

Targeted Protein Quantification in a Complex Mixture by Accurate Mass Traces

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Introduction

Proteins found in the discovery phase are called biomarker or drug target candidates. Those need to be validated in a 2nd stage of the discovery process. In contrast to the discovery which is a global approach, the validation of candidates allows a focused (or targeted) approach. Only the candidate proteins are considered in this validation process and any additional information produced at the same time is ignored. Most important for this validation step are quantitation accuracy, analysis speed (due biological and technical replicates) and analysis cost (as many samples are typically quantified for candidate validation). The MRM (multiple reaction monitoring) method in triple quads is a most cost-effective and fast approach for targeted quantitation. The mass chromatogram of the specific parent-to-fragment transitions is used for quantitation.

Here, a label-free approach with an ESI-TOF was used instead. Due to the mass accuracy of the ESI-TOF (independent of the peak intensity), high resolution extracted ion chromatograms (hrEIC) with a mass error of ca. 2 mDa can be generated (fig. 1). This allows for an outstanding selectivity of compounds vs. background and can be used for quantitation. Advantages of this novel concept are the unlimited number of target ions as well as the possible revision of data to quantify additional peptides, if needed at a later stage after the run (table 1).

In a feasibility study, the relative quantitation of 4 known proteins in two different samples against the complex protein background of A549 cells (adeno-carcinoma from lung tissue) was performed.

Methods

- Sample:**
 Described here are results we obtained in a study for the 2007 "Martinsried Workshop: Micro Methods in Protein Chemistry":
- 2 µg whole lysate of A549 cells (lung tissue adenocarcinoma) as protein background.
 - Four proteins spiked in sample A and B in different amounts: human Protein kinase A, mouse Prot. kinase A, Lipase, Myoglobin.
 - Identify these target proteins and determine their A/B ratio.
 - Report in HUPO-PSI standard format

- Experimental:**
- Dionex Ultimate 3000 nanoHPLC, PepMap 100 150mm*75µm column, 5mm*300µm pre-column; gradient: 5min preconcentration 30 µl/min 0.1% TFA; 120min. elution 300 nl/min 5-45% ACN/0.4 % ac. acid
 - MS Quantification: micrOTOF (Bruker Daltonics), 1/4 sample per run, 2 replicates each; Compass 1.2 data SW
 - MS/MS identification and validation: HCTultra (Bruker Daltonics)
 - WARP-LC for LC/MS visualization and sample comparison. BioTools for calculation of in-silico digests of the targets and generation of inclusion lists for the hrEICs as well as the MS/MS runs.

	hrEIC	MRM
Analysis selectivity	By MS accuracy	By MS/MS
# of target ions	Unlimited @ 2 mDa acc.	Several 100 targeted MS/MS
Retrospective revision of new info	yes	no
sensitivity	low fmol/µl	low fmol/µl

Table 1: Analytical comparison of the new hrEIC target analysis vs. the established triple quad MRM technology.

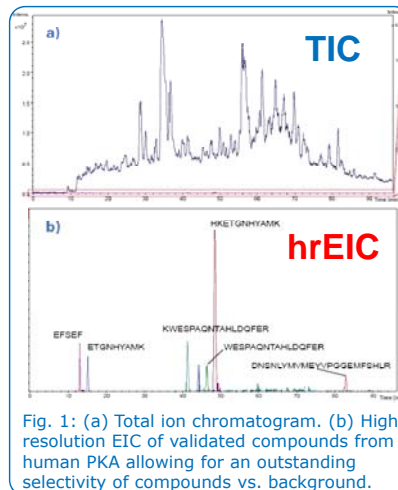


Fig. 1: (a) Total ion chromatogram. (b) High resolution EIC of validated compounds from human PKA allowing for an outstanding selectivity of compounds vs. background.

Results

The assay development is shown in fig. 2. Notably, the two PKA protein isoforms from human and mouse differ by four peptides only. All other peptides are identical in both isoforms and cannot be used for quantitation. The four relevant peptides were all successfully detected in the assay. Fig. 3 shows the contour plot of the entire LC/MS analysis with its high sample complexity. In total, 60,000 multiply charged species were detected, corresponding to 12,000 peptides. The result of the assay development is a list of known peptides that occur in the LC/MS analysis with known molecular weights and retention times that permit the proper definition of the analytical hrEICs. Finally, replicates were ran for each sample. The compound match criteria across the various runs were a mass tolerance of 2 mDa and a retention time tolerance of 5 sec. For the final hrEICs, all charge states and isotopes

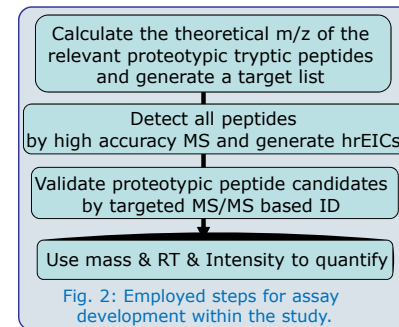


Fig. 2: Employed steps for assay development within the study.

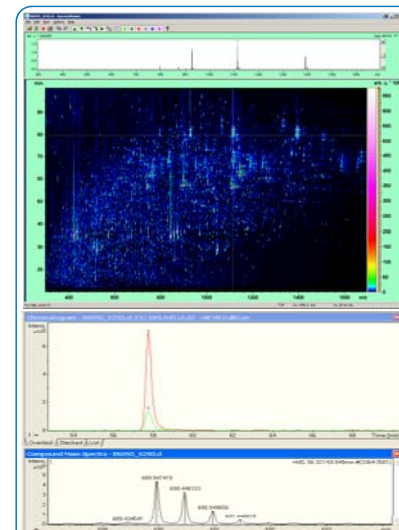


Fig. 3: Top: Contour plot of one LC-MS analysis showing the high complexity. In total, 12,000 peptides were detected. Bottom: a peptide hrEIC for relative quantitation. Notice the absence of any interfering peaks in the flat hrEIC baseline.

of the targeted peptides were taken into account (as shown in Fig. 3, bottom). Fig. 4 shows the summary of the quantitation in relation to the real values. With the exception of lipase, which was close to the limit of detection, all proteins were quantified within an average quantitation error of 4.8 %. Including the data for Lipase, the average quantitation error was still approx. 30%.

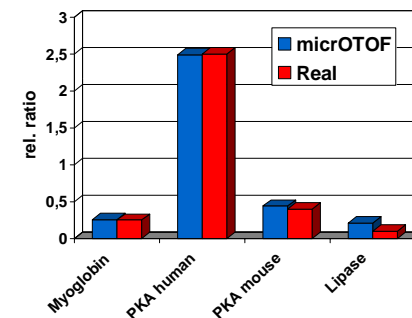


Fig. 4: A/B ratios of the 4 spiked proteins. Shown are the quantitation results obtained with the hrEIC approach in the micrOTOF in comparison to the real values.

Conclusions

A highly accurate quantitation assay based on hrEICs using an ESI-TOF is shown. High specificity hrEICs with 2 mDa mass errors were used to select the significant peptides for quantitation from the high matrix background. Isoform differentiation was achieved by selecting isoform-specific proteotypic peptides. The obtained full scan MS spectra can be used for extended analysis to any other existing compound in the crude mixture at a later stage of a discovery and validation study.

ESI-TOF