HPLC Method Development

Systematic Approach vs Random Walk

Improving the Efficiency of Method Development and Optimization

William Champion
Agilent Technologies, Inc.

1-800-227-9770, opt 3, opt 3, opt 2

lc-column-support@agilent.com

OBJECTIVE

- Demonstrate a systematic approach to method development
- Improve understanding of separation process
- Development of more robust methods
- More efficient than “random walk”

OUTLINE

- Definitions - column parameters

- Systematic Approach
  - Comments
  - Experimental Designs
  - Example

Some Basic Chromatography Parameters

- **Resolution (R_S)**
- **Retention Factor (k)**
- **Selectivity or Separation Factor (α)**
- **Column Efficiency as Theoretical Plates (N)**

Definition of Resolution

$$R_s = \frac{t_{R\text{-}2} - t_{R\text{-}1}}{(w_2 + w_1)/2} = \frac{\Delta t_R}{\bar{w}}$$

Resolution is a measure of the ability to separate two components

Resolution …

Determined by 3 Key Parameters – Efficiency, Selectivity and Retention

The Fundamental Resolution Equation

$$R_S = \frac{\sqrt{N}}{4} \frac{(\alpha - 1)}{\alpha} \frac{k}{(k+1)} = \frac{\Delta t_R}{\overline{w}}$$

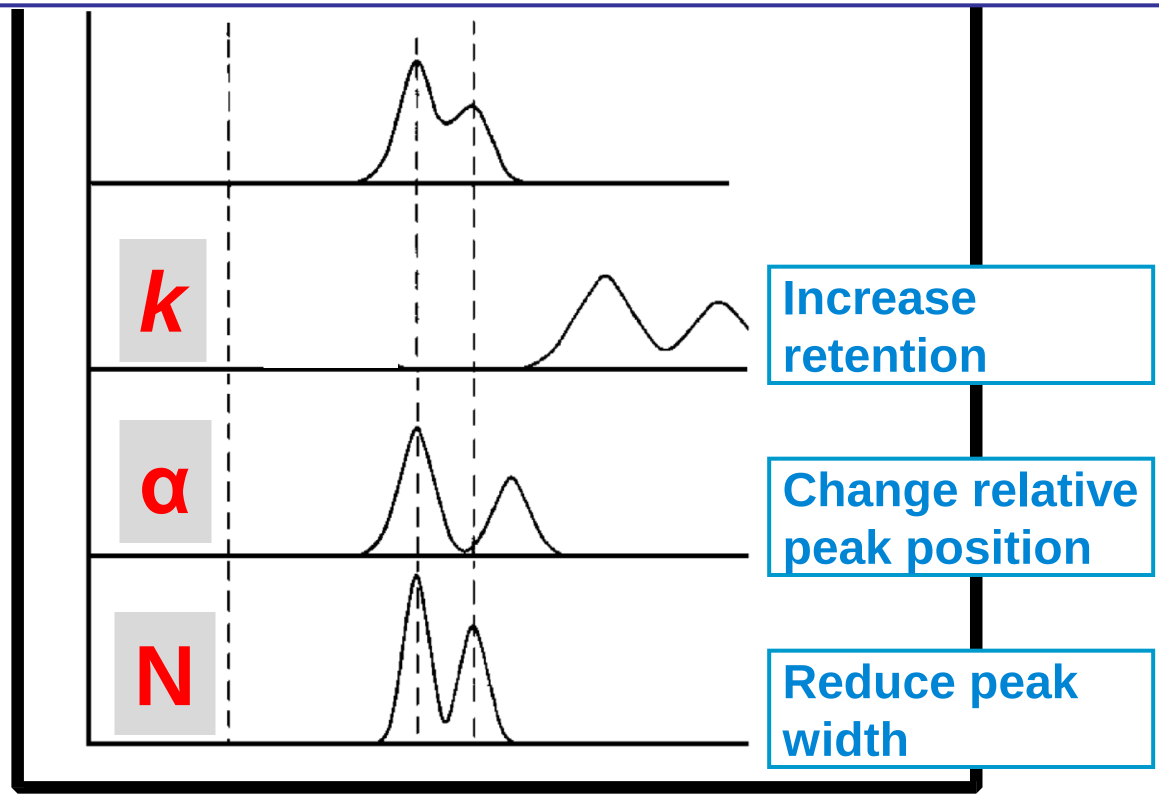
N = Column Efficiency – Column length and particle size

α = Selectivity – Mobile phase and stationary phase

***k* = Retention Factor** – Mobile phase strength

Factors that Improve Resolution

$$R_S = \frac{\sqrt{N}}{4}\,\frac{(\alpha-1)}{\alpha}\,\frac{k}{(k+1)} = \frac{\Delta t_R}{\overline{w}}$$



Factors that Improve Resolution

$$R_s = \frac{\sqrt{N}}{4}\,\frac{(\alpha-1)}{\alpha}\,\frac{k}{(k+1)} = \frac{\Delta t_R}{\bar{w}}$$

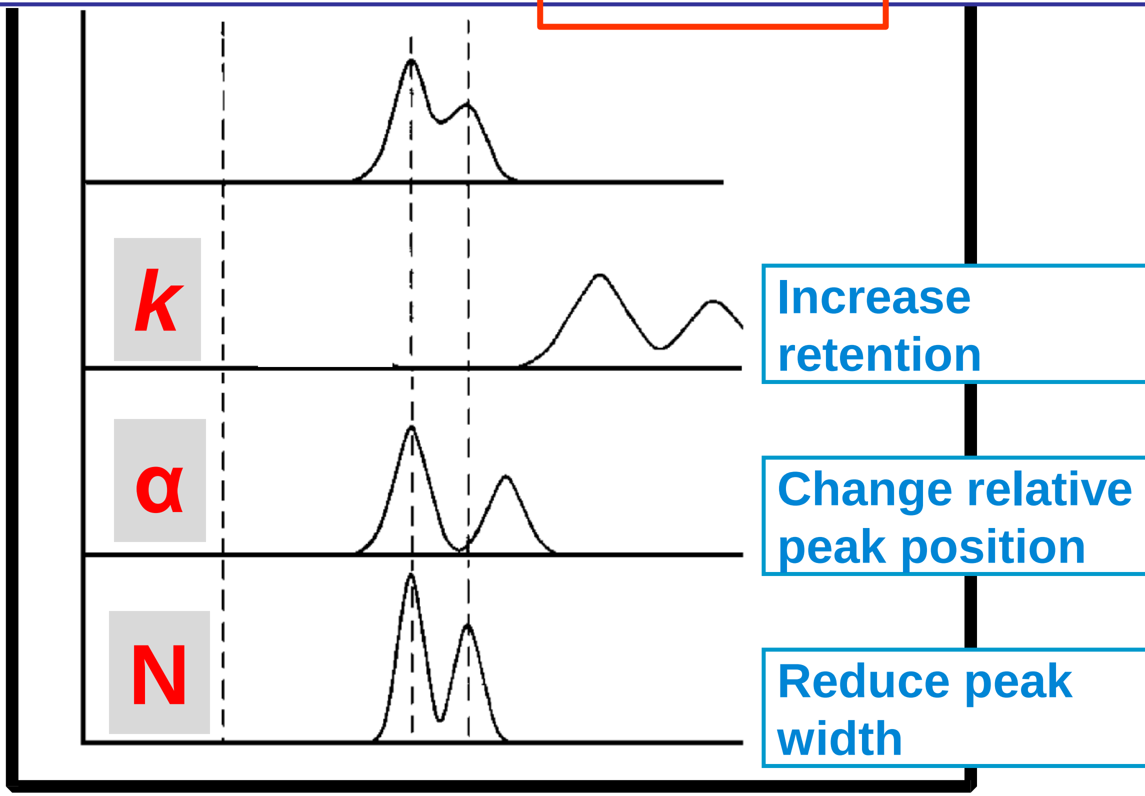
$k = (t_R - t_0) / t_0$

$k = t_s / t_m$

$\alpha = k_2 / k_1$

$\alpha = t_{s2} / t_{s1}$

$N = 16\,(t_R / w)^2$



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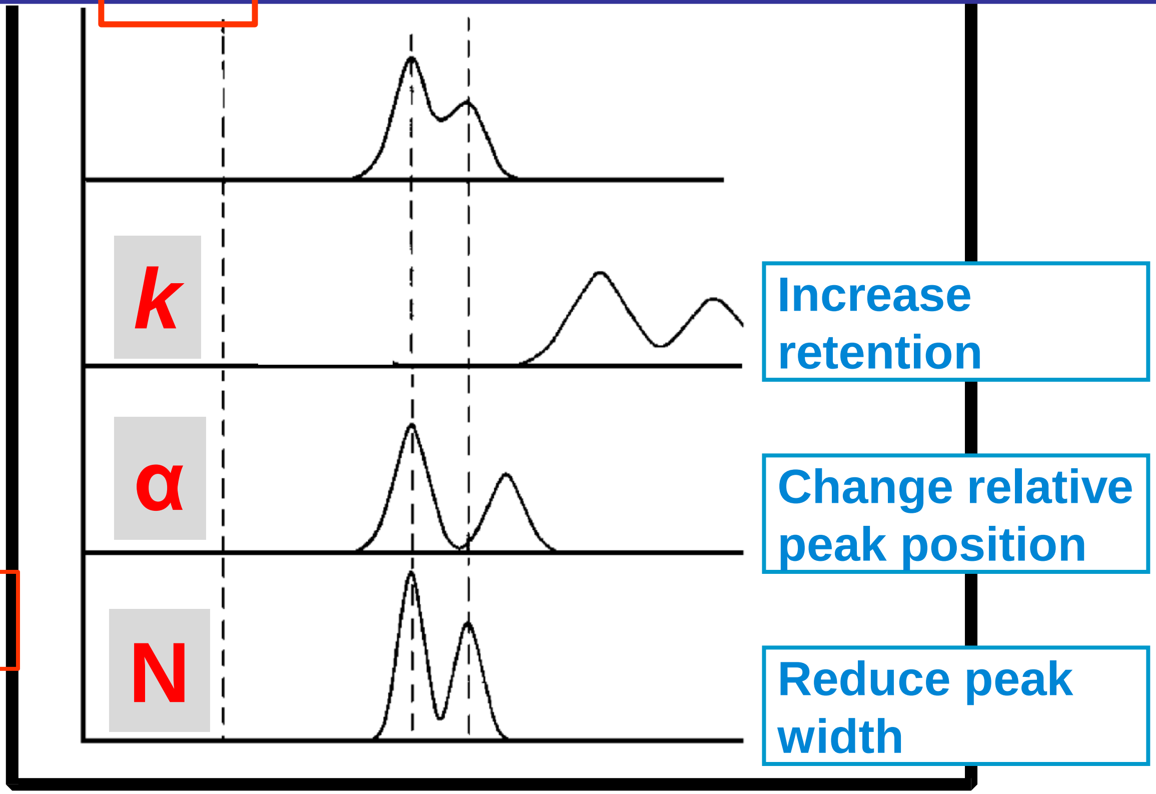

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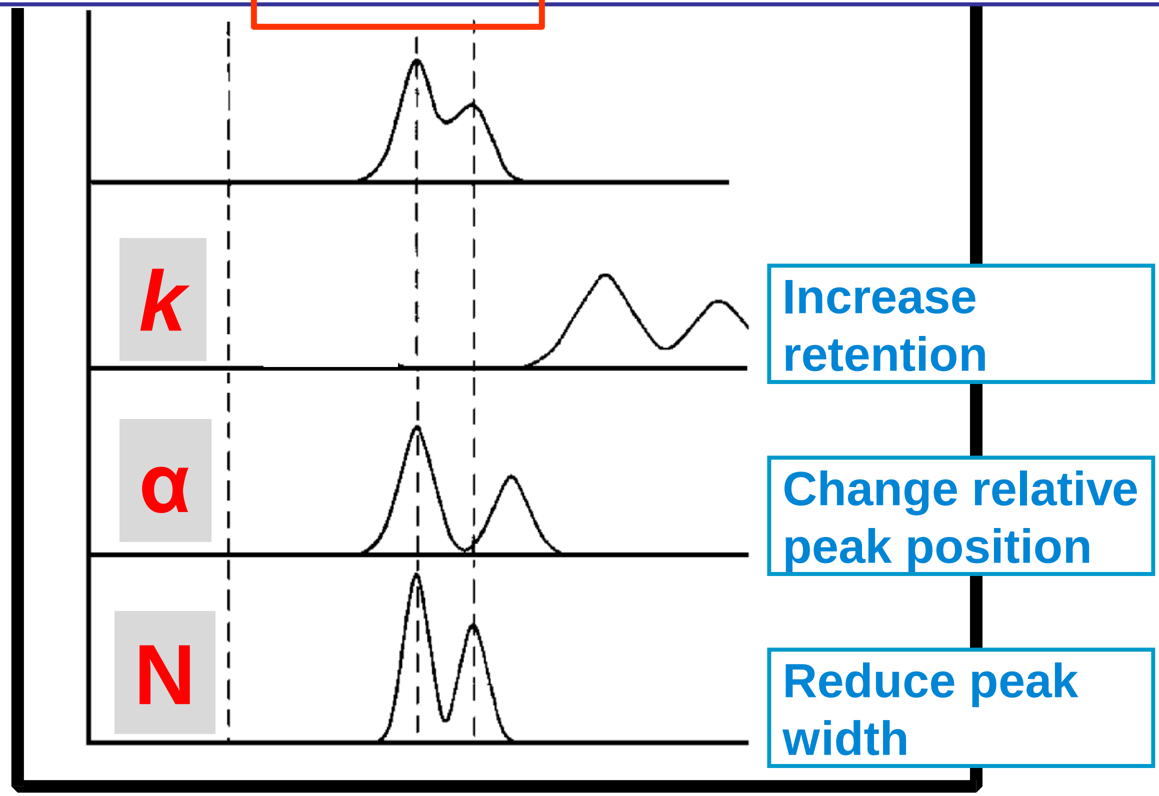

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- **Systematic Approach**
    - **Comments**
    - **Experimental Designs**
    - **Example**

SYSTEMATIC APROACH

Experiments Are "Front-end Loaded"

Experiments Provide Easier to Interpret Results

Insight into how to adjust conditions to affect separation

Pre-validation

SYSTEMATIC APROACH

“Front-end Loaded” Experiments:

- Know what experiments to perform
- Often can be run in groups or block (e.g. overnight)
- Can be intimidating because it looks like a lot of work
- More complicated designs looks like statistics

SYSTEMATIC APROACH

Results Easier to Interpret:

- Indicate where optimum is/is not
- Provides insight into trade-offs
- Tells about Sensitivity/Robustness to small parameter changes
- What conditions are important/what are not important (*e.g.*, buffer)
- Easier to follow confusing interactions ("connect the dots")

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“RANDOM WALK”

- Uncoordinated series of experiments
- Poor understanding of the interaction of parameters
- Can achieve acceptable separation - but not understand “why”
- Do not know if complicated conditions are necessary
- Does not provide insight into sensitivity of modifications to the conditions (*i.e.*, robustness)

SYSTEMATIC APROACHES

Algorithm driven or search techniques

Models based upon fundamental understanding of the chromatographic process

Experimental design

Experimental Designs

Gradient screening

Isocratic separation – vary mobile phase strength

Simple screening design

Factorial design

OUTLINE

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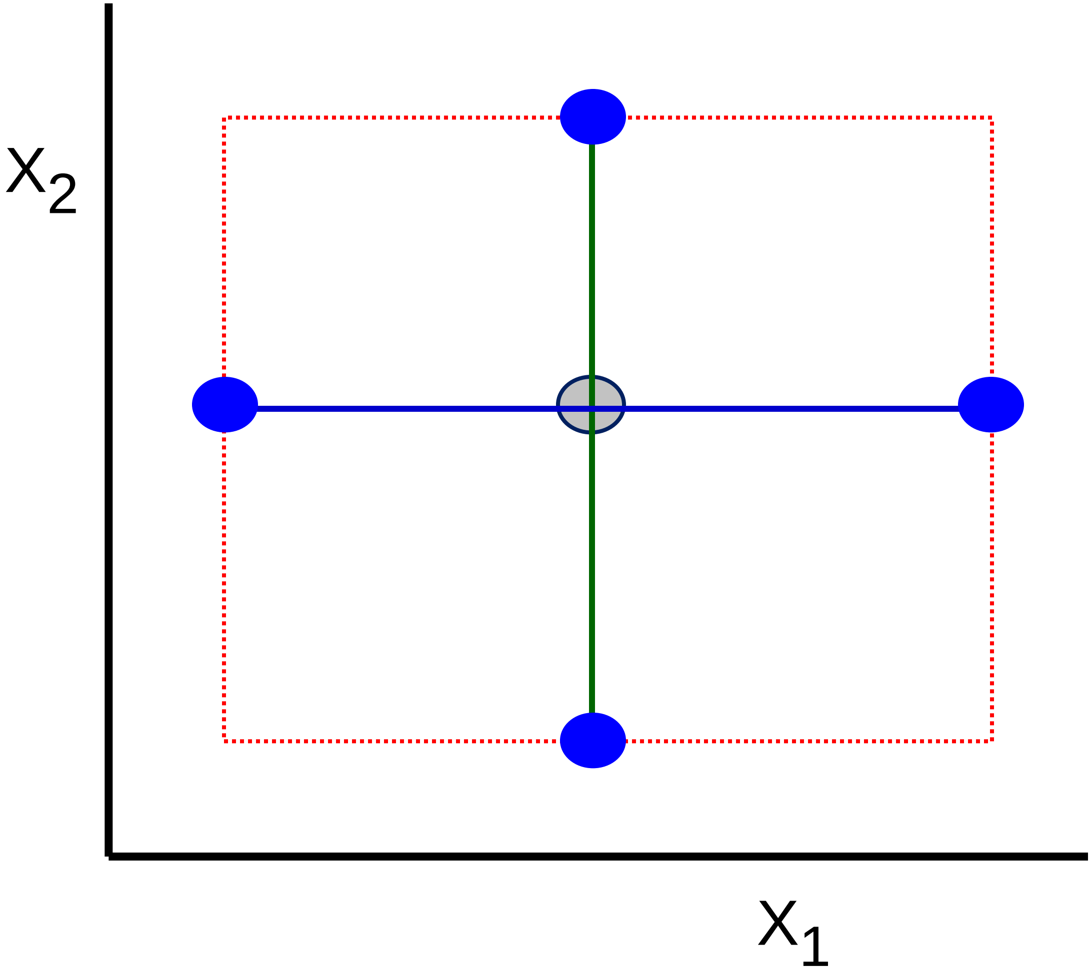
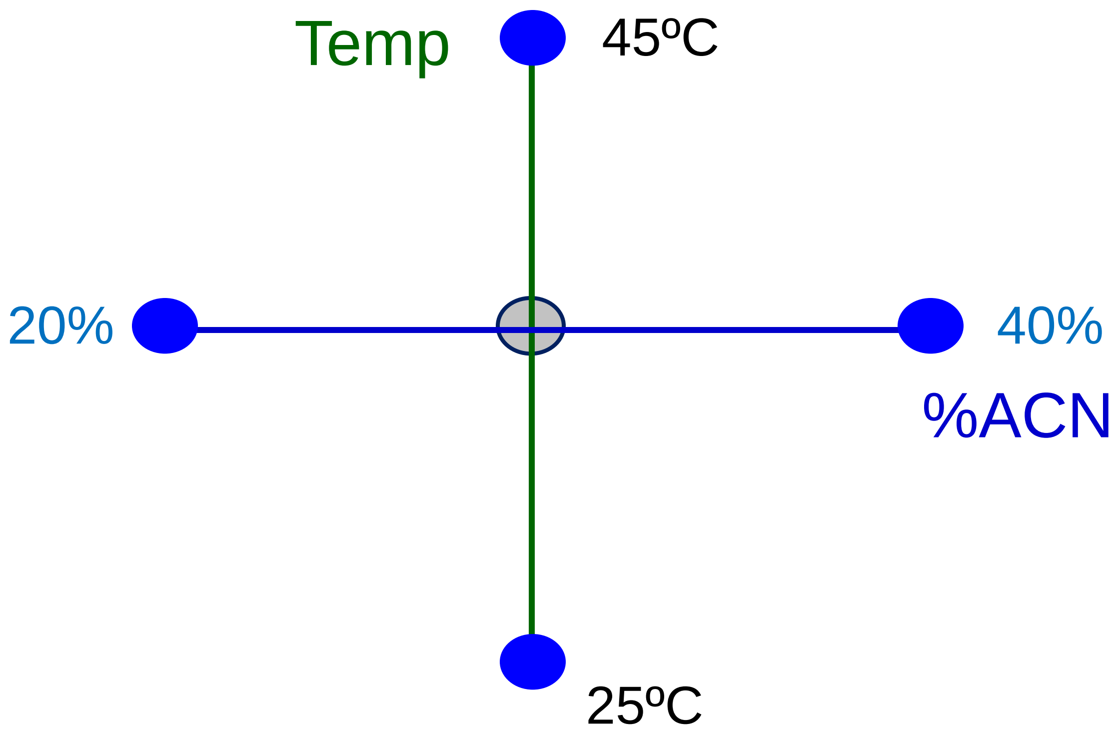
EXPERIMENTAL DESIGN

- Well understood
- Efficient, information rich
- Provides insight into fundamental behavior
- Experiments can be run in blocks
- Data easy to present and interpret
- Easy to compare effects of individual components
- Possible to determine if there is interaction

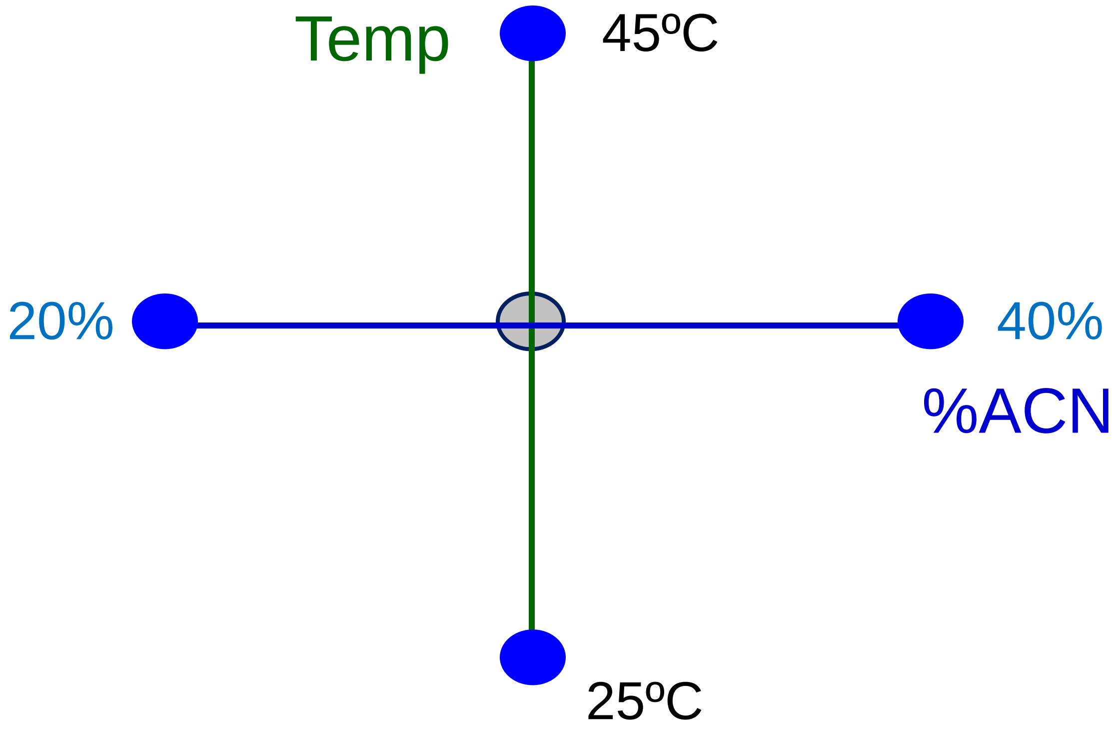
Main Effects – "One Factor at a Time"



Main Effects

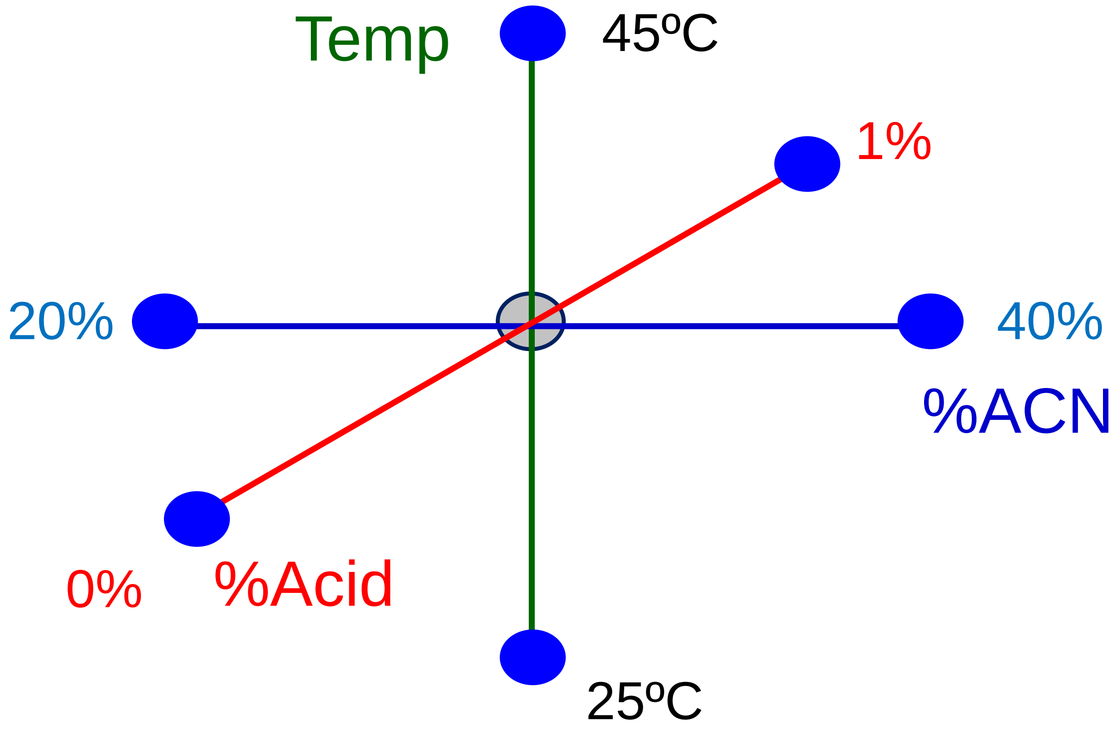


Main Effects



Number of Exp's = 2 x n = 4

Main Effects

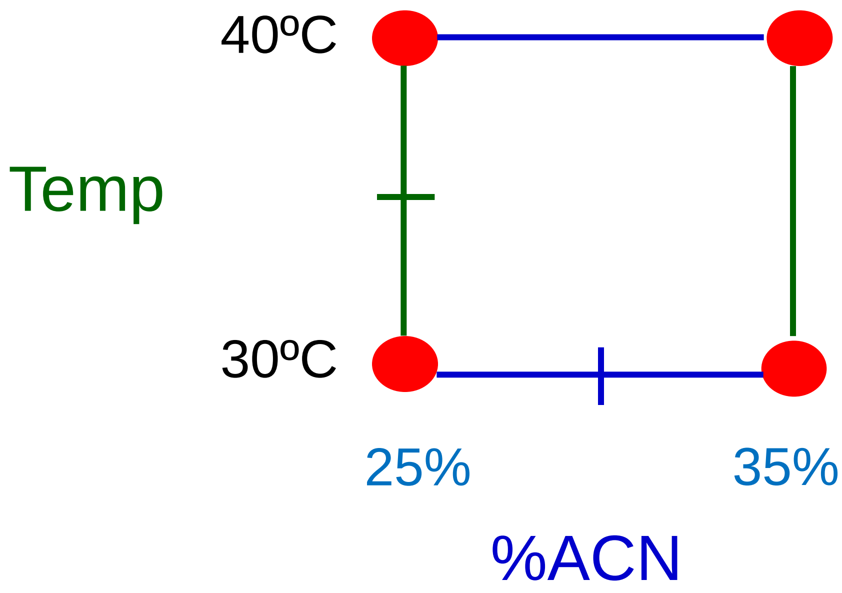


Number of Exp's = 2 x n = 6

Single Factor Design

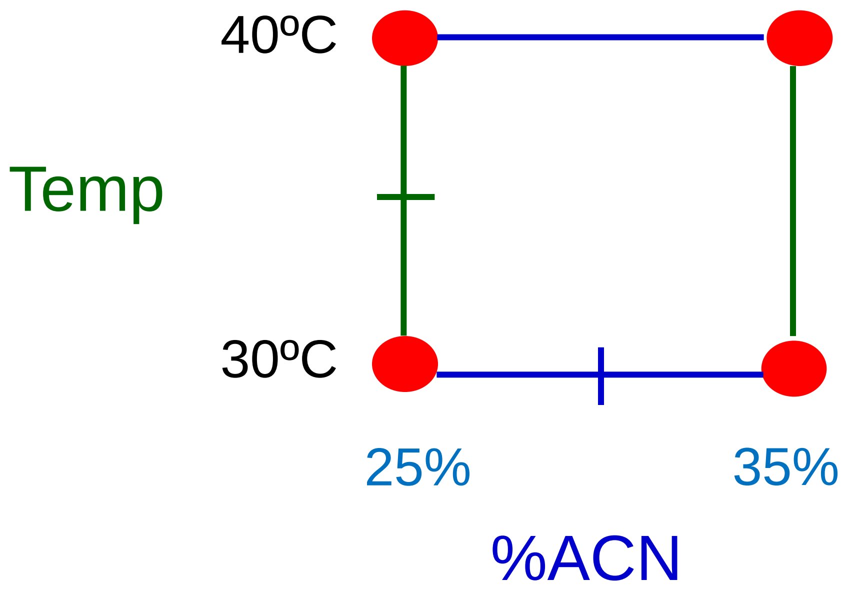
- Few experiments
- Adding a factors only adds two experiments
- Isolate the effect of each factor
- Data easy to present and interpret
- Good screening design
- Unfortunately, does not provide information on interaction of factors

Factorial Design for Two Factors

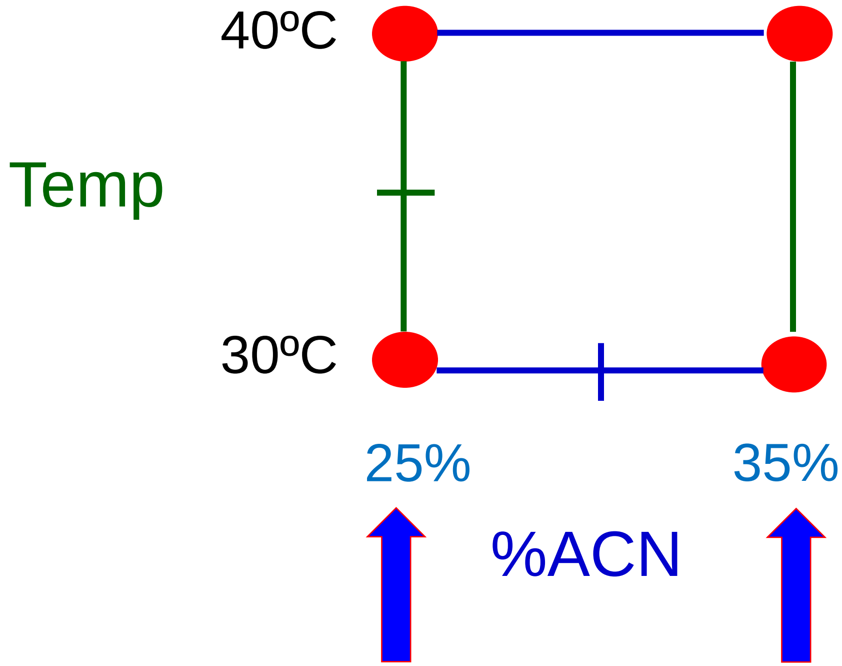


Factorial Design for Two Factors

Provides information at Different Levels

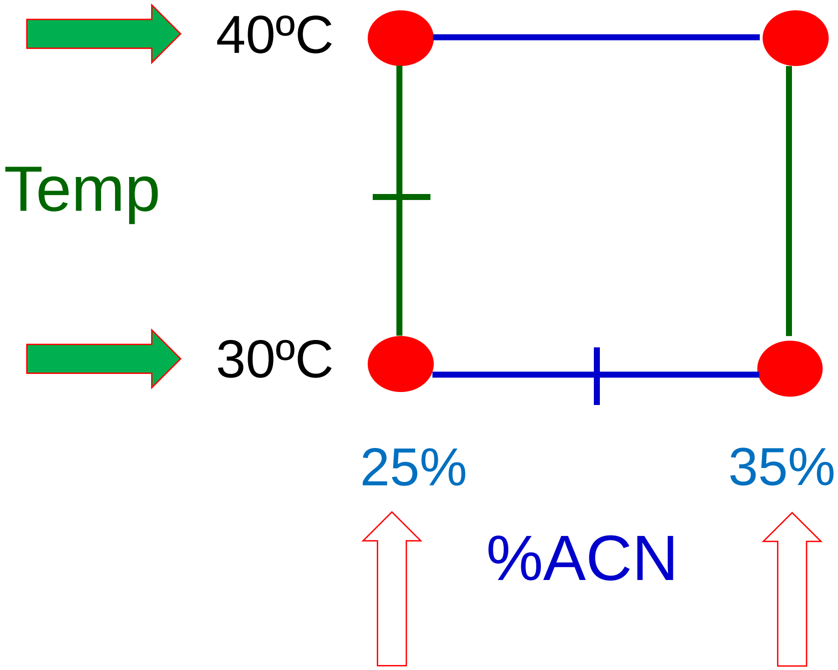


Factorial Design for Two Factors
Looking for Interaction of Factors



Effect of Temperature at 25% and 35% ACN

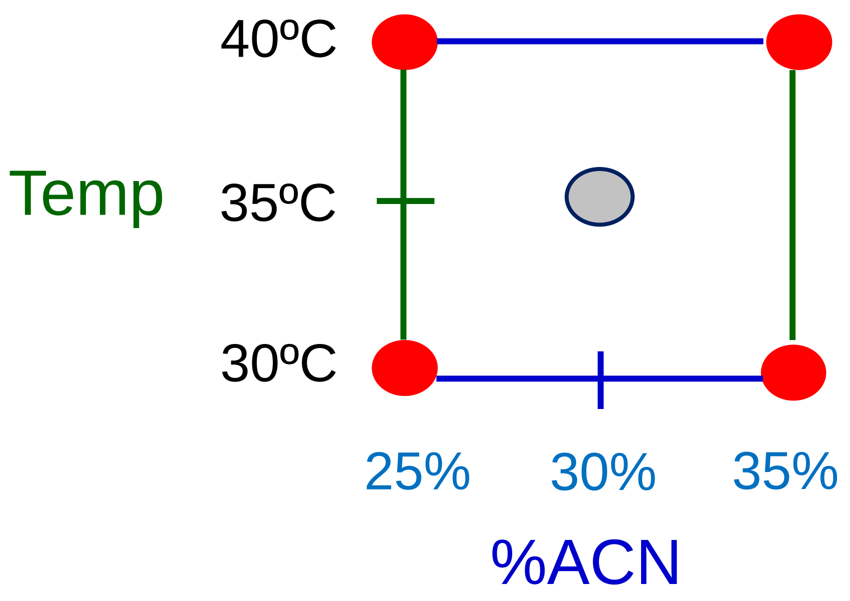
Factorial Design for Two Factors
Looking for Interaction of Factors



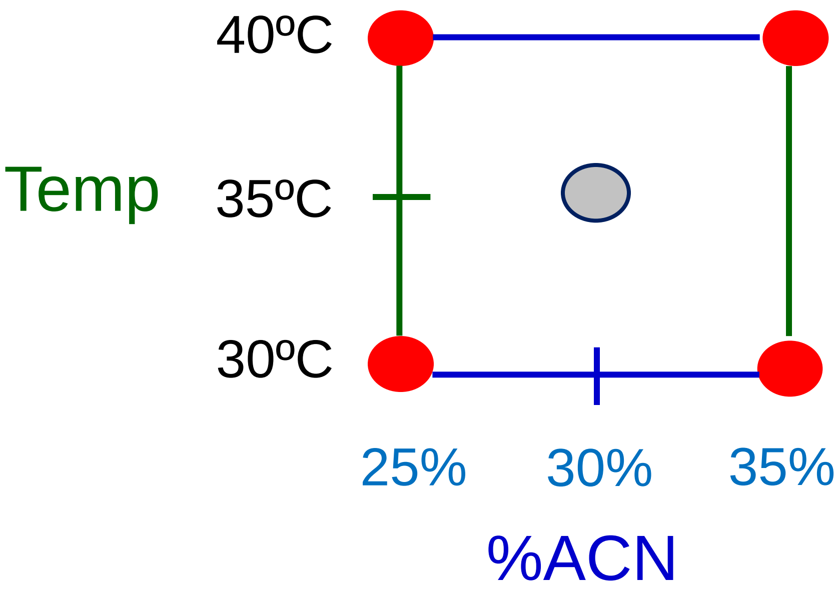
… And Effect of ACN at 30º and 40ºC

Factorial Design

For More Detailed Understanding

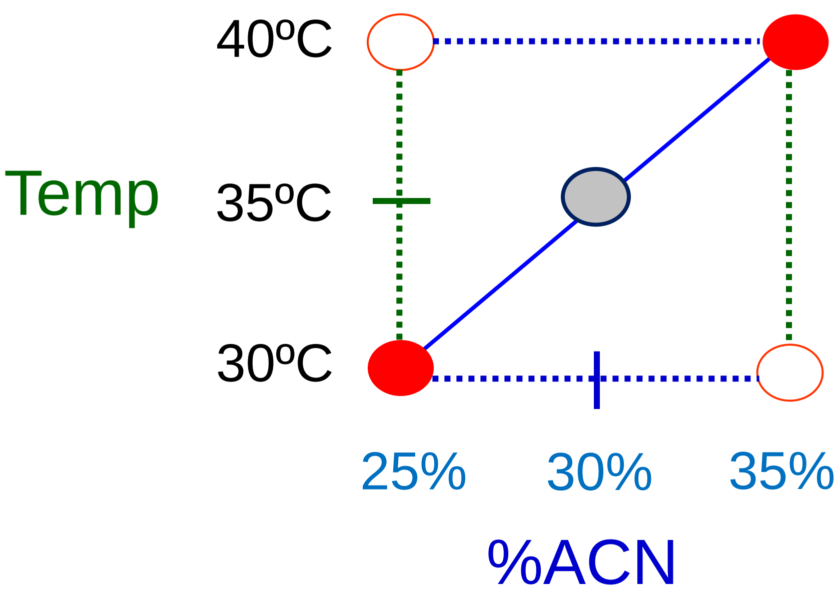


Factorial Design for Two Factors With Center-Point - Orthogonal Design

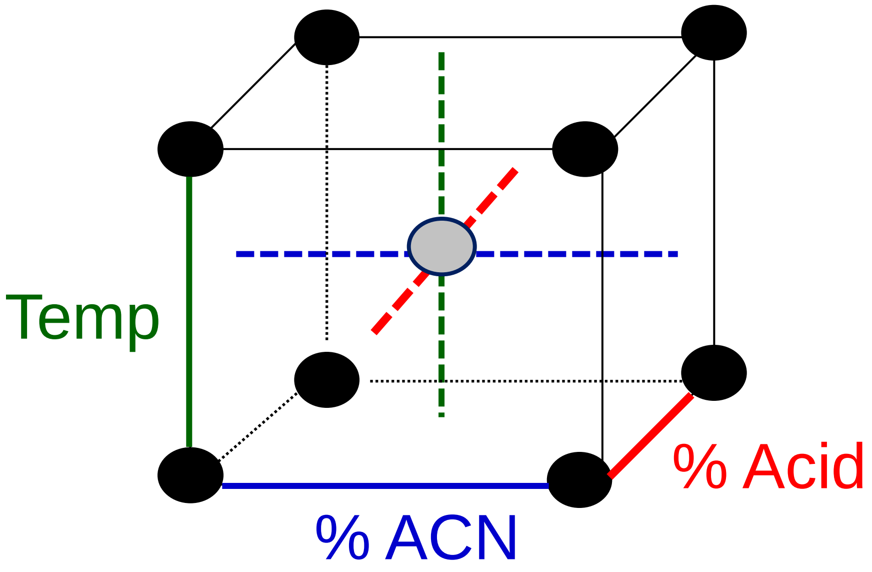


Non-Orthogonal Design for Two Factors

(25%, 30ºC), (30%, 35ºC) and (35%, 40ºC)



Factorial Design for Three Factors with Center Point



3 Factors, $2^3 + 1 = 9$

OUTLINE

- Definitions - column parameters
- **Systematic Approach**
  - Comments
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PROCEDURE

Decide upon the objective

What are the properties of the analyte(s)

Decide upon detector, column, mp, etc.

Literature search?

Perhaps perform a few familiarization experiments to get feel for behavior?

Gradient (e.g., 10% to 90% ACN/20 min)

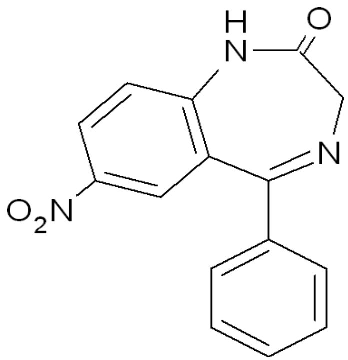
Plot effect of mobile phase on retention

Perform more detailed exp's if necessary

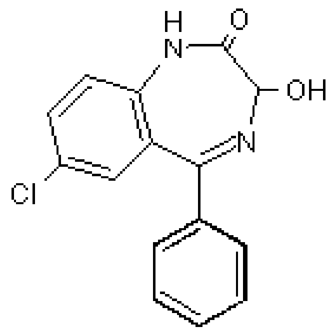
Decide upon the objective

- What is the objective?
  (assay, det. impurities, prep, trace level)
- What is important?
  (time, peak capacity, LOQ/LOD)
- What kinds of constraints?
  (equipment, sample properties)

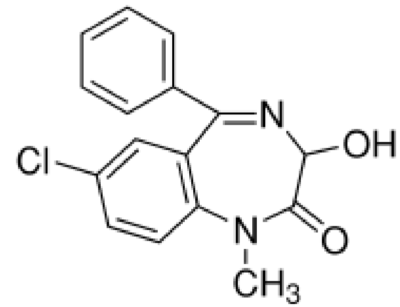
The Analytes - Benzodiazepines



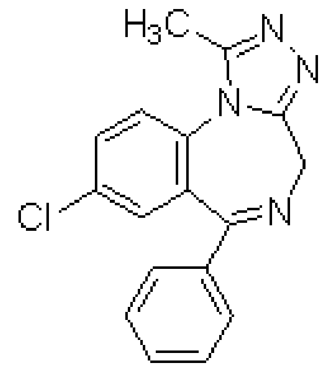
Nitrazepam



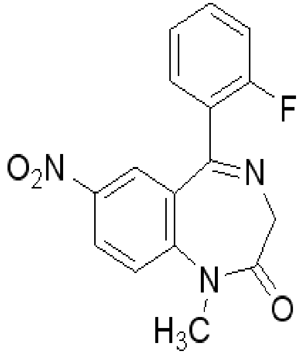
Oxazepam



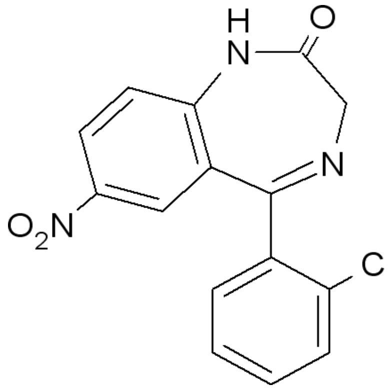
Temazepam



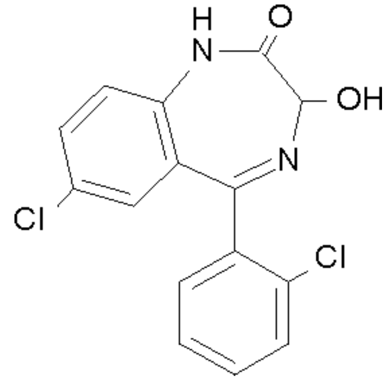
Alprazolam



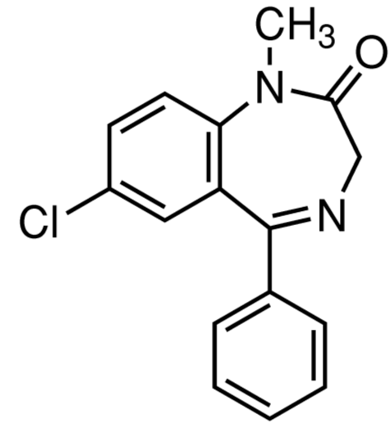
Flunitrazepam



Clonazepam



Lorazepam



Diazepam

Decide upon detector, column, mp, etc

- **Column (*e.g.*, C18, phenyl, CN, etc.)**
- **Mobile Phase Solvents (*e.g.*, MeOH, ACN)**
- **Acid (*e.g.*, HOAc, Formic, TFA, H_3PO_4, none)**
- **Buffer (*e.g.*, acetate, formate, none)**
- **Temperature**

Separation of Eight Benzodiazepines

Model Compounds

Single Column
(Poroshell 120 EC-C18, 3.0 x 100 mm)

Single Organic Solvent
(prefer ACN)

Simple Acid/Buffer
(formic acid, ammonium formate, H_3PO_4)

PROCEDURE

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Experimental Designs

Gradient screening

Isocratic separation – vary mobile phase strength

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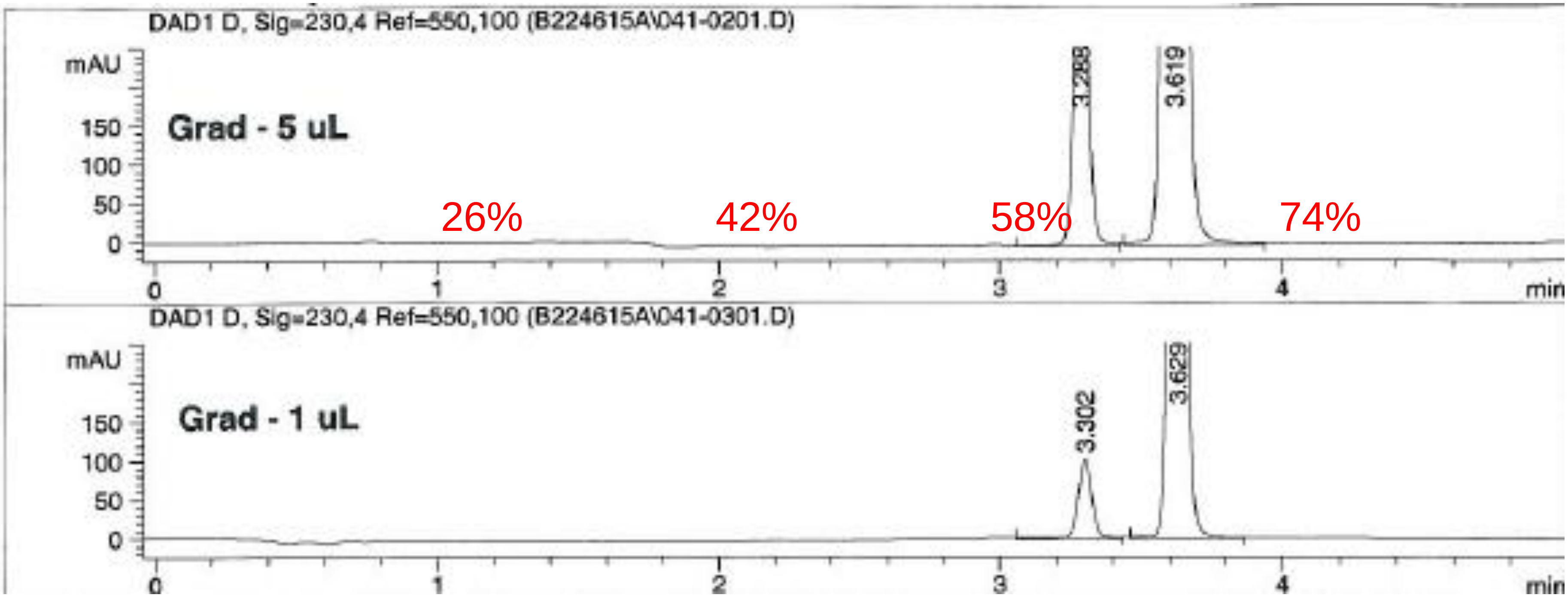
Factorial design

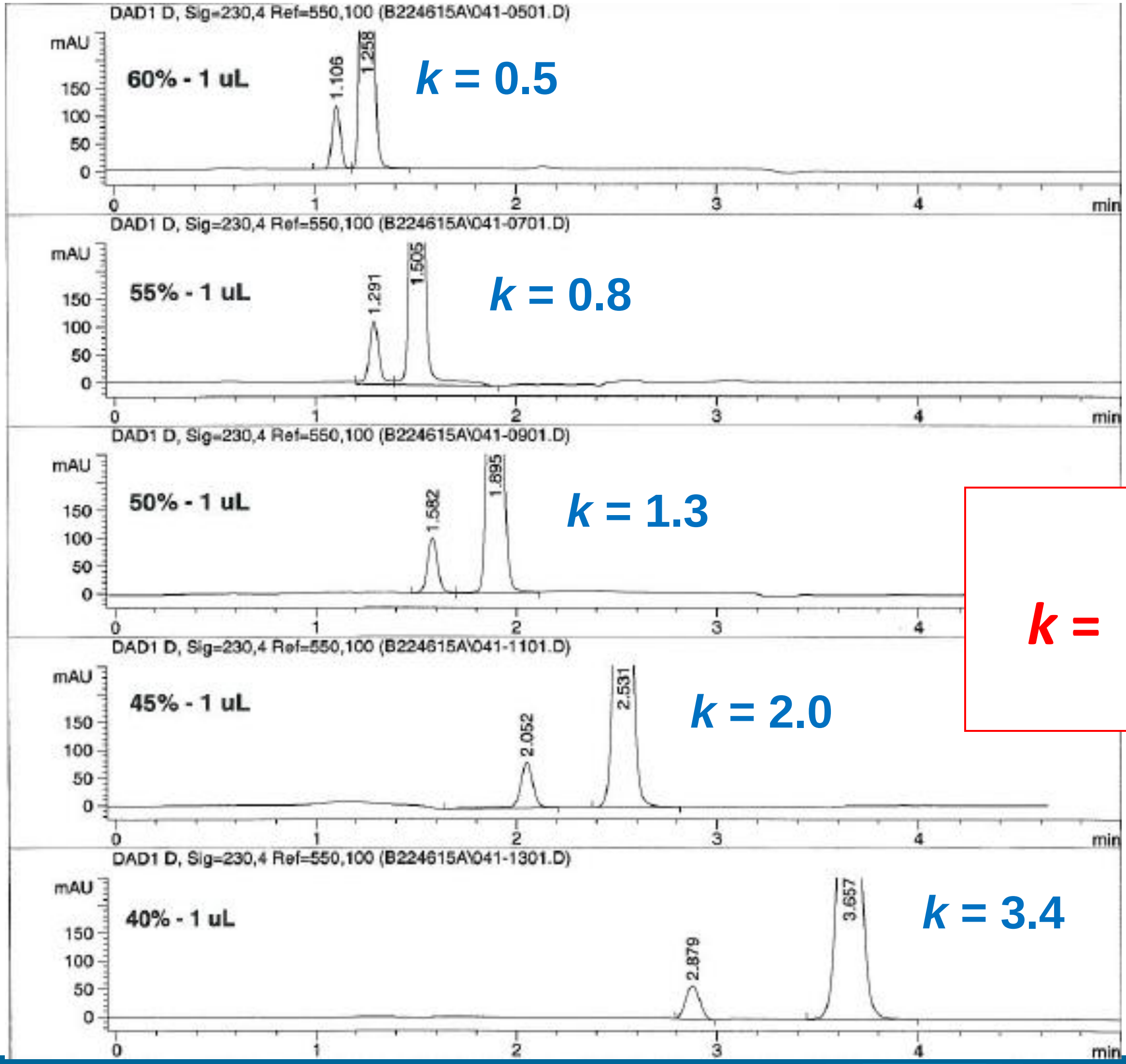
Initial Gradient

% B – at head of column

Gradient: 10% ACN to 90% in 5 min (16%/min)



Effect of Mobile Strength on Retention



$$k = \frac{(t_R - t_0)}{t_0}$$

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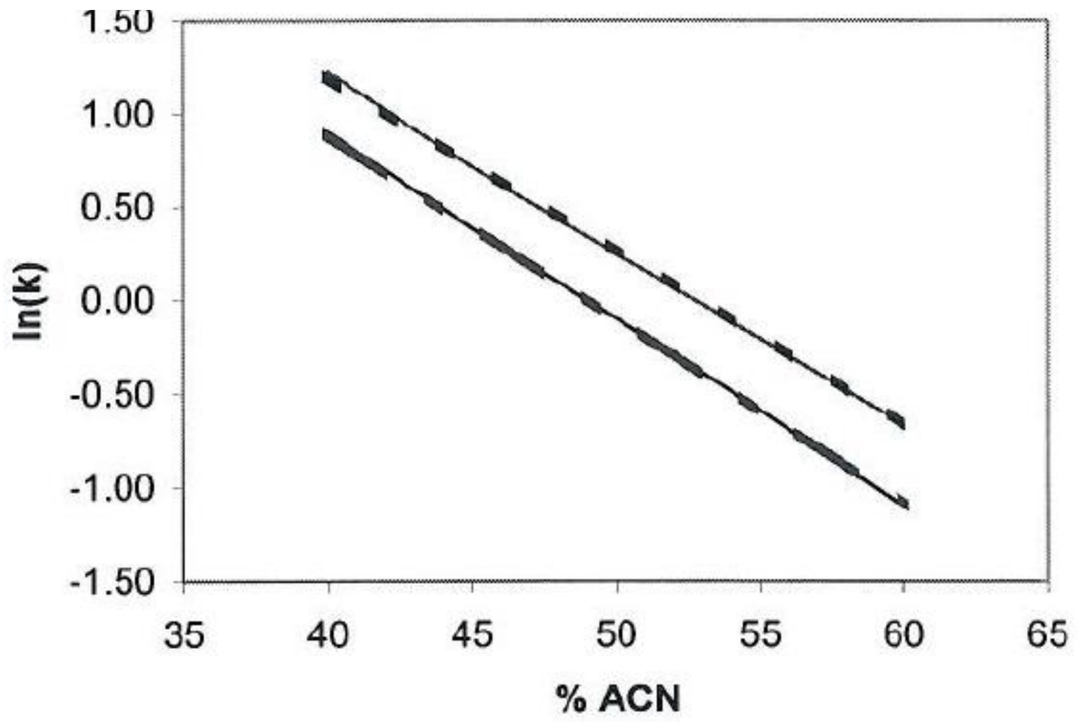
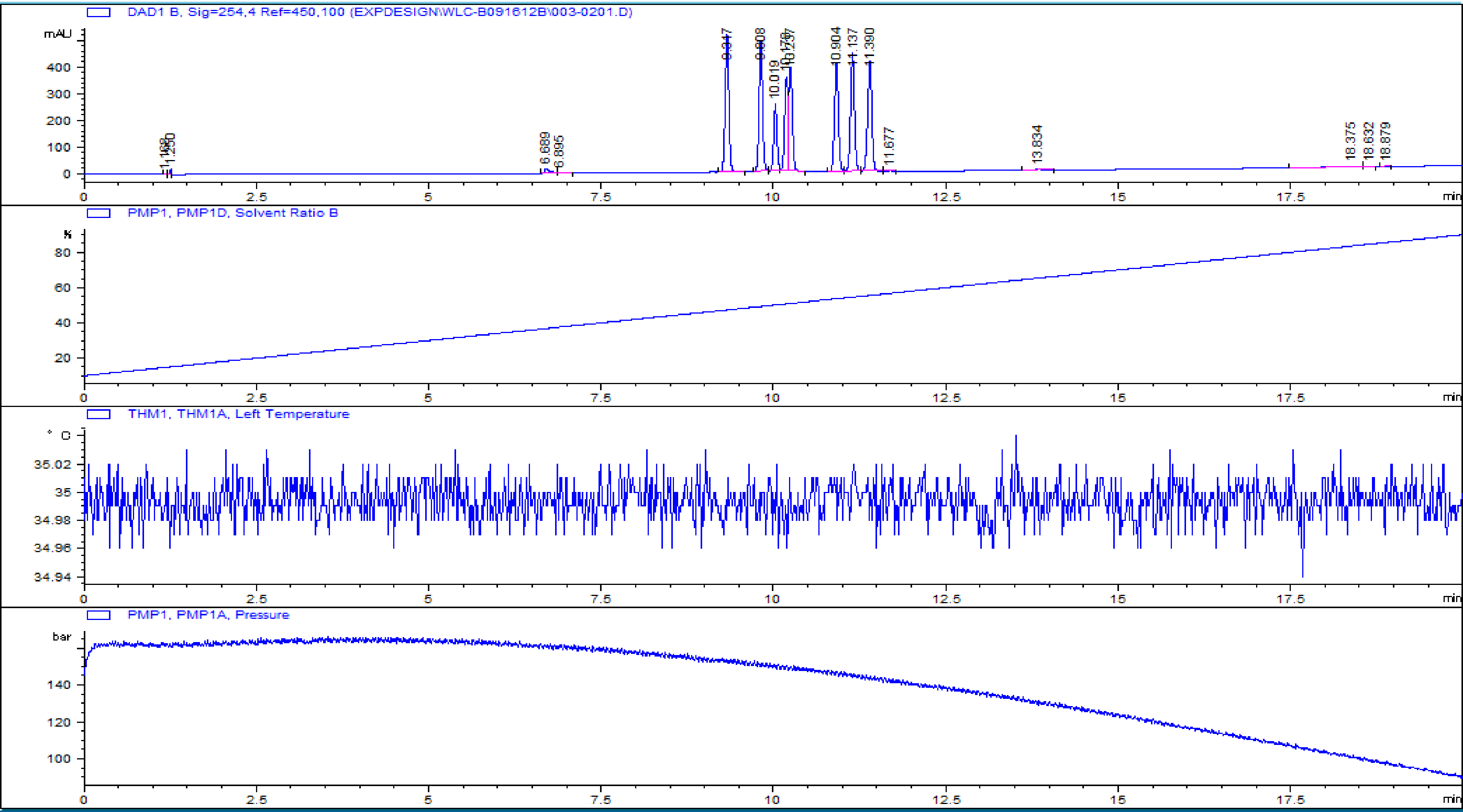
Isocratic Runs

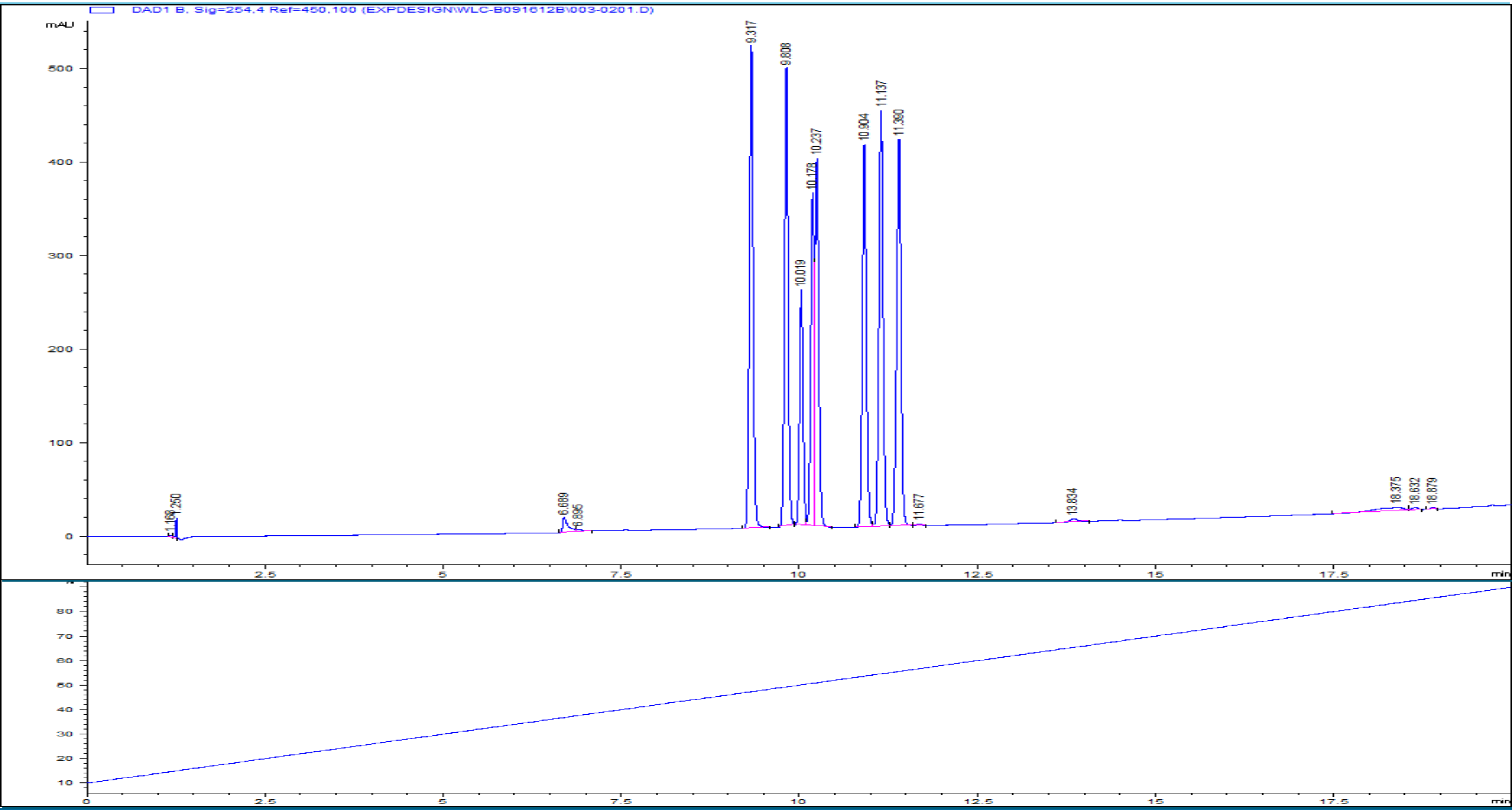
% ACN	tR-1	tR-2	k(1)	k(2)	ln(k1)	ln(k2)
60	1.11	1.26	0.33	0.52	-1.10	-0.66
55	1.29	1.51	0.56	0.81	-0.59	-0.21
50	1.58	1.90	0.91	1.28	-0.10	0.25
45	2.05	2.53	1.47	2.05	0.39	0.72
40	2.88	3.66	2.47	3.41	0.90	1.23



Initial Gradient
10% to 90% ACN/0.1% Formic Acid in 20 min



Initial Gradient
10% to 90% ACN/0.1% FA in 20 min



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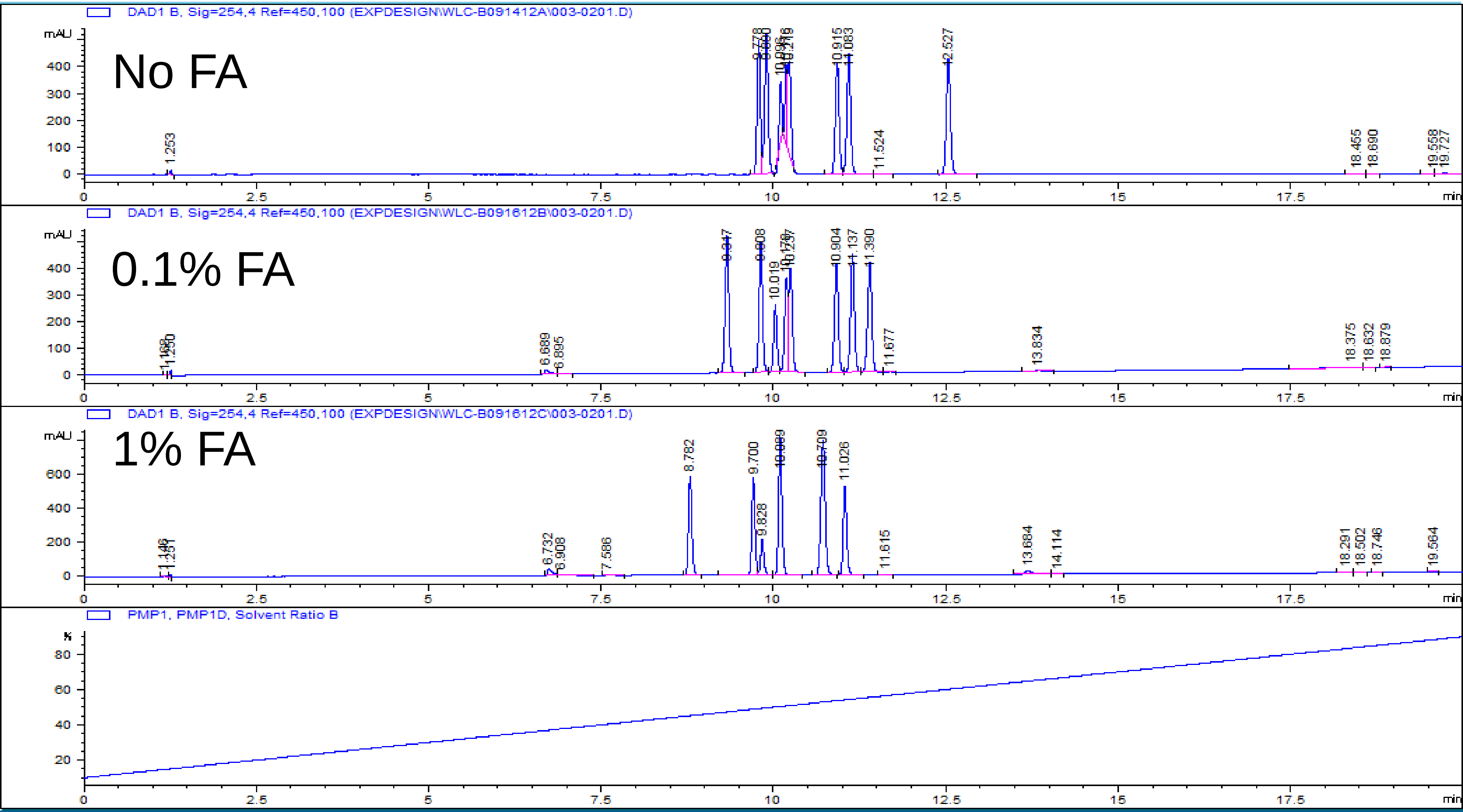
Gradient – Compare %Formic Acid
10% ACN to 90% in 20 min



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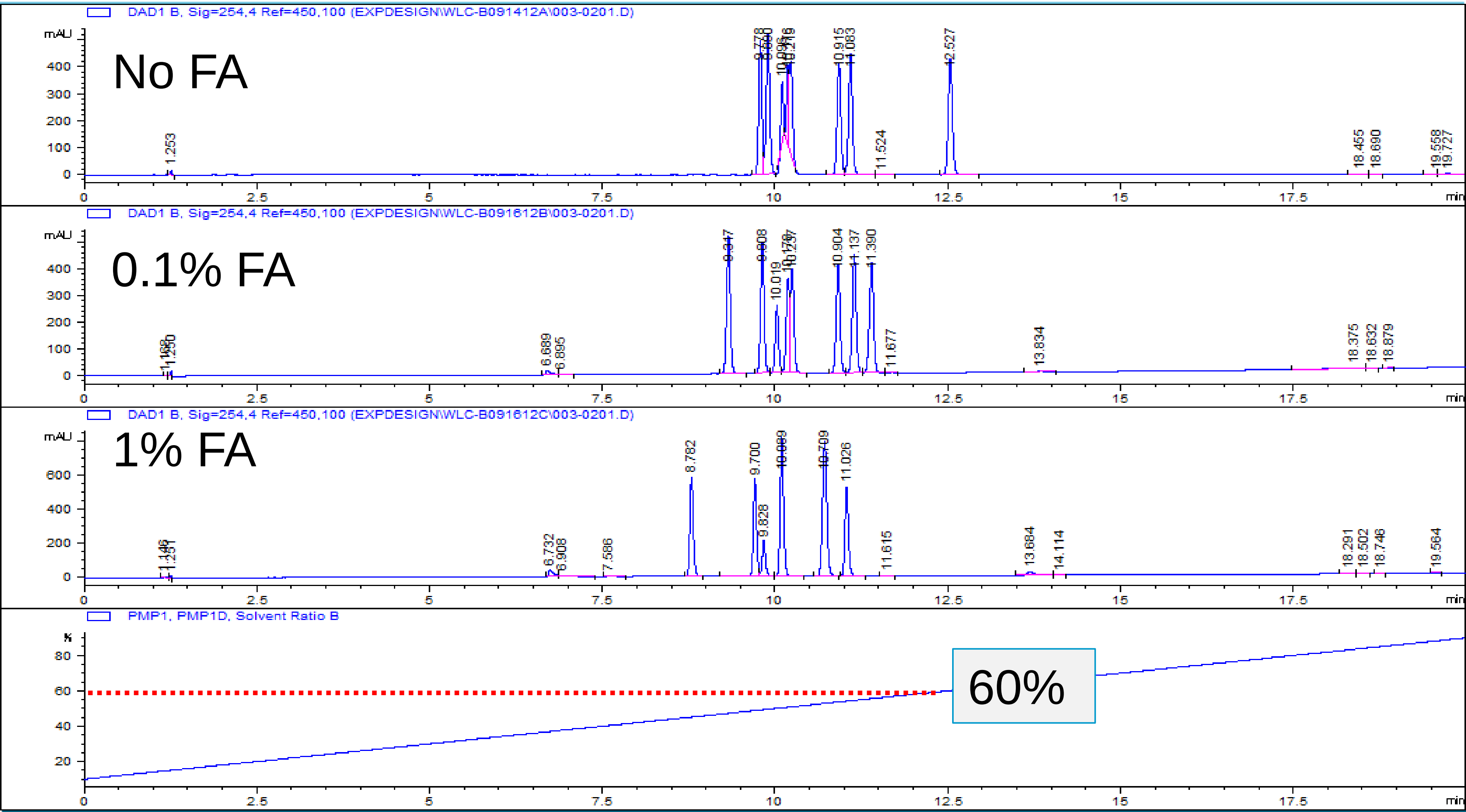

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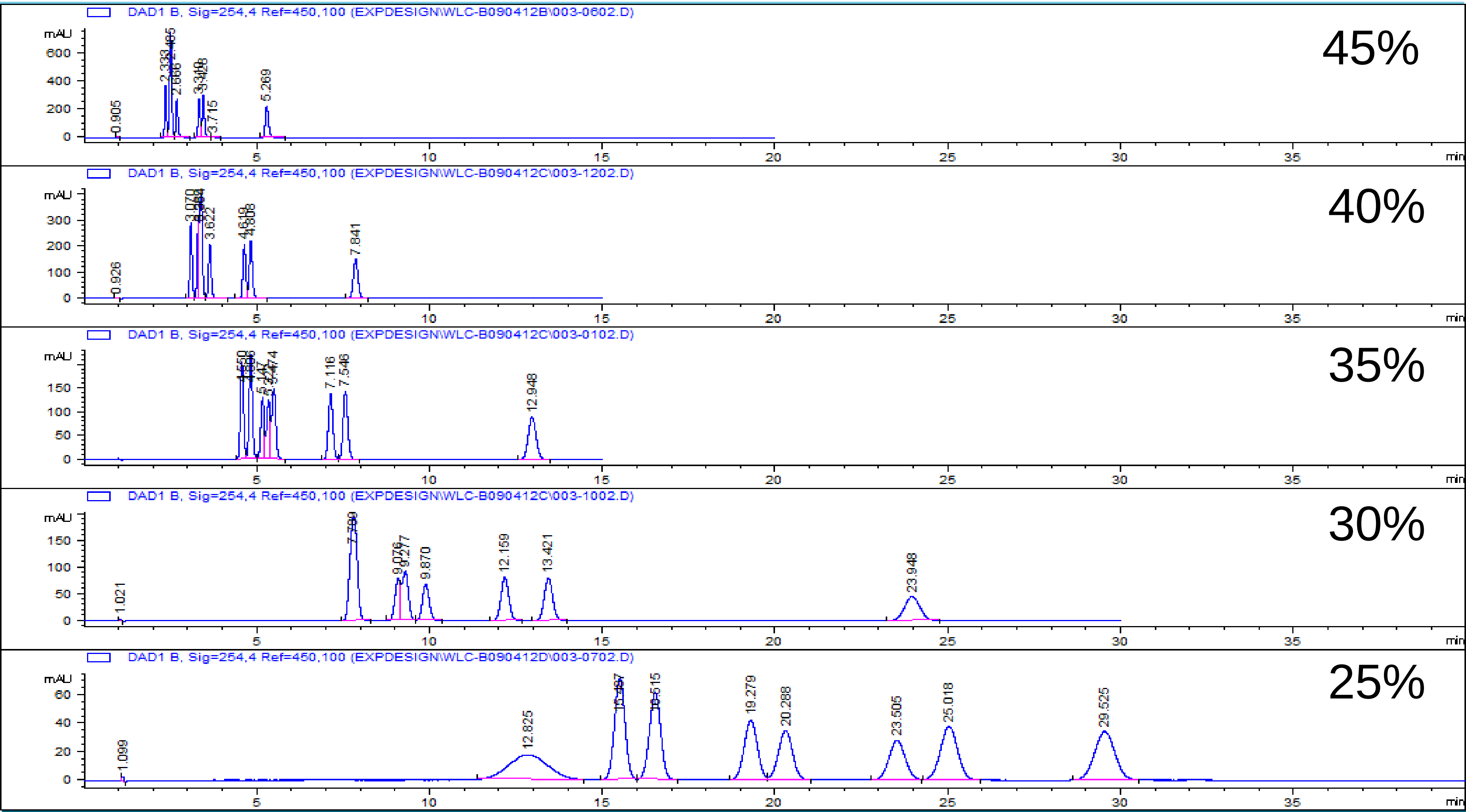
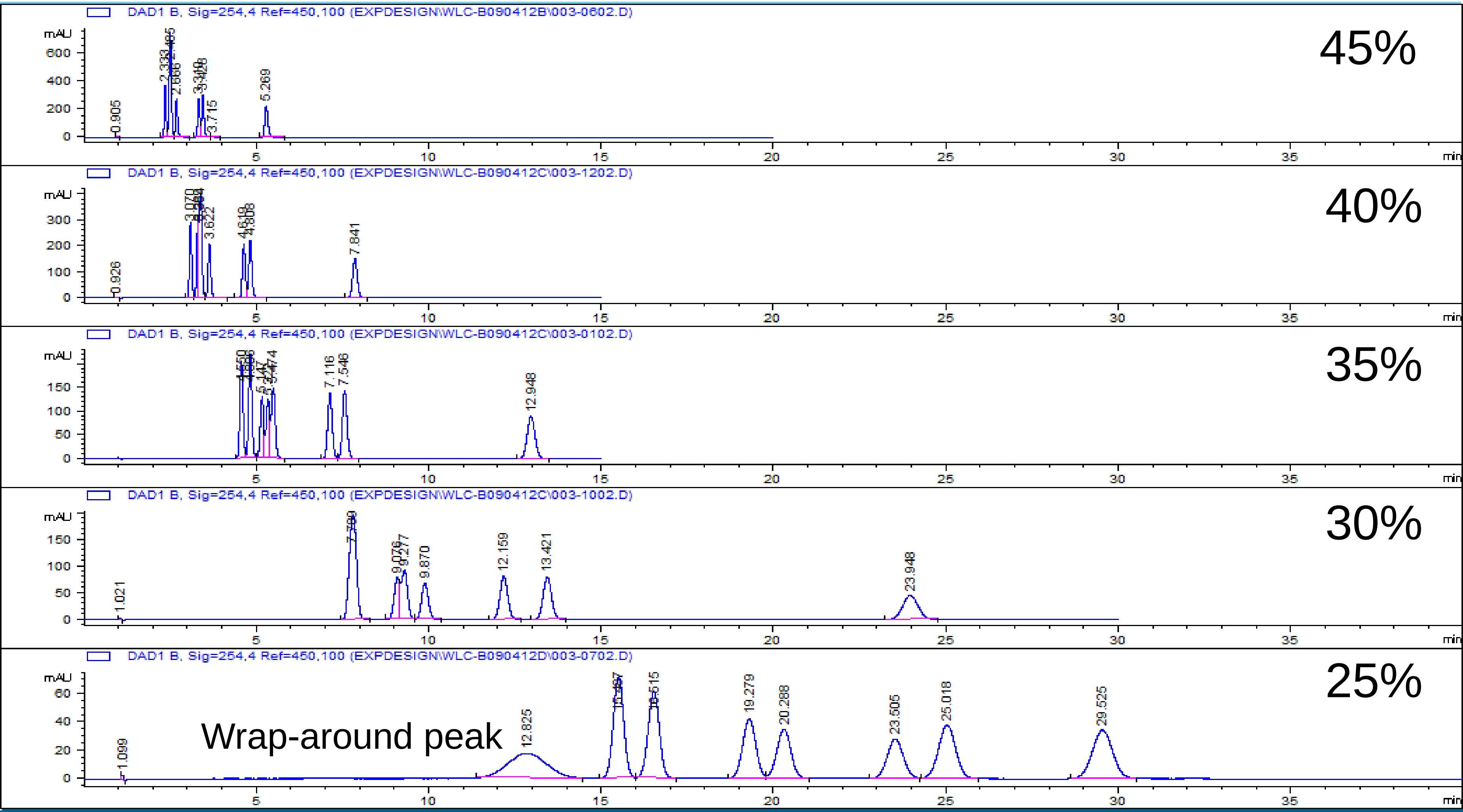
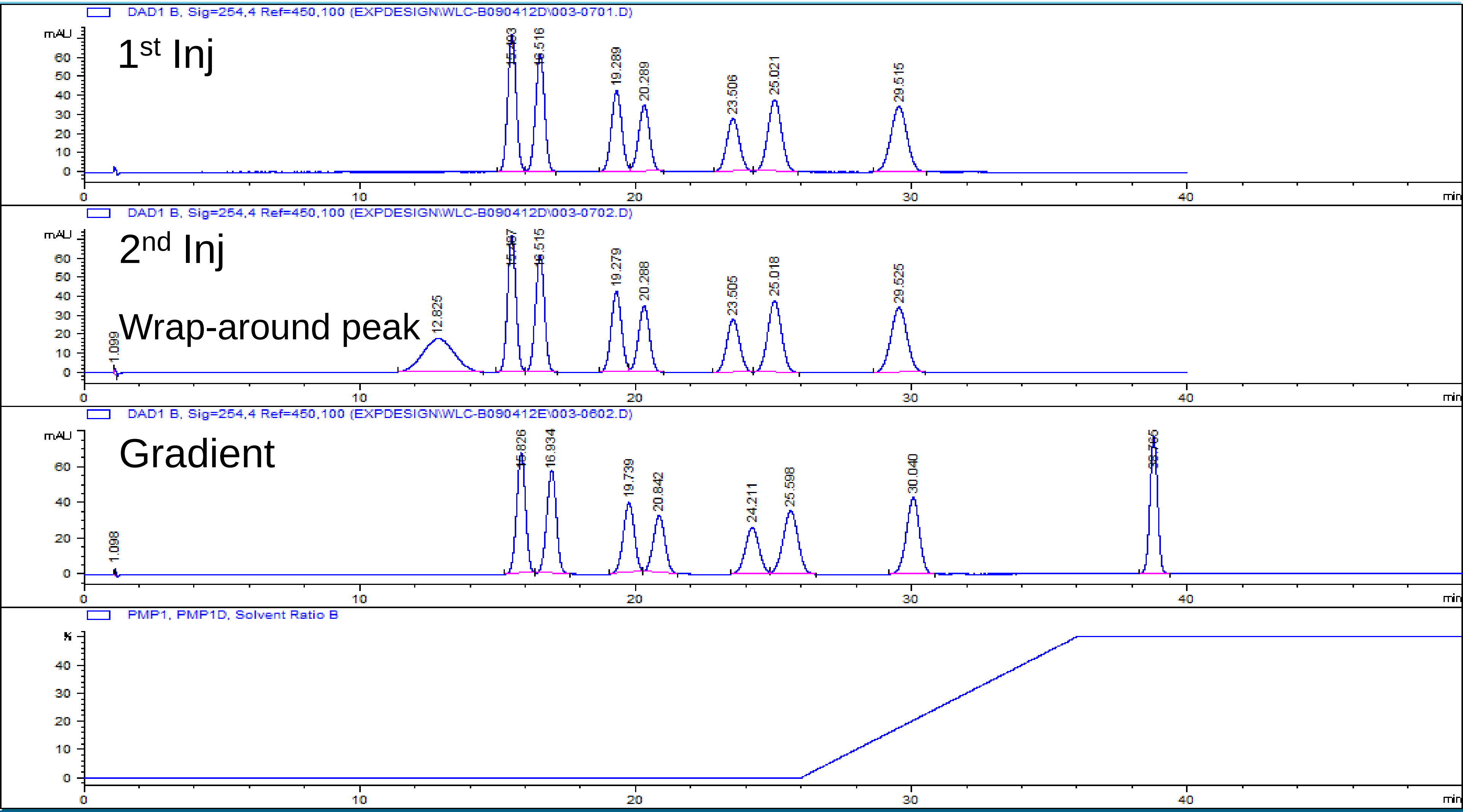
Isocratic Runs – 35°C, no acid



Isocratic Runs – 35°C, no acid



Isocratic Runs – 25% ACN, 35°C, no acid



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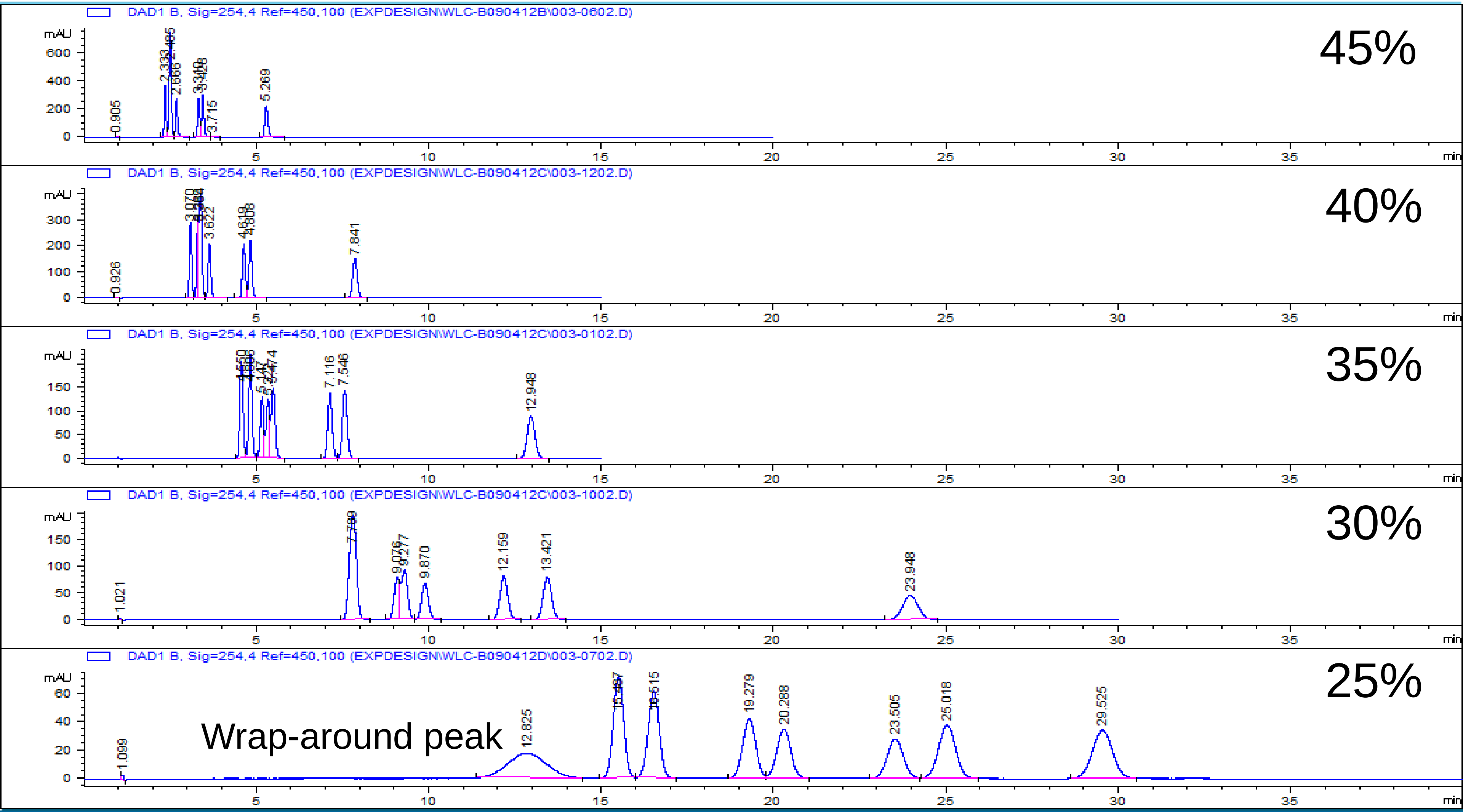

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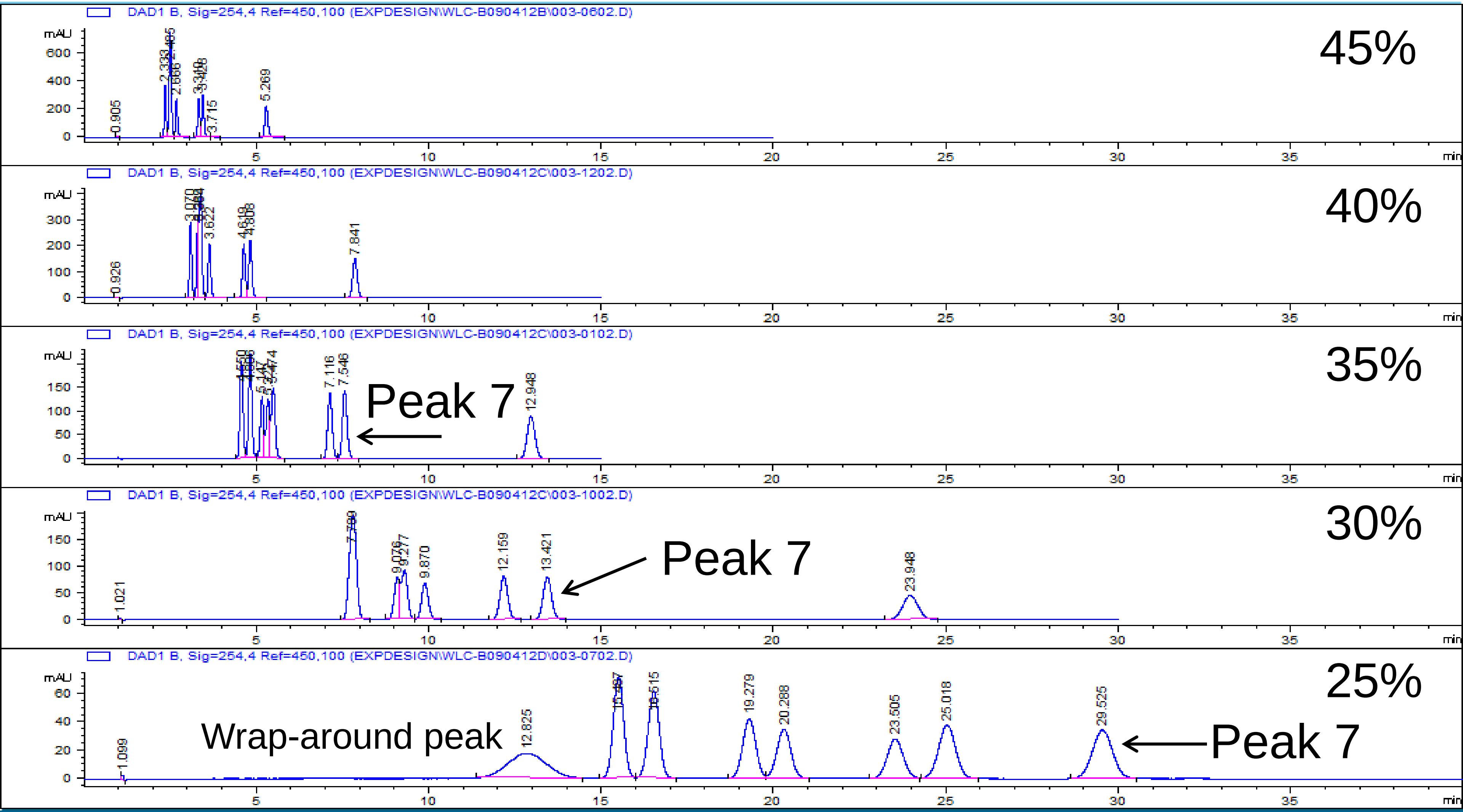

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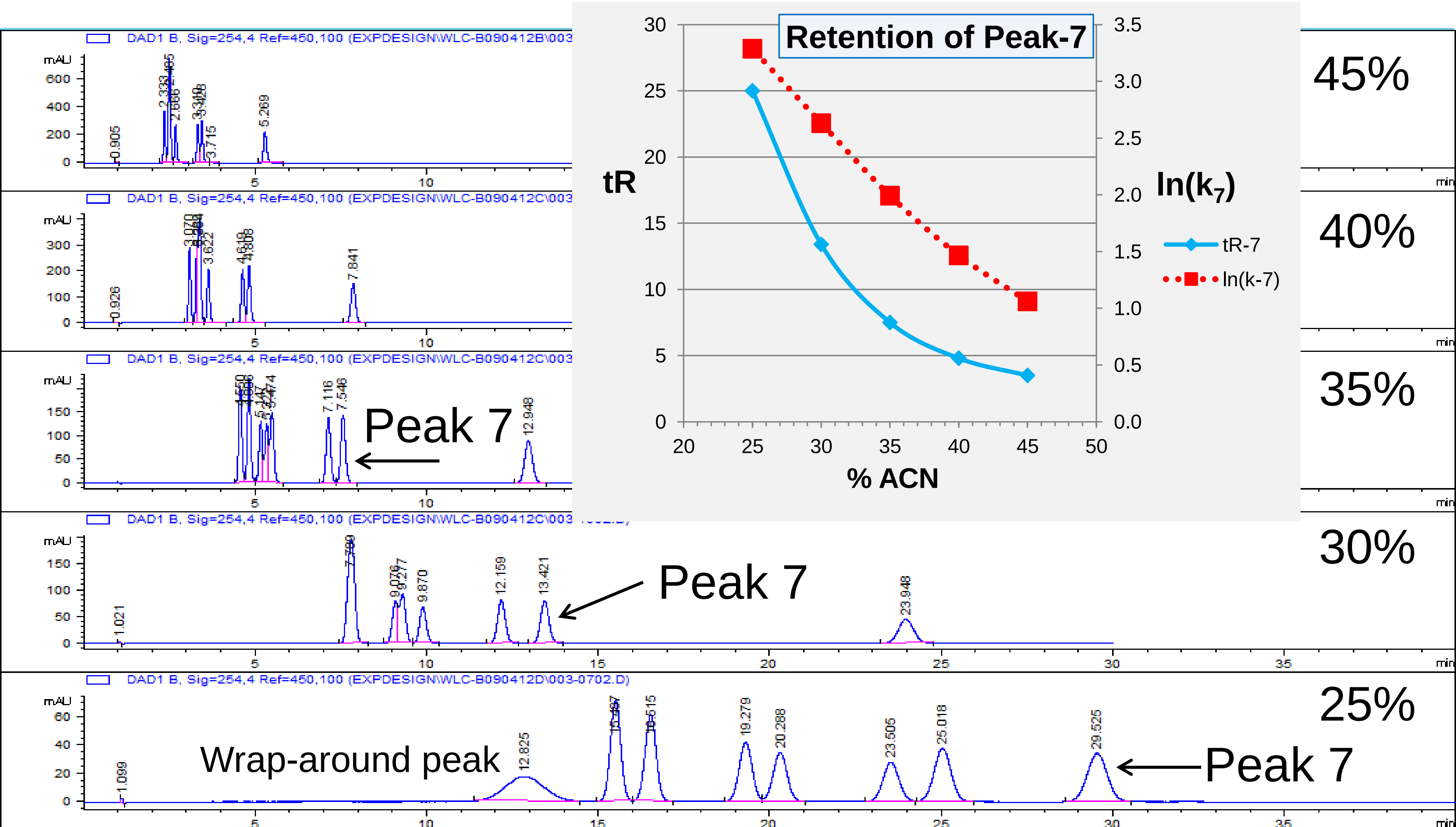

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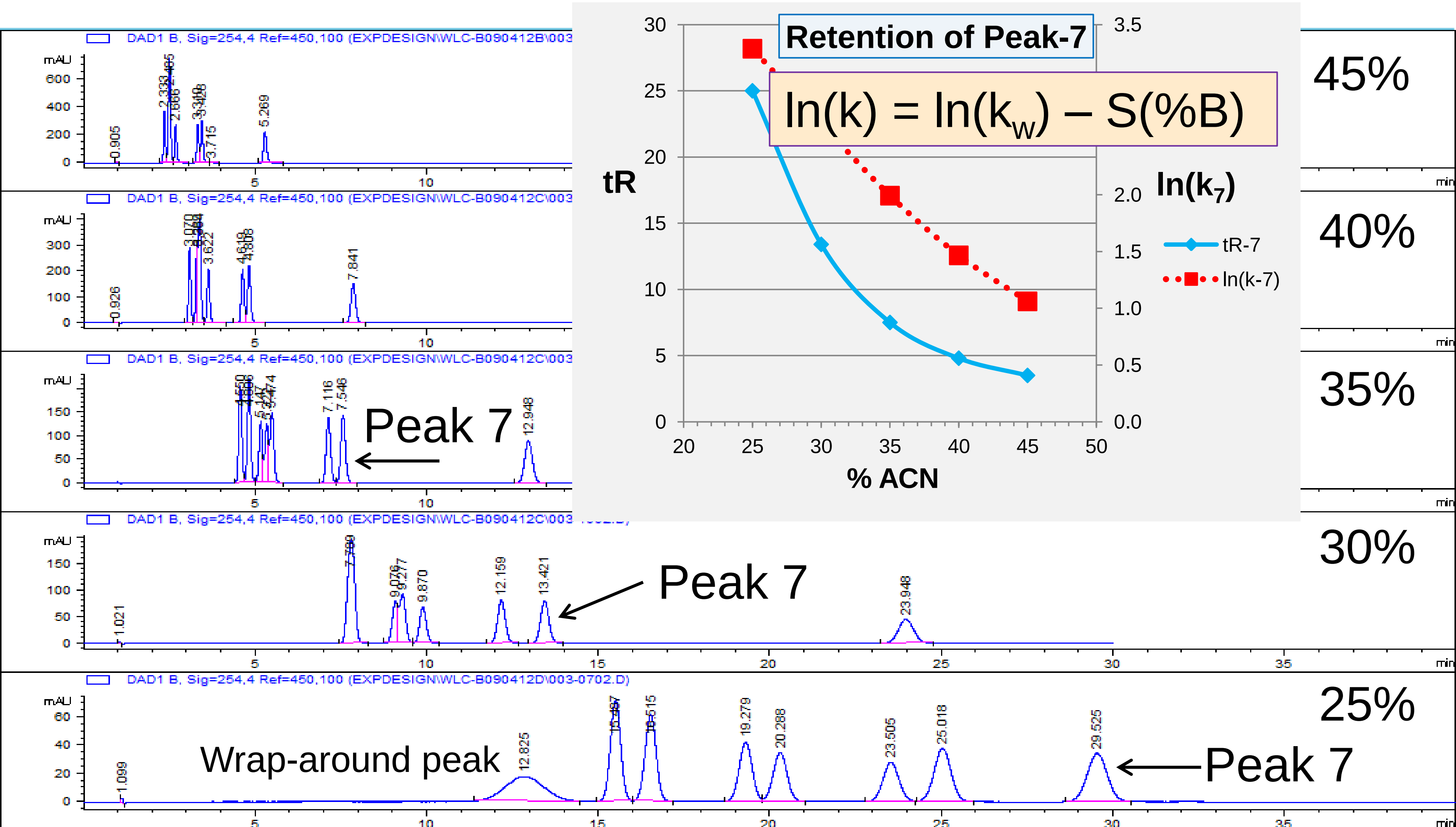
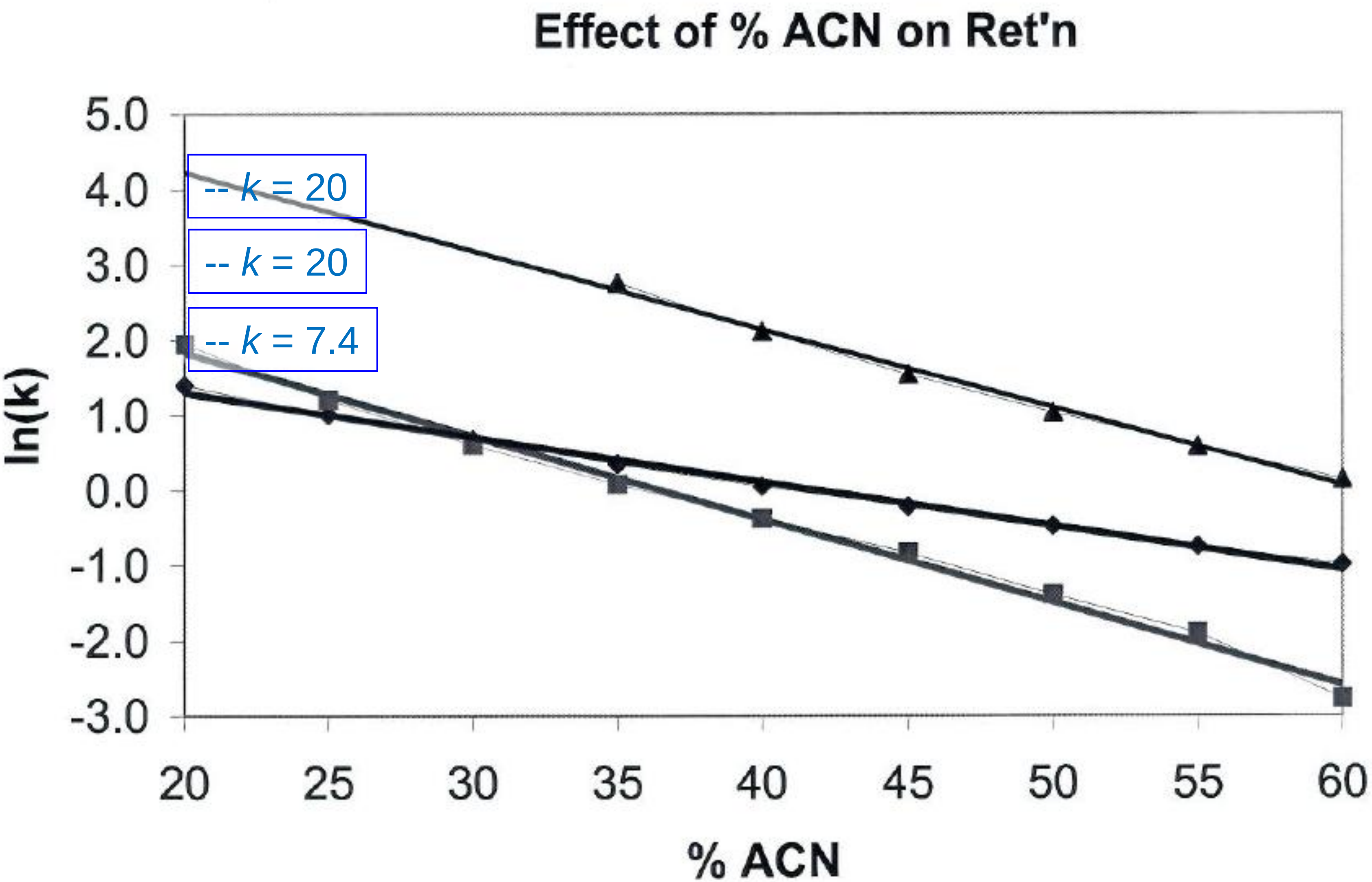
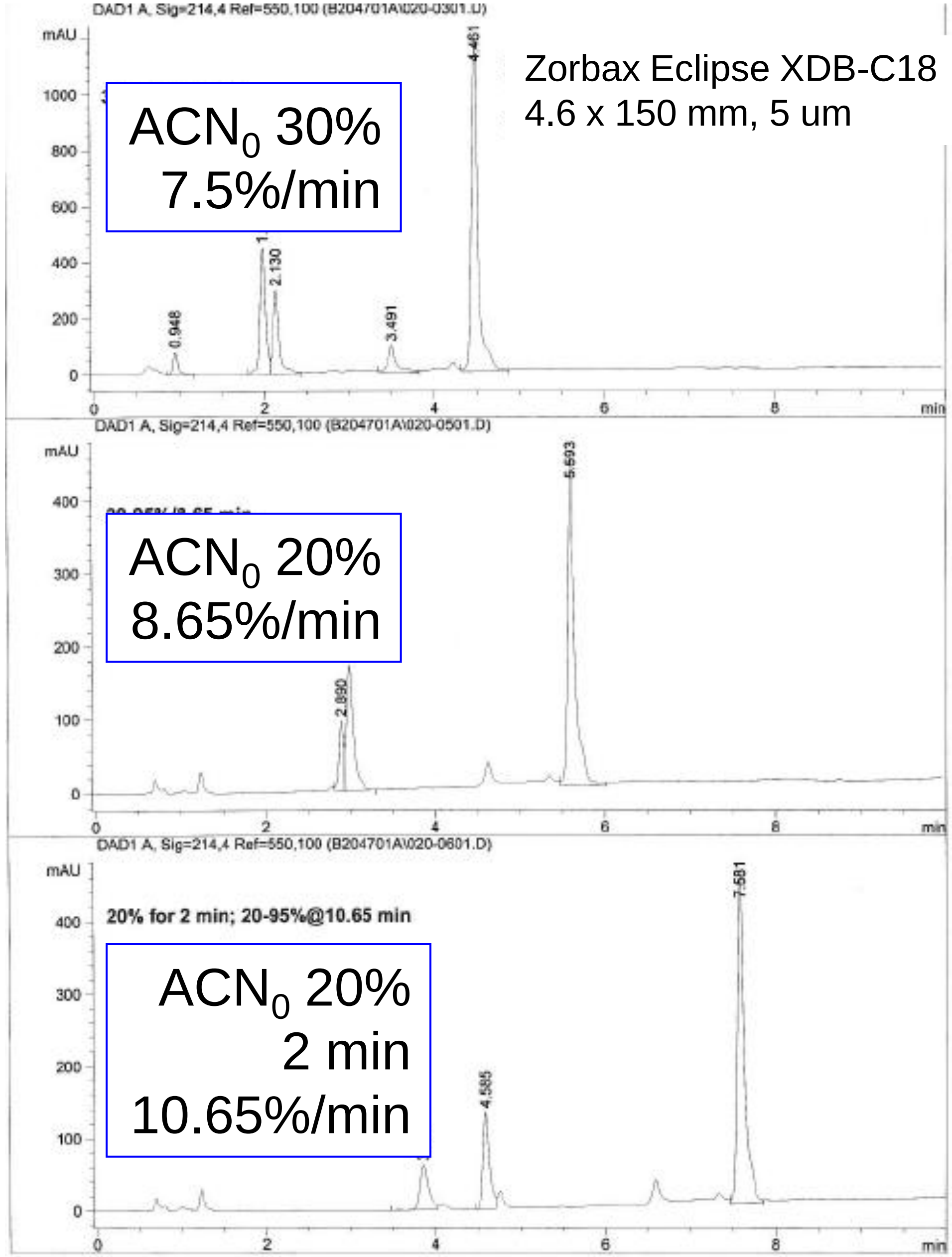


Change in Order of Elution





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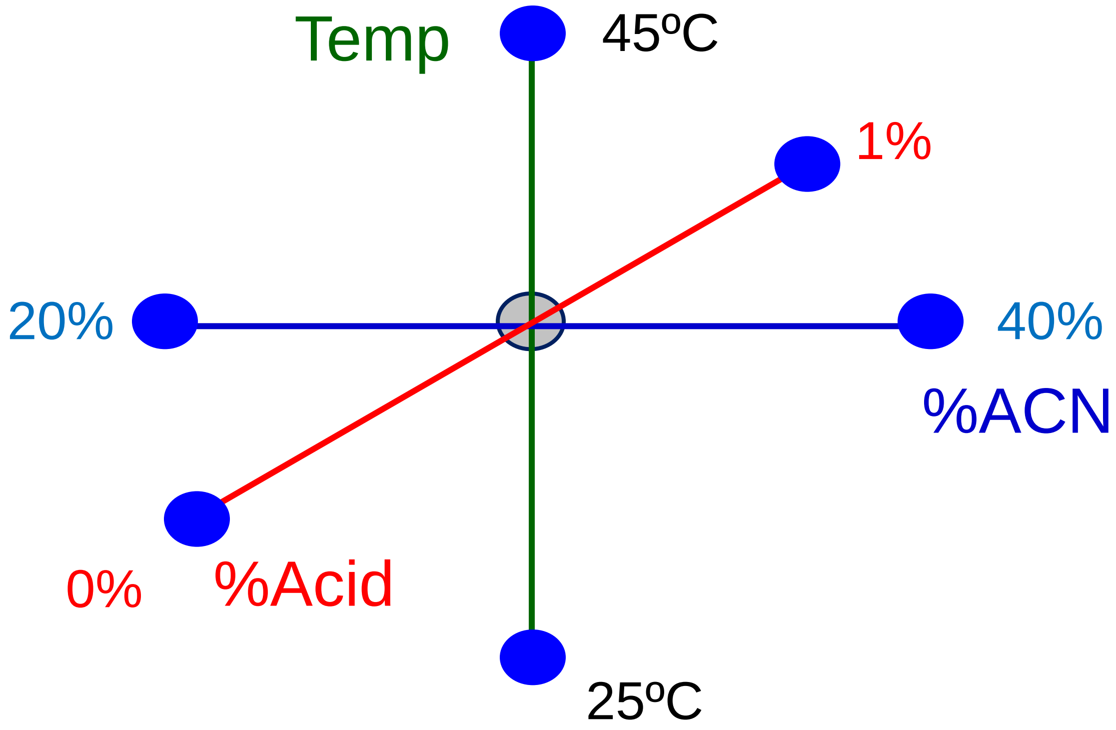
Screening for Main Effects/Star Points



Number of Exp's = 2 x 4 + 1= 9

Screening for Main Effects (Star Points)

Exp	% ACN	% FA	Temp	mM AF
1	30	0.1	35	10
2	**40**	0.1	35	10
3	**20**	0.1	35	10
4	30	**1.0**	35	10
5	30	**0**	35	10
6	30	0.1	**45**	10
7	30	0.1	**25**	10
8	30	0.1	35	**25**
9	30	0.1	35	**10**

Screening for Main Effects

Exp	% ACN	% FA	Temp	mM AF
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5	30	**0**	35	10
6	30	0.1	**45**	10
7	30	0.1	**25**	10
8	30	0.1	35	**25**
9	30	0.1	35	**10**

Screening for Main Effects

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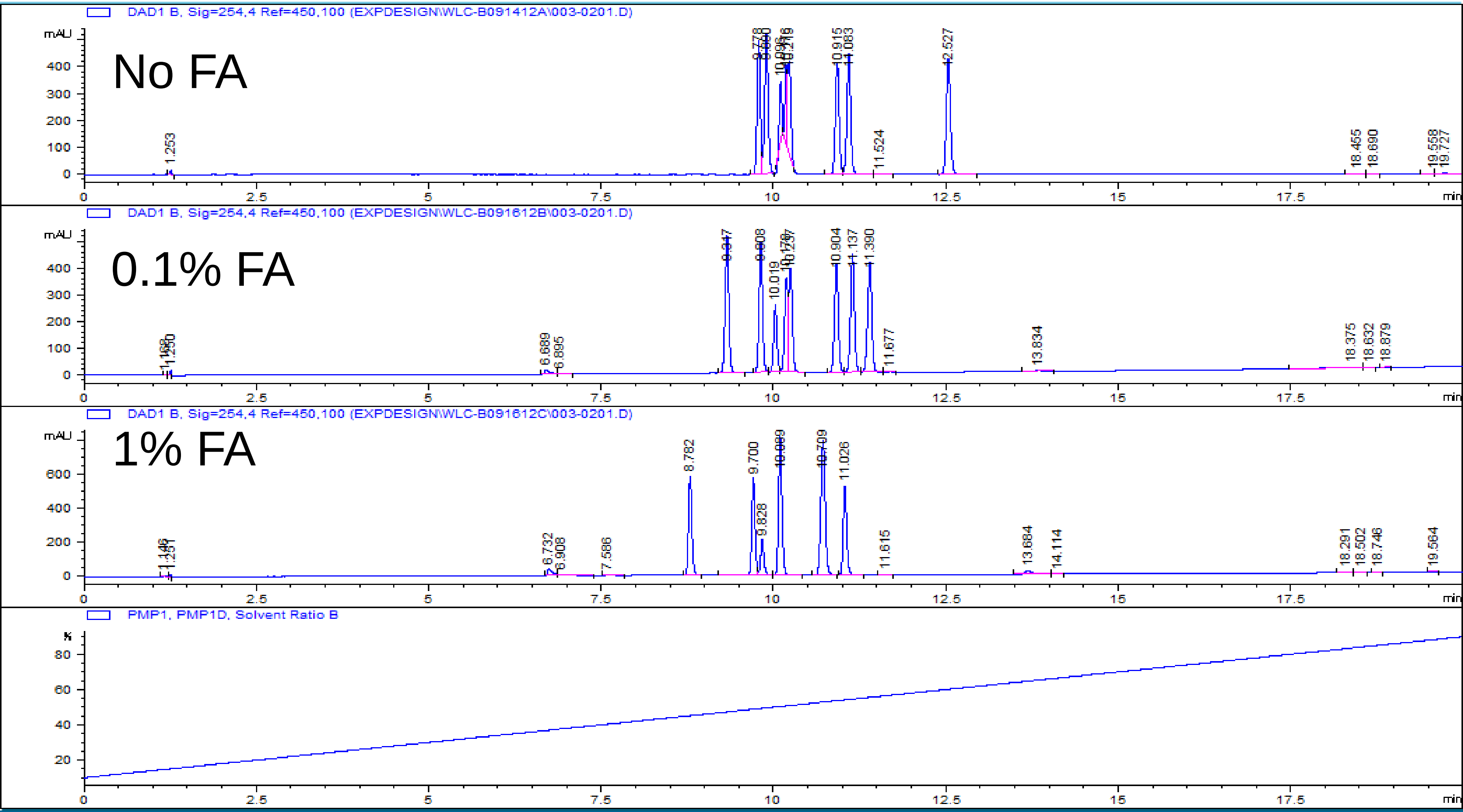
4	30	**1.0**	35	10
5	30	**0**	35	10
6	30	0.1	**45**	10
7	30	0.1	**25**	10
8	30	0.1	35	**25**
9	30	0.1	35	**10**

Gradient – Compare %Formic Acid

10% ACN to 90% in 20 min



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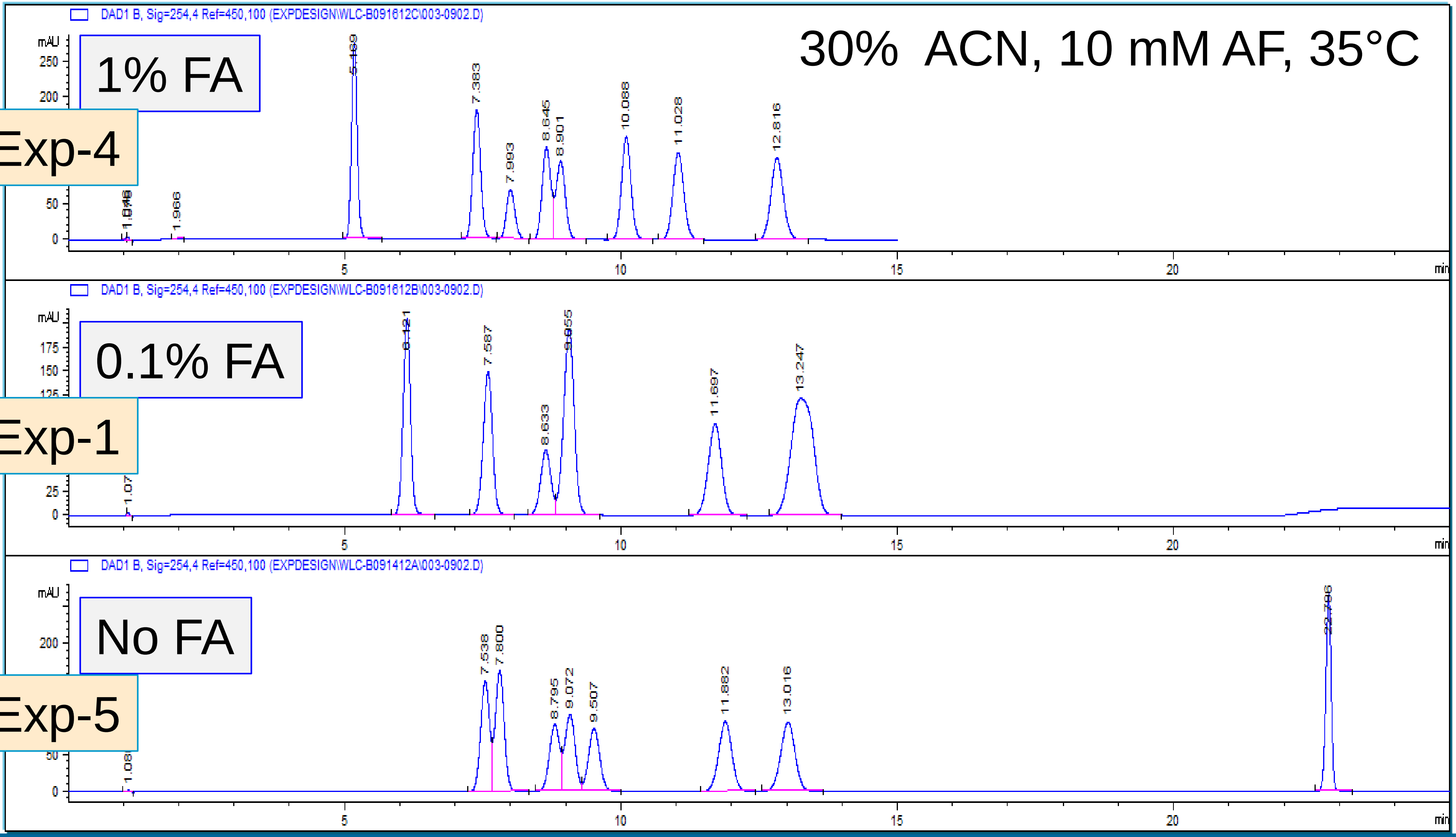
Compare %FA



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Star Points

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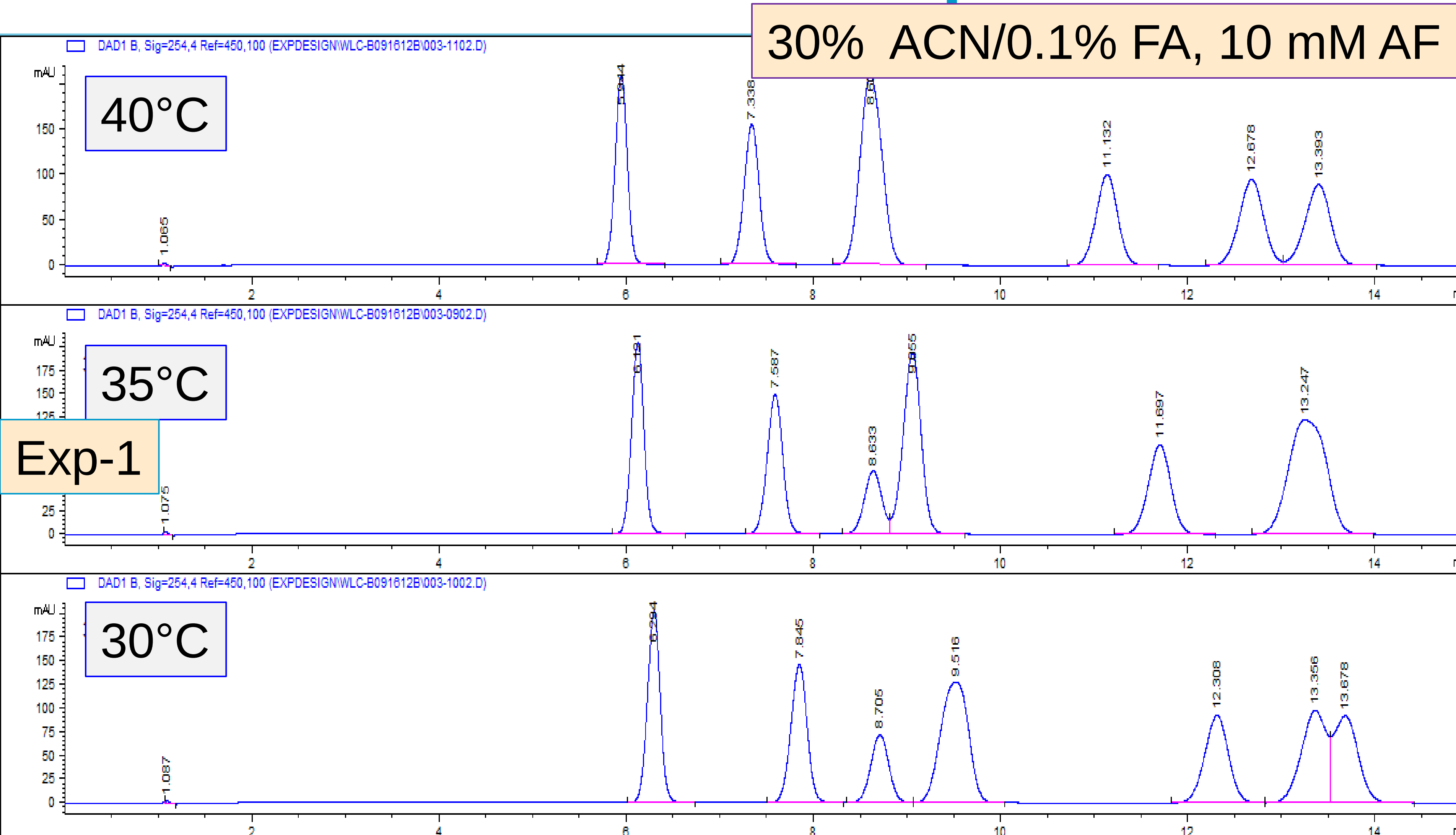
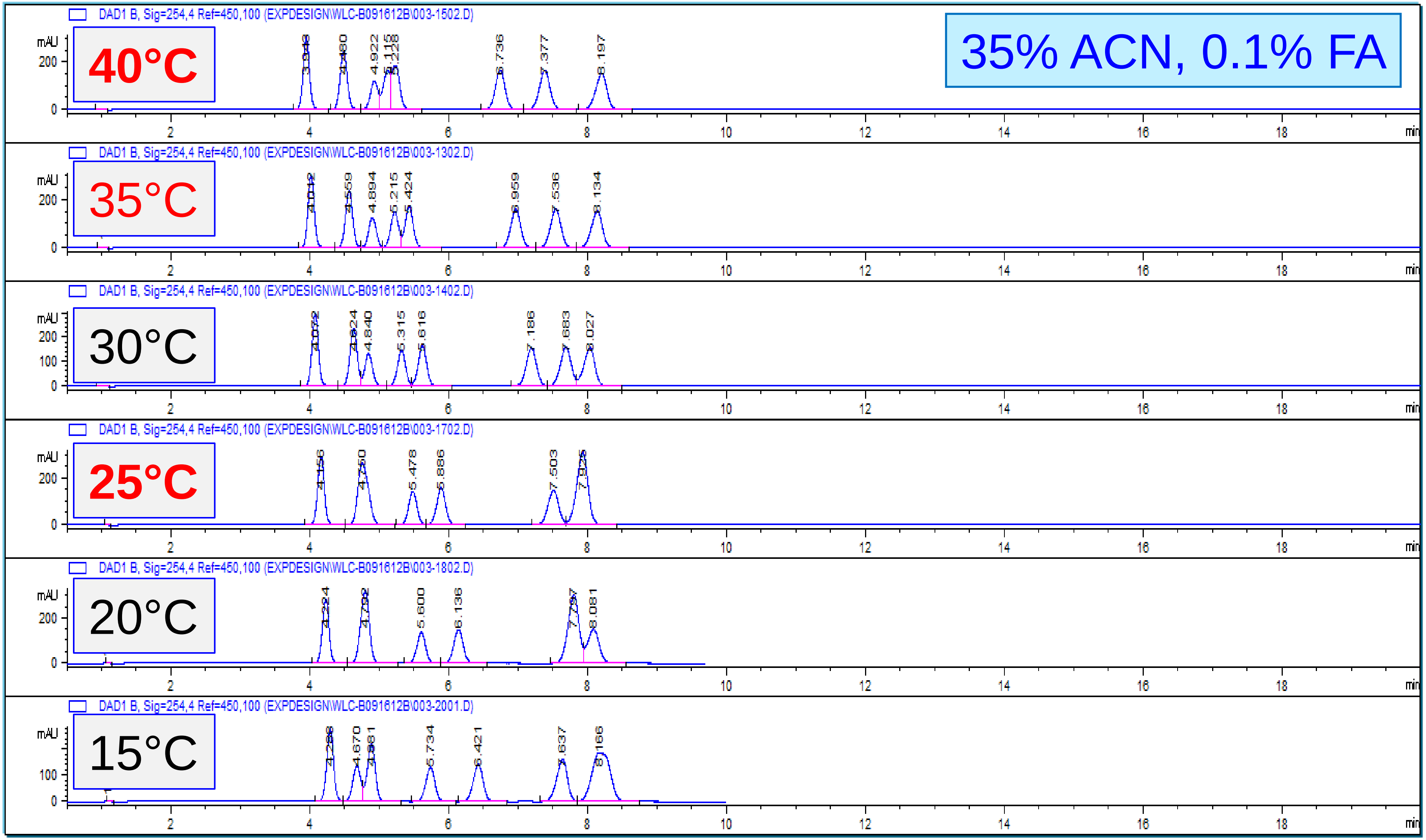
5	30	**0**	35	10
6	30	0.1	**45**	10
7	30	0.1	**25**	10
8	30	0.1	35	**25**
9	30	0.1	35	**10**

Effect of Temp



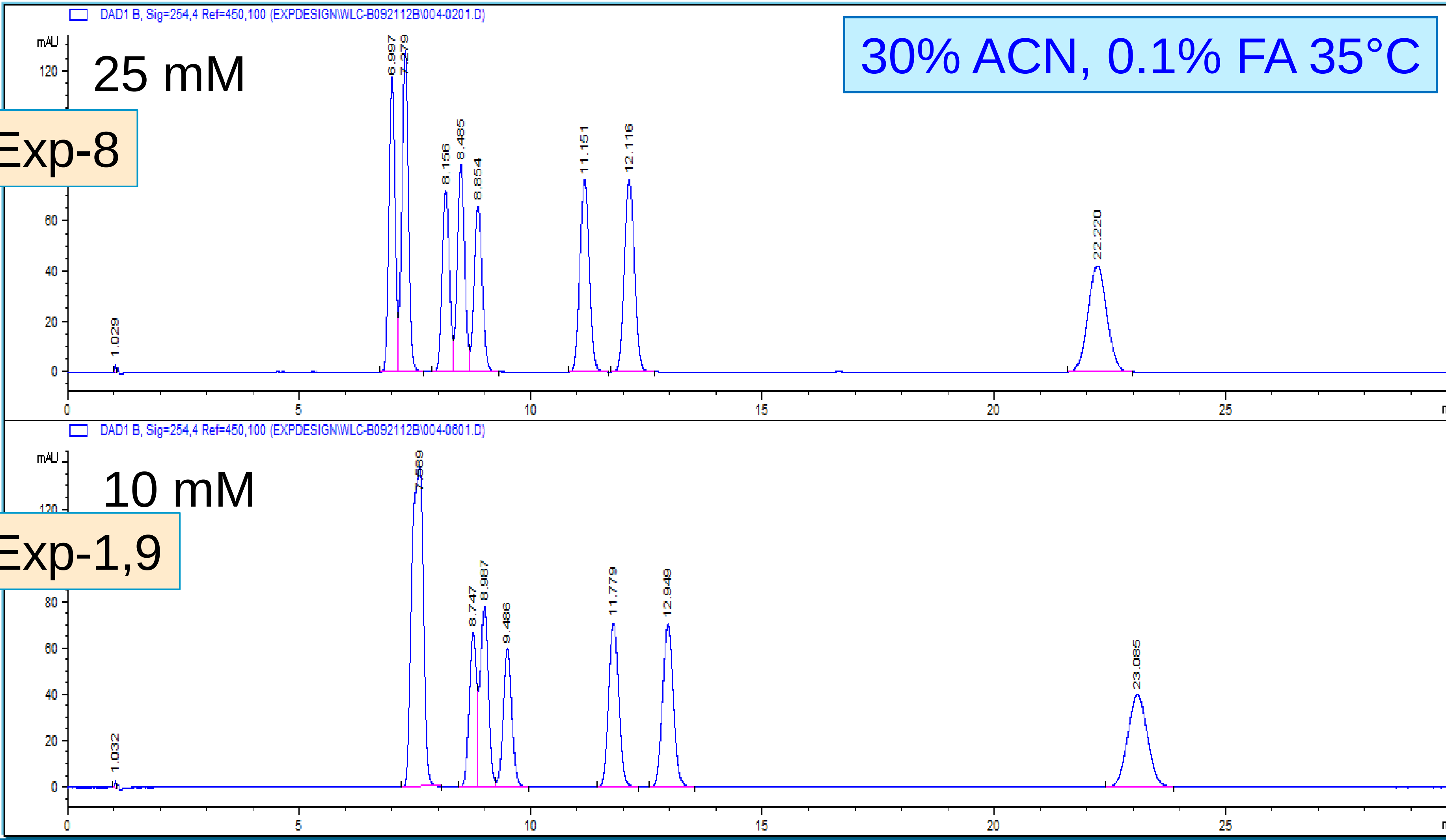
Temperature – Main Effect



Star Points

Exp	% ACN	% FA	Temp	mM AF
1	30	0.1	35	10
2	**40**	0.1	35	10
3	**20**	0.1	35	10
4	30	**1.0**	35	10
5	30	**0**	35	10
6	30	0.1	**45**	10
7	30	0.1	**25**	10
8	30	0.1	35	**25**
9	30	0.1	35	**10**

Effect of Ammonium Formate



Star Points

Exp	% ACN	% FA	Temp	mM AF
1	30	0.1	35	10
2	**40**	0.1	35	10
3	**20**	0.1	35	10
4	30	**1.0**	35	10
5	30	**0**	35	10
6	30	0.1	**45**	10
7	30	0.1	**25**	10
8	30	0.1	35	**25**
9	30	0.1	35	**10**

Star Points

Yes

Exp	% ACN	% FA	Temp	mM AF
1	30	0.1	35	10
2	**40**	0.1	35	10
3	**20**	0.1	35	10
4	30	**1.0**	35	10
5	30	**0**	35	10
6	30	0.1	**45**	10
7	30	0.1	**25**	10
8	30	0.1	35	**25**
9	30	0.1	35	**10**

Star Points

Exp	Yes % ACN	Yes % FA	Temp	mM AF
1	30	0.1	35	10
2	**40**	0.1	35	10
3	**20**	0.1	35	10
4	30	**1.0**	35	10
5	30	**0**	35	10
6	30	0.1	**45**	10
7	30	0.1	**25**	10
8	30	0.1	35	**25**
9	30	0.1	35	**10**

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Star Points

Exp	Yes % ACN	Yes % FA	Yes Temp	mM AF
1	30	0.1	35	10
2	**40**	0.1	35	10
3	**20**	0.1	35	10
4	30	**1.0**	35	10
5	30	**0**	35	10
6	30	0.1	**45**	10
7	30	0.1	**25**	10
8	30	0.1	35	**25**
9	30	0.1	35	**10**

Star Points

	Yes	Yes	Yes	Defer
Exp	**% ACN**	**% FA**	**Temp**	**mM AF**
1	30	0.1	35	10
2	**40**	0.1	35	10
3	**20**	0.1	35	10
4	30	**1.0**	35	10
5	30	**0**	35	10
6	30	0.1	**45**	10
7	30	0.1	**25**	10
8	30	0.1	35	**25**
9	30	0.1	35	**10**

Screening - Star Points Conclusions

- %ACN, Temp, FA, all affect the separation
- Order of elution reversals are observed - potentially very confusing
- Will choose to leave AF out of optimization
- FA affects separation, but not required for chromatography
- Sometimes find our conditions or very close using screen, but not in this case

Experimental Designs

Gradient screening

Isocratic separation – vary mobile phase strength

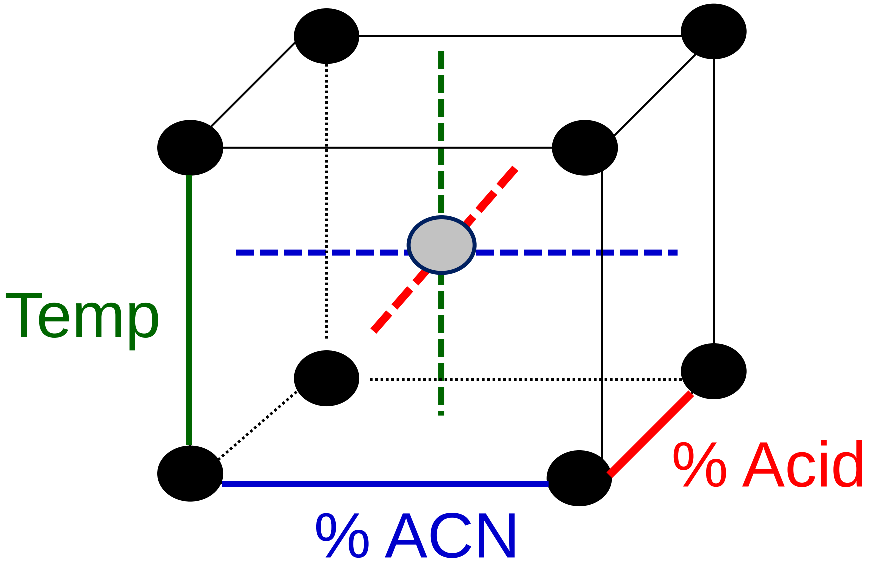
Simple screening design

Factorial design

Time/Effort

- **Initial Gradients: > Half Day**
- **Isocratic: > Half Day (overnight?)**
- **Screening: Set-up ~ 2 hrs**
- **Screening: Run overnight**

Factorial Design for Three Factors with Center Point



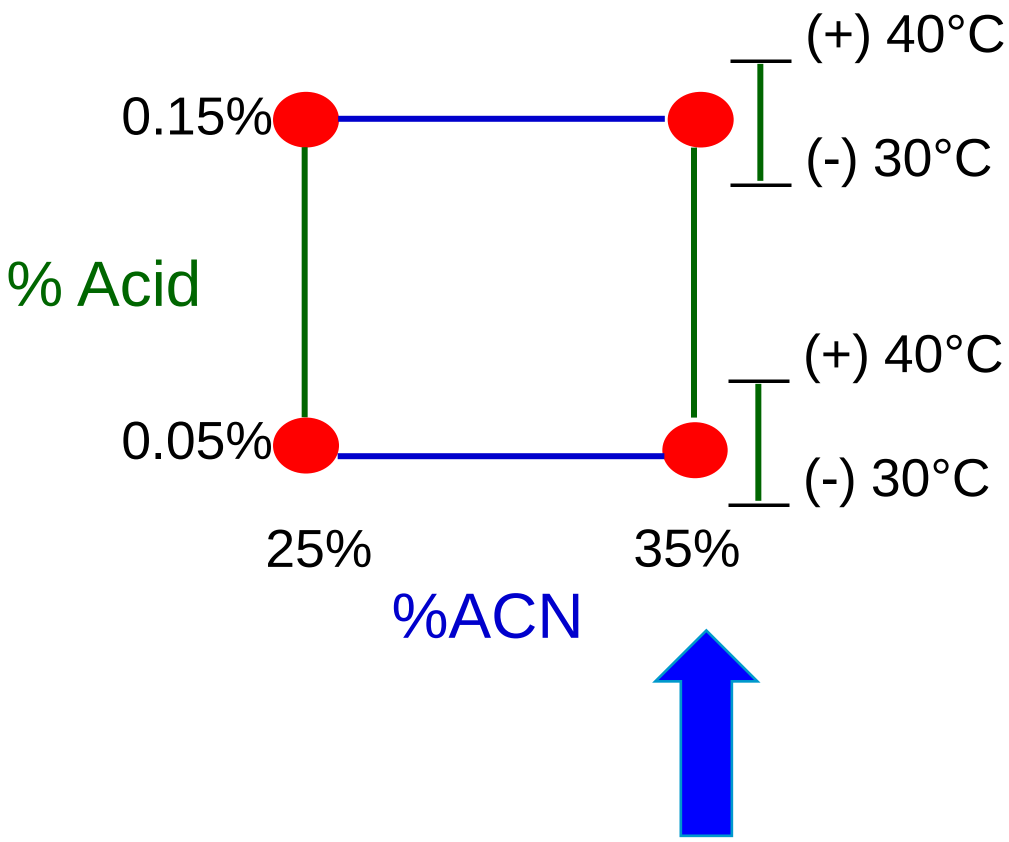
3 Factors, $2^3 + 1 = 9$

3 Factor Factorial Design

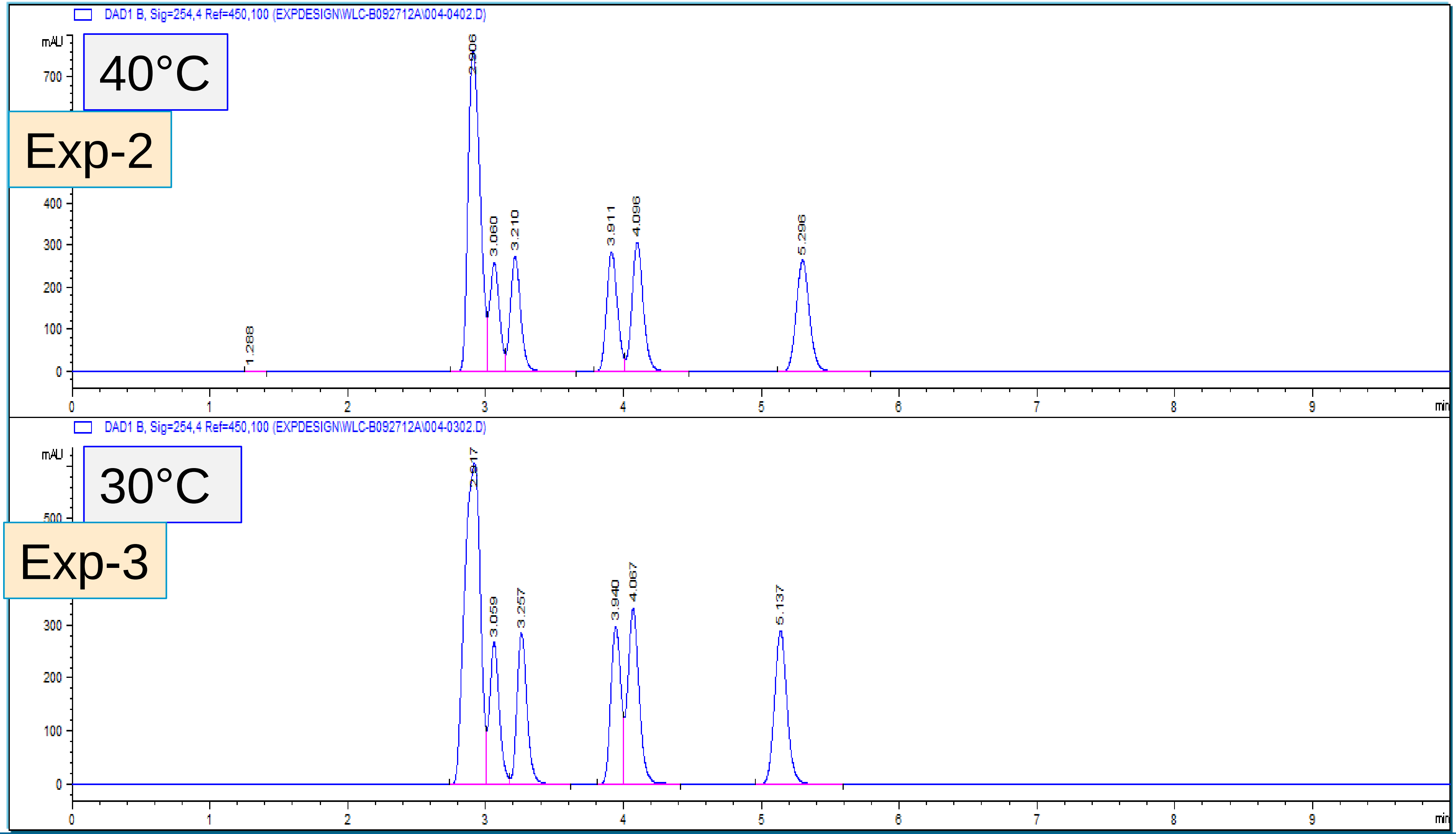
Exp	% ACN	% FA	Temp
1	30	0.10	35
2	35	0.15	40
3	35	0.15	30
4	35	0.05	40
5	35	0.05	30
6	25	0.15	40
7	25	0.15	30
8	25	0.05	40
9	25	0.05	30

Displaying Results

Displaying Results



0.15% Formic Acid, 35% ACN



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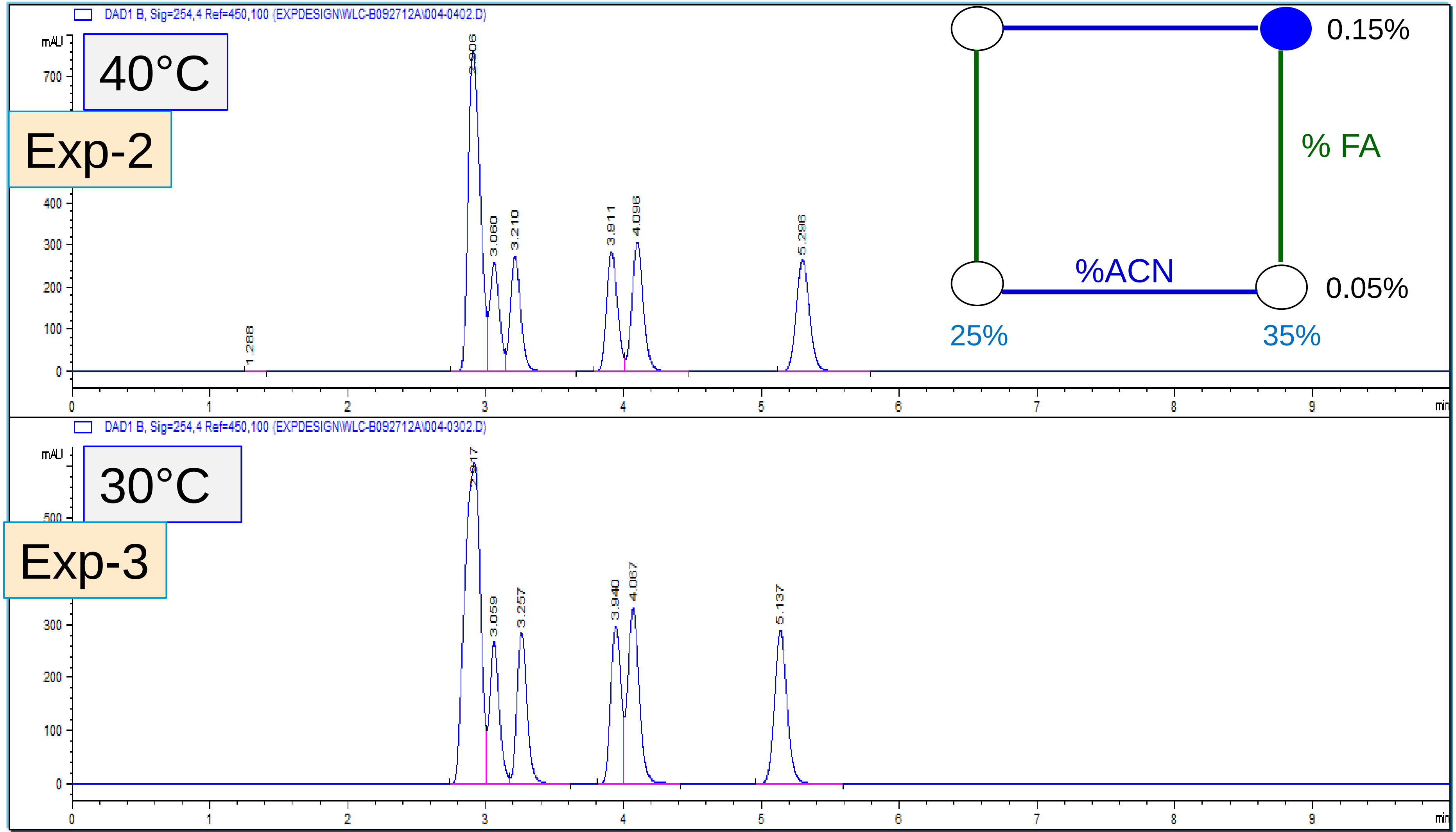
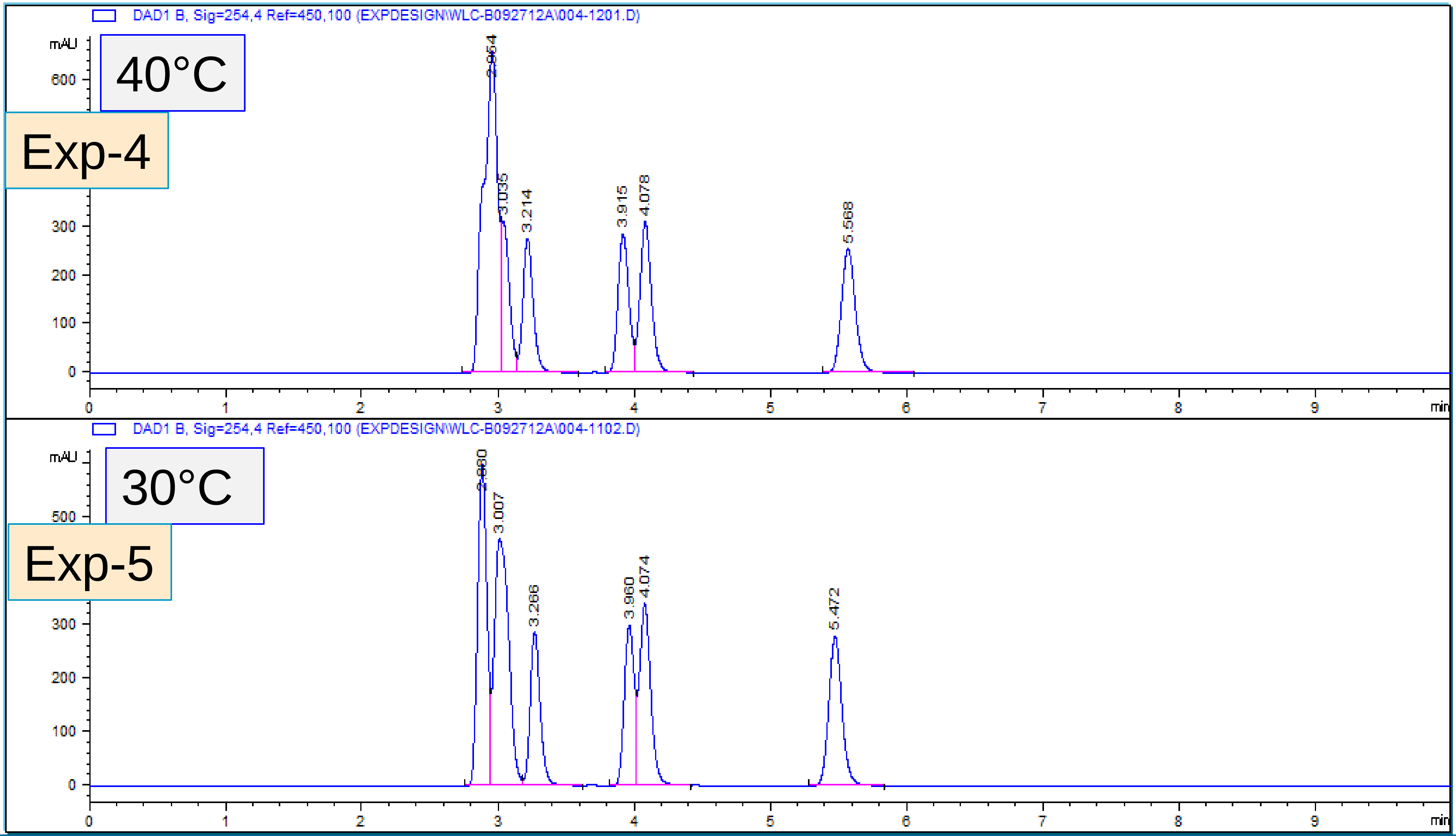
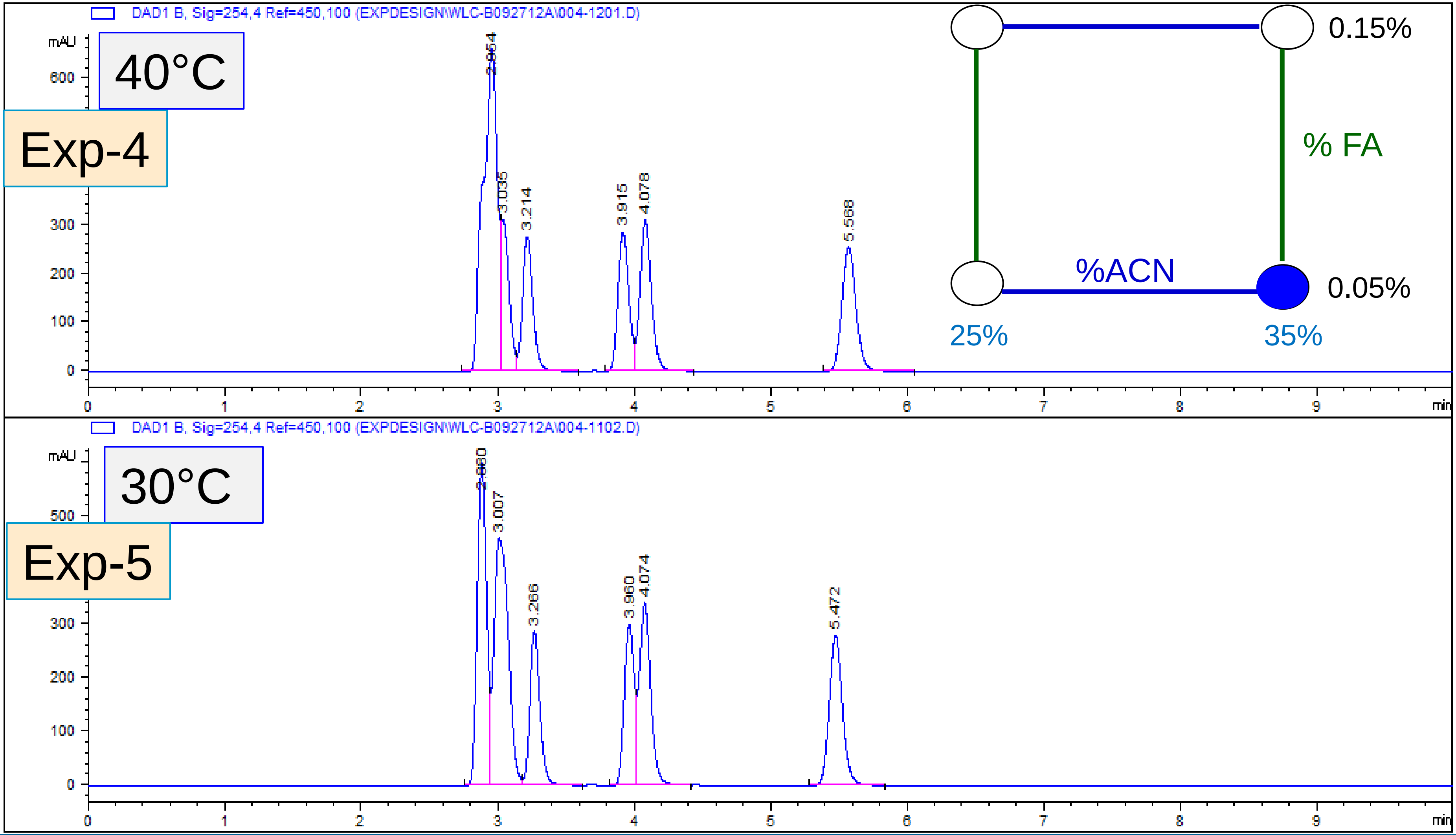
0.15% Formic Acid, 35% ACN



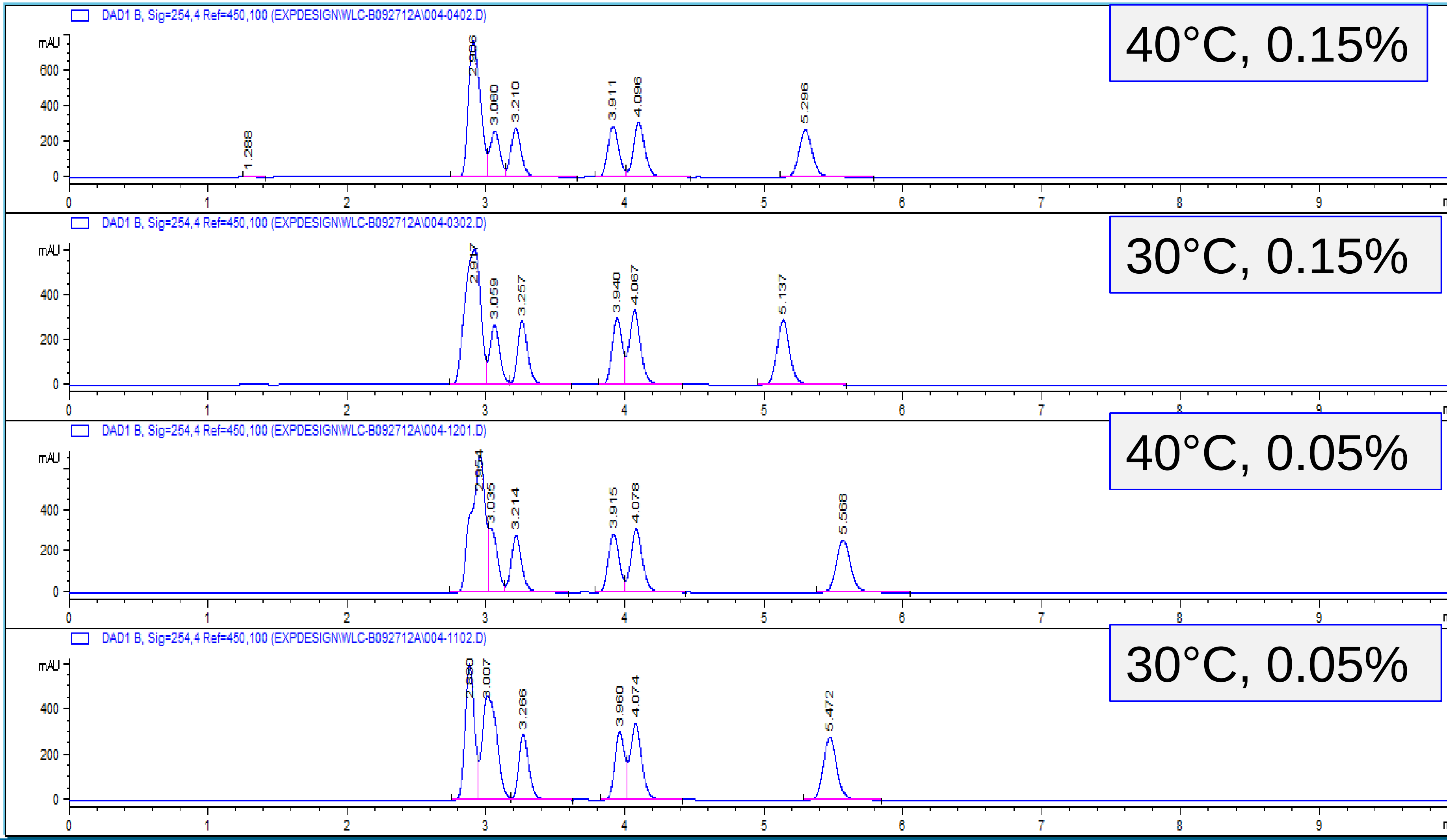
0.05% Formic Acid, 35% ACN



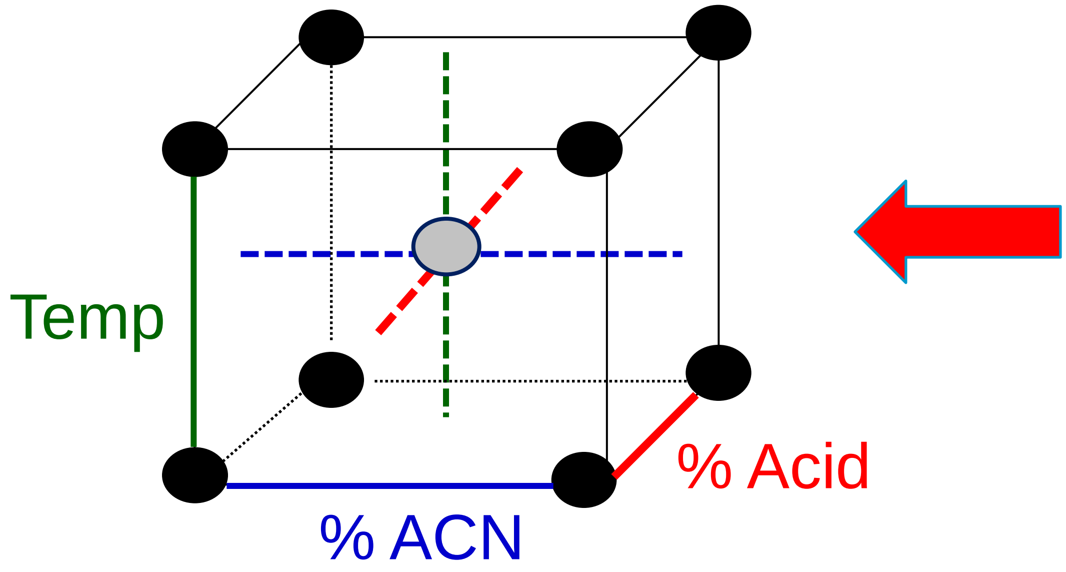
0.05% Formic Acid, 35% ACN



Temp, Formic Acid, 35% ACN

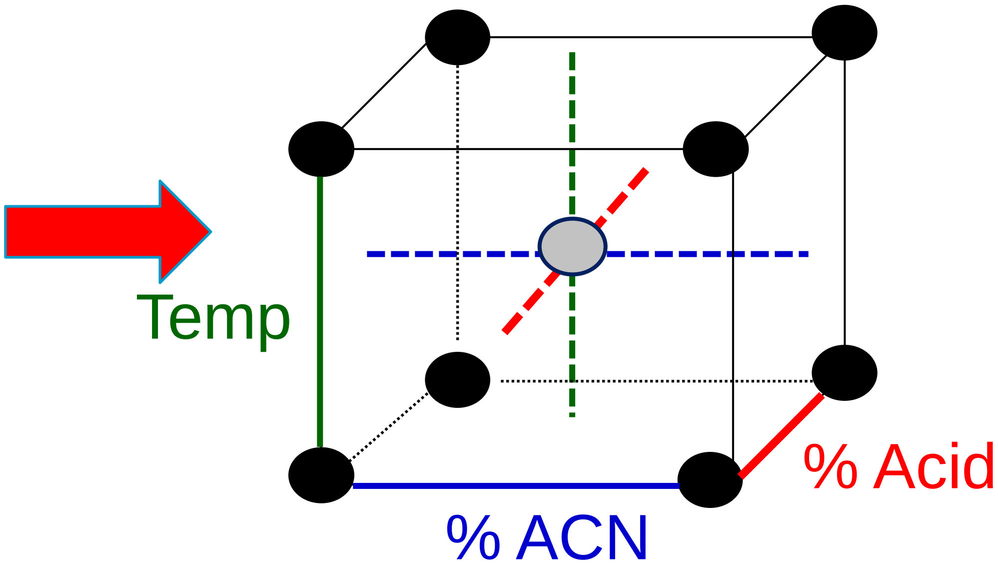


Factorial Design for Three Factors with Center Point



3 Factors, $2^3 + 1 = 9$

Factorial Design for Three Factors with Center Point

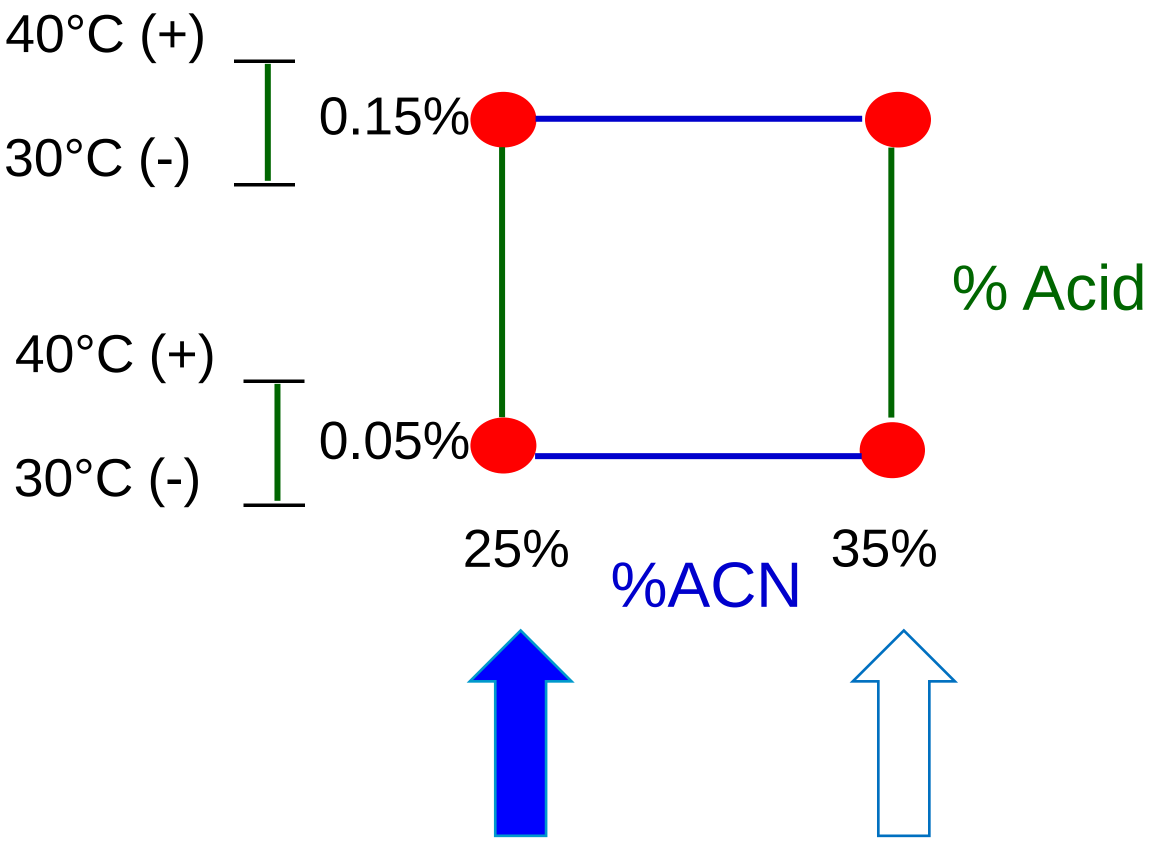


3 Factors, $2^3 + 1 = 9$

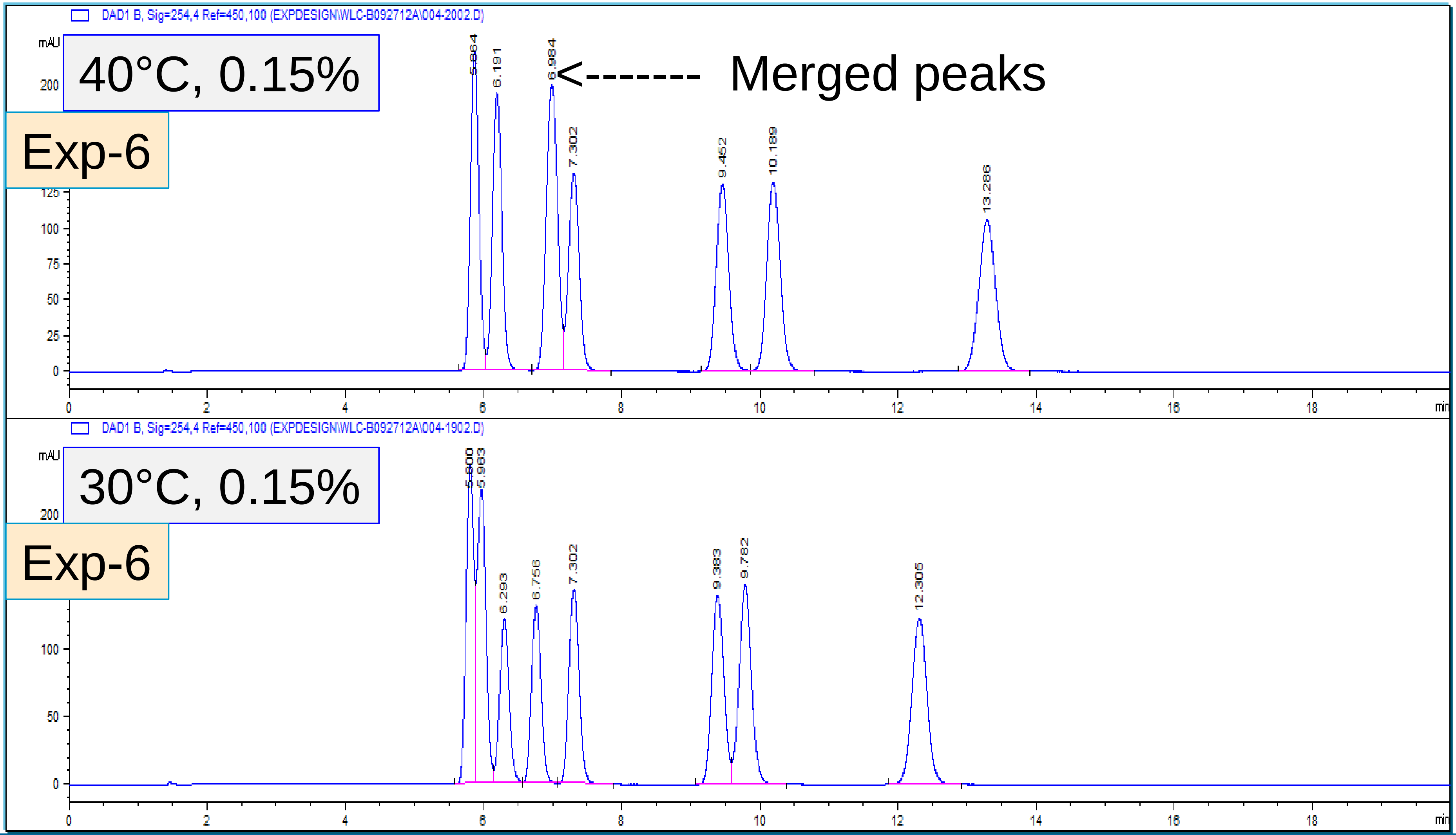
Factorial Design

Exp	% ACN	% FA	Temp
1	30	0.10	35
2	35	0.15	40
3	35	0.15	30
4	35	0.05	40
5	35	0.05	30
6	25	0.15	40
7	25	0.15	30
8	25	0.05	40
9	25	0.05	30

Displaying Results



0.15% Formic Acid; 25% ACN



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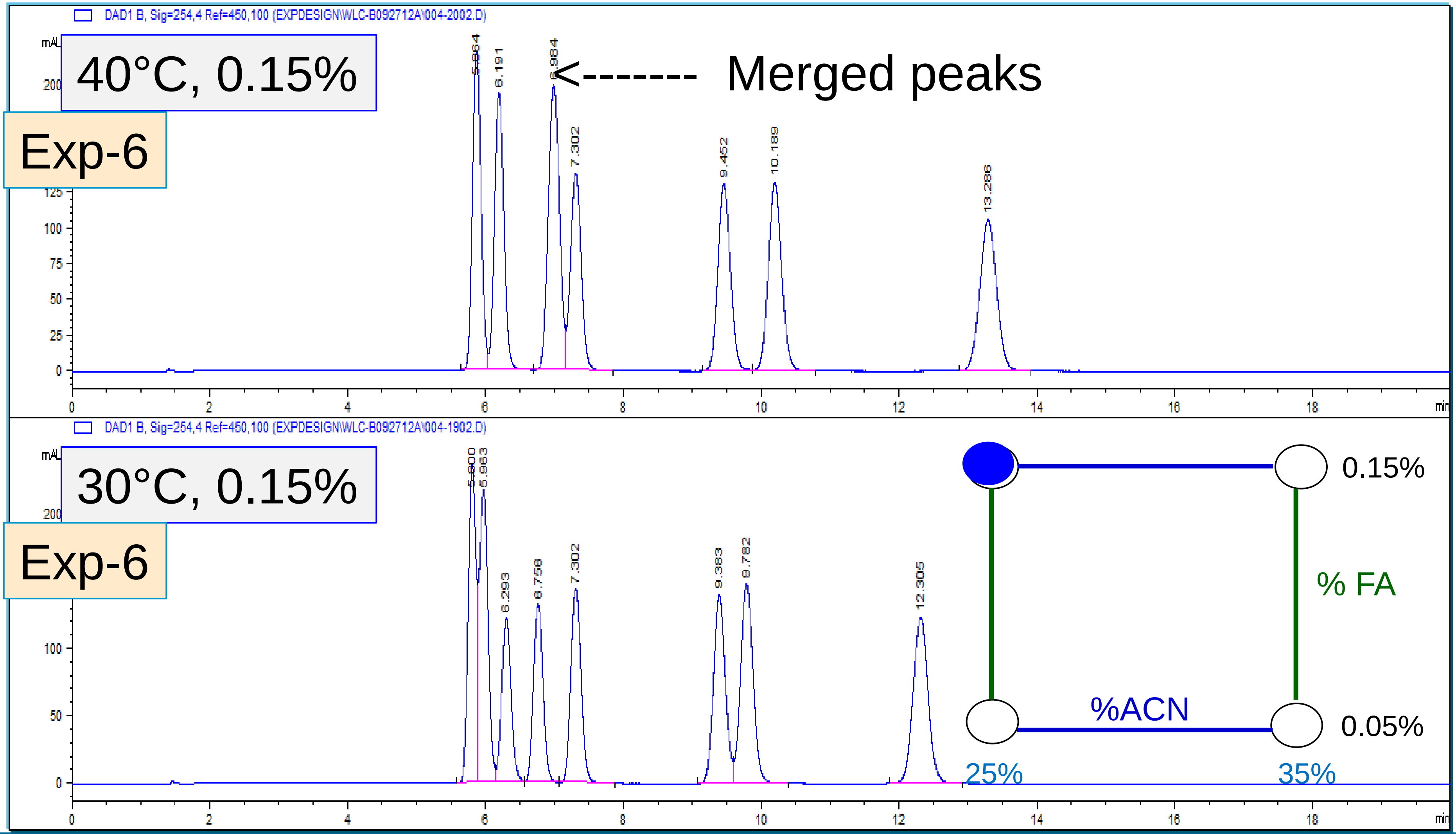
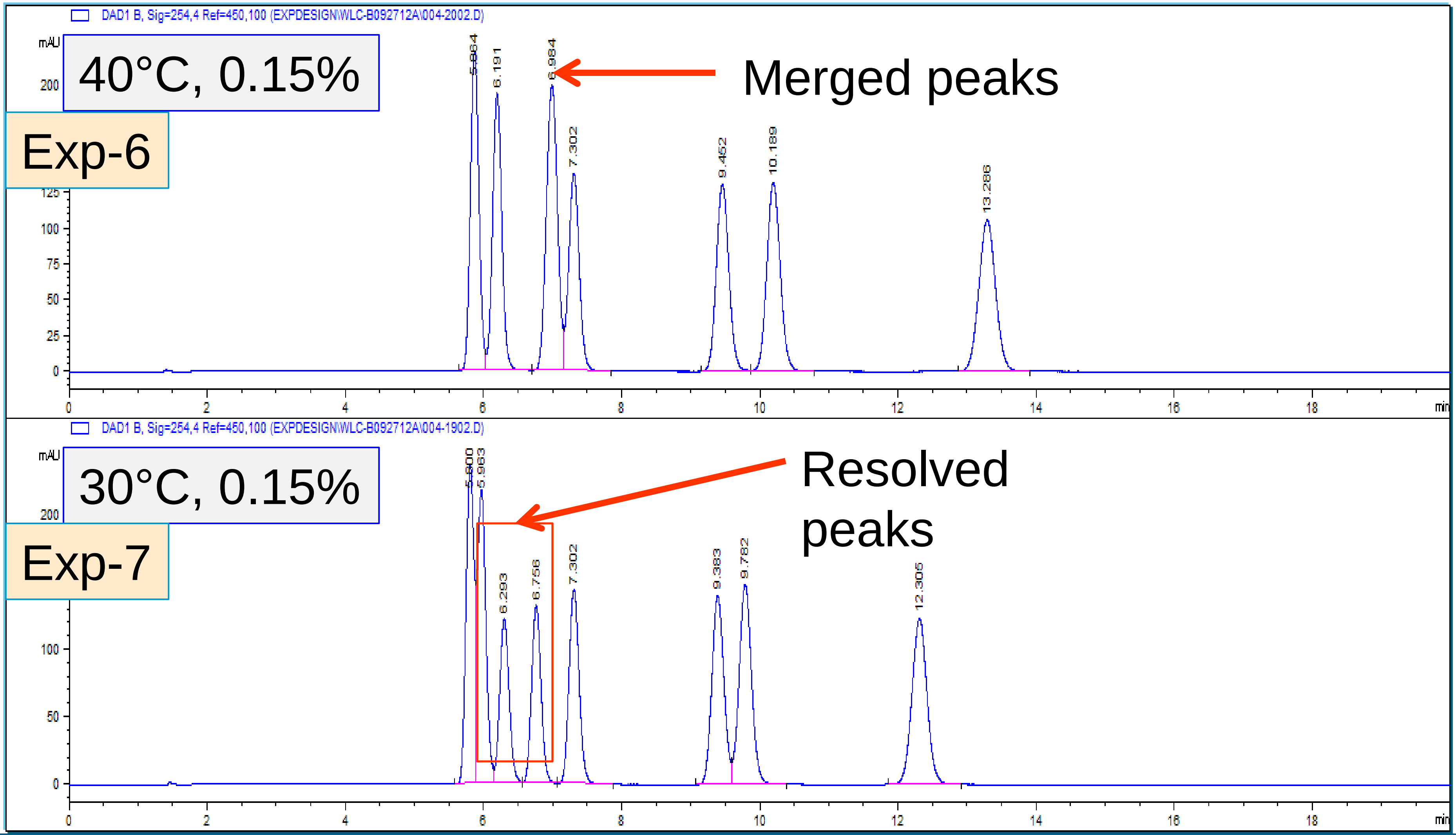
0.15% Formic Acid; 25% ACN

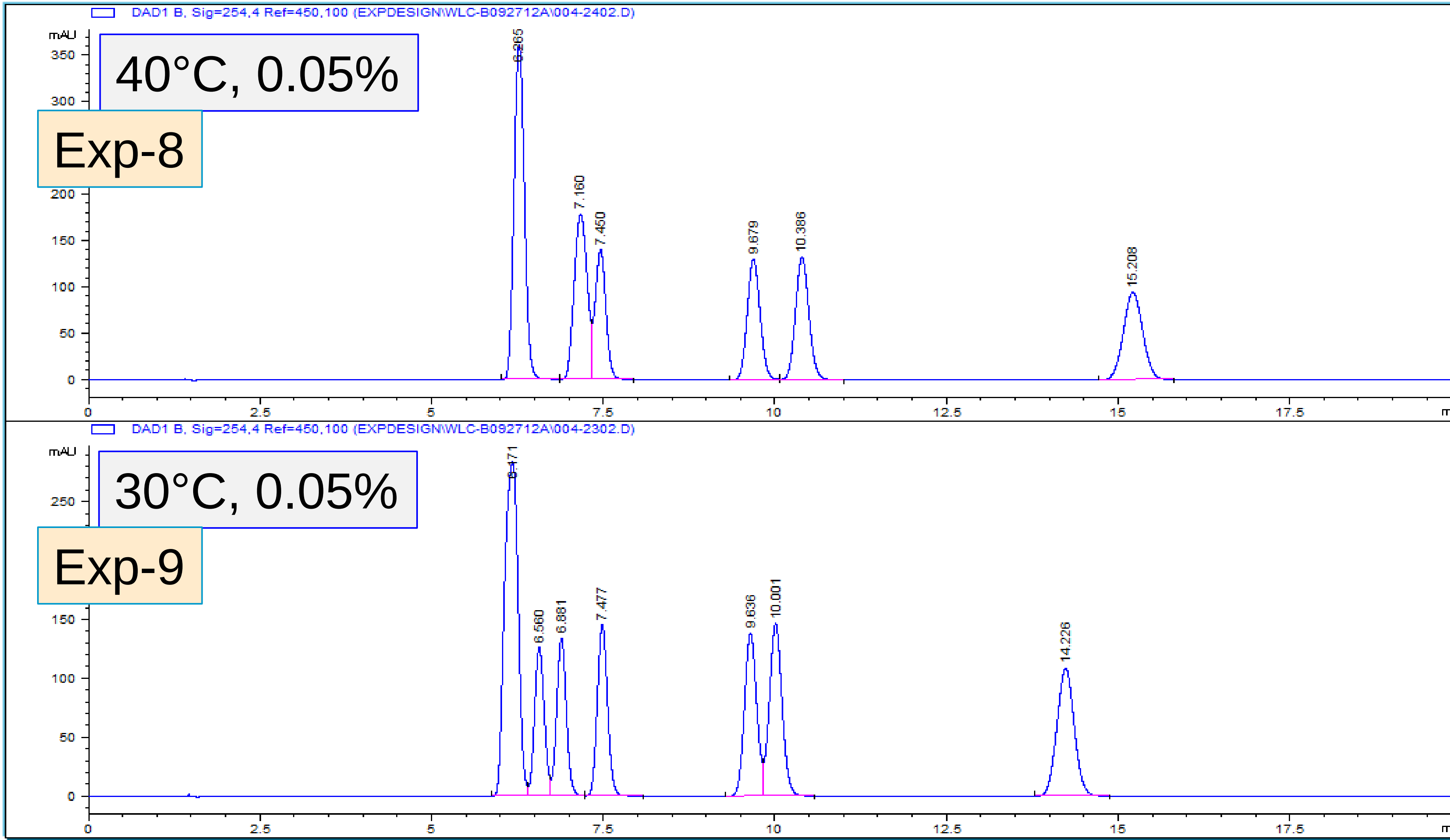


Agilent Technologies

0.15% Formic Acid; 25% ACN

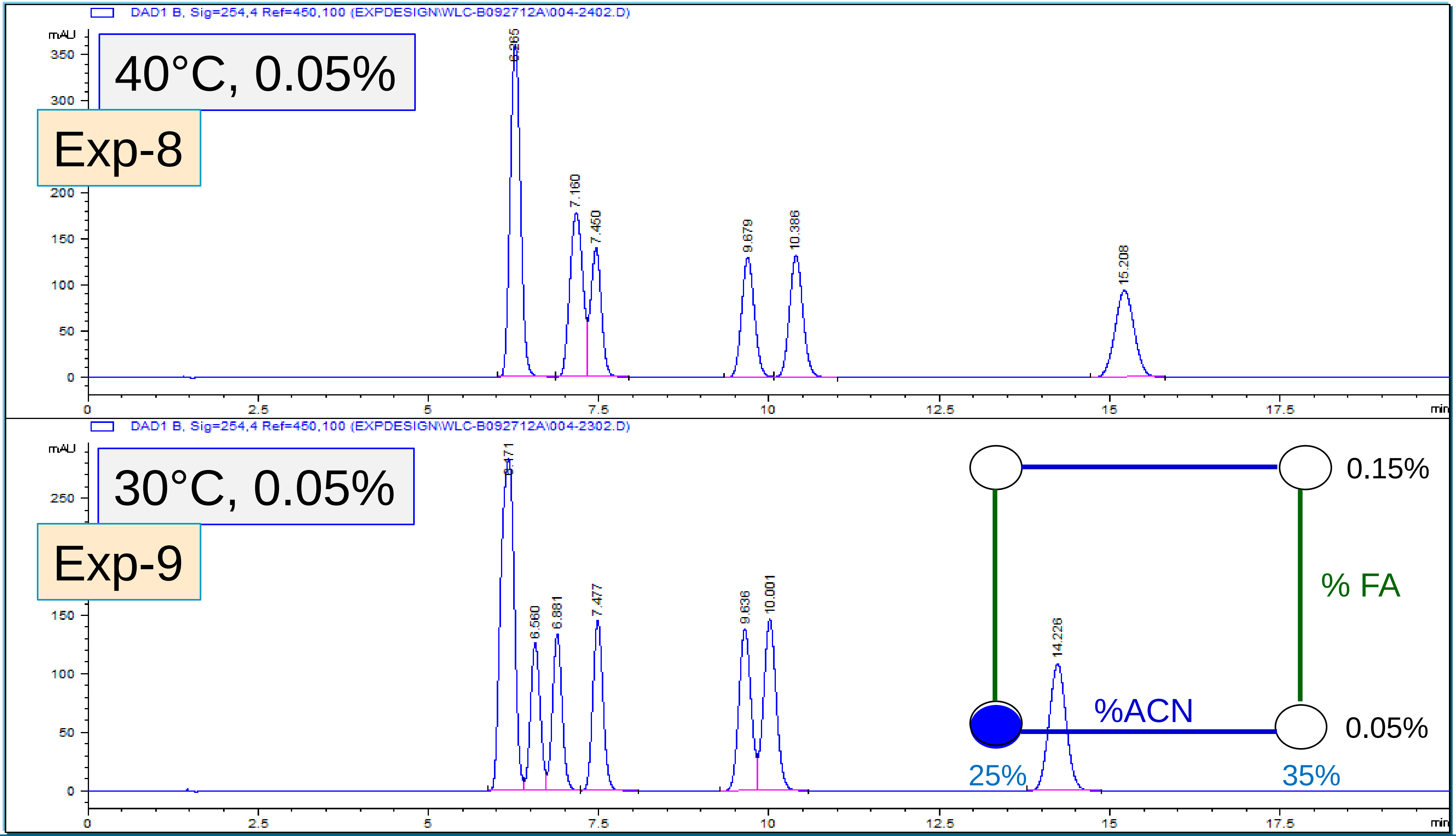


0.05% Formic Acid; 25% ACN



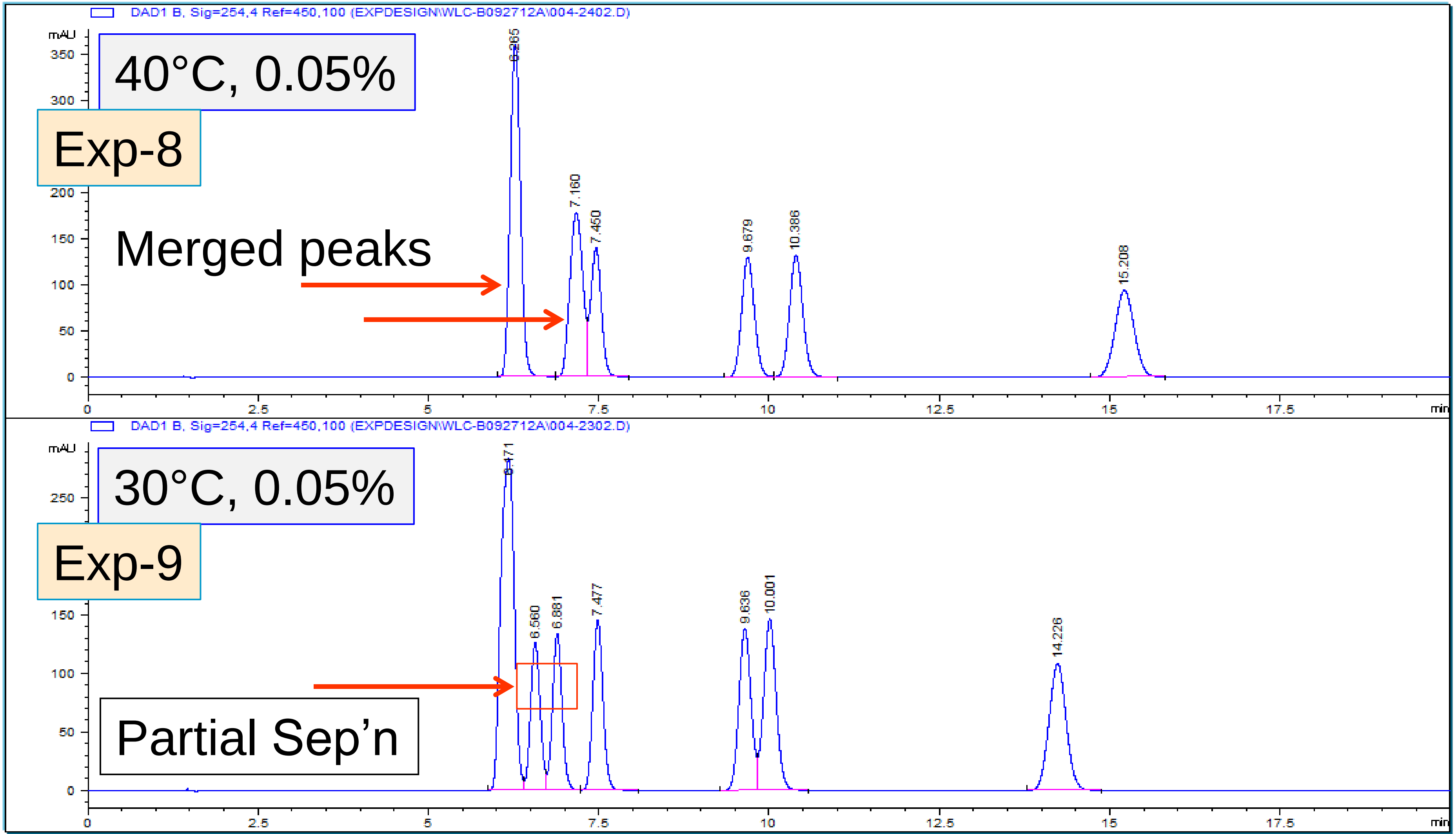
Agilent Technologies

0.05% Formic Acid; 25% ACN



Agilent Technologies

0.05% Formic Acid; 25% ACN



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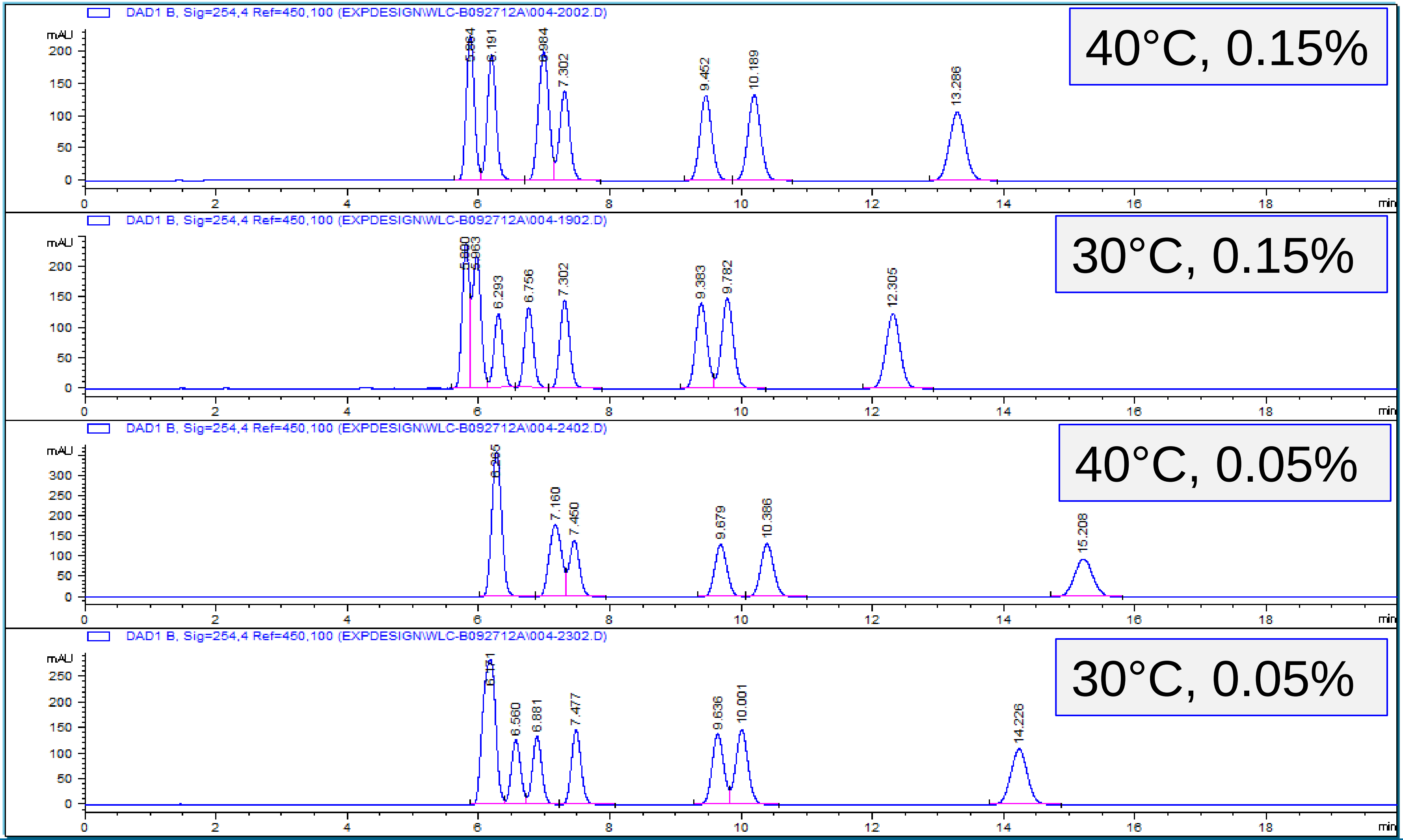
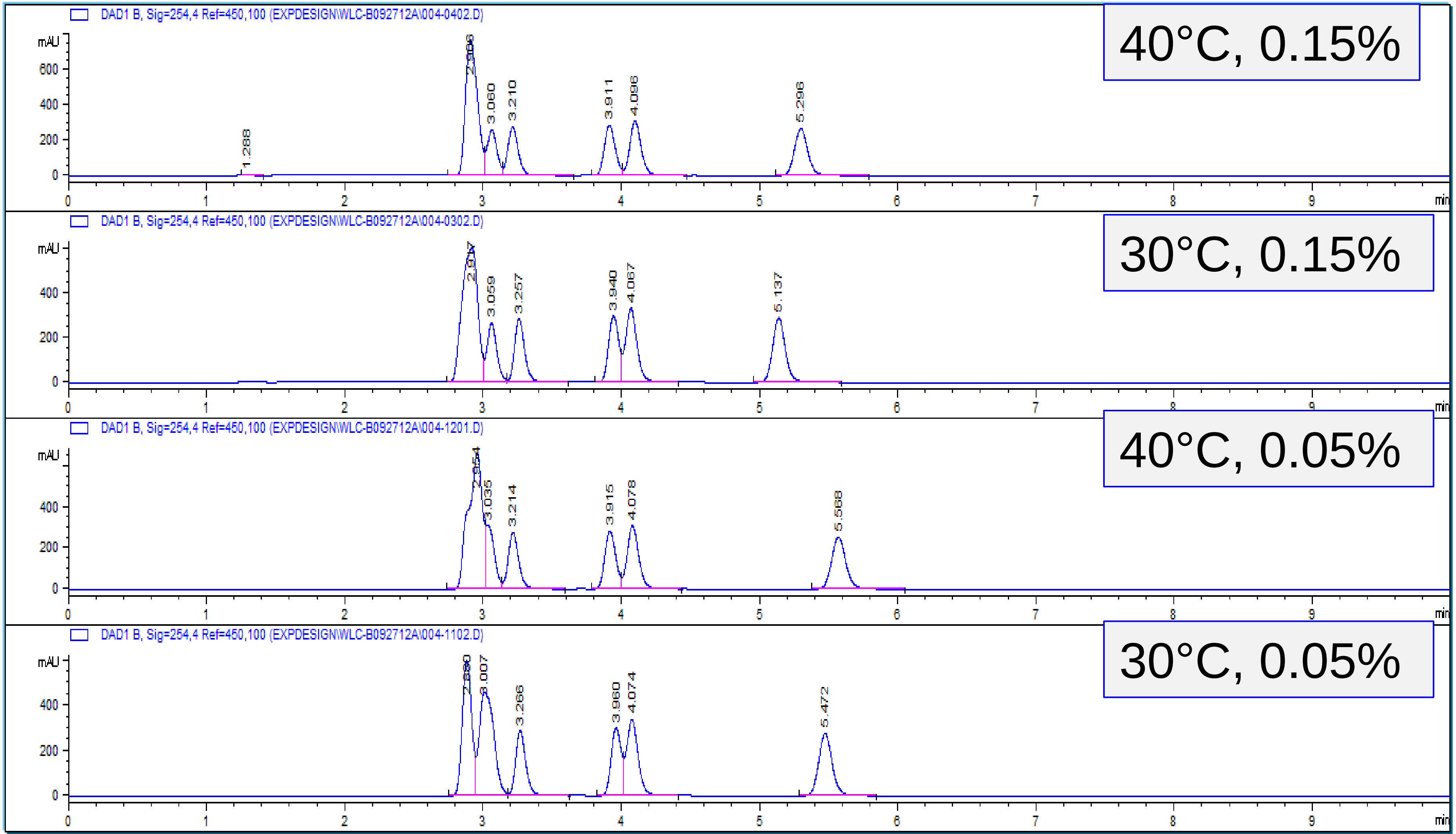
Temp, Formic Acid, 25% ACN



Temp, Formic Acid, 35% ACN



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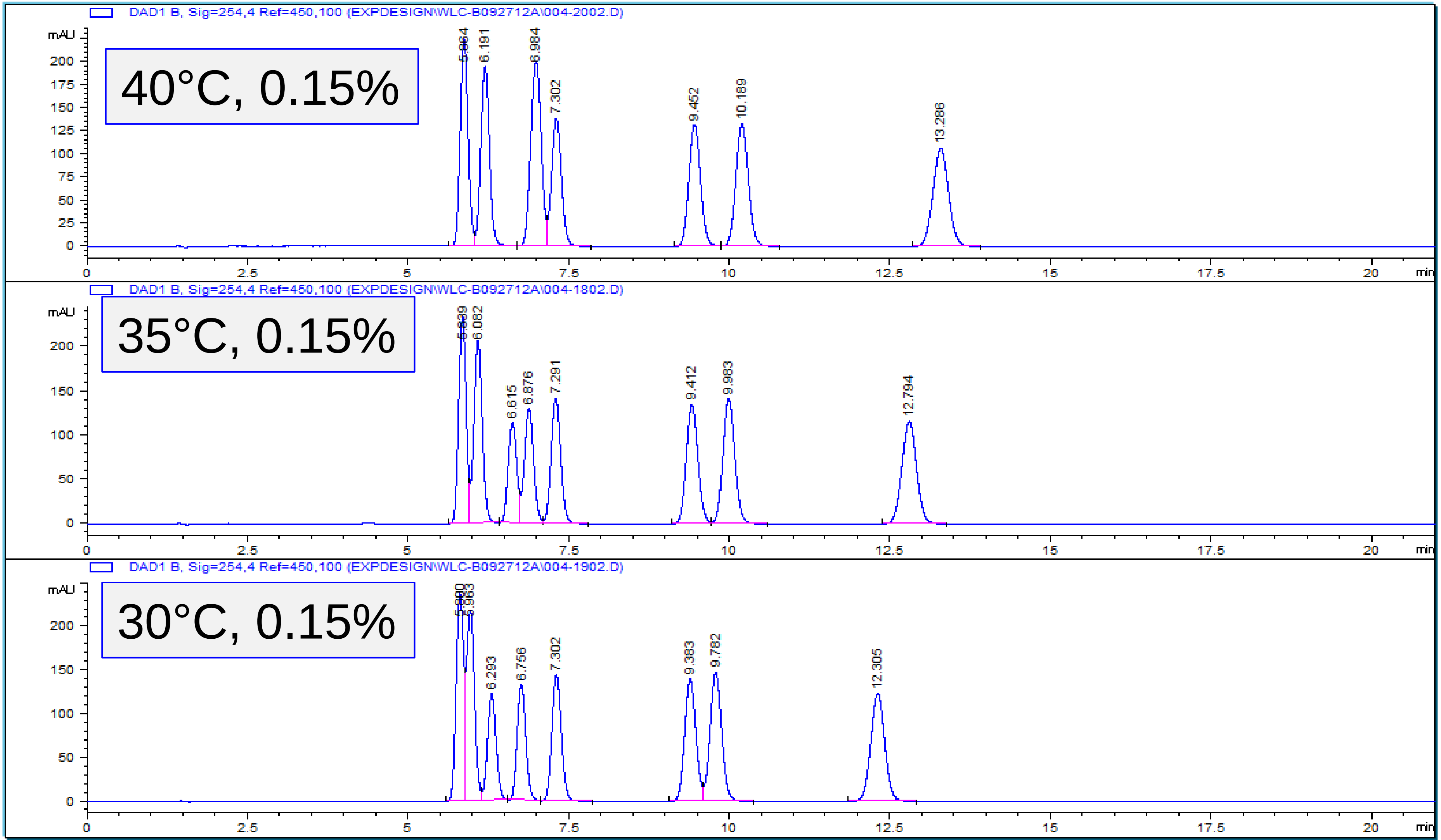
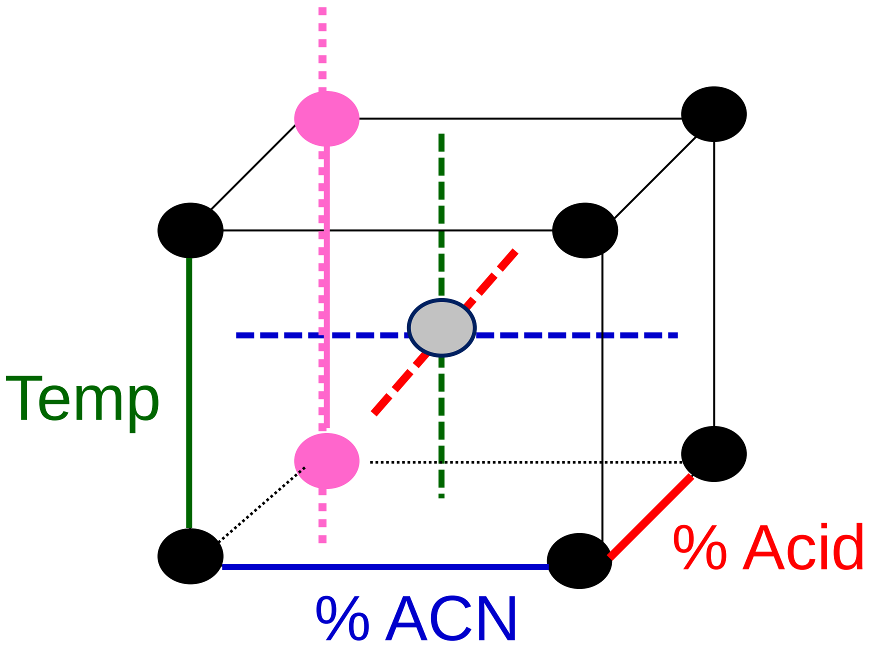
0.15% Formic Acid; 25% ACN



Factorial Design for Three Factors with Center Point

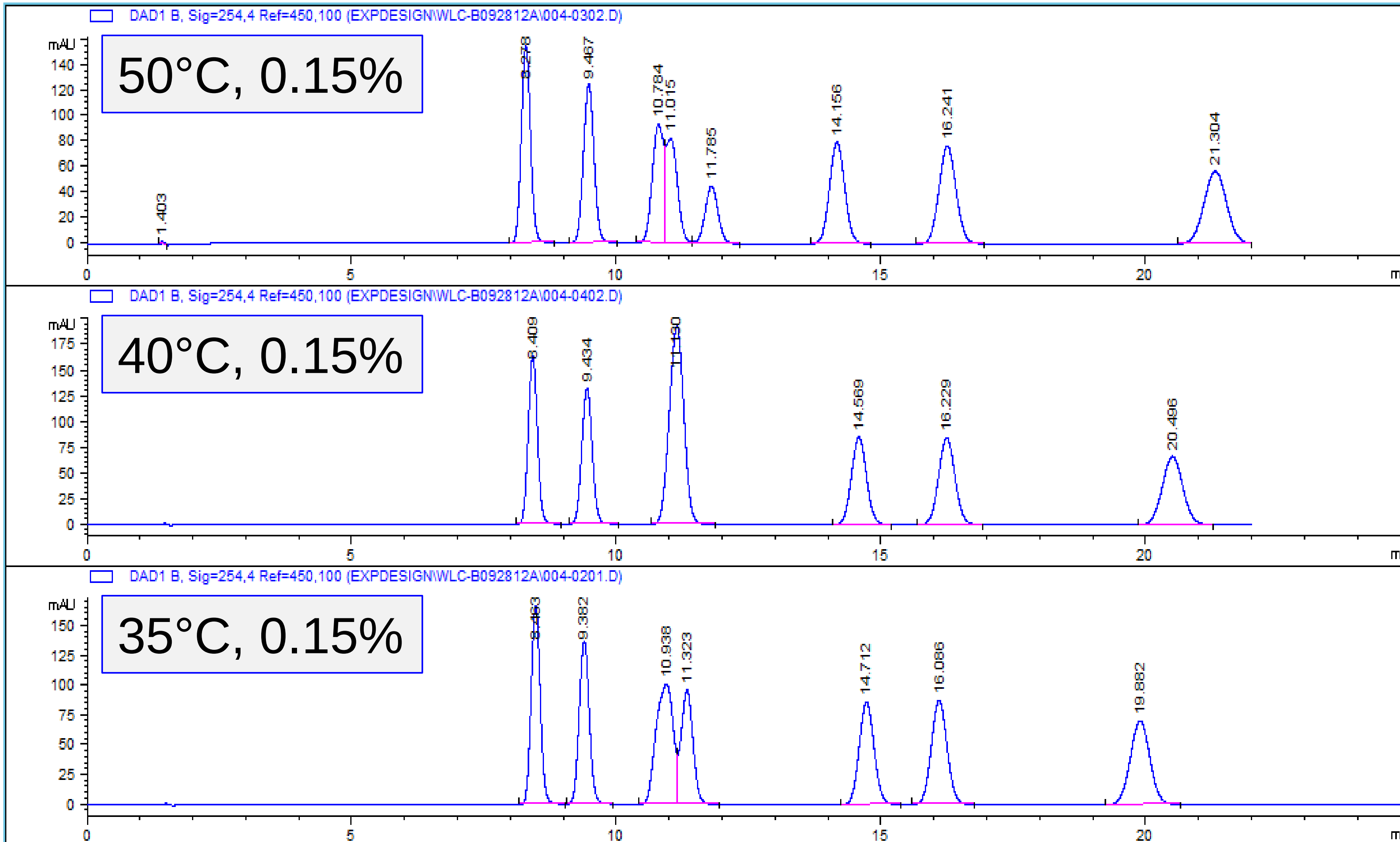


3 Factors, $2^3 + 1 = 9$

Factorial Design

Exp	% ACN	% FA	Temp
1	30	0.10	35
2	35	0.15	40
3	35	0.15	30
4	35	0.05	40
5	35	0.05	30
6	25	0.15	40
7	25	0.15	30
8	25	0.05	40
9	25	0.05	30

0.15% Formic Acid; 22% ACN



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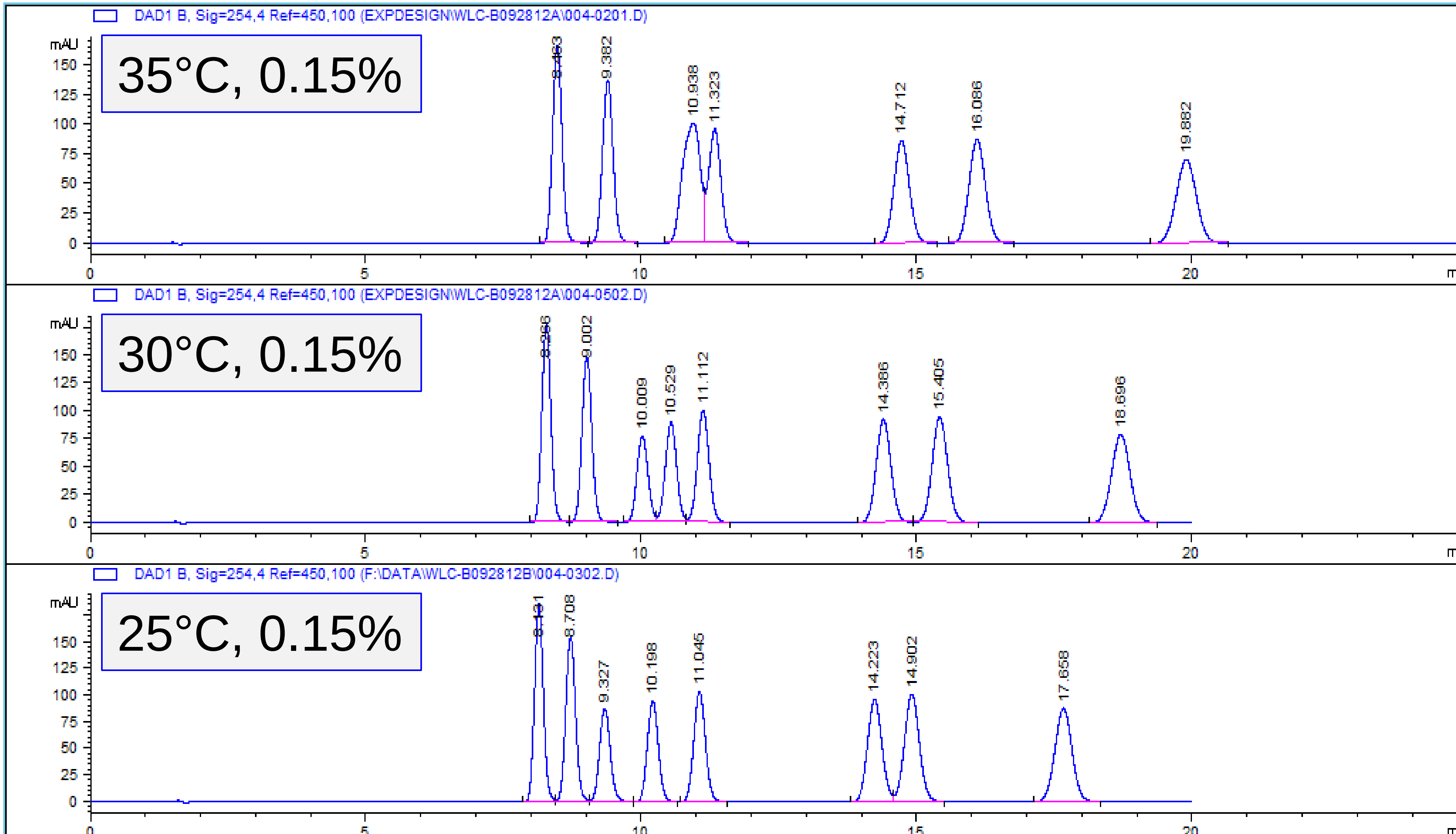
0.15% Formic Acid; 22% ACN



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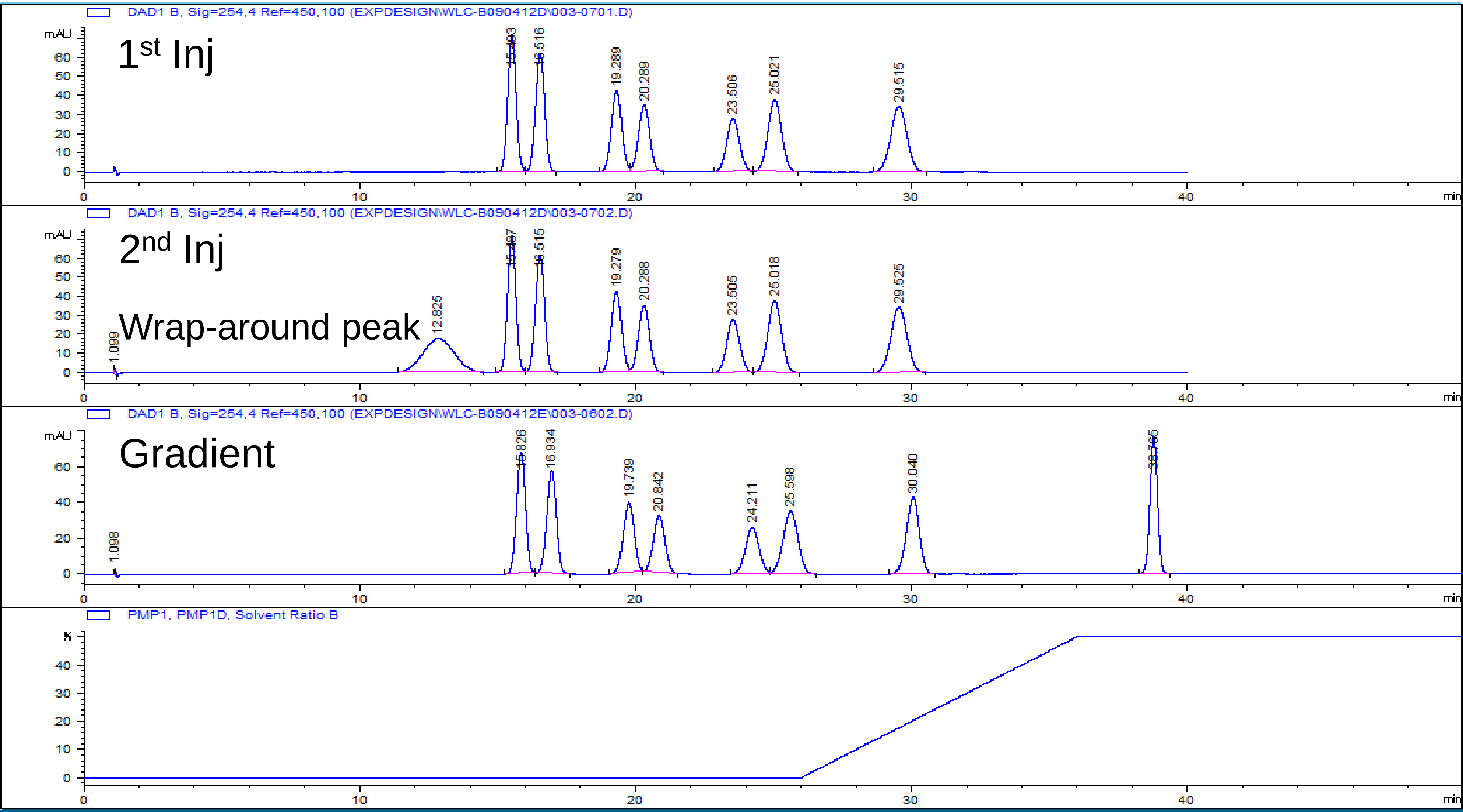
Isocratic Runs – 25% ACN, 35°C, no acid



OBSERVATIONS

- **Effect of small changes in acid**
- **Order of elution changes with % ACN**
- **Order of elution changes with Temp**
- **Similar polarity – similar retention**
- **22% ACN, 30°C and 25°C, 0.15% formic acid provide separation**
- **Expect changes in retention**

Experimental Designs – Increasing "Power"

Gradient screening

Isocratic separation – vary mobile phase strength

Simple screening design

Factorial design

Time/Effort

- Initial Gradients: > Half Day (3 exp's)
- Isocratic: Over-night (7 exp's)
- Screening: Set-up ~ 2 hrs
- Screening: Over-night (15 exp's)
- Factorial: Set-up ~ 2 hrs
- Factorial: Over-night (9 exp's)
- Optimization: > Half Day (6 exp's)

OBJECTIVE

- **Demonstrate a systematic approach to method development**
- **Improve understanding of separation process**
- **Development of more robust methods**
- **More efficient than “random walk”**

Thank you – Questions?

Bill Champion
800-227-9770, opt 3, LC Column Support
william_champion@agilent.com