Practical Method Development and Optimization for UHPLC and HPLC Separations of Peptides for Peptide Mapping Analyses Using Different Mobile Phase Additives

Thomas J. Waeghe[1], Benjamin Libert[2], Stephanie A. Schuster[2], and Barry E. Boyes[2]
[1]MAC-MOD Analytical, Inc., 103 Commons Court, Chadds Ford, PA 19317; [2]Advanced Materials Technology Inc., Wilmington, DE 19810

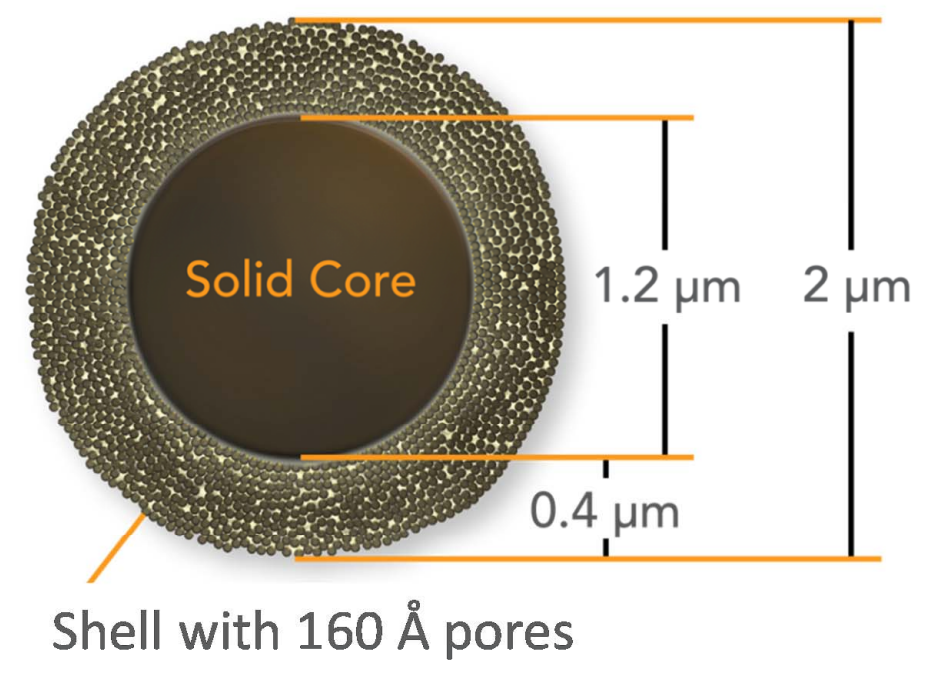
Presented at EAS 2016

Abstract

Peptide mapping is a critical competency and technique for both research and development and for quality assurance and product release for many new biopharmaceuticals including peptide drugs, monoclonal antibody drugs and antibody-drug conjugates (ADCs). New 2-µm superficially porous columns, capable of pressures up to 1000 bar, can deliver high resolution /peak capacity separations, and can be used at temperatures up to 60 °C. Moreover, a variety of mobile phase additives include trifluoroacetic acid (TFA), ammonium formate/formic acid buffers and a new additive, difluoroacetic acid (DFA), can be applied for effective development and optimization of rugged and robust separations of complex enzyme digests.

We will demonstrate the usefulness of these columns and additives in the development of peptide mapping separations, and will offer a stepwise method development and optimization strategy for such separations.

HALO® 2 Peptide ES-C18



Bonded phase:	di-isobutyloctadecylsilane
Endcapping:	None
Carbon Load:	4%
Surface Area:	65 m^2/gram
Max. Temp.:	60 °C at pH 1 to 3

Designed for:

- Peptide mapping (analysis of enzyme digests) for characterization and quality monitoring of synthetic protein and antibody drugs
- Analysis of therapeutic peptides and peptide biomarkers (protein surrogates)

Attributes

- Excellent for both fast and high resolution UHPLC separations
- High peak capacity
- Rugged, reliable performance
- Extremely stable up to 60˚C at low pH

Typical Peptide Mapping Conditions and Desirable Mobile Phase Additive Properties

Typical Conditions for RPLC Separations of Peptides

- 150 or 250 mm C18 column
- Mobile phase A: water with 0.02–0.2% (v/v) additive
- Mobile phase B: CH_3CN with same % additive or 10–30% lower than in A solvent
- Temperature: 30 to 60 °C
- Flow rate set so that linear velocity is ~2 mm/sec
- Initial % CH_3CN: ≤ 5%
- Final % CH_3CN: 40–60%
- Gradient slope: ranges from 0.1% to 2% CH_3CN per min.
- Gradient time: can vary from 30 to 240 min.

Desirable properties for additives

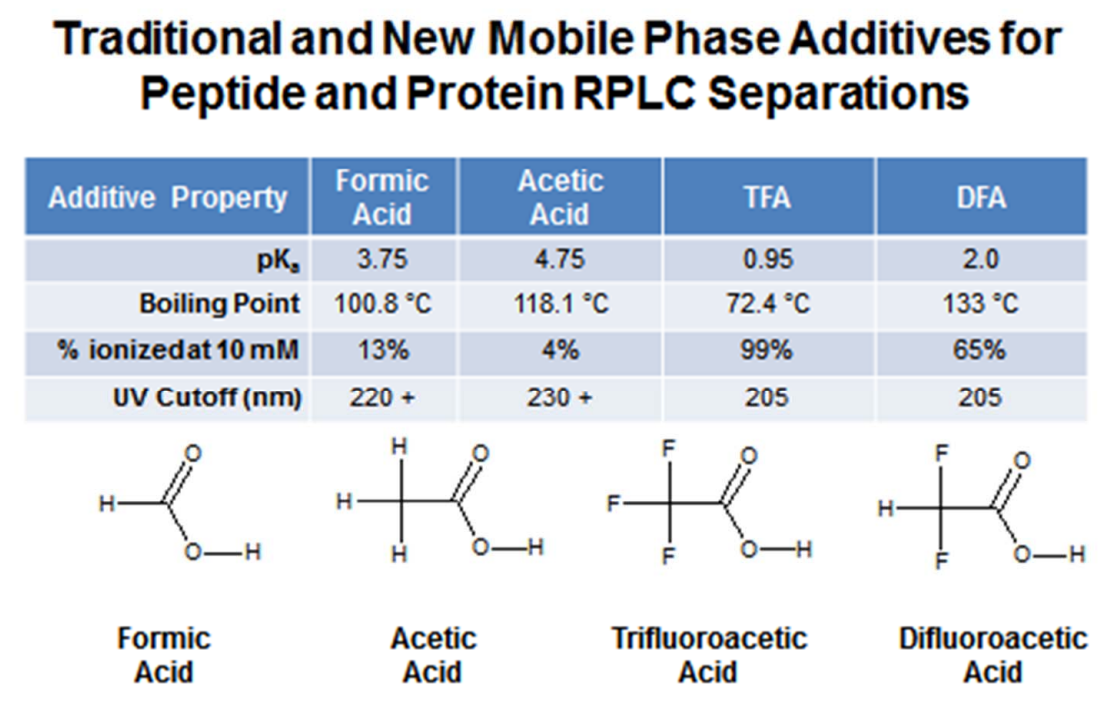
- Very high purity, quality, stability and reproducibility
- Low UV absorbance with acceptable baselines and blank runs
- Ability to provide good retention and symmetrical peak shapes for acidic, basic and zwitterionic analytes
- Volatile
- Acceptably low or no suppression of LC-MS signal
- Miscible with water and organic modifiers

Parameters That Affect Peptide Reversed-Phase Gradient Separations

Parameter	Determined by	Impacts performance
Organic modifier	Most labs choose acetonitrile for viscosity, mass transfer, efficiency reasons, UV transparency, ionization efficiency in MS	Peak width Selectivity Retention Peak capacity
Mobile phase additive type and concentration	UV transparency LC-MS sensitivity (S/N) Column stability at pH and temperature	Peak shape Peak width Loading (dynamic range) Selectivity LC-MS signal
Gradient steepness	User experience Sample complexity Experimentation and optimization software	Selectivity Peak capacity Peak height
Column temperature	Analyte requirements (recovery, stability) Column stability requirements Flow rate (thermal heating) Instrument capability and pressure limit	Selectivity Recovery Analysis time Peak width Peak capacity
Flow rate	User choice Column and Instrument pressure limits Column particle morphology and diameter	Selectivity Peak capacity
Pressure	Packing particle size Column length Flow rate Column temperature	Retention Selectivity

Assessment of LC-UV and LC-MS Performance With Various Mobile Phase Additives

- Experiments were carried out using a 2.1 x 100 mm, HALO 2 Peptide ES-C18 column with the following mobile phase additives :
  - HCOOH (20 mM)
  - NH_4COOH/HCOOH (10 mM each)
  - TFA (10 mM)
  - DFA (10 mM)
- Mobile Phase A: water with additive
- Mobile Phase B: CH_3CN with additive
- Column Temperature: 60 °C
- Gradient: 2 to 47% CH_3CN/water
- Flow Rate: 0.3 mL/min
- Instrument: Shimadzu Nexera
- Instrument dispersion: 15.1 µL^2

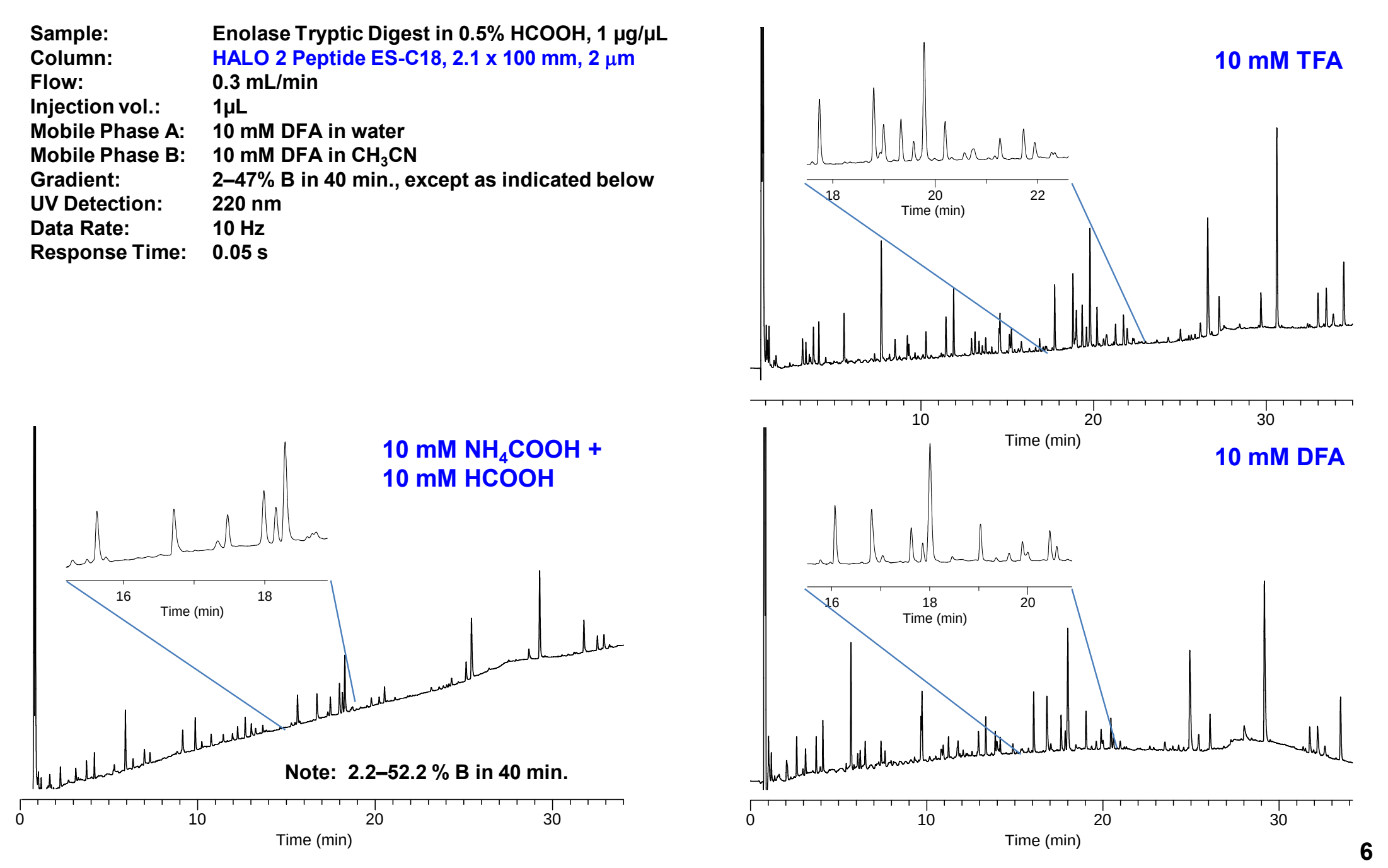


Traditional and New Mobile Phase Additives for Peptide and Protein RPLC Separations

Additive Property	Formic Acid	Acetic Acid	TFA	DFA
pK_a	3.75	4.75	0.95	2.0
Boiling Point	100.8 °C	118.1 °C	72.4 °C	133 °C
% ionized at 10 mM	13%	4%	99%	65%
UV Cutoff (nm)	220 +	230 +	205	205

LC-UV Separations of Enolase Tryptic Digest Using Different Mobile Phase Additives

Sample: Enolase Tryptic Digest in 0.5% HCOOH, 1 µg/µL
Column: HALO 2 Peptide ES-C18, 2.1 x 100 mm, 2 µm
Flow: 0.3 mL/min
Injection vol.: 1µL
Mobile Phase A: 10 mM DFA in water
Mobile Phase B: 10 mM DFA in CH_3CN
Gradient: 2–47% B in 40 min., except as indicated below
UV Detection: 220 nm
Data Rate: 10 Hz
Response Time: 0.05 s



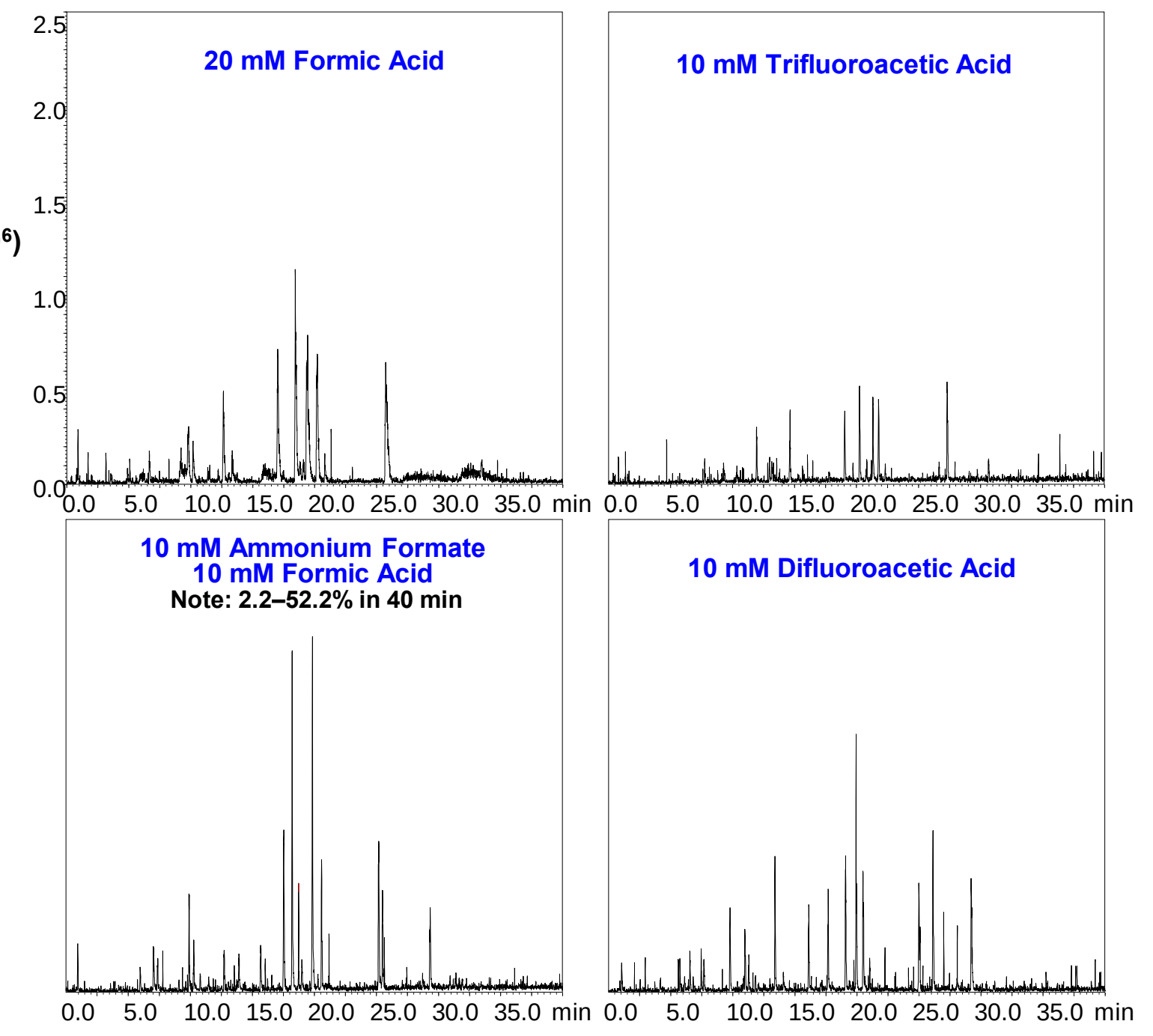
DFA, TFA and NH_4COOH/HCOOH give narrow peaks, similar retention but different selectivities for peptide mapping.

LC-MS Separations of Enolase Tryptic Digest Using Different Mobile Phase Additives

Same LC conditions as shown on previous panel for LC-UV chromatograms

MS Scan: 0.2 s (300–2000 m/z)

Multiple Ion Chromatogram scale (x 10^6)
All chromatogram are on the same scale.



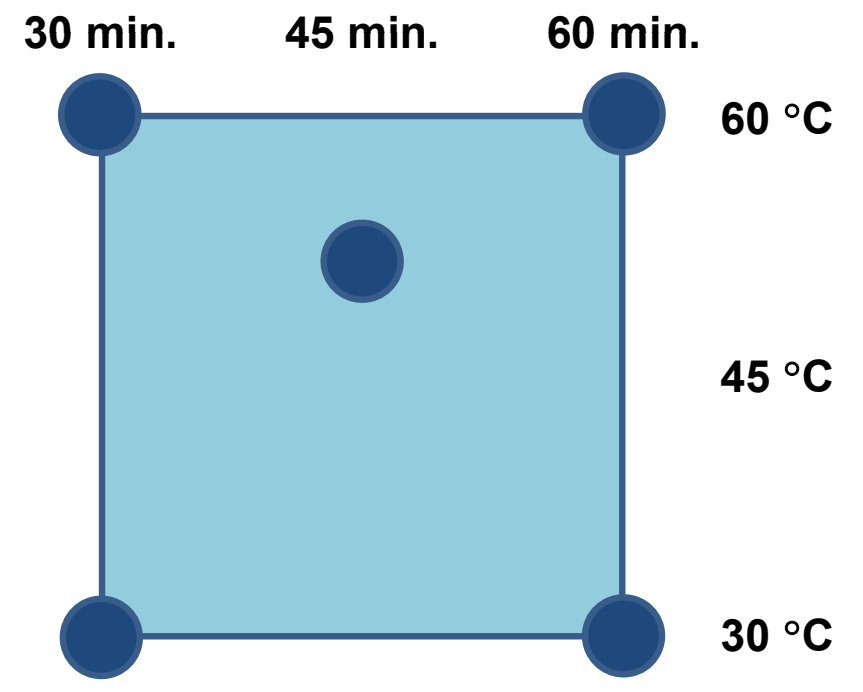
Comparison of multiple-ion chromatograms from LC-MS analyses of enolase tryptic digests using formic acid, ammonium formate/formic acid, trifluoroacetic acid, and difluoroacetic acid as mobile phase additives.

DFA offered 2–3 fold better signal intensity compared to TFA for LC-MS, with comparable narrow peaks and retention.

Method Development Approaches for Peptide Mapping Separations

Systematic Approach using Computer Simulation (DryLab® 4)

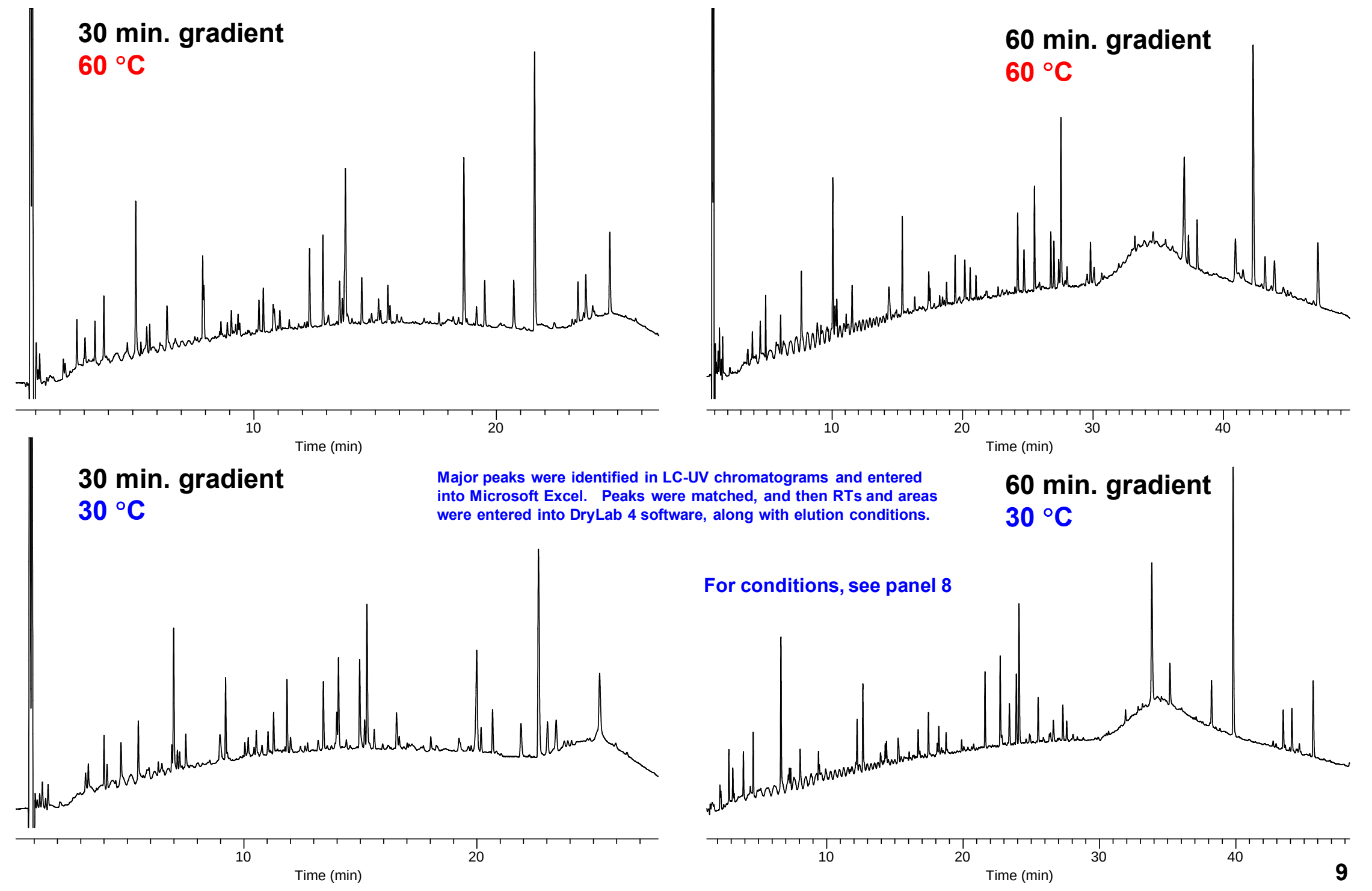
- Two-dimensional Design
  - t_G x T
  - 2–3 gradient steepnesses
  - 2–3 column temperatures



- Column
  - HALO 2 Peptide ES-C18 2.1 x 100 mm, 2 µm
- Mobile Phase
  - A Solvent: 0.1% DFA in water
  - B Solvent: 0.1% DFA in CH_3CN
  - $\Phi_{initial}$: 2% B
  - Φ_{final} : 50% B
- Column Temperatures
  - 30 and 60 °C
- Gradient Times
  - 30 and 60 min.
  - 45-min x 50 °C run used for peak matching

Optimization was carried out to identify a robust gradient time and temperature combination for a run time < 40 minutes

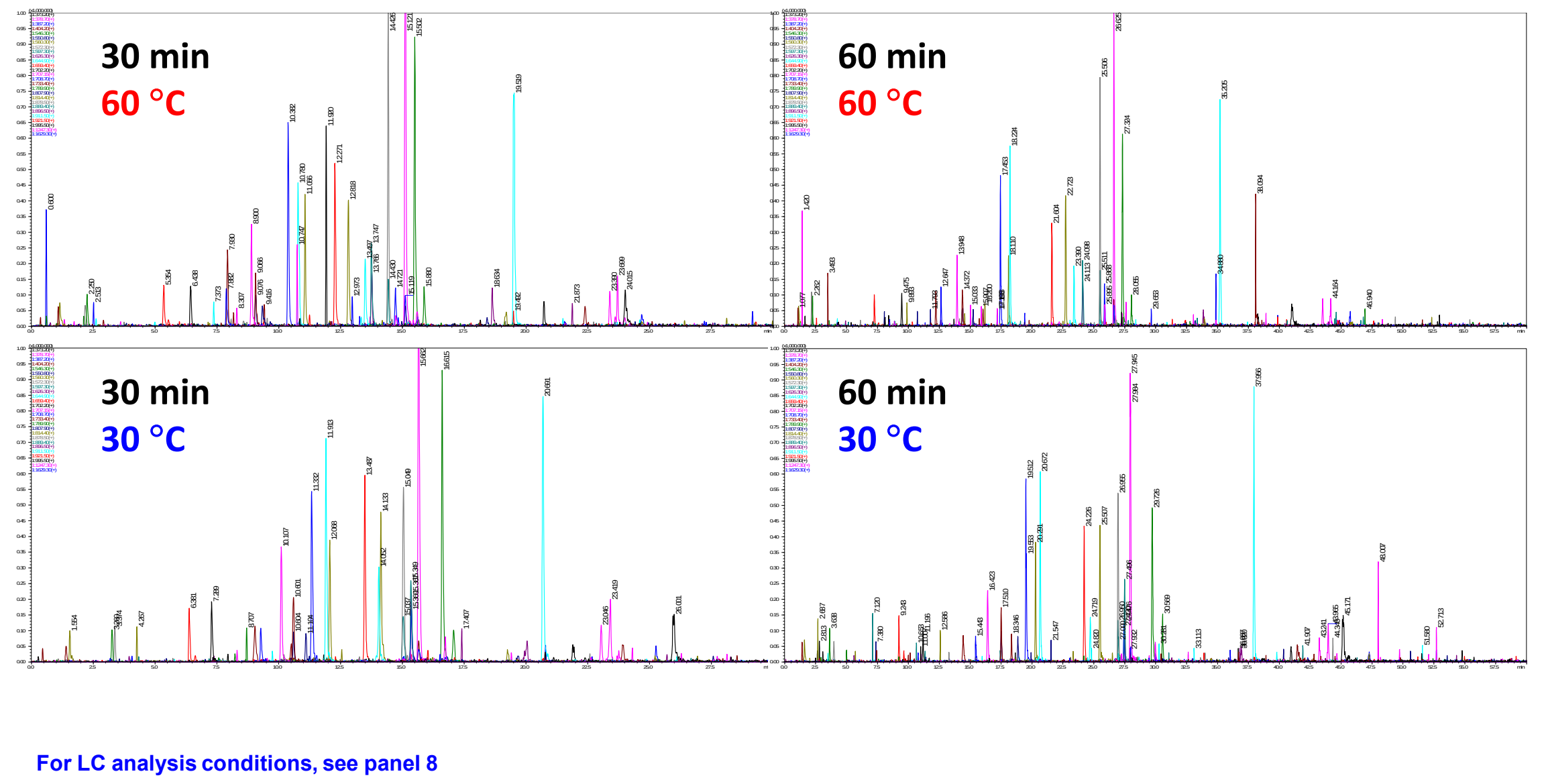
LC-UV Chromatograms for Gradient Input Runs at Two Temperatures For DryLab Input



Major peaks were identified in LC-UV chromatograms and entered into Microsoft Excel. Peaks were matched, and then RTs and areas were entered into DryLab 4 software, along with elution conditions.

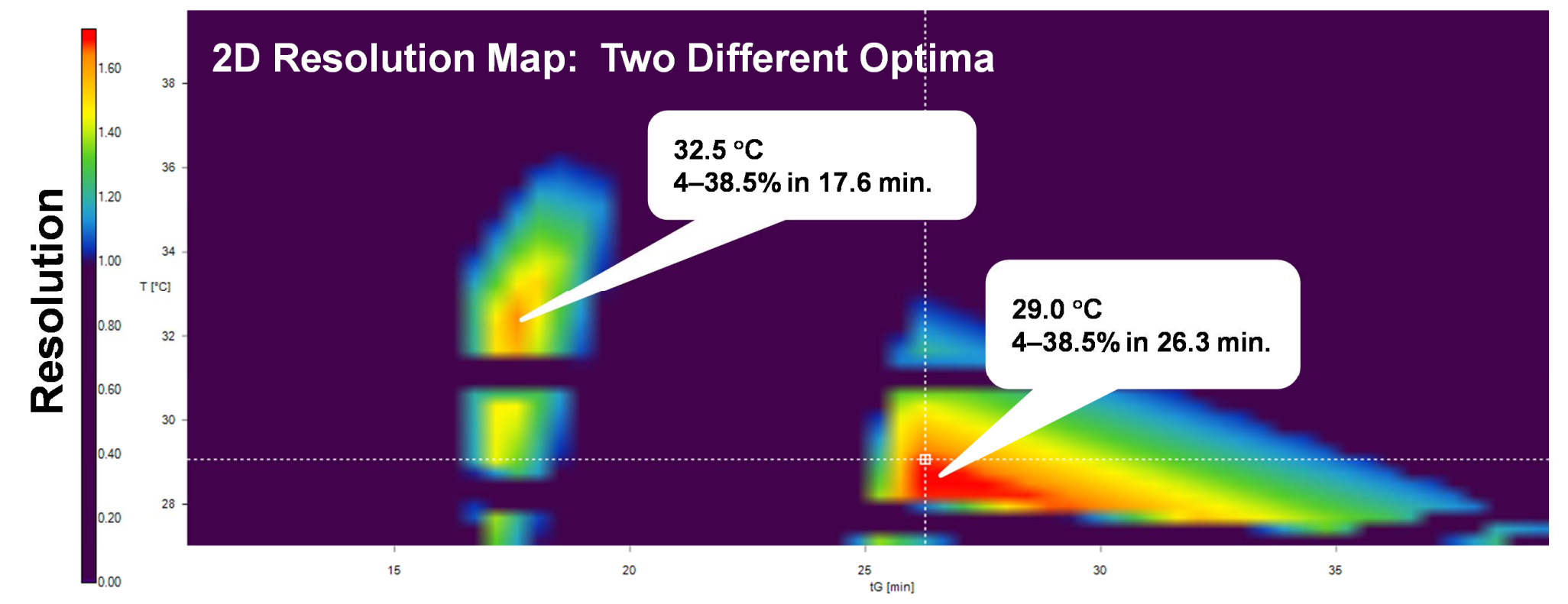
For conditions, see panel 8

LC-MS Extracted-Ion Chromatograms for Gradient Input Runs at Two Temperatures

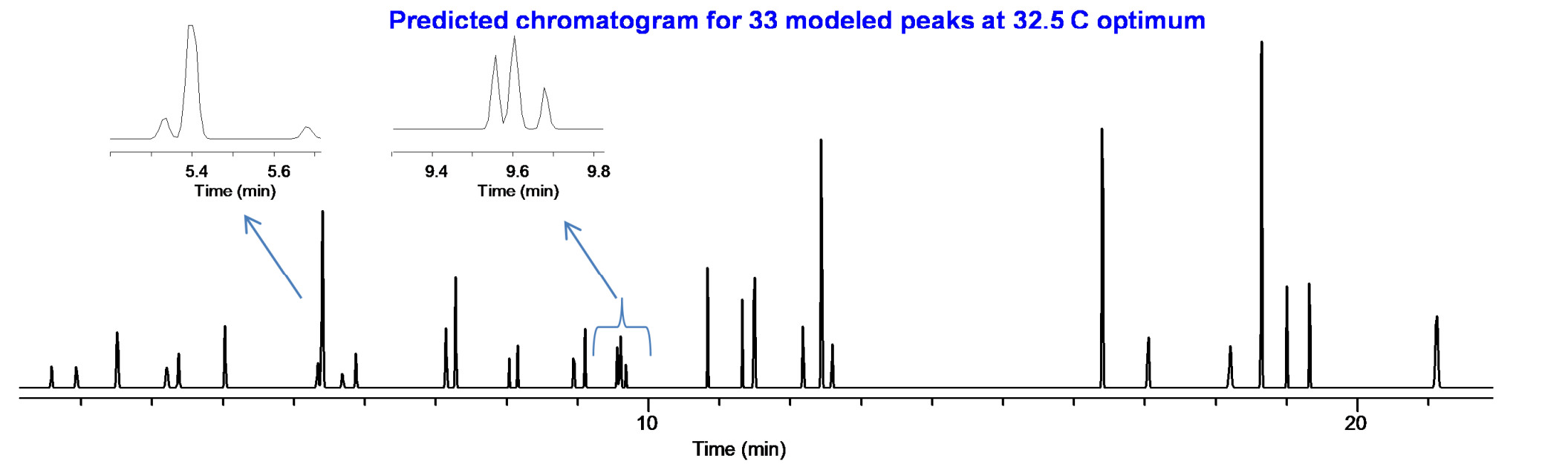


For LC analysis conditions, see panel 8

Input Data for DryLab Software and 2-D Resolution Map With Optima



Predicted chromatogram for 33 modeled peaks at 32.5 C optimum



Peptide Identification Using Proteome Discoverer Software

Confidence	Single Letter Amino Acid Sequence	RT [min] Sequest HT	Modifications	# PSMs	# Missed Cleavages	Theo. MH+ [Da]	XCorr Sequest HT	ΔM [ppm] Sequest HT
High	TGAPAR	1.99		1	0	572.31509	1.85	-1.32
High	ANIDVK	5.22		1	0	659.37227	1.99	-2.06
High	HLADLSK	5.59		2	0	783.43593	2.87	-0.89
High	IATAIEK	6.32		1	0	745.44543	2.26	-1.14
High	IGSEVYHNLK	7.76		2	0	1159.6106	2.98	0.04
High	DGKYDLDFKNPNSDK	8.46		3	2	1755.81842	5.11	-0.7
High	LNQLLR	8.75		2	0	756.47265	2.01	1.28
High	TFAEALR	8.94		1	0	807.43593	2.06	-1.23
High	YDLDFKNPNSDK	9.13		1	1	1455.67505	3.81	-0.89
High	DGKYDLDFK	9.22		2	1	1100.52587	2.52	-1.5
High	KAADALLLK	9.97		2	1	942.59824	2.61	-0.38
High	YDLDFK	10.10		1	0	800.3825	1.74	-1.28
High	GNPTVEVELTTEK	10.29		1	0	1416.72167	3.57	-0.73
High	VNQIGTLSESIK	10.68		2	0	1288.71071	3.67	-1.3
High	SIVPSGASTGVHEALEMR	10.76	1×Oxidation [M17]	1	0	1856.91709	4.23	-0.29
High	IEEELGDNAVFAGENFHHGDK	11.36		2	0	2328.05273	4.73	-2.91
High	AAQDSFAAGWGVMVSHR	11.71	1×Oxidation [M13]	1	0	1805.83878	4.72	-1.41
High	SIVPSGASTGVHEALEMR	12.17		2	0	1840.92217	4.22	-0.27
High	IEEELGDNAVFAGENFHHGDKL	12.70		1	1	2441.13679	5.34	-0.91
High	NVNDVIAPAFVK	13.40		2	0	1286.71031	2.67	-1.51
High	IGLDCASSEFFK	13.60	1×Carbamidomethyl [C5]	1	0	1373.64058	3.78	-0.46
High	AAQDSFAAGWGVMVSHR	13.63		1	0	1789.84386	5.28	-0.45
High	TAGIQIVADDLTVTNPK	14.31		2	0	1755.9487	4.97	0.18
High	LGANAILGVSLAASR	15.02		1	0	1412.82199	4.57	-1.33
High	AVDDFLISLDGTANK	15.40		3	0	1578.80098	5.83	-0.48
High	WLTGPQLADLYHSLMK	16.89	1×Oxidation [M15]	1	0	1888.96258	4.8	-1.3
High	WLTGPQLADLYHSLMK	18.55		2	0	1872.96767	5.32	-0.18
High	SGETEDTFIADLVVGLR	19.42		1	0	1821.92288	6.42	-0.33
High	RYPIVSIEDPFAEDDWEAWSHFFK	20.64		1	1	2984.38898	7.39	-1.18
High	YPIVSIEDPFAEDDWEAWSHFFK	21.51		2	0	2828.28787	5.46	-0.78
High	YGASAGNVGDEGGVAPNIQTAEEALDLIVDAIK	24.66		1	0	3257.61721	4.97	-0.88
Data Set was generated using the 30-min, 60 °C Xcalibur software raw file					PSM = Peptide Spectrum Match	XCorr = Cross Correlation Factor		

Total-ion Chromatogram Showing Enolase Tryptic Digest Fragments Obtained Using Near-Optimum Conditions

Tune File Values

Source Type:	HESI
Capillary Temp (C):	225.00
Source Heater Temp (C):	150.00
Sheath Gas Flow ():	35.00
Aux Gas Flow ():	10.00
POSITIVE POLARITY	
Source Voltage (kV):	2.20
Source Current (uA):	100.00
S-Lens RF Level (%):	69.00
FTMS Full Micro Scans:	1
FTMS Full Max Ion Time (ms):	50.00

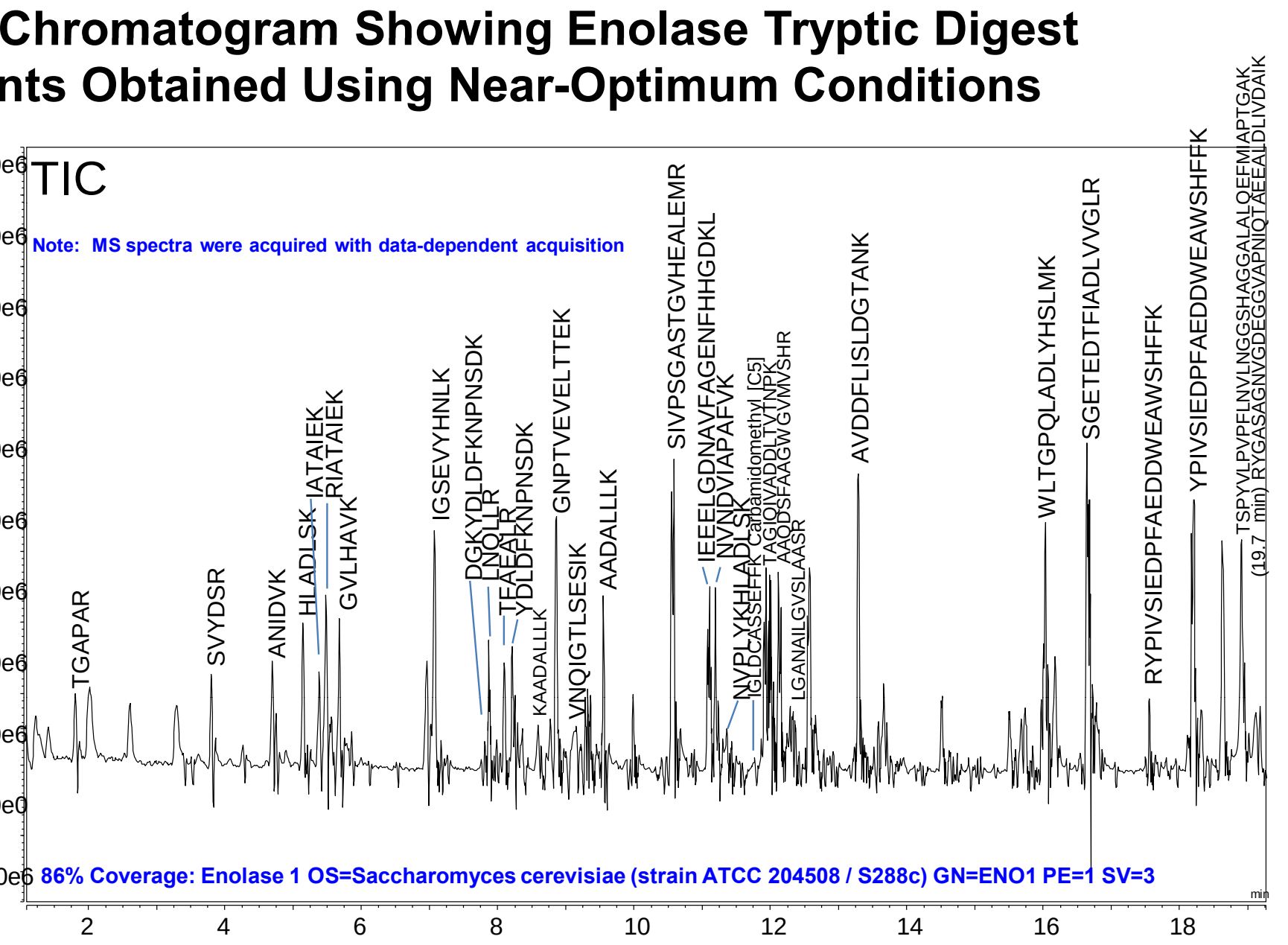
Data-Dependent Acquisition Settings

Scan Event Details:
1: FTMS + positive high res=30000
(200.0–2000.0)
Collision Voltage = 0.0V

2: ITMS + p norm Dep MS/MS
(110.0–2000.0)
Most intense ion from (1)

Activation Type: CID
Min. Signal Required: 500.0
Isolation Width: 4.00
Normalized Coll. Energy: 35.0
Default Charge State: 2
Activation Q: 0.250
Activation Time: 30.000
CV = 0.0V

Number of Scan Events: 6



Sample:	Enolase Tryptic Digest in 0.5% HCOOH, 2 µL of 1 µg/µL	Instrument:	Shimadzu Nexera UHPLC
Column:	HALO 2 Peptide ES-C18, 2.1 x 100 mm, 2 µm	MS:	Thermo Orbitrap Velos Pro ETD
Flow:	0.3 mL/min		
Mobile Phase A:	0.1% (v/v) DFA in water		
Mobile Phase B:	0.1% (v/v) DFA in CH_3CN		
Gradient:	4–40% CH_3CN in 18.34 min.		
Column temp.:	35 °C		
UV Detection:	220 nm		
Data Rate:	10 Hz		
Response Time:	0.05 s		

Recommended Method Development Strategies for RPLC-UV and RPLC-MS of Protein Enzymatic Digests

Systematic Approach (DryLab)

1. Select desired column geometry.
2. Choose initial flow rate based on column ID.
3. Select two different gradient times varying by 2- or 3-fold.
4. Select two column temperatures varying by 20–40 °C.
5. Set gradient range of 0–5 to 60% CH_3CN or 0–5 to 50% CH_3CN.
6. Carry out gradient runs at T1 and T2 with two different gradient times.
   - For example, 30 and 60 min. at 30 and 60 C.
7. Carry out an additional gradient run at intermediate temperature and gradient time.
   - For example, 45 min. at 45 C.
8. Enter RTs and areas into DryLab software.
   - Note: Peak matching can be tricky and difficult
9. Compare the predicted intermediate gradient run vs. actual intermediate run to assess peak matches.
10. Determine whether there are any optimum regions of robust resolution in the 2-D resolution map.
11. Optimize linear gradient in gradient editor or choose a two or three segment gradient to find optimum conditions.

Wang, Carr et al. Approach*

1. Choose desired column geometry.
2. Set flow rate for column max. pressure or 75% of max. pressure.
3. Set Φ_{final} so that last peak of interest elutes at very end of gradient.
4. Choose temperature based on column stability and “fluidity” 60C?
5. For example, use 2–50% CH_3CN for initial run.
6. Set gradient time for maximum allowable time.
7. Calculate %B at elution of last peak of interest (using RT, %B/min, t_0, and t_D).
8. Rerun sample with the 2–X %B limit for gradient time corresponding to that final %B.

* Xiaoli Wang, Dwight Stoll, Adam Schellinger, and Peter Carr Peak Capacity Optimization of Peptide Separations in Reversed-Phase Gradient Elution Chromatography: Fixed Column Format *Anal. Chem.* **2006, 78**, 3406-3416

Summary and Conclusions

- A sample of enolase was digested with trypsin, and was analyzed using gradient UHPLC using a 100-mm HALO 2 Peptide ES-C18 column.
- Various mobile phase additives were compared in terms of LC-UV and LC-MS signal, peak shape, peak width and retention.
- Difluoroacetic acid (DFA) offers peak shape, peak width and retention comparable to TFA and ammonium formate/formic acid, but with much better LC-MS ionization efficiency (signal-to-noise).
- Systematic method development for optimizing peptide separations allows one to find temperature and gradient time combinations for robust, effective analyses.
- HALO 2 Peptide ES-C18 columns facilitate high resolution peptide mapping of complex tryptic digests with short analysis times (20–30 minutes).

Acknowledgements: Supported in part by NIH Grant GM116224 (BEB). Opinions expressed are solely those of the authors. HALO and Fused-Core are registered trademarks of Advanced Materials Technology, Inc.