Comparison of Phenyl and C18 bonded-phases to obtain separation selectivity of peptide mixtures

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Agenda

Philadelphia

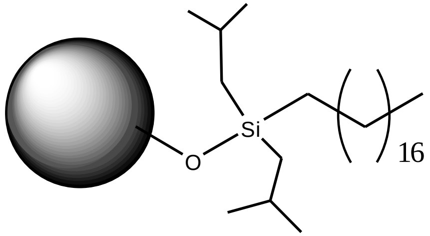
NASA Earth Observatory. J. Allen Landsat 8 - OLI

- Peptide separation selectivity options in RP versus standard alkyl bonded phase are often sought.
- Alternate selectivity could be obtained by use of phenyl bonded phases
- Aim to explore selectivity differences of φ-Hexyl, φ-Butyl, φ-Ethyl vs C18
- Selectivity Differences of Identified Peptides using Test System under various conditions
- Global vs specific selectivity differences
- Examples of utility for selected peptides

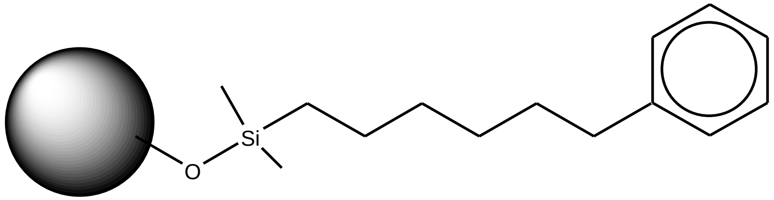
Bonded Phase	Surface Coverage $(\mu m/m^2)$	Endcapped
C18	1.7	No
φ-Hexyl	3.5	Yes
φ-Butyl	3.4	No
φ-Ethyl	2.1	No

[1] Surface Coverage based on %Carbon

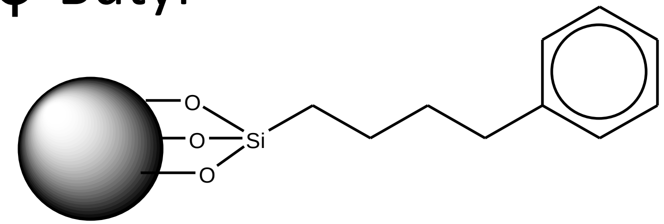
C18



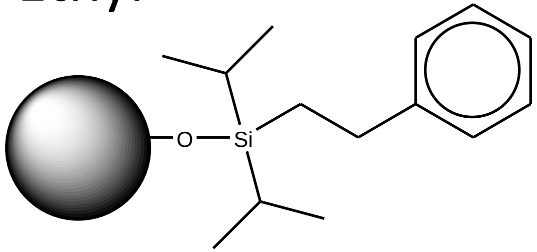
φ-Hexyl



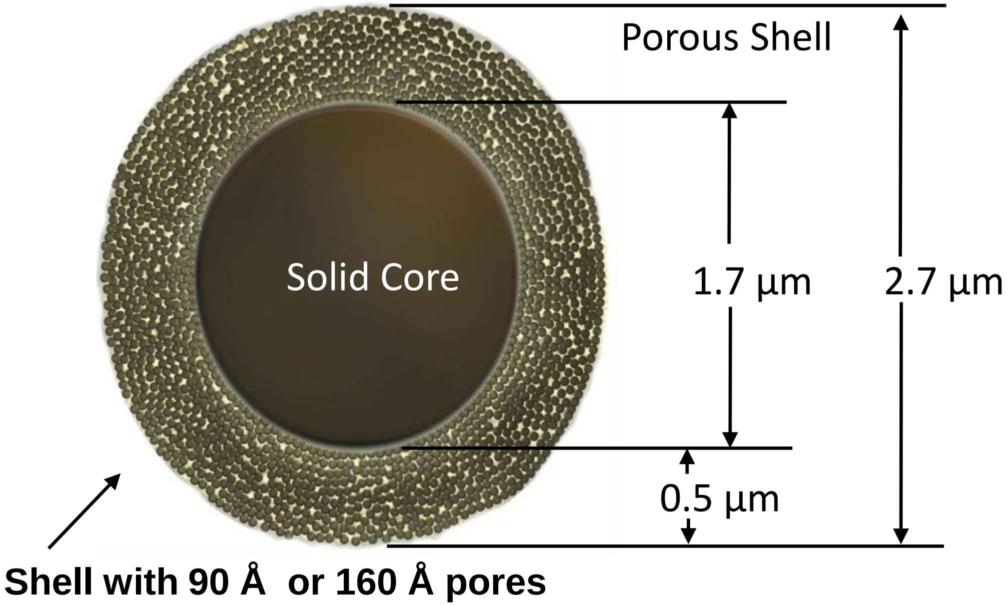
φ-Butyl

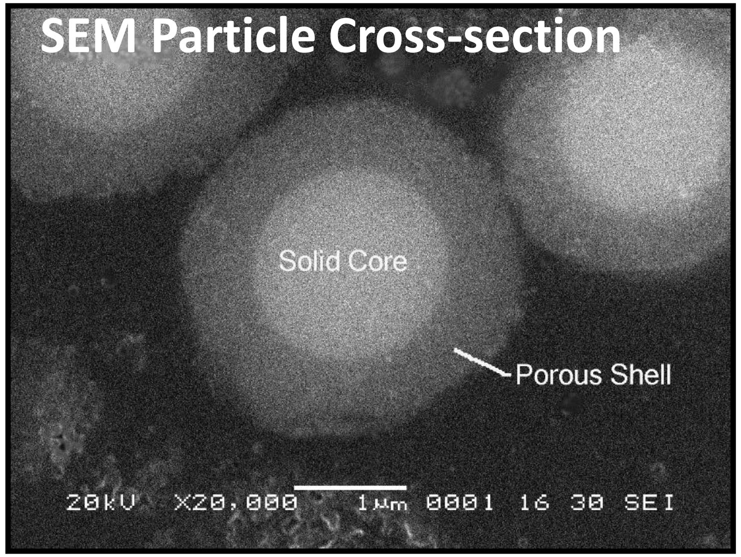


φ-Ethyl



Original Halo Superficially Porous Particles Fused-Core®





- Low back pressure due to the particle design (solid core with a porous shell)
- No need for specialized HPLC equipment
- Not necessary to filter samples and mobile phase since frits are not as small as needed for sub-2-µm
- High resolution is maintained at high flow rates (flat C-term in van Deemter plot)

Low pH Column Stability: φ-Hexyl
19,520 column volumes

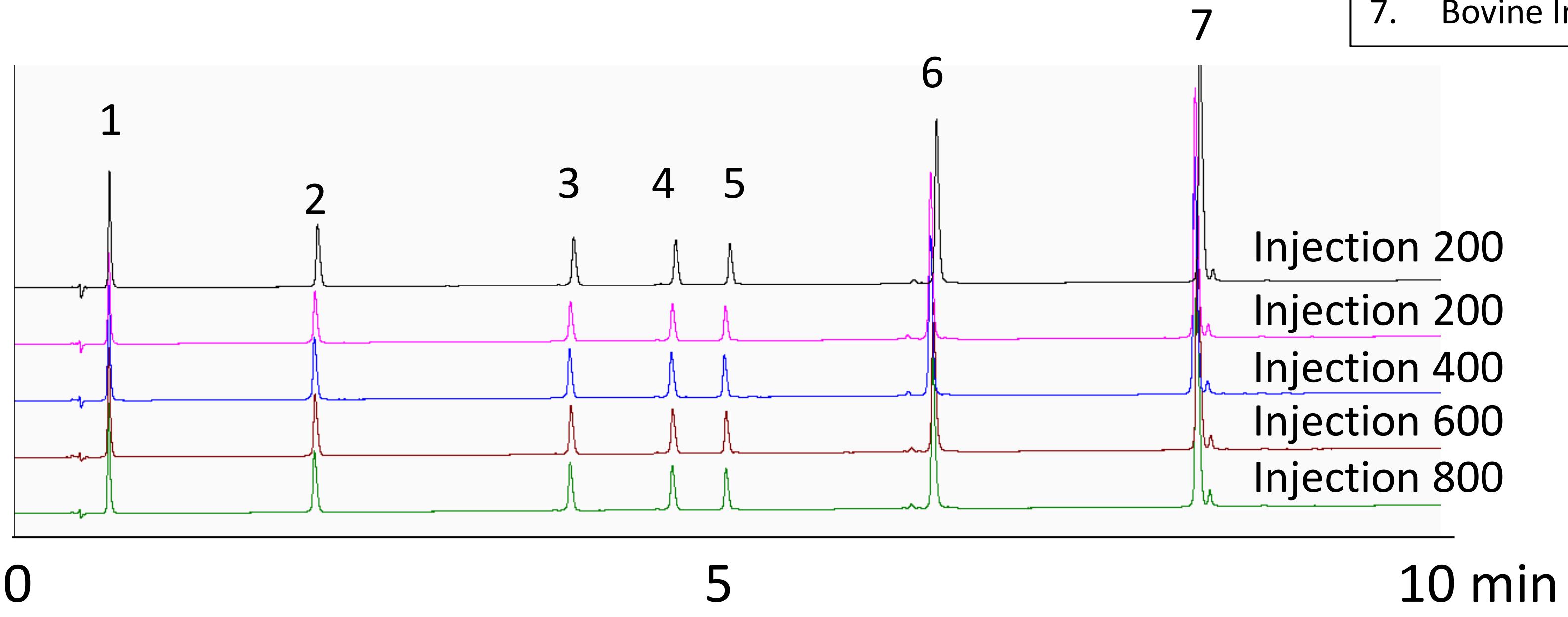
Column: HALO φ-Hexyl
2.7µm, 2.1x100mm 160Å
A: Water/ 0.1% Trifluoroacetic Acid
B: 70-30 ACN/ Water/ 0.1% Trifluoroacetic Acid
Gradient: 9-55% B in 10 min.

Flow: 0.5 ml/min
Detection: 220 nm
Temperature: 60 °C
Injection Volume: 2 µl

Standard Peptides/Proteins:

1. Gly-Tyr
2. Val-Tyr-Val
3. Methionine Enkephalin
4. Angiotensin II
5. Leucine Enkephalin
6. Bovine RNaseA
7. Bovine Insulin

Peak Shape Reproducible >>

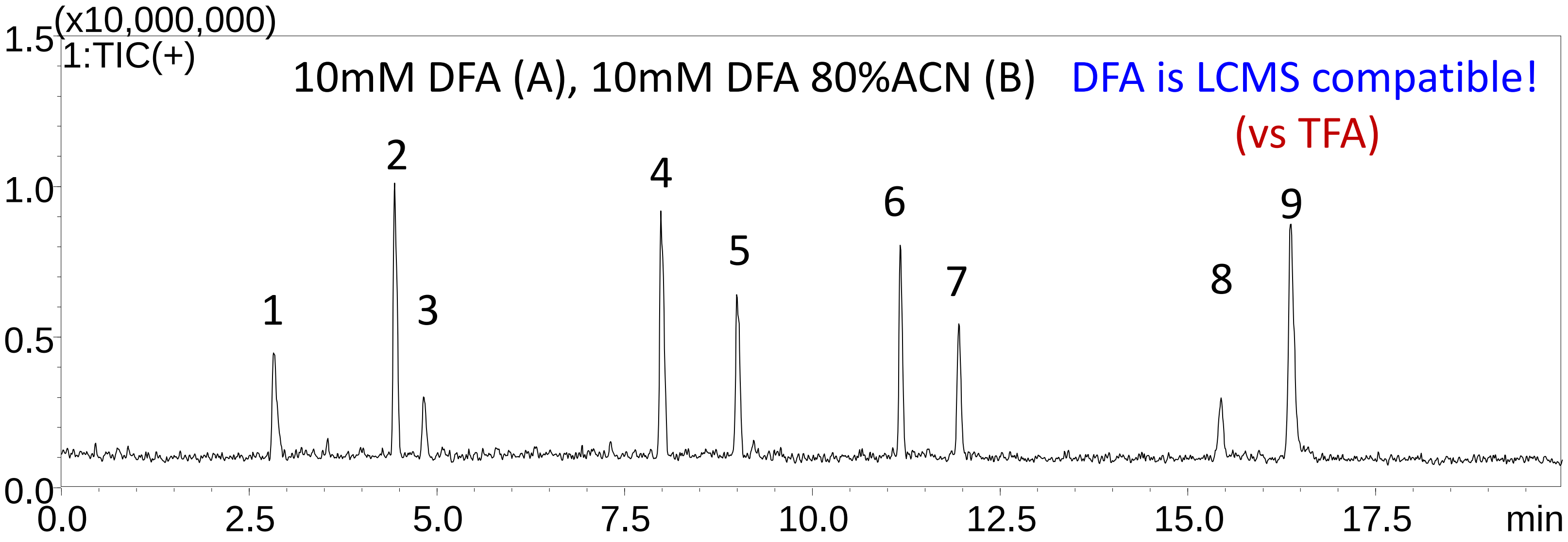


Butyl & Ethyl
low pH Stable

Mobile Phase Acid Modifier Selection for Bonded Phase Comparison:

Column: 2.1 x 150 mm Halo Peptide C18; Flow rate: 0.4 mL/min;
Gradient: 5 - 60% B in 20 min

Difluoroacetic acid (DFA)


10mM DFA (A), 10mM DFA 80%ACN (B)

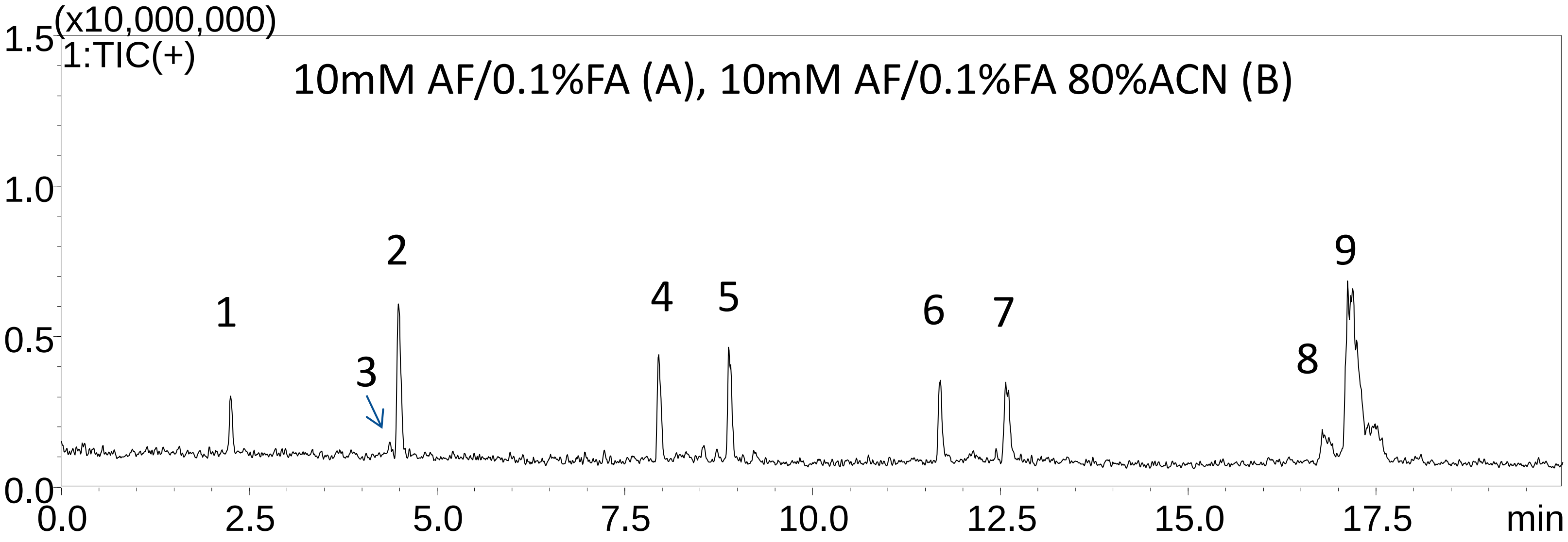
DFA is LCMS compatible! (vs TFA)

Standard Peptides

1. Asp-Phe
2. Angiotensin(1-7)amide
3. Tyr-Tyr-Tyr
4. Angiotensin(II)
5. Neurotensin
6. Angiotensin(1-2)human
7. β-endorphin
8. Sauvagine
9. Mellitin

Formic acid (FA)

Ammonium Formate (AF)


10mM AF/0.1%FA (A), 10mM AF/0.1%FA 80%ACN (B)

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WORK FLOW

Trastuzumab: (monoclonal antibody)
Reduced/Alkylated

⇩

Trypsin Digest

⇩

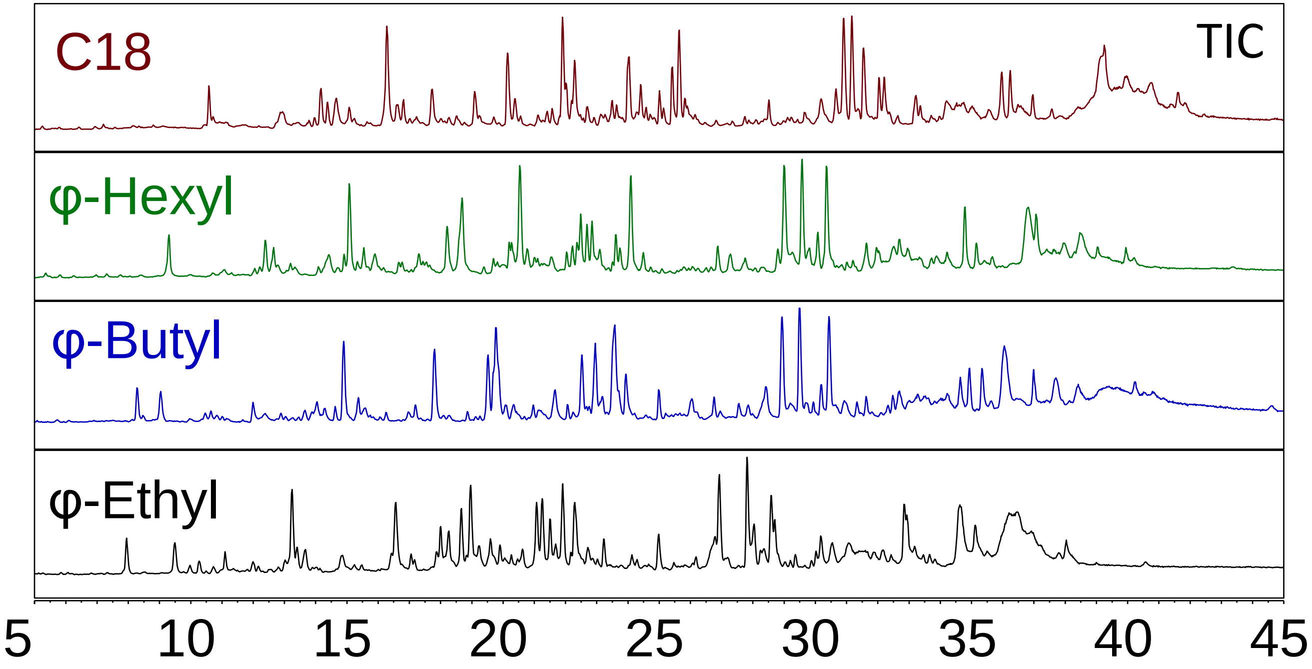
Shimadzu Nexera-UV coupled to
Orbitrap Velos Pro ETD

⇩

Peptide Identification by
MS^2 fragmentation spectra

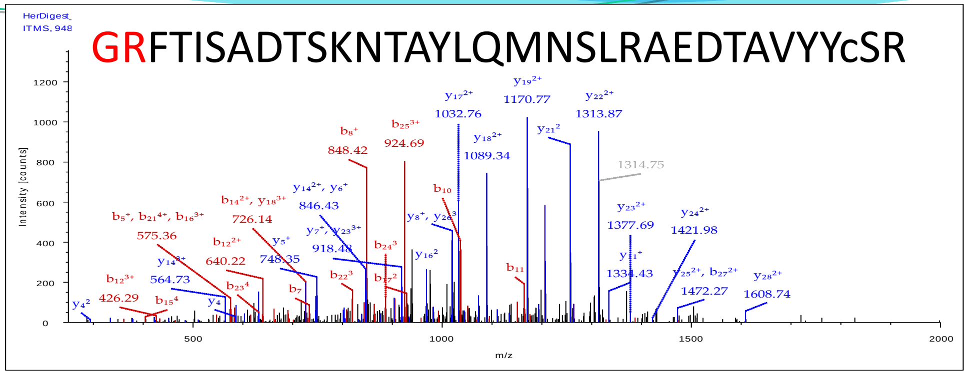
⇩

Extracted Ions Used to Measure Retention
and Peptide Pair Selectivity Differences

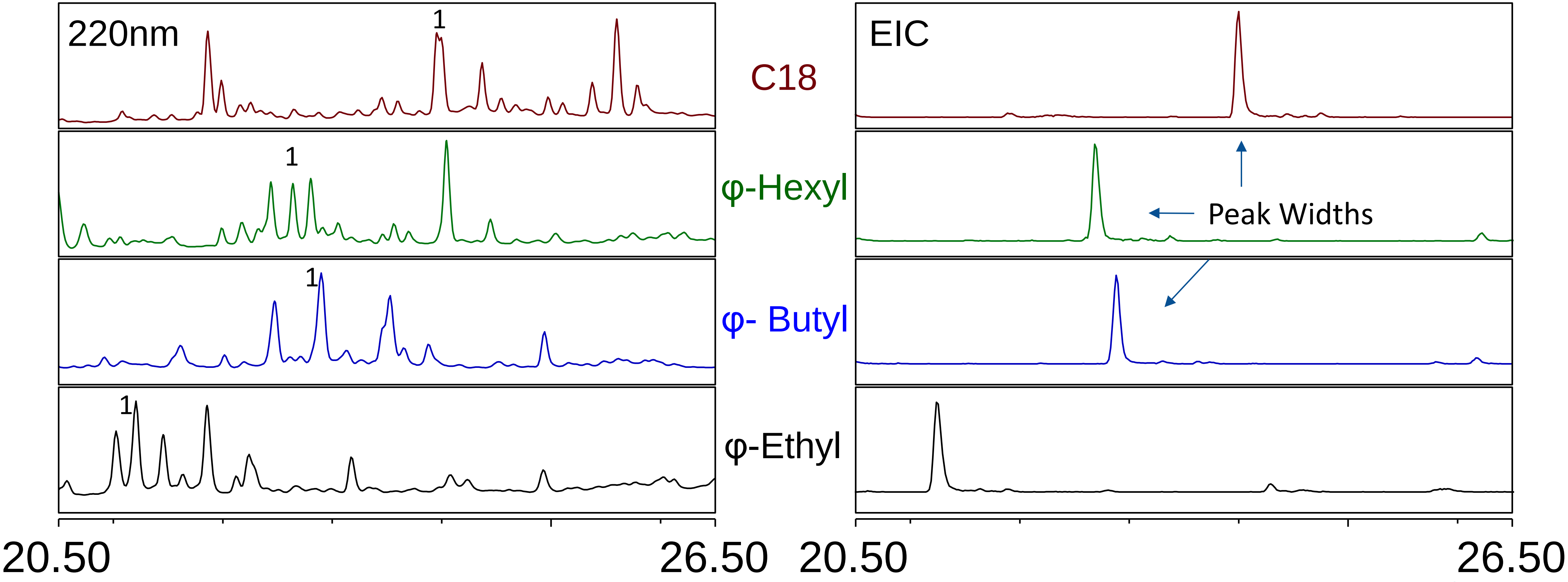


10 DFA

Specific Comparison: Identified Peptides



UNRESOLVED PAIR 24 min



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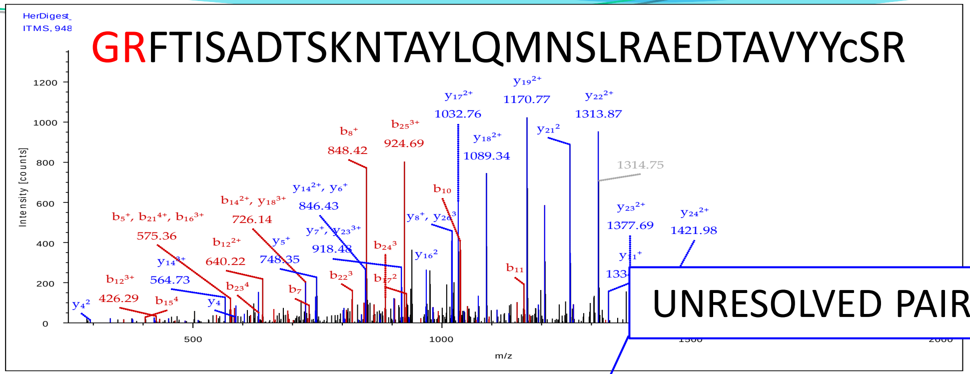
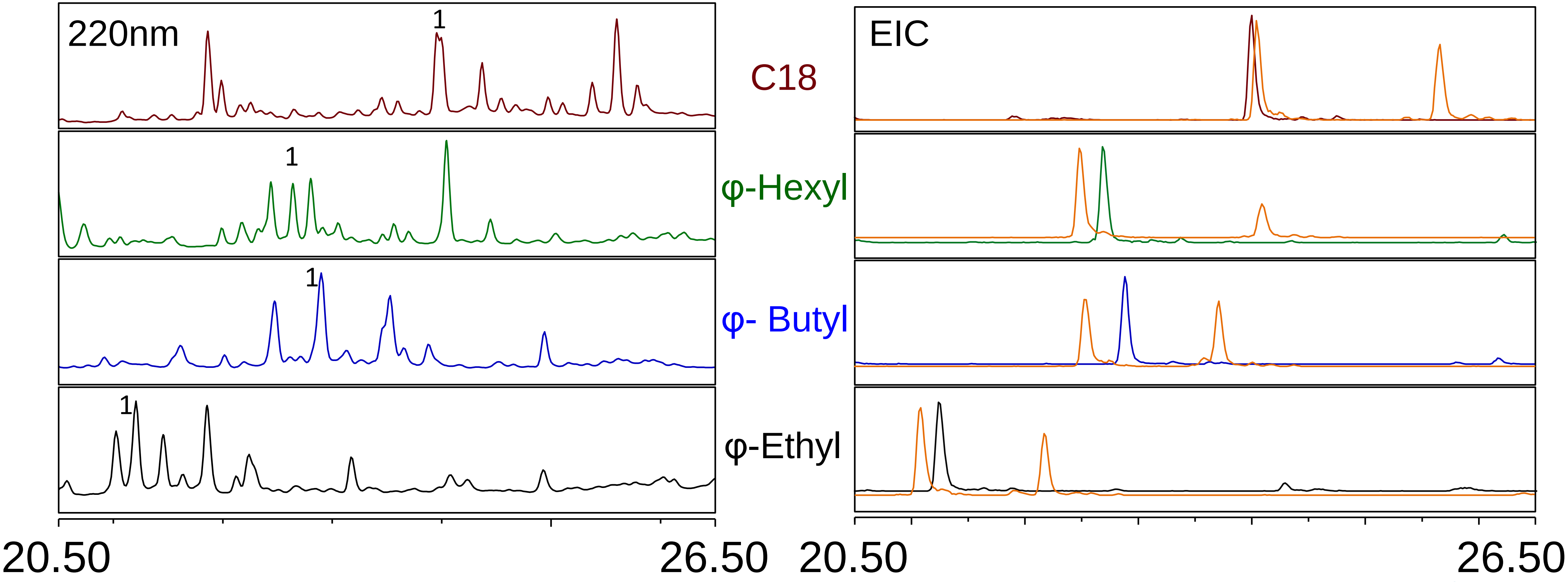


UNRESOLVED PAIR 24 min



10 DFA

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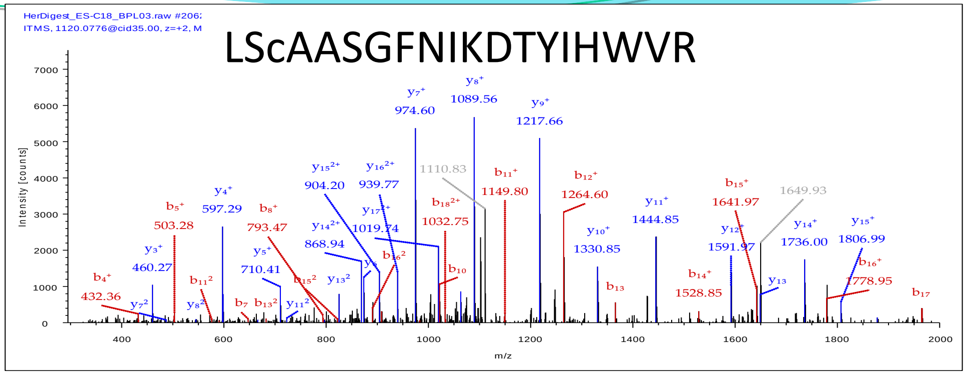

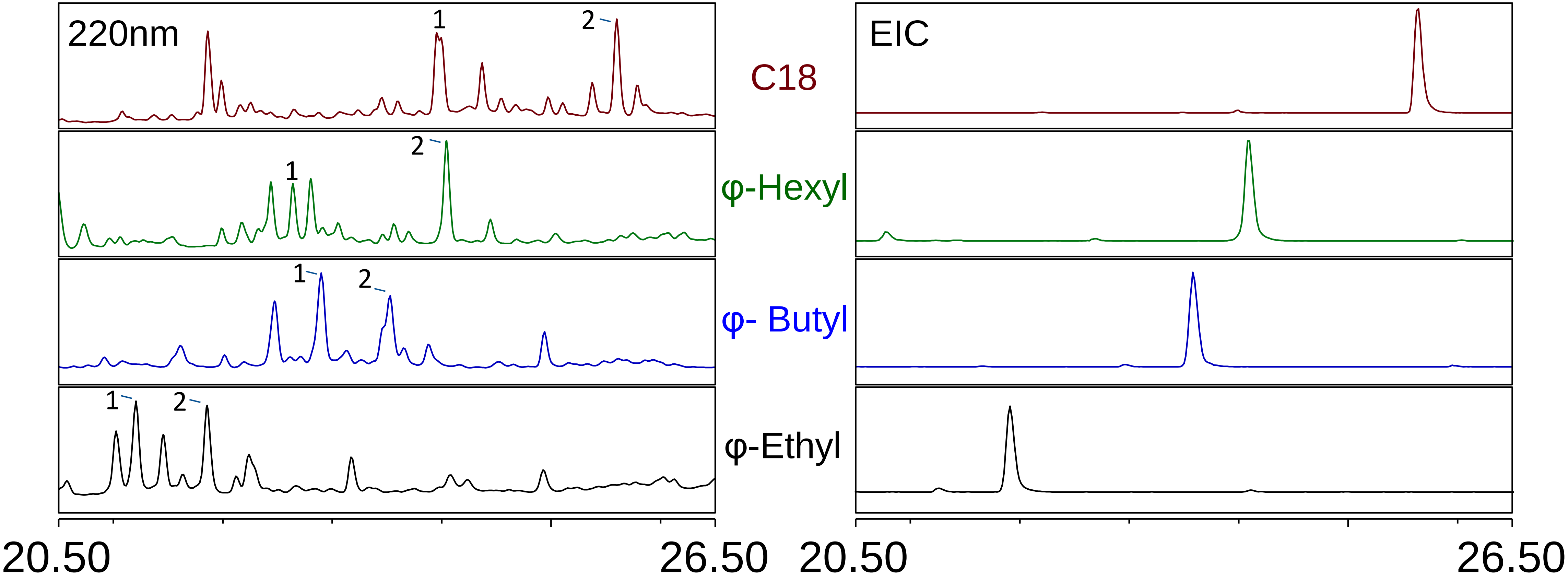

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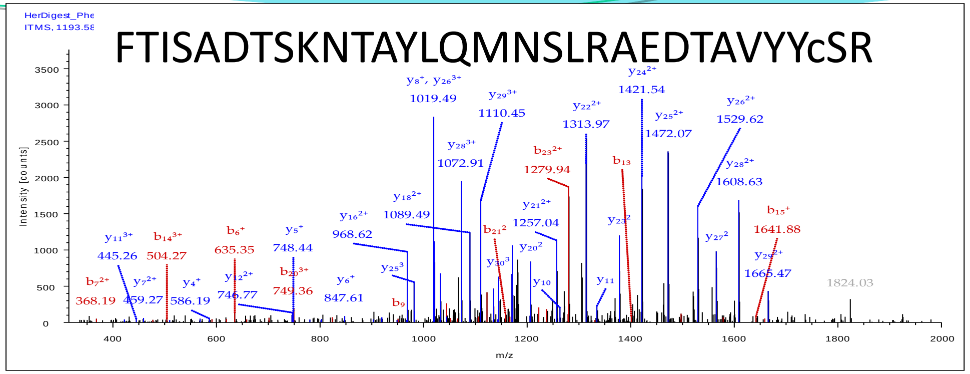
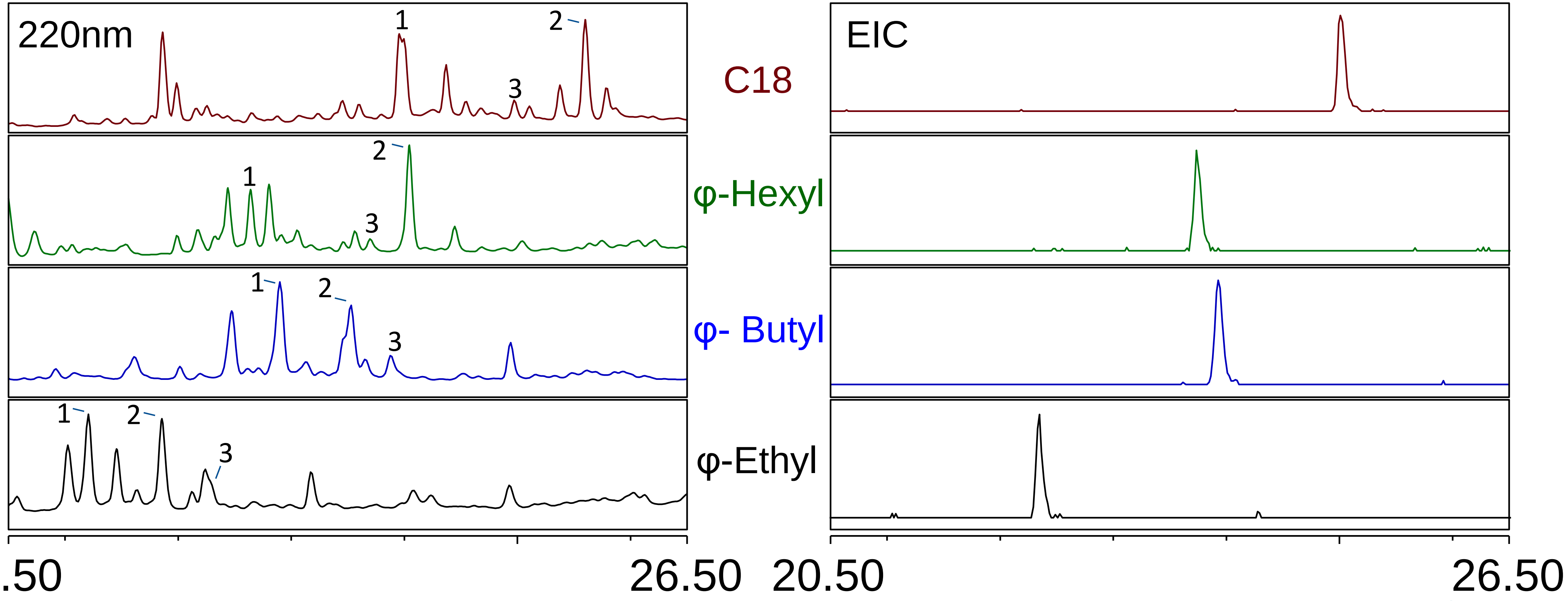
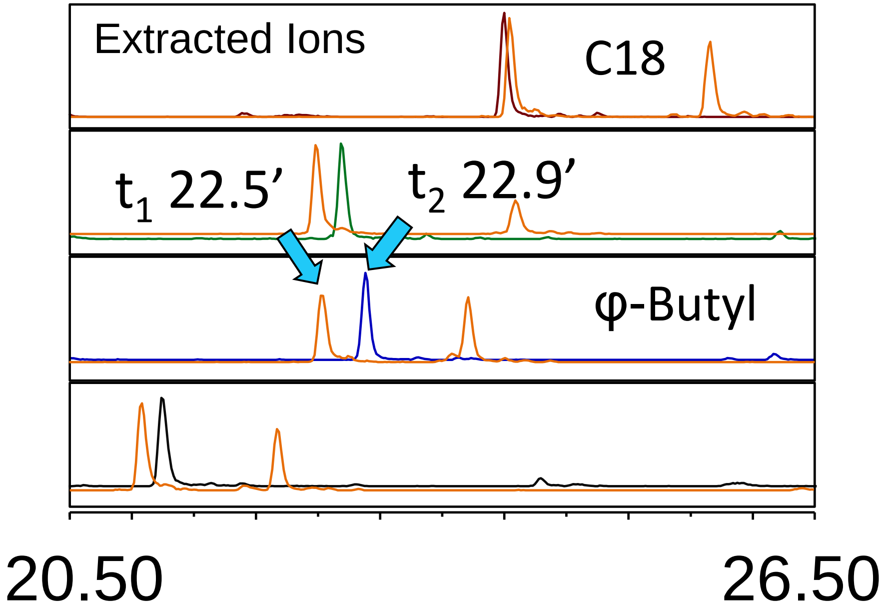
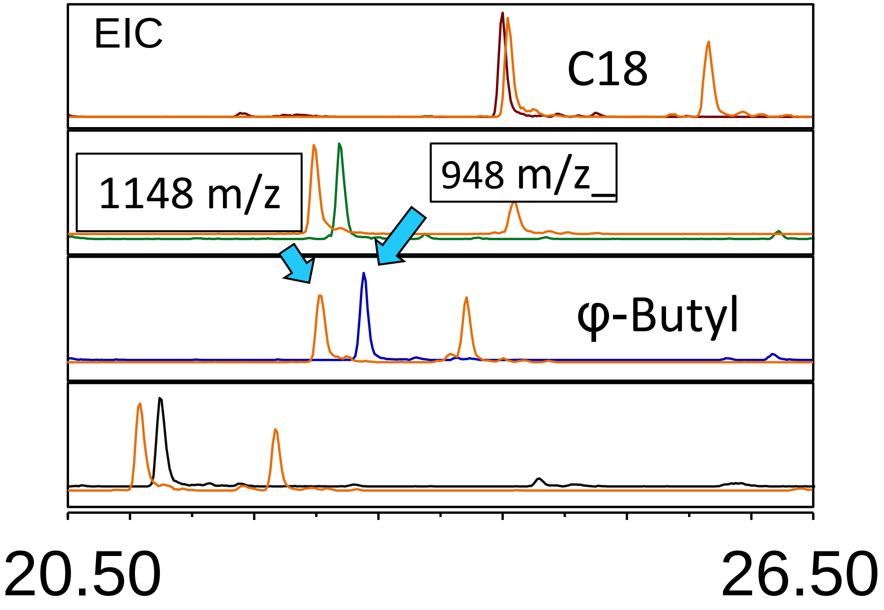




Chromatographic Selectivity (α)



MS Selectivity (α)



$k_1 = (t_1 - t_0)/t_0$ t_0 = void time

$$\alpha = k_2/k_1$$

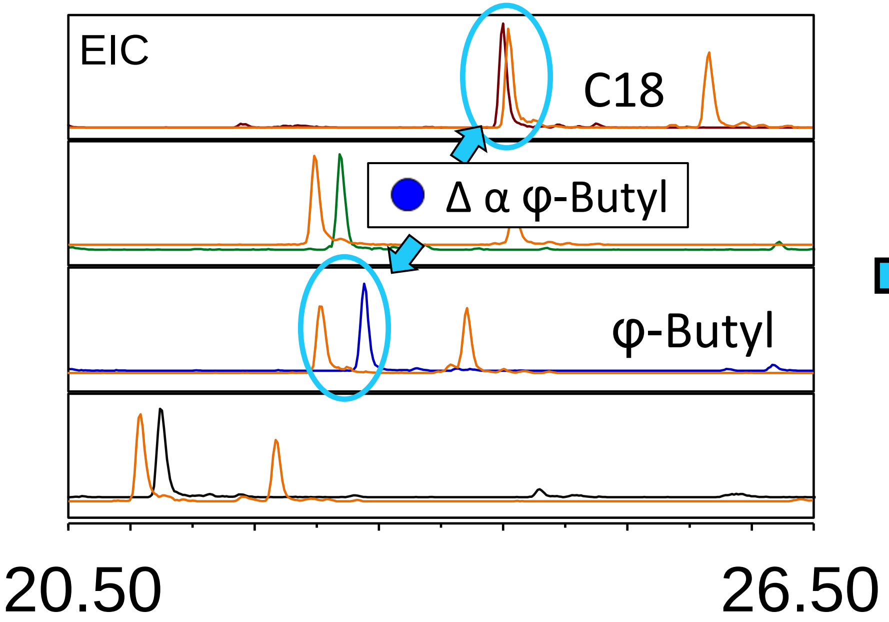
Measuring Differences in Selectivity for Peptide Pairs

$k = (t_1 - t_0)/t_0$

$\alpha = k_2/k_1$

$\Delta\,\alpha = \alpha_{phenyl} - \alpha_{c18}$

$|\Delta\,\alpha| = |\,\alpha_{phenyl} - \alpha_{c18}\,|$



$\Delta\,\alpha = \alpha_{\varphi\text{-butyl}} - \alpha_{c18}$

$|\Delta\,\alpha| = |\alpha_{\varphi\text{-butyl}} - \alpha_{c18}|$

Average (Δα):

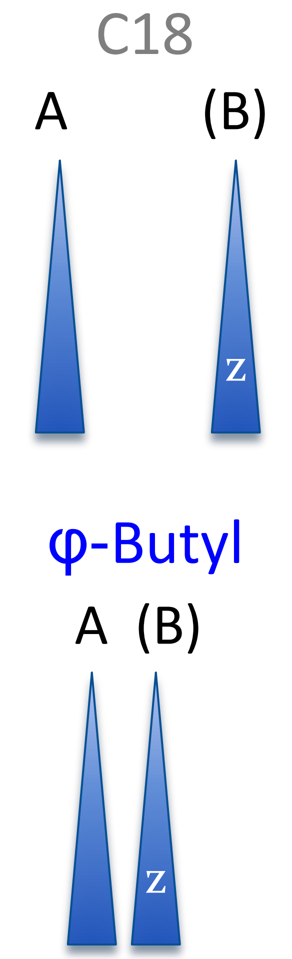
$$\frac{1}{(n-1)}\,\Sigma_{n-1}\,\Delta\,\alpha$$

Average Absolute Value (Δα):

$$\frac{1}{(n-1)}\,\Sigma_{n-1}\,|\,\Delta\,\alpha\,|$$

n = number of peptides; n-1 = number of peptide pairs

Selectivity Differences for 42 Peptide Pairs



C18

A (B)

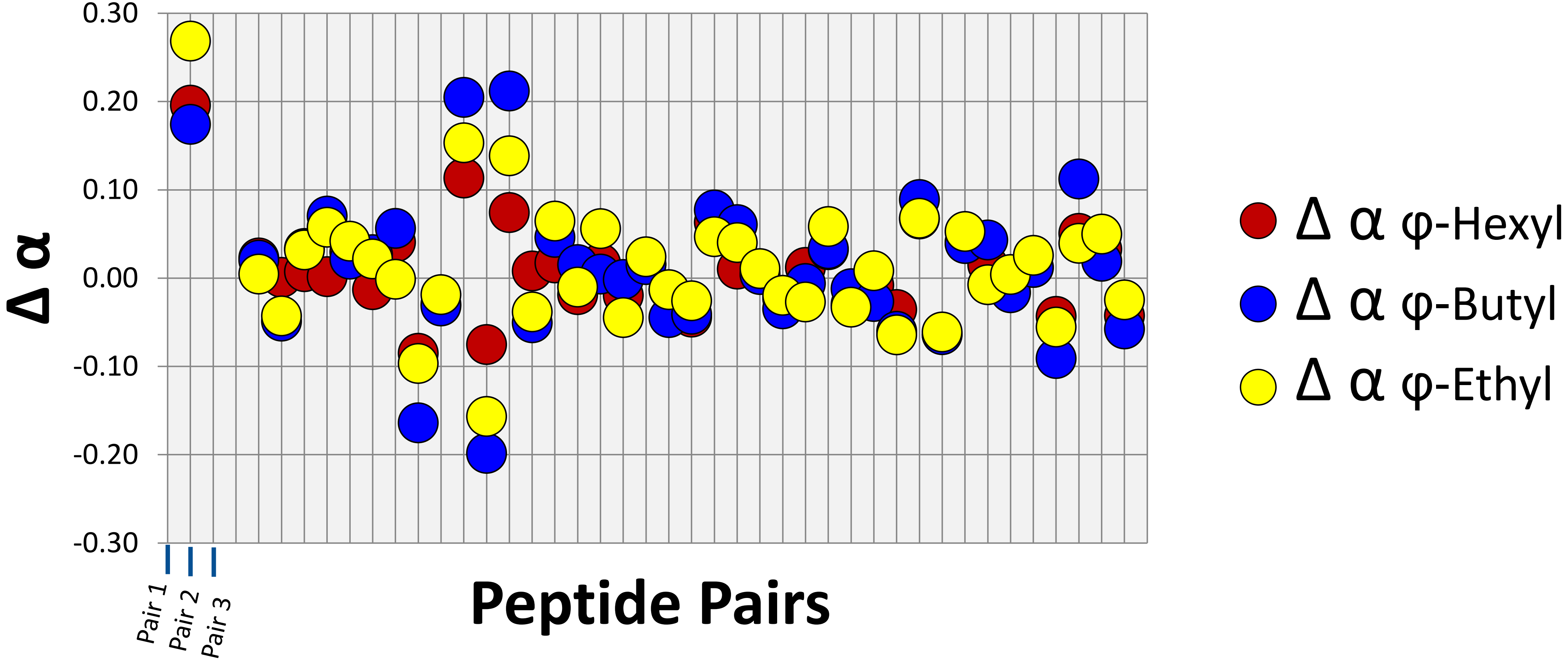
φ-Butyl

A (B)

$\alpha_{\varphi Butyl} - \alpha_{c18}$

Negative Δ α

$$\Delta\,\alpha = \alpha_{phenyl} - \alpha_{c18}$$



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Average Selectivity Differences for Peptide Pairs

Varied Bonded Phase (Same Mobile Phase)			
	φ-Hexyl:C18	φ-Butyl:C18	φ-Ethyl:C18
$1/(n-1)\ \Sigma_{n-1}\ \Delta\alpha$	0.017	0.025	0.028
$1/(n-1)\ \Sigma_{n-1}\ \lvert \Delta\alpha \rvert$	0.044	0.072	0.064

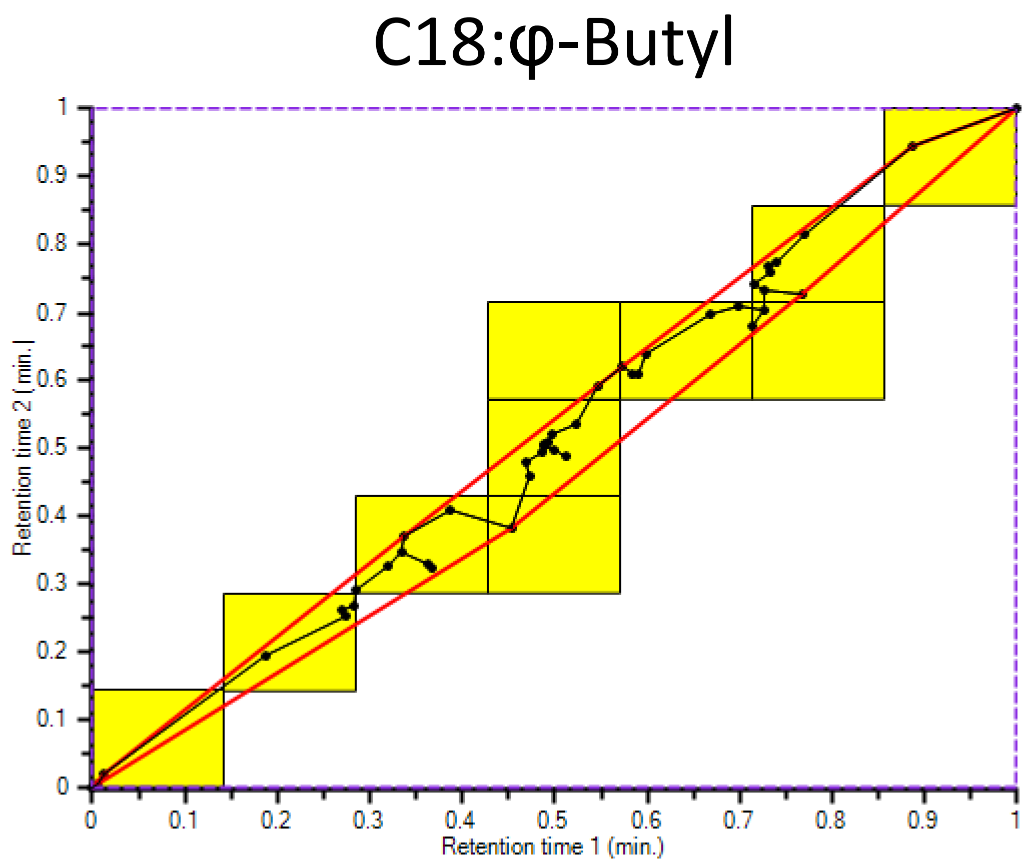
Pair-wise Comparison of Bonded Phase Orthogonality

Varied Bonded Phases (Same Mobile Phase)	Dim	R.H.A.	$1-r^2$
C18/φ-Hexyl	1.10	0.048	0.0045
C18/φ-Butyl	1.23	0.073	0.0123
C18/φ-Ethyl	1.19	0.057	0.0079
φ-Hexyl/φ-Butyl	1.21	0.040	0.0035
φ-Hexyl/φ-Ethyl	1.15	0.030	0.0024
φ-Butyl/φ-Ethyl	1.16	0.034	0.0026
"perfect Dim"	2		

Dim = Dimensionality

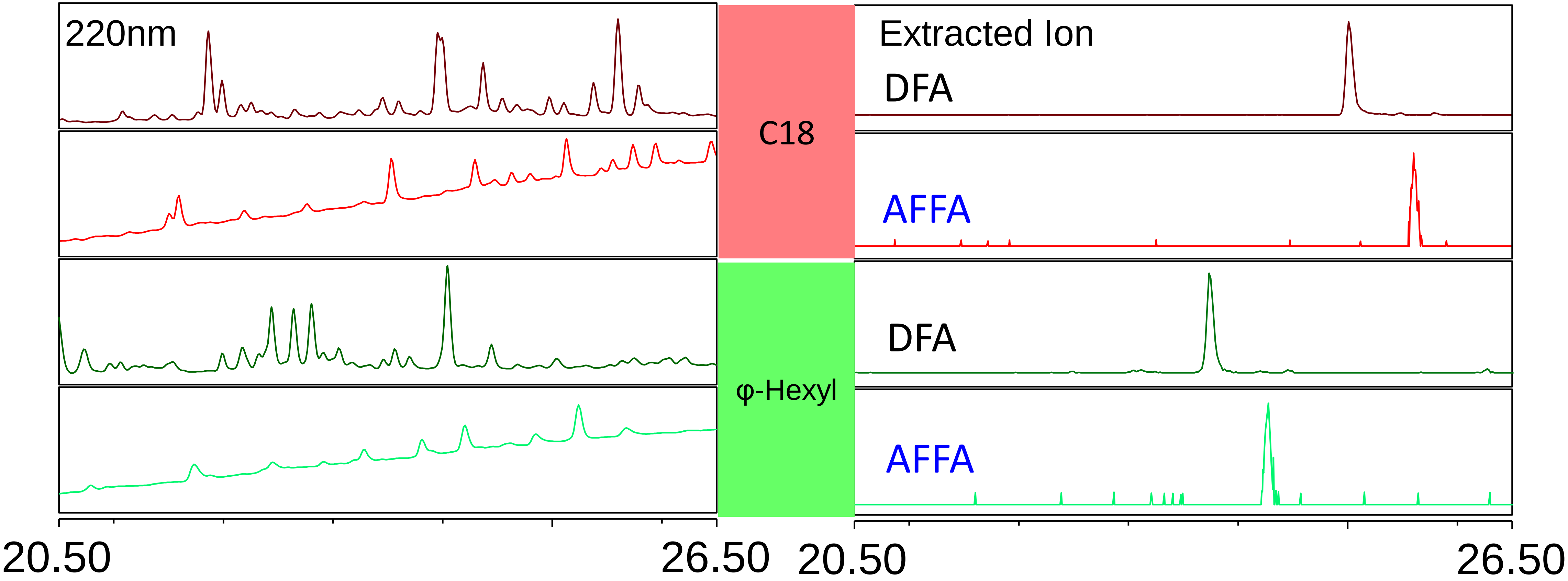
R.H.A. = Relative Hull Area

$1-r^2$ = Pearson Correlation Coefficient



M.R. Schure, J. M. Davis, Orthogonality measurements for multidimensional chromatography in three and higher dimensional separations, J. Chromatogr. A (2017)

Could You Observe Larger Selectivity Differences by Changing the Mobile Phase?



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Average Selectivity Differences for Peptide Pairs

Varied Mobile Phases (Same Bonded Phase)		
	C18:C18	φ-Hexyl:φ-Hexyl
$1/(n-1)\ \Sigma_{n-1}\ \vert\ \Delta\alpha\ \vert$	0.076	0.103

<< Improved!
φ-Hexyl:φ-Hexyl

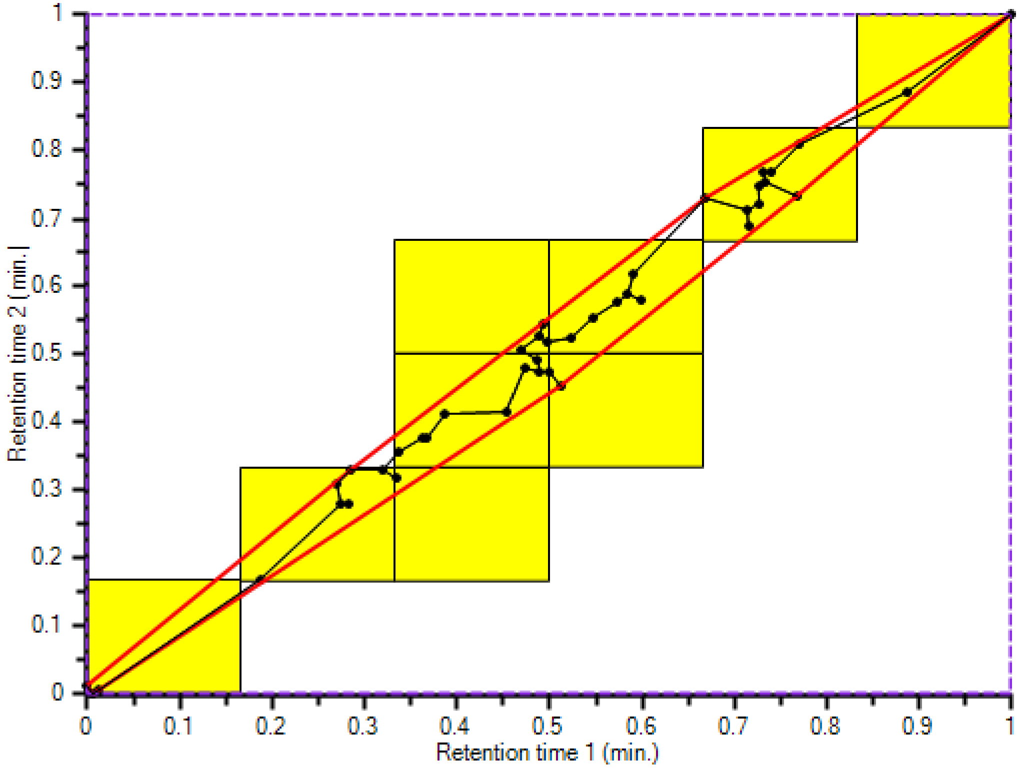
Varied Bonded Phases (Same Mobile Phase)			
	φ-Hexyl:C18	φ-Butyl:C18	φ-Ethyl:C18
$1/(n-1)\ \Sigma_{n-1}\ \vert\ \Delta\alpha\ \vert$	0.044	0.072	0.064

vs
φ-Butyl:C18

Pair-wise Comparison of Mobile Phase Orthogonality

	Varied Mobile Phase (Same Bonded Phase)			
		Dim	R.H.A.	$1-r^2$
C18	DFA:AFFA	1.206	0.070	0.012
φ-Hexyl	DFA:AFFA	1.187	0.068	0.012

DFA:AFFA (C18)



Dim = Dimensionality

R.H.A. = Relative Hull Area

$1-r^2$ = Pearson Correlation Coefficient

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Conclusions

- For identified peptides, the order of increasing **selectivity difference** relative to C18 was: **φ-Hexyl < φ-Ethyl < φ-Butyl**

- When the bonded phase was varied (same mobile phase), φ-*Butyl* demonstrated the greatest average selectivity difference relative to C18

- When the mobile phase was varied (same bonded phase), φ-*Hexyl* demonstrated the greater average selectivity difference than C18

- Models for measuring selectivity differences and for measuring orthogonality were in agreement; varied mobile phase comparison ongoing..

- A wider range of useful operating conditions (pH, temperature, etc.) could take advantage of improvements in bonded phases for HPLC and LCMS applications.

Thanks To:

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Will Miles
Ron Orlando
Jason Lawhorn

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Conditions and Procedure: Column Comparison

Trypsin Digest Sample: Reduced and alkylated trastuzumab (monoclonal antibody) was digested at 1:30 protein to enzyme for 4hrs in 50 mM Tris-HCl (pH 7.8)/1.5M Guanidine-HCl, followed by formic acid acidification and direct injection.

Instrument:	Nexera/Orbitrap Velos Pro ETD		
{Particle Size} µm:	2.7	**Bonded Phases:**	C18, Phenyl Hexyl, Phenyl Butyl, Phenyl Ethyl
{Pore Size} Å:	160	**{Column Size} mm:**	2.1x100mm
{Sample Conc.} mg/mL:	0.1mg/mL	**{Digest Injection V.} µL:**	10 µL
{Temperature} C^{o}:	60	**{Flow Rate} mL/min:**	0.3
Mobile Phase 1 (A):	10mM Difluoroacetic Acid (DFA)	**Mobile Phase 1 (B):**	10mM DFA in ACN
MP1 Gradient:	2-50% B		
Mobile Phase 2 (A):	10mM Ammonium Formate (AF)/10mM Formic Acid (FA)	**Mobile Phase 2 (B):**	10mM Ammonium Formate (AF)/ 10mM Formic Acid (FA) in 90% ACN
MP2 Gradient:	2.2-56% B		
{Gradient Time} min:	60		
% ACN/min:	0.8	**{Sampling Rate} Hz:**	10
MS Scan:	300-2000 m/z	**{Response} s:**	0.1
ESI Source:	3.5 kV	**Wavelength:**	220 nm

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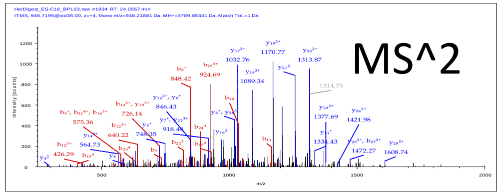
Global Comparison: Identified Peptides



High Confidence Peptide Spectral Match (above)

MS^2 used for identification of peptides

Compared ions that were identified using extracted ion current