Journal of proteome research

Technical Note

pubs.acs.org/jpr

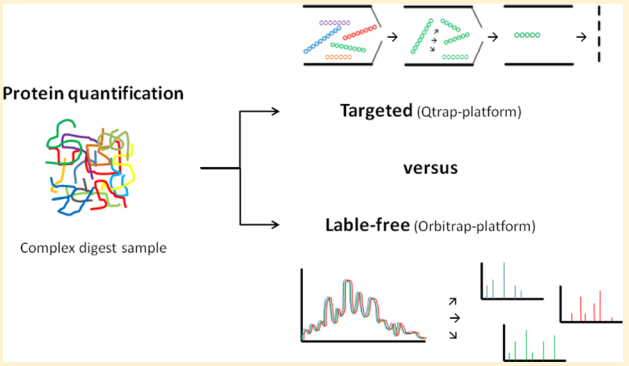
Comparative Study of Targeted and Label-free Mass Spectrometry Methods for Protein Quantification

Linda IJsselstijn, Marcel P. Stoop, Christoph Stingl, Peter A. E. Sillevis Smitt, Theo M. Luider, and Lennard J. M. Dekker*

Department of Neurology, Erasmus Medical Center, Rotterdam, The Netherlands

Supporting Information

ABSTRACT: We compared data acquired on an LTQ-Orbitrap MS used in a typical shotgun proteomics setting (optimized for protein identification) with data from a quadrupole ion trap MS operated in the MRM mode. Six relative abundant proteins were quantified in identical sets of serum and CSF samples by the following methods: a qual/quant method with and without use of internal standards and a quantitative method (MRM with use of internal standards). Comparison of these methods with an antibody-based method in CSF samples showed good linearity for both methods (R^2 of 0.961 and 0.971 for the qual/quant method with use of internal standards and the quantitative method, respectively). Besides its better linearity, the quantitative method was also more reproducible with lower CVs for all samples. Next to these comparisons we also explored why a qual/quant approach had typically a lower reproducibility compared to MRM analyses. We observed that modified peptides, or peptides with a cysteine or a methionine, yielded a significant increase in CV. Furthermore, a positive correlation was found between the length of the peptide and the CV. We conclude that qual/quant is an alternative for the quantification of abundant proteins and that the use of internal standards in qual/quant could be advantageous. Furthermore, the ongoing development in MS techniques increases the possibilities of qual/quant in protein quantification.



KEYWORDS: *protein quantification, targeted, qual/quant, multiple reaction monitoring, mass spectrometry*

INTRODUCTION

Mass spectrometry (MS) is a well-established tool in protein quantification and is increasingly used for the verification of biomarkers. Multiple reaction monitoring (MRM) has been a reference quantitative technique to analyze small molecules for more than 30 years,[1] but the past decade it has emerged as a tool in quantitative proteomics.[2,3] MRM can be used for relative quantification, in which a reference sample is labeled either metabolically (SILAC),[4] chemically (ICAT,[5] mTRAQ[6]) or enzymatically with stable isotopes (O^{18}).[7] By addition of internal standards, like synthetic stable-isotope labeled homologues[8] or glycine inserted peptides,[9] the absolute amount of the corresponding endogenous peptide can be determined. MRM coupled with stable isotope dilution MS has shown to be highly reproducible, within and across laboratories, and sensitive to low μg/mL protein concentrations in complex samples without enrichment of the proteins of interest.[10] At these concentration levels, immunoassays can be replaced by MRM assays in the verification of larger numbers of candidate biomarkers in a single experiment. In the biomarker discovery phase, often qualitative MS measurements are performed aiming at identifying large numbers of proteins for instance in shotgun proteomic experiments.[11] Due to higher accuracy and faster scan speeds of modern equipment, workflows have been developed that combine qualitative measurements with high-resolution, accurate mass measurements for quantitative purposes. These approaches that combine qualitative and quantitative aspects are called qual/quant. Qual/quant measurements have a considerable advantage over a targeted approach, because prior to the MS measurements no target proteins or peptides have to be selected. All observed peptides by MS are in principle available for quantification and can improve the quality of quantification.

In this study, we investigated whether qual/quant measurements performed on equipment routinely used for protein identification are of sufficient sensitivity and reproducibility to use for quantification of proteins in complex samples. Second, we examined why a qual/quant approach has typically a lower reproducibility and quantitative performance compared to MRM analyses. To answer these questions, six proteins (ranging from mg/mL to low μg/mL concentrations) were quantified in identical sets of serum and cerebrospinal fluid (CSF) digests, by qual/quant measurements using an Orbitrap-platform and by quantitative measurements using a Qtrap-platform. We used the two instruments operating in a general

Received: December 29, 2012
Published: March 6, 2013

way which has been described in previous publications (Orbitrap-platform for biomarker discovery and Qtrap-platform for biomarker verification/validation).[12−14] Stable-isotope labeled peptides were used as internal standards in the quantitative measurements. In addition, these standards were also spiked in the samples for the qual/quant analysis, in order to determine if this improves quantification.

MATERIALS AND METHODS

Sample Background

The serum samples used in this study were a selection of control samples from a previous biomarker study.[14] The CSF samples originated from a previous proteomics and metabolomics analysis of normal CSF samples.[15] Both studies were approved by the Erasmus University Medical Ethics Committee, and written informed consents were obtained from all participants.

Protein Selection

The six proteins selected for a quantitative analyses are proteins which were detected in previous proteomics studies in serum[14] as well as in CSF.[16] The concentrations of the proteins are in the mg/mL (albumin) to low μg/mL (galectin-3 binding protein) range. For each protein two peptides were selected for quantification. These peptides had to meet the following criteria; no internal trypsin cleavage sites, no potential modification sites (cysteine and methionine), no ragged ends, and a maximum of 30 amino acid residues. For each selected peptide, a stable-isotope labeled peptide standard was synthesized (Pepscan Presto, Lelystad, The Netherlands), in which the arginine (R) or lysine (K) at the C-terminus was replaced with the heavy form of the amino acid. The sequences of the selected peptides and the corresponding standards can be found in Table 1.

Albumin Concentration

Albumin concentrations were determined by the department of Clinical Chemistry, Erasmus Medical Center with an antibody-based method on the Immage 800 (Beckman Coulter, Brea, CA). Shortly, the samples were diluted, an albumin-specific antibody was added and the resulting immunoprecipitation was measured by nephelometry.

Preparation Spiked Digests

To prepare the spiked serum digests, serum samples (n = 20) were thawed on ice, 20 μL was taken and diluted 50 times in 50 mM ammonium bicarbonate. From this dilution 10 μL was further diluted by adding 90 μL of 0.1% RapiGest SF (Waters, Milford, MA). During this second dilution step, synthetic stable-isotope labeled peptide standards (Pepscan Presto, Lelystad, Netherlands) were added to yield final concentrations after digestion of 100 fmol/μL for the albumin peptides and 25 fmol/μL for all other peptides investigated. Subsequently, samples were reduced and alkylated with 1,4-dithiothreitol (DTT) and iodoacetamide. For the enzymatic digestion 10 μL of 100 μg/mL gold grade trypsin (Promega, Madison, WI) in 3 mM Tris-HCl was added and samples were incubated overnight at 37 °C. To inactivate trypsin and degrade RapiGest SF, trifluoroacetic acid (TFA; Biosolve, Valkenswaard, Netherlands) was added to a final concentration of 0.5%. Subsequently, samples were incubated for 30 min. The spiked serum digests were aliquoted to perform LC Orbitrap MS and LC MRM MS measurements on identical samples.

Table 1. Transitions Used in MRM Assay of the Selected Peptides and Their Standards[a]

protein	peptide	Q1 [m/z]	Q3 [m/z]
Albumin	SLHTLFGDK	509.27	579.31 (y5), 680.36 (y6), 817.42 (y7)
	SLHTLFGD**K**	513.27	587.33 (y5), 688.38 (y6), 825.43 (y7)
	FQNALLVR	480.78	500.36 (y4), 571.39 (y5), 685.44 (y6)
	FQNALLV**R**	485.79	510.36 (y4), 581.40 (y5), 695.44 (y6)
Complement C3	IWDVVEK	444.74	474.29 (y4), 589.32 (y5), 775.40 (y6)
	IWDVVE**K**	448.75	482.31 (y4), 597.33 (y5), 783.41 (y6)
	VVLVAVDK	421.77	432.25 (y4), 531.31 (y5), 644.40 (y6)
	VVLVAVD**K**	425.78	440.26 (y4), 539.33 (y5), 652.41 (y6)
Vitamin D binding protein	VLEPTLK	400.25	458.30 (y4), 587.34 (y5), 700.42 (y6)
	VLEPTL**K**	404.26	466.31 (y4), 595.35 (y5), 708.44 (y6)
	HLSLLTTLSNR	627.86	691.37 (y6), 917.54 (y8), 1004.57 (y9)
	HLSLLTTLSN**R**	632.87	701.38 (y6), 927.55 (y8), 1014.58 (y9)
Complement factor B	QLNEINYEDHK	701.83	805.35 (y6), 918.43 (y7), 1161.52 (y9)
	QLNEINYEDH**K**	705.84	813.36 (y6), 926.45 (y7), 1169.53 (y9)
	ISVIRPSK	450.29	600.38 (y5), 699.45 (y6), 786.48 (y7)
	ISVIRPS**K**	454.29	608.40 (y5), 707.47 (y6), 794.50 (y7)
Galectin-3 binding protein	LADGGATNQGR	530.26	575.29 (y5), 760.37 (y8), 875.40 (y9)
	LADGGATNQG**R**	535.27	585.30 (y5), 770.38 (y8), 885.41 (y9)
	YSSDYFQAPSDYR	799.84	338.18 (y2), 637.29 (y5), 708.33 (y6)
	YSSDYFQAPSDY**R**	804.85	348.19 (y2), 647.30 (y5), 718.34 (y6)
Apolipoprotein E	LGPLVEQGR	484.78	360.20 (y3), 588.31 (y5), 701.39 (y6)
	LGPLVEQG**R**	489.78	370.21 (y3), 598.32 (y5), 711.40 (y6)
	AATVGSLAGQPLQER	749.40	642.36 (y5), 827.44 (y7), 898.47 (y8)
	AATVGSLAGQPLQE**R**	754.41	652.37 (y5), 837.45 (y7), 908.48 (y8)

[a]For each peptide the transitions (Q1 and Q3) are given (all doubly charged). Peptides with a bold K (lysine) or R (arginine) and the C-terminus are stable-isotope labeled. Stable-isotope labeled amino acids K and R contained C13 and N15 in place of C12 and N14.

To prepare the spiked CSF digests, CSF samples (n = 21) were thawed on ice and 20 μL was taken. Twenty microliters of 0.2% RapiGest SF was added together with the synthetic peptide standards to yield final concentrations after digestion of 100 fmol/μL for the albumin peptides and 25 fmol/μL for all other peptides. After reduction and alkylation, 4 μL of trypsin was added for the enzyme digestion and the samples were incubated overnight at 37 °C and treated as described above for serum. The spiked CSF digests were aliquoted to perform LC Orbitrap MS and LC MRM MS measurements on identical samples.

dx.doi.org/10.1021/pr301221f | J. Proteome Res. 2013, 12, 2005−2011

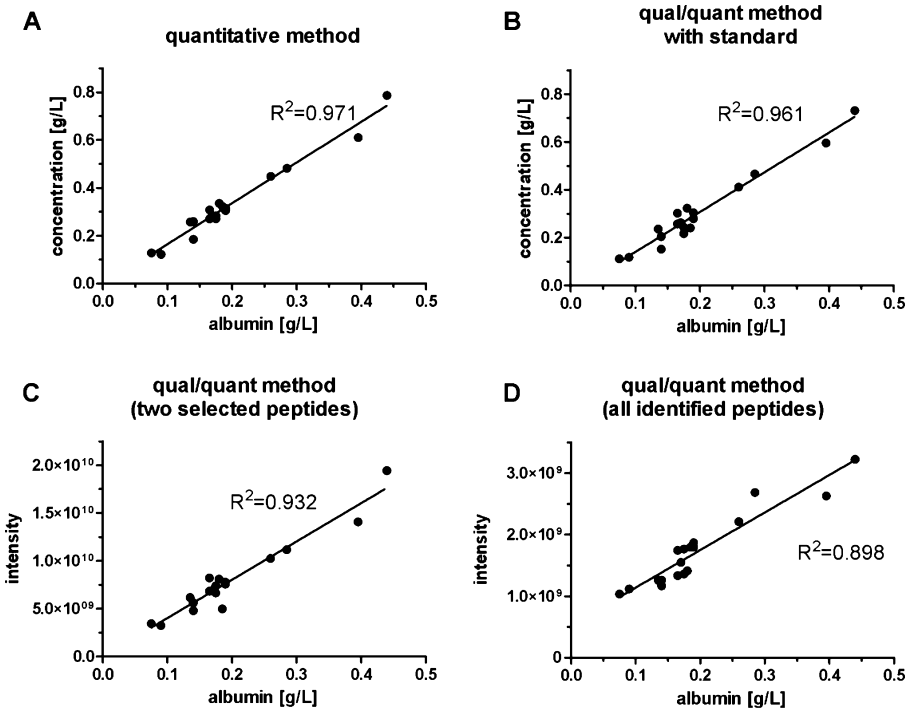


Figure 1. Albumin concentrations in CSF determined via various methods plotted against the concentration measured with the antibody based method on the *x*-axis. (A) Quantitative method (Qtrap-platform) with internal standards. (B) Qual/quant method (Orbitrap-platform) with internal standards. (C) Qual/quant method (Orbitrap-platform), average intensity displayed of two peptides used in the methods with internal standard. (D) Qual/quant method (Orbitrap-platform), average intensity displayed of all identified peptides for albumin.

LC Orbitrap MS

LC−MS measurements were carried out on a nano LC system (Ultimate 3000; Dionex, Amsterdam, Netherlands) online coupled to a hybrid linear ion trap/Orbitrap MS (LTQ Orbitrap XL; Thermo Fisher Scientific, Bremen, Germany). Four microliters of spiked serum digest or 5 μL of spiked CSF digest was injected onto the nano LC system, which held a C18 trap column (PepMap C18, 300 μm ID × 5 mm, 5 μm particle size and 100 Å pore size; Dionex) and a 25 cm long analytic column (PepMap C18, 75 μm ID × 500 mm, 3 μm particle size and 100 Å pore size; Dionex). A 90-min gradient with a 300 nL/min flow was run with solvent A (H_2O/acetonitrile (ACN) 98/2 (v/v), 0.1% formic acid (FA)) and solvent B (H_2O/ACN 20/80 (v/v), 0.1% FA): 0−25% solvent B in 60 min and 25−50% solvent B in the next 30 min. All solvents used were purchased from Biosolve (Valkenswaard, Netherlands). The separation of the peptides was monitored by a UV detector (absorption at 214 nm).

High resolution full scan MS was obtained from the Orbitrap (resolution 30000; AGC 1 000 000), MS/MS spectra were obtained by CAD fragmentation. MS/MS was performed on the top five masses in the full scan spectra. Dynamic exclusion was used, with a repeat count of one; exclusion duration was set at 3 min and exclusion width at ±5 ppm.

To check the performance of the system, one of the spiked serum or CSF digests was remeasured every 5 runs as a technical control.

LC MRM MS

Chromatographic separation of spiked digests, was performed on a nano LC system (Ultimate 3000; Dionex, Amsterdam, Netherlands). Two microliters of spiked serum or CSF digest was loaded onto a C18 trap column (PepMap100 C18, 300 μm ID × 5 mm, 5 μm particle size and 100 Å pore size; Dionex) and washed for 5 min at a flow rate of 20 μL/min 0.1% TFA in H_2O. Next, the trap column was switched in line with the analytic column (PepMap C18, 75 μm ID × 150 mm, 3 μm particle size and 100 Å pore size; Dionex). Peptides were eluted at a flow rate of 300 nL/min with the following gradient: 0−45% solvent B in 30 min, solvent A (H_2O, 0.1% formic acid (FA)) and solvent B (H_2O/acetonitrile 20/80 (v/v), 0.08% FA). All solvents used were purchased at Biosolve. The separation of the peptides was monitored by a UV detector (absorption at 214 nm).

MRM detection was performed by means of a quadrupole ion trap tandem mass spectrometer (4000 QTRAP; AB SCIEX, Concord, Ontario, Canada) in the positive ion mode. A scheduled MRM method was used with the MRM detection window set to 240 s and the target scan time to 3 s. The transitions used are listed in Table 1. The mass spectrometer was controlled using Analyst 1.5.1 (AB SCIEX).

To check the performance of the system, one of the spiked serum and CSF digests was remeasured every 5 runs as a technical control.

Calibration curves for the standards were made in digested serum and CSF. The following concentrations of standards were measured 0, 0.5, 2, 5, 20, 50, and 200 fmol/μL. Each concentration point for the calibration curve was measured in triplicate.

Data Analysis

The program Skyline v1.1[17] was used to analyze both LC Orbitrap MS and LC MRM MS data measured with internal standards. For quantification of Orbitrap data, we used the sum of the integrated areas of the extracted ion chromatograms (XIC) of the monoisotopic precursor and first ^{13}C isotope precursor. For the MRM data, the peak areas for all measured transitions were determined. The concentration of the peptides

dx.doi.org/10.1021/pr301221f | J. Proteome Res. 2013, 12, 2005−2011

was calculated using the ratio of the original peptide to its standard. The program Progenesis LC–MS (version 3.0; Nonlinear Dynamics Ltd., Newcastle-upon-Tyne, UK) was used additionally for analysis of LC Orbitrap MS data. After peptide detection a filter was used, yielding only features charged with two to eight protons and containing more than two isotope peaks. Of the whole experiment a matrix was generated, which includes all features with their corresponding intensities and (normalized) abundances. For peptide identification, MS/MS data was searched against the human SwissProt database (version July 23th 2009) using Mascot (version 2.3.01; Matrix Science Inc., London, UK). The following settings were used; enzyme: trypsin, fixed modifications: carbamidomethylation of cysteine (+57.021 u); variable modifications, oxidation of methionine (+15.995 u); peptide mass tolerance, ±10 ppm (#13C = 2); fragment mass tolerance, ±0.5 Da; maximally allowed missed cleavages, 2.

Statistical Analysis

To test for differences in CV of the six proteins between serum and CSF, a paired *t* test was used with a significance level of 0.05. For each peptide of the six proteins identified by the qual/quant method, the CV was calculated. To determine whether there was a significant difference in CV between identified peptides with and without a modification, a potential modification site present (cysteine or methionine), or a missed cleavage, a two-sided *t* test with a significance level of 0.05 was used. To test for the presence of a significant correlation between the CV and the number of amino acids, the intensity or the number of coeluting peptides within 0.1 min time window, the Pearson's correlation was used with a significance level of 0.05. The statistical tests were performed using the SPSS statistical software package (version PASW 17.0.2).

RESULTS

Linearity

The albumin concentrations in cerebrospinal fluid (CSF) measured by qual/quant and quantitative methods were compared with the albumin concentrations determined by an antibody-based method (Figure 1). The regression coefficient (R^2) was taken as a measure for the linearity of the methods. The quantitative method (Figure 1A) showed the best linearity (highest R^2) and the qual/quant method based on the average intensity of all identified peptides of albumin the worst linearity (Figure 1D). The use of an internal standard in the qual/quant method improved this linearity (Figure 1B). The albumin concentrations measured with both the quantitative and the qual/quant methods differed systematically from the antibody-based method with factor of 1.7.

The same methods were applied to determine the concentrations of albumin in serum. The results of these measurements were similar to the results obtained in CSF (see Supporting Information).

Reproducibility

The technical reproducibility of the different methods was determined by calculating the CV for each of the six proteins quantified in one serum and one CSF sample that was repeatedly measured during the sequence (Table 2). The quantitative method showed the lowest CV for all six proteins in serum and CSF expect for complement factor B measured in CSF. The CV calculated for the quantitative method increased when proteins became less abundant. The addition of internal standards to the sample using the qual/quant method resulted in lower CVs for the less abundant proteins, when only the peptides corresponding to the standards were analyzed. The CVs for the qual/quant method using all identified peptides per protein (last column of Table 2) showed lower CVs than the qual/quant methods based on only two peptides, with or without the addition of internal standards. The various methods did not show significant differences in CVs between serum and paired CSF sample, except for the CVs calculated for the qual/quant method where only two selected peptides for quantitation were included (*p*-value 0.029).

CVs for internal standards in just water were calculated for the quantitative method and the qual/quant method (Table 3).

Table 2. CVs for the Level of Six Different Proteins Measured in Serum and CSF by Quantitative and Qual/Quant Methods

proteins in serum	quantitative	qual/quant with standard	qual/quant (2 peptides)	qual/quant (all peptides)
Albumin	0.98	22.24	27.15	6.18
Complement C3	3.22	10.82	22.55	8.95
Vitamin D binding protein	5.50	21.27	26.15	6.61
Complement factor B	5.55	4.18	30.00	9.07
Apolipoprotein E	5.97	10.69	43.22	10.30
Galectin-3 binding protein	12.17	29.07	24.11	34.65
proteins in CSF	**quantitative**	**qual/quant with standard**	**qual/quant (2 peptides)**	**qual/quant (all peptides)**
Albumin	1.49	17.21	12.07	6.01
Complement C3	3.22	8.67	8.03	4.47
Vitamin D binding protein	3.84	7.97	24.16	3.92
Complement factor B	14.63	9.52	8.79	4.30
Apolipoprotein E	2.32	11.06	11.72	3.99
Galectin-3 binding protein	2.32	9.44	22.73	4.88
p-value[a]	0.712	0.188	**0.029***	0.130

[a]p-Value of paired *t* test to check for differences in CVs between serum and CSF (* significant difference $p < 0.05$).

Table 3. CVs for the Internal Standards Measured in Water by the Quantitative Method (Qtrap-platform) and by the Qual/Quant Method (Orbitrap-platform)

standards in water		quantitative	qual/quant
Albumin	SLHTLFGDK	5.82	10.33
	FQNALLVR	2.53	11.64
Apolipoprotein E	LGPLVEQGR	2.84	11.05
	AATVGSLAGQPLQER	2.87	8.97
Complement C3	IWDVVEK	3.21	13.01
	VVLVAVDK	4.62	6.96
Complement factor B	QLNEINYEDHK	4.62	17.30
	ISVIRPSK	2.08	13.07
Galectin-3 binding protein	LADGGATNQGR	3.90	7.51
	YSSDYFQAPSDYR	3.61	9.49
Vitamin D binding protein	VLEPTLK	2.10	9.88
	HLSLLTTLSNR	17.47	13.31

dx.doi.org/10.1021/pr301221f | J. Proteome Res. 2013, 12, 2005–2011

The CVs for the quantitative method varied between 2.08 and 5.82 for 11 of the 12 standards. Peptide HLSLLTTLSNR had a divergent CV of 17.47. For the qual/quant methods the CVs ranged from 6.96 to 17.30.

Influence on Reproducibility in the Qual/Quant Method

In the CSF samples, 242 peptides were identified belonging to the six proteins with an average CV for the normalized intensity of 12.9. The CV's of these peptides were used to determine which factors were of influence on the reproducibility of the qual/quant measurements. We examined the influence of the presence of modifications, the presence of a cysteine or the presence of a methionine and missed cleavages, the length of the peptide (number of amino acids), the number of coeluting peptides within 0.1 min and the intensity of the peptide (Table 4). When the peptide had one or more modifications, or when a cysteine or a methionine were present, a significant increase in the CV was observed (p = 0.005, p = 0.005 and p < 0.001, respectively). A positive correlation was found between the length of the peptide and the CV (Pearson correlation coefficient 0.238, p < 0.001).

Table 4. Influence of Various Factors on the CVs of Identified Peptides using the Orbitrap-platform

factor	CV_{pres}	CV_{abs}	p-value[a]
Modification	18.2	12.0	0.005
Cysteine	16.5	11.5	0.005
Methionine	18.6	11.4	<0.001
Missed cleavage	14.1	12.2	0.25
factor		correlation coefficient	p-value[b]
Number of amino acids		0.238	<0.001
Number of coeluting peptides (within 0.1 min)		−0.054	0.05
Intensity of the peptide		−0.043	0.51

[a]A t test was used to test for differences in CVs of peptides with the presence (CV_{pres}) or absence (CV_{abs}) of the factor (average CVs are listed). [b]For these factors, the presence of a correlation was determined using Pearson's correlation (correlation coefficients are listed).

To confirm the influence of these factors in practice, we used data from two other studies. In these studies both qual/quant (Orbitrap-platform) and quantitative (Qtrap-platform) measurements have been performed. In one study apolipoprotein E levels were determined in 70 serum digests and in the other study haptoglobin levels were determined in 34 CSF digests (unpublished data). In both studies we determined the correlation (R^2) between the intensity measured of each individual peptide using the Orbitrap-platform and the concentration determined using the Qtrap-platform. In Figure 2, the R^2 for the individual peptides are plotted for both the filtered and the unfiltered situation. We observed that the average correlation improved when the peptide list was filtered using the criteria described above and when peptides were removed containing missing values in the qual/quant approach, since these were the peptides with the lowest R^2 values.

DISCUSSION

In this study, we investigated the differences in quantitative performance between a purely quantitative method (Qtrap-platform) and a qual/quant method with and without the use of internal standards and partially cross-validated these methods with an antibody-based technique that is routinely used in clinical chemistry laboratories. The Qtrap-platform is a dedicated system that is optimized for quantification. On the other hand, the settings for the Orbitrap MS were intentionally not optimized for quantitative measurements because this instrument is intended for identification. Yet, we were interested in its quantitative performance next to its (optimized) qualitative performance. Identical samples were used throughout all the measurements to ensure a fair comparison between all quantification methods.

The linearity of the measured albumin concentrations in CSF compared to the antibody-based technique was best for the quantitative method, followed by the qual/quant method with use of internal standards and without use of internal standards. Although the linearity of the qual/quant method without prior knowledge of the identified peptides was the least accurate method, there was still a regression coefficient (R^2) of 0.898 in CSF when compared to the concentrations determined using an antibody-based method. We therefore conclude that qual/quant is a good method to give a first and rather accurate quantitative impression. The factor 1.7 observed between the concentrations measured by MS methods and the antibody-based method can be explained by the amino acid content of the used internal standards, which is in general 60−80% of the

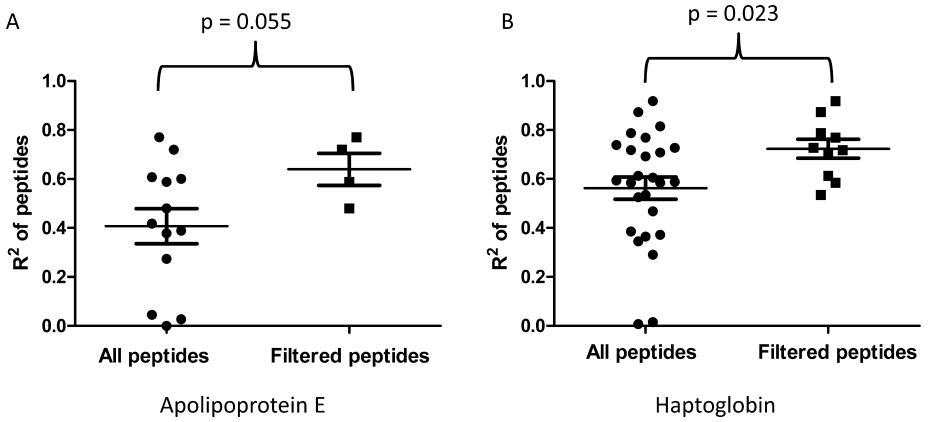


Figure 2. Plot of the correlation coefficients of the concentration determined on the Qtrap-platform and the intensity measured on the Orbitrap-platform for each individual peptide. Plot A shows the values for apolipoprotein E in serum and plot B for haptoglobin in CSF. In the plots the R^2 for all the peptides are shown and the peptides that have been filtered based on the criteria proposed in this manuscript.

dx.doi.org/10.1021/pr301221f | J. Proteome Res. 2013, 12, 2005−2011

weighted mass of the standard. If one corrects for this percentage, the values correlate within acceptable ranges.

When comparing the three qual/quant analysis (with internal standards and without internal standards taken two peptides or all identified peptides into account), the analysis using the internal standards showed a better reproducibility, but the best reproducibility for the qual/quant methods were observed for the analysis performed without internal standards and when all peptides were taken into account, so measurements without prior knowledge of the identified peptides. This was to be expected since the intensity of the protein is dominated by its most intense peptides which are not necessarily the best signature peptides for the protein to be used in a quantitative method.

The CVs were only significantly lower in CSF than in serum when measured by the qual/quant method without internal standards taken only two peptides into account. All other methods also showed lower CVs for CSF but these differences were not significant. To determine the lowest possible CVs, we calculated the CV for the pure internal standards without biological sample added. For the quantitative method, the CVs in water for the highest abundant serum/CSF proteins are in the same range as the CVs for these proteins in serum or CSF. For the qual/quant method on the other hand the CVs in water are clearly lower indicating a larger influence of the other compounds present in the biological sample on the CV.

We examined why peptide measurements by qual/quant approaches typically have a lower reproducibility than peptide measurements using a targeted quantitative approach. To this end, we examined peptide properties that influence digestion and chromatographic separation, as well as the intensity of the measured peptides for their influence on the CV. Factors that showed a significant influence on the CV were the presence of modifications, the presence of a cysteine and the presence of a methionine. The presence of missed cleavages did not have a significant influence per se, but the closely related length of the peptide was of significant influence on the CV. Related to the chromatographic separation the influence of coeluting peptides was examined, which was found not to be significant. The CV was surprisingly also not influenced by the intensity of the peptides. This could be due to the fact that it was based on a very high abundant protein (albumin). With exception of the last two factors mentioned, the described factors are also the factors taken into account when peptides for MRM assays are selected.[18] Applying these criteria to two independent data sets resulted in an improved average correlation between MRM determined protein concentration and average peptide intensity in the qual/quant measurements. In addition, we observed that the peptides with the lowest R^2 were removed from the data set. We therefore conclude that peptides fulfilling the general criteria for peptide selection for MRM are also the most reproducible peptides in qual/quant measurements and that the quantitative performance of qual/quant measurements is significantly improved when these criteria in combination with the removal of peptides with missing values is applied to a data set.

Recently, new instrumentation has become available which is even better suited for qual/quant approaches. Both the new types of Q-TOF instruments and the Q-Orbitrap (Q-Exactive) have improved quantitative capabilities.[19] The advantages of these instruments are that the resolution for both MS and MS/MS is improved, which generates theoretically an additional selectivity. New mass spectrometry methods like all ion fragmentation and MS^E on a high resolution instrument are used on these instruments to perform fragmentation on all peptides present in an MS scan.[20,21] The high resolution of all ion fragment spectra can be used to quantify peptides based on specific fragments. For the quantitation of small molecules a shift is already observed from MRM to qual/quant using high resolution MS.[22,23] At the moment, however, quadrupole ion trap instruments remain the mass spectrometer of choice for quantitation of peptides in complex samples.

The data presented in this manuscript confirms that the most reproducible quantitative results are obtained using a Qtrap-platform. Also in terms of sample throughput this platform outperformed the Orbitrap-platform using the qual/quan method. However, we showed that the quantitative capabilities of a qual/quan approach can considerably be improved by applying previously proposed filtering criteria for peptides and the addition of internal standards. The quantitative performance for the proteins for which internal standards have been spiked in the samples is improved and absolute quantitation for spiked peptides/proteins is possible. The quantitative results obtained from a limited number of proteins can be used for normalization of the samples, to decrease the run-to-run variability in long samples sequences. Additionally, internal standards can be used to monitor the quality of the data in time. Mass spectrometry response, retention time reproducibility and other chromatographic parameters can even be fully automatically determined for each individual sample using the signals of the internal standards. Besides the quantitative data of proteins of interest also a large shotgun proteomics data set remains which can easily be used for reanalyzes of other proteins.

We have shown that qual/quant in combination with modern LC and high resolution measurements could offer an alternative for the quantification of abundant proteins in complex samples. In the near future, we foresee an improvement in specifications of qual/quant methods and qual/quant becoming an equivalent method to pure quantitative methods. The largest challenge in this technology is still to reach the ng/mL range.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +31 10 7044522. Fax: +31 10 7044365. E-mail: l.dekker@erasmusmc.nl.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was sponsored by Eurostars project Pepspex. The isotopic labelled peptides were kindly provided by Pepscan Presto B.V. (Lelystad, The Netherlands). L.D. was supported by the Virgo consortium, funded by the Dutch government project number FES0908, and by the Netherlands Genomics Initiative (NGI) project number 050-060-452.

REFERENCES

(1) Baty, J. D.; Robinson, P. R. Single and multiple ion recording techniques for the analysis of diphenylhydantoin and its major metabolite in plasma. *Biomed. Mass Spectrom.* **1977**, *4* (1), 36−41.

(2) Gallien, S.; Duriez, E.; Domon, B. Selected reaction monitoring applied to proteomics. *J. Mass Spectrom.* **2011**, *46* (3), 298−312.

(3) Anderson, L.; Hunter, C. L. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol. Cell. Proteomics* **2006**, *5* (4), 573−88.

(4) Ong, S. E.; Blagoev, B.; Kratchmarova, I.; Kristensen, D. B.; Steen, H.; Pandey, A.; Mann, M. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* **2002**, *1* (5), 376−86.

(5) Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **1999**, *17* (10), 994−9.

(6) DeSouza, L. V.; Taylor, A. M.; Li, W.; Minkoff, M. S.; Romaschin, A. D.; Colgan, T. J.; Siu, K. W. Multiple reaction monitoring of mTRAQ-labeled peptides enables absolute quantification of endogenous levels of a potential cancer marker in cancerous and normal endometrial tissues. *J. Proteome Res.* **2008**, *7* (8), 3525−34.

(7) Yao, X.; Freas, A.; Ramirez, J.; Demirev, P. A.; Fenselau, C. Proteolytic 18O labeling for comparative proteomics: model studies with two serotypes of adenovirus. *Anal. Chem.* **2001**, *73* (13), 2836−42.

(8) Gerber, S. A.; Rush, J.; Stemman, O.; Kirschner, M. W.; Gygi, S. P. Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (12), 6940−5.

(9) IJsselstijn, L.; Dekker, L. J.; Koudstaal, P. J.; Hofman, A.; Sillevis Smitt, P.; Breteler, M. M.; Luider, T. Serum clusterin levels are not increased in presymptomatic Alzheimer's disease. *J. Proteome Res.* **2011**, *10* (4), 2006−10.

(10) Addona, T. A.; Abbatiello, S. E.; Schilling, B.; Skates, S. J.; Mani, D. R.; Bunk, D. M.; Spiegelman, C. H.; Zimmerman, L. J.; Ham, A. J.; Keshishian, H.; Hall, S. C.; Allen, S.; Blackman, R. K.; Borchers, C. H.; Buck, C.; Cardasis, H. L.; Cusack, M. P.; Dodder, N. G.; Gibson, B. W.; Held, J. M.; Hiltke, T.; Jackson, A.; Johansen, E. B.; Kinsinger, C. R.; Li, J.; Mesri, M.; Neubert, T. A.; Niles, R. K.; Pulsipher, T. C.; Ransohoff, D.; Rodriguez, H.; Rudnick, P. A.; Smith, D.; Tabb, D. L.; Tegeler, T. J.; Variyath, A. M.; Vega-Montoto, L. J.; Wahlander, A.; Waldemarson, S.; Wang, M.; Whiteaker, J. R.; Zhao, L.; Anderson, N. L.; Fisher, S. J.; Liebler, D. C.; Paulovich, A. G.; Regnier, F. E.; Tempst, P.; Carr, S. A. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat. Biotechnol.* **2009**, *27* (7), 633−41.

(11) Domon, B.; Aebersold, R. Options and considerations when selecting a quantitative proteomics strategy. *Nat. Biotechnol.* **2010**, *28* (7), 710−21.

(12) de Groot, C. J.; Guzel, C.; Steegers-Theunissen, R. P.; de Maat, M.; Derkx, P.; Roes, E. M.; Heeren, R. M.; Luider, T. M.; Steegers, E. A. Specific peptides identified by mass spectrometry in placental tissue from pregnancies complicated by early onset preeclampsia attained by laser capture dissection. *Proteomics Clin. Appl.* **2007**, *1* (3), 325−35.

(13) Guzel, C.; Ursem, N. T.; Dekker, L. J.; Derkx, P.; Joore, J.; van Dijk, E.; Ligtvoet, G.; Steegers, E. A.; Luider, T. M. Multiple reaction monitoring assay for pre-eclampsia related calcyclin peptides in formalin fixed paraffin embedded placenta. *J Proteome Res* **2011**, *10* (7), 3274−82.

(14) IJsselstijn, L.; Dekker, L. J.; Stingl, C.; van der Weiden, M. M.; Hofman, A.; Kros, J. M.; Koudstaal, P. J.; Sillevis Smitt, P. A.; Ikram, M. A.; Breteler, M. M.; Luider, T. M. Serum levels of pregnancy zone protein are elevated in presymptomatic Alzheimer's disease. *J Proteome Res* **2011**, *10* (11), 4902−10.

(15) Stoop, M. P.; Coulier, L.; Rosenling, T.; Shi, S.; Smolinska, A. M.; Buydens, L.; Ampt, K.; Stingl, C.; Dane, A.; Muilwijk, B.; Luitwieler, R. L.; Sillevis Smitt, P. A.; Hintzen, R. Q.; Bischoff, R.; Wijmenga, S. S.; Hankemeier, T.; van Gool, A. J.; Luider, T. M. Quantitative proteomics and metabolomics analysis of normal human cerebrospinal fluid samples. *Mol. Cell. Proteomics* **2010**, *9* (9), 2063−75.

(16) Stoop, M. P.; Singh, V.; Dekker, L. J.; Titulaer, M. K.; Stingl, C.; Burgers, P. C.; Sillevis Smitt, P. A.; Hintzen, R. Q.; Luider, T. M. Proteomics comparison of cerebrospinal fluid of relapsing remitting and primary progressive multiple sclerosis. *PLoS One* **2010**, *5* (8), e12442.

(17) MacLean, B.; Tomazela, D. M.; Shulman, N.; Chambers, M.; Finney, G. L.; Frewen, B.; Kern, R.; Tabb, D. L.; Liebler, D. C.; MacCoss, M. J. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* **2010**, *26* (7), 966−8.

(18) Han, B.; Higgs, R. E. Proteomics: from hypothesis to quantitative assay on a single platform. Guidelines for developing MRM assays using ion trap mass spectrometers. *Brief Funct. Genomic Proteomic* **2008**, *7* (5), 340−54.

(19) Gallien, S.; Duriez, E.; Crone, C.; Kellmann, M.; Moehring, T.; Domon, B. Targeted proteomic quantification on quadrupole-orbitrap mass spectrometer. *Mol. Cell. Proteomics* **2012**, *11* (12), 1709−23.

(20) Geiger, T.; Cox, J.; Mann, M. Proteomics on an Orbitrap benchtop mass spectrometer using all-ion fragmentation. *Mol. Cell. Proteomics* **2010**, *9* (10), 2252−61.

(21) Levin, Y.; Hradetzky, E.; Bahn, S. Quantification of proteins using data-independent analysis (MSE) in simple and complex samples: a systematic evaluation. *Proteomics* **2011**, *11* (16), 3273−87.

(22) Ramanathan, R.; Jemal, M.; Ramagiri, S.; Xia, Y. Q.; Humpreys, W. G.; Olah, T.; Korfmacher, W. A. It is time for a paradigm shift in drug discovery bioanalysis: from SRM to HRMS. *J. Mass Spectrom.* **2011**, *46* (6), 595−601.

(23) Kaufmann, A.; Butcher, P.; Maden, K.; Walker, S.; Widmer, M. Quantitative and confirmative performance of liquid chromatography coupled to high-resolution mass spectrometry compared to tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2011**, *25* (7), 979−92.