RapiGest precipitation depends on peptide concentration

Peter R. Mosen | Robert Hardt | Dominic Winter

Institute for Biochemistry and Molecular Biology, Medical Faculty, University of Bonn, Bonn, North Rhine-Westphalia, Germany

Correspondence
Dominic Winter, Institute for Biochemistry and Molecular Biology, Medical Faculty, Rheinische Friedrich-Wilhelms-University of Bonn, Nussallee 11, 53115 Bonn, NRW, Germany.
Email: dominic.winter@uni-bonn.de

Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: FOR2625

Abstract
The mass spectrometry-compatible surfactant RapiGest promotes the enzymatic digestion of proteins by facilitating their unfolding while retaining enzymatic activity. RapiGest consists of a hydrophilic head and a hydrophobic tail, which can be separated by acid hydrolysis. This allows for removal of RapiGest prior to mass spectrometric analysis via precipitation and solid phase extraction. During in-solution digestion experiments with RapiGest, we noticed a high variability in the formation of precipitates after acid hydrolysis, implying that RapiGest precipitation is sample-dependent. We show that RapiGest hydrolyses efficiently under acidic conditions and that differences in precipitation are solely due to protein/peptide concentration. Furthermore, we demonstrate that RapiGest precipitation can be triggered by the addition of intact proteins, providing a strategy for its efficient removal from highly diluted samples. Data are available via ProteomeXchange with identifier PXD025982.

KEYWORDS
in solution digestion, precipitation, proteomics, RapiGest, surfactant

1 INTRODUCTION

For the analysis of proteins by mass spectrometry (MS)-based proteomics, the vast majority of studies utilizes the so-called bottom-up approach, for which one of the key steps is the generation of peptides by proteolytic digestion [1]. Over the years, a large variety of digestion strategies has been developed. In principle, three types of approaches can be distinguished: in gel digestion [2], on surface digestion (beads, e.g., single-pot solid-phase-enhanced sample preparation, SP3 [3]; or membranes [4]), and in solution digestion (either in tubes [5]; spin filters, e.g., filter aided sample preparation, FASP [6]; or tips, e.g., in-StageTip, iST [7]). Especially for the preparation of complex samples, such as whole cell lysates, on bead, and in solution digestion approaches are used frequently. For such experiments, many protocols include a step for denaturing of samples in order to provide proteases access to the respective cleavage sites. A crucial factor for these protocols is that the compounds used for denaturation must not interfere with the following MS analyses [3,6]. Therefore, in solution digestion strategies frequently employ denaturing agents which can be separated from the generated peptides. Commonly used agents are either chaotropes, such as urea [5,8–10] or guanidinium chloride (GCl) [9], as well as MS-compatible removable surfactants, such as deoxycholate [8,9], ProteaseMAX [5,11], or RapiGest [5,8,10,12]. The latter are structurally similar to sodium dodecyl sulphate (SDS), being composed of a hydrophobic tail and a polar head group [11,12]. The separation of samples and MS-compatible surfactants is based on distinct processing steps. While deoxycholate can be removed by liquid phase extraction [9,13], ProteaseMax and RapiGest require a step that separates their hydrophobic and hydrophilic moieties, which is achieved by tryptic cleavage [11] or acid hydrolysis [12], respectively. Subsequently, the hydrophobic tail of the corresponding surfactant

Abbreviations: ABC, ammonium bicarbonate; AcOH, acetic acid; BLG, β-lactoglobulin; CAH, carbonic anhydrase; CASA, α-casein; CASB, β-casein; FA, formic acid; FASP, filter aided sample preparation; GCl, guanidium hydrochloride; HCD, higher-energy C-trap dissociation; iST, in-StageTip; LC-MSMS, liquid chromatography tandem mass spectrometry; MS, mass spectrometry; RT, room temperature; SP3, single-pot solid-phase-enhanced sample preparation; UHPLC, ultra-high performance liquid chromatography

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2021 The Authors. Proteomics published by Wiley-VCH GmbH

https://doi.org/10.1002/pmic.202100129
During the tryptic digestion of protein samples with RapiGest, we observed that acid hydrolysis and centrifugation does not always result in the formation of a pellet at the bottom of the sample tube. The precipitate, which consists of the hydrophobic moiety of the molecule (Figure 1A) [12], was sometimes not visible, while in other cases, it was either floating on top of the sample or distributed along the tube wall.

As we always used the same concentration of RapiGest, we investigated several parameters that could influence RapiGest precipitation. Concerning the location of precipitated RapiGest on the top, middle, or bottom of the tube, we were able to identify the sample temperature as the decisive factor. Acid hydrolysis at 1% TFA for 45 min and 37°C, followed by centrifugation at 20,000 × g for 10 min at room temperature (RT), often resulted in a floating layer on top of the sample. Incubation of those samples for 45 min on ice and centrifugation at 20,000 × g for 10 min at 4°C, on the other hand, resulted in a solid pellet at the bottom of the tube (Figure 1B). For the complete lack of precipitation, we argued that either incomplete RapiGest hydrolysis or precipitation could be the reason. First, we tested whether quantitative hydrolysis of RapiGest was achieved. Therefore, we selected the most extreme situation, a solution of 0.1% RapiGest, 100 mM NH₄HCO₃ pH 7.8 (ABC) without any peptides and performed acid hydrolysis as well as centrifugation. During the tryptic digestion of protein samples with RapiGest, ABC at a concentration of 2.6 mg/mL, reduced and alkylated disulphide bonds with 5 mM DTT (45 min at 56°C) and 20 mM acrylamide (30 min at RT), respectively, and quenched the reaction with 5 mM DTT (15 min at RT). After addition of trypsin (Promega, Mannheim, Germany) at an enzyme to sample ratio of 1:50 (w/w), samples were digested at a final protein concentration of 2 mg/mL and RapiGest concentration of 0.1% at 37°C overnight. Complete digestion was confirmed by combination of the sample with Laemmli buffer (1x final concentration) [14], SDS-PAGE, and staining of gels with Coomassie Brilliant Blue (Page Blue Protein Staining Solution, Thermo Fisher, Waltham, MA, USA) (Figure 1D). Using this sample, we tested different buffer (50, 100, and 200 mM ABC) and acid concentrations (0.5%, 1%, and 2% TFA), as well as differences in sample viscosity/density (0%, 5%, 10%, and 20% ACN). For all conditions, we did not observe any effects on RapiGest precipitation. Finally, we tested the effect of varying peptide concentrations obtained by dilution of the RapiGest digest with a 0.1% RapiGest solution (final concentration of 0.1 to 2 mg/mL), which showed a clear correlation with RapiGest pellet sizes (Figure 1E).

This effect may be related to an interaction of peptides and the hydrophobic tail of RapiGest, implying the possibility of co-precipitation, which was suggested previously [12]. In such a scenario, increasing amounts of RapiGest should co-precipitate increasing amounts of peptides, resulting in a reduction of peptide amount in the clear supernatant. To investigate potential peptide losses, we precipitated increasing amounts of RapiGest in a constant amount of solution of 0.1% RapiGest (Waters, Milford, MA, USA), 100 mM NH₄HCO₃ pH 7.8 (ABC) without any peptides and performed acid hydrolysis as well as centrifugation. This did not result in the formation of any pellet (or oily layer on top of the sample). To assess RapiGest cleavage in this sample, we analysed it by thin layer chromatography (TLC) on a heat-activated silica plate (silica gel 60 HPTLC, Merck, Darmstadt, Germany) at RT, using chloroform/methanol/water (70:30:4, v/v/v), followed by iodine staining. This analysis revealed complete cleavage of RapiGest, implying that only its precipitation is decisive for the observed effect (Figure 1C).

We subsequently evaluated several parameters, which may affect the formation of a RapiGest pellet using a proteolytic bovine serum albumin (BSA, Carl Roth, Karlsruhe, Germany) digest. For this, we dissolved BSA in 0.13% RapiGest (Waters, Milford, MA, USA), 100 mM ABC at a concentration of 2.6 mg/mL, reduced and alkylated disulphide bonds with 5 mM DTT (45 min at 56°C) and 20 mM acrylamide (30 min at RT), respectively, and quenched the reaction with 5 mM DTT (15 min at RT). After addition of trypsin (Promega, Mannheim, Germany) at an enzyme to sample ratio of 1:50 (w/w), samples were digested at a final protein concentration of 2 mg/mL and RapiGest concentration of 0.1% at 37°C overnight. Complete digestion was confirmed by combination of the sample with Laemmli buffer (1x final concentration) [14], SDS-PAGE, and staining of gels with Coomassie Brilliant Blue (Page Blue Protein Staining Solution, Thermo Fisher, Waltham, MA, USA) (Figure 1D). Using this sample, we tested different buffer (50, 100, and 200 mM ABC) and acid concentrations (0.5%, 1%, and 2% TFA), as well as differences in sample viscosity/density (0%, 5%, 10%, and 20% ACN). For all conditions, we did not observe any effects on RapiGest precipitation. Finally, we tested the effect of varying peptide concentrations obtained by dilution of the RapiGest digest with a 0.1% RapiGest solution (final concentration of 0.1 to 2 mg/mL), which showed a clear correlation with RapiGest pellet sizes (Figure 1E).

This effect may be related to an interaction of peptides and the hydrophobic tail of RapiGest, implying the possibility of co-precipitation, which was suggested previously [12]. In such a scenario, increasing amounts of RapiGest should co-precipitate increasing amounts of peptides, resulting in a reduction of peptide amount in the clear supernatant. To investigate potential peptide losses, we precipitated increasing amounts of RapiGest in a constant amount of an equimolar mixture of the tryptic digest of standard proteins BSA, β-lactoglobulin (BLG), ovalbumin (OVA), carbonic anhydrase (CAH), α-casein (CAS), and β-casein (CASB, all from Sigma-Aldrich, St. Louis, MO, USA). To exclude any effects due to the RapiGest concentration during proteolytic digestion, we first generated peptides separately by an alternative method, digestion in 0.8 M urea, 100 mM HEPES pH 7.5 (1 mg/mL protein concentration) at an enzyme to sample ratio of 1:50 (w/w). After desalting of peptides (3cc SepPak C₁₈ cartridges, Waters), dried eluate fractions were resuspended in different concentrations of RapiGest (0.1%, 0.5%, 1%, and 2%), 100 mM ABC pH 7.8. Following
RapiGest precipitation with 1% TFA and desalting of the clear supernatants using StageTips [15], we analysed the samples by LC-MSMS. Measurements were performed with a Dionex Ultimate 3000 nano UHPLC system coupled to an Orbitrap Fusion Lumos mass spectrometer (both Thermo Fisher Scientific, Bremen, Germany) using in-house-prepared 40 cm × 100 μm C18 analytical columns [10]. Peptides were loaded at a flow rate of 600 nL/min with 100% solvent A (water with 0.1% FA) and eluted with a 30 min linear gradient from 1% solvent B (90% ACN, 0.1% FA) to 35% solvent B at a flow rate of 300 nL/min. Eluting peptides were ionized in the positive ion mode and all scans performed in the Orbitrap analyser. After each survey scan (330–1500 m/z, resolution of 60,000), the most abundant peptides (m/z 2–7) were isolated at m/z 1.4 in the quadrupole, fragmented by HCD (30% collision energy), and analysed in the top speed mode with a cycle time of 2 s (fragment ion spectrum resolution of 30,000) and dynamic exclusion of 15 s. Acquired data were analysed with MaxQuant 1.6.5 [16], utilizing a database containing the six standard proteins in combination with the default contaminants list. Oxidation (M), acetylation (protein N-terminus), and deamidation (NQ) were set as variable modifications, and propionamide (C) was defined as fixed modification. Trypsin/P was set as enzyme, up to two missed cleavages were allowed, and standard settings were used for mass tolerances. Due to the small size of the database, FDR filtering was deactivated and a fixed Andromeda score cut-off of 40 was applied.

Comparison of the summed peptide intensities of the individual proteins did not reveal strong effects between the different conditions, with the exception of CASA, for which we observed for all RapiGest concentrations a trend towards decreased signal intensities relative to the control sample (Figure 2A, Table S1). This effect could be related to a stronger interaction of CASA-derived peptides with the hydrophobic part of RapiGest, resulting in their co-precipitation.

The lack of RapiGest precipitation in low concentrated samples suggests that sufficient reduction of sample volume, and therefore an increase of peptide concentration to at least 0.5 mg/mL (Figure 1E), could allow for efficient precipitation. As volume reduction can only be performed to a certain extend before it becomes impracticable to separate the RapiGest pellet from the supernatant, this strategy is, however, limited by the sample amount. Alternatively, peptide concentration could be increased by the addition of external peptides. Such peptides would, however, most likely mask low abundant co-eluting sample peptides, and block a significant amount of column binding capacity. This is, however not the case for intact proteins, as they can be separated from peptides based on their chemical properties.

We therefore tested the removal of hydrolysed RapiGest by adding intact “trigger proteins”. In these experiments, we added either BSA or BLG to a low concentrated tryptic BSA digest (peptide concentration of 0.25 mg/mL), which previously did not result in the formation of a proper pellet (Figure 2B, top), followed by acid hydrolysis of RapiGest and centrifugation. Both supplementation with BSA and BLG (1 mg/mL final concentration), which differ in size and hydrophobicity (GRAVY scores [17]/molecular weights of -0.43/69 kDa and -0.01/20 kDa, respectively), resulted in the precipitation of RapiGest with a slightly bigger pellet in the case of BLG (Figure 2B). Investigation of the protein distribution in the individual fractions of these samples by SDS-PAGE showed that the majority of intact proteins was present in the pellet (Figure 2C), implying that most of the trigger protein is removed during RapiGest precipitation [12]. We did, however, observe residual leftovers of trigger protein in the supernatant (Figure 2C, middle lanes).

As the presence of residual intact trigger proteins in a low concentrated peptide sample could interfere with its subsequent LC-MSMS analysis, we directed our attention towards its removal. In theory,
proteins should still be retained by C$_8$ reversed phase material at ACN concentrations where the vast majority of peptides is found in the flowthrough. We therefore determined the maximum ACN concentration at which BSA and BLG are retained by C$_8$ tip columns. After binding of proteins resuspended in 100 mM ABC, 1% TFA to Empore C$_8$ StageTips (3M, Saint Paul, MN, USA), we eluted the tips with increasing concentrations of ACN/0.5% acetic acid (AcOH). Eluate fractions were dried using a vacuum centrifuge, resuspended in 4% SDS, and the protein content was determined using the DC protein assay (Bio-Rad, Hercules, CA, USA). For BSA, we observed an earlier elution (25% ACN) compared to BLG (30% ACN), indicating a weaker interaction with the stationary phase (Figure 2D). As BLG also resulted in more efficient precipitation of RapiGest (Figure 2B), we selected it as trigger protein. As not the elution of BLG from C$_8$ material but its ability to bind to it at a certain concentration of ACN is decisive for its separation from peptide samples, we further tested at which maximal ACN level BLG is still retained by C$_8$ material. We loaded BLG, which was dissolved in buffers containing different amounts of ACN, on C$_8$ StageTips and quantified protein levels in the flowthrough fractions. These experiments showed, that BLG is efficiently retained by C$_8$ material at sample ACN concentrations of up to 30% (Figure 2E).

We subsequently assessed possible peptide losses due to the C$_8$ material in samples of low and high complexity (tryptic digests of BSA and HeLa whole cell lysate). For this purpose, HeLa cells were cultured in DMEM supplemented with 10% FCS, penicillin (100 IU/mL)/streptomycin (100 μg/mL) and 2 mM L-glutamine, washed with 1x PBS, harvested by scraping, and centrifuged at 1000 $\times$ g for 5 min at 4°C. The supernatant was discarded, the pellet resuspended in 4% SDS, 100 mM HEPES pH 7.5, and cells were lysed by incubation at 95°C for 10 min, sonication for 3 x 30 s at 60% duty cycle using an Ultrasonic Sonifier (Branson, Brookfield, CT, USA), and a second heating step. After centrifugation at 20,000 $\times$ g for 30 min at RT, the clear supernatant was recovered, the protein content determined, and a sample containing 1 mg of protein was transferred to a new microtube. Proteins were precipitated by chloroform-methanol (2:1 v/v) precipitation, and the air-dried protein pellet was solubilized in 0.3% RapiGest, 100 mM ABC pH 7.8 for 15 min at 95°C. Trypsin was added at an enzyme to sample ratio of 1:500 (w/w) and the sample was incubated for 60 min. Further sample processing was performed as previously described for the standard proteins and the digest was carried out overnight at a final concentration of 0.1% RapiGest.

After precipitation of RapiGest, supernatant volumes corresponding to either 100 fmol of BSA or 10 μg of HeLa digest were adjusted to 30% ACN and passed over a C$_8$ StageTip (50 fmol of BSA peptides and 2.5 μg of HeLa digest per C$_8$ layer, respectively). The flowthrough fraction was collected, desalted using C$_{18}$ StageTips, and analysed by
LC-MSMS using a 30 or 120 min gradient for BSA and HeLa whole cell lysate digests, respectively. The BSA experiment was carried out once, HeLa experiments were performed in three independent replicates. For data analysis of HeLa samples, Swiss-Prot human (release date: 2021-07 with 20,371 entries) was used for database searching, and results were filtered at an FDR of 0.01. For BSA, we plotted values for individual peptides between C8 exposed and control samples, revealing an excellent correlation (Figure 3A). For HeLa samples, data were further processed with Perseus v1.6.15 [18]. We considered only peptides quantified with three valid values in at least one of the two conditions and performed missing value imputation based on a down-shifted normal distribution. We then performed a two-sided Student’s t-test with an FDR of 0.05 and applied a fold-change cut-off of 1.5-fold for the determination of up-/downregulated peptides (Table S1). On the peptide level, we observed losses in signal intensity for ~ 4% of peptides in the sample exposed to C8 material while ~ 1% of peptides showed higher values (Figure 3B). This translated on the protein level to significant changes for 3 out of 3497 proteins (Figure 3C). Furthermore, we observed that the dataset has an asymmetric distribution for a subpopulation of peptides, whose change in signal intensity is below the applied cut-off. These data imply, that the exposure of the sample to C8 material under the experimental conditions used (2.5 μg of peptides/disk in 30% ACN) do result in certain peptide losses. Therefore, it should be carefully considered if a C8 material clean-up is necessary, for example, by analysis of a small fraction of the sample by SDS-PAGE and silver staining, as residual amounts of trigger protein will most likely not have a negative effect on the subsequent LC-MSMS analyses.

We performed the same experiment for the addition of trigger proteins, to address if the addition of BLG also results in peptide losses due to an increased co-precipitation of peptides with RapiGest. Utilizing the urea digested mixture of six standard proteins as well as the HeLa whole cell lysate digest, we precipitated RapiGest with/without addition of BLG, followed by C8 StageTip treatment and LC-MSMS analysis. No statistically significant peptide loss was observed for the six standard protein digest (Figure 3D, Table S1). We did, however, observe trends opposing the results obtained for precipitation of the six standard proteins digest without addition of BLG as trigger protein (Figure 2A), with a slight decrease in signal intensity for BLG and OVA, and an increase for CASA and CASB. For CASA, the increase in signal intensity might result from hydrolysed RapiGest, which does not co-precipitate with CASA peptides anymore but rather with intact BLG, resulting in a better recovery of these peptides. For the HeLa whole cell lysate digest, we did not observe a trend towards loss or gain of signal intensity for individual peptides (54 and 57 out of 18,401 peptides had a lower/higher signal intensity due to the addition of BLG) and no changes on the protein level (Figure 3E,F). Furthermore, we did not observe any trends in sample distribution, as it was the case for exposure to the C8 material, indicating that the addition of BLG as trigger protein has no negative effects on the sample.

In conclusion, we show that the precipitation of the acid-cleavable surfactant RapiGest depends on the peptide/protein concentration of the sample. To ensure proper pellet formation, we advocate for a peptide concentration of at least 1 mg/mL. For lower concentrated samples, we propose the addition of trigger proteins to the digest, which co-precipitate with the hydrophobic RapiGest cleavage product. This provides a strategy to reliably induce RapiGest precipitation, and to remove it from low concentrated samples.

ACKNOWLEDGMENTS
The authors thank Norbert Roesel for technical support. The workflow presented in the Table of Contents graphic was created with BioRender.com and the chemical structure was created with ChemDraw 16, a registered trademark of PerkinElmer Informatics. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD025982. [19].

Open access funding enabled and organized by Projekt DEAL.

AUTHOR CONTRIBUTIONS
Dominic Winter conceived the study and designed the experiments. Peter R. Mosen and Robert Hardt performed the experiments, acquired and analysed the data. Dominic Winter drafted the manuscript. Dominic Winter, Peter R. Mosen and Robert Hardt revised the manuscript. All authors have given approval to the final version of the manuscript.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are openly available in ProteomeXchange Consortium via the PRIDE partner repository at https://www.ebi.ac.uk/pride/, reference number PXD025982.

ORCID
Peter R. Mosen https://orcid.org/0000-0001-5922-3805
Robert Hardt https://orcid.org/0000-0003-2939-7657
Dominic Winter https://orcid.org/0000-0001-6788-6641

REFERENCES
1. Aebersold, R., & Mann, M. (2016). Mass-spectrometric exploration of proteome structure and function. Nature, 537 (7620), 347–355.
2. Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., & Mann, M. (2006). In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nature Protocol, 1 (6), 2856–2860.
3. Hughes, C. S., Foehr, S., Garfield, D. A., Furlong, E. E., Steinmetz, L. M., & Krijgsved, J. (2014). Ultrasensitive proteome analysis using paramagnetic bead technology. Molecular Systems Biology, 10 (10), 14.
4. Bennike, T. B., Bellin, M. D., Xuan, Y., Stensballe, A., Møller, F. T., Beilman, G. J., Levy, O., Cruz-Monserrat, Z., Andersen, V., Steen, J., Conwell, D. L., & Steen, H. (2018). A cost-effective high-throughput plasma and serum proteomics workflow enables mapping of the molecular impact of total pancreatectomy with islet autotransplantation. Journal of Proteome Research, 17 (5), 1983–1992.
5. Winter, D., & Steen, H. (2011). Optimization of cell lysis and protein digestion protocols for the analysis of HeLa S3 cells by LC-MS/MS. Proteomics, 11 (24), 4726–30.
6. Wisniewski, J. R., Zougman, A., Nagaraj, N., & Mann, M. (2009). Universal sample preparation method for proteome analysis. *Nature Methods*, 6(5), 359–62.

7. Kulak, N. A., Pichler, G., Paron, I., Nagaraj, N., & Mann, M. (2014). Minimal, encapsulated proteomic-sample processing applied to copy-number estimation in eukaryotic cells. *Nature Methods*, 11(3), 319–324.

8. Leon, I. R., Schwammle, V., Jensen, O. N., & Sprenger, R. R. (2013). Quantitative assessment of in-solution digestion efficiency identifies optimal protocols for unbiased protein analysis. *Molecular & Cellular Proteomics*, 12(10), 2992–3005.

9. Proc, J. L., Kuzyk, M. A., Hardie, D. B., Yang, J., Smith, D. S., Jackson, A. M., Parker, C. E., & Borchers, C. H. (2010). A quantitative study of the effects of chaotropic agents, surfactants, and solvents on the digestion efficiency of human plasma proteins by trypsin. *Journal of Proteome Research*, 9(10), 5422–5437.

10. Ponnaiyan, S., Akter, F., Singh, J., & Winter, D. (2020). Comprehensive draft of the mouse embryonic fibroblast lysosomal proteome by mass spectrometry based proteomics. *Scientific Data*, 7(1), 68–81.

11. Saveliev, S. V., Woodroofe, C. C., Sabat, G., Adams, C. M., Klaubert, D., Wood, K., & Urh, M. (2013). Mass spectrometry compatible surfactant for optimized in-gel protein digestion. *Analytical Chemistry*, 85(2), 907–914.

12. Yu, Y. Q., Gilar, M., Lee, P. J., Bouvier, E. S. P., & Gebler, J. C. (2003). Enzyme-friendly, mass spectrometry-compatible surfactant for in-solution enzymatic digestion of proteins. *Analytical Chemistry*, 75(21), 6023–6028.

13. Masuda, X. T., Tomita, T., & Ishihama, Y. (2008). Phase transfer surfactant-aided trypsin digestion for membrane proteome analysis. *Journal of Proteome Research*, 7(2), 731–740.

14. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage-T4. *Nature*, 227(5259), 680–685.

15. Rapaport, J., Ishihama, Y., & Mann, M. (2003). Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Analytical Chemistry*, 75(3), 663–670.

16. Cox, J., & Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnology*, 26(12), 1367–72.

17. Kyte, J., & Doolittle, R. F. (1982). A Simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology*, 157(1), 105–132.

18. Tyanova, S., Temu, T., Sinitsyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., & Cox, J. (2016). The Perseus computational platform for comprehensive analysis of (pro)teomics data. *Nature Methods*, 13(9), 731–740.

19. Vizcaíno, J. A., Deutsch, E., Wang, R., Csordas, A., Reisinger, F., Rios, D., Dianes, J. A., Sun, Z., Farrah, T., Bandeira, N., Binz, P., Xenarios, I., Eisenacher, M., Mayer, G., Gatto, L., Campos, A., Chalkley, R. J., Kraus, H., Albar, J. P., & Hermjakob, H. (2014). ProteomeXchange provides globally coordinated proteomics data submission and dissemination. *Nature Biotechnology*, 32(3), 223–226.

**SUPPORTING INFORMATION**

Additional supporting information may be found online [https://doi.org/10.1002/pmic.202100129](https://doi.org/10.1002/pmic.202100129) in the Supporting Information section at the end of the article.

**How to cite this article:** Mosen, P. R., Hardt, R., & Winter, D. (2021). RapiGest Precipitation Depends on Peptide Concentration. *Proteomics*, 21, e2100129. [https://doi.org/10.1002/pmic.202100129](https://doi.org/10.1002/pmic.202100129)