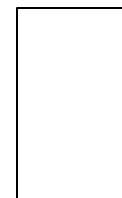


**Integrated approach to HPLC method
development:
Using all the tools in the
chromatographer's toolbox**

Rosario LoBrutto

Pharmaceutical and Analytical Development
Novartis Pharmaceuticals



Overall Strategy

- Determine aim of analysis
- Look at structure (estimate pK_a) or use ACD (Advanced Chemistry Development)
- Try to use shorter columns for scouting experiments (5cm x 4.6 mm)- 3 um or use Acquity system with 10 cm x 2.1 mm, 1.7 um particles
- Use 35 - 45C as starting temperature.
- Run probe linear gradient with hold at high organic on short column, 5cm column
- Run pH studies isocratically to determine optimal pH (5 cm column)
 - Acquity system very effective for doing so
- Run linear gradient with hold at high organic with optimized pH on 5 cm x 4.6 mm column
- If sufficient resolution Finished
- If need more resolution then go to 15 cm x 3 mm id column
- If resolution obtained- Finished
- If desired resolution not obtained use AMDS system (need to optimize gradient and selectivity)
- Use AMDS system for gradient optimization on 15 cm x 3 mm id column. (2 organics, steep/shallow gradient)- we will discuss today.

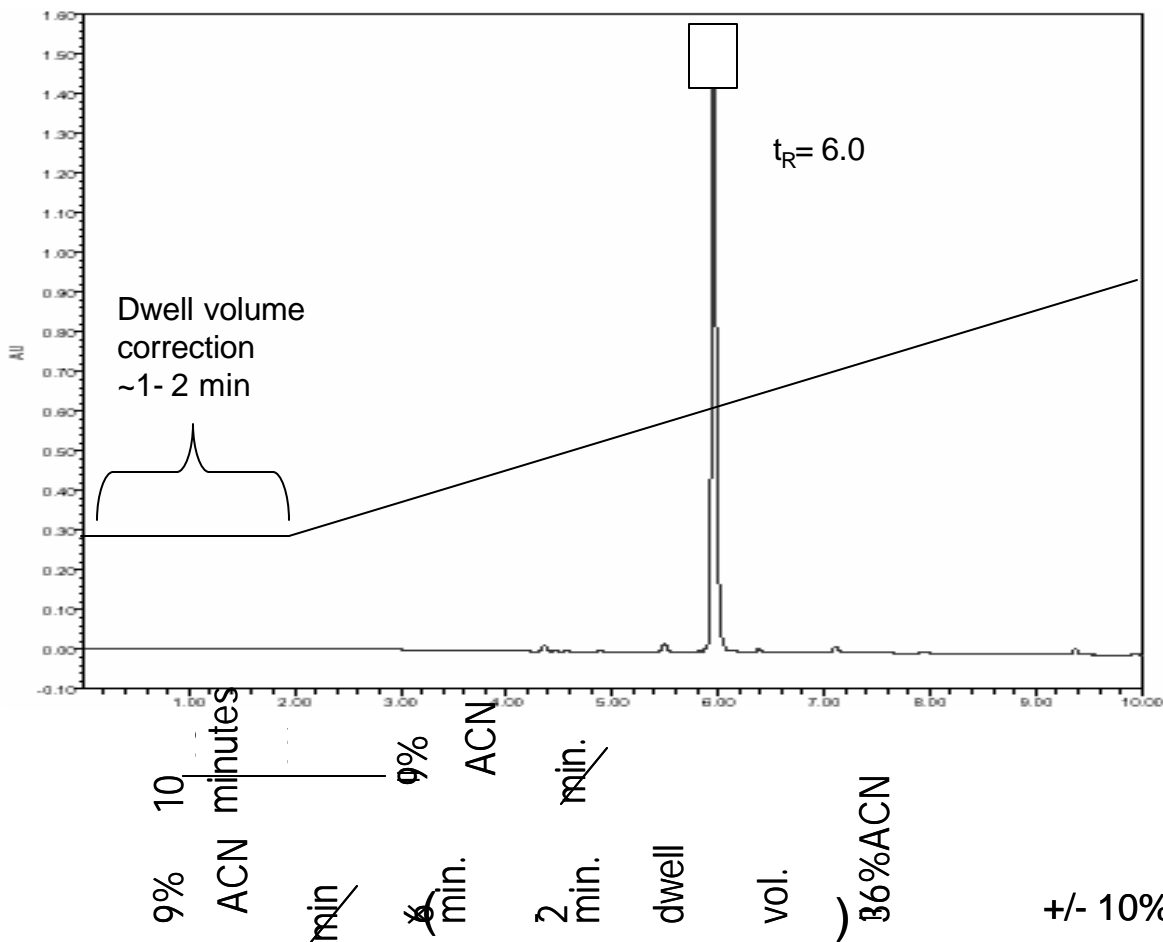
Sample Prep

- Use methanol as sample prep solvent if using second dilution. (check for sample reactivity)
- If using methanol for only dilution look at impact on peak shape (diluent/mobile phase mismatch for components with $k' < 2$)
- Sample prep constitutes apx. 70% of solvent usage, try to use methanol if possible.

First Step: pK_a Estimation Using ACD Software

- Product T is a diprotic base with two pK_a s 3.3 and 5.3 estimated by ACD
- It has two pK_a values and at mobile phase pH values between 3 and 5, multiple species exist.
- Two equilibria could be written for this amphoteric species
- Since pK_a 's are close to one another (<4 pK_a units apart) the inflection points overlap making titration and or chromatographic pK_a prediction difficult.
- At pH=4.3 basic site (A) will be predominately neutral (90%) and the other basic site (B) will be predominately ionized (90%).

Estimating Isocratic Conditions



Column: Luna C8(2) 150x4.6 mm
 Inj. Vol.: 10 μ L
 Flow: 1.0 mL/min
 Wavelength: 300 nm
 Col. Temp.: 35 C

	Time	Flow	%A	%B
1		1	95	5
2	10	1	5	95

%A – 0.2% H_3PO_4 (v/v) pH 1.90
 %B - Acetonitrile

Initially a steep gradient was run to estimate the isocratic elution conditions in order to study the effect of pH on analyte retention.

Retention Dependence on Mobile Phase pH

Chromatographic conditions:

Column: Phenomenex Luna 3u C8(2)

[150x4.6mm, 3 μ m]

MP: 10 mM K₂HPO₄:ACN (71:29,v/v)

pH adj. w/ H₃PO₄

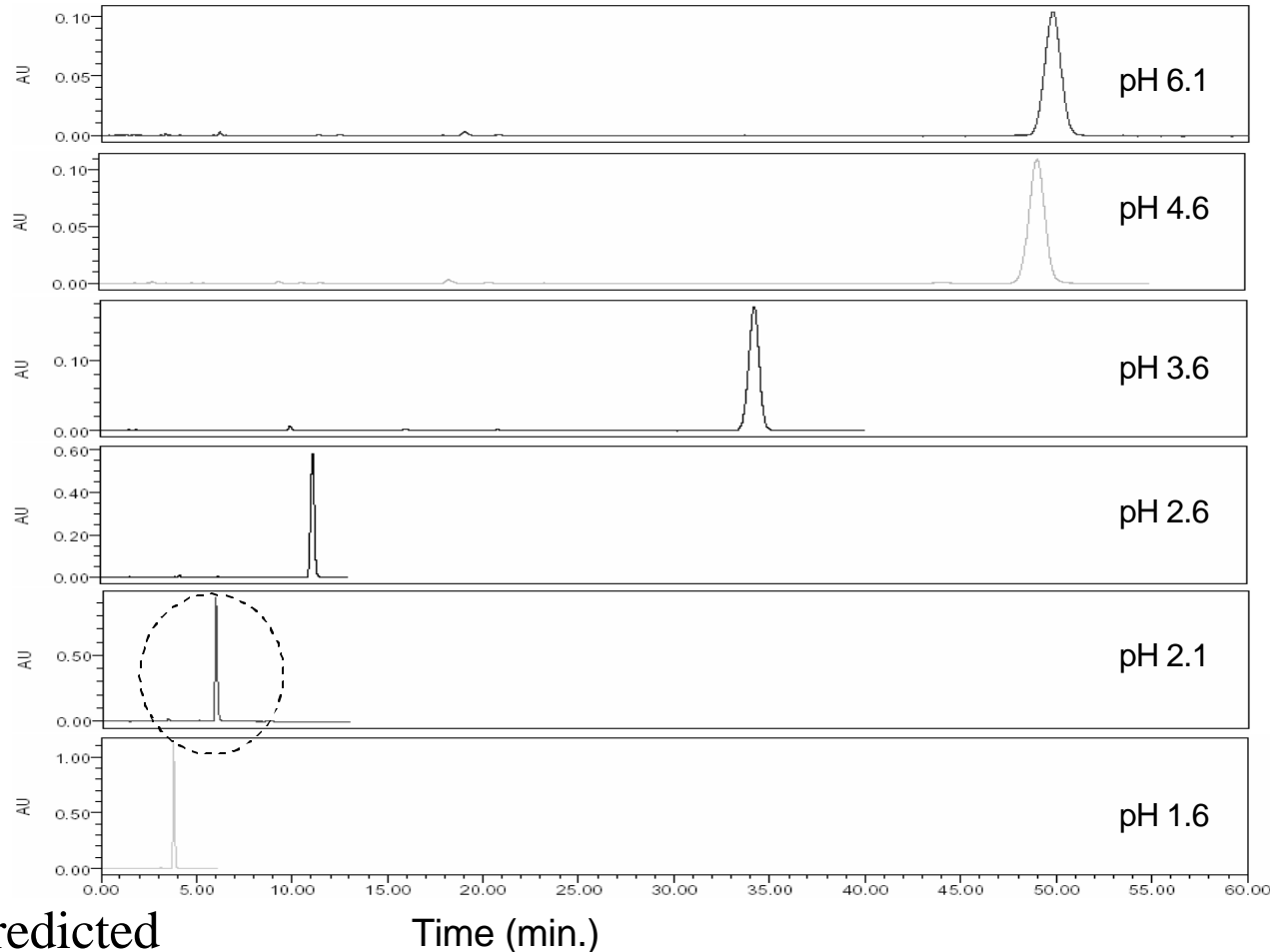
Flow rate: 1.0 mL/min

Injection Vol.: 10 μ L

Wavelength: 247 nm

Column Temp.: 35 C

pH	tR
6.1	49.8
4.6	49.1
3.6	34.3
2.6	11.2
2.1	6.0
1.6	3.8



- Initial isocratic conditions predicted from probe gradient run correlates very well with actual retention obtained at isocratic conditions.
- Try 5 cm x 4.6 mm column on conv. HPLC system
- Try 10 cm x 2.1 mm column on Acquity system
- pH 6 is the optimal pH to run for the analysis.
- Check which wavelength gives the best sensitivity

What should the starting pH be in order to analyze this molecule in its neutral form?

- *The downward pK_a shift for basic analytes must be accounted for.*
- *The working pH should be at least 2 pH units above the basic analyte pK_a to be fully neutral.*
- *The upward pH shift of the aqueous acidic buffer upon addition of the organic must be accounted for.*

Example: The higher pK_a of Product T is 5.3 and initial eluent conditions are: 30% MeCN and 70% Buffer.

What should the pH of the buffer be in order to obtain the basic analyte in its neutral form?

$5.3 - (3 * 0.2) = 4.7$ Downward analyte pK_a shift.

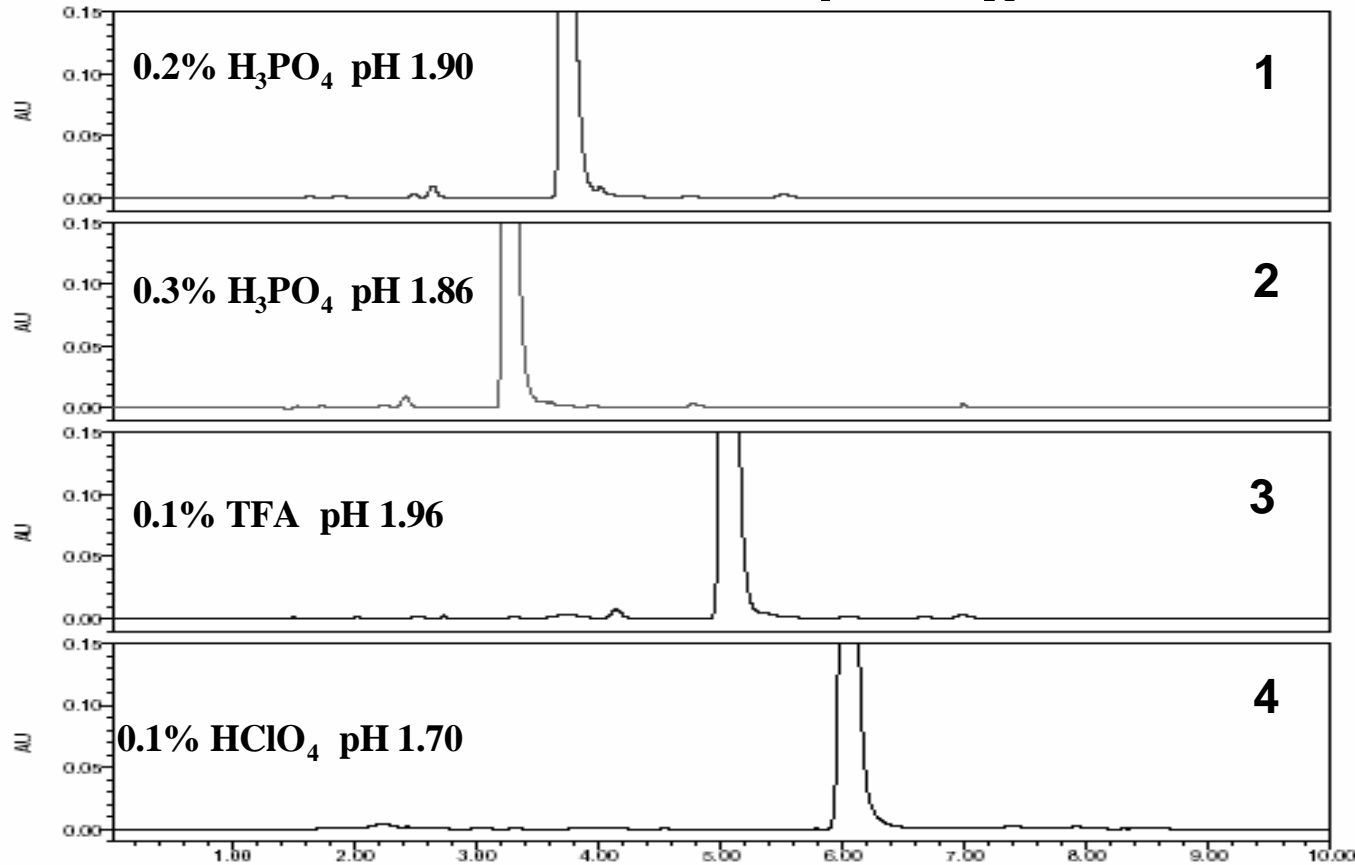
$4.7 + 2 = 6.7$ pH at which basic analyte would be neutral

$3 * 0.2 = 0.6$ Upward pH shift of aqueous acidic buffer upon addition of organic

$6.7 - 0.6 = 6.1$ Max pH of buffer in order to have analyte in fully neutral form.

This prediction agrees well with the experimental!!

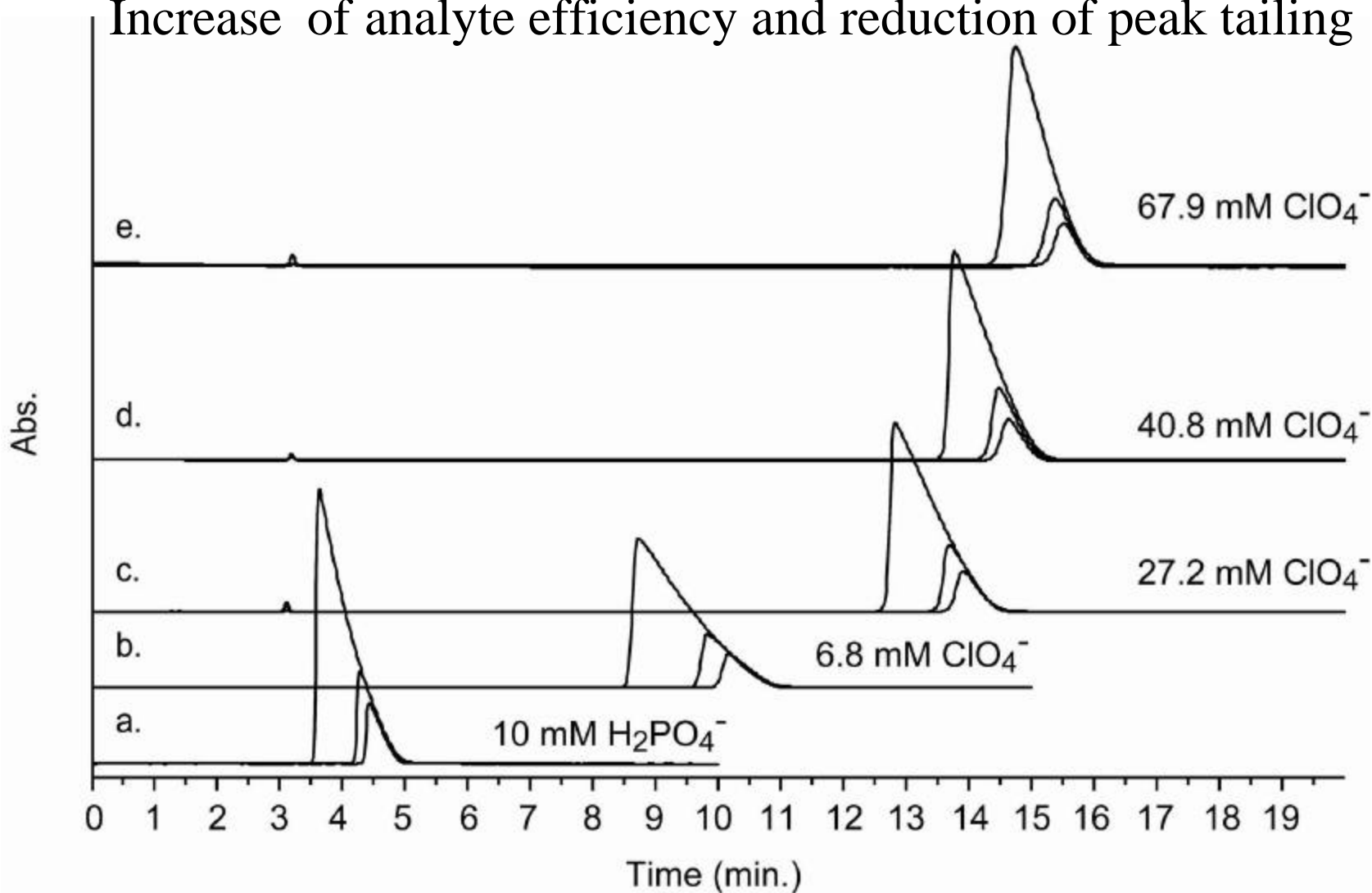
(Option 1) Chaotropic Effect: Increasing retention in low pH region



Chromatographic Conditions
Column: Luna C8(2) 150x4.6 mm
MP: Aqueous :ACN (71:29, v/v)
Wavelength: 247 nm
Col. Temp.: 35 C
Inj. vol.: 10 μ L

- Chaotropic anions, TFA and HClO₄ interact with positively charged analyte and lead to an increase in the analyte retention.
- Retention decreases from pH 1.90 to 1.86 for MP with H₃PO₄. Indicates analyte not completely ionized in this pH region.
- TFA is MS compatible however is not the best MS mobile phase additive to employ (ion suppression in gas phase) so went to high pH analysis.

Increase of analyte efficiency and reduction of peak tailing



Chromatographic overlays of Labetalol analyzed at different analyte concentrations using increasing mobile phase concentration of perchlorate anion

Chromatographic conditions: Analyte load: 3.3, 6.5, 31.2 mg , (a) 75%: 0.1 v/v% H₃PO₄: 25% acetonitrile, (b) 75%: 0.05 v/v% HClO₄: 25% acetonitrile, (c) 75%: 0.2 v/v% HClO₄: 25% acetonitrile, (d) 75%: 0.4 v/v% HClO₄: 25% acetonitrile, (e) 75%: 0.5 v/v% HClO₄: 25% acetonitrile

Increase of analyte efficiency and reduction of peak tailing

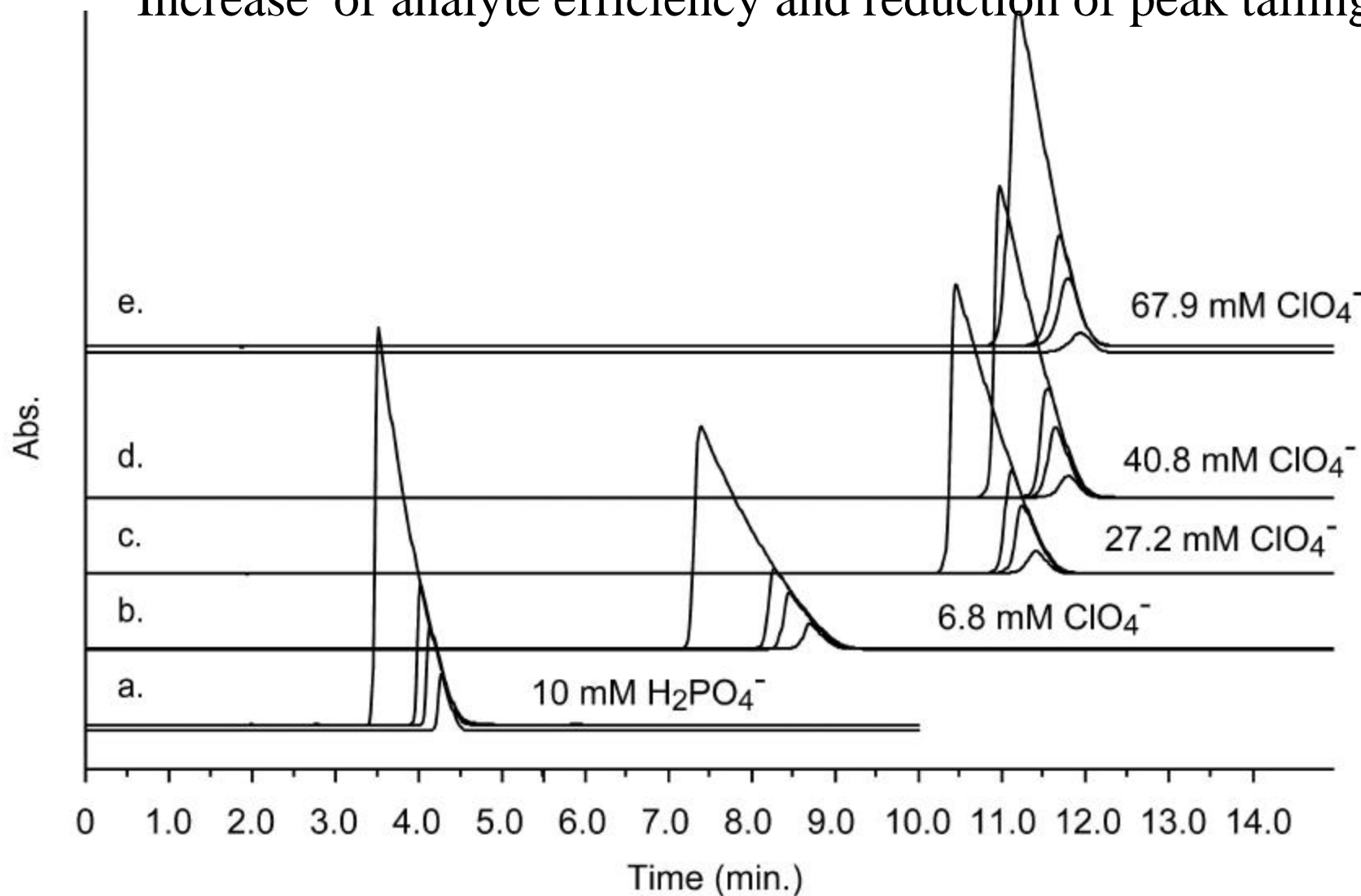
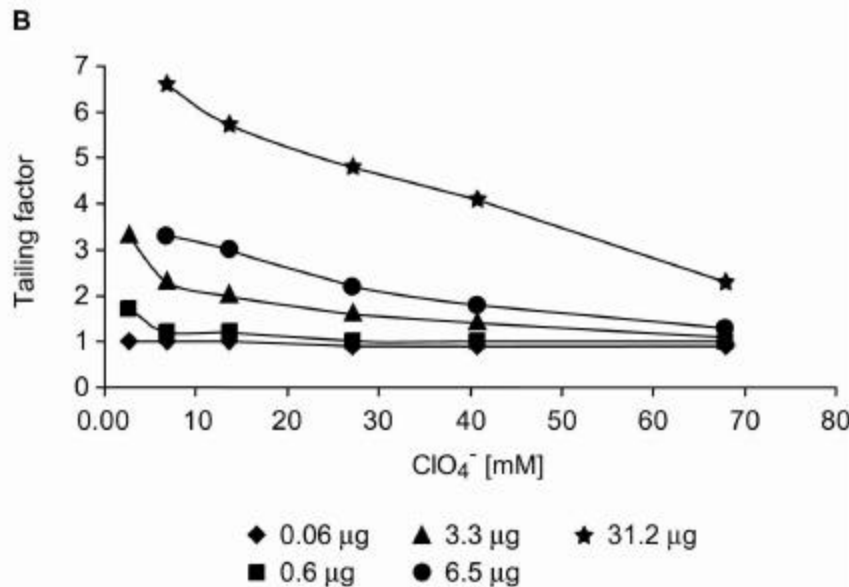
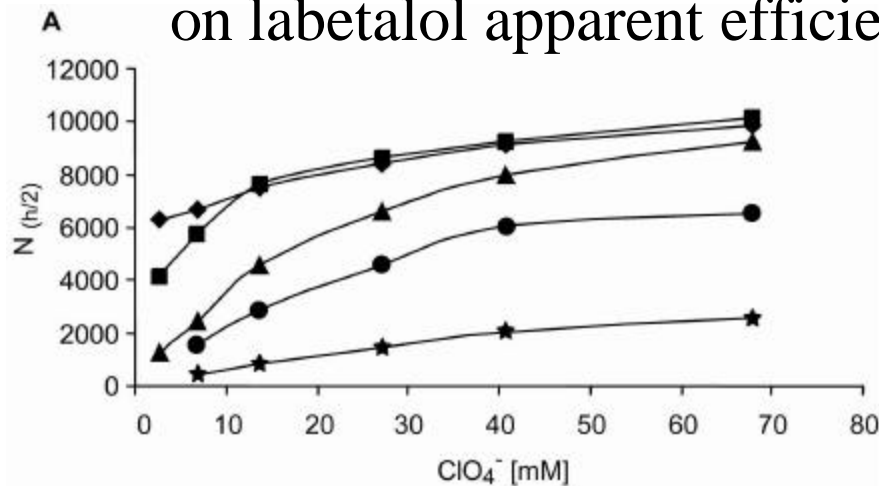


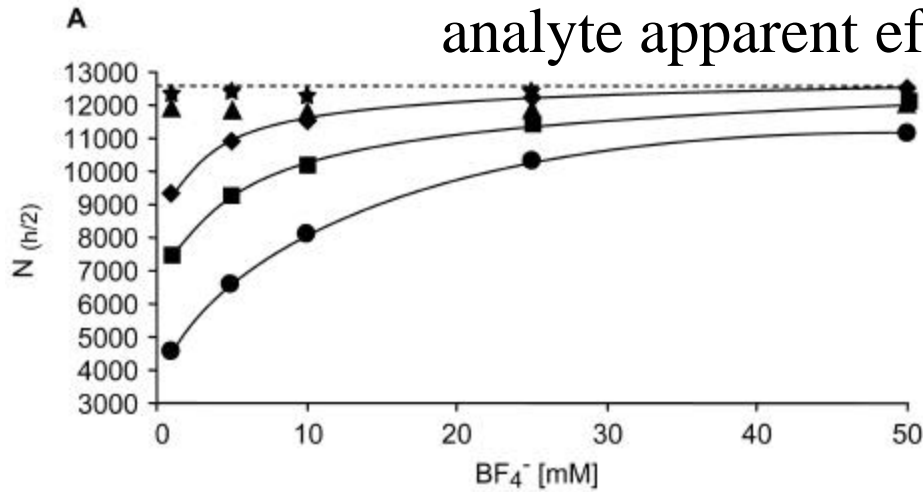
Fig. 5. Chromatographic overlays of Dorzolamide HCl analyzed at different analyte concentrations using increasing mobile phase concentration of perchlorate anion
Chromatographic conditions: Analyte load: 1.4, 5.2, 9.2, 48 mg, (a) 90%: 0.1 v/v% H₃PO₄: 10% acetonitrile, (b) 90%: 0.05 v/v% HClO₄: 10% acetonitrile, (c) 90%: 0.2 v/v% HClO₄: 10% acetonitrile, (d) 90%: 0.4 v/v% HClO₄: 10% acetonitrile, (e) 90%: 0.5 v/v% HClO₄: 10% acetonitrile

Effect of analyte load and perchlorate counteranion conc. on labetalol apparent efficiency and tailing factor.

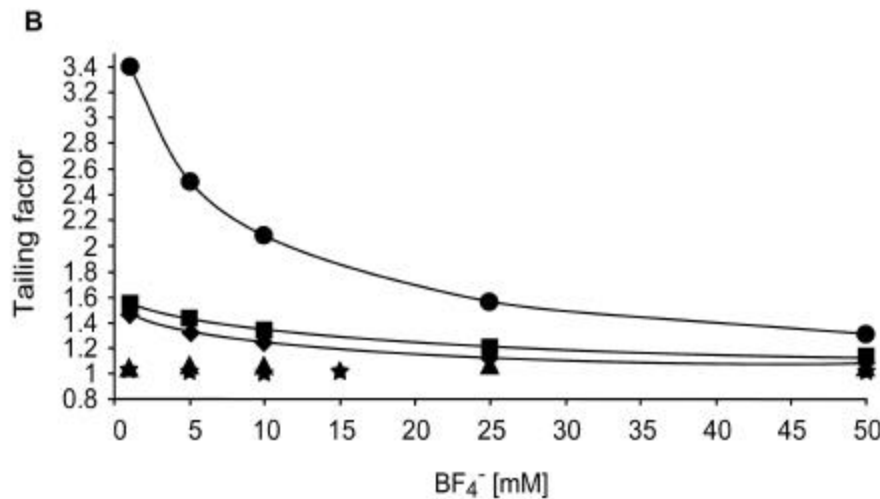


Chromatographic conditions:
 Analyte load: 0.06 - 31.2 mg , 75%
 water: 25% acetonitrile. Water
 adjusted with 0.025 - 0.5 v/v%
 HClO₄. **(A)** N (h/2) vs. perchlorate
 concentration. **(B)** Tailing factor vs.
 perchlorate concentration.

Effect of tetrafluoroborate concentration on analyte apparent efficiency and tailing factor.

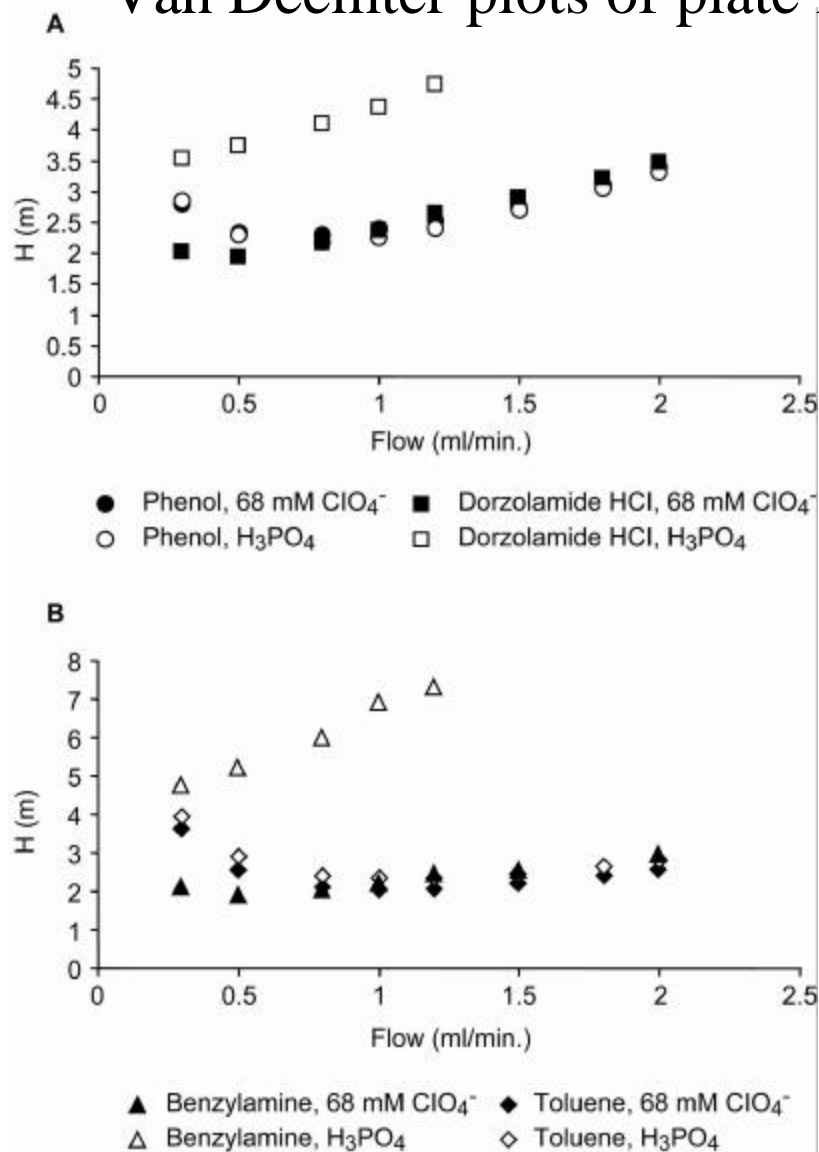


Mobile phase: 0.1 v/v% phosphoric acid + xBF₄ [1 mM – 50 mM]: acetonitrile, Ophthalmic compounds (10% acetonitrile), phenols (25% acetonitrile), (A) N(h/2) vs. tetrafluoroborate concentration. (B) Tailing factor vs. tetrafluoroborate concentration.



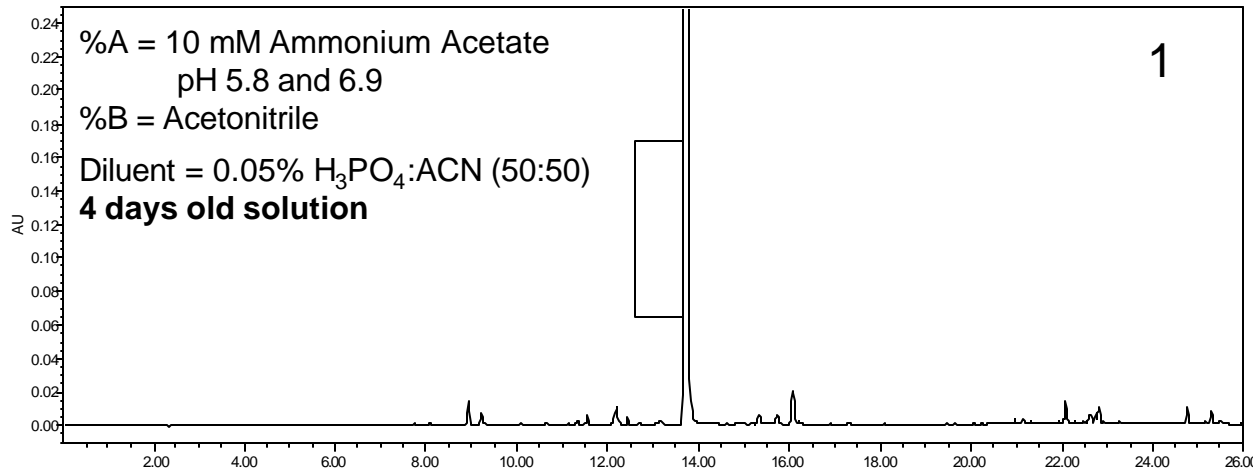
- ▲ 4-nitrophenol ■ Dorzolamide HCl ● Timolol Maleate
- ★ 2-nitrophenol ◆ Compound X

Van Deemter plots of plate height (H) versus flow rate.

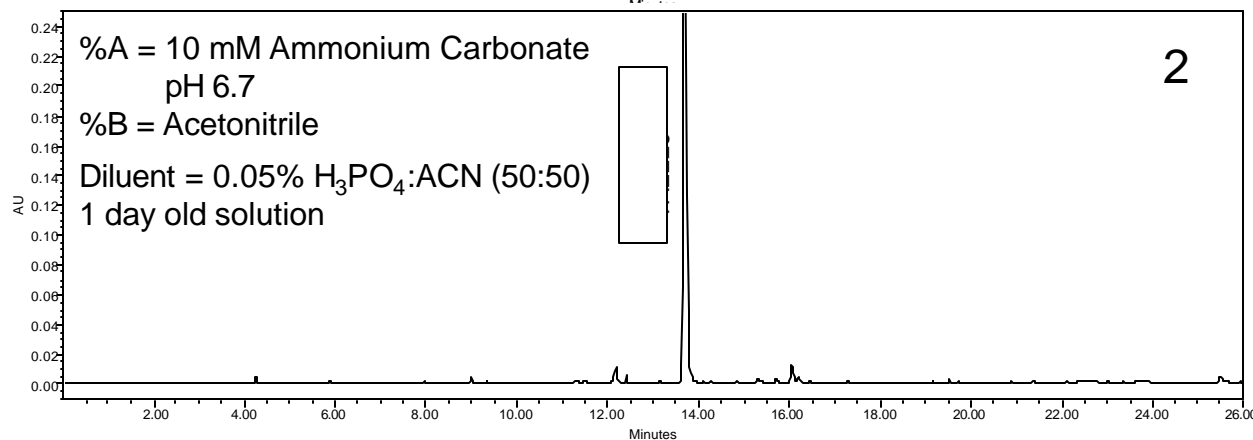


Mobile phases (A) Dorzolamide HCl: 90% water: 10% acetonitrile. Water adjusted with either 0.1 v/v% phosphoric acid or 0.5 v/v% HClO₄, Phenol: 75% water: 25% acetonitrile. Water adjusted with either 0.1 v/v% phosphoric acid or 0.5 v/v% HClO₄. (B) Benzylamine: 95% water: 5% acetonitrile. Water adjusted with either 0.1 v/v% phosphoric acid or 0.5 v/v% HClO₄, Toluene: 50% water: 50% acetonitrile. Water adjusted with either 0.1 v/v% phosphoric acid or 0.5 v/v% HClO₄.

Option 2- Analysis of Analyte (Free Base) as Neutral Species



Chromatographic Conditions:
Column: Luna C8(2) 150x4.6 mm
MP: Aqueous :Acetonitrile
Wavelength: 247 nm
Col. Temp.: 35 C
Flow: 1 mL/min
Inj. Vol.: 10 μ L

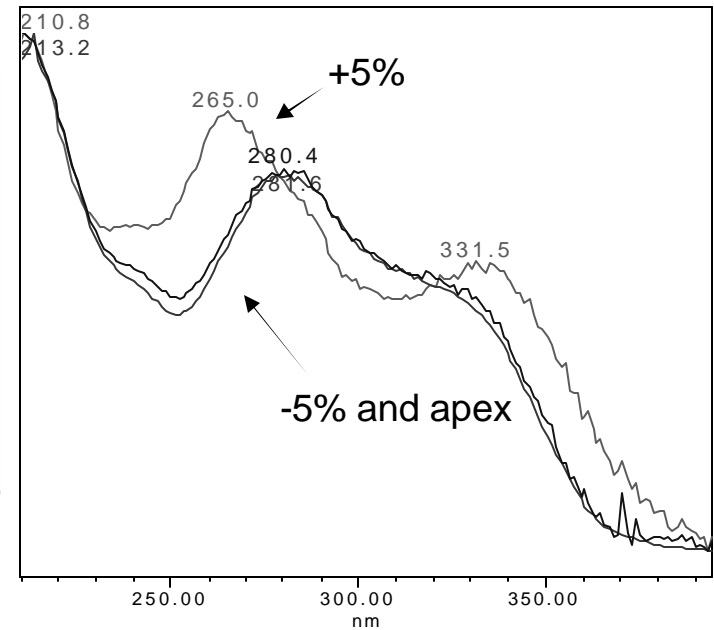
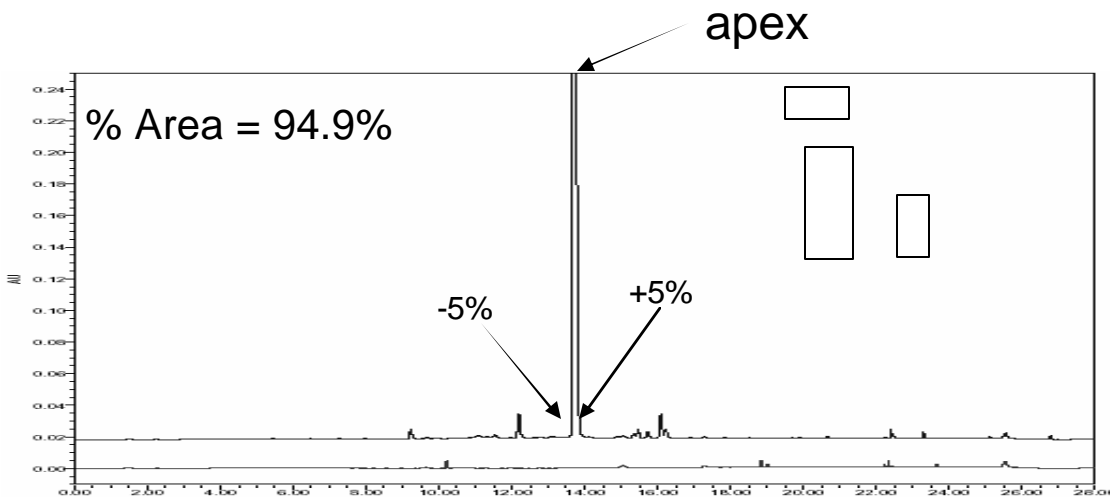


	Time	Flow	%A	%B
1		1	95	5
2	20	1	5	95
3	23	1	5	95

%A: Aqueous
%B: ACN

- Analyte is in neutral form at the mobile phase pH of the aqueous phases used.
- Ammonium Carbonate and ammonium acetate have a UV cutoff < 220 nm.
- Both buffers are compatible with LC-MS.
- Ammonium carbonate buffer may be undesired because it can form CO₂ and alter the mobile phase pH.

Spectral Purity: Diode array



- Diode array spectra show that this main peak is not spectrally homogenous.
- Need to perform method optimization experiments to resolve the impurity from active.
- LC-MS studies were also done in parallel.

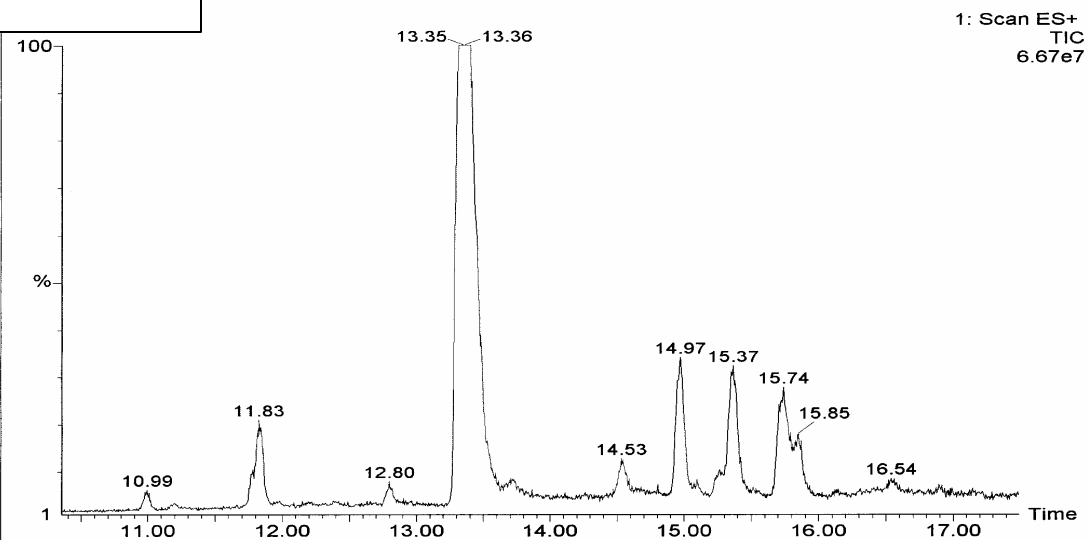
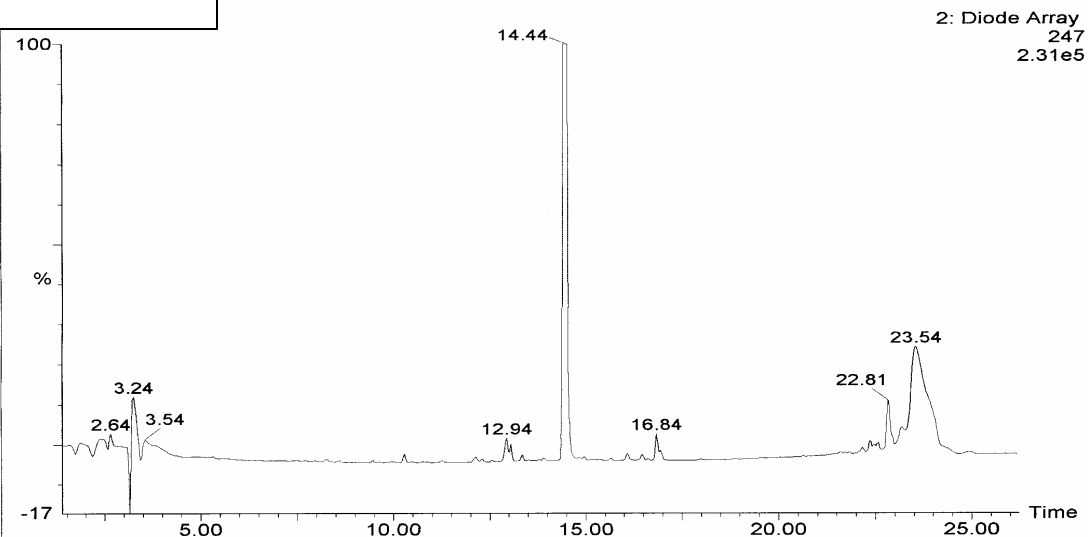
Chromatographic Conditions:
 Column: Luna C8(2) 150x4.6 mm
 MP: 10 mM NH₄OAc:Acetonitrile
 Col. Temp.: 35 C
 Flow: 1.0 mL/min
 Inj. Vol.: 10 µL

	Time	Flow	%A	%B
1		1.0	95	5
2	20	1.0	5	95
3	23	1.0	5	95

%A: 10 mM NH₄OAc, pH 5.8
 %B: ACN

Spectral Purity: MS

Product T free base



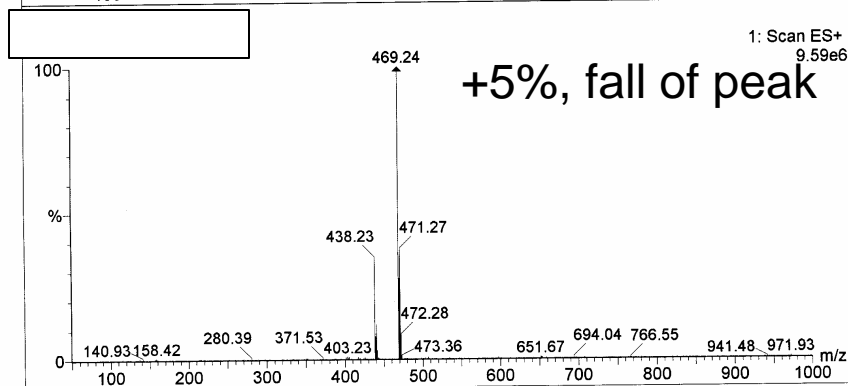
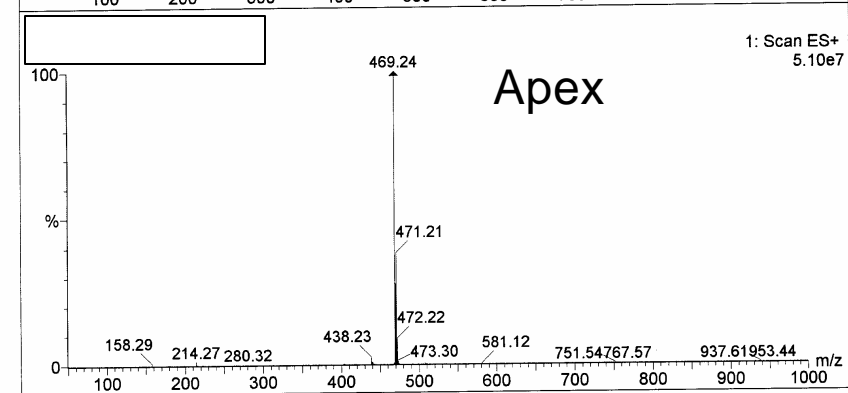
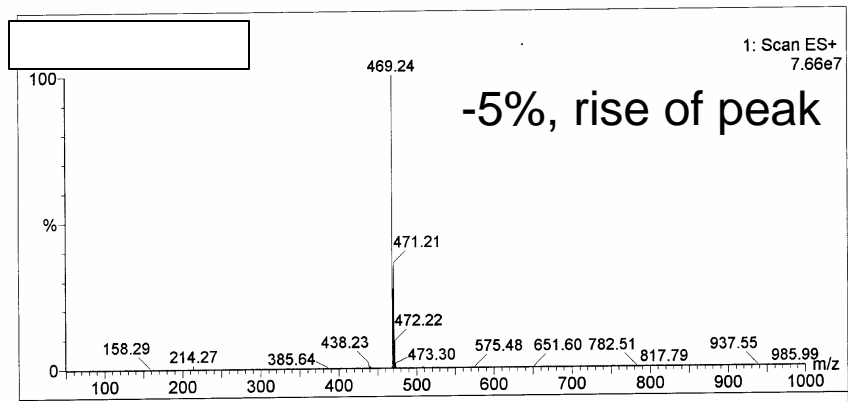
Chromatographic Conditions:
Column: Luna C8(2) 150x4.6 mm
MP: Aqueous :Acetonitrile
Wavelength: 247 nm
Col. Temp.: 35 C
Flow: 1 mL/min, split 10:1
Inj. Vol.: 10 µL

	Time	Flow	%A	%B
1		1	95	5
2	20	1	5	95
3	23	1	5	95

%A: 10 mM Amm. Acetate, pH 5.8
%B: ACN

ESI: + ion mode
Single Quadrupole, Open access
Capillary: + 3.5 kV
Cone: 25 V
Source Temp: 150C
Cone Temp: 20C
Desolvation Temp: 400C
Cone gas flow: 113 L/hr
Desolvation gas flow: 419 L/hr

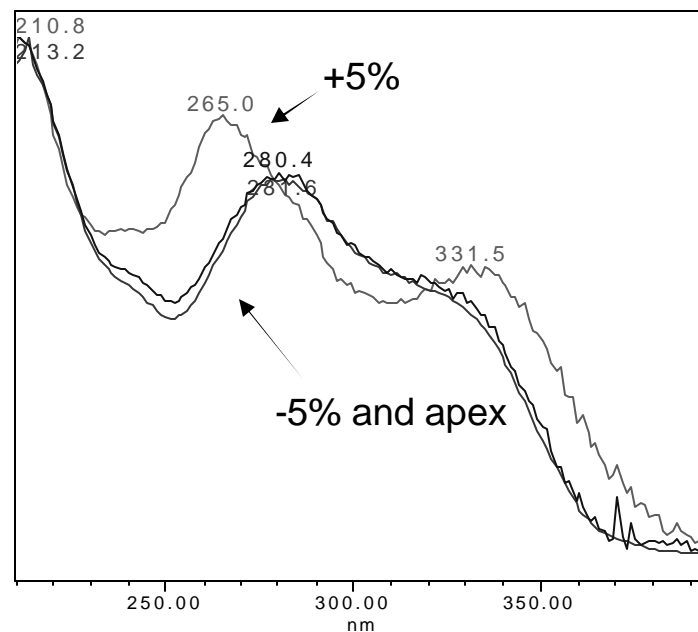
Using open access MS and diode array spectra in parallel



Indicates peak is not spectrally homogenous.

- [M+H] ion of active = 469
- [M+H] ion of impurity = 438

Indicates impurity has odd number of nitrogens.



We will revisit the mass spectra once we separate!!!

Waters Automated Method Development System (AMDS)

The Waters AMDS was used for method optimization by varying several chromatographic parameters in order to satisfy a users set criteria.

AMDS varies the following chromatographic parameters:

- Organic solvent
- Gradient
- Column temperature
- Column

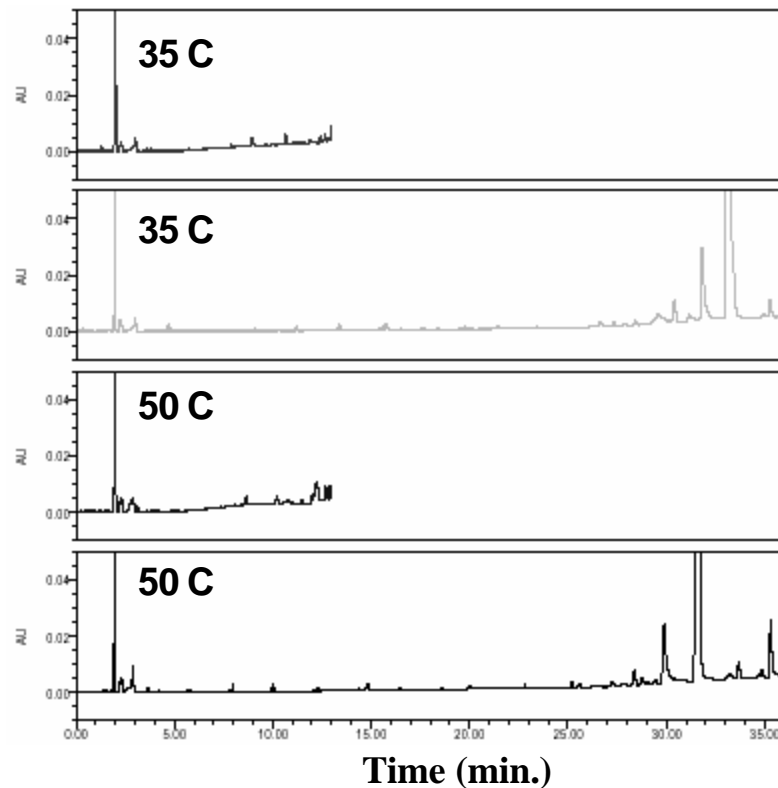
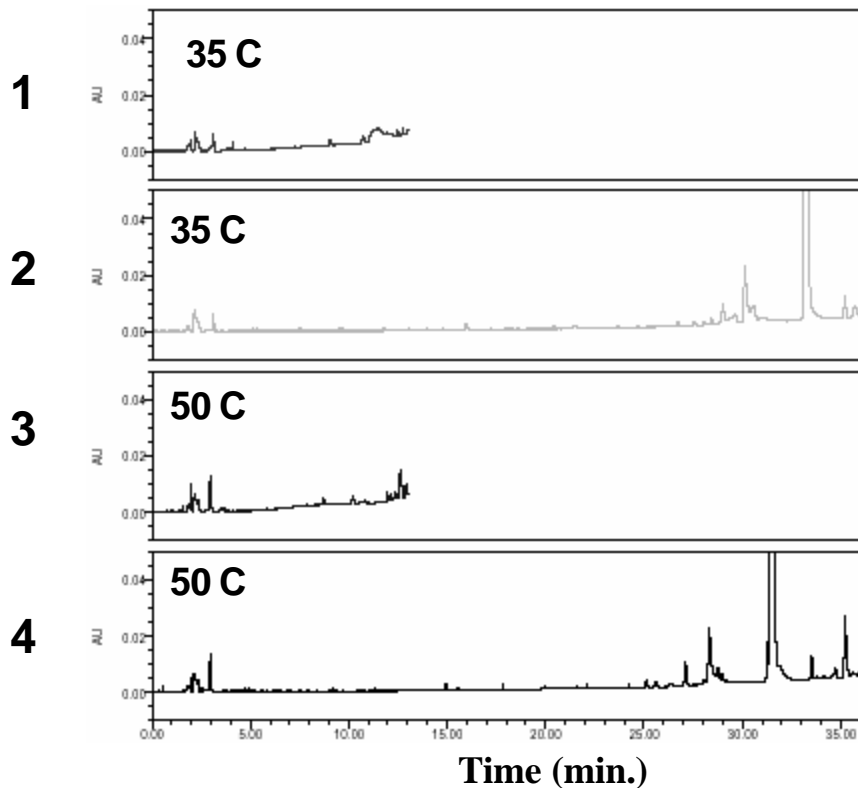
Analytical goals for the Analysis of Product T on the AMDS:

- Organic solvents: Methanol, Acetonitrile
- Aqueous: 10 mM NH₄OAc, pH 5.8
- Columns: Luna C18 (2) 3μm(150x4.6 mm), Sunfire C18 3.5μm (150x4.6 mm)
- Resolution more important than run time
- Runtime < 10 min.
- Minimum Resolution > 3.0 (between identified peaks)
- Pressure < 3000 psi

AMDS for Method Optimization

Luna C18(2), 3 μ m, 150x4.6 mm

Sunfire C18, 3.5 μ m, 150x4.6 mm



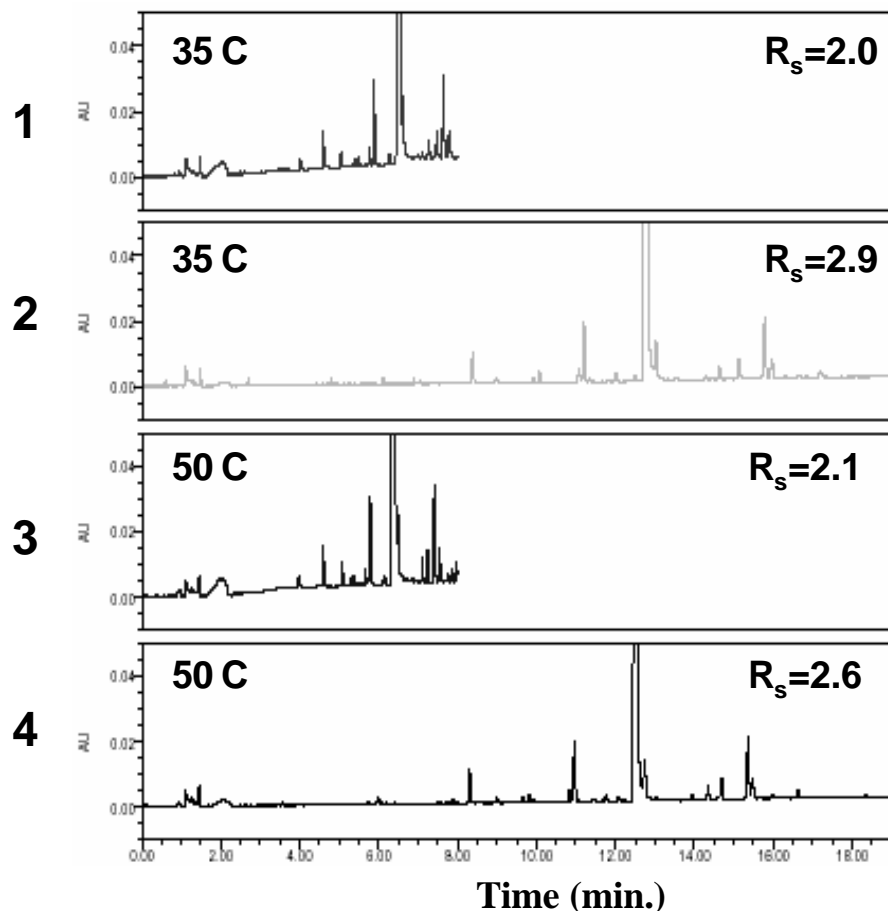
- 1 – (Steep gradient) 5% - 90% Methanol in 6 min
- 2 – (Shallow gradient) 5% - 90% Methanol in 17 min
- 3 – (Steep gradient) 5% - 90% Methanol in 6 min
- 4 – (Shallow gradient) 5% - 90% Methanol in 17 min

Flow rate = 0.8 mL/min

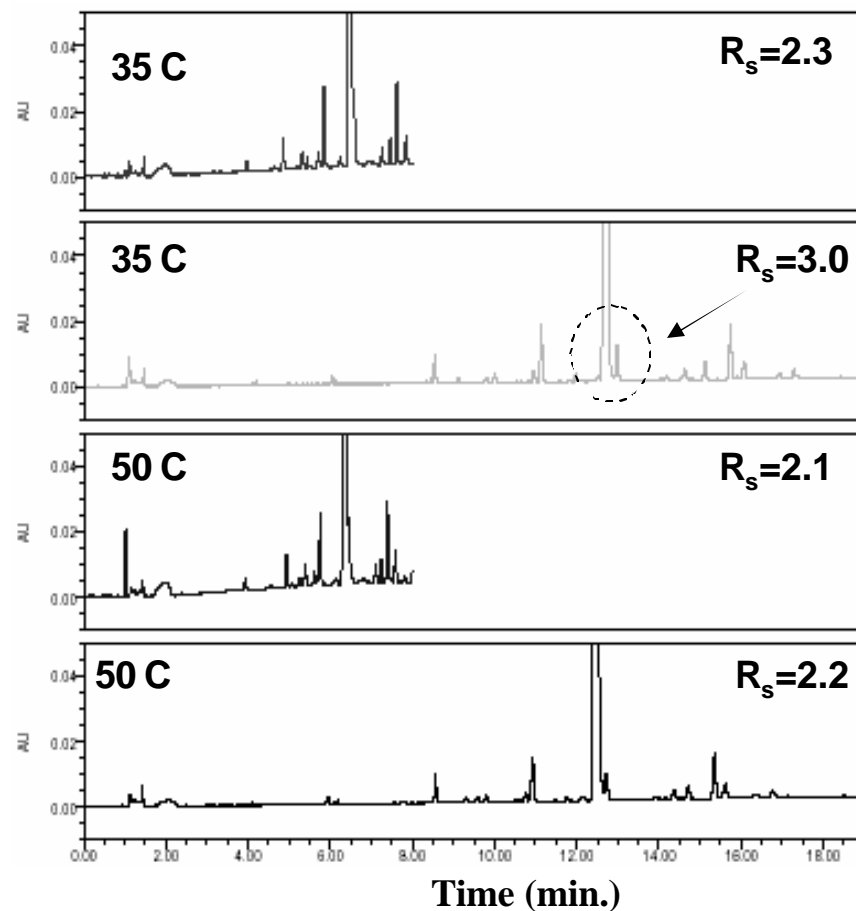
Future suggestion: Gradients used should be 15 – 95% Methanol

AMDS for Method Optimization

Luna C18(2), 3 μ m, 150x4.6 mm



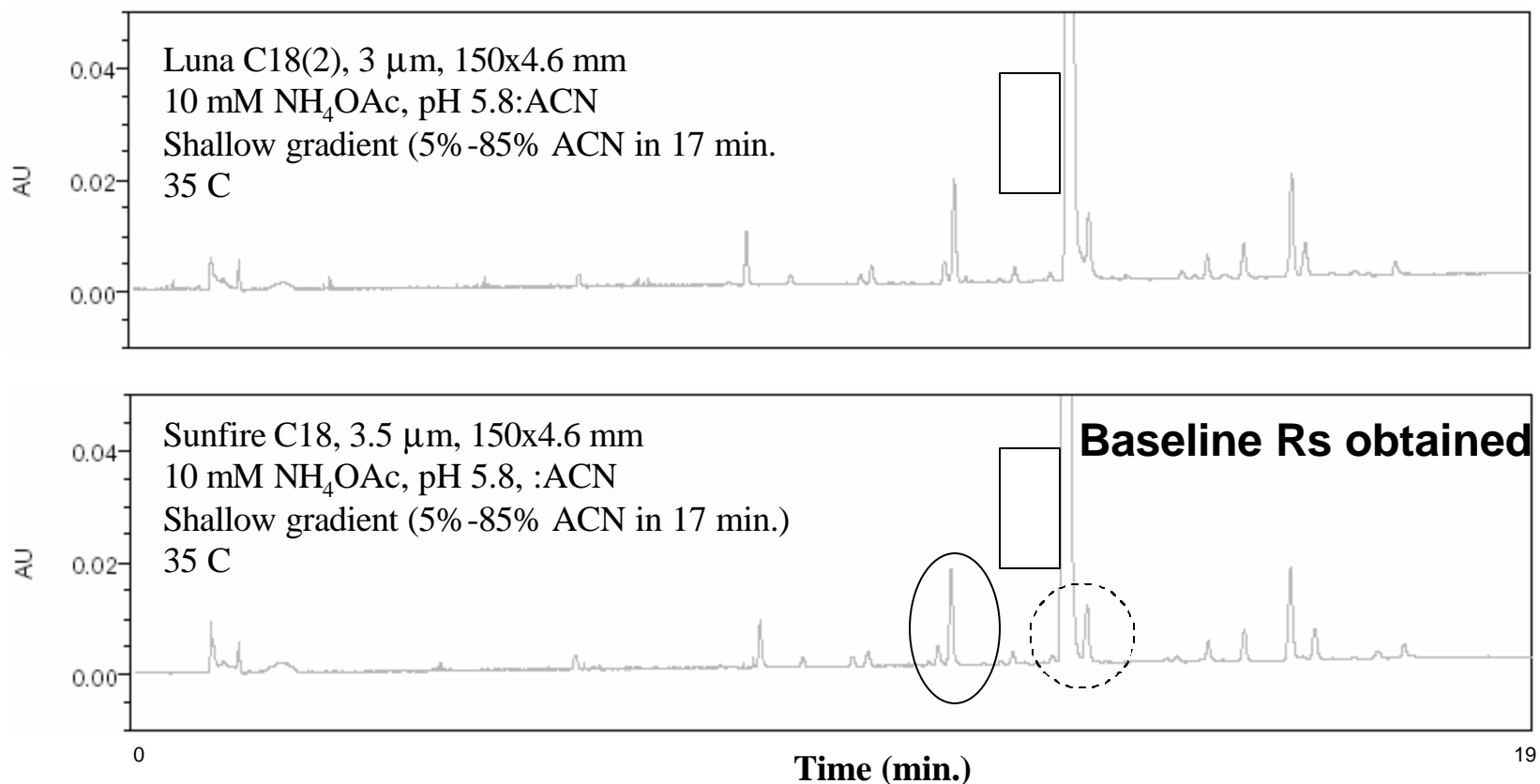
Sunfire C18, 3.5 μ m, 150x4.6 mm



- 1 – (Steep gradient) 5% - 85% Acetonitrile in 6 min
- 2 – (Shallow gradient) 5% - 85% Acetonitrile in 17 min
- 3 – (Steep gradient) 5% - 85% Acetonitrile in 6 min
- 4 – (Shallow gradient) 5% - 85% Acetonitrile in 17 min

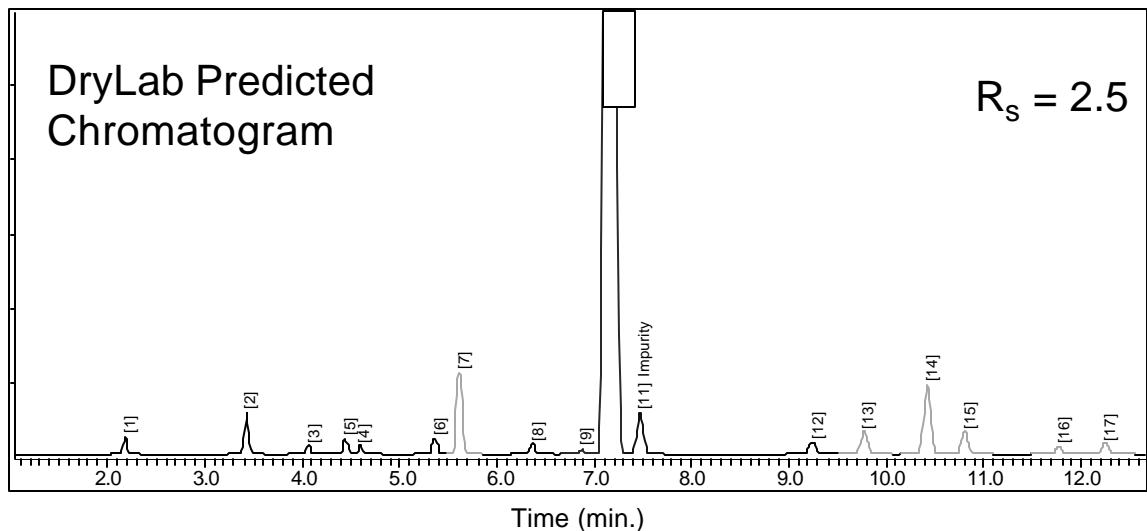
Flow rate=1.5 mL/min

AMDS for Method Optimization



- Acetonitrile is the preferred organic solvent for this separation.
- Greater resolution between the critical pair is observed on the Sunfire column.
- Further optimization is necessary to shorten run time.

Predicting Method Conditions Using DryLab



Gradient Conditions

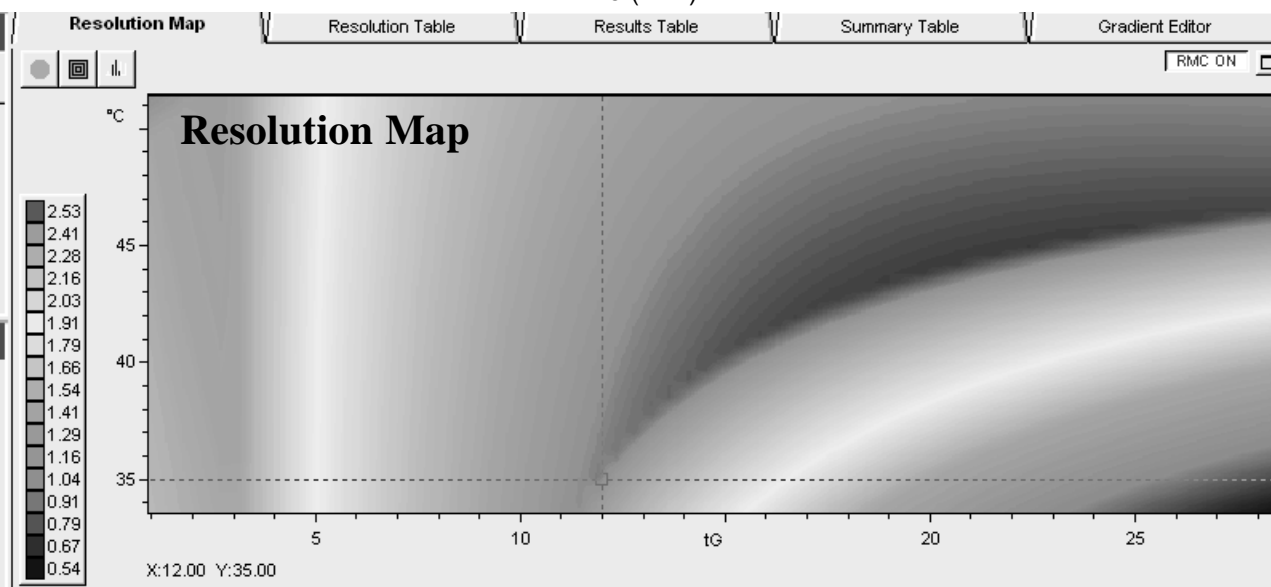
Time	%B
0	35
10	75
12	75

Col. Temp. = 35° C

Flow Rate = 1.5 mL/min

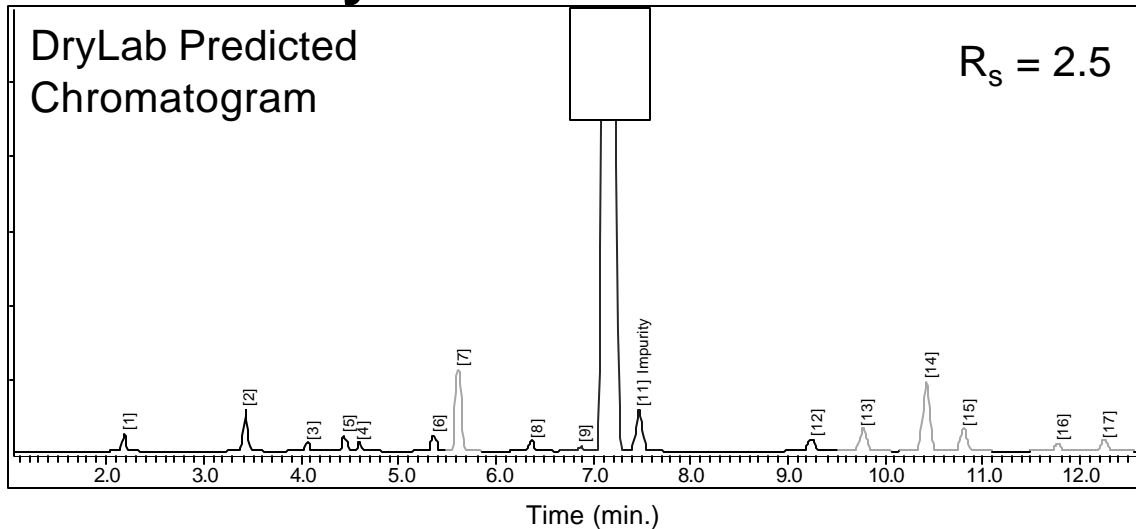
Sunfire C18, 3.5 μ m, 150x4.6 mm

10 mM NH_4OAc , pH 5.8, :ACN



- Entered chromatographic results into DryLab to predict minimum resolution between active and impurity.
- Used the resolution map to determine optimal gradient run time and temperature condition for analysis.

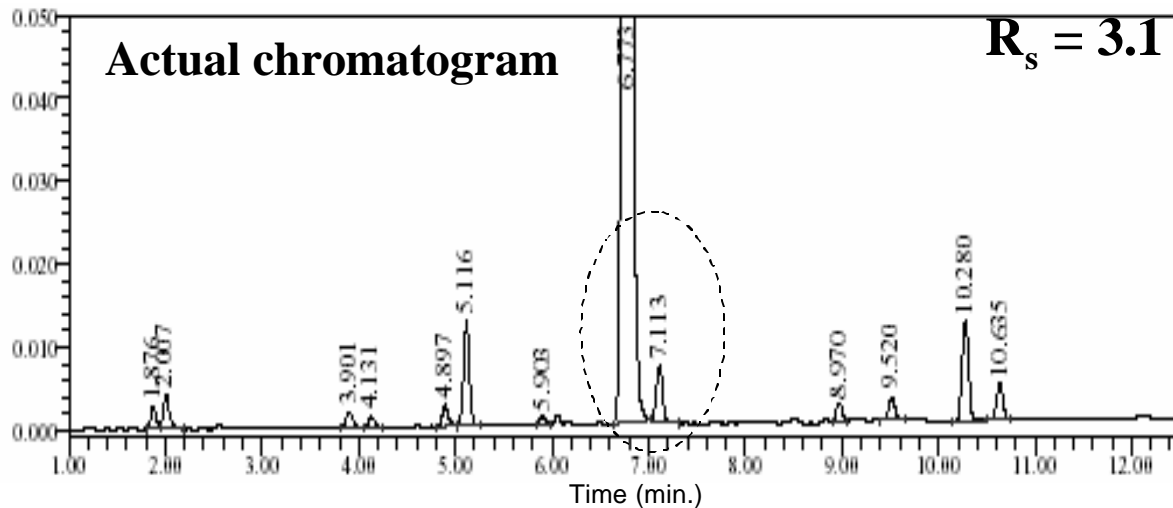
DryLab Prediction vs. Actual Chromatogram



Chromatographic Conditions:
Column: Sunfire C18, 3.5um, 150x4.6 mm
MP: Aqueous:Acetonitrile
Wavelength: 247 nm
Col. Temp.: 35° C
Flow: 1.5 mL/min
Inj. Vol.: 10 µL

	Time	Flow	%A	%B
1		1.5	65	35
2	10	1.5	25	75
3	12	1.5	25	75

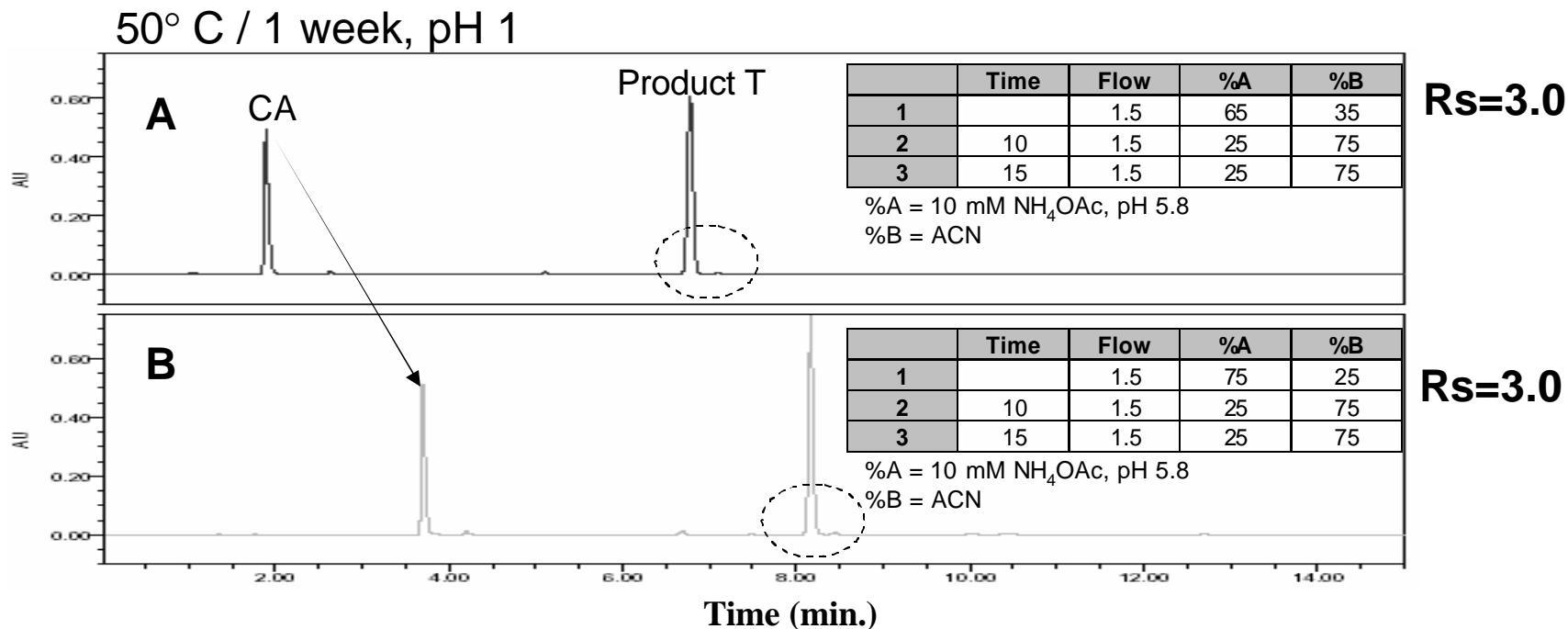
%A: 10 mM NH_4OAc , pH 5.8
%B: ACN



- DryLab predicted chromatogram and actual chromatogram are similar.
- Note that Drylab gives accurate predictions only if the analyte ionization state is not changing with increasing organic
- The run time was decreased without compromising the resolution between the active and the impurity.

Predict/Confirm possible deg. products of your analyte

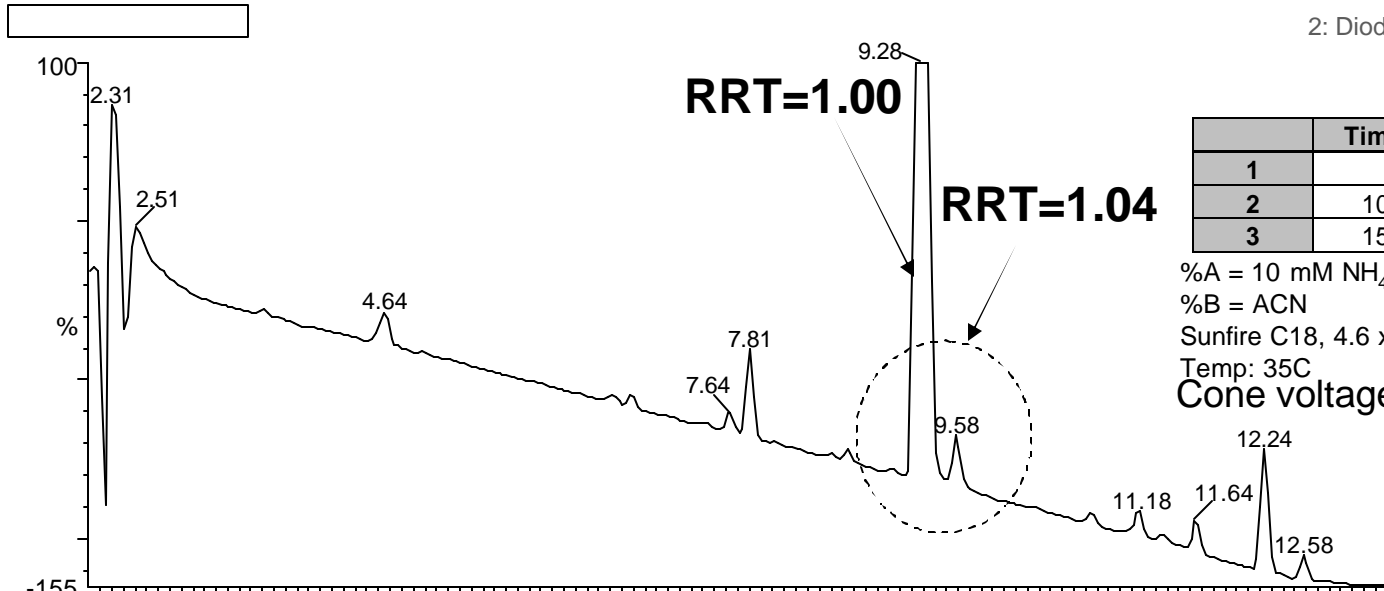
Forced Deg studies



- Sample was stressed at 50°C for 1 week in pH 1 diluent.
- Decreased the initial organic composition of the gradient from 35% to 25%.
- Carboxylic acid impurity has enhanced retention at lower organic.
- Resolution between active and impurity eluting after main component was not compromised.

MS analysis of Product T

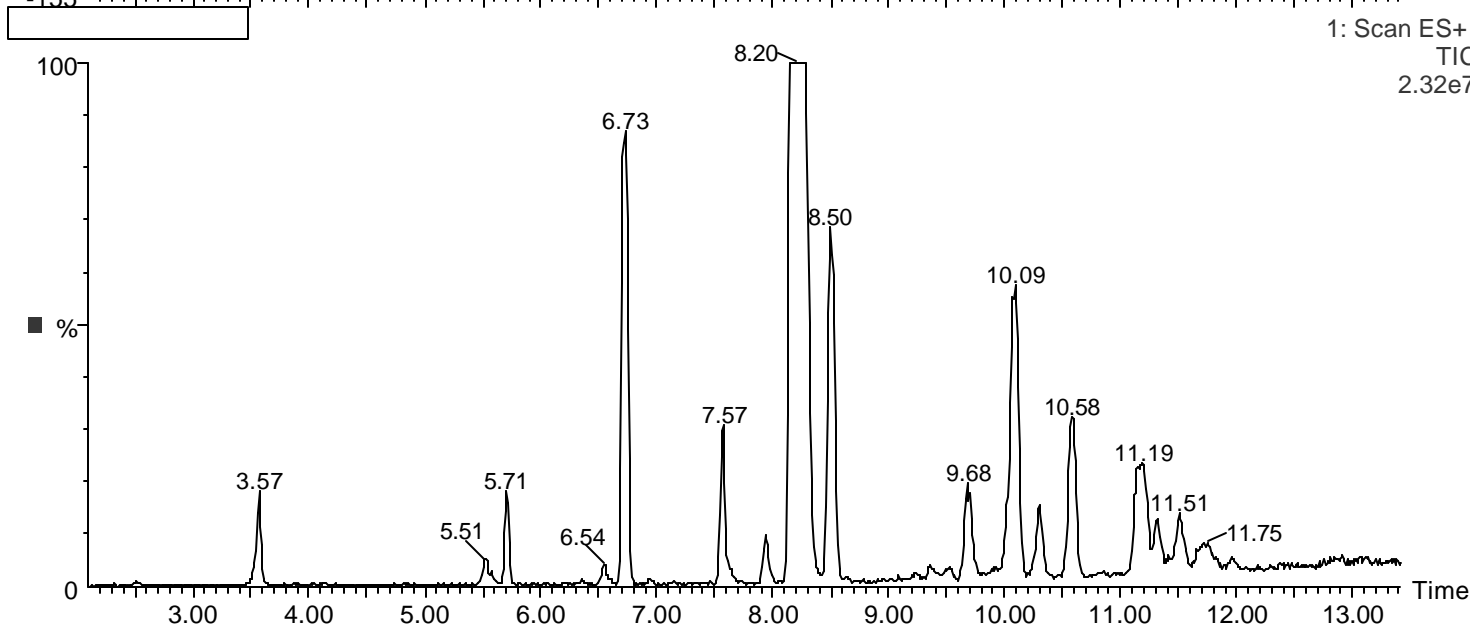
FB- 25CV +



2: Diode Array
TIC
3.67e6

	Time	Flow	%A	%B
1		1.5	75	25
2	10	1.5	25	75
3	15	1.5	25	75

%A = 10 mM NH₄OAc, pH 5.8
%B = ACN
Sunfire C18, 4.6 x 150 mm, Flow 1.5 ml/min, split 10:1
Temp: 35C
Cone voltage: 25 V, Single quad.

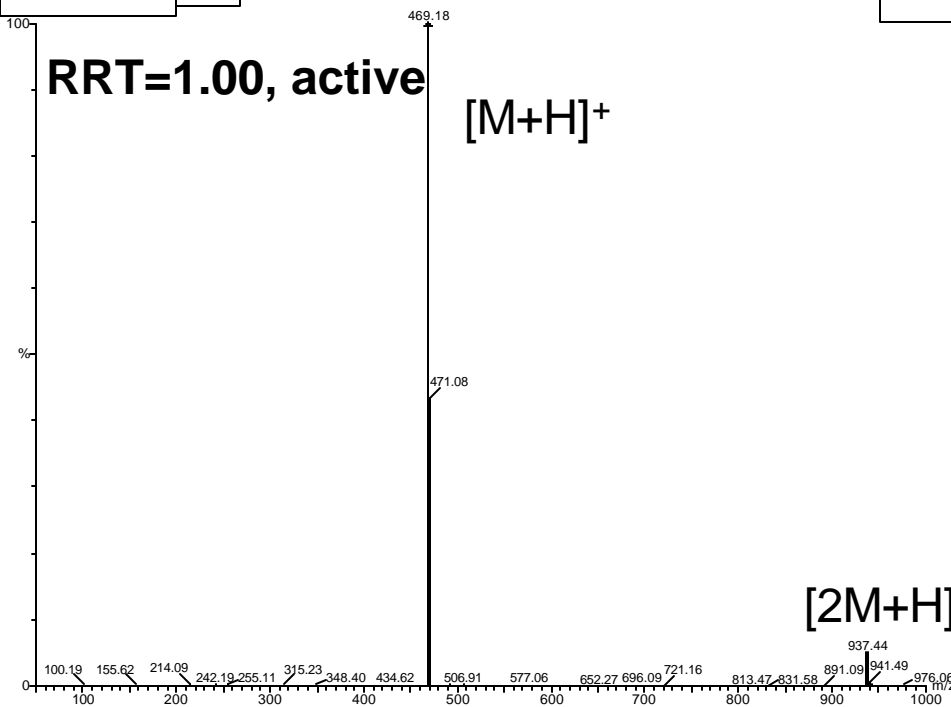


1: Scan ES+
TIC
2.32e7

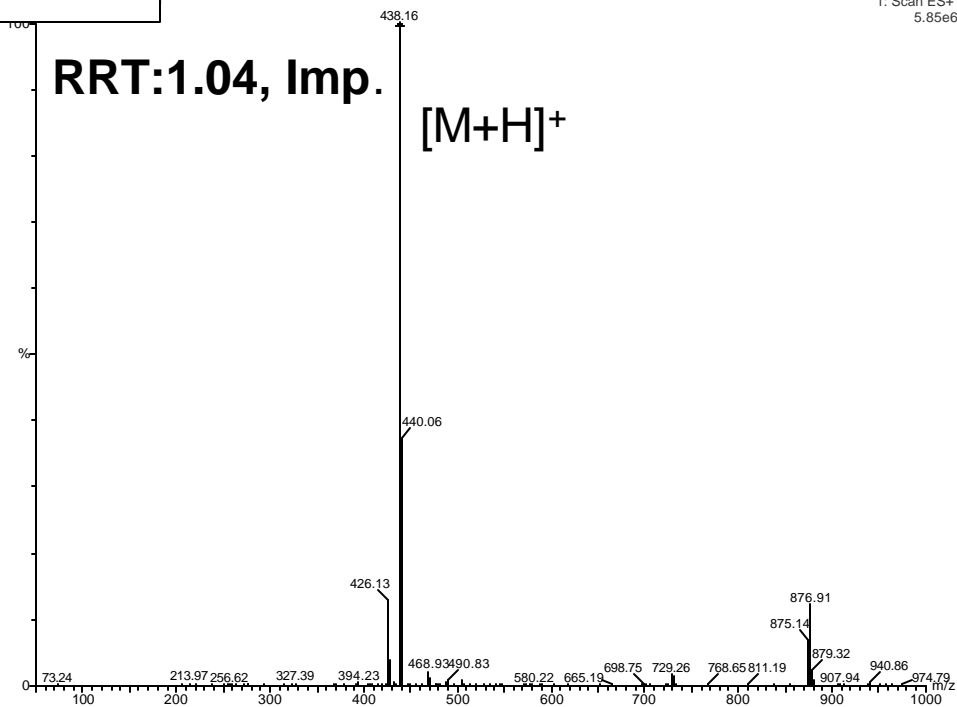
MS analysis confirmed the separation of the active from the MW 437 impurity

[Redacted]

1: Scan ES+
5.85e6



[Redacted]

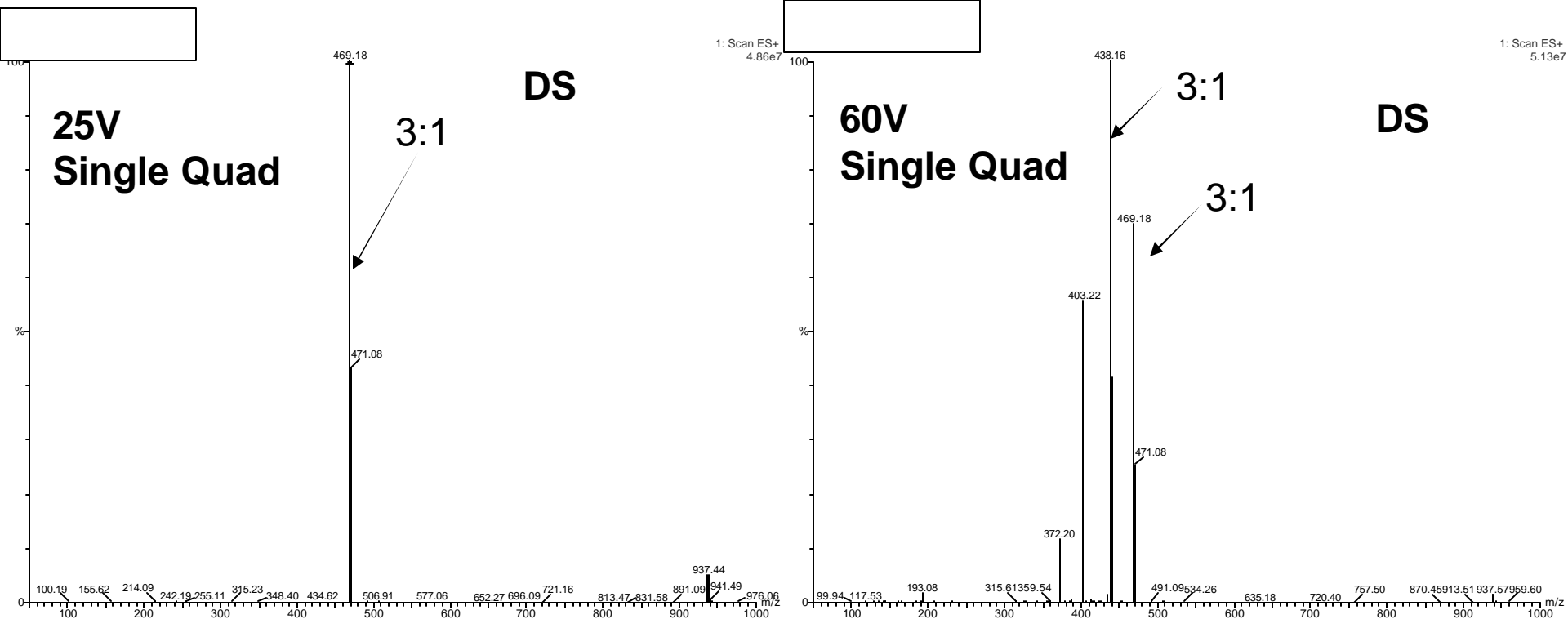


%A = 10 mM NH₄OAc, pH 5.8
%B = ACN
Sunfire, 4.6 x 150 mm, Flow 1.5 ml/min, split 10:1
Cone voltage: **25 V, Single quad.**

Product T
Monoisotopic mass: 468.2

Impurity at RRT 1.04
Monoisotopic mass: 437.2
[Redacted]

Evaluate the fragmentor voltage!!



- DS loses methyl amine (31 dalton) to form stable ion (m/z 438).
- Note if too high a fragmentor voltage used fragment could not be deconvoluted from RRT:1.04 impurity [M+H]= 438.
- The proposed fragmentation pattern was confirmed by MS/MS analysis and deuterated experiments.

Conclusion

- Use ACD to estimate pK_a of molecule and predict optimal pH for analysis
- Run steep gradient to predict isocratic conditions for further pH optimization
- Determine retention behavior of active as function of pH (isocratic)- use Acquity if possible
- Determine best mobile phase pH for further experiments
- Co-elution of impurities
 - Determine spectral purity with PDA spectra
 - LC-MS to elucidate spectral homogeneity (watch out for isomers)
 - Run all intermediates, precursors, forced deg. samples
- Method optimization
 - Use AMDS/Dry Lab for method optimization
 - Use MS to confirm the separation of active from possible co-eluting species
- MS/MS analysis can be performed for structural elucidation of impurities
- Deuterated experiments can be performed to support structural assignments

Acknowledgements

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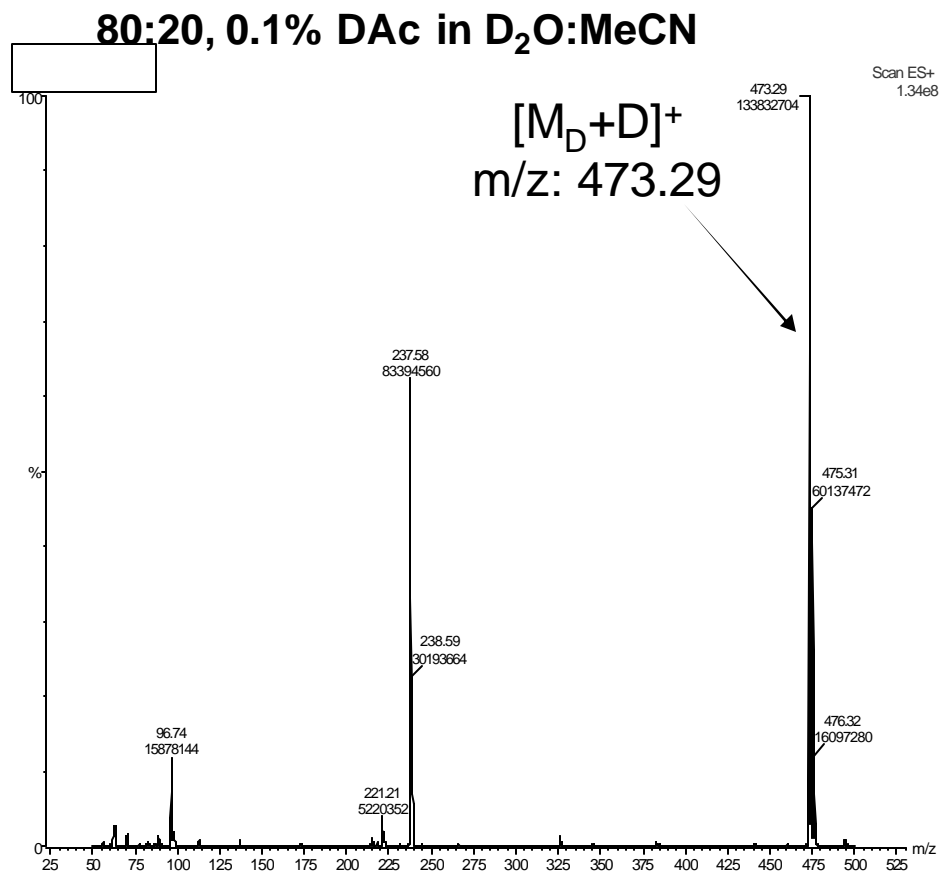
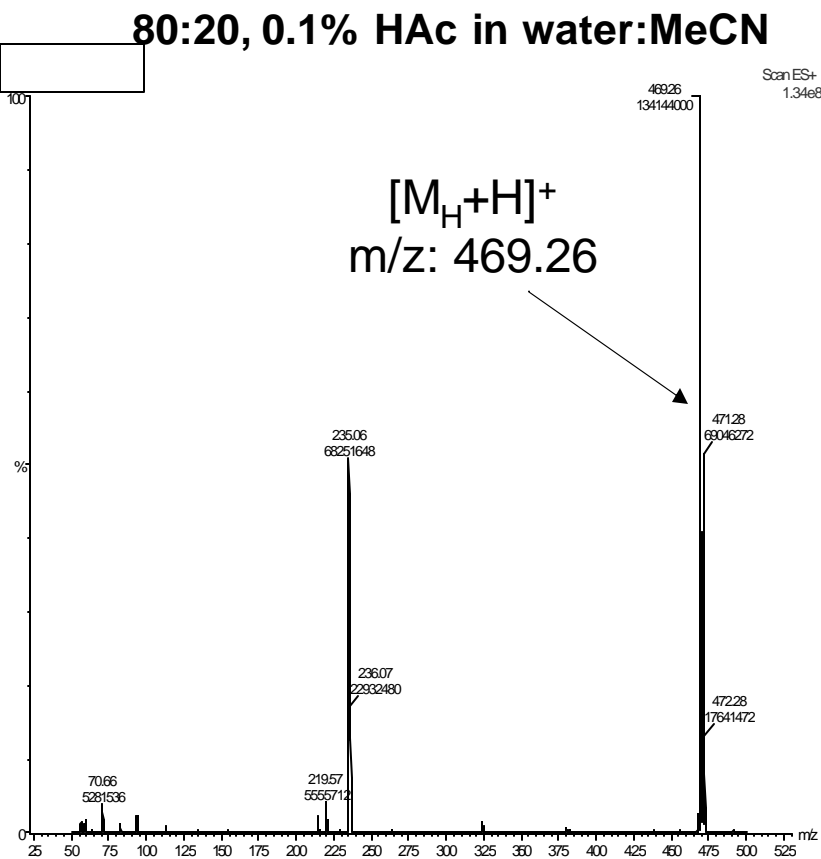
Background Slides

Deuterated experiments

- Deuterium-exchange experiments can also be used to further elucidate and define structural detail.
- The fine structural details of analytes could be further defined by a deuterium-exchange experiment that measures the number of exchangeable protons in each molecule.
- The number of exchangeable protons in a molecule can be determined based on the mass shift.
- This technique allows an understanding of which protons are susceptible to exchange, but also can be used to differentiate compounds of the same molecular weight that have a different number of exchangeable protons.

H/D Exchange ESI (+)

Product T: MW: 468

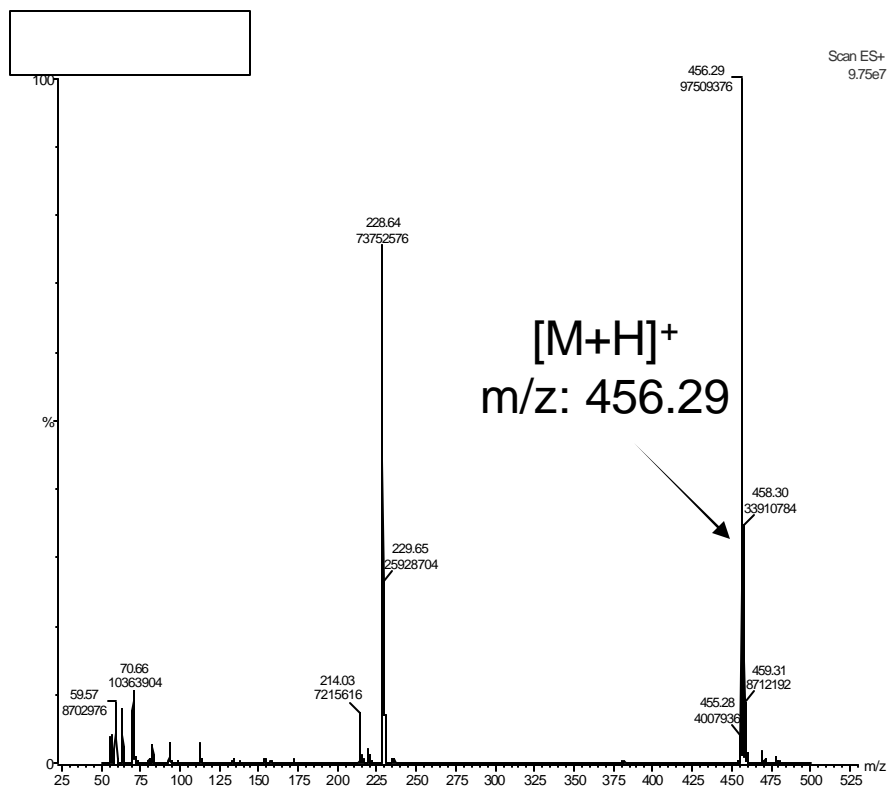


Deuterated exchange provides strong evidence to support degradation product and synthetic by-product elucidation.

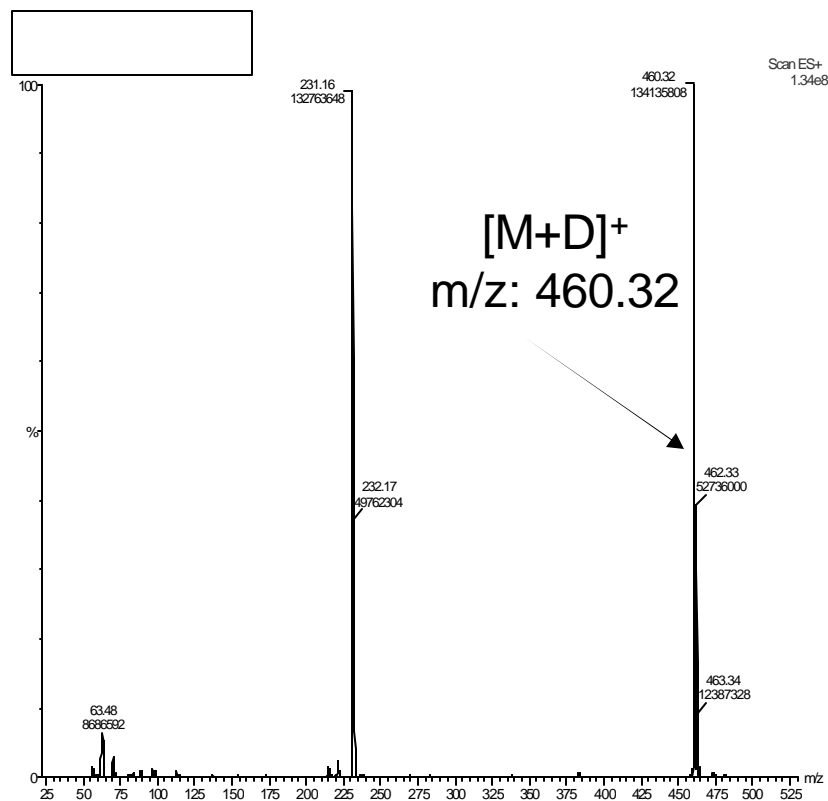
H/D Exchange ESI (+)

Product T COOH
Impurity MW: 455

80:20, 0.1% HAc in water:MeCN



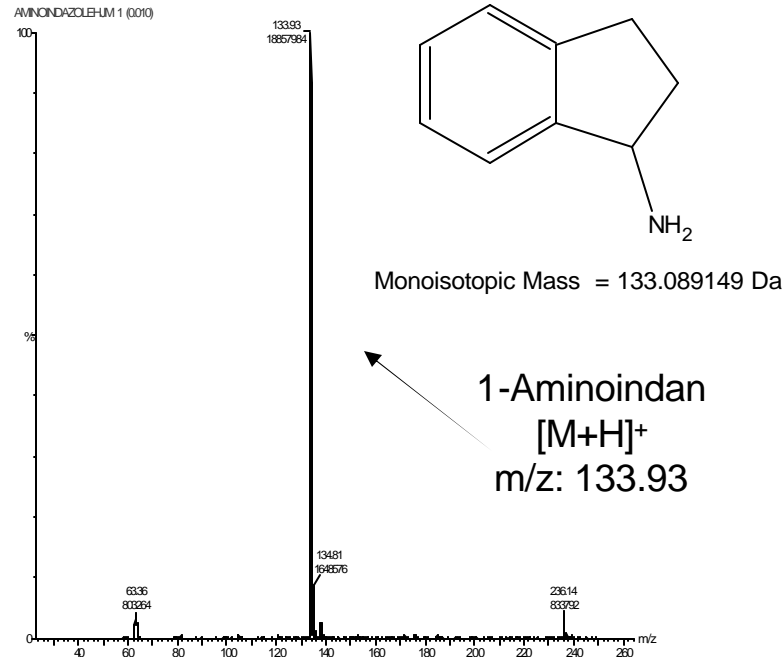
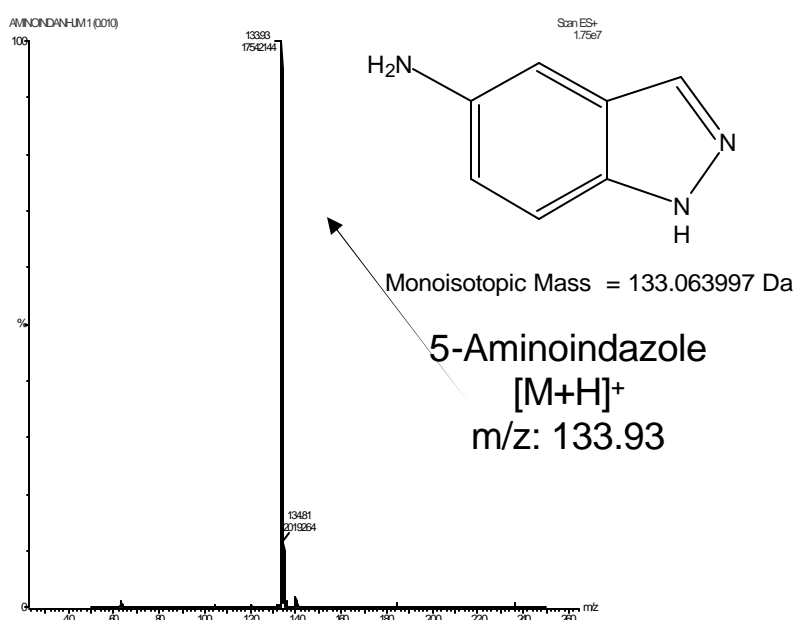
80:20, 0.1% DAc in D₂O:MeCN



Deuterated exchange provides strong evidence to support degradation product and synthetic by-product elucidation.

Case Study 1

Structural elucidation of compounds with the same mass

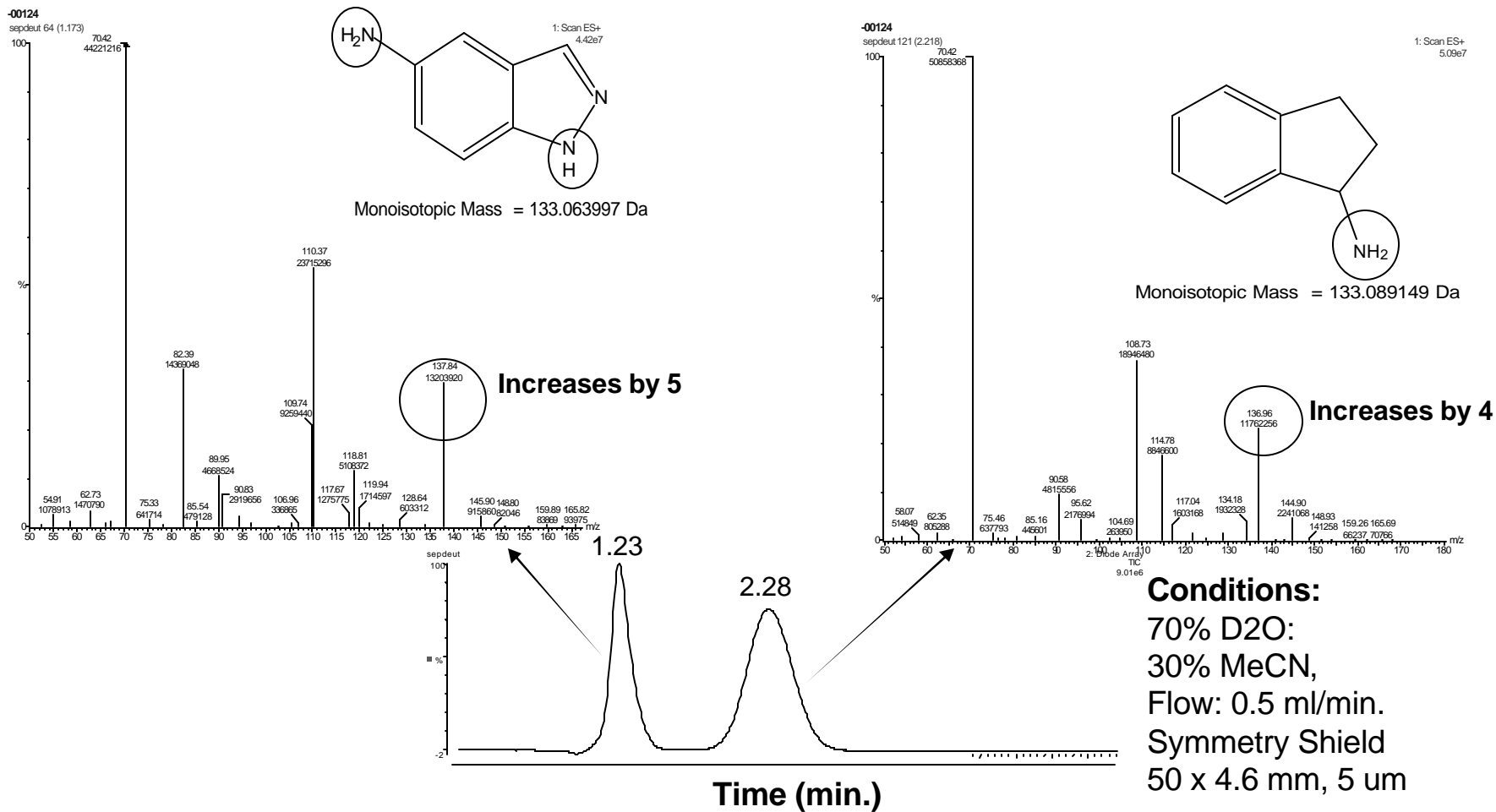


Mass spectra of these different analytes are similar and the same [M+H]⁺ ion obtained. Application of the “nitrogen rule” does not help (odd number of nitrogens in both).

Deuterium exchange might help in structural elucidation since 5-aminoindazole has 3 exchangeable protons and 1-Aminoindan has only two exchangeable protons.

80:20, 0.1% HAc in water:MeCN

HPLC analysis using on-line H/D exchange



- The mass of the first component on the chromatogram increases by 5 therefore 3 exchangeable protons are present, which suggests that this analyte is 5-aminoindazole.
- The mass of the second compound increases by 4 therefore only 2 exchangeable protons are present.
- Knowledge of the number of labile H atoms in a molecule is useful for comparing proposed impurity structures with that of the parent drug to determine the presence or absence of these functional groups.

HPLC References

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