

# Using high mass accuracy to perform high throughput identification and quantification of targeted proteins

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## Overview

**Purpose:** Using high mass accuracy to identify and quantify targeted proteins.

**Methods:** A panel of 59 synthetic peptides were spiked-into a digested plasma sample and then analyzed on a stand-alone Exactive mass spectrometer based on Orbitrap™ technology. Identification and quantification of all peptides was accomplished in a single 30 minute LCMS separation.

**Results:** We show an extremely simple workflow that can be used to identify and quantify peptides and proteins in one single LCMS run.

## Introduction

To date, high-mass accuracy mass spectrometry data have been used mainly for the discovery of new biomarkers. We propose an entirely different workflow where high-resolution parent and product *m/z* are used to perform targeted quantification peptide mass fingerprinting. Using information from the initial discovery experiment, we can characterize target proteins and peptides, their isotopic distribution patterns, LC elution times and charge states. This information can then be carried forward to a quantitative experiment, performed on the same platform, using full scan MS only. For multiple isobaric compounds having close elution times, workflows involving SILAC and AQUA can assist in differentiating those isobaric compounds. An additional benefit of this workflow is that the same platform can be used for both discovery and targeted quantification experiments. Our goal is to only quantify the targeted list of proteins and peptides, and not annotate every full scan peak, thus making the problem much more tractable.

## Methods

**Sample:** A panel of 59 synthetic peptides was spiked at a concentration of 50 fmol on column into a background of 0.5 µg plasma digest.

**LC-MS/MS:** The mixture was analyzed on a Thermo Scientific Exactive mass spectrometer. For the LC separation, a Thermo Scientific Hypersil Gold 50X1 column with a particle size of 3 µm was used. The LC gradient was: 0 min 98:2 water:ACN with 0.1 formic acid (FA) to 35% ACN with 0.1% FA in 30 min @ 100 µl/min, 10ul loop. The probe used was HESI-2 with the parameters, Spray voltage = 3500, Capillary temp = 275 °C, sheath gas = 50, Aux gas = 5, spare gas = 5, and heater temp = 365 °C. The instrument was programmed to monitor full scan MS (scan range:250 to 1500) at ultrahigh resolution (100,000), with AGC target values at 3e6. In addition, we also monitored full scan MS/MS using HCD at 30eV as the fragmentation mode, scan range: 250 to 1500, medium resolution (25,000), and AGC target values of 250,000. Figure 1 shows the total ion chromatogram of the RAW data acquired.

FIGURE 1. Chromatogram of the RAW data. RAW file has two scan filters, one for full scan MS, and the other for full scan MS/MS, with scan range for both set to 250-1500 *m/z*

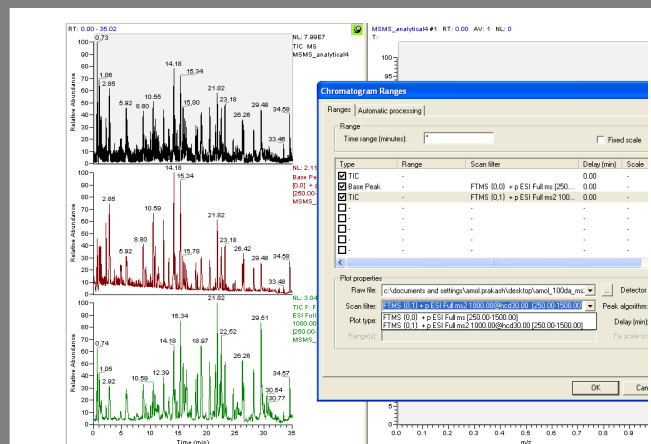
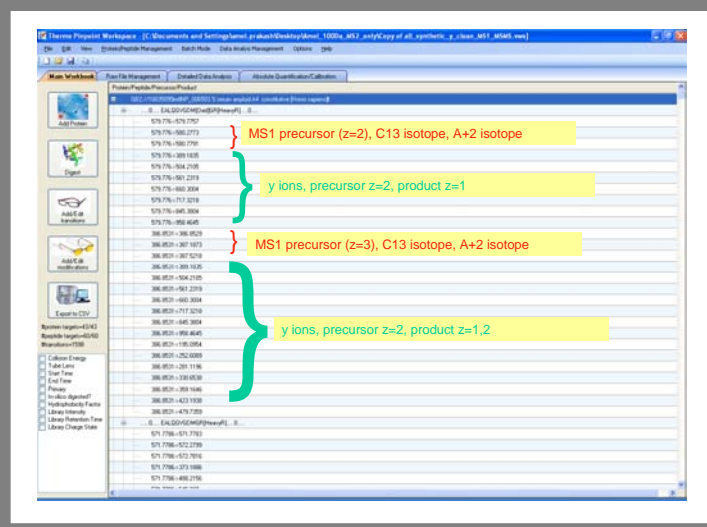


FIGURE 2. Individual transitions extracted from the RAW file. The red annotated transitions show the mass ranges extracted from full scan MS (precursor ions of various charge states, and their isotopes) and the green annotated transitions show the mass ranges extracted from the full scan MS/MS. Here we extracted the various product ions (y and b for various charge states).



## Methods – continued

**Data analysis:** The sample was acquired in triplicate. Thermo Scientific Pinpoint software was used to process all the RAW files, and identify and quantify the 59 peptides. The software allowed extraction of the chromatogram (with a 10ppm accuracy) around the MS1 precursor ion, its C13 isotope, and its A+2 isotope for precursor charge states +2 and +3 for each peptide. In addition, the chromatogram for each theoretical product ion (b and y) was extracted from the full-scan MS/MS scans. As there was no precursor-selection (no data dependent scans), the resulting extraction resulted in a complete chromatogram (from the start of the run to the end). An example of transitions extracted from a RAW file is shown in Figure 2. The transitions extracted from full scan MS are shown in red, and the transition extracted from full-scan MS/MS is shown in green.

After extracting the transitions, the retention times were identified based upon the co-elution of the various transitions. An example is shown in Figure 3 for the synthetic peptide EALQGVDMGR[Heavy], which is identified as eluting at 12.6 minutes. The green chromatogram is for the +2 precursor, purple is for the +2 precursor C13 isotope, and the remaining for the various product ions. Co-elution of precursors and products confirmed the identity of the peptide. The peak area (peptide quantification) was computed using the +2 precursor chromatogram, because it was the most sensitive. Triplicate runs for this peptide had a CV of 11% (shown in the middle graph).

Figure 4 shows the benefit of having the MS/MS scan interleaved with the MS1 scan. Using only precursor ion information from full scan MS (10ppm), there are multiple possibilities for the peptide LSITGYDLK[Heavy]. Even when using the C12 and C13 isotope ratios, we were not able to differentiate between those four options and in addition, two of them even have very similar retention times. The only way to confirm the peptide identity is to analyze the MS/MS product ions. Situations like these are extremely common in a complex matrix like plasma.

FIGURE 3. Synthetic peptide EALQGVDMGR[Heavy], is identified as eluting at 12.6 minutes based upon co-elution of its various fragments. The green chromatogram is for the +2 precursor, purple is for the +2 precursor C13 isotope, and the remaining for the various product ions. Co-elution confirms the identity of the peptide. The peak area is computed by using the +2 precursor chromatogram because it is the most sensitive. This provides peptide quantification with a CV of 11% for triplicate runs (shown in the middle graph).

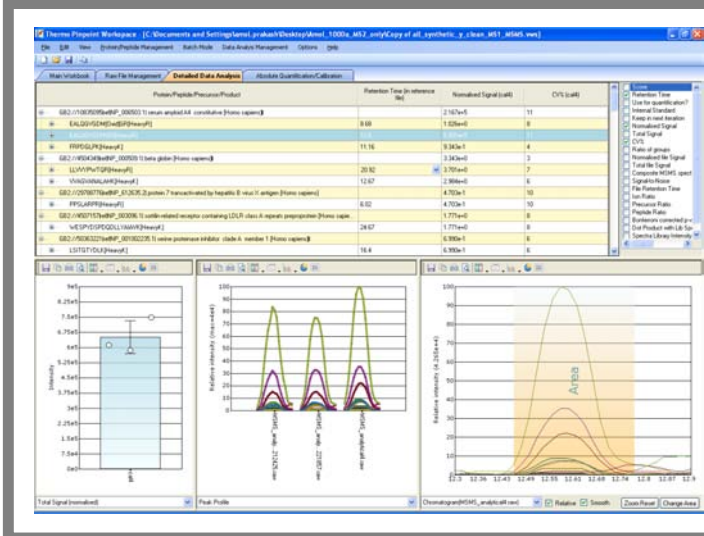
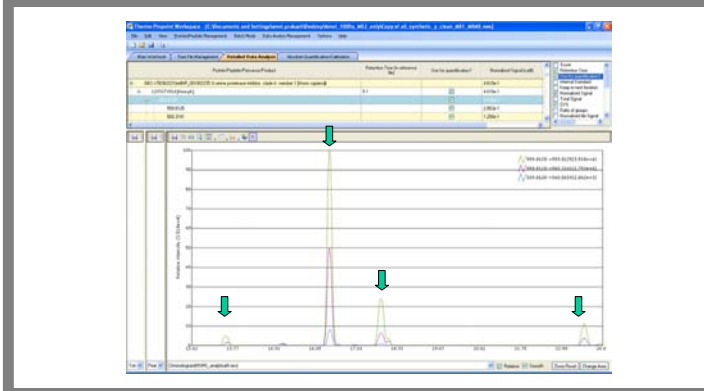


FIGURE 4. This plot demonstrates the benefit of interleaved MS/MS scan analysis. For example, consider the synthetic peptide LSITGYDLK[Heavy]. If accurate mass precursor, isotopes and isotope ratios are the only data considered for identification, then there are multiple possibilities for retention times. However, by adding MS/MS scan information, identification can be confirmed with co-eluting fragment ions.



## Results

Using the strategy outlined above, we were able to identify and quantify all 59 synthetic peptides in a background matrix of plasma with a CV of less than 20% (triplicate analysis). Most peptides had a CV of less than 10%. The list of peptides, their identified retention times and CVs are given in Table 1. Further confidence is lent by the linear correlation between the Krokhin's hydrophobicity factors (1) and the identified retention times (Figure 5).

FIGURE 5. Linear regression between Krokhin's hydrophobicity factor<sup>1</sup> and identified retention time

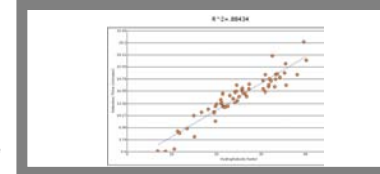


TABLE 1. 59 peptides identified and quantified in a single LCMS run in the background of serum. The identified retention time and the corresponding CVs are shown.

Annotation	RT	CV(%)	Annotation	RT	CV(%)
EALQGVDMGR[Heavy]	12.6	11	LNLSMER[Heavy]	1.29	21
FRPDPGLK[Heavy]	11.16	4	LMHNLGK[Heavy]	4.56	11
LLVYPWTQR[Heavy]	20.92	7	SVSEIQLMHLNGLK[Heavy]	17.1	6
VVAGVANALAHK[Heavy]	12.67	6	WDATATELNALNQLAR[Heavy]	23.76	11
PPSLARPR[Heavy]	6.02	10	AQAGLLEAEHQALIR[Heavy]	16.36	7
WESPYDSDPDQLLYAIAVK[Heavy]	24.67	8	LGGVAIVK[Heavy]	12.62	1
LSITGYDLK[Heavy]	16.45	6	NFQVIVQANAHGQK[Heavy]	14.67	6
FLVGPDPIM[Oxid][Heavy]	18.06	3	FSISWAR[Heavy]	18.08	11
ALLSTPVR[Heavy]	13.36	8	LQDTYGGWANR[Heavy]	13.57	3
FWDYLR[Heavy]	18.21	4	NYVNEALK[Heavy]	11.85	3
LAVYQAGAR[Heavy]	10.16	19	QLESPLNR[Heavy]	11.05	9
LAVQFTNR[Heavy]	13.23	17	SSALFYQK[Heavy]	12.46	1
VTIASLPR[Heavy]	14.38	5	FQTFEGDLK[Heavy]	15.21	3
IFFYDSENPASEVLR[Heavy]	20.84	6	QLAEELYR[Heavy]	15.21	6
ATLVNLYLPK[Heavy]	18.32	5	QLSLPETGDSATLK[Heavy]	19.19	13
VGESLYIGR[Heavy]	16.32	9	OSTLVLPFGDLR[Heavy]	21.3	12
GAYPLSIEPIGVR[Heavy]	19.73	5	QWVYTGASVGLGPR[Heavy]	19.66	9
TYPFPHDLSHGSAQVK[Heavy]	17.69	9	SLGALLLQK[Heavy]	22.29	4
DGYLFQLLR[Heavy]	29.54	1	IAPQLSTEELVSLGK[Heavy]	20.17	11
ATVYQGER[Heavy]	6.74	5	GFGGLTQIYAALSTAK[Heavy]	25.84	15
TGISPLALK[Heavy]	20.84	7	DAVEDESIVGK[Heavy]	15.89	6
LLDNWDSVTSTFSK[Heavy]	21.04	9	ENAGEDPGLAR[Heavy]	5.51	14
GWVTDGFSSTLK[Heavy]	19.35	6	GHFSSIPVK[Heavy]	17.12	7
LKFGNTLEDK[Heavy]	12.46	8	YVYRIVK	0.67	14
NILTSNNDIVK[Heavy]	15.46	1	CLNRLS[Phosphoryl][SGVSEIR]	16.3	4
SKEQLTPLIK[Heavy]	13.6	0	TTSQVRPR	0.73	5
LGPAGDVEGHSFLFK[Heavy]	17.63	7	RPPGFSFPR	14.11	5
ESLSSYWESAK[Heavy]	16.94	6	YGGFMR	10.89	8
			DRVYIHPF	15.84	2

## Conclusions

- Identification and quantification of 59 target synthetic peptides in a background of plasma digest was achieved in a single LC-MS/MS run by using co-elution of isotopes, charge states, and product ions based upon full-scan MS and MS/MS data.

## References

- V. Krokhin, R. Craig, V. Spicer, W. Ens, K. G. Standing, R. C. Beavis, J. A. Wilkins. An improved model for prediction of retention times of tryptic peptides in ion-pair reverse-phase HPLC: its application to protein peptide mapping by off-line HPLC-MALDI MS. *Molecular and Cellular Proteomics* 2004 3:908-19