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Direct infusion-SIM as fast and robust method for absolute protein quantification in complex samples

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ABSTRACT

Relative and absolute quantification of proteins in biological and clinical samples are common approaches in proteomics. Until now, targeted protein quantification is mainly performed using a combination of HPLC-based peptide separation and selected reaction monitoring on triple quadrupole mass spectrometers. Here, we show for the first time the potential of absolute quantification using a direct infusion strategy combined with single ion monitoring (SIM) on a Q Exactive mass spectrometer. By using complex membrane fractions of *Escherichia coli*, we absolutely quantified the recombinant expressed heterologous human cytochrome P450 monooxygenase 3A4 (CYP3A4) comparing direct infusion-SIM with conventional HPLC-SIM. Direct-infusion SIM revealed only 14.7% (±4.1 (s.e.m.)) deviation on average, compared to HPLC-SIM and a decreased processing and analysis time of 4.5 min (that could be further decreased to 30 s) for a single sample in contrast to 65 min by the LC–MS method. Summarized, our simplified workflow using direct infusion-SIM provides a fast and robust method for quantification of proteins in complex protein mixtures.

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1. Introduction

Quantification of proteins is a common approach in proteomics. Using heavy isotope labeled peptides, absolute quantification of specific proteins by selected reaction monitoring (SRM), a targeted mass spectrometric approach measured on a triple quadrupole mass spectrometer, is a well-established method [1–6]. Although SRM measurements are sensitive and highly selective, a triple quadrupole does not show high resolution and high mass accuracy. Furthermore, absolute quantification with SRM requires method optimization for every new protein of interest or peptides representing the protein with regard to selection of transitions and collision energy optimization. Utilizing the Q Exactive mass spectrometer (Thermo Scientific), which combines a quadrupole mass filter and an orbitrap mass analyzer, targeted approaches can be carried out with high resolution and accurate mass (HR/AM)

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detection [7]. Furthermore, quantification can be performed by single ion monitoring (SIM) at MS level or parallel reaction monitoring (PRM) at MS/MS level [8,9]. In the SIM mode, the quadrupole isolates the precursor mass of interest in a narrow window of e.g. 2 *m/z*. Then, ions are accumulated in the C-Trap and analyzed in the orbitrap. In a standard proteomics workflow prior to mass spectrometry (MS) measurement an HPLC is used for pre-fractionation of the peptide mixture, which usually takes about 1–2 h depending on sample complexity. So far, the direct infusion MS setup is applied for characterization and quantification of metabolites, for example in lipid profiling to investigate the progression of colorectal cancer [10] or to detect metabolite changes in liver extracts from mice after exposure to polychlorinated biphenyls [11]. To our knowledge a direct infusion-SIM approach has not been used to absolutely quantify proteins from complex biological samples until now. This method might be of special interest e.g. for protein quantification in biotechnological systems, such as whole-cell biocatalysts as well as other biological systems like cells or tissues. In order to establish an efficient biocatalyst and process setup, a fast and reliable absolute quantification of the target protein from the biological matrix is important to define or monitor optimal reaction conditions to achieve high amounts of active protein. Here, we applied the direct infusion-SIM approach to quantify the heterologously expressed human cytochrome P450 monooxygenase 3A4 (CYP3A4), which was produced in *Escherichia coli*. Human cytochrome P450 enzymes are a family of membrane-bound heme-containing monooxygenases responsible for the metabolism of many endogenous compounds as well as xenobiotics, mainly in the liver [12,13]. The enzymes are of special interest as biocatalysts in order to synthesize gram-scale amounts of drug metabolites [14]. The overall aim of this study was to develop a fast, robust and reproducible method for protein quantification that can be used to (absolutely) quantify proteins from complex protein mixtures, e.g. *E. coli* membrane fractions. We performed direct infusion-SIM with the Q Exactive mass spectrometer and compared results to data obtained with a conventional HPLC-SIM approach. Direct infusion-SIM appeared to be a fast and accurate tool for the determination of absolute abundance of proteins in complex biological samples.

2. Experimental procedures

2.1. Reagents

$^{13}C_6/^{15}N_2$-Lys labeled peptides were purchased from Thermo. Solvents used for mass spectrometry were all HPLC/MS grade. Acetonitrile (ACN) was purchased from Biosolve, trifluoroacetic acid (TFA) and formic acid (FA) from Sigma Aldrich.

2.2. E. coli cultivation and CYP3A4 synthesis

E. coli DH5α [15] containing the genes encoding human CYP3A4 and cytochrome P450 reductase on the plasmid pCW3A4 [16] was used. *E. coli* DH5α (pCWori(+)) was used as control strain without CYP3A4. A single colony of *E. coli* DH5α from an LB agar plate [17] was used to inoculate 3 ml LB medium, which were grown overnight at 37 °C and 200 rpm to a biomass concentration of about 1 $g_{CDW}\,l^{-1}$. The precultures were diluted to a biomass concentration of 33 $mg_{CDW}\,l^{-1}$ in 40 or 100 ml TB medium (0.89 M KPO_4 buffer (potassium phosphate buffer), pH 7.4, 24 $g\,l^{-1}$ yeast extract, 12 $g\,l^{-1}$ tryptone, 2 $g\,l^{-1}$ peptone, 4 $ml\,l^{-1}$ glycerol, 1 mM thiamin, 25 $\mu l\,l^{-1}$ trace elements and 100 $mg\,l^{-1}$ ampicillin) [18] in non-baffled, capped 250 ml Erlenmeyer flasks. The cultures were incubated at 30 °C and 200 rpm (rotary incubator) until a biomass concentration of 83 $mg_{CDW}\,l^{-1}$ was obtained. Then, gene expression was induced by the addition of 1 mM IPTG, 0.5 mM δ-aminolevulinic acid (δ-ALA), and 25 μM $FeCl_3$ and incubation was continued for another 24 h. Cells were harvested by centrifugation (20 min at 4700 × *g* and 4 °C) and resuspended in 100 mM KPO_4 (pH 7.4) containing 20% glycerol, 1 mM EDTA, 0.1 mM dithiothreitol (DTT), and 0.2 mM Pefabloc (Sigma–Aldrich) at 17 $g_{CDW}\,l^{-1}$ and stored at −20 °C until usage.

2.3. Membrane isolation

Cell lysis was achieved by two passages through a French Press, followed by centrifugation (20 min, 4 °C, 4700 × *g*) and ultracentrifugation of the supernatant (2 h, 4 °C, 40,800 × *g*). Membranes were resuspended in 100 mM KPO_4 buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA and 0.1 mM dithiothreitol (DTT) and stored at −20 °C.

2.4. Sample preparation

Membrane fractions containing human CYP3A4 were subjected to tryptic digestion. For each tryptic digest 10 μg protein were used and 110 $fmol\,\mu l^{-1}$ of each heavy peptide were spiked. Samples were reduced for 20 min at 56 °C with 5 mM DTT and alkylated with 15 mM iodoacetamide in the dark for 15 min. Digestion was carried out over night at 37 °C with trypsin (trypsin:protein ratio 1:5.5) in the presence of 0.01% (wt/vol) ProteaseMAX (Promega), 15 mM bicine and 30 mM NH_4HCO_3. After stopping the digestion by addition of 0.5% TFA, samples were purified using OMIX columns (Agilent Technologies) and eluted in water containing 55% ACN and 0.1% TFA. Eluates were concentrated in a vacuum concentrator and resolved in 100 μl water containing 40% ACN/0.1% FA.

2.5. Direct infusion-SIM with HESI source

Samples were loaded in a 250 μl Hamilton syringe, injected by a syringe pump with a flow rate of 5 $\mu l\,min^{-1}$ into the HESI (heated electrospray ionization) source and measured for 4.5 min with a SIM method on a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific). For ionization a spray voltage of 3.6 kV and capillary temperature of 320 °C was used and sheath gas flow rate was set to 6 units. The acquisition method consisted of two scan events, Full MS and targeted SIM (t-SIM). The Full MS was monitored from *m/z* 350–1400, with an orbitrap resolution of 35,000 (at *m/z* 200), a maximum injection time of 120 ms and an automatic gain control (AGC) value of 3e6. The t-SIM was performed with a resolution of 70,000 (at *m/z* 200), a maximum injection time of 250 ms and an automatic gain control (AGC) value of 1e5.

The isolation window was 2 *m/z* for the respective masses of light and heavy peptides.

2.6. Direct infusion-MS/MS with HESI source

Samples were injected into the mass spectrometer and ionized as described above. In order to manually acquire MS/MS spectra of the respective peptides, the following scan parameters were set in the Tune software (Thermo Scientific). The scan type was Full MS-SIM and in the scan range the center *m/z* was set to the *m/z* of interest with an isolation window of 2 *m/z*. The isolated *m/z* was fragmented with a normalized collision energy of 27 in the HCD (higher-energy collisional induced dissociation) cell and fragment spectra were monitored from *m/z* 150–1300, with an orbitrap resolution of 70,000 (at *m/z* 200).

2.7. Direct infusion-SIM with nano source

For direct infusion with the nano source the Offline Nano ES kit (Thermo Scientific) was used. Samples were loaded in a Borosilicate Offline Emitter (Thermo Scientific), injected by means of the static air pressure device and measured for 4.5 min with a SIM method on a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific). For ionization a spray voltage of 1.5 kV and capillary temperature of 250 °C was used. Subsequently, measurement was performed as described in direct infusion-SIM with HESI source.

2.8. HPLC-SIM

Samples were diluted 1:15 in 0.1% TFA. 15 μl were injected via the autosampler of an RSLC nano system (Thermo Scientific), concentrated on a C_{18} trapping column (2 cm length, 100 μm i.d., 5 μm particle size, Thermo Scientific), and separated on a C_{18} analytical column (50 cm length, 75 μm i.d., 2 μm particle size, Thermo Scientific) heated at 60 °C before being emitted via a coated silica tip (FS360-20-10-D-20, New Objective) of the nano-electrospray source. The HPLC separation was performed with a gradient method of in total 65 min gradient consisting of: 6 min of loading the sample and washing the column with 0.1% TFA at a flow rate of 30 $\mu l\,min^{-1}$ on the trapping column, followed by separation applying a linear gradient at a flow rate of 400 $nl\,min^{-1}$ with the solvents A (0.1% FA in HPLC grade water) and B (84% ACN/0.1% FA in HPLC grade water) starting from 4% B to 40% B in 34 min on the heated analytical column, a linear gradient of 40% B to 95% B in 3 min, washing for 5 min with 95% B. Finally, a gradient was applied from 95% B to 4% B in 1 min followed by equilibration for 16 min with 4% B. For ionization a spray voltage of 1.6 kV and capillary temperature of 250 °C was used. The acquisition method was the same as described above in the direct infusion-SIM with HESI source section. The t-SIM scans were time scheduled and multiplexed: *m/z* values of the respective light and heavy CYP3A4 peptides were scanned only in a specific time window and the ions were isolated sequentially, stored together in the C-Trap and detected in the orbitrap in one single scan.

2.9. Data evaluation

Data evaluation was performed with the XCalibur software (Thermo Scientific) and Microsoft Excel (Microsoft Corporation). After direct infusion-SIM, raw files were loaded in the XCalibur Qual Browser, the plot type was set to base peak (as with the base peak chromatogram the noise level is decreased compared to the total ion chromatogram) and the scan filter was set to the respective masses. Then the intensity of this mass was averaged over the whole measurement time and the intensity of the monoisotopic peak was transferred in Excel. For comparison, intensities of respective masses were averaged over 30 s (minute 1.5–minute 2.0) and the intensities of the monoisotopic peaks were transferred in Excel. After HPLC-SIM, raw files were also loaded in the XCalibur Qual Browser and the scan filter was set to the respective multiplexed masses. Then, the intensities were averaged over the elution profile, which borders were set manually with the same elution span for each peptide and the intensity of each monoisotopic peak was again transferred in Excel. The ratios of light/heavy CYP3A4 peptide were calculated and a linear curve was established by plotting the ratios of the intensities of light/heavy peptide against the amounts of spiked light peptide. The linear equation and regression coefficient were calculated for each peptide (supporting information Fig. S-1a and b). Using the respective linear equation, the amounts of expressed CYP3A4 were calculated.

3. Results and discussion

3.1. Selection of appropriate peptides for direct infusion-SIM

Peptides that are used for quantification need to be chosen carefully and should meet the following criteria: (1) they need to be proteotypic for the quantified protein, (2) they should not contain post-translational modifications, (3) they should contain neither methionine nor cysteine, which tend to be chemically modified, and (4) they should not show any miscleavages [19]. Furthermore, for direct infusion approaches it is important that peptide masses are unique. According to this two tryptic CYP3A4 peptides (LQEEIDAVLPNK, *m/z* 684.8721; YWTEPEK, *m/z* 476.7242) met the described specifications and were selected for the setup of a direct infusion-SIM method using the Q Exactive. We set the instrument to SIM scan mode with the respective monoisotopic masses (*m/z* 684.8721 (peptide LQEEIDAVLPNK) or *m/z* 476.7242 (peptide YWTEPEK)) and an isolation window of 2 *m/z*. The peptide LQEEIDAVLPNK (*m/z* 684.8271) could be detected (Fig. 1a). We used the same isolation window for collection and fragmentation of the ions resulting in the corresponding MS/MS spectrum for the peptide LQEEIDAVLPNK with most of the y-ions annotated (Fig. 1b). For the peptide YWTEPEK and the related spiked heavy peptides (which both incorporate a $^{13}C_6$, $^{15}N_2$ isotopically labeled lysine that causes a mass difference of 8 Da), SIM scans and MS/MS spectra are given in supporting information Fig. S-2a–f. All peptides could be verified by direct infusion-SIM and were used for further analyses. As these peptides were

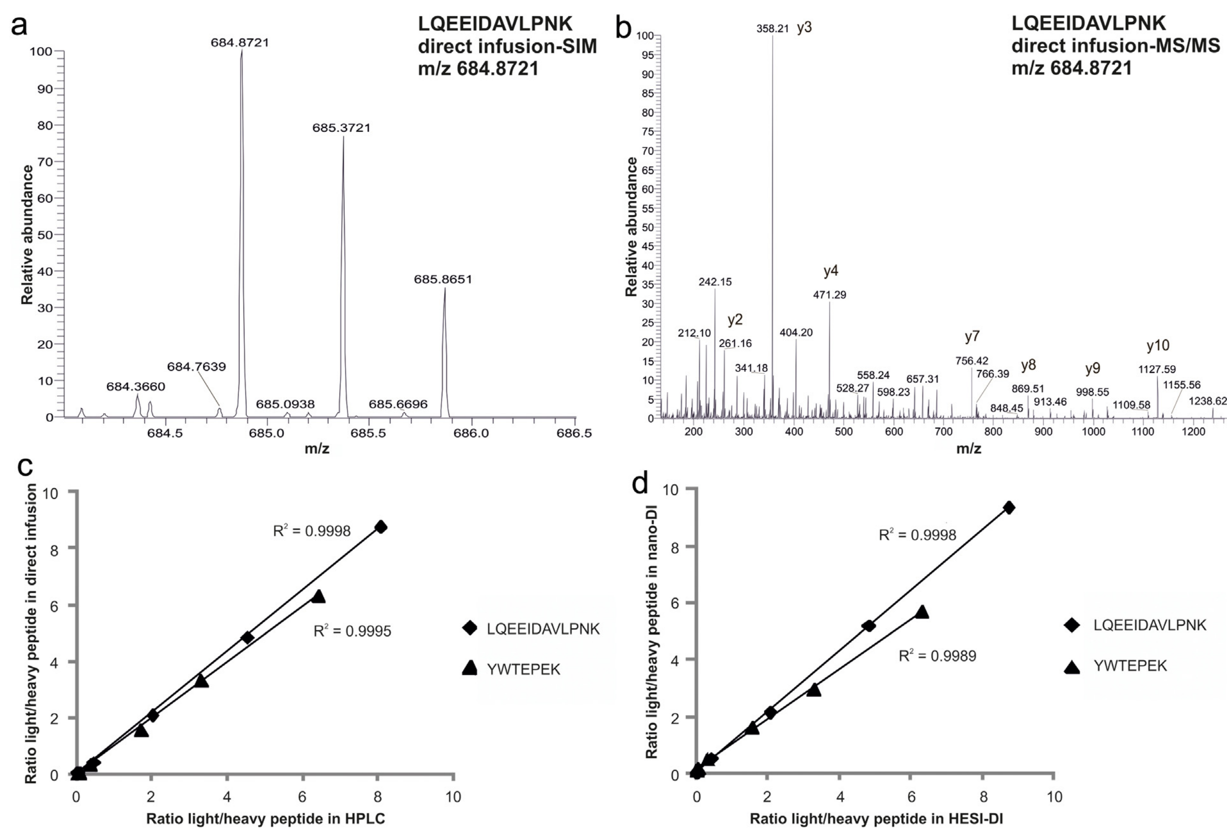

Fig. 1 – Peptide verification in the direct infusion-SIM setup and correlation of linearity studies. (a) Direct infusion-SIM scan and (b) corresponding MS/MS scan for *m/z* 684.8721 (peptide LQEEIDAVLPNK) and an isolation window of 2 *m/z* from 0.2 μg/μl *E. coli* membrane fraction containing CYP3A4. The peptide could be detected using the direct infusion-SIM approach. (c) Ratios of light/heavy peptides determined by direct infusion-SIM and HPLC-SIM and (d) determined by direct infusion-SIM with the HESI source and the nano source are highly correlated for the peptide LQEEIDAVLPNK and the peptide YWTEPEK. Therefore, direct infusion-SIM is as suitable for absolute quantification as HPLC-SIM.

merely detected in the 2+ charge state, only the corresponding 2+ *m/z*-values were considered for subsequent analyses.

3.2. *Calibration curve*

A standard approach for absolute protein quantification in proteomics is to use generated tryptic peptides from the protein of interest to quantify the protein amount within the sample. In order to perform absolute quantification of CYP3A4, we prepared linearity curves for the respective peptides in triplicate and used them for the calculation of absolute amounts in real samples. For both CYP3A4 peptides we measured calibration curves in a range from 2 fmol to 2 pmol (supporting information Fig. S-1a and b). Therefore we used an *E. coli* DH5α (pCWori(+)) membrane fraction as matrix in that we spiked varying amounts (2 fmol μl^{-1}, 20 fmol μl^{-1}, 100 fmol μl^{-1}, 500 fmol μl^{-1}, 1000 fmol μl^{-1}, 2000 fmol μl^{-1}) of each light CYP3A4 peptide along with a constant amount of 110 fmol $\mu l^{-1}$of each heavy peptide. We analyzed these samples using direct infusion-SIM as described as well as HPLC-SIM for comparison. With direct infusion-SIM linearity of measurements was achieved and the resulting linear equation showed a regression coefficient (R^2) larger than 0.99 (supporting information Fig. S-1a). As expected, also HPLC-SIM measurements were linear with an R^2 value larger than 0.99 (supporting information Fig. S-1b). Slopes were different for the different peptides and methods (LQEEIDAVLPNK: 0.0044 (direct infusion-SIM) and 0.0041 (HPLC-SIM), YWTEPEK: 0.0032 (both approaches); supporting information Fig. S-1a and b).

In order to correlate the data for both methods, we plotted obtained ratios for light and heavy peptides from both approaches against each other. For both peptides we observed high correlation between both approaches with regression coefficients larger than 0.99 (Fig. 1c).

Precision of both methods was compared for different amounts of peptides by calculating the coefficient of variation (CV) (supporting information Tab. S-1). For low amounts as 2 fmol μl^{-1} CVs were ≥30% with higher CVs for the HPLC-SIM approach. For amounts of 20 fmol μl^{-1}–2000 fmol μl^{-1} the HPLC-approach showed mainly smaller CVs with <20% for 20 fmol μl^{-1} and <10% for 100 μl^{-1} fmol–2000 fmol μl^{-1} compared to direct infusion-SIM with >15% for 20 and 100 fmol μl^{-1} and <15% for 500 fmol μl^{-1}–2000 fmol μl^{-1}. This shows that in the low fmol range both approaches are less precise compared to the high fmol range. Nevertheless, in the high fmol range also the direct infusion-SIM approach reveals high precision indicating that it can be used as alternative technique for HPLC-SIM measurements.

We successfully demonstrated linearity for direct infusion-SIM and HPLC-SIM with similar regression coefficients for both techniques (supporting information Fig. S-1a and b). However, it was obvious that for direct infusion-SIM, especially signals from peptides in the low fmol range (2–20 fmol) were less resolved compared to the high fmol range of 1000–2000 fmol making data evaluation more difficult (data not shown). In HPLC-SIM measurements peptides showed fully resolved signals in the whole measured range from 2 to 2000 fmol. Nevertheless, measured ratios of light and heavy peptides correlated very well between both techniques showing that direct infusion-SIM is comparably suitable for absolute quantification.

3.3. *Application of direct infusion-SIM and HPLC-SIM to real samples*

After showing a good correlation between the direct infusion-SIM and HPLC-SIM approach, we used both methods to determine absolute amounts of heterologous CYP3A4 samples derived from *E. coli* DH5α (pCW3A4). Therefore, we investigated membrane fractions from nine independent cultivations that were known to express different amounts of CYP3A4 (unpublished data). In each sample we determined the ratio of the intensity of light/heavy peptide and after considering the graph equation of each peptide we calculated the absolute amount of light peptide:

$$\text{fmol Light Peptide} = \frac{(\text{Intensity Light Peptide}/\text{Intensity Heavy Peptide}) - y\,\text{Intercept}}{\text{Slope of Calibration Curve}}$$

As expected, the different membrane fractions revealed varying amounts of CYP3A4. Absolute amounts of CYP3A4 ranged from 34 fmol/0.1 μg total protein (±16.81 (s.e.m.; standard error of the mean) (34 nM) to 1876 fmol/0.1 μg total protein (±88.78 (s.e.m.)) (1876 nM) for direct infusion-SIM and 24 fmol/0.1 μg total protein (±0.24 (s.e.m.)) (34 nM) to 1697 fmol/0.1 μg total protein (±3.12 (s.e.m.)) (1697 nM) for HPLC-SIM (Fig. 2a and b) showing that quantification in the low nM range is achievable also with direct infusion-SIM.

The use of the two peptides with direct-infusion SIM resulted in higher error bars for the determined amounts of CYP3A4 compared to HPLC-SIM (Fig. 2a and b). Whereas we used spiked light CYP3A4 peptides for creating the calibration curve, in samples expressing CYP3A4 the endogenous/light peptides were released upon digestion. Hence, the diverging results for the two peptides may result from incomplete digestion or different peptide decay during digestion [20]. The fact that the HPLC measurements were more consistent for the two peptides might be due to a more accurate measurement caused by a peptide pre-separation and therefore less disturbing/suppressing co-measured ions in the SIM. Especially, the peptide YWTEPEK with an *m/z* of 476.7242 was always accompanied by an *m/z* of 476.7638 in the direct infusion-SIM approach (supporting information Fig. S-2a), that was of course not detected in the HPLC-SIM approach. Even though the calculated amounts for CYP3A4 were slightly different for the two methods, amounts obtained with direct infusion-SIM only deviated on average of 14.7% (±4.1 (s.e.m.)) from data obtained with HPLC-SIM. This is a small variance regarding that the comparison of HPLC-SRM and HPLC-SIM for the quantification of transferrin in urine samples revealed less than 10% difference between the approaches [8]. Furthermore, comparison of LC-MRM and direct infusion-MRM of heat shock proteins showed errors of up to >30% [21]. With the direct infusion-SIM approach absolute quantification is even more robust due to an averaged error of <15%. Moreover, SIM measurements are simpler than MRM measurements because no optimization of transitions is needed. Another advantage of direct infusion-SIM over direct infusion-MRM is that no correction factor for quantification is needed.

3.4. *Data evaluation for shortened analysis time*

After showing that direct infusion-SIM and HPLC-SIM revealed similar absolute amounts of CYP3A4, data evaluation for the direct infusion-SIM approach was performed for only 30 s of the analyzed time and compared to HPLC-SIM results in order to show that analysis time could be further decreased. In this case, absolute amounts of the CYP3A4 within 100 ng of total protein sample ranged from 36 fmol (±14.02 (s.e.m.)) to 1926 fmol (±303.84 (s.e.m.)) corresponding to 36–1926 nM CYP3A4 (supporting information Fig. S-3). Using the 30 s analysis time revealed only a variance of 16.7% ((±4.0 (s.e.m.)) compared to HPLC-SIM, confirming that analysis time can be

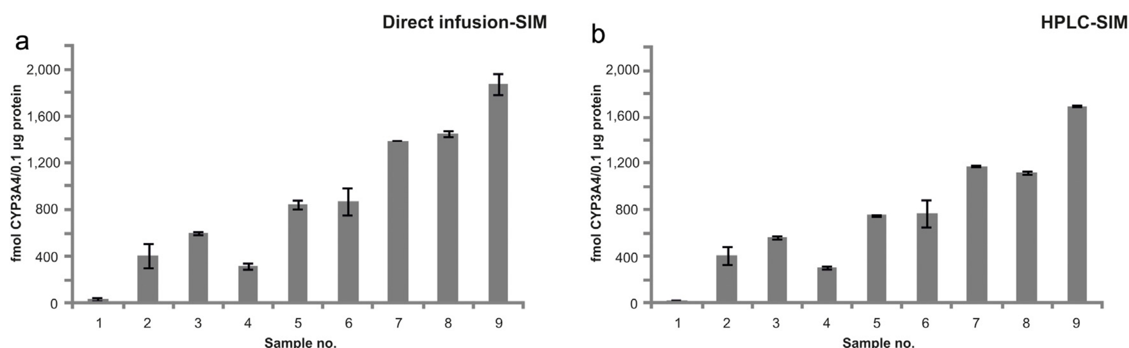

Fig. 2 – Comparison of quantification results in real samples. (a) Absolute quantification of CYP3A4 in *E. coli* DH5α (pCW3A4) membranes derived from nine independent cultivation experiments (1–9) was performed with direct infusion-SIM and (b) HPLC-SIM. Calculated amounts for both peptides were averaged and showed similar results for both approaches.

further decreased. Provided that spray stability is high analysis times of 30 s are sufficient to gain similar results as compared to 4.5 min analysis time with only a slight increase in the variance between direct infusion-SIM and HPLC-SIM.

3.5. *Comparison of HESI-direct infusion vs. nano-direct infusion*

Due to the extremely short measuring time with direct infusion-SIM of 4.5 min or even 30 s per sample compared to 65 min for nano HPLC-SIM, sample throughput is much higher and allows fast comparison of samples. Besides this advantage, a remaining drawback of direct infusion-SIM exists with respect to sample consumption. Relatively high sample amounts are needed to prepare the samples for the injection via a syringe pump and HESI source. Whereas in direct infusion-SIM we measured sample concentrations of 0.1 $\mu g\,\mu l^{-1}$ with a flow rate of 5 $\mu l\,min^{-1}$, for HPLC-SIM measurements 0.1 μg sample on column is sufficient. In order to reduce the required sample amount a nano source application was used for direct infusion-SIM. First measurements showed promising results so that for future applications sample volume will only be a few microliter (1–10 μl). With the nano direct infusion-SIM setup a further linearity study was performed. Obtained data (ratios for light and heavy peptides from both approaches, HESI direct infusion-SIM vs. nano direct infusion-SIM) were plotted against each other in order to correlate the data for either method. For both peptides we observed high correlation between the two approaches with R^2 values larger than 0.99 (Fig. 1d). These first measurements already revealed comparable results for direct infusion with the HESI source or nano source. In addition, the use of the nano static spray will bring further advantages regarding the accuracy of the measurements. With this, the lower signal-to-noise ratios in direct infusion-SIM compared to HPLC-SIM will partly be overcome. Higher signal to noise ratios lead to better resolved SIM signals in direct infusion-SIM for the values between 2 and 20 fmol (data not shown).

4. Conclusion

It was shown that direct infusion-SIM can be used as an alternative method for absolute quantification, also in complex biological samples. This method allows a reproducible (similar to that of HPLC-SIM) quantification as shown here for human CYP3A4 in *E. coli* membrane fractions down to an amount of 34 fmol/0.1 μg protein (34 nM). When using this method some requisites need to be fulfilled: (1) quantified peptides need to have unique masses within the sample, (2) quantified peptides should be present in the lower nM range or higher and (3) sample amounts of 1 μg should be available.

The successful application of direct infusion-SIM for absolute quantification of proteins in complex samples consisting of microbial membranes as shown in this study might in future also be used for quantification of abundant and/or pre-enriched proteins in complex samples from different origin. Furthermore, it can also be used for a fast relative quantification, e.g. to determine the influence of different expression conditions on several proteins within one sample. Another advantage of direct infusion-SIM over HPLC-based methods is that when it comes to peptide selection for targeted protein quantification, also highly hydrophilic and hydrophobic, or rather long peptides (30–50 amino acids), that usually are not analyzable using conventional C18-reversed phase HPLC, can be detected with the direct infusion approach and thus be used for protein quantification by direct infusion-SIM. First results for this application of direct infusion-SIM have shown to be promising (unpublished data). However, to achieve more accurate results peptides should be selected with *m/z* values that are not too close to *m/z* values from other peptides in the sample, as was discussed for the peptide YWTEPEK. A final advantage of direct infusion-SIM over HPLC-SIM is the fact that resolution can be easily increased without losing acquisition of data points (although it was not done in this study in order to keep analysis parameters comparable for HPLC and direct infusion). Scanning with higher resolution is associated with longer scanning times. In HPLC-SIM measurements peptides elute for a certain time in that they are alternately collected and scanned. Longer scanning times increase the cycle time so that less data points will be acquired over the elution peak which makes data evaluation less accurate, especially for low abundant proteins. In direct infusion-SIM all peptides are injected throughout the whole acquisition time. Therefore, longer cycle times due to increased resolution can be compensated by longer acquisition times.

Altogether, we here demonstrate the potential of a fast, easy (no need for optimization of analysis parameters or data evaluation), robust and reproducible direct infusion-SIM approach and its applicability in protein/peptide quantification in complex samples with promising efficiency for future biological as well as clinical applications.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2015.03.001.

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