Technical note

The effect of peptide adsorption on signal linearity and a simple approach to improve reliability of quantification

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**Abstract**

Peptide quantification using MS often relies on the comparison of peptide signal intensities between different samples, which is based on the assumption that observed signal intensity has a linear relationship to peptide abundance. A typical proteomics experiment is subject to multiple sources of variance, so we focussed here on properties affecting peptide linearity under simple, well-defined conditions. Peptides from a standard protein digest were analysed by multiple reaction monitoring (MRM) MS to determine peptide linearity over a range of concentrations. We show that many peptides do not display a linear relationship between signal intensity and amount under standard conditions. Increasing the organic content of the sample solvent increased peptide linearity by increasing the accuracy and precision of quantification, which suggests that peptide non-linearity is due to concentration-dependent surface adsorption. Using multiple peptides at various dilutions, we show that peptide non-linearity is related to observed retention time and predicted hydrophobicity. Whereas the effect of adsorption on peptide storage has been investigated previously, here we demonstrate the deleterious effect of peptide adsorption on the quantification of fresh samples, highlight aspects of sample preparation that can minimise the effect, and suggest bioinformatic approaches to enhance the selection of peptides for quantification.

**Biological significance**

Accurate quantification is central to many aspects of science, especially those examining dynamic processes or comparing molecular stoichiometries. In biological research, the quantification of proteins is an important yet challenging objective. Large-scale quantification of proteins using MS often depends on the comparison of peptide intensities with only a single-level calibrant (as in stable isotope labelling and absolute quantification).
There are many published MS-based approaches for peptide quantification [1–10]. Most of these methodologies rely on the comparison of peptide signal intensities between different samples. Quantification approaches include isotopic labelling, which can be performed at the MS (precursor) or MS/MS level of analysis (e.g. stable isotope labelling by amino acids in cell culture [1] or iTRAQ [2], respectively), allowing samples under comparison to be combined and analysed in a single MS run. Alternatively, label-free quantification involves the comparison of samples analysed in sequential MS runs, which avoids an unfavourable increase in sample complexity [3–5]. Quantification may be targeted, whereby specific target peptides are selected beforehand and quantified, commonly using optimised parameters [6–8]. Targeted quantification may be performed at the MS level of analysis, using extracted ion chromatogram data to monitor the signal of the peptide precursor [9]. Alternatively, some targeted approaches rely on quantification at the MS/MS level of analysis, performed with or without isotopic labelling, such as MRM [10]. For both labelled and label-free approaches, successful peptide quantification requires that the signal intensity observed for a specific peptide or fragment ion has a linear relationship to the abundance of that species in the sample tested. This requirement is of particular relevance to many proteomics methodologies, which do not use calibration curves but instead rely on a single-point calibration. This study tests this critical assumption, demonstrates its impact on the reliability of peptide quantification by MS and provides a simple solution to overcome peptide non-linearity that is compatible with mainstream proteomic approaches.

There are many factors that affect variability in a typical proteomics experiment [11,12]. To reduce the effects of variables that would otherwise confound the interpretation of the relationship between peptide amount and observed signal, we used a low-complexity, commercially available standard in all tests. To test a simple approach to reduce surface adsorption of peptides and improve peptide quantification, we diluted the six-protein mix digest in 0.1% (v/v) formic acid containing 2.5% (v/v) or 5% (v/v) ACN. We used ACN because this organic modifier is routinely used for LC separations upstream of MS analysis, and it results in reduced ion suppression compared to aprotic solvents such as DMSO. The maximum concentration of ACN tested was 5% (v/v) in order to reduce loss of hydrophobic peptides during LC separation. All samples were analysed identically and in triplicate. The addition of 2.5% (v/v) ACN increased the observed linearity of detection of all peptides, which increased further in the presence of 5% (v/v) ACN (Fig. 1D–F; Supporting Information Fig. S1; Supporting
Fig. 1 – Relationship between peptide amount on column and signal intensity measured by MRM MS. (A–C) Relative signal intensity is displayed for the three transitions measured for each of three BSA peptides in 0.1% (v/v) formic acid (A), 2.5% (v/v) ACN (B) or 5% (v/v) ACN (C). Peptide sequences are indicated. (D–F) Relative signal intensity is displayed for all 54 transitions measured for all selected peptides in 0.1% (v/v) formic acid (D), 2.5% (v/v) ACN (E) or 5% (v/v) ACN (F). All measurements were acquired in triplicate; mean points are plotted, and error bars represent SD. Lines are shaded transparent grey to visualise overlapping lines.

Fig. 2 – Effect of organic modifier on accuracy and precision of peptide quantification by MS. (A) Distribution of $R^2$ values of all peptides in the presence or absence of ACN. $R^2$ values were calculated from the signal intensities of each transition as a function of peptide amount on column using the least-squared method. Mean $R^2$ values were generated for each peptide. (B) Distribution of RSD values of all transitions in the presence or absence of ACN. RSD values were calculated from the signal intensities for each transition. (C and D) Mean $R^2$ values of all peptides in the presence or absence of ACN were compared to the hydrophobicity of each peptide as determined by the LC retention time (C) or the GRAVY score (D). More hydrophobic peptides have a longer retention time and a higher GRAVY score; more hydrophilic peptides have a shorter retention time and a lower GRAVY score.
Information Fig. S2). The proportion of peptides with an $R^2$ value of 0.95 or less was reduced from 35% in formic acid alone to 0% in the presence of 5% (v/v) ACN, whereas the proportion of peptides with an $R^2$ value of greater than 0.99 was increased from 35% in formic acid alone to 83% in the presence of 5% (v/v) ACN (Fig. 2A). The addition of 5% (v/v) ACN was sufficient to correct the linearity of detection of the majority of peptides tested, although the most hydrophobic of peptides may require higher concentrations of ACN. These data indicate that, in the presence of organic solvent, the peptide mixtures were more accurately quantified. Furthermore, the proportion of measurements with an RSD of 5% or less was 41% in formic acid alone, compared to 67% in the presence of 5% (v/v) ACN (Fig. 2B). This indicates that the presence of organic modifier increased the precision of measurements by MS.

Peptide hydrophobicity has been shown to influence the detectability of peptides using MS [19–22], but its effect on accurate quantification is not clear. To examine the relationship between peptide hydrophobicity and non-linearity of detection, we calculated a number of parameters of peptide properties. The LC retention time of each peptide was recorded as an indicator of hydrophobicity; peptides with longer retention times were considered to be more hydrophobic [23]. In addition, the GRAVY score [24] of each peptide was calculated using the ProtParam tool (http://expasy.org/tools/protparam.html) on the expert protein analysis system proteomics server from the Swiss Institute of Bioinformatics [25]. Notably, the more hydrophobic peptides (with longer retention times and higher GRAVY scores) generally had lower linearity of detection as determined by lower $R^2$ values (Fig. 2C, D). Moreover, the linearity of detection of all peptides was increased in the presence of organic solvent, including the most hydrophobic peptides. Six out of seven peptides with a GRAVY score less than 0.1 had an $R^2$ value of greater than 0.99 in the presence of 2.5% (v/v) ACN, whereas peptides with a GRAVY score greater than 0.1 generally had a lower $R^2$ value (Fig. 2D). In the presence of 5% (v/v) ACN, 15 out of 18 peptides had an $R^2$ value of greater than 0.99 (Fig. 2C, D). Our data suggest that more hydrophilic peptides should be selected for MS studies in order to provide more reliable quantification. Of the peptides tested here, the addition of 2.5% (v/v) ACN was sufficient to allow reliable quantification of peptides with a GRAVY score of less than 0.1. Increasing the concentration of ACN to 5% (v/v) allowed the majority of peptides to be reliably quantified. These data show that observed peptide retention time and predicted peptide hydrophobicity provide an indication of reliability for linearity of detection, which may direct the selection of peptides for reliable quantification by MS. Computational tools have been developed to predict readily detectable peptides as surrogates for protein quantification [19–22], but such tools do not assess the reliability of peptide quantification. We propose that the evaluation of peptide hydrophobicity could be used as a predictor of the linearity of peptide detection and thus provide a useful selection criterion for peptides most likely to be reliably quantified in targeted MS experiments. Furthermore, we present a general method for reducing non-linearity of peptide detection, which provides a straightforward solution to improve the reliability of peptide quantification in MS-based quantification studies.

**Conflict of interest**

The authors declare no conflict of interest.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.04.034.

**REFERENCES**

[1] Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, et al. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics 2002;1:376–86.

[2] Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, et al. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 2004;3:1154–69.

[3] Wang W, Zhou H, Lin H, Roy S, Shaler TA, Hill LR, et al. Quantification of proteins and metabolites by mass spectrometry without isotopic labeling or spiked standards. Anal Chem 2003;75:4818–26.

[4] Liu H, Sadygov RG, Yates III JR. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem 2004;76:4193–201.

[5] Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, et al. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics 2005;4:1265–72.

[6] Kirkpatrick DS, Gerber SA, Gygi SP. The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications. Methods 2005;35:265–73.

[7] Domon B, Aebersold R. Challenges and opportunities in proteomics data analysis. Mol Cell Proteomics 2006;5:1921–6.
Wolf-Yadlin A, Hautaniemi S, Lauffenburger DA, White FM. Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. Proc Natl Acad Sci U S A 2007;104:5860-5.

Chelius D, Bondarenko PV. Quantitative profiling of proteins in complex mixtures using liquid chromatography and mass spectrometry. J Psychiatr Res 2002;1:317–23.

Anderson L, Hunter CL. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. Mol Cell Proteomics 2006;5:573–88.

Russell MR, Lilley KS. Pipeline to assess the greatest source of technical variance in quantitative proteomics using metabolic labelling. J Proteomics 2012;77:441–54.

Piehowski PD, Petyuk V, Orton DJ, Xie F, Moore RJ, Ramirez-Restrepo M, et al. Sources of technical variability in quantitative LC-MS proteomics: human brain tissue sample analysis. J Proteome Res 2013;12:2128–37.

Horinek D, Serr A, Geisler M, Pirzer T, Slotta U, Lud SQ, et al. Peptide adsorption on a hydrophobic surface results from an interplay of solvation, surface, and intrapeptide forces. Proc Natl Acad Sci U S A 2008;105:2842–7.

John H, Walden M, Schäfer S, Genz S, Forssmann WG. Analytical procedures for quantification of peptides in pharmaceutical research by liquid chromatography–mass spectrometry. Anal Bioanal Chem 2004;378:883–97.

Van Midwoud PM, Rieux L, Bischoff R, Verpoorte E, Niederländer HA. Improvement of recovery and repeatability in liquid chromatography–mass spectrometry analysis of peptides. J Proteome Res 2007;6:781–91.

Pezeshki A, Vergote V, Van Dorpe S, Baert B, Burvenich C, Popkov A, et al. Adsorption of peptides at the sample drying step: influence of solvent evaporation technique, vial material and solution additive. J Pharm Biomed Anal 2009;49:607–12.

Hyenstrand P, Metcalf JS, Beattie KA, Codd GA. Effects of adsorption to plastics and solvent conditions in the analysis of the cyanobacterial toxin microcystin-LR by high performance liquid chromatography. Water Res 2001;35:3508–11.

Kraut A, Marcellin M, Adrait A, Kuhn L, Louwagie M, Kieffer-Jaquinod S, et al. Peptide storage: are you getting the best return on your investment? Defining optimal storage conditions for proteomics samples. J Proteome Res 2009;8:3778–85.

Mallick P, Schirle M, Chen SS, Flory MR, Lee H, Martin D, et al. Computational prediction of proteotypic peptides for quantitative proteomics. Nat Biotechnol 2007;25:125–31.

Sanders WS, Bridges SM, McCarthy FM, Nanduri B, Burgess SC. Prediction of peptides observable by mass spectrometry applied at the experimental set level. BMC Bioinforma 2007;8:S23.

Fusaro VA, Mani DR, Mesirov JP, Carr SA. Prediction of high-responding peptides for targeted protein assays by mass spectrometry. Nat Biotechnol 2009;27:190–8.

Eyers CE, Lawless C, Wedge DC, Lau KW, Gaskell SJ, Hubbard SJ. CONSeQuence: prediction of reference peptides for absolute quantitative proteomics using consensus machine learning approaches. Mol Cell Proteomics 2011;10 [M110.003384].

Meek JL. Prediction of peptide retention times in high-pressure liquid chromatography on the basis of amino acid composition. Proc Natl Acad Sci U S A 1980;77:1632–6.

Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. J Mol Biol 1982;157:105–32.

Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res 2003;31:3784–8.