Supporting Information

Retention time prediction for TMT-labeled peptides in proteomic LC-MS experiments

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Supplementary Experimental procedures

Supplementary Figure S4. The distribution of SSRCalc predicted hydrophobicity values for *in-silico* digest of human proteome (peptides 6 or more amino acids long and 0 missed cleavages).

Supplementary Experimental procedures

Second dimension LC-MS/MS

The first set of experiments (second dimension RPLC). An EASY-nLC1000 Liquid Chromatography system (Thermo Fisher Scientific, Waltham, MA), using a Thermo Fisher Scientific™ EASY Spray column (PepMap™, RSLC C18, 2µm, 100Å, 75µm × 50cm, operated at 35°C) was coupled online to a The Thermo Scientific™ QExactive™ HF-X hybrid quadrupole-Orbitrap™ mass spectrometer through EASY spray source interface. Q-Exactive HF-X mass spectrometer was used using following settings: positive ion mode, an MS1 resolution 60,000, AGC target of 1e6, maximum injection time of 45 ms, and a scan range of 350 to 1400 m/z; MS2 settings were 15,000 resolution, AGC target of 1e5, isolation window of 1.4 m/z, and scan range of 200 to 2000 m/z, NCE = 24, 31 and 31 for NL, TMT and TMTpro peptides respectively. MS2 scans were done +2 to +5 charge states. Previously targeted parent ions were excluded for 10 seconds from repetitive MS/MS acquisition.

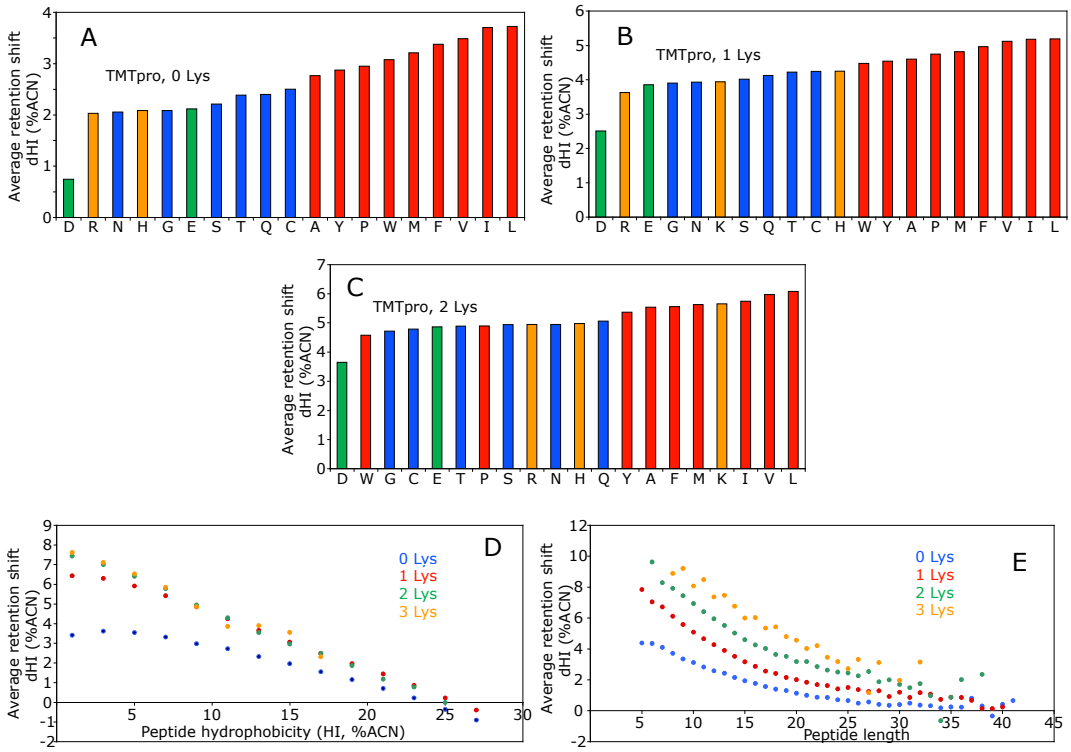
The second set of experiments (various columns in the first dimension). 2D LC Ultra system (Eksigent, Dublin, CA) was used, delivering buffers A and B through a 100µm x 200mm analytical column packed with 3 µm Luna C18(2) (Phenomenex, Torrance, CA) at 500nL/min flow rate. Samples we loaded on a 300 µm x 5 mm PepMap 100 trap-column (Thermo Fisher). The gradient program consisted of following steps: a linear increase from 0.5 to 35% buffer B (acetonitrile) in 49 minutes, 3 minutes at 80% B and then 8 minutes at 0.5% B for column equilibration (60 min total analysis time). Both eluents A (water) and B (acetonitrile) contained 0.1% formic acid. Data-dependent acquisition using a TripleTOF5600 mass spectrometer (Sciex, Concord, ON) in standard MS/MS mode was used. The following settings were applied: 250 ms survey MS spectra (m/z 370-1500) followed by up to 20 MS/MS measurements on the most intense parent ions (300 counts/sec threshold, +2 through +5 charge state, m/z 100-1500 mass range for MS/MS, 100 ms each). Previously targeted parent ions were excluded for 12 seconds from repetitive MS/MS acquisition.

Supplementary Table S1. Hydrophobicity Index values (HI, % acetonitrile) for standard peptides used for retention time alignment.

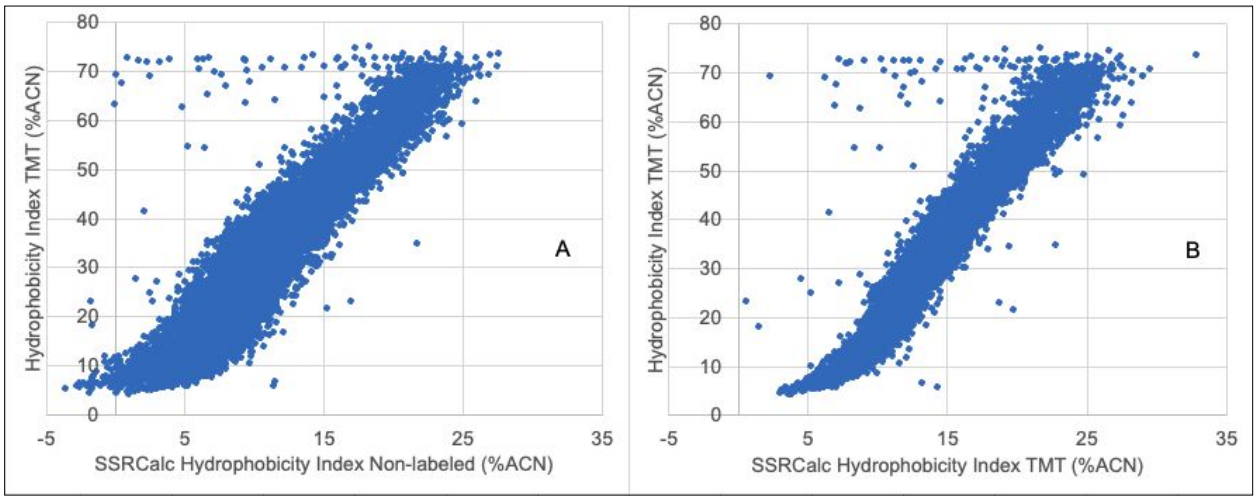
Peptide	Sequence	Retention time (min)*	HI (% ACN)*
University of Manitoba P1-P6 standards[1]			
P2	LGGGGGGDFR	27.79	3.88
P3	LLGGGGDFR	38.73	7.85
P4	LLLGGDFR	51.46	12.48
P5	LLLLDFR	65.86	17.72
P6	LLLLLDFR	74.02	20.68
Pierce™ LC-MS/MS system suitability standard mix (Thermo Fisher Scientific)			
TF1	GISNEGQNASIK	26.15	3.28
TF2	IGDYAGIK	34.33	6.25
TF3	TASEFDSAIAQDK	40.41	8.46
TF4	ELGQSGVDTYLQTK	47.41	11.01
TF5	SFANQPLEVVYSK	52.68	12.92
TF6	LTILEELR	57.22	14.58
TF7	ELASGLSFPVGFK	64.71	17.3

* - measurements for 35 °C column temperature.

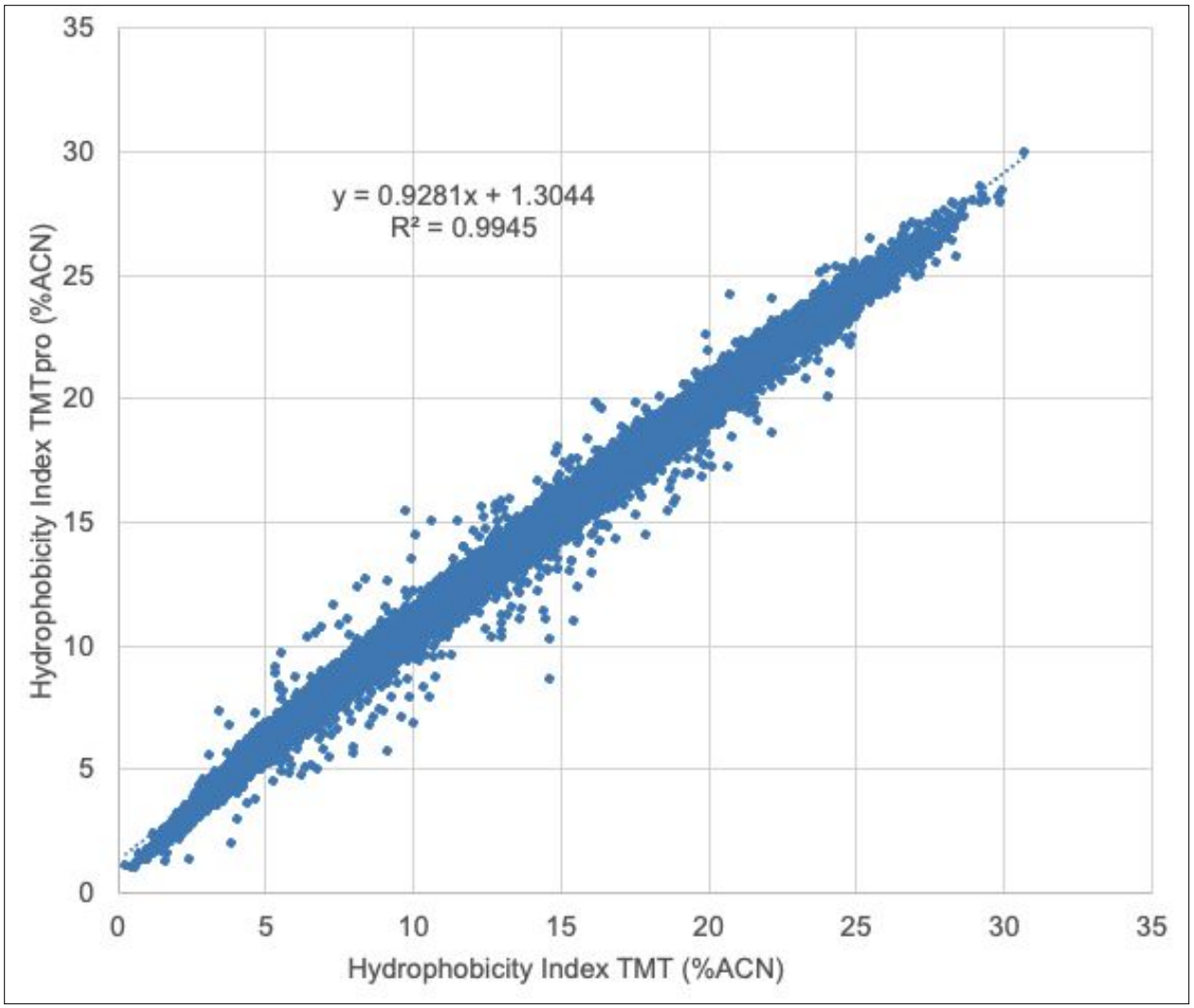
Krokhin, O. V.; Spicer, V., Peptide retention standards and hydrophobicity indexes in reversed-phase high-performance liquid chromatography of peptides. *Anal Chem* **2009,** 81, (22), 9522-30.



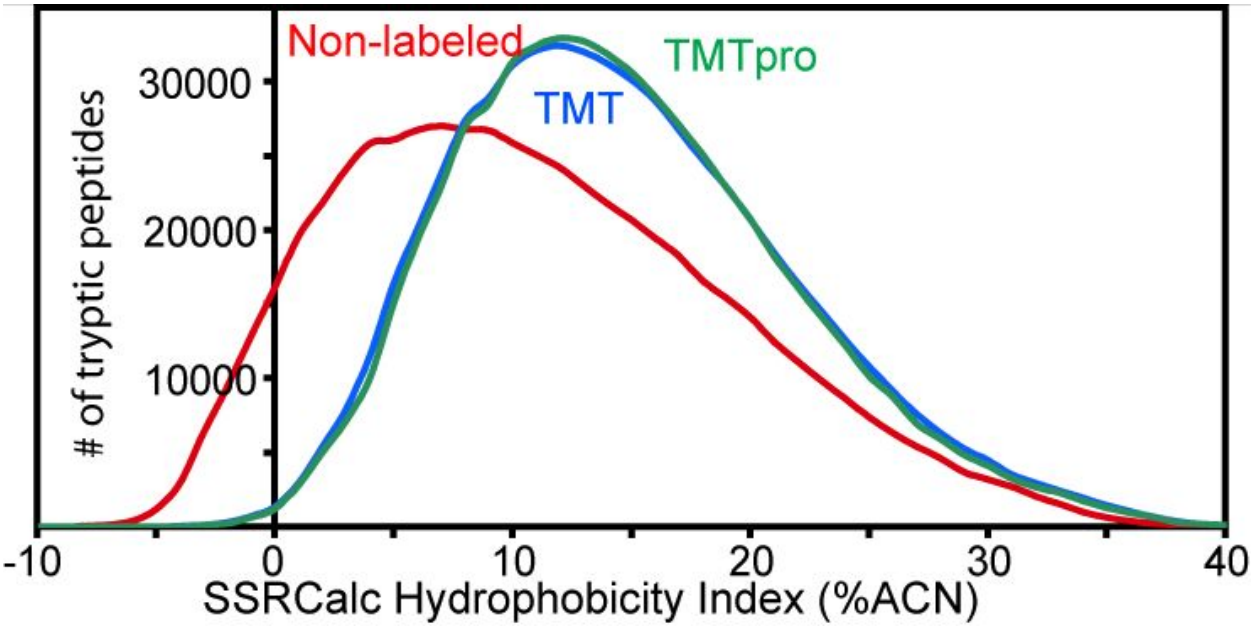
Supplementary Figure S1. Composition and sequence dependent features driving RT shifts upon TMTpro labeling. A-C - average retention time shifts for different residues in position #1 for 0 Lys, 1 Lys, and 2 Lys peptides, respectively; D, E – dependence of average retention shifts from peptide hydrophobicity and length.



Supplementary Figure S2. Retention time prediction for TMT labeled murine peptides (external data) using non-modified (A) and TMT adjusted (B) SSRCalc model. Retention data information from a single 2D fraction was used to generate these plots (from Popow, O.; Liu, X.; Haigis, K. M.; Gygi, S. P.; Paulo J. A., A Compendium of Murine (Phospho)Peptides Encompassing Different Isobaric Labeling and Data Acquisition Strategies *J Proteome Res* 2021, 20, (7), 3678−3688.).



Supplementary Figure S3. Correlation between experimental retention values for TMT and TMTpro labeled peptides. (96895 peptides found in both TMT and TMTpro formats).



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