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Application Note # LCMS-89

High quantification efficiency in plasma targeted proteomics with a full-capability discovery Q-TOF platform

Abstract

Targeted proteomics for biomarker verification/validation is a challenge for MS instrumentation as it requires maintaining the sensitivity and dynamic range over a large number of samples to precisely quantify specific subsets of peptides from complex proteomes. These verification/validation tasks have traditionally been performed on triple quadrupole instruments, using multiple (or selected) reaction monitoring (MRM or SRM) with stable isotope-labeled standards (SIS). In this study, we investigate the efficiency of an ultra-high resolution benchtop Q-TOF platform, typically used for discovery approaches, for targeted quantitation of peptides in a plasma tryptic digest. Two different quantification methods were investigated enabling quantification at either the precursor (HR-XIC) or fragment (DIA) ion level.

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	impact HD

Introduction

Shotgun mass spectrometry is a popular tool for candidate protein biomarker discovery. This global profiling method is used to gain qualitative and quantitative information of several thousands of peptides in complex proteomic digests. Subsequent validation/verification of detected biomarkers requires accurate measurements of protein target abundances in biological samples. Recent advances in the speed, robustness, and quality of the chromatographic separation and mass spectrometric detection have enabled the verification of discovered protein biomarker candidates in complex biological matrices, such as blood plasma. These tasks are typically performed through a targeted quantitative proteomic approach involving MRM with SIS peptides or proteins. This approach requires *a priori* knowledge of the target precursor/product ion pairs (referred to as transitions) and time-consuming method preparation, but prevents post-analysis data mining. Furthermore, when working with highly complex biological samples, such as human plasma, a wide dynamic range needs to be covered with high sensitivity. High resolution
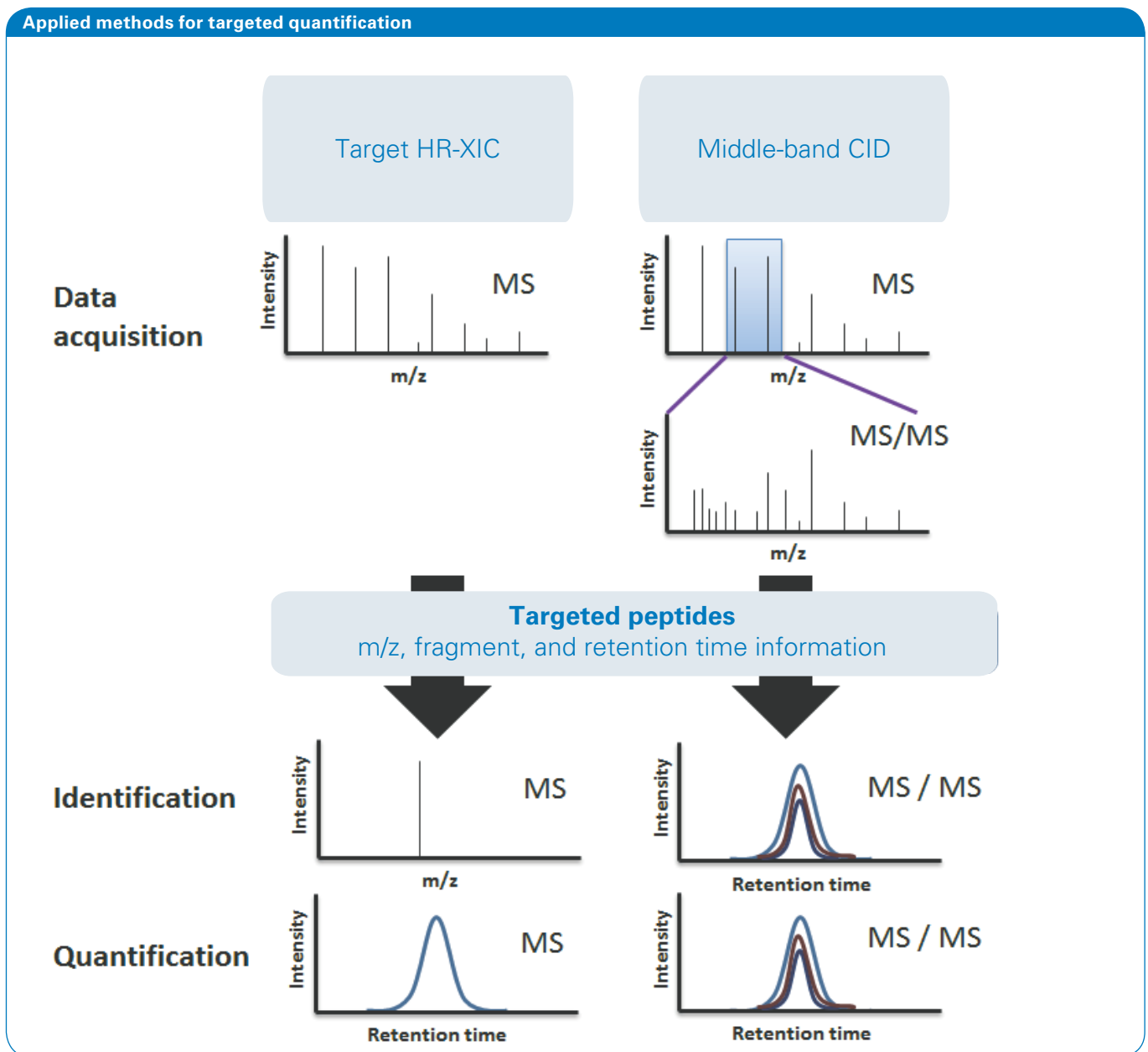
Applied methods for targeted quantification

Figure 1: Methods used for targeted plasma proteomics on the impact UHR-Q-TOF. For targeted high resolution extracted ion chromatogram (HR-XIC), data acquisition is accomplished in the full-scan MS mode without any fragmentation events. Identification and quantification is based on the MS1 signals from the *m/z* value and retention time. In the DIA approach, broad isolation of sequential windows (26 and 52 Da) is used for data acquisition. Qualification and quantification is based on the fragment signals.

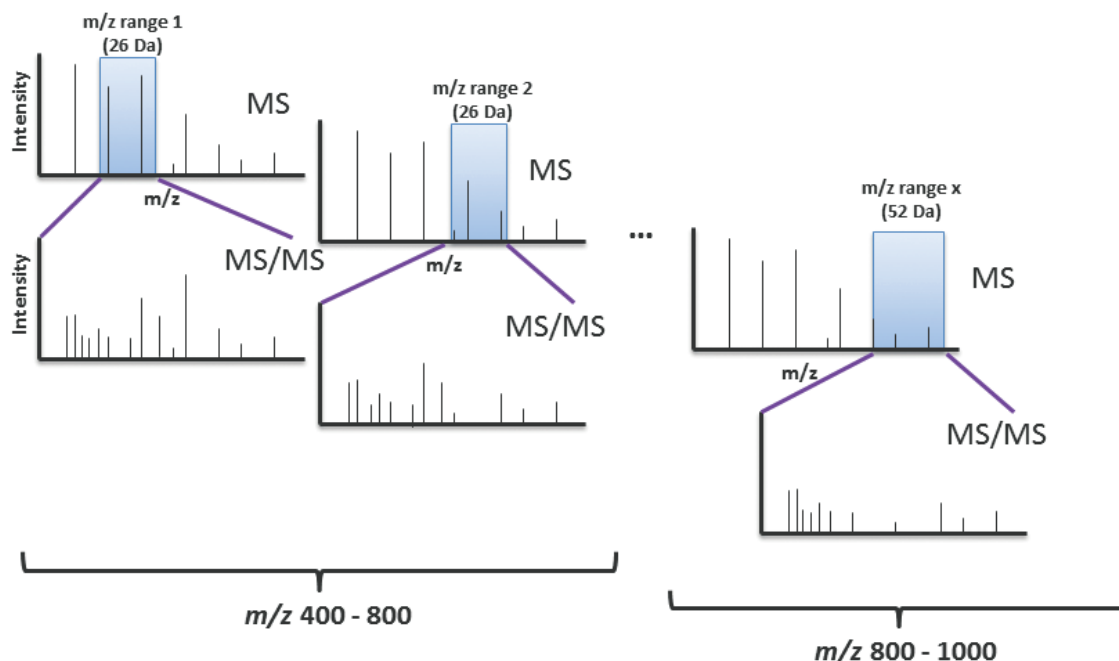**Schematic representation of the DIA method**

Figure 2: Illustration of the DIA method. Data acquisition was performed from m/z 400 to 1000, with smaller overlapping isolation windows of 26 Da from m/z 400 to 800 and larger 52 Da windows from m/z 800 – 1000.

systems, such as Q-TOFs, are now able to address the sensitivity and dynamic range requirements for targeted proteomic measurements, while providing comparable selectivity. Moreover, the high-resolution based approaches supported by the Q-TOF architecture enable all detectable ions to be recorded, therefore enabling retrospective data mining. We report here the evaluation of an ultra-high resolution benchtop Q-TOF system for the targeted quantitation of peptides in a plasma tryptic digest.

Experimental

A reference kit (#1 – the instrument performance kit for daily QC) for targeted proteomics containing freeze-dried plasma tryptic digests that were spiked with a 43 SIS peptide mixture were provided by Dr Andrew Percy (UVic–Genome BC Proteomics Centre). The lyophilized samples (7 in total, standards A to G) were re-suspended in 0.1% trifluoroacetic acid and anlysed in quadruplicate by LC-MS. The samples contained a constant concentration of endogenous (natural or NAT) peptides and a variable concentration of SIS (spanning a 10,000-fold range). Standard E reflects the 1:1 level where the concentration of SIS approximates that of NAT. In the LC-MS analysis, peptides were first trapped on a Pepmap pre-column (2 cm x 100 µm; Thermo Scientific) and separated on a PepMap UHPLC column (25 cm x 75 µm, 2 µm particles; Thermo Scientific) with a 60 min multi-step ACN gradient (mobile phase A: 0.1% FA; mobile phase B: 100% ACN in 0.1% FA). A CaptiveSpray nanoBooster source, with acetonitrile as a dopant, was used to interface the LC system to the impact HD benchtop UHR-Q-TOF system (Bruker Daltonics). The standard equipment of the instrument includes a new 10 bit (50 Gbits/sec) digitizer that makes it possible to increase the dynamic range by a factor of 3-4 in comparison with the previous generation of instruments. The instrument was operated in the high resolution extracted ion chromatogram (HR-XIC) or Data Independent Analysis (DIA) modes (see Figure 1). In the HR-XIC approach, full scan MS spectra were acquired at a spectra rate of 0.5 Hz. DIA measurements were performed by setting in total 20 windows with 26 Da width from m/z 400-800, and 52 Da width from m/z 800-1000 in a slightly overlapping fashion (see Figure 2). Acquisition speed was at 8 Hz resulting in a 2.5 s cycle time. MS precursors and MS/MS products were acquired over a 400-1000 m/z range. A MS spectrum was acquired before each MRM cycle for data qualification. All results were processed (in terms of peak detection and integration) in Skyline software (v. 2.1; University of Washington). An AutoMS/MS data set of the matrix-free, balanced SIS mix was used as the library in Skyline. Quantitation was performed on the MS level for HR-XIC and on the MS/MS level for DIA using exact m/z and retention time information. For the DIA approach three typical high intense fragment ions were used as quantifier
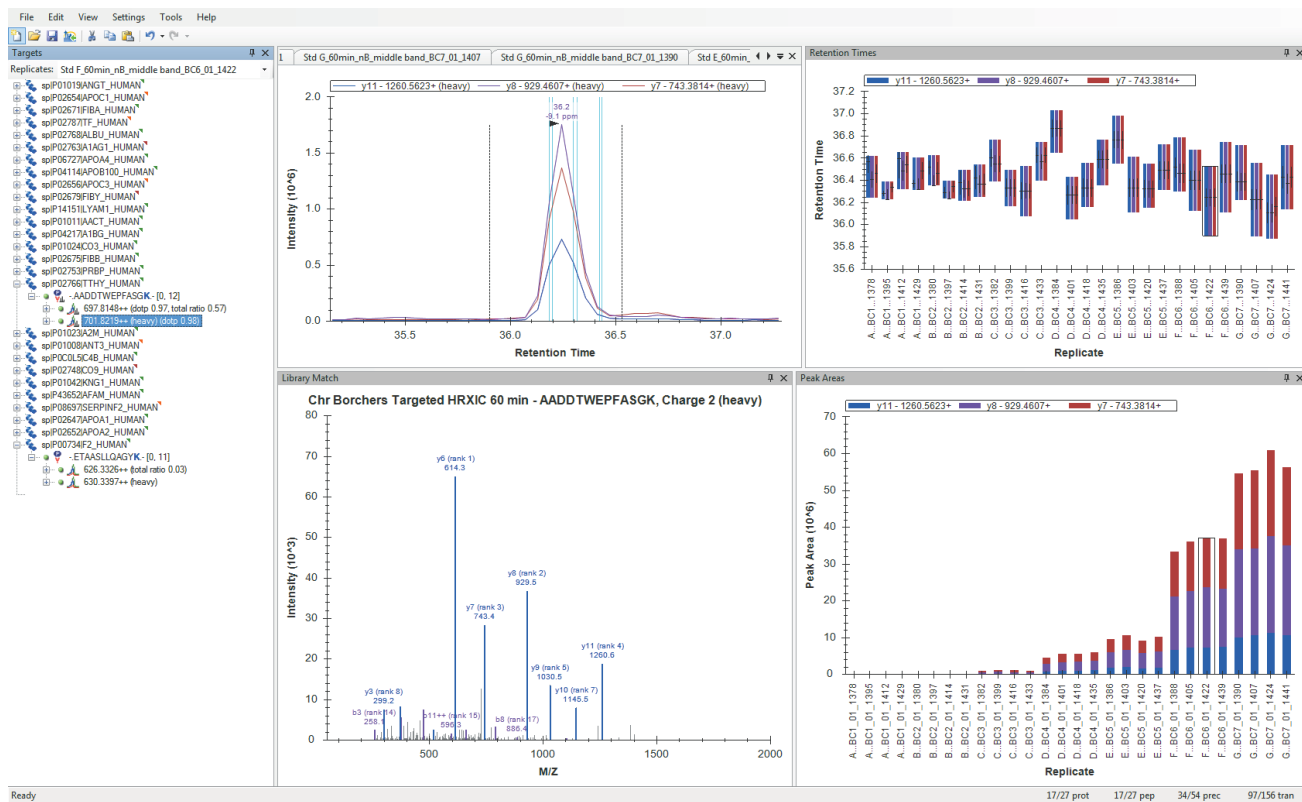
Figure 3: Quantification of y11, y8, and y7 fragments of transthyretin (P02766) using Skyline is shown as an example of the DIA approach. Quantitative and qualitative information is obtained out of the data of the three specific fragments.

and qualifier ions (see Figure 3). Results were exported and calibration curves for SIS peptides were fitted to a $1/x^2$ weighted linear regression model using R. Data points used for calibration were filtered for precision ($\leq$ 20% average CV between concentration level replicates) and accuracy (within 80 to 120% of theoretical concentration, on average for the replicates of each level). In order to generate a curve, a minimum of 3 consecutive concentration levels must have all replicates being both precise and acurate. Concentrations (in ng/mL) were determined by averaging the product of each qualified level's relative response and its corresponding reported SIS concentration then factoring in the protein molecular weight a conversion factor.

Results and Discussion

We investigated the efficiency of a discovery ultra-high resolution bench top Q-TOF platform for targeted proteomics using plasma samples spiked with a 43 SIS peptide mixture. Two different approaches were evaluated in this study – HR-XIC and DIA (see Figure 1). On the left side HR-XIC approach is displayed for which MS full-scan data are acquired. Thus this approach does not require specific parameter optimizations. The DIA approach

(middle-band CID) is displayed on the right side in Figure 1. For this approach sequential broad isolation windows (26 to 52 Da) are used for MS/MS fragmentation. Identification and quantification are done based on MS/MS signals. The collision energies of the peptides of interest were first optimized for LC-MS/MS by infusing the SIS peptides in the absence of matrix. Isolation windows were adapted according to the number of detectable peptides. As the average number of peptides decreased from *m/z* 800, isolation windows were increased from 26 to 52 Da. The instrument platform was composed of a nanoLC system coupled to an impact HD UHR-Q-TOF system due to the high reproducibility (in terms of retention times and peak responses) it was previously found to confer. For instance, the peak variation in a HeLa cell lysate analysis was found to be below 10% CV across 55 analytical replicates when analysing 10 peptides eluting over a 90 min gradient.

Optimal gradient length was determined by injecting the balanced SIS mix in the absence (*i.e.*, SIS in buffer) and presence (*i.e.*, SIS in plasma using standard E) of matrix. We optimized the nanoLC gradient for rapidity, while maintaining a comparable quantitative output to longer
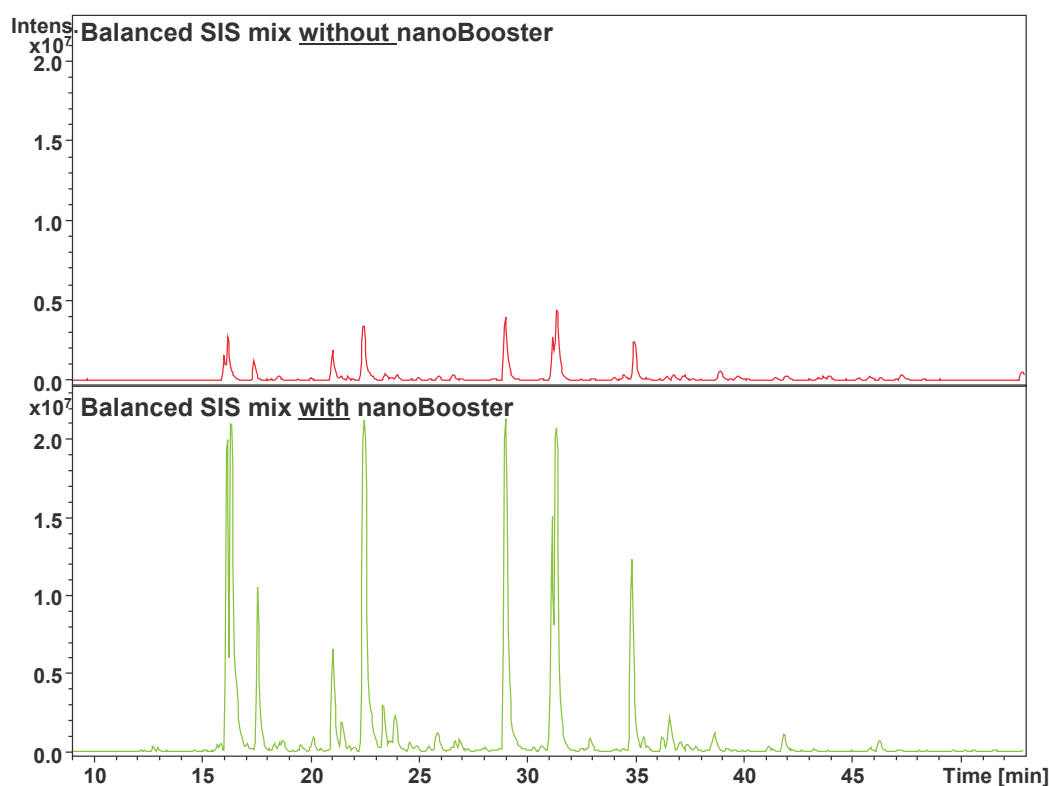
Figure 4: Investigation of the effect of the nanoBooster on the SIS peptides. Upper panel shows a base peak chromatogram of the balanced SIS mix without nanoBooster using standard lab air setup, while the lower panel highlights the utility of the nanoBooster with acetonitrile as the dopant.

gradients. An extended gradient of 90 min was not used since it did not enhance the quantification efficiency of the SIS peptides.

The CaptiveSpray source was coupled to the nanoBooster, which enabled organic solvent to be added to the gas inlet system for enhanced peptide ionization (more information can be found in [2]). Acetonitrile was used as the dopant, which provided an up to 5-fold improvement in the target peptides signal compared to air (Figure 4).

The system setup enabled reproducible quantification at nano-flow rates with an average CV of 4.5% for the peak areas of the 43 target peptides calculated for NAT and SIS peptides over three technical replicates. This amounts to retention time variability of approximately 15 s over a 60 min gradient. The maximum linear range of the assay was 10^4 for both approaches, which coincides with the maximum range covered by the kit. Nevertheless, quantification on MS signals using HR-XIC revealed a higher dynamic range, on average, compared to quantification on MS/MS signals using DIA. This finding is related to the lower overall sensitivity of the DIA method, which thereby reduces the obtainable range of the analyzed kit. For both approaches, a broad

quantification range was obtained, with the endogenous concentrations ranging from 230 to 180,000 ng/ml for targeted HR-XIC and from 360 to 210,000 ng/ml for DIA. While HR-XIC reveals higher dynamic range, on average, for quantification, the DIA approach is advantageous if interference occurs on the MS-level. This is the case with highly complex samples (especially if separated over short gradients), for which quantification on MS1 level can be disturbed by the occurrence of co-eluting precursors with similar or isobaric m/z values. In these cases, quantification at the fragment level delivers a higher specificity that compensates for the small loss in absolute sensitivity. In the presented study, interference in the MS-level analysis of alpha-2-antiplasmin was overcome with the DIA approach (see Figure 5).

In total, 35 interference-free peptides were quantified with the targeted HR-XIC approach and 30 with the DIA approach. To assess quantitative accuracy, the results of the quantifiable peptides were compared to the known values reported in the kit [1]. Incidentally, these were obtained from the MRM with SIS peptide approach. The comparison revealed the UHR-Q-TOF system (operated in the HR-XIC or DIA mode) to be capable of returning

DIA method results in higher specificity for quantification
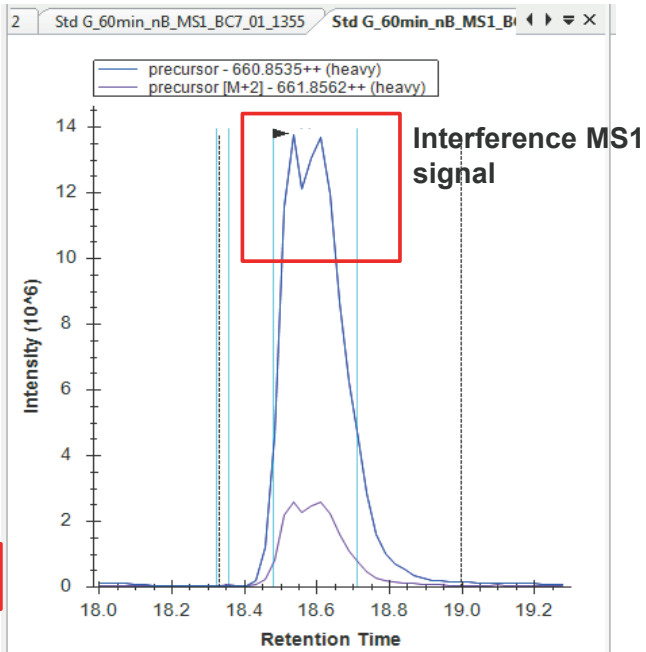
DIA
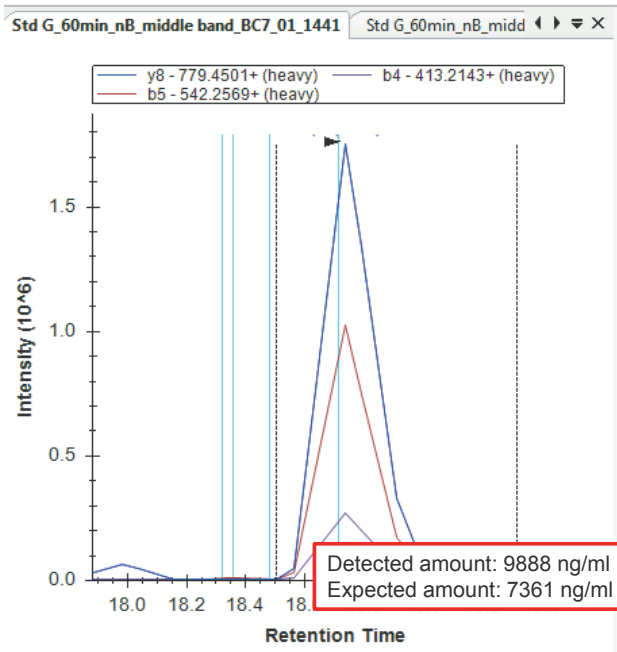
Targeted HR-XIC

Figure 5: Comparison of the extracted ion chromatograms of peptide LGNQEPGGQTALK from alpha-2-antiplasmin (P08697) using fragment ion information for the DIA approach (y8, b4, and b5), and precursor ion information (m/z 660.8535 and m/z 661.8562) for the targeted HR-XIC approach.

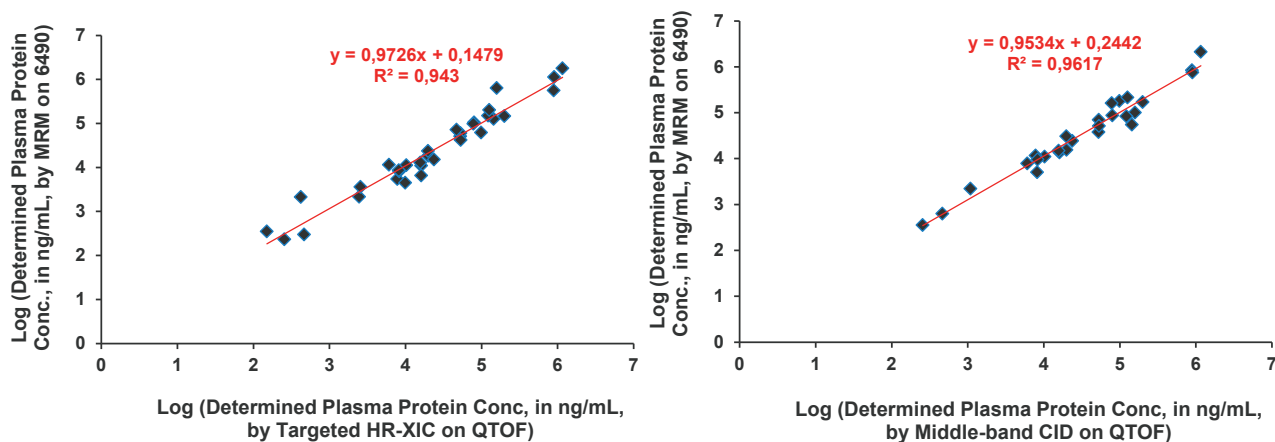## Comparison of the determined plasma concentration with reference

Figure 5: Comparison of the determined plasma protein concentrations from targeted HR-XIC (left panel) and DIA (right panel) with the sample kit reference values obtained with a MRM-based acquisition method.

equivalent endogenous protein concentrations (Figure 6), which supports the validity of both approaches. We note that higher correlation was observed for the DIA approach (0.96 vs. 0.94) due to enhanced selectivity when quantifying fragment ions.

For some of the proteins no quantitative information could be obtained. Albumin belongs to those proteins, since its endogenous concentration lied above the ULOQ (upper limit of quantitation). In addition, vitamin D-binding protein, alpha-1-acid glycoprotein 1, and serum amyloid P-component could not be quantified in both approaches due to low or absent NAT signals. Interestingly, these proteins have been rejected from quantification in previous studies due to peptide instability and poor proteolytic digestion efficiency [1]. For the targeted HR-XIC approach, alpha-2-macroglobulin was rejected from quantification because it possessed too few calibration points based on the strict qualification criteria. Further, the peptide is reportedly unstable in solution.
Additional peptides (including antithrombin-III and vitronectin) could not be quantified with the DIA approach due to an insufficient number of calibration points or fragments usable for quantification.

Conclusion

The evaluated DIA and HR-XIC modes represent an effective way to precisely quantify peptides in plasma on a typical full-discovery Q-TOF platform. Quantification of labelled spiked-in peptides covered a 4 order-of-magnitude range, which was the maximum dynamic range of the samples analyzed. The quantified concentrations of natural plasma peptides were highly comparable with each other

and with previous MRM results obtained on the same sample, which demonstrates the excellent capability of the Q-TOF instrument for targeted proteomics. The two approaches make use of the capabilities which are specific to the Q-TOF architecture. Specifically, the HR-XIC method offers a very simple way to proceed to targeted proteomics experiments, with virtually no method development and a full compatibility with very sharp UHPLC peaks. The only limitation of this approach is the potential of interferences from isobaric co-eluting ions in very complex sample digests. The DIA approach overcomes this difficulty with a much higher specificity obtained by performing the quantitation at the MS/MS level after having isolated and fragmented a subset of the *m/z* range. That increased specificity comes at the price of a reduced absolute sensitivity and of a slower method, suppressing its compatibility with very fast LC separation. Nevertheless, when compared to the classical MRM and SRM approach, both methods require less effort in instrument optimization and enable post-analysis data mining. In the end, the two methods offer an ideal complementarity that makes it possible to use the Q-TOF capabilities to their fullest.

References

[1] Percy et al., Method and platform standardization in
     MRM-based quantitative plasma proteomics, Journal
     of Proteomics, Volume 95, 2013, pp 66 -76

[2] Kaspar et al., Increasing peptide identification rates for
    proteomics samples by controlling peptide charge states
    using CaptiveSpray nanoBooster, #TN44

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