**TECHNICAL BRIEF**

**GOAT – A simple LC-MS/MS gradient optimization tool**

*David C. Trudgian¹*, Roman Fischer²*, Xiaofeng Guo¹**, Benedikt M. Kessler² and Hamid Mirzaei¹

¹ Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX, USA
² Target Discovery Institute, Nuffield Department of Medicine, University of Oxford, Oxford, UK

Modern nano-HPLC systems are capable of extremely precise control of solvent gradients, allowing high-resolution separation of peptides. Most proteomics laboratories use a simple linear analytical gradient for nano-LC-MS/MS experiments, though recent evidence indicates that optimized non-linear gradients result in increased peptide and protein identifications from cell lysates. In concurrent work, we examined non-linear gradients for the analysis of samples fractionated at the peptide level, where the distribution of peptide retention times often varies by fraction. We hypothesized that greater coverage of these samples could be achieved using per-fraction optimized gradients. We demonstrate that the optimized gradients improve the distribution of peptides throughout the analysis. Using previous generation MS instrumentation, a considerable gain in peptide and protein identifications can be realized. With current MS platforms that have faster electronics and achieve shorter duty cycle, the improvement in identifications is smaller. Our gradient optimization method has been implemented in a simple graphical tool (GOAT) that is MS-vendor independent, does not require peptide ID input, and is freely available for non-commercial use at http://proteomics.swmed.edu/goat/

**Keywords:**
Bioinformatics / Gradient / LC-MS/MS / Liquid chromatography / Optimization / Separation

Additional supporting information may be found in the online version of this article at the publisher’s web-site

The introduction of non-split flow nano-HPLC systems to LC-MS/MS proteomics workflows has allowed improved coverage of samples via high-resolution separation of peptides. Modern nano-HPLC systems are capable of accurately delivering extended solvent gradients at low-flow rates and high pressures through long capillary columns packed with low pore-size resins [1]. These capabilities increase the peak capacity of the online reversed-phase (RP) LC separation, simplifying the mixture of peptides delivered to the mass-spectrometer at a point in time during an LC-MS/MS analysis and allowing deeper coverage of the sample [2]. The accuracy of solvent delivery for very shallow gradients in the nanoliter per minute flow range using modern binary pumps allows multi-hour LC-MS/MS analyses to be performed successfully. Various studies have made use of gradients up to 6 h in length to identify thousands of proteins from unfraccionated lysates [3–5].

Despite the improvements in coverage of unfraccionated samples resulting from LC and MS advances, fractionation prior to MS analysis is required for deepest coverage. This pre-fractionation is commonly performed at the peptide-level, after the sample has been digested. Increased coverage is due to the fact that the pre-fractionation method employed is orthogonal to the RP separation in the LC-MS/MS analysis. Techniques commonly applied include column-based methods such as strong-anion exchange (SAX), strong-cation exchange, and high-pH RP [6], as well as IEF [7]. The resulting fractions are subjected to LC-MS/MS typically in the same manner as unfraccionated samples—using a simple linear gradient.

*These authors contributed equally to this work.

**Current address: Dr. Xiaofeng Guo, Center for Sleep, University of Pennsylvania, 124 South 31st Street, Pennsylvania, PA 1904-3403, USA

Correspondence: Dr. H. Mirzaei, Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390–9038, USA
E-mail: Hamid.Mirzaei@UTSouthwestern.edu
Fax: +1-214-648-3524

Abbreviation: SAX, strong-anion exchange
Hydrophobicity bias in peptide-level fractionation. A 14-lysate digest was fractionated using a high-pH RP fractionation of Jurkat cell lysate that was not distributed evenly. Flow-through and elutions using 10, 20, and 50% ACN in ammonium formate at pH10. Conditions were chosen with reference to Gilar et al. [10]. LC-MS/MS analysis was performed using a nanoAcquity UPLC system (Waters, Milford, MA, USA), coupled to an LTQ-Orbitrap Velos or a U3000-RLSCnano system (Dionex, Sunnyvale, CA, USA) on a Q Exactive mass-spectrometer (Thermo Fisher, Bremen). Data were acquired using a standard analytical gradient of 0–40% ACN in 60 or 120 min (see Supporting Information Methods for details). In the second laboratory, at UT Southwestern Medical Center, a separate HeLa digest was fractionated using a tip-based SAX protocol into eight fractions with elution at pH 2, 3, 4, 6, 8, and 11, and finally 80% ACN. LC-MS/MS analysis was performed using a U3000-RLSCnano system coupled to an Orbitrap Elite MS (Thermo Fisher, Bremen). The standard analytical gradient was 2–25% ACN in 100 min. Full details of fractionation and MS are given in the Supporting Information Methods. The diversity of pre-fractionation methods and mass-spectrometers covers some of the most common methods and platforms used in proteomics facilities, demonstrating that GOAT is useful for a variety of laboratories and workflows.

Based on data acquired from the standard gradients, optimised gradients were computed using our GOAT tool. Our intention was to create a tool that did not require MS1 peak-picking or peptide-identification input to drive gradient optimization. MS1 peak-picking can accurately identify peptide precursor ions visible in a sample, whether or not they have been identified. However, peak-picking algorithms are computationally complex and must be optimized for the characteristics of different MS instruments [11]. Peptide identification information for gradient optimization can be obtained by searching data acquired on a standard gradient. However, peptide ID results are produced by different search engines in a variety of formats, requiring multiple import filters or manual manipulation to use in a generic optimization tool. Additionally, many workflows do not report retention times for peptides in their output. In silico gradient optimization, as performed by Moruz et al. would be complex for fractionated or enriched samples, as the set of peptides used for per-fraction optimization must accurately reflect the separation or enrichment employed.

To obtain a simple set of points for optimization, we make the assumption that a certain percentage of spectra in an LC-MS/MS run can be identified (i), and that these identifiable
MS/MS spectra have the highest TICs in the sample. To create an optimized gradient, we extract the retention time for all MS/MS scans and then filter this set to the highest 5% by TIC. The MS retention time is mapped to a% solvent B value using the user-provided standard gradient, LC dead-volume delay, and an optional MS start delay. The result is a list of N MS/MS spectra and the% solvent B at which they eluted. A non-linear gradient is constructed from linear steps, at intervals of T minutes. The% solvent B at each step is chosen so that all steps contain an equal fraction of the N MS/MS spectra acquired in the standard gradient. This MS/MS scan based optimization allows gradient optimization to be performed extremely quickly, without the need for peak-picking optimization or peptide IDs.

To create an optimized gradient with our GOAT tool, the user must provide a raw format or MzXML/MzML data file generated using a standard gradient, details of that gradient, and the LC dead volume delay. Note that Moruz et al. provided a simple procedure to obtain an accurate value for the LC dead volume delay. Three optimization parameters can be adjusted, with sensible defaults (used in this study) provided. MS/MS scans with precursor charge states below a specified charge can be excluded from the optimization. By default, we exclude singly charged precursors as many are chemical contaminants. The user may also specify a value for i—the percentage of MS/MS spectra typically identified. Here we used 50%, which is a typical value for complex lysate fractions on Orbitrap and Q Exactive instruments in our experience. The resolution of the optimization T can also be altered and is 5 min by default. The optimization procedure is illustrated in Fig. 2A, with the standard and optimized gradient shown in Fig. 2B for the pH3 elution of the SAX separation. Our software uses the Proteowizard MSAccess utility [12] to extract scan information from raw files in a vendor-independent manner.

To assess the improvement offered by gradient optimization, we analyzed each dataset using our in-house central proteomics facilities pipeline [13]. Briefly, all data were searched against the UniProtKB whole proteome sequence database (release 2012_04), considering up to three missed cleavages, and oxidation of Met as a variable modification. Ion tolerances were 20 ppm for precursors and 0.1 Da or 0.5 Da for fragments on Q Exactive and Orbitrap data, respectively. Peptide to spectrum match, unique peptide sequence, and protein group counts were obtained at a 1% FDR using the concatenated target-decoy method [14]. Full details of data processing, protein identifications, and gradients are given for each dataset in the Supporting Information Methods and Supporting Information Tables 1-15.

GOAT optimization of gradients improves the distribution of peptides throughout the length of the analysis, as seen in Fig. 3A, which displays base-peak chromatograms for the pH 3 SAX elution. In the standard gradient, the majority of material elutes close to the end of the run, while the GOAT gradient makes use of a far greater portion of the analysis. Figure 3B plots the increase in peptide to spectrum matches (total spectral counts), and protein groups for each dataset. We observed the greatest increase in identifications for short 1-h gradients on previous generation Orbitrap Velos instrumentation, and the smallest improvement for data acquired using a current Q Exactive instrument. The magnitude of the improvement correlates with the instrument duty cycle (Supporting Information Fig. 1). The slower Orbitrap Velos undersamples on the standard gradient, and identifications increase when peptides are distributed more evenly through the runs. The Q Exactive instrument is sampling sufficiently fast that there is little improvement in IDs. The Orbitrap Elite has a slightly longer duty cycle than the Q Exactive, and shows some increase in peptide and protein ID. These findings are in agreement with Moruz et al. who observed greater
improvements in unfractionated lysate for 2-h gradients on slower LTQ-Orbitrap XL instrumentation than 4-h gradients using a Q Exactive.

Qualitatively, our results suggest that optimized gradients are beneficial for the analysis of peptide-fractionated samples, particularly on slower duty cycle mass spectrometers. We have demonstrated that by using only MS/MS retention time and TIC information, we can successfully increase peptide and protein identifications. Though it is not practical to run standard and optimized gradients for all samples, GOAT may be used as part of the development of a standardized pre-fractionation method. Working from a standard protein mixture, optimized gradients can be developed for each fraction, which can then be re-used on similar experimental samples. Laboratories may use GOAT to assemble a library of gradients for each pre-fractionation approach employed, and use these gradients without the need to perform optimization on each sample. On slower instrumentation, such as the Orbitrap Velos and earlier, significant gains in peptide and protein identification can be achieved. Despite the low ID improvement on more recent faster instrumentation, we believe the method would be applicable to experiments where enrichment methods deliver samples that are heavily biased toward hydrophobic or hydrophilic peptides. There are also applications in targeted proteomics: in complex scheduled SRM studies, which assay a large number of peptides, elution must be uniform throughout the run to minimize duty cycle. Gradient optimization from shotgun data may be useful for this task. Outside of proteomics, GOAT can be used to optimize the separation of any mixture of molecules studied in a data-dependent LC-MS/MS experiment. Since gradient optimization is based on acquired MS/MS scan timings and TIC, it is not specific to peptides, and could be applied to metabolites, lipids, etc.

Our GOAT software is simple to use. It is a graphical application for Windows XP and above, which requires the .NET framework (v4) and an installation of the ProteoWizard tools with vendor support. Because optimization is performed using MS/MS scan information, our tool is instrument and vendor independent. The simplicity of the procedure allows gradients to be generated quickly from large RAW files. All parameters are adjusted in the application via simple controls. Gradients are visualized within GOAT and can be copied to the clipboard for transfer into LC control software. Tab-separated and Dionex Chromeleon command formats are supported. Download at: http://proteomics.swmed.edu/goat/

DCT and HM are supported by Cancer Prevention and Research Institute of Texas Grants RP120613 and R1121 (to HM). RF is supported by Action Medical Research and a British Medical Association award to Paul Bowness and BMK. BMK is supported by a Biomedical Research Centre (NIHR) RCF, and a John Fell OUP Award. We acknowledge the Computational Biology Research Group, Nuffield Department of Medicine, Oxford for use of their services in this project. We thank Luminita Moruz and Lukas Käll for their helpful discussion.

The authors have declared no conflict of interest.

References

[1] Ye, M. L., Jiang, X. G., Feng, S., Tian, R. J., Zou, H. F., Advances in chromatographic techniques and methods in shotgun proteome analysis. Trac-Trend. Anal. Chem. 2007, 26, 80–84.
[2] Liu, H., Finch, J. W., Lavallee, M. J., Collamati, R. A. et al., Effects of column length, particle size, gradient length and flow rate on peak capacity of nano-scale liquid chromatography for peptide separations. J. Chromatogr. A 2007, 1147, 30–36.
[3] Thakur, S. S., Geiger, T., Chatterjee, B., Bandilla, P. et al., Deep and highly sensitive proteome coverage by LC-MS/MS without prefractionation. Mol. Cell. Proteomics 2011, 10, M110 003699.
[4] Nagaraj, N., Kulak, N. A., Cox, J., Neuhauer, N. et al., System-wide perturbation analysis with nearly complete
coverage of the yeast proteome by single-shot ultra HPLC runs on a bench top Orbitrap. Mol. Cell. Proteomics 2012, 11, M111.013722.

[5] Cristobal, A., Hennrich, M. L., Giansanti, P., Goerdalay, S. S. et al., In-house construction of a UHPLC system enabling the identification of over 4000 protein groups in a single analysis. Analyst 2012, 137, 3541–3548.

[6] Nakamura, T., Kuromitsu, J., Oda, Y., Evaluation of comprehensive multidimensional separations using reversed-phase, reversed-phase liquid chromatography/mass spectrometry for shotgun proteomics. J. Proteome Res. 2008, 7, 1007–1011.

[7] Essader, A. S., Cargile, B. J., Bundy, J. L., Stephenson, J. L., A comparison of immobilized pH gradient isoelectric focusing and strong-cation-exchange chromatography as a first dimension in shotgun proteomics. Proteomics 2005, 5, 24–34.

[8] Gilar, M., Olivova, P., Daly, A. E., Gebler, J. C., Orthogonality of separation in two-dimensional liquid chromatography. Anal. Chem. 2005, 77, 6426–6434.

[9] Moruz, L., Pichler, P., Stranzl, T., Mechtler, K., Kall, L., Optimized nonlinear gradients for reversed-phase liquid chromatography in shotgun proteomics. Anal. Chem. 2013, 85, 7777–7785.

[10] Gilar, M., Olivova, P., Daly, A. E., Gebler, J. C., Two-dimensional separation of peptides using RP-RP-HPLC system with different pH in first and second separation dimensions. J. Sep. Sci. 2005, 28, 1694–1703.

[11] Hoopmann, M. R., Finney, G. L., MacCoss, M. J., High-speed data reduction, feature detection and MS/MS spectrum quality assessment of shotgun proteomics data sets using high-resolution mass spectrometry. Anal. Chem. 2007, 79, 5620–5632.

[12] Kessner, D., Chambers, M., Burke, R., Aguasand, D., Mallick, P., ProteoWizard: open source software for rapid proteomics tools development. Bioinformatics 2008, 24, 2534–2536.

[13] Trudgian, D. C., Thomas, B., McGowan, S. J., Kessler, B. M. et al., CPFP: a central proteomics facilities pipeline. Bioinformatics 2010, 26, 1131–1132.

[14] Elias, J. E., Gygi, S. P., Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat. Meth. 2007, 4, 207–214.