Optimized Peptide Retention Time Standards for Targeted Proteomics

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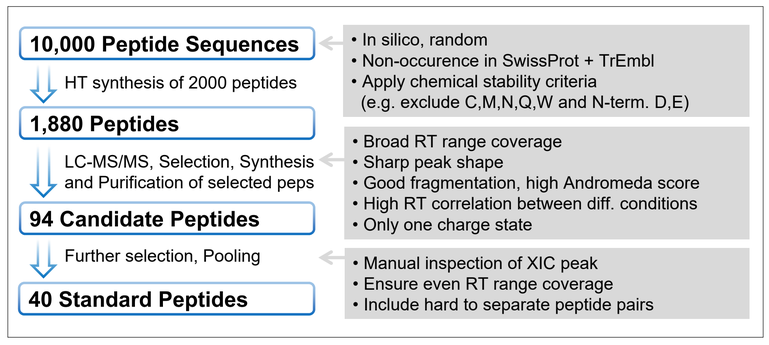
Introduction

Targeted proteomics (e.g. the development of SRM/MRM assays) is dependent on the definition of HPLC retention time (RT) windows for the selected proteotypic peptides.[1] Although a number of RT standards have been described, many of them display limitations like relatively small peptide numbers and the use of naturally occurring sequences which is disadvantageous when the standards are spiked into biological samples.

Methods and Results

Suitable peptides for an optimized set of RT standards were identified through an iterative selection process. The process started from 10,000 *in silico* generated non-naturally occurring peptide sequences followed by iterative steps of synthesis and experimental examination by LC-MS/MS on an Orbitrap Fusion Lumos (Thermo) instrument (Scheme 1).



Scheme 1: Selection process for the new RT standard.

Comprehensive examination of each peptide resulted in a set of 40 optimized peptides (Table 1). The peptides were synthesized in high purity and pooled in aliquots of approx. 10 or 100 pmol/peptide.

Table1: List of the selected peptide retention time standards.

No.	Sequence	No.	Sequence	No.	Sequence
1	YSAHEEHHYDK	15	GFLDYESTGAK	29	VYAETLSGFIK
2	HEHISSDYAGK	16	ALFSSITDSEK	30	GFVIDDGLITK
3	TFAHTESHISK	17	FVGTEYDGLAK	31	GASDFLSFAVK
4	ISLGEHEGGGK	18	YALDSYSLSSK	32	FFLTGTSIFVK
5	LSSGYDGTSYK	19	HDTVFGSYLYK	33	VSSIFFDTFDK
6	FGTGTYAGGEK	20	YFGYTSDTFGK	34	GDFTFFIDTFK
7	VGASTGYSGLK	21	HFALFSTDVTK	35	LFISALVDFFK
8	TASGVGGFSTK	22	TFTGTTDSFFK	36	SLFFIIDGFVK
9	SYASDFGSSAK	23	VSGFSDISIYK	37	IDVYILALLLK
10	LYSYYSSTESK	24	TFGTETFDTFK	38	SILAFLYLYFK
11	LYTGAGYDEVK	25	TSIDSFIDSYK	39	SLIFFLSTLLK
12	TLIAYDDSSTK	26	ASDLLSGYYIK	40	FLISLLEEYFK
13	HLTGLTFDTYK	27	FLFTGYDTSVK		
14	FLASSEGGFTK	28	GIFGAFTDDYK		

Typical LC-MS chromatograms of the newly developed peptide set are shown in Figure 1. All peptides yielded good LC-MS characteristics (sharp peak shapes), good detectability (Andromeda score >60, data not shown), similar intensities, and broad and even RT coverage.

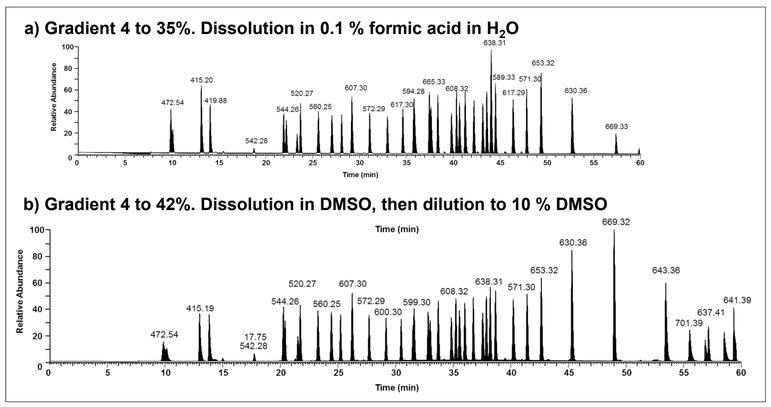


Figure 1: LC-MS chromatograms of the peptide RT standard (3 µm C18 LC column, column length 47 cm, solvent A: 1 % formic acid in H_2O, solvent B: 5 % DMSO in MeCN).
a) 4 to 35 % solvent B in 60 min. b) 4 to 42 % solvent B in 60 min.

To appraise the RT coverage of the new RT standard, the SwissProt human proteome was digested *in silico* and the hydrophobicity index (HI)[2] of the resulting proteotypic peptides calculated (Figure 2, top). 95.8% of the human proteome peptides lie within the RT range of the new RT standard set, which is significantly higher compared to other sets (Figure 2, bottom).

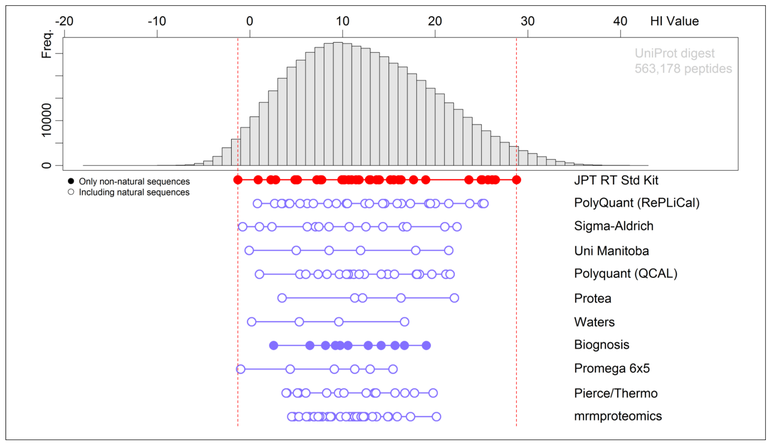


Figure 3: Predicted Hydrophobicity index (HI)[2] for a theoretical digest of the Swissprot human proteome and different RT standards.

The application of the standard peptides to RT normalization is dependent on high reproducibility of the associated RTs. Multiple injections under the same conditions showed that the measured RTs were indeed highly repeatable (Figure 3a).

In addition to RT normalization the peptide set has also been used for the evaluation of HPLC column performance. This is exemplified in Figure 3b where two pairs of peptides could not be separated with an old column (upper trace) but were separable by using a new column (lower trace).

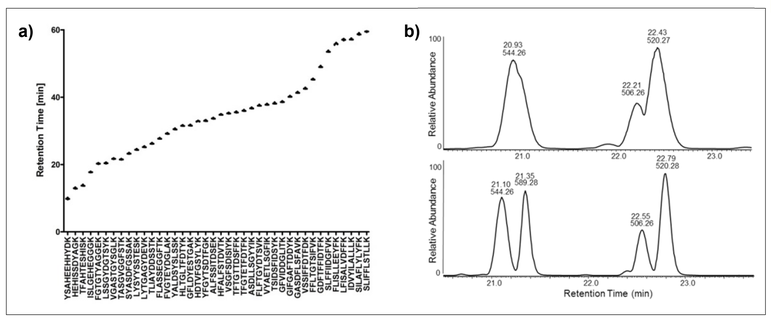


Figure 3: a) RT stability of all RT standards over 10 LC-MS injections (all data points shown). b) EIC peaks of four RT standard peptides using an old (top) and a fresh (bottom) HPLC column.

Based on the observed favorable properties a subset of the RT standard peptides was spiked into every of the >1200 peptide pools generated in the course of the ProteomeTools project.[3] This allowed the calculation of RT indices for all peptides thus ensuring the transferability of RTs between laboratories.

Conclusion

An optimized set of HPLC reference standard peptides with several favorable properties was developed.

- **Non-naturally occurring peptides (allowing application in all sorts of biolog. samples)**
- **Broader LC gradient coverage and higher number of peptides than any other RT kit**
- **Very stable RTs across multiple injections**
- **Good detectability, sharp peak shapes**
- **Similar intensities**
- **Chemical stability criteria**
- **Use for column performance evaluation**
- **Intensive use in ProteomeTools project**

References

(1) Picotti, P. et al., *Nat. Methods* **2012**, *9(6)*, 555-566.
(2) http://hs2.proteome.ca/SSRCalc/SSRCalcQ.html.
(3) Zolg, D. P., Wilhelm, M., Schnatbaum, K., Zerweck, J., Knaute, T., Delanghe, B., Bailey, D.J., Gessulat, S., Ehrlich, H. C., Weininger, M., Yu, P., Schlegl, J., Kramer, K., Schmidt, T., Kusebauch, U., Deutsch, E. W., Aebersold, R., Moritz, R. L., Wenschuh, H., Moehring, T., Aiche, S., Huhmer, A., Reimer, U., Kuster, B. *Nat. Methods* **2017**, *14(3)*, 259-262.

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