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SMARTER CHROMATOGRAPHY

Using Selectivity to Optimize Resolution for Improved LC and LC–MS Method Development with Superficially Porous Particles

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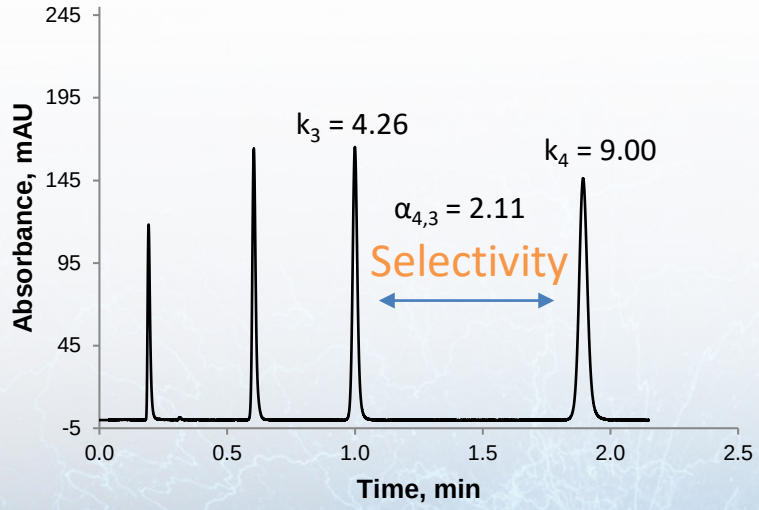
Outline

- Selectivity
- Parameters that Influence Selectivity
- Method Screening
- Summary/Conclusions

What is Selectivity?

- Selectivity (α) (also known as separation factor)
  - space between peaks in an HPLC separation
  - ratio of retention factors (k) between neighboring peaks and is always 1.0 or greater: $\alpha = k_2 / k_1$



TEST CONDITIONS
Column: 2.1 mm x 50 mm HALO 2 C18
Mobile Phase:85/15 ACN/Water
Flow rate: 0.5 mL/min
Temperature: Ambient (~25 °C)
Detector: UV (254 nm)
Sample Volume: 0.2 µL
Peak Identities:

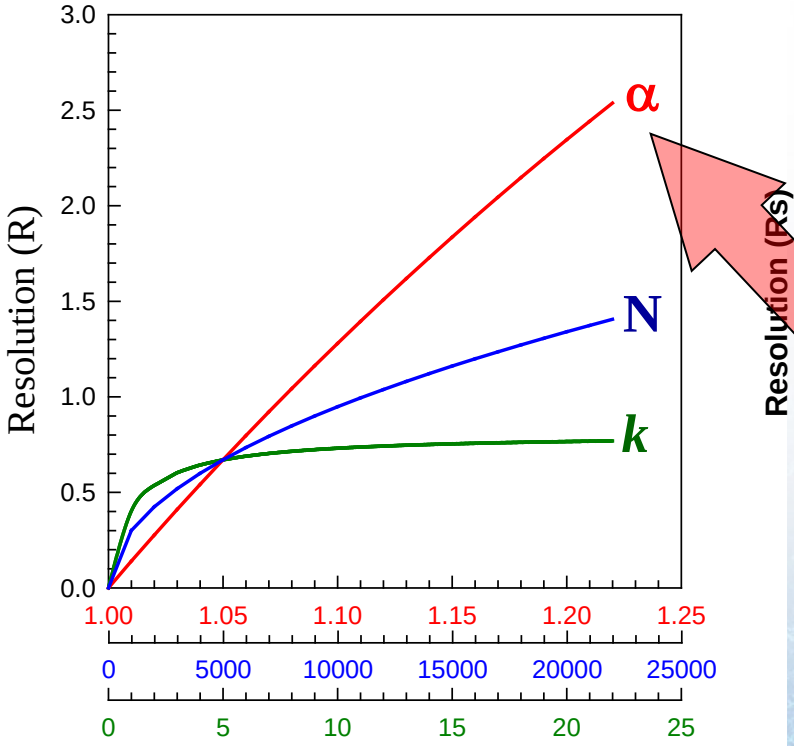
1. Uracil	27 µg/mL
2. Pyrene	67 µg/mL
3. Decanophenone	267 µg/mL
4. Dodecanophenone	467 µg/mL

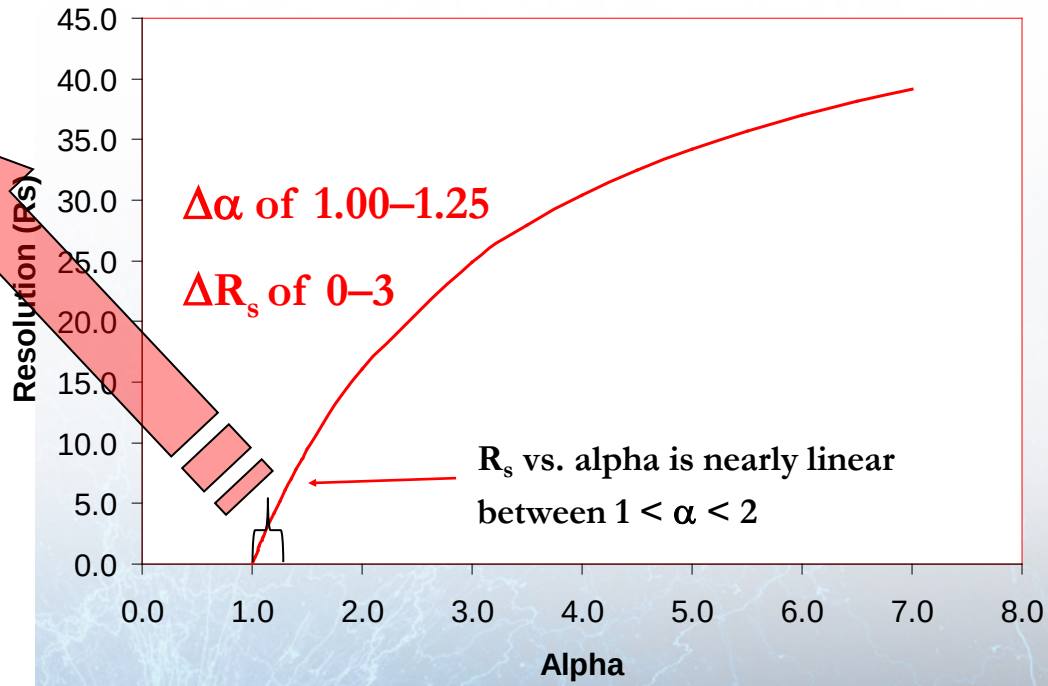
Effect of Selectivity on Resolution

$$R_S = \left(\frac{\sqrt{N}}{4}\right) x \left[\frac{(\alpha - 1)}{\alpha}\right] x \left[\frac{k_2}{(1+k_2)}\right]$$

Efficiency Selectivity Retention

N = plates
α = selectivity
k = retention factor





Source: Jun Mao, PhD Thesis with Professor Peter Carr, U. of Minnesota, 2001

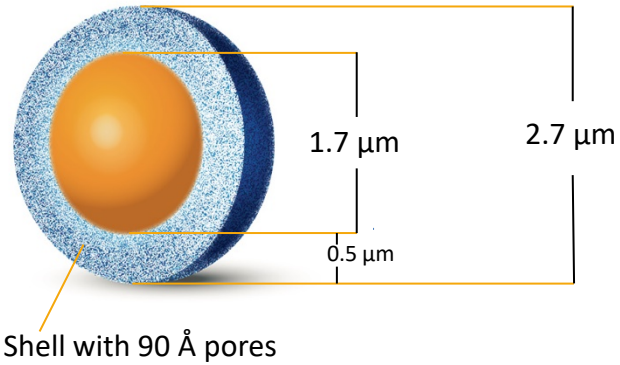
Parameters that Influence Selectivity

- Mobile phase* 
- Column type* 
- pH* 
- Temperature 

- Ion-pair concentration*
- % B (organic) solvent/gradient slope
- Buffer concentration

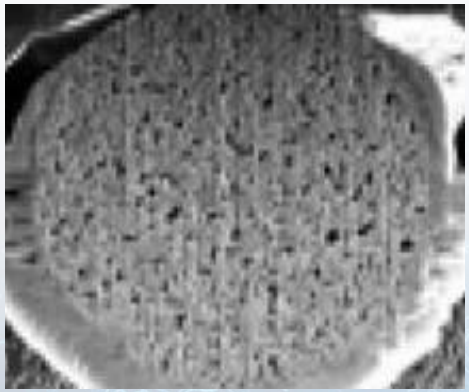
*More effective at influencing selectivity

Superficially Porous Particles

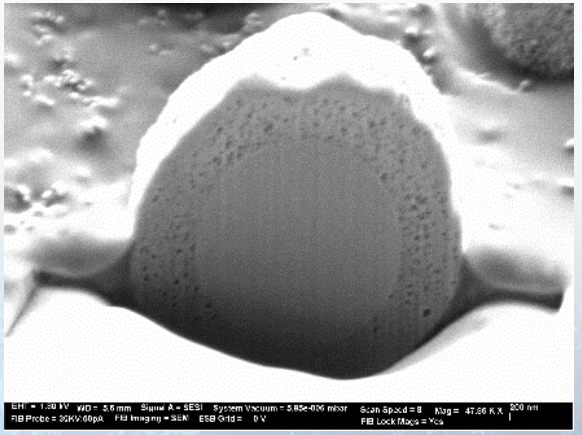


- Solid silica core
- Porous silica shell
- Shell thickness and pore size are tightly controlled

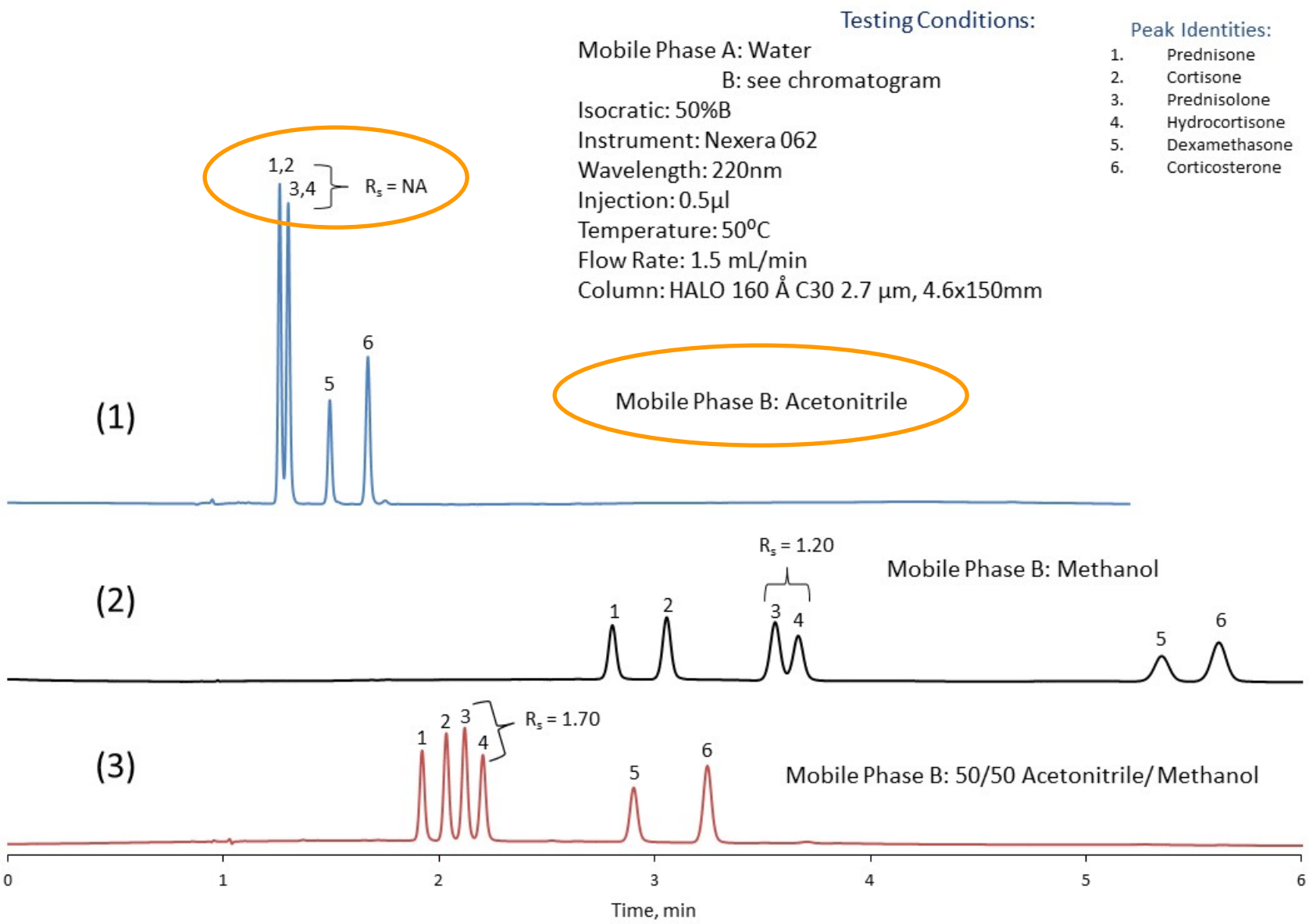
Fully Porous Particle (FPP)



HALO 90 Å, 2.7 µm



Effect of Mobile Phase on Selectivity



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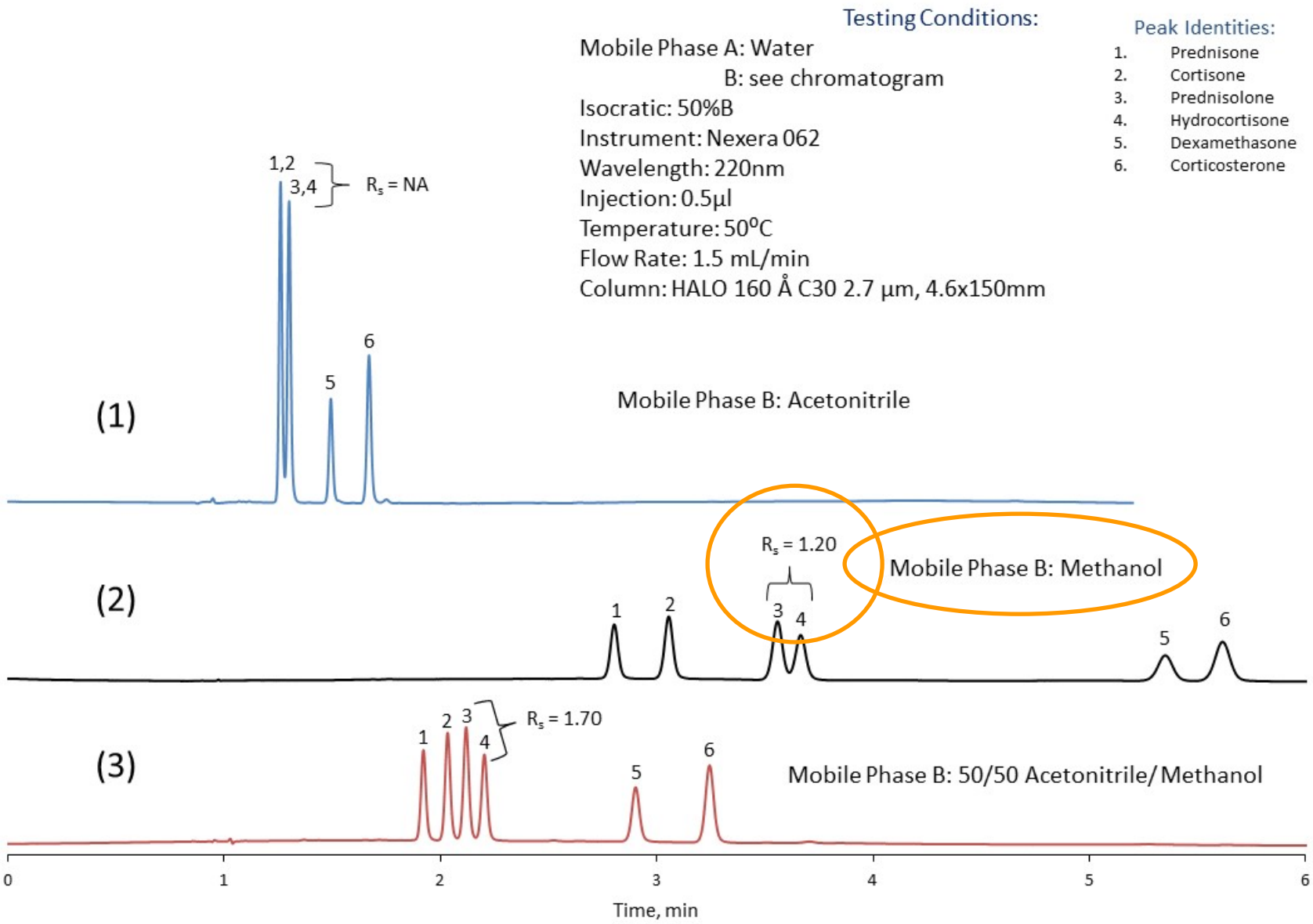


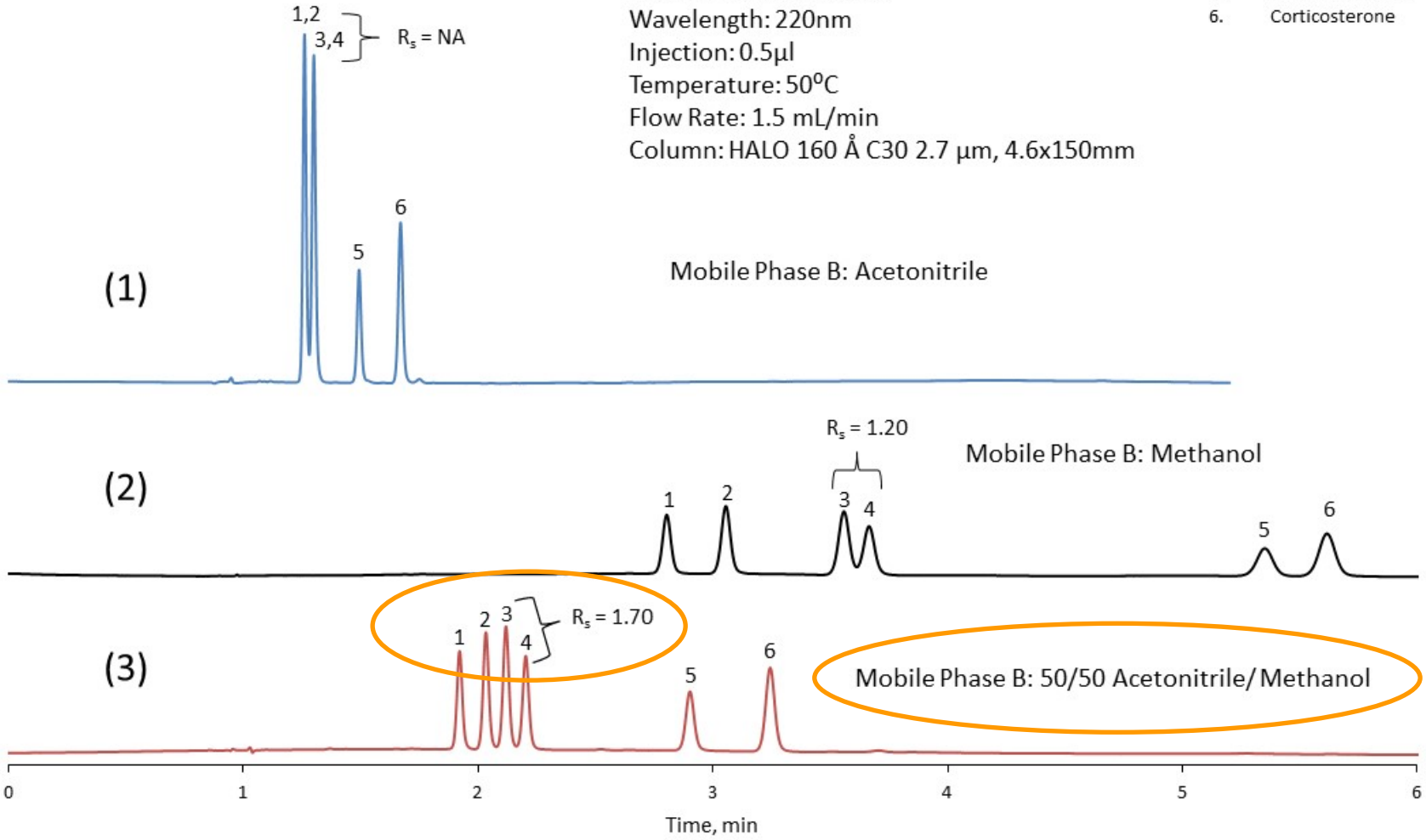
Effect of Mobile Phase on Selectivity

Testing Conditions:

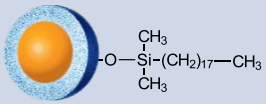
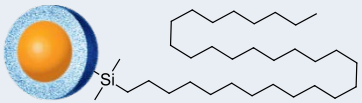
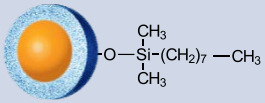
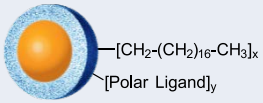
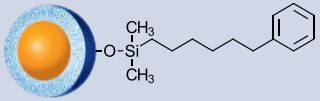
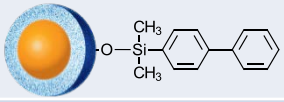
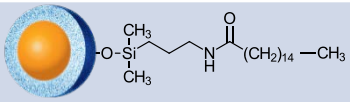
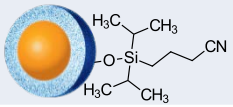
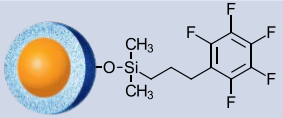
Mobile Phase A: Water
B: see chromatogram
Isocratic: 50%B
Instrument: Nexera 062
Wavelength: 220nm
Injection: 0.5µl
Temperature: 50°C
Flow Rate: 1.5 mL/min
Column: HALO 160 Å C30 2.7 µm, 4.6x150mm

Peak Identities:

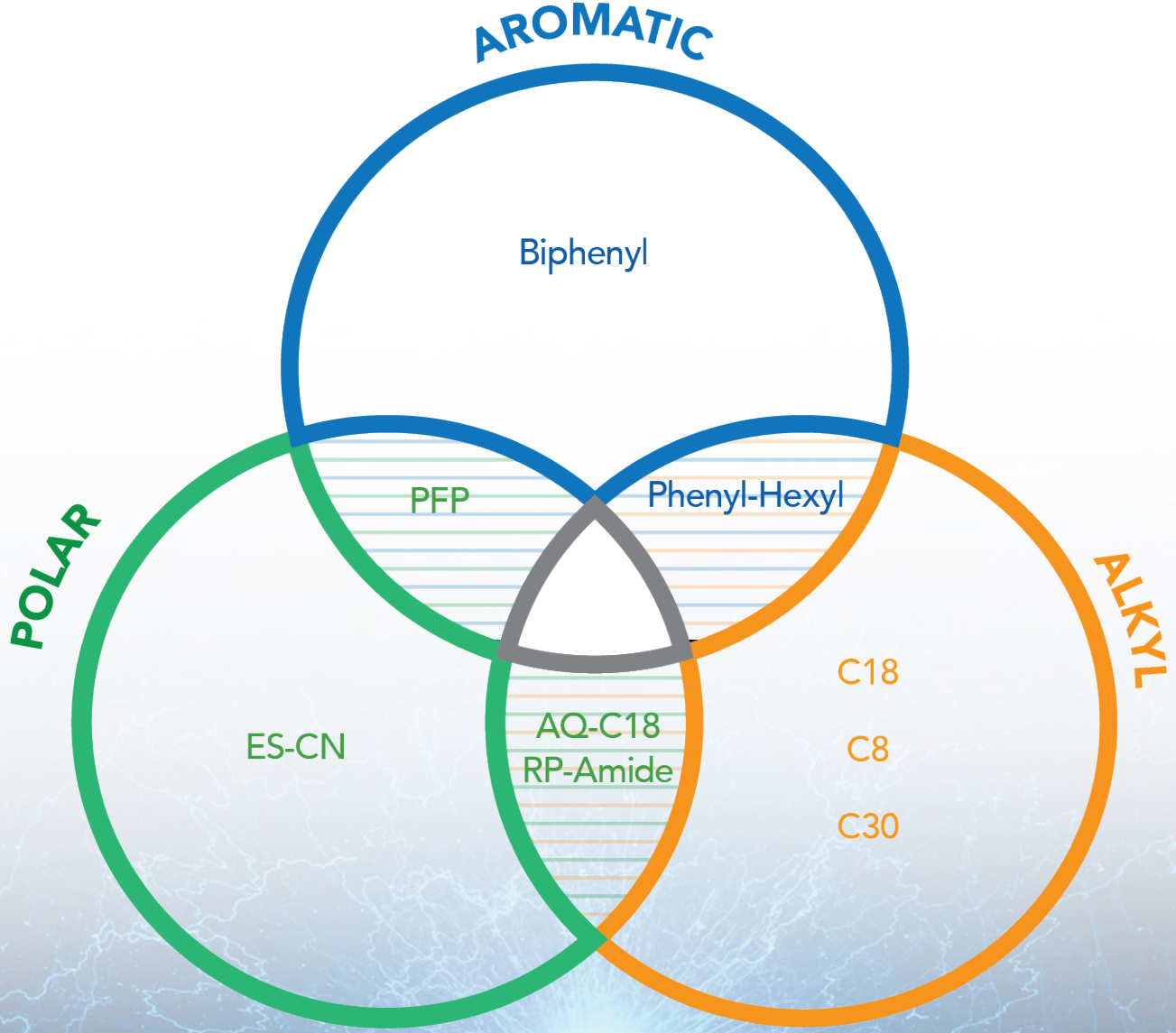
1. Prednisone
2. Cortisone
3. Prednisolone
4. Hydrocortisone
5. Dexamethasone
6. Corticosterone



HALO® Phases for Reversed-Phase HPLC and UHPLC

HALO® Bonded Phase	Structure	Chemistry	Types of Interactions
C18		Alkyl	• Hydrophobic
C30		Alkyl	• Hydrophobic
C8		Alkyl	• Hydrophobic
AQ-C18		Alkyl Polar	• Mainly hydrophobic • Some dipole-dipole
Phenyl-Hexyl		Aromatic Alkyl	• Hydrophobic • π - π
Biphenyl		Aromatic	• π – π • Hydrophobic
RP-Amide		Polar Alkyl	• Hydrogen Bonding • Hydrophobic
ES-CN		Polar	• Dipole-dipole • Hydrophobic
PFP		Aromatic Polar	• Hydrophobic • π - π • Dipole-dipole • Hydrogen bonding

HALO® Phases by Chemical Classification



Selectivity Differences Among HALO® Phases

Hydrophobic Subtraction Model

$$\log\left(\frac{k_x}{k_{EB}}\right) = \eta H - \sigma S^* + \beta A + \alpha B + \kappa C$$

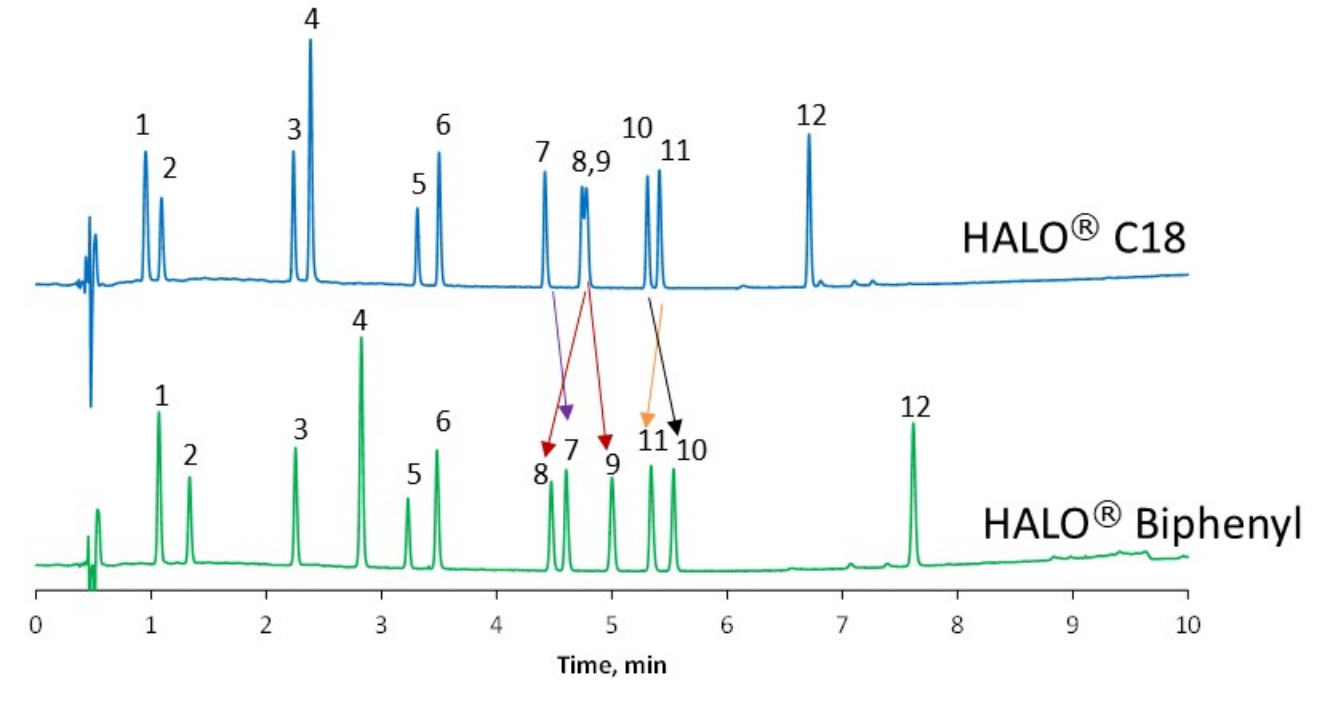
Decreased Similarity to C18

Fs	Phase	USP type	H	S*	A	B	C (pH 2.8)	C (pH 7.0)
0	HALO C18	L1	1.100	0.040	0.000	-0.050	0.050	0.040
10.04	HALO C8	L7	0.910	0.020	-0.130	0.000	-0.010	0.180
12.07	HALO AQ-C18	L1	1.000	-0.036	0.099	-0.048	0.156	0.864
17.35	HALO Phenyl-Hexyl	L11	0.780	-0.090	-0.230	0.000	0.100	0.450
17.43	HALO C30	L62	0.938	-0.046	-0.140	0.023	0.170	0.350
22.78	HALO ES-CN	L10	0.566	-0.110	-0.344	0.021	0.126	1.150
26.76	HALO Biphenyl	L11	0.708	-0.183	-0.279	0.028	0.047	0.990
52.83	HALO RP-Amide	L60	0.850	0.080	-0.380	0.190	-0.410	0.310
94.45	HALO PFP	L43	0.702	-0.117	-0.073	-0.062	1.170	0.972

*Note HSM values listed are for 2.7 µm particle size.

$$F_s = \sqrt{\left(w_H(H_1 - H_2)\right)^2 + \left(w_S(S^*_1 - S^*_2)\right)^2 + \left(w_A(A_1 - A_2)\right)^2 + \left(w_B(B_1 - B_2)\right)^2 + \left(w_{C_{2.8}}\left(C_{2.8_1} - C_{2.8_2}\right)\right)^2}$$

Effect of Bonded Phase on Selectivity

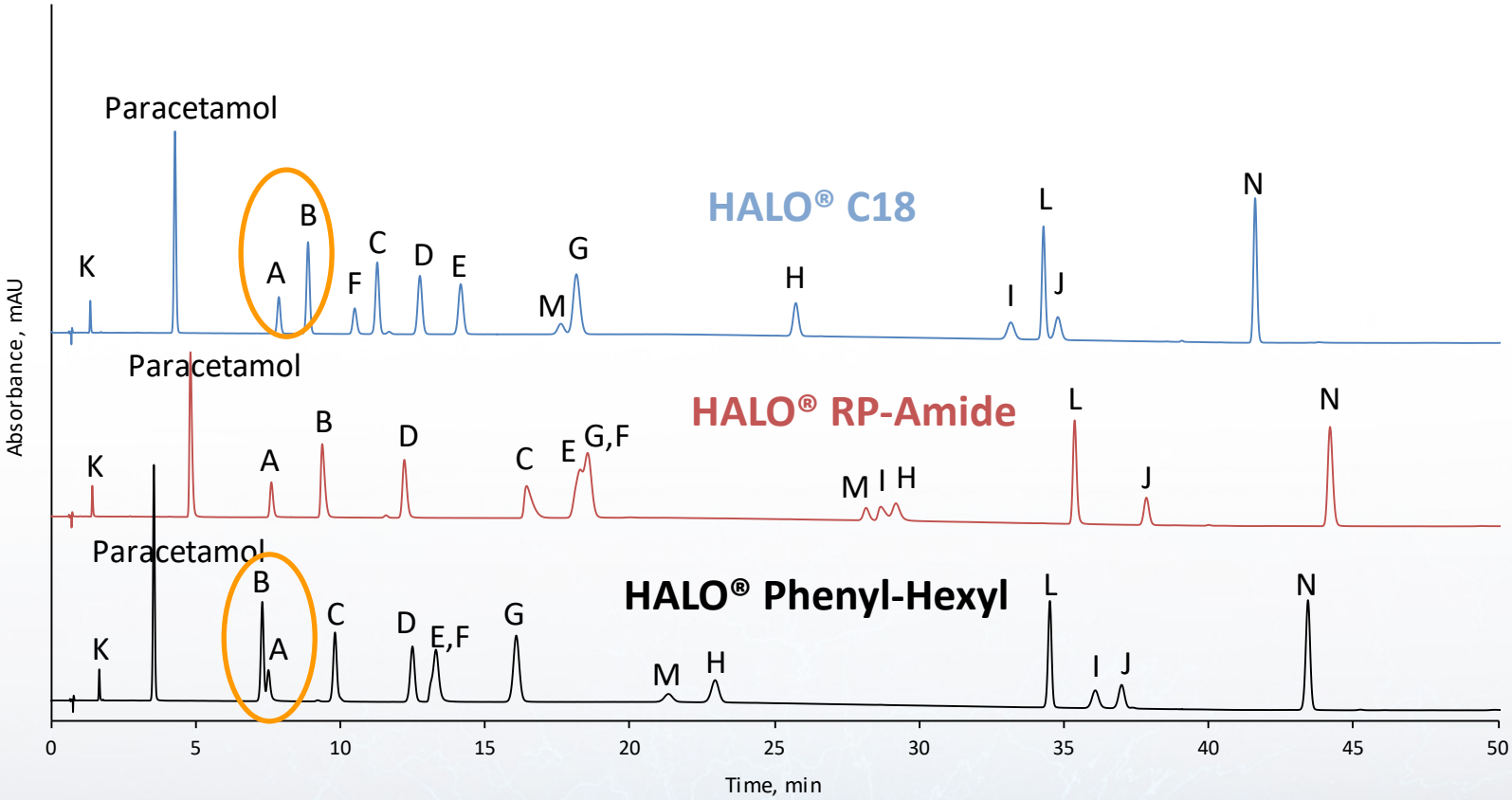


Peak Identities:

1. Atenolol
2. Sotalol
3. Nadolol
4. Pindolol
5. Acebutolol
6. Metoprolol
7. Oxprenolol
8. Bisoprolol
9. Labetalol
10. Propranolol
11. Alprenolol
12. Carvedilol

2.7 µm HALO, phases as indicated, 2.1 x 100 mm SPP columns
1 µL, 0.50 mL/min, 35 °C, 220 nm
Gradient from 10-50% CH_3CN/water/0.1% TFA in 10 min

Phase Screening with Constant Conditions



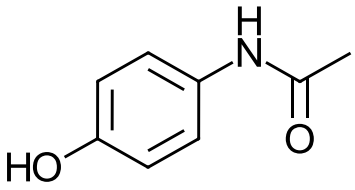
TEST CONDITIONS:

Column: HALO 90 Å, 2.7 µm, 2.1 x 100 mm, phase as indicated
Mobile Phase A: pH 7 Phosphate Buffer (1.7 g. potassium dihydrogen phosphate and 1.8 g. dipotassium hydrogen in 1000 mL)
Mobile Phase B: Methanol

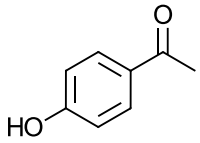
Gradient:

Time	%B
0.0	5
1.0	5
10.0	10
20.0	10
40.0	34
50.0	34

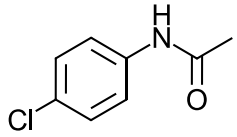
Flow Rate: 0.3 mL/min
Initial Pressure: 246 bar
Temperature: 30 °C
Detection: 254 nm, PDA



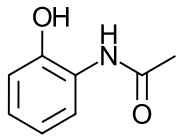
Paracetamol



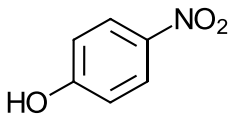
E. 1-(4-hydroxyphenyl) ethan-1-one



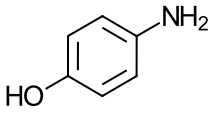
J. N- (4-chlorophenyl) acetamide



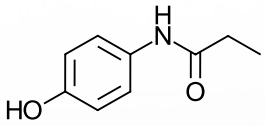
A. N-(2-hydroxyphenyl) acetamide



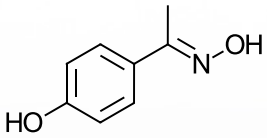
F. 4- nitrophenol



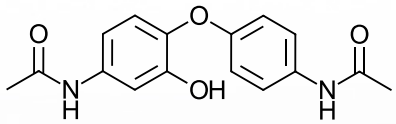
K. 4- aminophenol



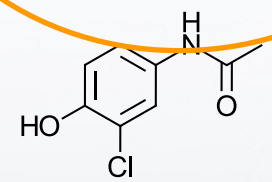
B. N- (4-hydroxyphenyl) propanamide



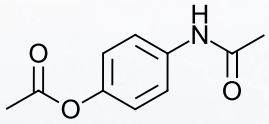
G. [1-(4-hydroxyphenyl) ethylidene] hydroxylamine



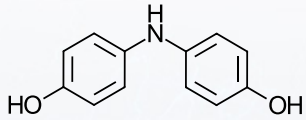
L. N- [4-(4-acetamido-2-hydroxyphenoxy) phenyl] acetamide



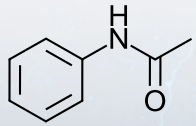
C. N-(3-chloro-4-hydroxyphenyl) propanamide



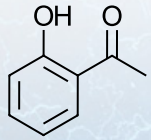
H. 4- acetamidophenyl aceate



M. 4,4'- azanediyldiphenol



D. N- phenylacetamide



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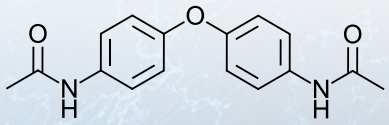
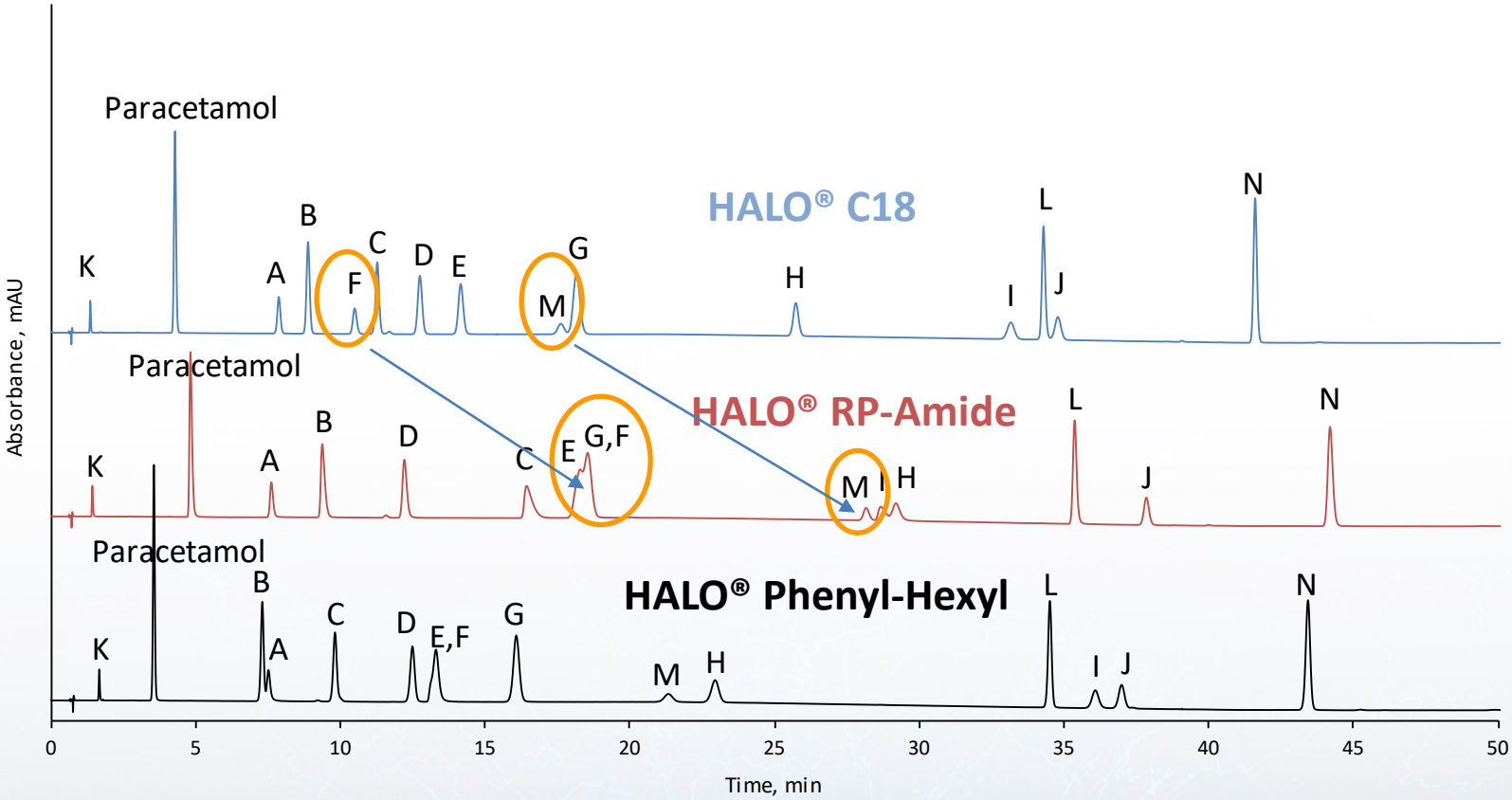


N. N,N'- [oxydi (4,1- phenylene)] diacetamide

Phase Screening with Constant Conditions



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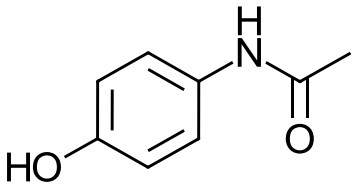
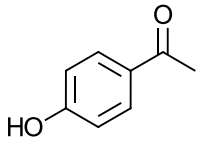


Paracetamol



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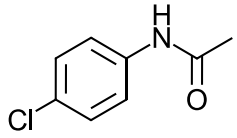

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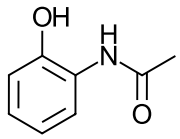
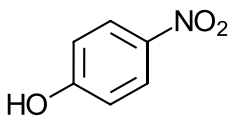
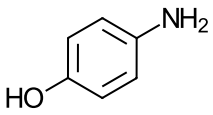


A. N-(2-hydroxyphenyl) acetamide



F. 4- nitrophenol



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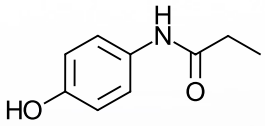
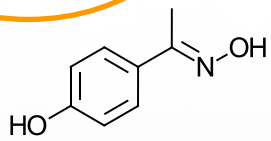
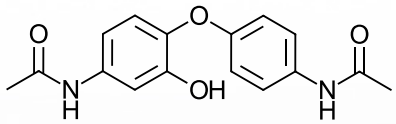


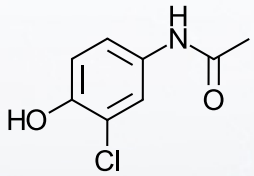
B. N- (4-hydroxyphenyl) propanamide



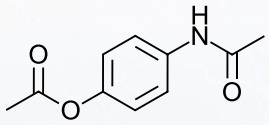
G. [1-(4-hydroxyphenyl) ethylidene] hydroxylamine



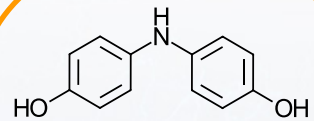
L. N- [4-(4-acetamido-2-hydroxyphenoxy) phenyl] acetamide



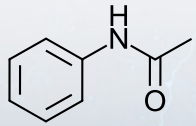
C. N-(3-chloro-4-hydroxyphenyl) propanamide



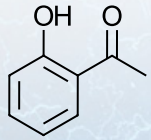
H. 4- acetamidophenyl aceate



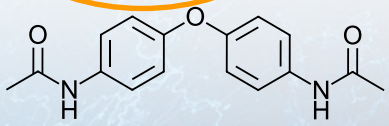
M. 4,4'- azanediyldiphenol



D. N- phenylacetamide



I . 1-(2-hydroxyphenyl) ethan-1-one



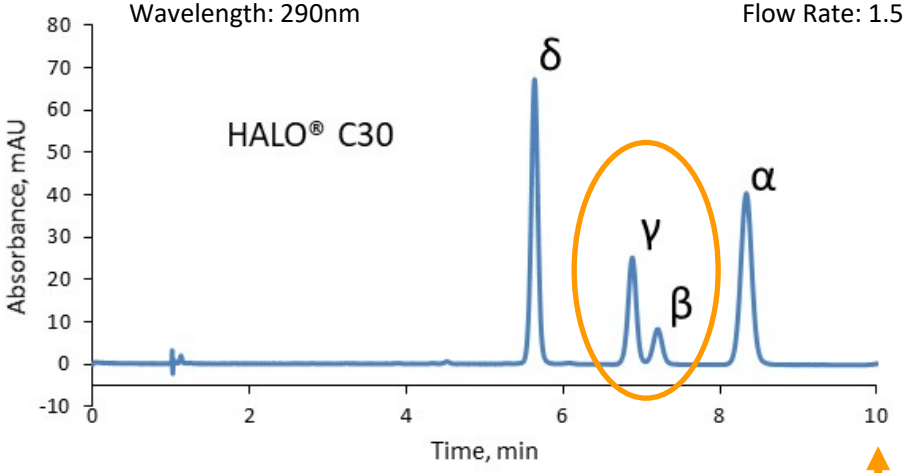
N. N,N'- [oxydi (4,1- phenylene)] diacetamide

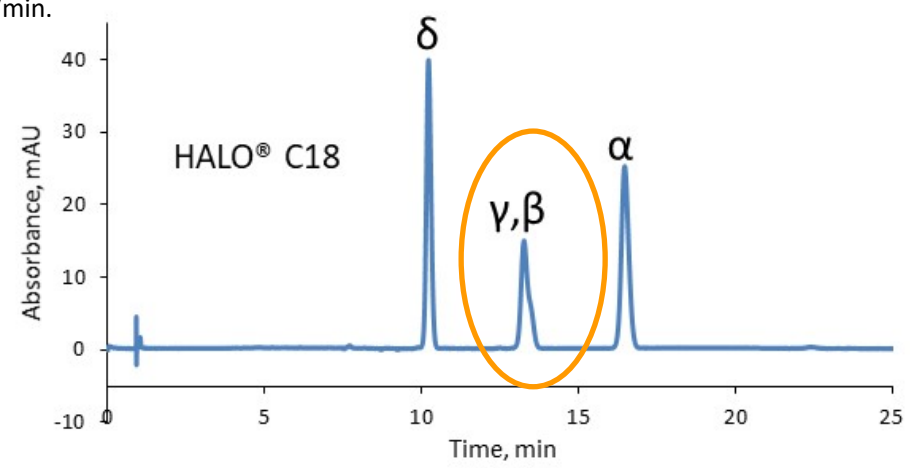
Phase Screening with Constant Conditions
α, β, γ, and δ-Tocopherols

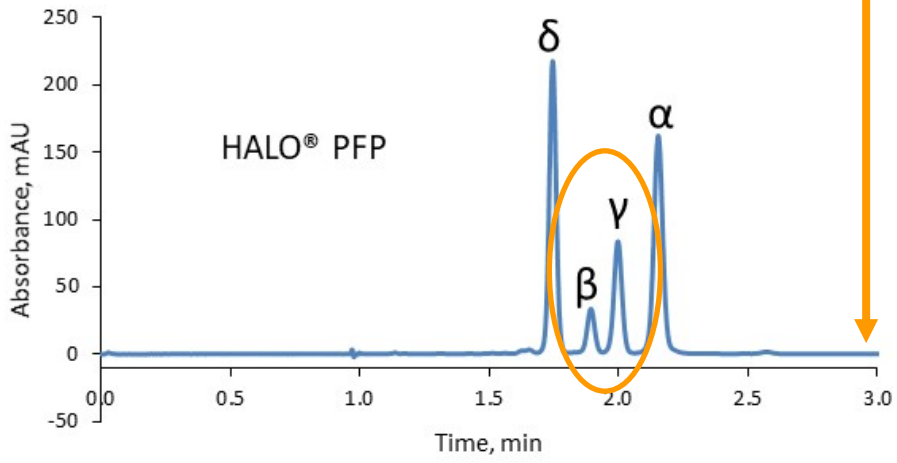
Testing Conditions:

Columns: HALO 4.6 x 150 mm, phase as indicated
Isocratic: 5/95 water/methanol
Wavelength: 290nm

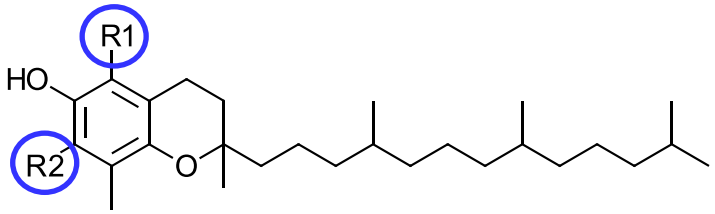
Injection: 1.5 µL
Temperature: 10°C
Flow Rate: 1.5 mL/min.





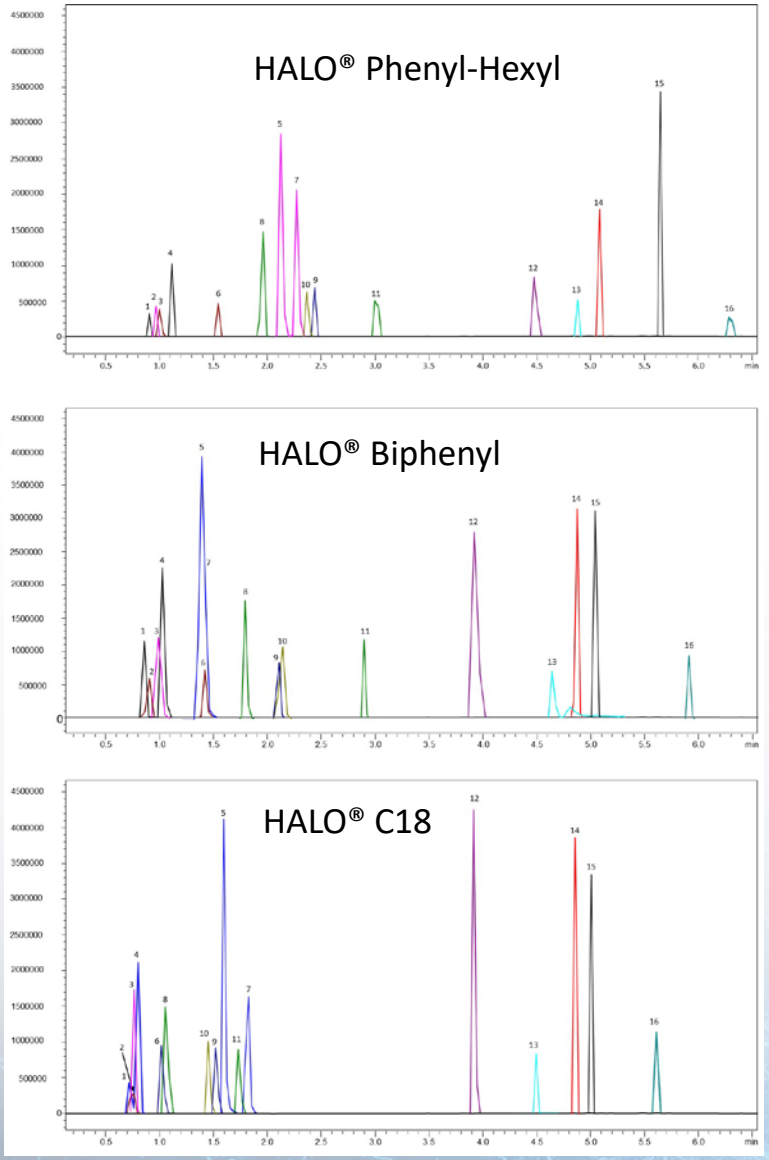


Generic Tocopherol (Vitamin E) Structure:



Tocopherol	R1	R2
Alpha (α)	CH_3	CH_3
Beta (β)	CH_3	H
Gamma (γ)	H	CH_3
Delta (δ)	H	H

LC-MS Screening of Drugs of Abuse and Metabolites: Phenyl-Hexyl, Biphenyl and C18



Test Conditions:

Columns: HALO 90 Å phase as indicated, 2.7 µm, 2.1 x 100 mm

Mobile Phase A: water/0.1% formic acid

B: methanol/0.1% formic acid

Gradient:

Time	%B
0.00	10
3.00	20
6.00	80
7.00	80
7.01	10
9.00	10

Flow Rate: 0.4 mL/min

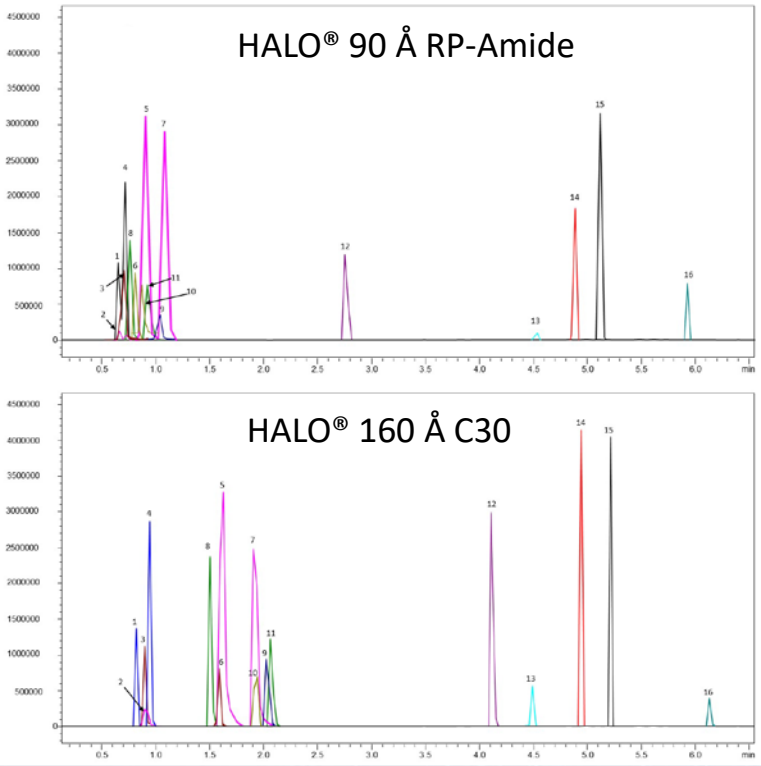
Temperature: 30 °C

Detection: MS

Injection Volume: 0.2 µL of 1 ng/mL drugs of abuse and metabolites

Peak Number	Compound	m/z
1	Hydromorphone	286
2	Oxymorphone	302
3	Noroxycodone	302
4	Morphine	286
5	Methamphetamine	150
6	Naloxone	328
7	Phentermine	150
8	Codeine	300
9	Naltrexone	342
10	Oxycodone	316
11	Hydrocodone	300
12	Meperidine	248
13	Fentanyl	337
14	Buprenorphine	468
15	Methadone	310
16	THC	304

LC-MS Screening of Drugs of Abuse and Metabolites: RP-Amide and C30



Test Conditions:

Columns: HALO 90 Å or 160 Å phase as indicated, 2.7 µm, 2.1 x 100 mm

Mobile Phase A: water/0.1% formic acid

B: methanol/0.1% formic acid

Gradient:

Time	%B
0.00	10
3.00	20
6.00	80
7.00	80
7.01	10
9.00	10

Flow Rate: 0.4 mL/min

Temperature: 30 °C

Detection: MS

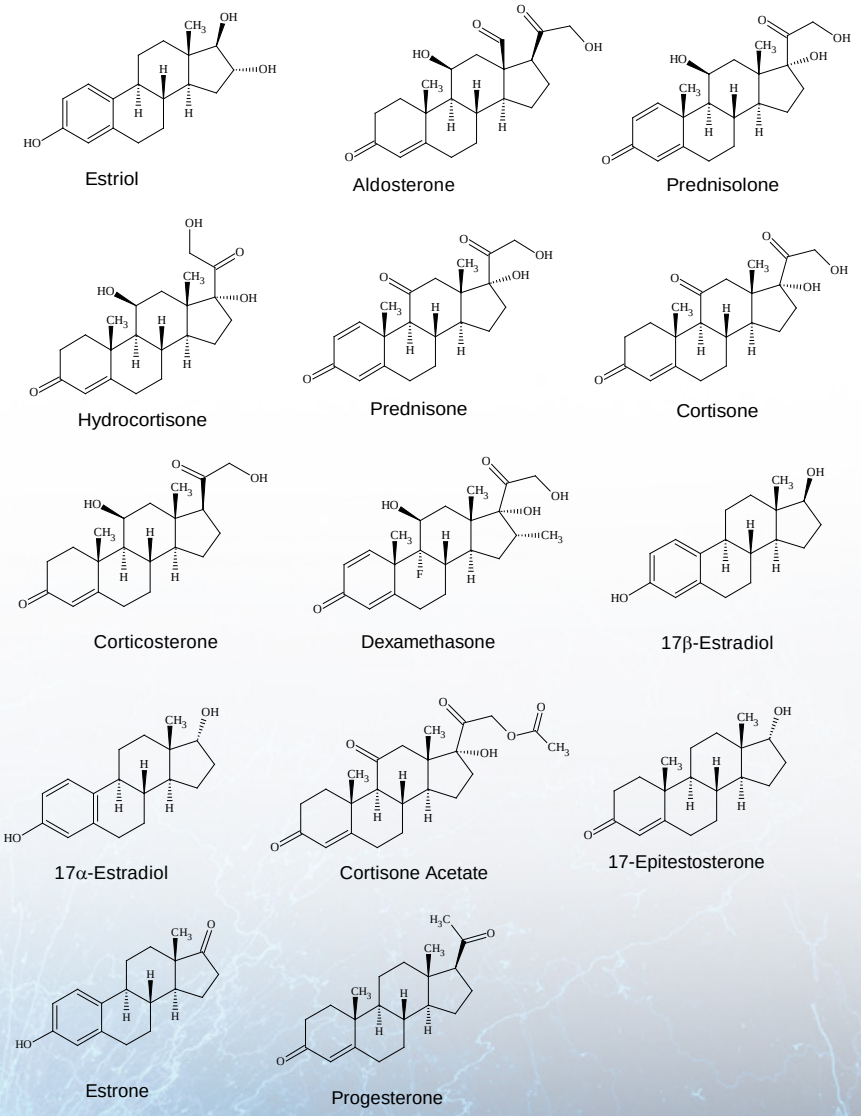
Injection Volume: 0.2 µL of 1 ng/mL drugs of abuse and metabolites

Peak Number	Compound	m/z
1	Hydromorphone	286
2	Oxymorphone	302
3	Noroxycodone	302
4	Morphine	286
5	Methamphetamine	150
6	Naloxone	328
7	Phentermine	150
8	Codeine	300
9	Naltrexone	342
10	Oxycodone	316
11	Hydrocodone	300
12	Meperidine	248
13	Fentanyl	337
14	Buprenorphine	468
15	Methadone	310
16	THC	304

Instrument and Conditions for Screening

Agilent 1200, 600 bar system

- Low delay volume configuration with pulse dampener bypassed
- Automatic delay volume reduction (ADVR) on
- 0.005” (0.127 mm) ID tubing in minimum lengths (except needle seat 0.007”)
- 2 µL Micro flow cell, path length 6 mm
- Injection volume, 1 µL
- 14 steroids



Steroid Mixture

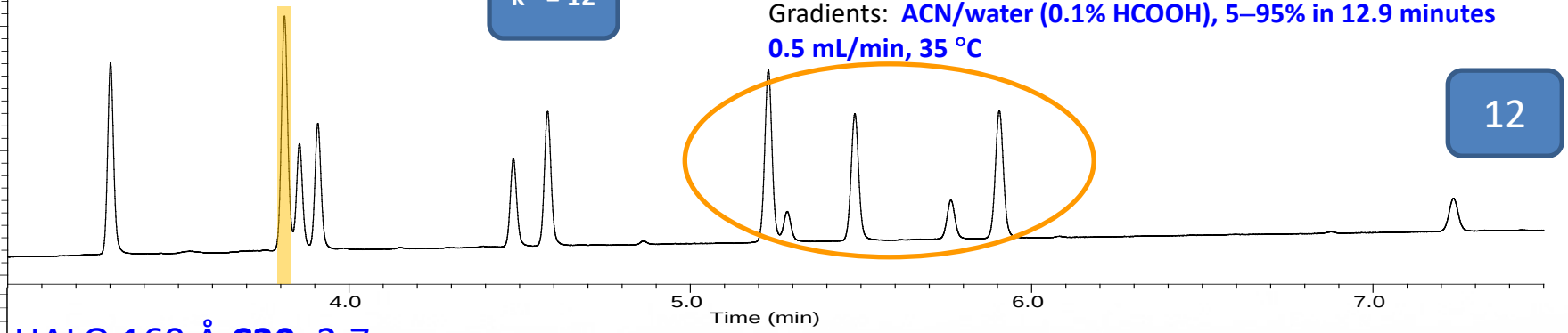
Screening gradients with ACN/water

HALO 90 Å **C18**, 2 µm

k* = 12

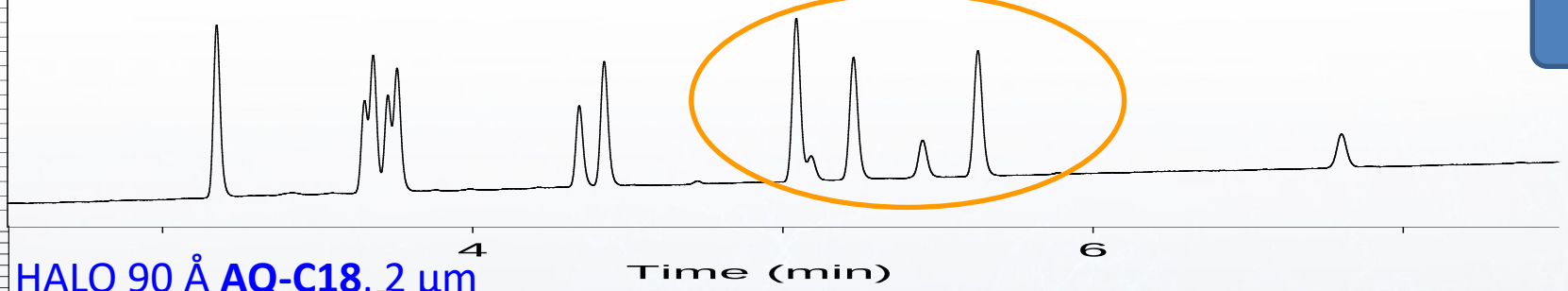
Columns: 2.1 x 50 mm, 2 µm
Gradients: **ACN/water (0.1% HCOOH), 5–95% in 12.9 minutes**
0.5 mL/min, 35 °C

12



HALO 160 Å **C30**, 2.7 µm

13

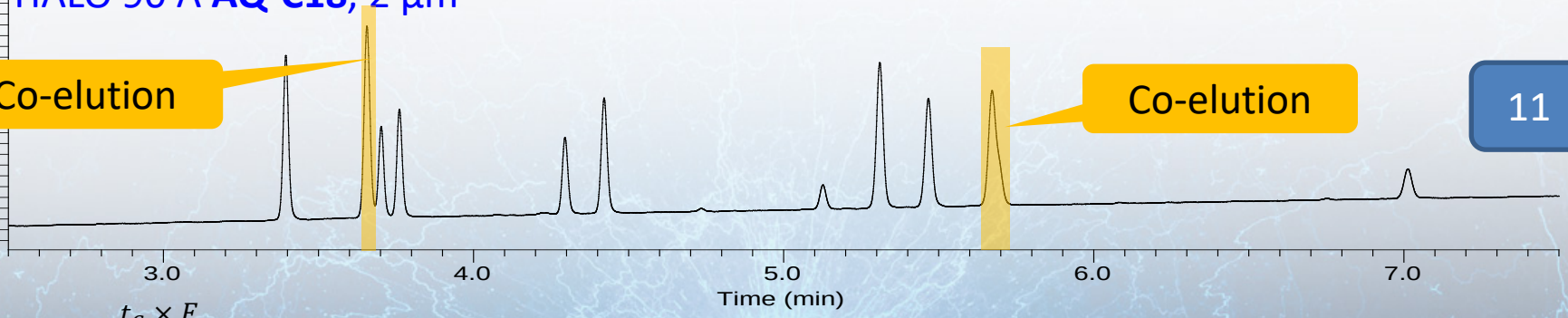


HALO 90 Å **AQ-C18**, 2 µm

Co-elution

Co-elution

11



$$k^* = \frac{t_G \times F}{1.15 \times \Delta \times V_M \times S}$$

Steroid Mixture

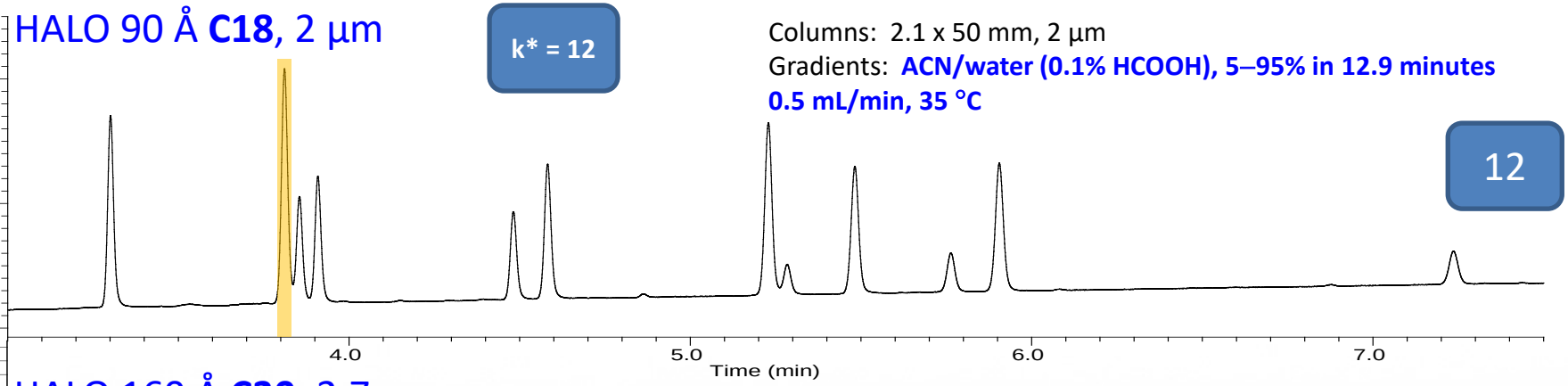
Screening gradients with ACN/water

HALO 90 Å **C18**, 2 µm

k* = 12

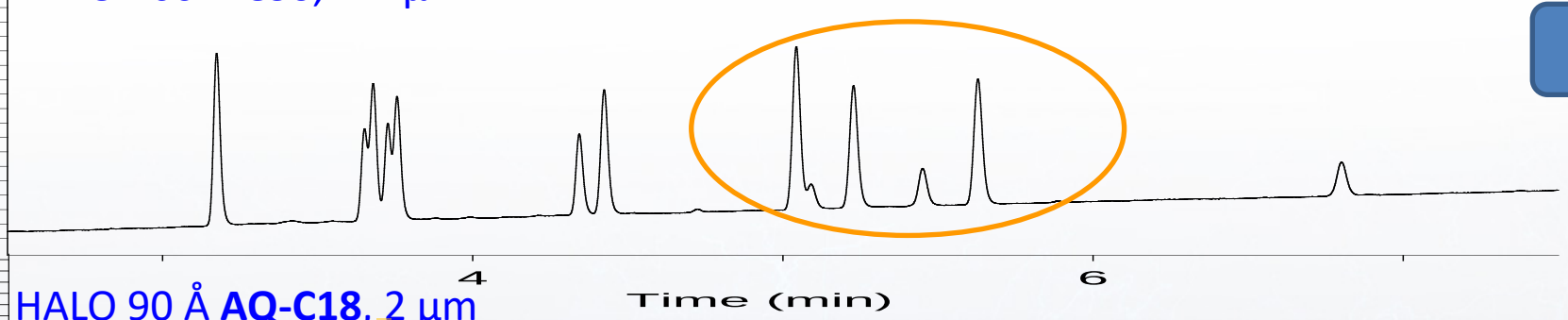
Columns: 2.1 x 50 mm, 2 µm
Gradients: **ACN/water (0.1% HCOOH), 5–95% in 12.9 minutes**
0.5 mL/min, 35 °C

12



HALO 160 Å **C30**, 2.7 µm

13

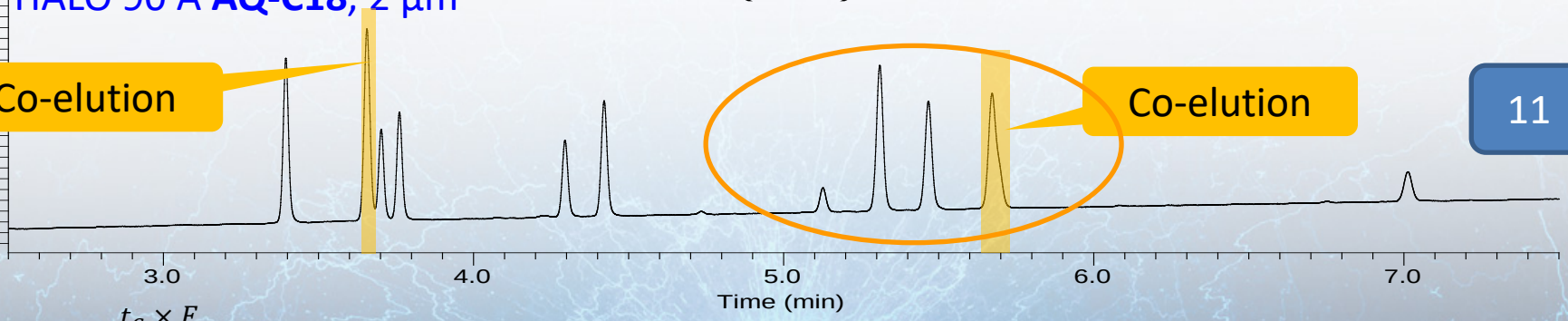


HALO 90 Å **AQ-C18**, 2 µm

Co-elution

Co-elution

11



$$k^* = \frac{t_G \times F}{1.15 \times \Delta \times V_M \times S}$$

Steroid Mixture

Screening gradients with ACN/water

Columns: 2.1 x 50 mm, 2 µm, except as noted for HALO PFP

Gradients: **ACN/water (0.1% HCOOH), 5–95% in 12.9 minutes**

HALO 90 Å **RP-Amide**, 2 µm

k* = 12

13

4.0 5.0 6.0 7.0

HALO 90 Å **Phenyl-Hexyl**, 2 µm

12

3.0 4.0 5.0 6.0 7.0

Time (min)

HALO 90 Å **PFP**, 2 µm, 2.1 x 30 mm

5-95% in 7.74 min **(same k*)**

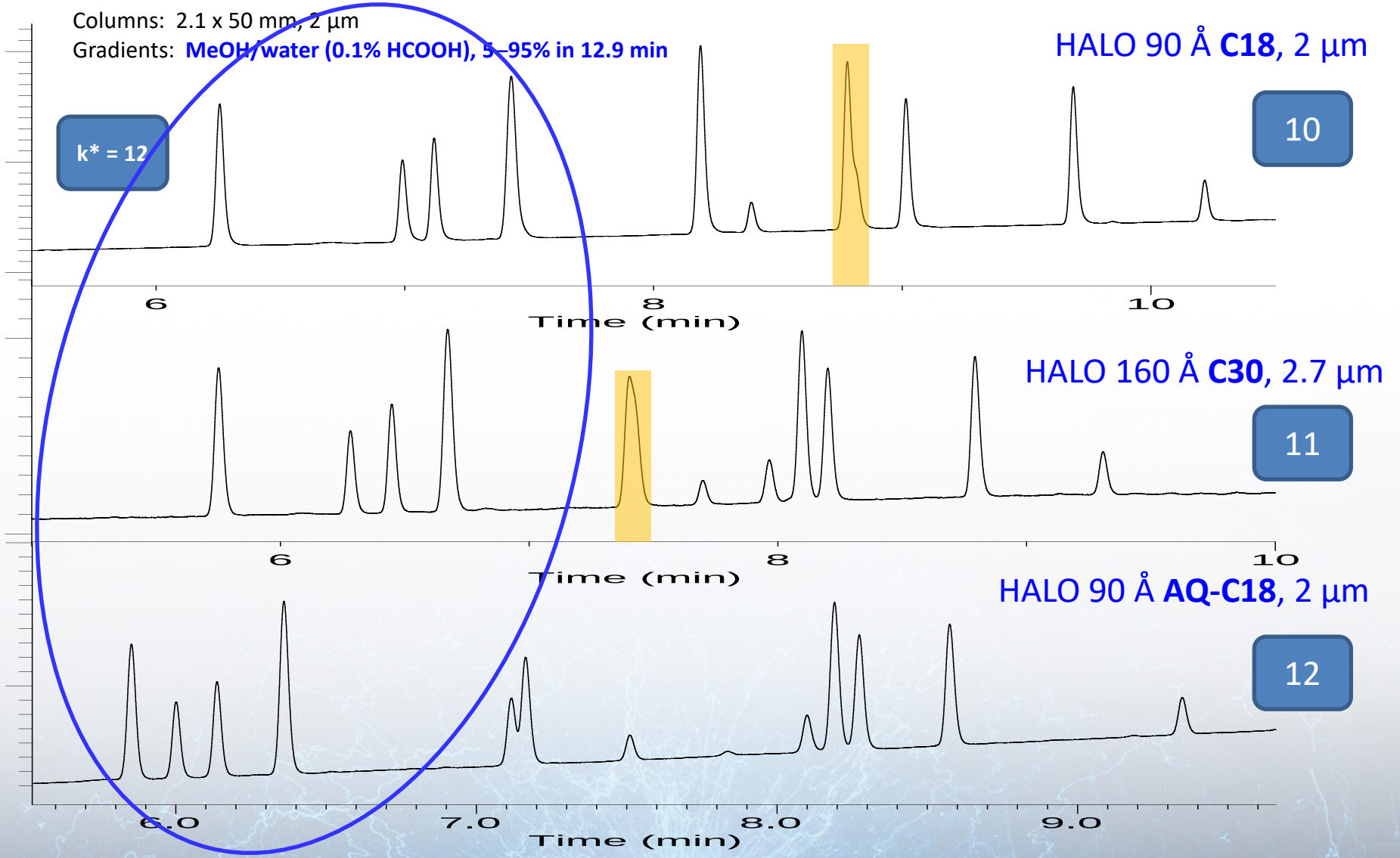
11

2.0 3.0

Time (min)

Steroid Mixture

Screening gradients with MeOH/water

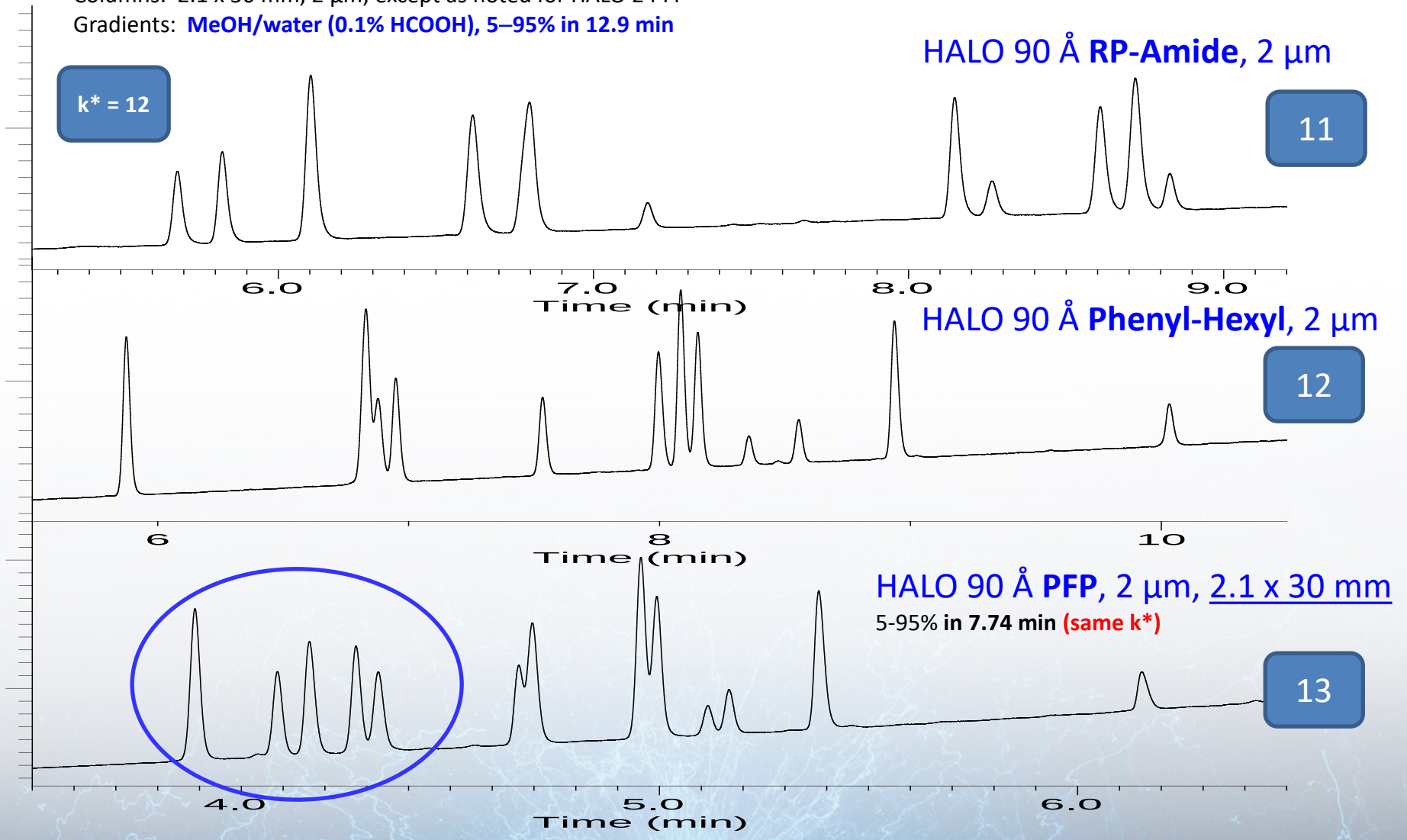


Steroid mixture

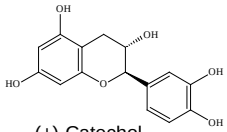
Screening gradients with MeOH/water

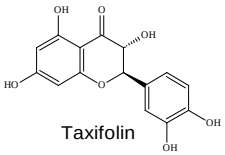
Columns: 2.1 x 50 mm, 2 µm; except as noted for HALO 2 PFP

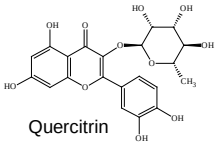
Gradients: **MeOH/water (0.1% HCOOH), 5–95% in 12.9 min**

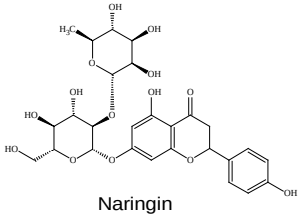


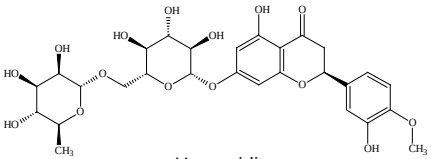
Flavonoid Sample: 19 Components

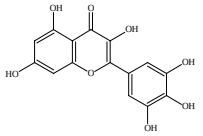

(+)-Catechol

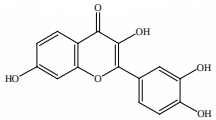

Taxifolin

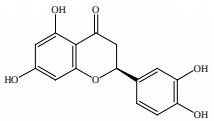

Quercitrin

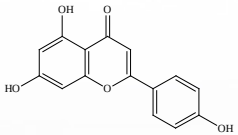

Naringin

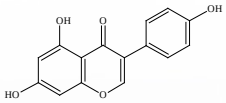

Hesperidin

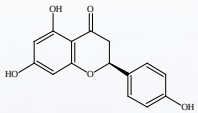

Myricetin

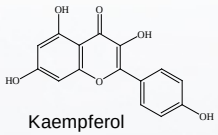

Fisetin

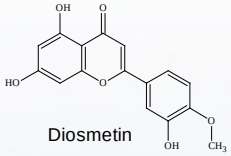

Eriodictyol

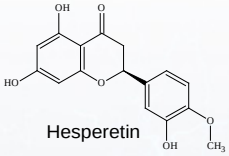

Apigenin

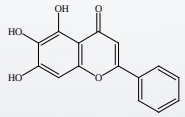

Genistein


Naringenin

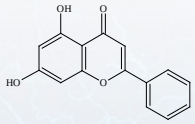

Kaempferol

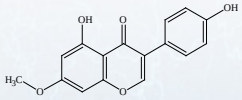

Diosmetin

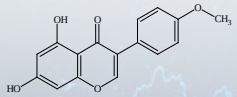

Hesperetin


Baicalein


Formononetin


Chrysin


Prunetin

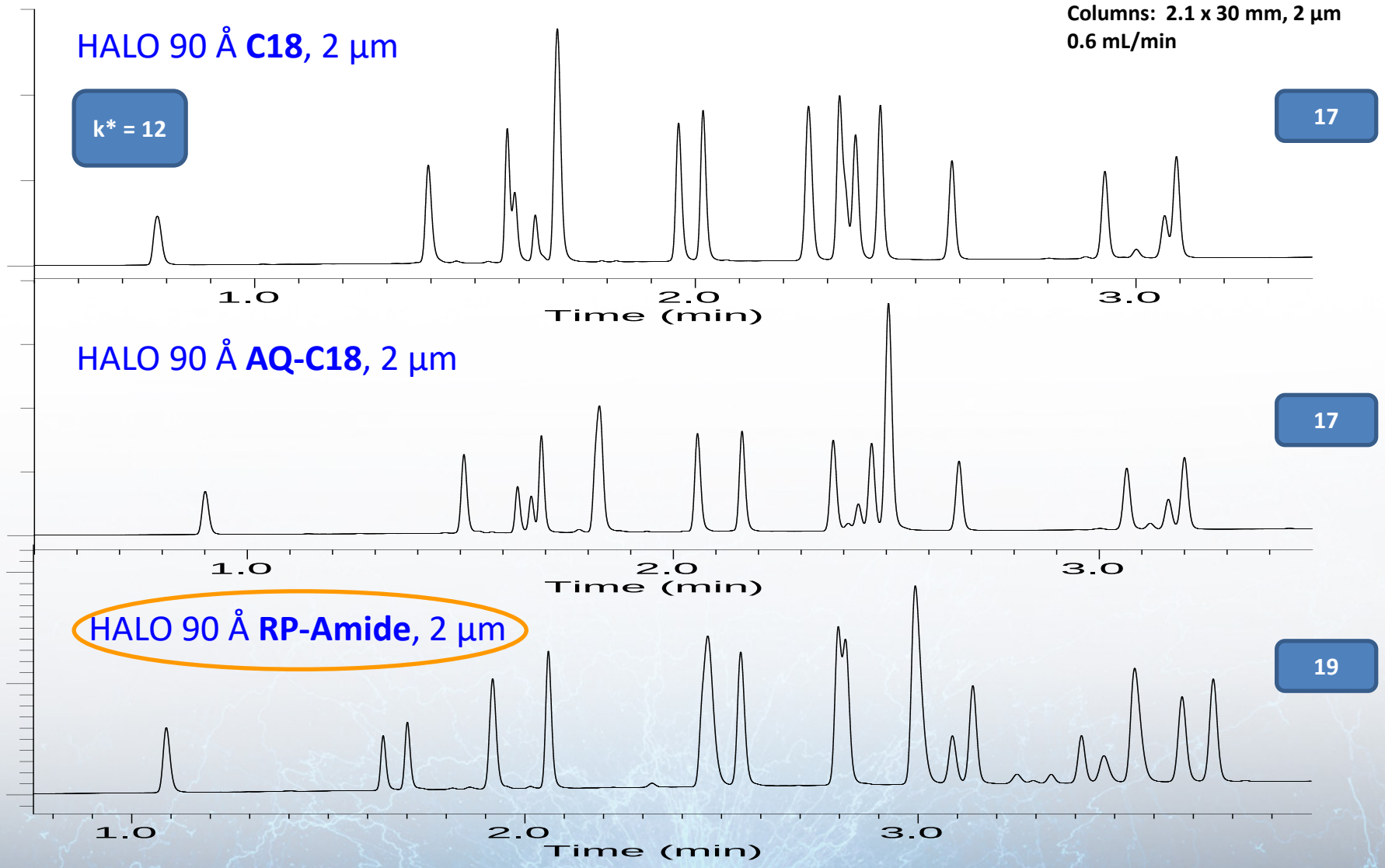

Biochanin A

Elution order C18	Flavonoids	MW	Log P
1	Catechin +/-	290.27	0.88
2	taxifolin	304.25	0.95
3	Quercitrin	448.38	2.16
4	Naringin	580.53	-0.44
5	Hesperidin	610.56	-0.31
6	Myricetin	318.24	2.94
7	fisetin	286.24	1.97
8	eriodictyol	288.25	2.02
9	**Quercetin**	**302.24**	**1.82**
10	apigenin	270.24	3.02
11	Genistein	270.24	3.04
12	Naringenin	272.26	2.52
13	Kaempferol	286.24	3.11
14	diosmetin	300.26	3.10
15	**Hesperetin**	**302.28**	**2.60**
16	baicalein	270.24	3.31
17	formononetin	268.26	2.79
18	chrysin	254.24	2.94
19	prunetin	284.26	2.88
20	BIOCHANIN A	284.26	2.88

Isobaric flavonoids highlighted

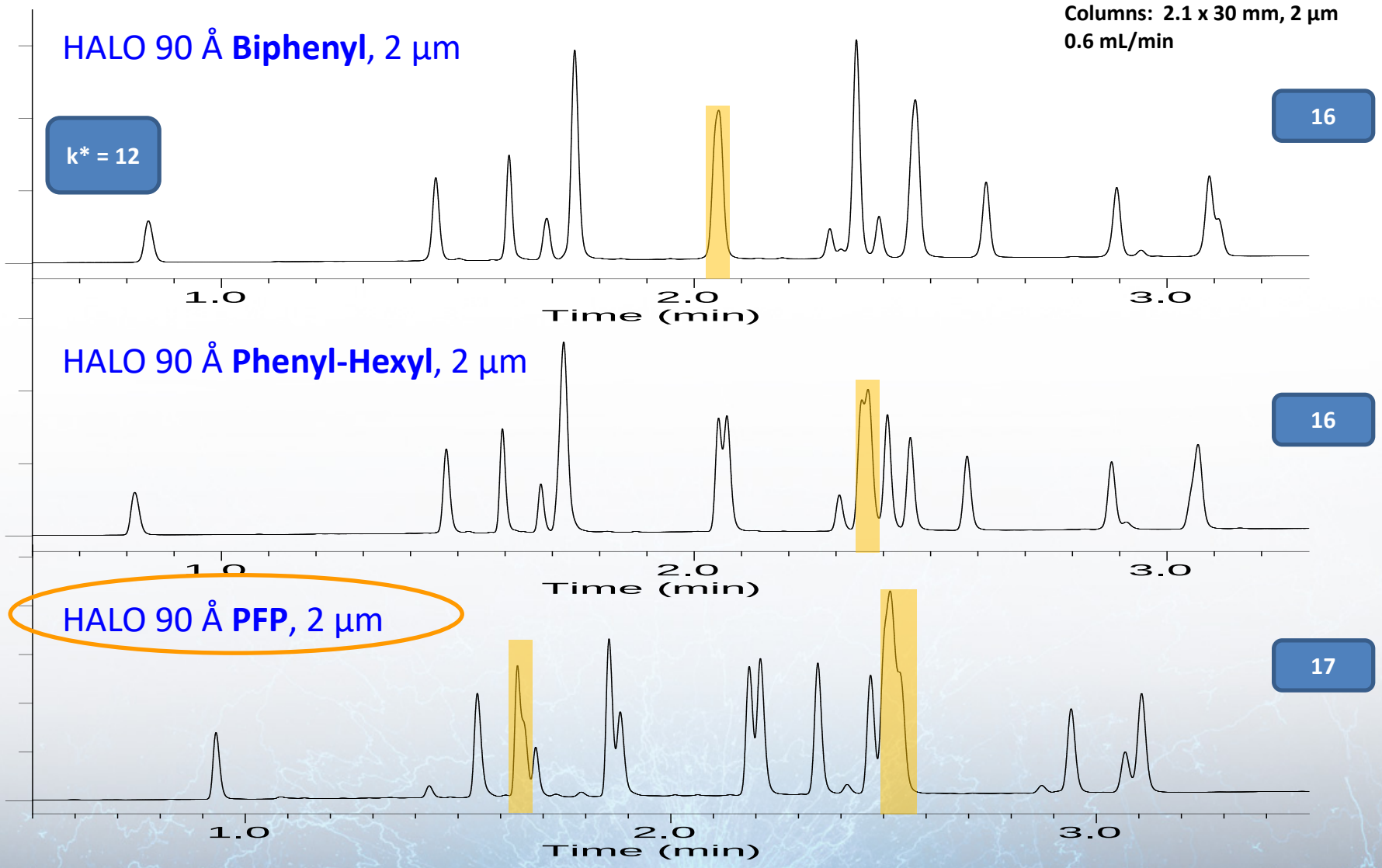
Flavonoid Sample

Gradient screening ACN/water, 5–90% in 6.1 min, 25 °C



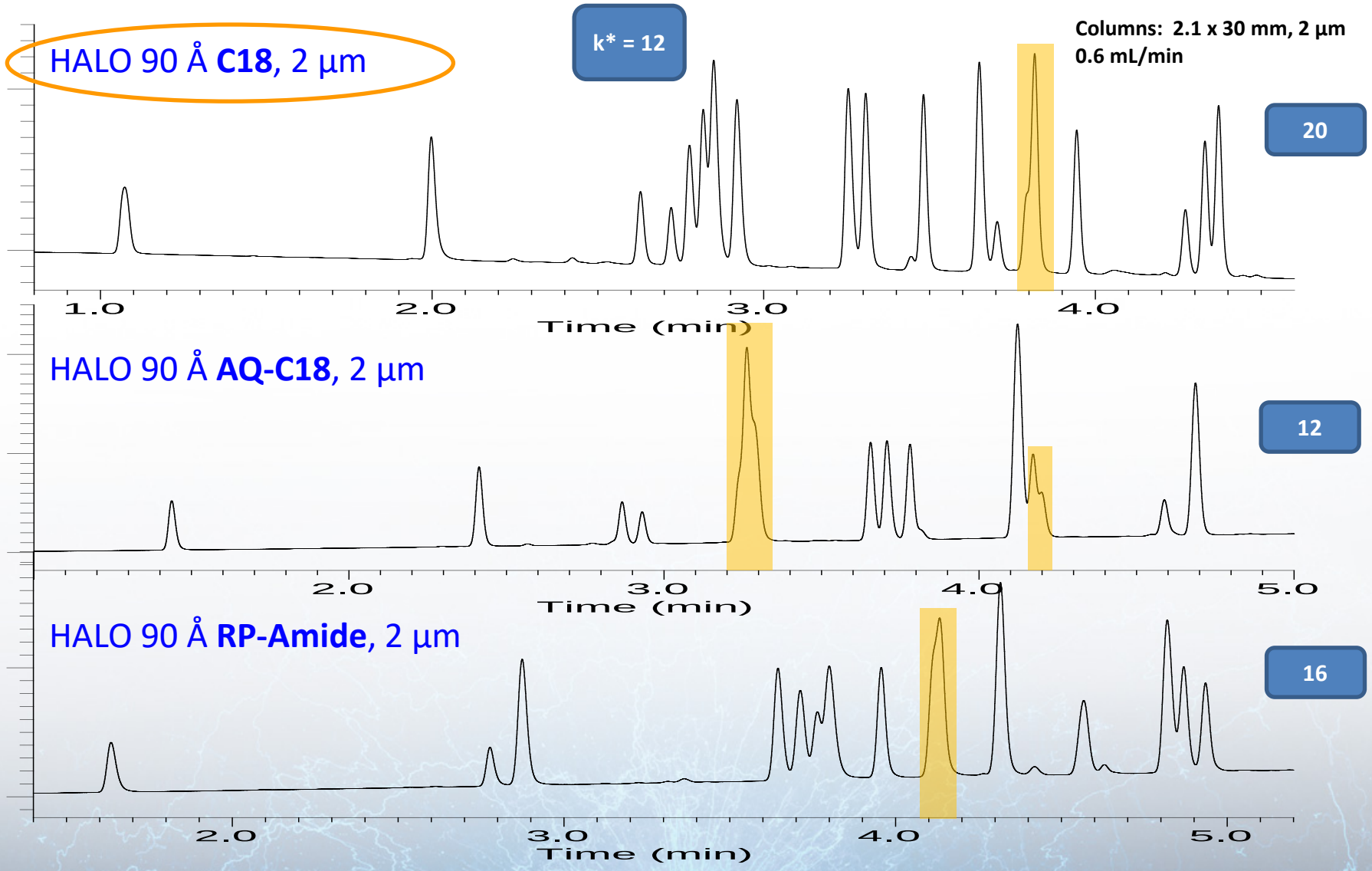
Flavonoid Sample

Gradient screening ACN/water, 5–90% in 6.1 min, 25 °C



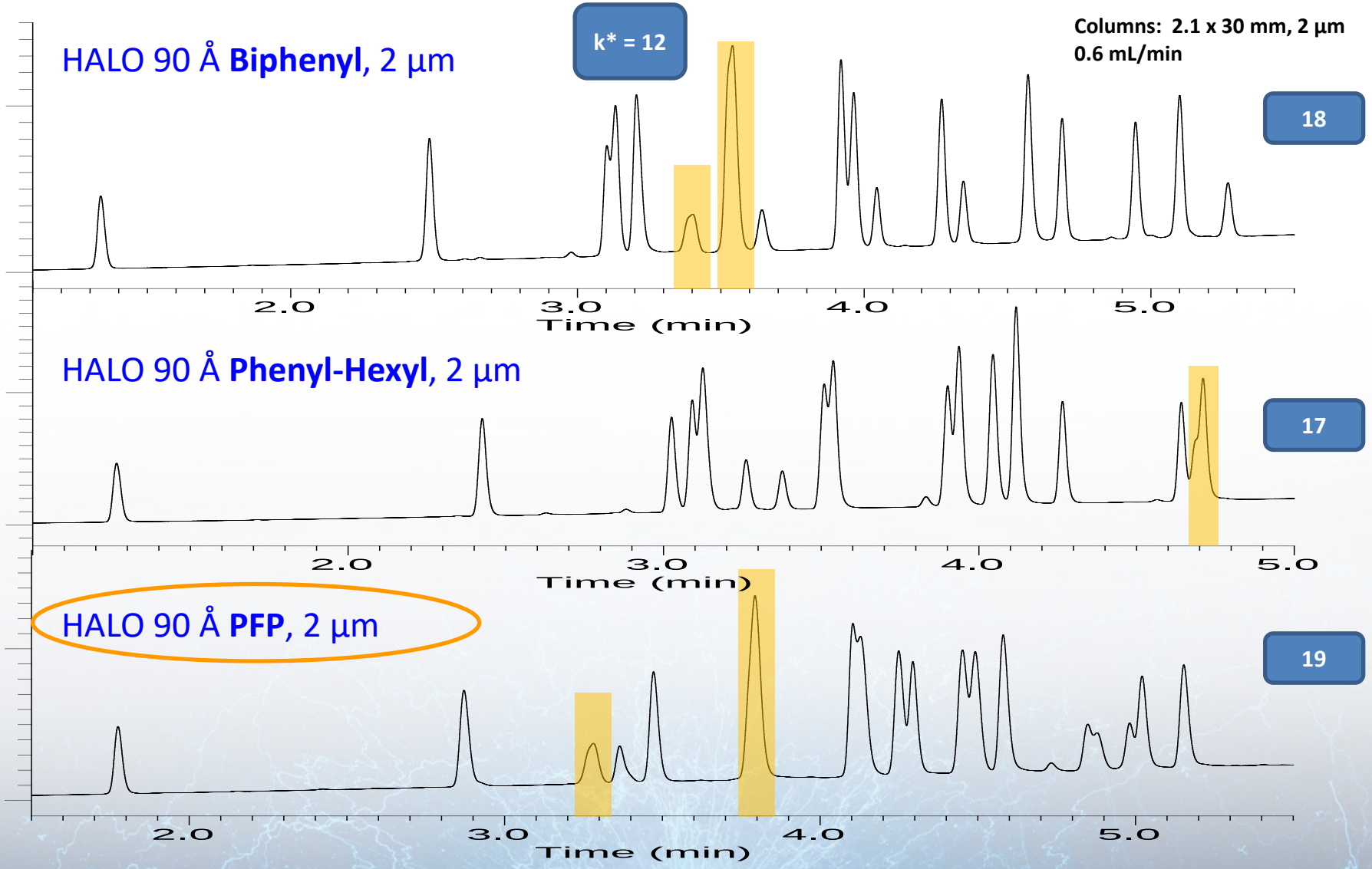
Flavonoid Sample

Gradient screening MeOH/water, 5–90% in 6.1 min, 25 °C



Flavonoid Sample

Gradient screening MeOH/water, 5–90% in 6.1 min, 25 °C



Effect of pH on Selectivity

Peak Identities:

1. uracil
2. 3-nitrobenzoic acid
3. fenuron

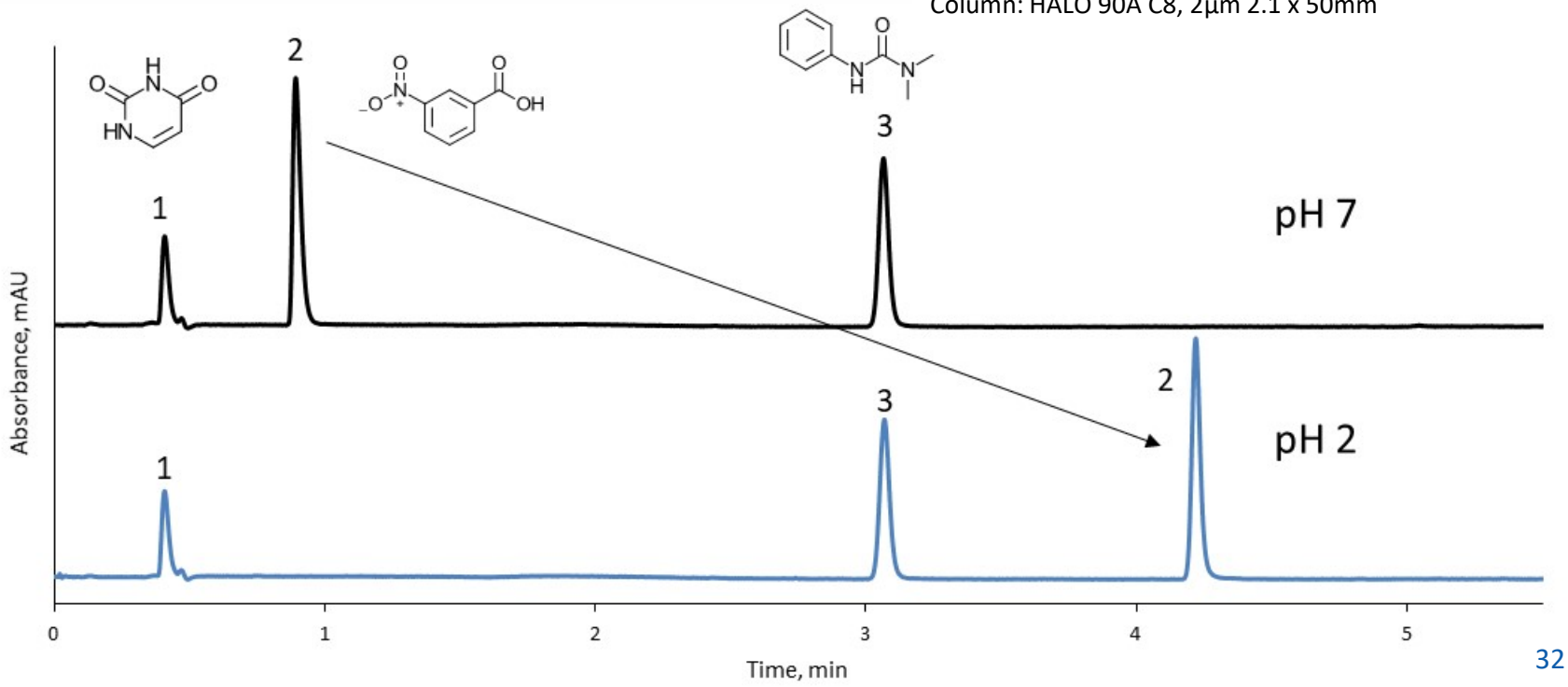
Testing Conditions:

Mobile Phase A: 20mM Potassium Phosphate buffer, pH 2 and 7
B: Acetonitrile

Gradient:

Time (min.)	%B
0.0	10
0.5	10
5.5	50

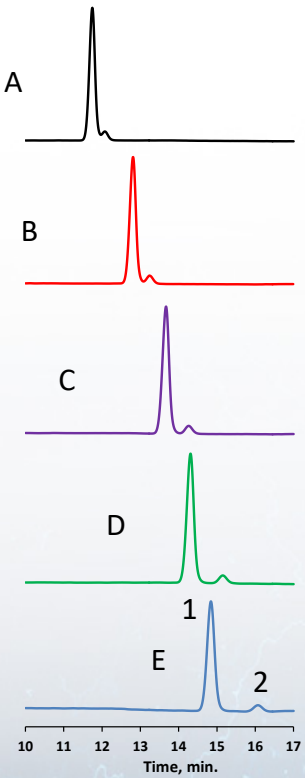
Instrument: Agilent 1200
Wavelength: 254nm
Injection: 1 µl
Temperature: 30°C
Flow Rate: 0.3 mL/min.
Column: HALO 90Å C8, 2µm 2.1 x 50mm

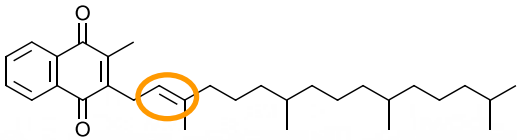


Effect of Temperature on Selectivity

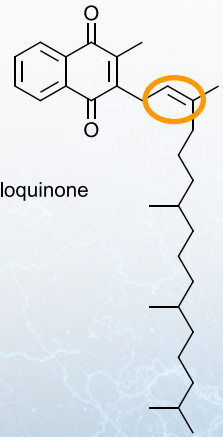
PEAK IDENTITIES:

1. 2,3-*trans*-phylloquinone (Vitamin K1)
2. *cis*-phylloquinone (K1)





Vitamin K1: 2,3-*trans*-phylloquinone



Vitamin K1: *cis*-phylloquinone

Column: HALO 160 Å C30, 2.7 µm, 4.6 x 150 mm
Part Number: 92114-730
Mobile Phase A: Water
Mobile Phase B: Methanol
Isocratic: 95% B
Flow Rate: 1.5 mL/min
Initial HALO Pressure: 341 bar
Detection: 280 nm, PDA
Injection Volume: 1.0 µL
Sample Solvent: Methanol

	Temperature (°C)	Resolution
A	35	1.53
B	30	1.58
C	25	1.78
D	20	2.20
E	15	3.03

Summary and Conclusions

- Screening both acetonitrile- and methanol-containing mobile phases with one column or a set of orthogonal columns is a good start for method development
- Selection of an appropriate pH for the analytes of interest is critical for ionizable compounds
- Once the stationary phase, organic solvent, and pH, are selected, vary temperature and gradient time
  - Use of a commercially available method optimization software package is recommended
- While C18 is a good all-purpose phase for initial method development, the other phases offer alternate selectivities
- It is good practice to screen multiple phases to ensure the best possible separation

Technical Resources

- Mac-Mod Analytical, Inc.
  - Exclusive distributor of HALO® columns in the USA
  - www.mac-mod.com

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