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Label-free protein quantification on tandem mass spectra in an ion trapping device

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ABSTRACT

Label free quantification using liquid chromatography and electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) is widely used in quantitative proteomics. However, data-dependent bottom-up proteomics suffers from low reproducibility due to semi-random selection of precursor ions for tandem mass spectrometry. In addition, this acquisition mode is biased towards abundant peptides. To overcome these problems, alternative precursor-ion selection methods were developed, such as data-independent acquisition and *pseudo*-multiple selected reaction monitoring (p-mSRM). With these methods, several tandem mass spectra are acquired over the chromatographic elution time of precursor ions. In this report, we investigated if the acquired tandem mass spectra can be used for label-free quantification. For this, extracted fragment ion currents were correlated to relative protein concentration. A linear relationship between ion current and proteins concentration was observed over five orders of magnitude. Thus, we conclude that relative label-free peptide and protein quantification can be performed in an ion trap using the data-independent acquisition mode.

Keywords: *pseudo*-multiple selected reaction monitoring; quantitative analysis, ion trap, peptides.

Abbreviations

B-LG: β-Lactoglobulin; **CID:** Collision induced dissociation; **DDA:** Data-dependent acquisition; **DIA:** Data-independent acquisition; **LC-ESI-MS/MS:** Liquid chromatography electrospray tandem mass spectrometry; **mSRM:** Multiple selected reaction monitoring; **PAcIFIC:** Precursor Acquisition Independent From Ion Count; **p-mSRM:** *Pseudo*-multiple selected reaction monitoring; **SD:** Standard deviation; **SRM:** Selected reaction monitoring; **Trp II:** Trypsin inhibitor type II .

1. Introduction

Over the past years, direct interfacing of liquid chromatographs with tandem mass spectrometers (LC-MS/MS) has become a very popular tool for qualitative and quantitative analysis of complex peptide mixtures, such as enzymatic digestion product of complex protein samples. In particular, data-dependent acquisition (DDA), or on-the-fly precursor ion selection for isolation and subsequent activation and tandem mass analysis is widely used [1]. MS survey scans are acquired over the full mass range of peptide precursor ions and over the entire chromatographic elution time. The peptide precursor ion signals can then be used for label-free relative quantification [2,3]. With this quantification method, ion currents from identical peptides from different samples are directly compared to each other.

Although dynamic exclusion [4] prevents redundant acquisition of the most abundant peptides, DDA is still biased towards abundant species. In addition, the selection contains a random component limiting reproducibility of identified peptides [5,6]. Thus, a major effort is focused on developing alternative precursor-ion selection methods. With the so-called "Precursor Acquisition Independent from Ion Count" (PAcIFIC) or "Data-Independent

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Acquisition" (DIA) mehods, continuous, non overlapping mass-to-charge ratio *(m/z)* windows are selected for isolation and subsequent activation [6,7]. Alternatively, no isolation is performed and all precursor ions are co-fragmented. The precursor-fragment ion lineage is then reconstituted post-acquisition using the chromatographic elution profile of all species [8,9]. A major advantage of these data-independent methods is that several tandem mass spectra are acquired over the entire chromatographic elution profile of all ions. Thus, fragment ion currents can be extracted for each precursor ion and used for quantification. Similarly, precursor-fragment ion transitions can be reconstructed post-acquisition, a term referred to as "*pseudo* -multiple selected reaction monitoring" or p-mSRM [10,11]. This mode of operation is described in Figure 1.

The term *"pseudo"* refers to the computer calculated post-acquisition reconstruction of the transition. Indeed, the full fragment ion spectrum is recorded here, by opposition to classical mSRM acquired in triple quadrupole instruments. *Pseudo*-mSRM has been showed to provide excellent quantitative measurement over a large dynamic range for small molecules [12] and peptides [13,14] in simple matrices and for non-complex mixtures. Here, we show that label-free MS2-based quantification is possible over at least five orders of magnitudes in very complex matrices, *e.g.* digested human plasma.

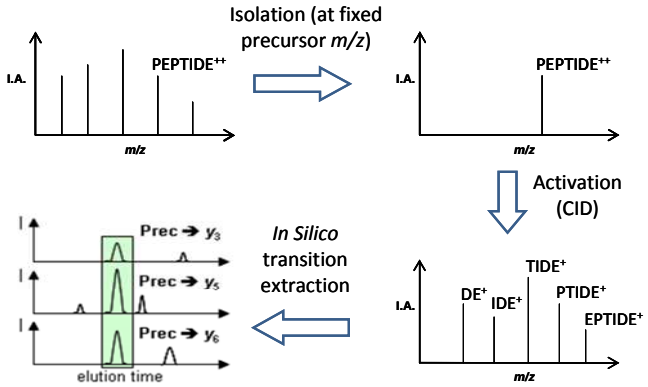

Figure 1. Principle of Selected Reaction Monitoring (SRM) and *pseudo-multiple* Selected Reaction Monitoring (p-mSRM). From a complex mixture of peptides, precursor ions are isolated and activated, typically by CID. All product ions are collected in the ion trap and scaned out according to their *m/z* ratio. This operation is repeated during the entire chromatographic elution of the peptides. Once all the data is collected, the ion currents corresponding to all desired transitions are reconstructed *in silico*.

2. Materials and methods

Liquid plasma, β-Lactoglobulin (B-LG), trypsin inhibitor type II (Trp II), iodoacetamide (IAA) and acetonitril (AcN) were purchased from Sigma (St.louis, MO, USA). Urea, ammonium bicarbonate (AB), dithioerythritol (DTE) and water for chromatography and dilution were from Merck (Darmstadt, Germany). Porcine trypsin and formic acid (FA) was respectively from Promega (Madison, WI, USA) and Biosolve (Valkenswaard, the Netherlands). Stationary phases for columns were from Michrom (Auburn, CA). Analytical column (OD = 375 µm, ID = 75 µm, L=150 mm) and pre-column (OD = 375 µm, ID = 100 µm, L=20 mm) was made from fused silica tubing from BGB Analytik AG (Boeckten, Switzerland)

Human plasma and standard proteins were digested as previously reported [15]. In short, 500µg of B-LG and Trp II were dissolved in 200µl of 6M Urea and 50mM AB. 10µl of DTE 38mM was added and the solution was incubated at 37° C for 60 min (reduction). Then 20µl of IAA 108mM was added for alkylation during 60 min in the dark. Liquid digestion was performed overnight, by adding 25µl of trypsin (0.2µg/µl). The digested solution was desalted with a C18 micro-spin column (Harvard Apparatus, Holliston, MA, USA) and dried. In order to have aliquots of 10pmol/µl, dried solutions were dissolved in AcN/FA/H_2O 5/0.1/94.9%. These digests were spiked at various concentrations in trypsin digested, non-depleted human plasma. The concentrations were adjusted so that a total amount of 1, 10, 100 atomoles, 1, 10, 100 femtomoles and 1 picomole standard proteins were injected in our LC-MS/MS system. The injected plasma amount was kept constant of 0.126 µg per injection (corresponding approximately to one picomole of albumin).

The LC-MS/MS system consisted of a NanoAcquity chromatograph (Waters, Milford, MA) interfaced with an LTQ-Orbitrap velos mass spectrometer (Thermo Scientific, San Jose, CA). Peptides were trapped on a home-made, 20 mm long precolumn of 100 µm inner diameter and separated on a 150 mm analytical column of 75 µm inner diameter. The analytical separation was run for 65 min using a gradient of H_2O/FA 99.9%/0.1% (solvent A) and CH_3CN/FA 99.9%/0.1% (solvent B). The gradient was run as follows: 0–1 min 95% A and 5% B, then to 65% A and 35% B at 55 min, and 20% A and 80% B at 65 min at a flow rate of 220 nL/min.

All samples were analyzed from the most diluted to the most concentrated, and new chromatographic columns were used for each technical replicate. The mass spectrometer was operated in the following conditions: for DDA, full MS spectra were acquired in the Orbitrap detector from *m/z* = 400 – 2000. The target ion population was 500,000 ions. Tandem mass spectra were acquired in a data-dependent manner in the linear ion trap on the five most abundant precursors (if present). Dynamic exclusion was set to one minute. Precursor isolation window was set to 2.0 *m/z* units. Normalized collision energy was set to 35%. For *p*-mSRM acquisitions, full MS spectra were acquired in the Orbitrap detector from *m/z* = 400 – 2000. The target ion population was 500,000 ions. Tandem mass spectra were acquired on the three β-lactoglobulin peptide precursor ions and on the three trypsin inhibitor peptide precursor ions listed in table 1, in a data independent manner. In other words, the precursor-ion *m/z* was isolated and fragmented over the full chromatographic analysis, no matter if precursor ions were present or not. The scan sequence was Full MS1 (orbitrap acquisition), CID of

m/z=858.406, CID of *m/z*=545.929, CID of *m/z*=623.294, CID of *m/z*=588.316, CID of *m/z*=600.858, CID of *m/z*=928.475. All CID spectra were acquired in the linear ion trap. The target ion population was set to 10,000 ions. The precursor isolation window was set to 2.0 *m/z* units. Normalized collision energy was set to 35%. All analysis were run in technical replicates (n=2). The first three DDA analyses, spiked with respectively 10 and 100 amol of standard proteins were used for database search.

Protein identification peak lists were generated from raw data using the embedded software from the instrument vendor (extract_MSN.exe). The monoisotopic masses of the selected precursor ions were corrected using an in-house written Perl script [16]. The corrected mgf files were searched against the SwissProt/Uniprot database (release 15.10 of 03-Nov-2009) using Phenyx (GeneBio, Geneva, Switzerland). Homo sapiens taxonomy was specified for database searching (34785 sequences) and the two standard protein sequences were added. The parent ion tolerance was set to 10 ppm. Variable amino acid modification was oxidized methionine. Fixed amino acid modification was carbamidomethylation of cysteins. Trypsin was selected as the enzyme, with one potential missed cleavage, and the normal cleavage mode was used. Only one search round was used with selection of "turbo" scoring. The peptide p value was 1 E-2 for LTQ-OT data. False-positive ratios were estimated using a reverse decoy database [17]. All datasets where searched once in the forward and once in the reverse database. Separate searches were used to keep the database size constant. Protein and peptide score were then set up to maintain the false positive peptide ratio below 1%. This resulted in a slight overestimation of the false-positive ratio [17]. For all analyses, only proteins matching two different peptide sequences were kept.

Quantitative data (extracted ion chromatograms and chromatographic peak integration) were extracted with Xcalibur 2.6 (Thermo Scientific). Base-to-base peak integration was performed manually using the "add peak" function of Xcalibur. The peak area value calculated by the software was used.

3. Results and discussion

In a first experiment, digested human plasma and the two digested standard proteins â-lactoglobulin (B-LG) and trypsin inhibitor type II (Trp II) were separately analyzed by DDA ESI-LC-MS/MS. This resulted with the identification of an average of 926 (SD=12) unique peptides from human plasma, corresponding to 413 (SD=18) unique proteins identified with at least two peptides. These numbers demonstrate the quality of the plasma digestion and the reproducibility of the data-dependent analysis. Then, for each standard protein the three peptides giving raise to the three most abundant precursor ion signals were selected for further analysis (Table 1). These 3 peptides correspond to sequence coverage of 15% for B-LG and 19% of Trp II.

In the next experiment, the digested proteins were mixed with digested plasma so that the injected amount varied from one atomole to one picomole. The quantity of plasma was kept constant, at 0.126 ug per injection (corresponding to aproximatively 1 pmol of injected albumin). The peptide mixture was then analyzed using DDA and *pseudo*-mSRM. For each concentration, the acquired MS1 spectra were inspected for the presence of the three peptide precursor of each standard protein. The isotopic cluster corresponding to peptide LFSNPTQLEEQCHI was visible at injected amounts of 1 femtomole and above. The two other peptides were dis-

Table 1. Observed peptides for MS1 and MS2-based quantification and their detection limit

Protein	Peptide	MS1 detection limit	p-SRM transition	MS2 detection limit	retention time (min)
β-Lactoglobulin	LFSNPTQLEEQCHI	1 fmol	$[M+2H]^{++} \rightarrow y_6^+$	100 amol	43,04
			$[M+2H]^{++} \rightarrow y_7^+$	100 amol	43,04
			$[M+2H]^{++} \rightarrow y_{10}^+$	10 amol	43,04
β-Lactoglobulin	TPEVDDEALEKFDK	10 fmol	$[M+3H]^{+++} \rightarrow y_{10}^{++}$	100 amol	33,97
			$[M+3H]^{+++} \rightarrow y_{11}^{++}$	100 amol	33,97
			$[M+3H]^{+++} \rightarrow y_{12}^{++}$	10 amol	33,97
β-Lactoglobulin	TPEVDDEALEK	10 fmol	$[M+2H]^{++} \rightarrow y_7^+$	100 amol	26,56
			$[M+2H]^{++} \rightarrow y_8^+$	100 amol	26,56
			$[M+2H]^{++} \rightarrow y_{10}^{++}$	100 amol	26,56
Trypsin Inhibitor	NELDKGIGTIISSPYR	1 fmol	$[M+3H]^{+++} \rightarrow y_5^+$	100 amol	39,98
			$[M+3H]^{+++} \rightarrow y_6^+$	100 amol	39,98
			$[M+3H]^{+++} \rightarrow y_{14}^{++}$	1 fmol	39,98
Trypsin Inhibitor	NKPLVVQFQK	10 fmol	$[M+2H]^{++} \rightarrow y_5^+$	100 amol	28,27
			$[M+2H]^{++} \rightarrow y_6^+$	100 amol	28,27
			$[M+2H]^{++} \rightarrow y_8^+$	10 amol	28,27
Trypsin Inhibitor	AAPTGNERCPLTWQSR	100 fmol	$[M+2H]^{++} \rightarrow y_8^+$	1 fmol	28,00
			$[M+2H]^{++} \rightarrow y_9^+$	1 fmol	28,00
			$[M+2H]^{++} \rightarrow y_{10}^{++}$	1 fmol	28,00

played clear isotopic clusters at 10 femtomoles and above. Similarly, one trypsin inhibitor peptide was visible at 1 femtomole of injected protein, the other two at 10 and 100 femtomoles. Thus, we concluded that relative quantification using an MS1 based label-free approach can be performed from amounts of 1 femtomoles and above. If the upper limit is arbitrarily set to 1 pmol, relative quantification could be done over a concentration range of three orders of magnitude (1 femtomole to 1 picomole).

Individual fragment ion currents were extracted for the same standard protein peptides from the *pseudo*-mSRM data. For three out of the six peptides, fragment ions were clearly visible at injected amounts of 10 atomoles and above (Table 1). The extracted fragment ion chromatograms for β-lactoglobulin peptide LFSNPTQLEEQCHI are shown in Figure 2.

Next, we investigated if the extracted fragment ion chromatograms could be used for label-free MS2-based relative quantification. However, this strategy is complicated by the fact that multiple fragment-ion chromatograms can be reconstructed from a single peptide precursor ion. Thus, we applied an empirical method similar to the one showed by Silva and co-workers. Indeed, this group showed that the average MS signal response of the three most abundant peptides is constant for each protein [9]. Based on this, the sum of the three most abundant fragment ions was calculated for all three peptides per standard protein. The protein abundance values calculated with this method showed excellent linearity with protein concentration (Figure 3).

Coefficients of correlation are above 0.98 for both measured proteins and all replicates over the full range of concentration. This implies that relative quantification using an MS2-based label-free approach can be performed at lowest protein concentrations. With an arbitrary upper limit of one

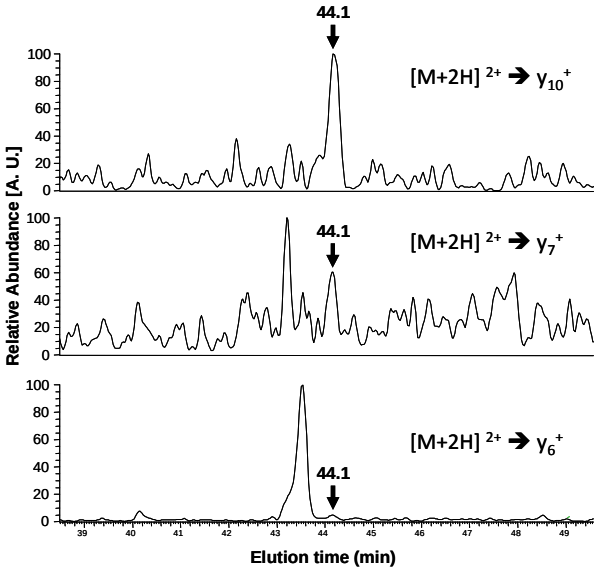

Figure 2. Extracted *p*-mSRM transitions ion currents from 10 amol of β-lactoglobulin peptide LFSNPTQLEEQCHI spiked into digested human plasma. The peptide elutes at 44.1 minutes.

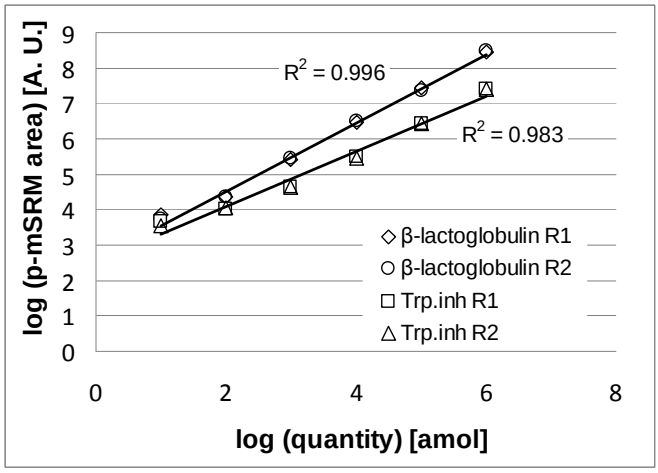

Figure 3. Combined *pseudo*-mSRM area as function of protein concentration for β-lactoglobulin and trypsin inhibitor spiked into human plasma. R1 = replicate 1, R2 = replicate 2. The correlation coefficient R^2 is calculated from the $\log_{(10)}$ values of *p*-mSRM area and concentration.

picomole, relative quantification could be done over five orders of magnitude (10 atomoles to 1 picomole). It represents an increase in dynamic range of two orders of magnitude compared to DDA acquisition, traditionally used for label-free quantification. Indeed, if the DDA mode is used on a very complex sample, the dynamic range of the analysis is close to the intra-spectrum dynamic range of the analyzer (in our case the Orbitrap analyzer). Makarov and co-workers have shown that the intra-spectrum dynamic range of an orbitrap was around 5,000 for full MS1 survey scans [18]. By contrast, the high dynamic range using the *pseudo*-mSRM mode can be explained by the combined dynamic range of the mass analyzer (in this case the linear ion-trap) and the ion injection time to fill the trap. The dynamic range of an ion trap is around 10^2 – 10^3, with a varying injection time between 0.1-100 ms. Consequently, the resulting dynamic range with *pseudo*-mSRM is the product of this two values. This indicates that a dynamic range of five orders of magnitude should be possible, which corresponds to the value found in our experiments.

4. Conclusions

Our data shows that relative label-free quantification is possible in ion trapping devices, using the data-independent or *p*-mSRM mode of operation. Quantification can be performed directly, in complex samples over a large dynamic range. Moreover, combination of large-scale data-independent protein identification and label-free quantification is also possible. Data-independent acquisition strategies such as the recently published PAcIFIC provide good results in terms of number of identifications and dynamic range. The data format itself corresponds to nothing else than a large-scale *pseudo*-mSRM experiments. The obtained data can therefore be directly used for MS2-based relative quantification.

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