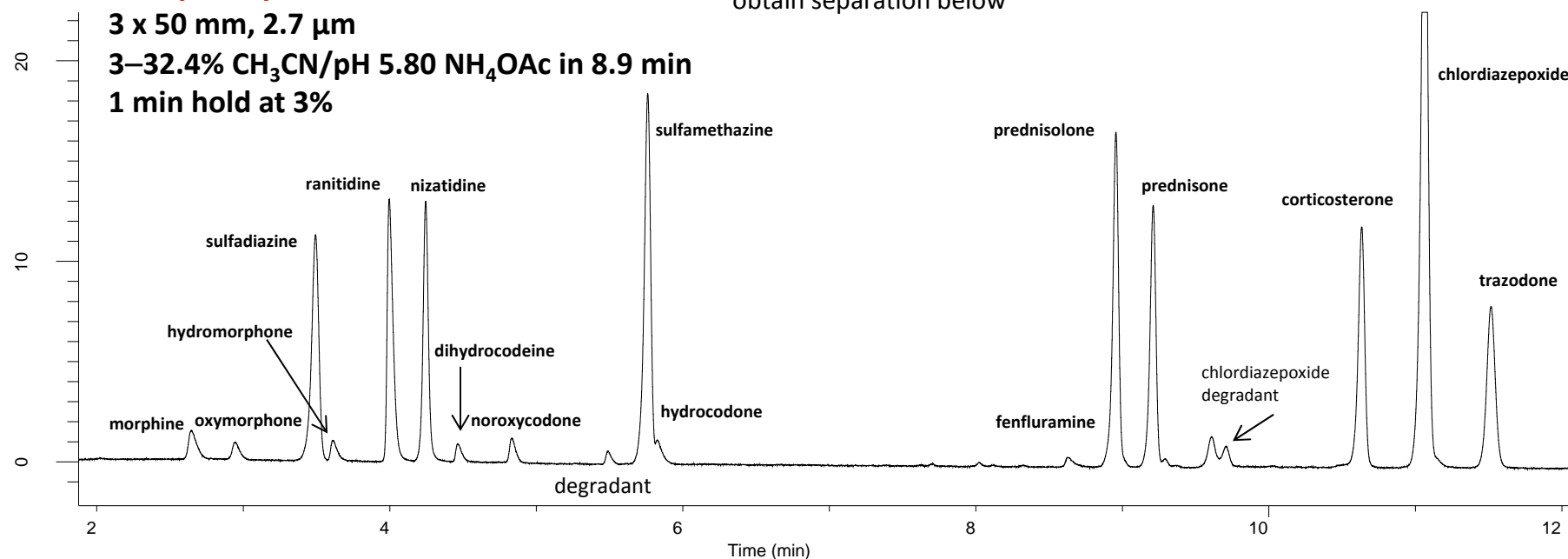
### Phenyl-Hexyl

3 x 50 mm, 2.7  $\mu$ m

3–32.4% CH<sub>3</sub>CN/pH 5.80 NH<sub>4</sub>OAc in 8.9 min

1 min hold at 3%

Carried out 10 and 30 min gradients and used DryLab 4 to obtain separation below

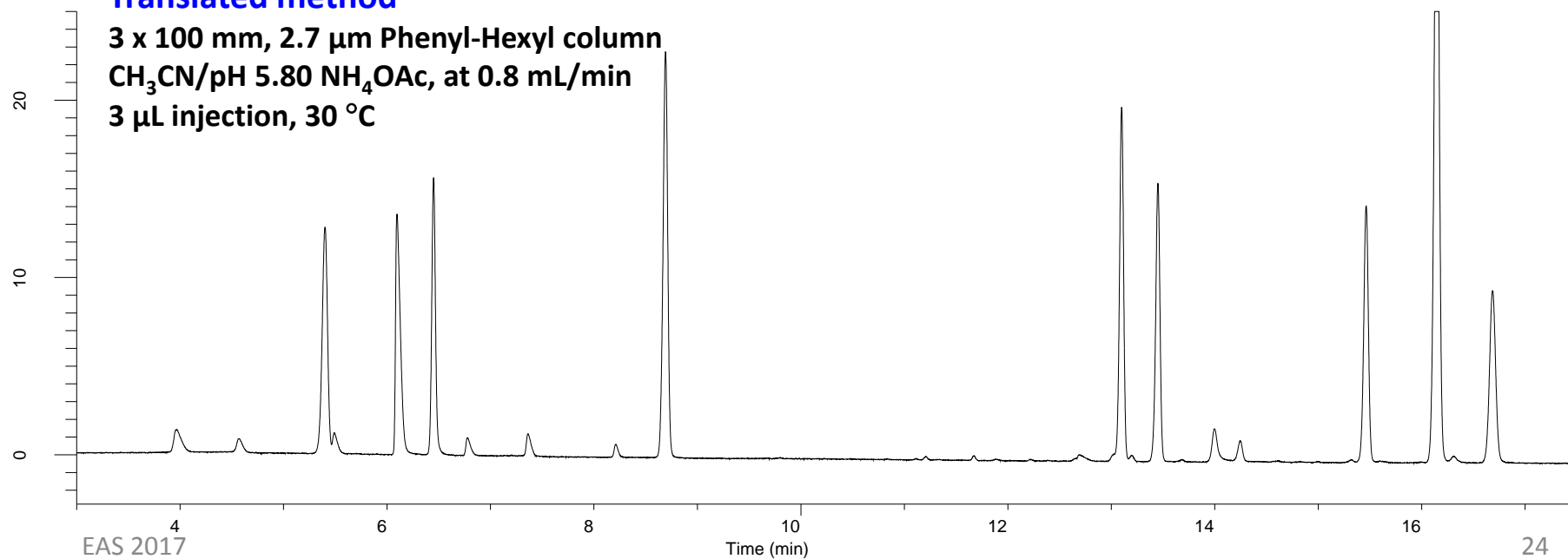


### Translated method

3 x 100 mm, 2.7  $\mu$ m Phenyl-Hexyl column

CH<sub>3</sub>CN/pH 5.80 NH<sub>4</sub>OAc, at 0.8 mL/min

3  $\mu$ L injection, 30  $^{\circ}$ C



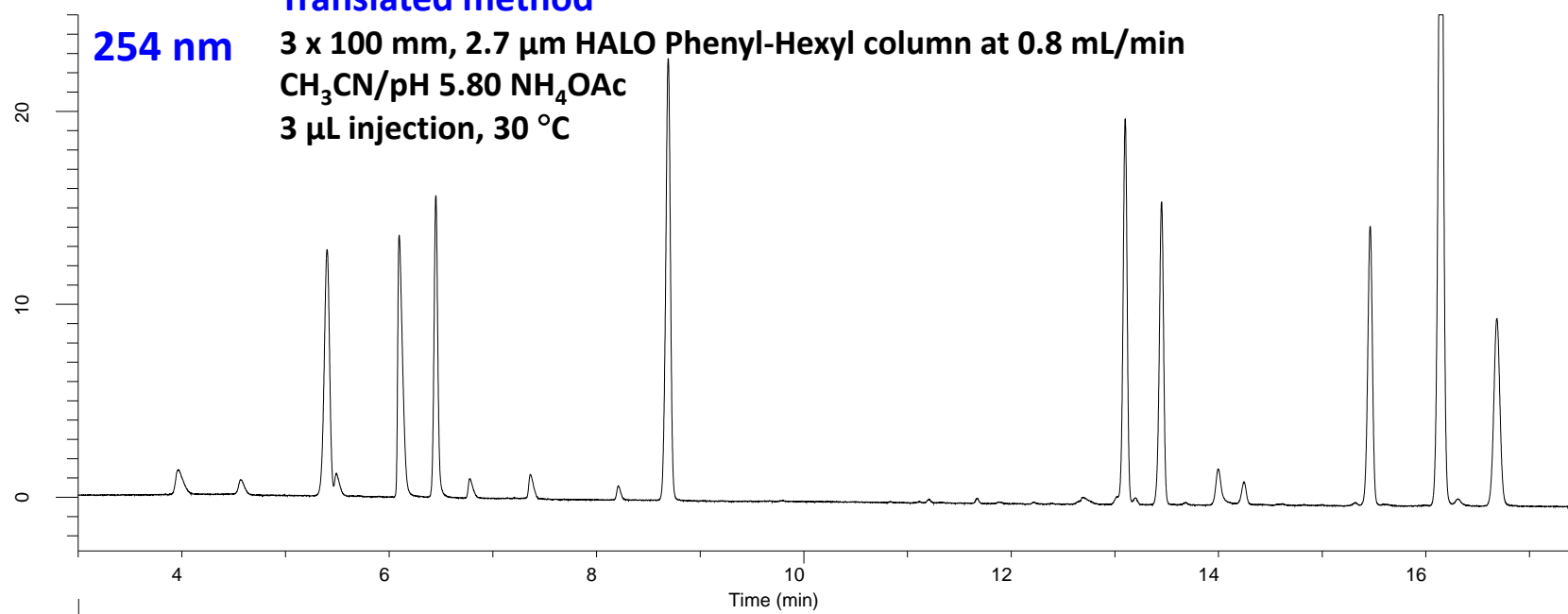
### Translated method

254 nm

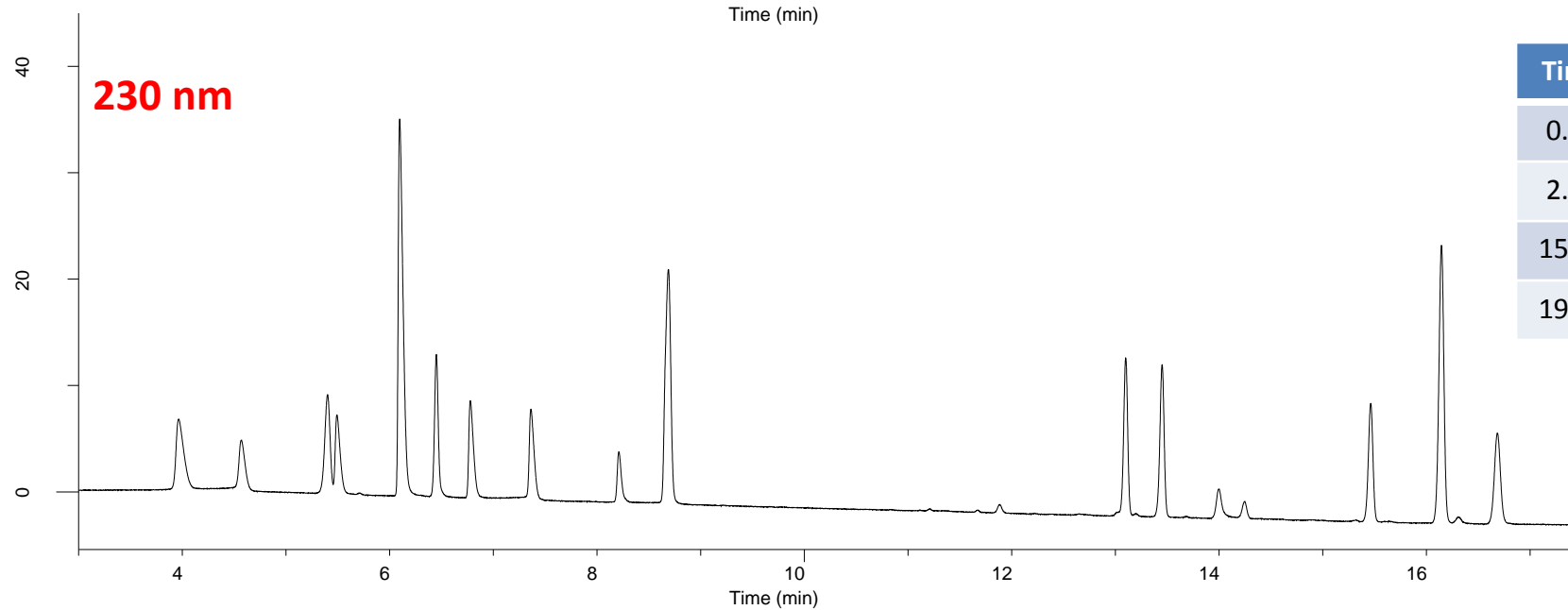
3 x 100 mm, 2.7  $\mu$ m HALO Phenyl-Hexyl column at 0.8 mL/min

CH<sub>3</sub>CN/pH 5.80 NH<sub>4</sub>OAc

3  $\mu$ L injection, 30  $^{\circ}$ C



230 nm



| Time  | %B   |
|-------|------|
| 0.00  | 3    |
| 2.42  | 3    |
| 15.02 | 32.4 |
| 19.67 | 32.4 |