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Rapid LC−MS Method for Accurate Molecular Weight Determination of Membrane and Hydrophobic Proteins

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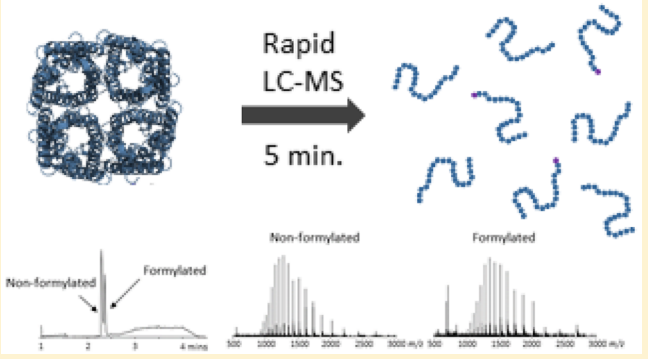
Supporting Information

ABSTRACT: Therapeutic target characterization involves many components, including accurate molecular weight (MW) determination. Knowledge of the accurate MW allows one to detect the presence of post-translational modifications, proteolytic cleavages, and importantly, if the correct construct has been generated and purified. Denaturing liquid chromatography−mass spectrometry (LC−MS) can be an attractive method for obtaining this information. However, membrane protein LC−MS methodology has remained relatively under-explored and under-incorporated in comparison to methods for soluble proteins. Here, systematic investigation of multiple gradients and column chemistries has led to the development of a 5 min denaturing LC−MS method for acquiring membrane protein accurate MW measurements. Conditions were interrogated with membrane proteins, such as GPCRs and ion channels, as well as bispecific antibody constructs of variable sizes with the aim to provide the community with rapid LC−MS methods necessary to obtain chromatographic and accurate MW measurements in a medium- to high-throughput manner. The 5 min method detailed has successfully produced MW measurements for hydrophobic proteins with a wide MW range (17.5 to 105.3 kDa) and provided evidence that some constructs indeed contain unexpected modifications or sequence clipping. This rapid LC−MS method is also capable of baseline separating formylated and nonformylated aquaporinZ membrane protein.



Membrane proteins are responsible for multiple cellular functions[1] and have been linked to many disease states, such as cystic fibrosis, multiple cancers, and retinitis pigmentosa,[2−4] making them prime therapeutic targets.[5,6] Having a rapid method for determining their accurate molecular weight (MW) would be of great utility to the pharmaceutical industry for drug design and development. However, their characterization via traditional biophysical techniques, such as SPR or NMR, is often difficult[7−11] due to the challenges associated with expression, purification, and solubilization, as well as overall sample amounts.[12] Native mass spectrometry (native-MS) has been an enabling technique for the investigation of numerous membrane protein systems,[13] as it allows for the study of intact membrane protein complex stoichiometry and structure, as well as interactions with various lipids and small molecules, using relatively small amounts of material.[14−16] However, membrane protein complexes can afford native-MS spectra that may include multiple nonvolatile adducts, bound ligands, and/or protein stoichiometries,[9] which can complicate accurate MW determination. Accurate MW measurements can provide insight into potential proteolytic cleavage(s) and/or low MW modifications, and importantly, whether the correct construct has been purified. Denaturing liquid chromatography−mass spectrometry (LC−MS) is an attractive technique to attain this information, as it involves the removal of noncovalently bound lipids or small molecules that may remain using other techniques. Also, it provides dissociation of membrane protein complexes, avoiding potential spectral interpretation issues.

Nevertheless, a widely incorporated high-throughput denaturing LC−MS method for membrane proteins has remained somewhat elusive throughout the literature. Early methods (1983−1998) utilized C8, C18, or polystyrene-divinylbenzene copolymer (PLRP/S) columns.[17−19] These methods involve long gradients (40+ minutes) and preinjection sample acidification in formic acid (FA) or trifluoroacetic

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acid (TFA). Methods from the early 2000s also employ PLRP/S columns.[7,20] These methods utilized lower concentrations of TFA or FA; however, they still require lengthy gradients (55+ minutes), preinjection sample acidification, and in some cases, require a second elution with 60% FA to elute larger subunits.[20] The column particle size employed for these methods is 5 μm. The most current methodology, to our knowledge, employs ZORBAX 300SB-C3 columns in multiple lengths (achieved by tandemly linking multiple 50 mm columns).[21] These gradients are 12−40 min and use 0.1% FA as the mobile phase additive. Here, the protein stock is diluted in 1% FA prior to injection. While the column particle size used for this work is not reported, only 3.5 or 5 μm particle size are commercially available from the manufacturer for the column dimensions reported in the article.[21]

Many of these gradients employ solvents, such as acetonitrile, 2-propanol, and methanol, with varying concentrations of ion pairing agents. It has also been reported that denaturing LC−MS and non-native size exclusion chromatography (SEC) have been used prior to top-down MS of membrane proteins.[21−24] These methods employ unique solvents, such as mixtures of chloroform and methanol.[24,25] While all of these methods have provided quality data, they require extensive gradient lengths and/or preinjection sample handling. Additionally, most of these methods are quite extensive, and their main purpose was the chromatographic separation of complex mixtures or separation of membrane protein complex subunits. The need to rapidly screen membrane protein constructs for MW determination or confirmation does not require such extensive separation. Instead, a more high-throughput method is optimal for this type of analysis, especially in a biopharmaceutical environment.

To extend denaturing LC−MS of membrane proteins and other hydrophobic proteins to an industrial platform method where high-throughput analysis is highly desirable, we systematically interrogated conditions to reduce method length, eliminate preinjection sample handling, and extend column lifetime. Here, we present a medium- to high-throughput denaturing rapid LC−MS method for membrane protein accurate MW determination. Development of this 5 min method involved investigation of several column chemistries, mobile phases, different ion pairing agents, and gradients (all investigated without preinjection sample handling). Furthermore, we have also explored bispecific antibody constructs to assess this method with other hydrophobic molecules. The goal of this work was to provide a rapid method for screening the quality of prepared and purified protein via accurate MW determination, not the separation of the protein from multiple solution contaminants (as the protein constructs analyzed herein are previously purified). For brevity, only the optimal chromatography conditions will be discussed. Refer to the Supporting Information for details regarding the other chromatography conditions investigated.

EXPERIMENTAL SECTION

Membrane Protein Preparation for Rapid Denaturing LC−MS. Protein stock preparation for all membrane proteins is detailed in the Supporting Information. Concentrations were determined using A_{280} measurements. For denaturing LC−MS experiments, the bacteriorhodopsin (bR) mutants L111A, T47A, P50A, and P50A/T46A were diluted for 10 μg injections. Proteolytic digestion conditions and instrument settings for bR P50A are detailed in the Supporting Information. Wild-type (WT) and W14A aquaporinZ (AqpZ) were diluted for 2 μg of monomer injections. Both WT and W14A AqpZ were also analyzed with covalent green fluorescent protein (GFP) tags. AqpZ-GFP samples were diluted for 1.5 μg injections of both the WT and W14A monomers. All AqpZ and bR samples were diluted in 200 mM ammonium acetate/1.1% (w/v) *n*-octyl-β-D-glucoside (OG). Coq10 was analyzed as received at 6 μg per injection. The ammonia transporter AmtB fused with maltose-binding periplasmic protein (AmtB-MBP) was received at 9 mg/mL[26] and was diluted in 200 mM ammonium acetate/0.017% (w/v) *n*-dodecyl-β-D-maltopyranoside (DDM) for 6 μg of monomer per injection. AmtB was also analyzed at 6 μg of monomer after TEV (91636; Invitrogen; Carlsbad, CA) cleavage to remove MBP. This cleavage required overnight incubation of AmtB-MBP and TEV in a 100:1 ratio with 5 μM β-mercaptoethanol at 4 °C. KcsA−Kv1.3 (K−K) was analyzed, as prepared, in *n*-decyl-β-D-maltopyranoside (DM), at 4 μg of monomer per injection after removal of the N-terminal His-tag by caspase3 (made in house) cleavage. This cleavage was executed by incubation of 350 μg of K−K with 6 μg of caspase3 at 4 °C for 6−8 h. Completion of the cleavage reactions was confirmed via the LC−MS method described herein. Optimization of starting % B was achieved with injections of WT AqpZ (polyphenol column) and caspase3 cleaved K−K (C3 column) under increasing starting % B from 5 to 40%. To demonstrate reproducibility, WT AqpZ-GFP was measured 10 additional times. Polyphenol column limit of detection studies were performed with K−K (0.2−15 μg on column). Limit of detection studies on the C3 column were performed with both bR P50A T46A (0.2−15.2 μg) and K−K (0.075−20 μg on column). Five Fc-provided bispecific antibody constructs (D6Q, K8T, M3X, X2W, and J8R) were analyzed at 10 μg on column. A non-Fc-provided bispecific antibody construct (construct A) was analyzed at 20 μg on column at 5 and 30% B. All constructs and concentrations were analyzed in triplicate in positive ionization mode.

Rapid Denaturing LC−MS Conditions. An Acquity UPLC (Waters MS-Technologies; Manchester, UK) with a 450 Å, 2.7 μm, 2.1 mm × 50 mm BioResolve RP mAb Polyphenol column (186008944; Waters; Milford, MA), which is compatible with both UPLC and HPLC systems, was employed. Classically, the term UPLC refers to sub-2 μm particle size columns.[27] As stated above, the particle size used in this method is 2.7 μm. However, according to manufacturer specifications, this column is compatible with UPLC systems. Additionally, this method is run on an Acquity UPLC system operated at typical UPLC pressures (>6000 psi). A 5 min method (including gradient and re-equilibration) was applied using a mix of 0.1% (v/v) FA/0.1% (v/v) TFA in water as mobile phase A and in 90% *n*-propanol as mobile phase B. The gradient was maintained at 30% B from 0 to 1 min, increased to 95% B from 1 to 2.5 min, and it was held for 1 min. The gradient was then reduced to 30% B from 3.5 to 4 min and held for an additional minute at 30% B. The flow rate was 500 μL/min with a column temperature of 65 °C. The same method was also employed on a 2.1 mm × 50 mm, 1.8 μm ZORBAX RRHD 300SB-C3 column (857750-909; Agilent; Santa Clara CA), and these results are discussed in the Supporting Information. This C3 column is representative of a standard UPLC particle size column.

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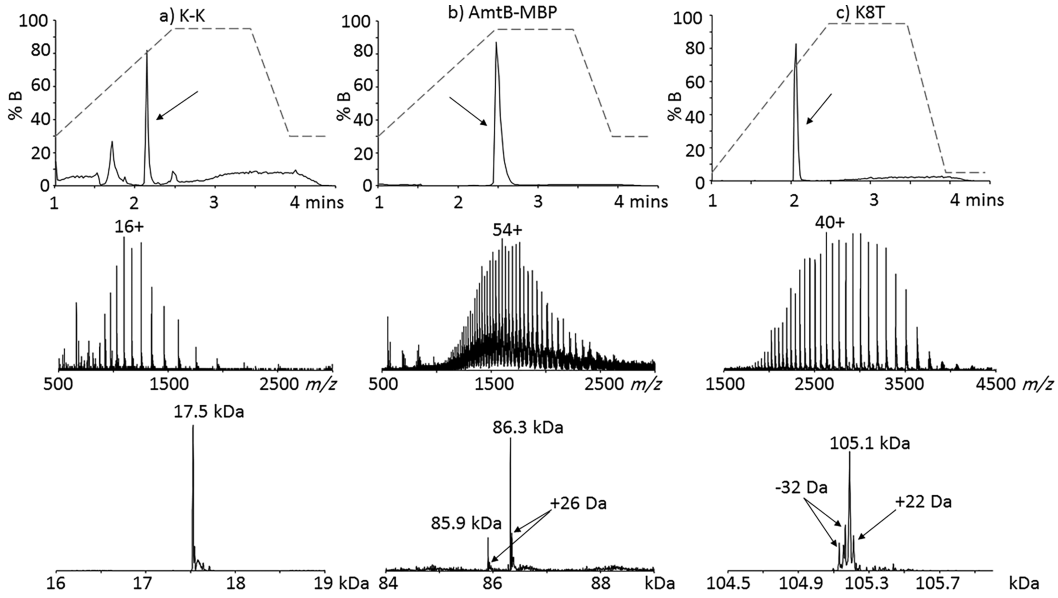

Figure 1. Representative reconstructed UPLC ion chromatograms (RICs) (upper) from the polyphenol column, MS spectra (middle) and deconvoluted MS spectra (lower) of the membrane proteins (a) K−K (~17.5 kDa); (b) AmtB-MBP (~86.3 kDa) and (c) Fc-provided antibody construct K8T (~105.1 kDa). The black arrow in each chromatogram denotes where the protein elutes from the column. The Y-axis for each chromatogram is the % B gradient profile (dashed line), starting at 30% B for the membrane protein samples and at 5% B for the bispecific antibody constructs. The most intense charge state is labeled in each MS spectrum. The 85.9 kDa species observed for AmtB-MBP corresponds to an N-terminal clip, (vide infra). The +26 Da and +22 Da species present in the deconvoluted spectra for AmtB-MBP and K8T, respectively, are presumably salt adducts. The Y-axis for the spectra represents intensity with arbitrary units.

The Acquity UPLC was run in-line with a Xevo Q-ToF mass spectrometer (Waters MS-Technologies). MS data was collected over the m/z 500−5000 range. For instrument tune settings and pressures refer to the Supporting Information. Zero-charge accurate mass measurements were determined using MaxEnt[28] in the MassLynx software (MassLynx 4.1, Waters MS-Technologies). MaxEnt parameters can be found in the Supporting Information. Limit of detection studies used the total ion counts from the area-centered deconvoluted spectra.

RESULTS AND DISCUSSION

The amphipathic nature of membrane proteins (hydrophilic-extracellular regions and hydrophobic-transmembrane regions) makes them intrinsically difficult to characterize by traditional LC−MS methods. Bispecific antibody constructs represent another class of hydrophobic molecules difficult to characterize by traditional LC methods. A bispecific antibody construct is composed of two single-chain variable fragments (scFvs) joined using short protein linkers.[29,30] One example of a bispecific antibody construct is blinatumomab, a second-line treatment for Philadelphia chromosome-negative relapsed or refractory acute lymphoblastic leukemia. Blinatumomab consists of two fused scFvs against CD19 and CD3.[30] Bispecific antibody constructs are roughly one-third the MW of traditional monoclonal antibodies; however, they are significantly more hydrophobic and have the propensity to aggregate.[29,30] Due to the similarity of hydrophobic characteristics, we felt that bispecific antibody constructs could also be used to assess this rapid LC−MS method meant for molecules exhibiting significant hydrophobic characteristics.

Reversed-phase HP(UP)LC commonly employs a C18 bonded silica stationary phase for small molecules.[31] However, membrane proteins would likely be retained during a short gradient given their hydrophobic character, and thus, C18 columns were not tested during this study and are not recommended. During method development, C3, C4, C8, cyano (CN), and polyphenol columns were investigated. Only the C8 column proved to be too retentive for the proteins under all of the gradient conditions investigated, which was apparent from the lack of protein signal and increasing column back pressure. Investigation of the C3, C4, CN, and polyphenol columns, which share similar short chain bonding, revealed that multiple gradient conditions resulted in protein elution. Thus, it can be inferred that short chain bonding is an ideal column chemistry for hydrophobic protein denaturing LC(UP)−MS; this is a logical inference considering minimization of column interactions with short chain lengths, thus decreasing unwanted retention. On the basis of the results, the polyphenol column operated at UPLC pressures at 65 °C (column temperature limited to 65 °C by manufacturer) was determined to provided optimal chromatography (see the Supporting Information for discussion of the other column chemistries).

Optimization of Chromatography Conditions. Historically, denaturing LC−MS methods for membrane proteins involved high concentrations of acid in the mobile phases (isopropanol, $CHCl_3$/methanol, methanol) and/or long gradients. Some gradients employed TFA in place of FA, but they remained lengthy (40+ minutes). Additionally, no direct comparison of TFA and FA chromatography for the same membrane protein has been performed. While recent efforts have reduced the mobile phase FA concentration,[21] the multiple methods presented in that work run upward of 12 min. Here, method development was aimed at decreasing the method length and minimizing preinjection sample handling through investigation of various FA and TFA concentrations, mobile phases, and column chemistries (Supporting Informa-

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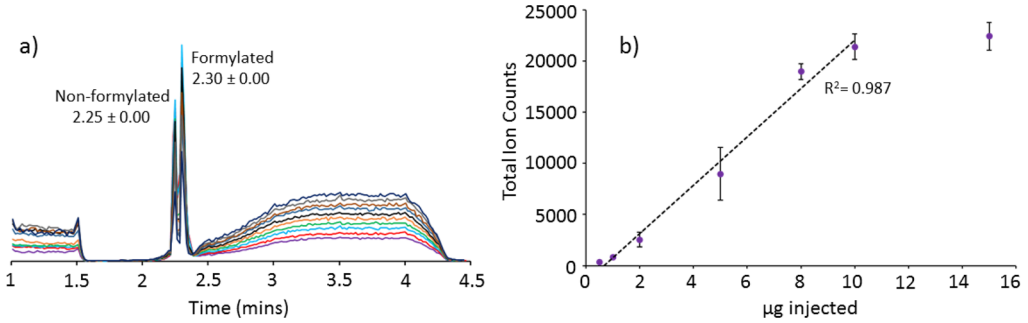

Figure 2. Polyphenol column reproducibility and limit of detection experiments. Reproducibility tests (a) with WT AqpZ-GFP at 1.6 μg/injection. Each run is represented with a different color and shows an overall reproducibility of retention time of 2.25 ± 0.00 and 2.30 ± 0.00 min for the nonformylated and formylated WT AqpZ-GFP, respectively. The *y*-axis for the spectra represents intensity with arbitrary units. Limit of detection studies (b) with K−K, injection amounts based on A_{280} readings ranged from 0.2 to 15 μg for K−K. Outliers existed at 0.2 and 15 μg. Total ion counts on the *y*-axis were obtained from MaxEnt deconvolution of the LC integrated peak area.

tion), similar to previous HPLC-MS method development for monoclonal antibody (mAb) characterization.[32,33]

Representative chromatograms and spectra obtained with the final optimized method conditions using 90% *n*-propanol are shown in Figure 1. Acetonitrile, methanol, isopropanol, and 90% *n*-propanol were evaluated with both TFA and FA as ion pairing agents. Methanol and isopropanol resulted in higher back pressure, poor chromatography, and/or low protein signal intensity. While contrary to previous reports,[21] employing acetonitrile with either 0.1% or 1% FA produced high quality chromatography and protein signal (data not shown). However, extreme carryover issues were observed with as little as 0.25 μg of protein. Replacing acetonitrile with 90% *n*-propanol, which is used for antibody reversed-phase chromatography, ameliorated the carryover issues without compromising protein signal.[33] Method development for mAb characterization reported by Dillon et al. demonstrated that employment of *n*-propanol for mobile phase B reduced peak tailing and increased resolution in comparison to acetonitrile, methanol, and isopropanol.[33] This was attributed to the high eluotropic coefficient of *n*-propanol, as compared to the other solvents, which helped to reduce column interactions.[33] Here, employment of 90% *n*-propanol as mobile phase B for membrane protein UPLC−MS analysis, as expected, aided in reducing column interactions, decreasing peak tailing, and eliminating carryover as compared to other organic solvents investigated.[33]

Addition of 0.1% TFA to both mobile phases (water and 90% *n*-propanol) resulted in good chromatography with no observable carryover. TFA is a commonly utilized solvent additive for reversed-phase HP(UP)LC because it is an excellent ion pairing agent, increases the surface tension of water, and improves peak shape.[34,35] However, it is not preferred for LC−MS as electrospray of solutions with high surface tension and conductivity ultimately results in analyte signal suppression.[35,36] Thus, as expected, protein ionization during these experiments was inferior when compared to those performed with FA.[35] Use of FA as a solvent additive is generally not recommended for HP(UP)LC of proteins due to potential *O*-formylation of amino acids during exposure.[17] However, protein exposure to FA during the experiments detailed within was limited to the 5 min method. Additionally, only the four AqpZ constructs analyzed displayed N-terminal formylation (vide infra), which was previously detected by native-MS and LC−MS/MS for the WT construct.[37]

Adding 1% FA to both mobile phases resulted in good chromatography and MS performance, but significant protein carryover was observed in blank runs between protein injections (data not shown). Lowering to 0.1% FA decreased the carryover while maintaining the strong ion signal. However, optimal results were achieved with a mix of 0.1% FA and 0.1% TFA. Upon mixing, superior chromatography was observed on the polyphenol column, with the separation of the nonformylated and formylated AqpZ monomers (Figures 2 and S1).

For all AqpZ constructs, the formylated construct elutes an average of 0.05 min after the nonformylated construct on the 5 min gradient. Though some separation was clearly achieved within 5 min, improved separation was attained with 7 and 15 min gradients (Figure S2). Increased separation of the two species in the 0.1% FA mobile phases was also achieved with the same 7 and 15 min gradients (Figure S2). Chromatographic separation of a nonformylated and formylated protein has been previously reported for human S100b, with a retention time shift of <0.5 min.[38] Additionally, a shift in retention time (mean value of 2.8 min) upon N-terminal formylation has also been reported for small peptides.[39] Formylation alters the N-terminal amine to an amide, causing the loss of a chargeable basic site, which could account for the retention time shift. All runs were performed at a column temperature of 65 °C, which minimized baseline fluctuation and protein carryover as compared to 40 °C (data not shown). Previous work done by Dillon et al. demonstrated that increased column temperature (60−75 °C) decreased column fouling and peak tailing, while improving resolution and recovery.[33,40] Additionally, mixing TFA and FA decreased the level of signal suppression observed on both the polyphenol and C3 columns with TFA alone (Figures S1 and S3, respectively).

In general, these proteins are very amphiphilic and some constructs could be retained longer than others. Thus, regardless of the gradient conditions tested, some carryover was always observed in blank injections run post protein injection (<10% carryover). However, carryover was never observed in proteins analyzed back-to-back. It has been previously reported that some membrane proteins elute with limited efficiency, thus yielding "ghost peaks" in subsequent runs.[22,41] Conversely, with optimization of injection concentration, these "ghost peaks" were not detected throughout these experiments. Additionally, column backing pressure remained stable throughout this entire work, indicating

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Table 1. Average Mass Accuracy (PPM) of Membrane Proteins and Bispecific Antibody Constructs, Average MWs, Retention Times, and Monomer Transmembrane (TM) Regions for the 12 Membrane Proteins[a]

(a)[b]

	nonformylated			formylated				
protein	theoretical mass (Da)	measured mass (Da)	PPM	theoretical mass (Da)	measured mass (Da)	PPM	retention time (min)	monomer TM regions
WT AqpZ	24 268.56	24 268.30 ± 0.09	10.53	24 296.57	24 296.05 ± 0.25	21.53	2.29 ± 0.01 2.35 ± 0.01	6#
W14A AqpZ	24 153.43	24 153.14 ± 0.13	11.81	24 181.44	24 180.65 ± 0.30	32.52	2.28 ± 0.01 2.34 ± 0.01	6#
WT AqpZ-GFP	49 861.42	49 862.29 ± 0.36	−17.35	49 889.43	49 890.45 ± 0.42	−20.45	2.25 ± 0.00 2.30 ± 0.00	6#
W14A AqpZ-GFP	49 746.29	49 747.20 ± 0.07	−18.42	49 774.30	49 774.93 ± 0.27	−12.76	2.22 ± 0.00 2.27 ± 0.00	6#

(b)[c]

	retinal unbound			retinal bound				
protein	theoretical mass (Da)	measured mass (Da)	PPM	theoretical mass (Da)	measured mass (Da)	PPM	retention time (min)	monomer TM regions
bR L111A	26 741.53	26 740.40 ± 0.49	42.25	27 007.96	27 007.66 ± 0.83	11.15	2.40 ± 0.00	7
bR T47A	26 753.59	26 753.44 ± 1.05	5.44	27 020.02	27 019.28 ± 0.28	27.24	2.42 ± 0.00	7
bR P50A	26 757.57	26 756.36 ± 0.64	45.54	27 024.00	27 023.39 ± 0.18	43.19	2.42 ± 0.00	7
bR P50A T46A	26 727.55	26 727.01 ± 0.29	20.16	26 993.98	26 992.94 ± 0.55	38.35	2.42 ± 0.00	7

(c)[d]

	clipped			unclipped				
protein	theoretical mass (Da)	measured mass (Da)	PPM	theoretical mass (Da)	measured mass (Da)	PPM	retention time (min)	monomer TM regions
Coq10*	18 307.43	18 306.96 ± 0.11	25.55	20 302.61	20 302.30 ± 0.09	15.42	1.79 ± 0.00	unknown
K−K	17 528.23	17 527.78 ± 0.12	25.64					2#
AmtB-MBP	85 905.27	85 905.23 ± 0.44	0.42	86 321.77	86 321.28 ± 0.91	5.68	2.51 ± 0.00	11
AmtB				42 263.89	42 263.58 ± 0.61	7.29	2.54 ± 0.00	11

(d)[e]

bispecific antibody constructs	theoretical mass (Da)	measured mass (Da)	PPM	retention time (min)
D6Q	105 123.77	105 123.21 ± 1.04	5.31	2.05 ± 0.01
K8T	105 190.86	105 191.10 ± 1.39	−2.20	2.04 ± 0.01
M3X	105 193.86	105 191.68 ± 1.09	20.79	2.05 ± 0.00
X2W	105 065.73	105 065.31 ± 1.89	4.07	2.06 ± 0.00
V2T	105 332.08	105 332.72 ± 1.00	−6.04	2.04 ± 0.01
construct A	54 635.20	54 634.61 ± 0.80	10.88	2.05 ± 0.01

[a]Theoretical MWs are the expected protein average MW and account for any protein modifications known prior to the work described herein. The theoretical MWs for the bR constructs consider the N-terminal pyroglutamate and the loss of aspartate from the C-terminus. The theoretical MW of construct A considered an N-terminal pyroglutamate and 4 disulfide bonds. Each MW measurement is an average of triplicate runs. Reported retention time and mass accuracy for Coq10* is from 25% B data. # denotes two additional half-membrane or intramembrane regions. An overall RMS error of 22.17 PPM (0.80 Da) was measured for all constructs. [b]The formylated and nonformylated AqpZ constructs. [c]The retinal bound and unbound bR constructs. [d]The clipped and unclipped membrane protein constructs. [e]Bispecific antibody construct mass accuracy and retention time data.

minimal protein retention over time. Since membrane proteins can be challenging to express and purify in high quantities, minimization of the protein concentration necessary to obtain quality chromatographic and accurate mass measurements was a key part of method development.

During method development, it was observed that chromatographic separation between the detergent and the protein for DDM solubilized proteins decreased as the method length was decreased to 5 min. In cases of detergent signal interference, a 7 or 9 min method, detailed in the Supporting Information, is suggested. Additionally, it is worth noting, that proteins are often diluted in mobile phase A for denaturing HPLC−MS injections.[31] However, injections of bR mutants from protein stock diluted in mobile phase A resulted in very low chromatographic protein signal, thus none of the samples were diluted in mobile phase A prior to injection. Therefore, none of the membrane proteins were denatured when injected, and instead existed in their "native-state" detergent micelle environment; this suggests that the proteins fully denatured only when subjected to the LC−MS gradient.

Initial 5 min method experiments at 5% B showed the nonformylated and formylated AqpZ partially resolved, but they were highly overpowered by the detergent signal. Optimization of starting % B was performed by increasing it from 5 to 50% B to determine the starting % needed to maximize chromatographic resolution. As shown in Figures 2 and S4, the chromatographic resolution of the two AqpZ species was easily observed at ≥30% B due to decreased chromatographic detergent signal. This separation was not observed on the C3 column, so optimization of starting % B (Figure S5) was performed with membrane protein K−K because it eluted near the detergent signal, and multiple chromatographic peaks containing various low MW species were detected. Both columns demonstrated that 30% B provided optimal chromatography for resolution of modified constructs, separation from detergent peaks, and decreased the chromatographic and spectral intensity of coeluting species. Hence, 30% B was used for membrane protein analysis. Chromatograms and spectra for K−K, AmtB-MBP, and the Fc-provided bispecific antibody construct K8T are shown in

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Figure 1. The gradient profiles have been overlaid on these respective chromatograms, demonstrating the difference in starting % B for the membrane protein samples as compared to the bispecific antibody constructs. Representative chromatograms and spectra for the remaining membrane protein samples on the polyphenol and C3 columns are shown in Figures S6 and S7, respectively. Bispecific antibody constructs were run only on the polyphenol column (Figure S8).

It should be noted, that the bR mutants provided a higher intensity MS signal on the C3 column as opposed to the polyphenol column (compare Figures S6 and S7). On the polyphenol column, monitoring protein elution by UV absorbance clearly showed that each of the bR mutants eluted at 2.4 min. Furthermore, Coq10 eluted at 30% from the polyphenol and C3 columns; however, optimal chromatography was at 25 or 20% B for the polyphenol and C3 column, respectively. Results for Coq10 at 25 and 20% B are shown in Figures S5 and S6, respectively. Data acquired at 30% B on both columns are provided in Figure S9. If a protein is not chromatographically separating from the detergent peak, reducing the starting % organic mobile phase is suggested to rectify the issue.

Analysis of the bispecific antibody constructs revealed that construct A was not retained on the column at 30% B (data not shown). Exploration of lower starting % B yielded data for the non-Fc-provided construct A and Fc-provided bispecific antibody constructs at under 15% B, with optimal chromatography at 5% B. These observations suggest that, while these species have been reported to exhibit hydrophobic characteristics, they may not be as hydrophobic as membrane proteins, which provide optimal chromatography at higher starting % B. However, additional non-Fc-provided bispecific antibody constructs will need to be tested to further support this inference. The Fc-provided bispecific antibody constructs all displayed similar chromatographic results to construct A.

Reproducibility and Limit of Detection. Triplicate injections of each membrane protein showed minimal retention time variability (Table 1a–c). To further demonstrate reproducibility, ten additional WT AqpZ-GFP injections were run, showing negligible retention time variability (Figure 2). Additionally, with a single blank between each WT AqpZ-GFP injection, only low levels of carryover were detected (<5%). Injection amounts were optimized to maintain observable protein signal in the presence of high chromatographic signals observed from the solubilization detergents. In general, it is known that detergents will preferentially ionize by ESI in comparison to proteins, thus high detergent concentration can suppress analyte ionization.[31] Limit of detection studies with caspase3-cleaved K–K showed detection to be as low as 0.2 μg on column. However, both the chromatographic resolution and ion signal were optimal at or above 2 μg. As shown in Figure 2, saturation occurred around 20 000 total ion counts (10 μg of K–K). C3 column reproducibility, with WT AqpZ-GFP, and limit of detection studies, with bR P50A T46A and K–K, are provided in Figure S10.

Mass Accuracy. Overall mass accuracy for the 12 membrane proteins and the 6 bispecific antibody constructs are shown in Table 1, with an overall measured RMS error of 22.17 ppm (0.80 Da). Several proteins investigated showed readily observable post-translational modifications or proteolytic cleavages. For example, during previous WT AqpZ experiments,[37] N-terminal methionine formylation was detected, which was observed for each of the AqpZ constructs herein (Table 1a). N-terminal formylation of AqpZ was observed due to expression of this protein in *E. coli*; however, the nonformylated species was observed because of incomplete post-translational removal of the formyl group. Additionally, when expressed in its native *Halobacterium* host, bR is known to undergo proteolytic cleavage on both the N- and C-termini.[24] Proteolytic digestion of bR mutants with chymotrypsin followed by LC–MS/MS collision-induced dissociation (CID) (Supporting Information) verified both N-terminal pyroglutamate formation and a loss of aspartate from the C-terminus. Loss of aspartate had occurred in roughly 95% of the individual construct (Figure S11). These findings are accounted for in the theoretical MWs reported in Table 1b. Additionally, the Schiff-linked retinal (+266.43 Da) species was also observed with each of the mutants. Roughly <20% of each mutant did not retain the Schiff-linked retinal.

Furthermore, Coq10 manifested two main species, one of which correlated to within 15.42 ppm (0.31 Da) of its intact theoretical mass (Figure S11). The second species corresponded to a clipped sequence, which, on the basis of MW, could have been either an N- or C-terminal clip (Table 1c). In the deconvoluted spectrum (Figure S12), several species were observed corresponding to additions of ∼510 Da, which were determined to be DDM adducts. This was the only protein to show detergent adduction upon deconvolution. Through Glu-C proteolytic digestion followed by LC–MS/MS (Supporting Information), it was determined to be an N-terminal cleavage (Figure S13). Also, the initial deconvoluted spectra for K–K revealed several species. To reduce heterogeneity, caspase3 cleavage was employed to remove the His-tag used for purification. Post caspase3 cleavage revealed only two species, the fully cleaved (most abundant) and another cleaved product, mis-cleaved by a single amino acid (less abundant). Of the two, the mis-cleaved product provided poorer mass accuracy. For AmtB-MBP, the clipped sequence corresponds to an N-terminal clip from the MBP of *–MDIG*. The Fc-provided bispecific antibody constructs are known to be processed and include N-terminal pyroglutamate formation as well as C-terminal lysine cleavage. These sequence characteristics were considered in the reported theoretical masses.

■ CONCLUSIONS

The ability to obtain accurate MW measurements of membrane proteins is an important aspect of construct characterization. Here, we have demonstrated that this is possible with a 5 min denaturing LC–MS method. This 5 min method (including column re-equilibration) employs a mix of 0.1% (v/v) TFA/0.1% (v/v) FA in water as mobile phase A and in 90% *n*-propanol as mobile phase B, with a flow rate of 500 μL/min and a column temperature of 65 °C. Using this rapid LC–MS method, we are able to derive a MW measurement RMS error of 22.17 ppm (0.80 Da) for 12 membrane proteins and 6 bispecific antibody constructs, ranging in MW from 17.5 to 105.3 kDa on a time-of-flight MS system operating at a modest resolution setting (10 000 fwhm)

Additionally, we demonstrate chromatographic separation of nonformylated and formylated AqpZ constructs. Minimal testing of a C8 column yielded no protein elution, which given the hydrophobicity of these multiple transmembrane region-containing proteins is not surprising. Thus, avoidance of column chemistries similar to C8 and C18 for development of other denaturing UPLC–MS and rapid LC–MS methods for

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membrane and hydrophobic proteins is recommended. However, with some optimization, C3, C4, and CN columns similar to those investigated here could be employed for high-throughput denaturing HPLC and UPLC−MS of membrane and other hydrophobic proteins. Although not investigated in this work, monolithic columns may also be useful for rapid denaturing LC−MS of hydrophobic proteins, as the large pore size of these columns would help minimize unwanted column retention. Contrary to previous opinions regarding the hydrophobicity of non-Fc-provided bispecific antibody constructs, thought to be similarly hydrophobic to membrane proteins, they did not exhibit similar chromatographic properties when analyzed by rapid LC−MS. The observation that chromatography of bispecific antibody constructs showed drastic improvement when decreasing mobile phase B from 30 to 5% supports a hypothesis that these molecules are, in general, not as hydrophobic as membrane proteins. Again, more non-Fc-provided bispecific antibody constructs would need to be analyzed to further support this hypothesis.

Furthermore, the authors acknowledge the considerable interest in identifying co-purified lipids in membrane protein purification reactions. While low MW species were detected in the chromatograms presented here, none of the species were pursued for further identification, as the focus of this method development was to provide a rapid screening method for accurate MW determination.

Furthermore, the optimized method requires no preinjection sample acidification or resuspension. The polyphenol column method development was performed using a single column, over 150 runs in total, with no loss in column viability. On the basis of the results presented herein using a short 5 min method on a commercially available polyphenol column, we expect that denaturing LC−MS of membrane proteins can indeed be a high-throughput characterization tool within both the industrial and academic-research setting. Additionally, the authors recognize that the 2.7 μm particle size is not standard for UPLC−MS analysis. However, this column is not yet available in 1.7 μm particle size. If made available, improved chromatographic separation and potentially even shorter method times could be achieved.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b03843.

Membrane protein construct preparation and purification protocols, poteolytic digestion conditions, alternative gradient conditions, Xervo Q-ToF tune settings and pressures, comparison of ion pairing agents, AqpZ chromatography, optimization of %B, representative chromatograms, Coq10 chromatography, reproducibility and limit of detection, deconvoluted mass, and proteolytic digestion (PDF)

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Supporting Information

A Rapid LC-MS Method for Accurate Molecular Weight Determination of Membrane and Hydrophobic Proteins

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The material supplied as Supporting Information is as follows:

- Membrane protein construct preparation and purification protocols for bR mutants, AqpZ constructs, K-K, Coq10 and AmtB-MBP
- Proteolytic digestion conditions for bR mutant and Coq10
- Alternative gradient conditions
- Xevo Q-ToF tune settings and pressures
- Comparison of ion pairing agents for the polyphenol and C3 columns
- AqpZ chromatography on 7 and 15-minute gradients
- Optimization of % B on both the polyphenol and C3 columns
- Representative chromatograms from both the polyphenol and C3 columns for all protein constructs
- Coq10 chromatography at 30% B on the polyphenol and C3 columns
- Reproducibility and limit of detection for the C3 column 5-minute method
- Deconvoluted mass for Coq10
- Proteolytic digestion followed by fragmentation for bR P50A and Coq10

bR mutants preparation and purification. The L111A, T47A, P50A and P50A/T46A bacteriorhodopsin (bR) mutants were purified from *Halobacterium*. The naturally-forming crystalline sheets in the membrane, referred to as purple membrane, were separated from other components by a sucrose gradient using the density of the purple membrane. For denaturing LC-MS, the bR mutants were diluted in 200 mM ammonium acetate/ 1.1% w/v β-Octyl Glucoside (OG).

WT, W14A and GFP-tagged AqpZ preparation and purification. The WT and W14A AqpZ were prepared as briefly described here. The gene encoding *E. coli* AqpZ was cloned in a pET29b expression vector to express the protein fused to a C-terminal hexahistidine tag cleavable with thrombin. The AqpZ-GFP construct was generated by Gibson assembly of the AqpZ and superfolder GFP genes and introduced into a pRSF vector to express an AqpZ-GFP fusion protein with a C-terminal hexahistidine tag cleavable with thrombin; a Gly-Ser linker connects the AqpZ and GFP proteins. The W14A point mutation was introduced using the QuikChange mutagenesis protocol. AqpZ was expressed in C43 (DE3) cells grown in 2x LB media at 37 °C. Protein expression was induced by adding β-D thiogalactopyranoside (IPTG) at 0.8 mM final concentration when cultures reached an OD600 of 0.5; induction was carried out for 5 hours at 37 °C before cells were harvested and washed. Cells were lysed in 500 mM NaCl/ 20 mM Tris-HCl pH 8.0/ 10 % glycerol/ 0.1 mM PMSF and 2.8 mM β-mercaptoethanol (β-ME) using a C3-Emulsiflex (Avestin) pressurized at 15,000 psi. Following lysis, the extract was clarified by low-speed centrifugation at 10,000 g for 30 minutes at 4 °C prior to separating the bacterial membranes by ultracentrifugation at 120,000 g for 2 hours at 4 °C. Bacterial membrane pellets were then solubilized in lysis buffer supplemented with 200 mM OG at 4 °C overnight. Insoluble material was pelleted by ultracentrifugation at 120,000 g for 1 hour at 4 °C. Detergent-solubilized AqpZ was then purified by immobilized-nickel affinity chromatography. After loading the solubilized extract, the resin was washed with 25 column volumes (CV) of 500 mM NaCl/ 20 mM Tris-HCl pH 8.0/ 10% glycerol/ 25 mM imidazole/ 40 mM OG/ 0.1 mM PMSF and 1.4 mM β-ME; AqpZ was then eluted in 500 mM NaCl/ 20 mM Tris-HCl pH 8.0/ 250 mM imidazole/ 40 mM OG/ 0.1 mM PMSF and 1.4 mM β-ME. AqpZ was then desalted using a PD-10 desalting column equilibrated in 150 mM NaCl/ 20 mM Tris-HCl pH 8.0/ 5% glycerol/ 1.4 mM β-ME. Following desalting, AqpZ was treated with thrombin for 16 hours at ambient temperature to remove the His-tag, then purified on a Superdex 200 HR10/30 size exclusion column (GE Healthcare) equilibrated in 150 mM NaCl/ 20 mM Tris-HCl pH=8.0/ 5% glycerol and 40 mM OG. Pure *E. coli* AqpZ eluted as a single peak corresponding to the homotetramer. AqpZ fractions were pooled and passed across a small Ni-IMAC column (250 µL resin) to remove any remnants of tagged protein. The protein was then concentrated to 6.5 mg/ml for subsequent analysis. A similar process was also performed to obtain AqpZ-GFP fusion and the W14A mutant forms.

KcsA Kv1.3 preparation and purification. KcsA Kv1.3 (K-K) fused to a N-terminal hexahistidine tag was expressed in transformed *E. coli* M15 pREP4 cells. Protein expression was induced by adding IPTG at 0.5 mM final concentration; induction was carried out for 2 hours at 30 °C before cells were harvested and washed. The cells were harvested by centrifugation at 4,500 *g* for 10 min at 4 °C and lysed with 2 passes through a cell disruptor in lysis buffer consisting of 20 mM Tris-HCl pH 7.5/ 5 mM imidazole/ 150 mM KCl. The crude lysate was centrifuged at 20,000 *g* for 1 hour at 4 °C. The supernatant was removed and centrifuged at 100,000 *g* for 1 hour at 4 °C, homogenized with a Potter-Elvehjem homogenizer and then frozen. The frozen lysate was then thawed and solubilized with 3% (w/v) (final concentration) styrene maleic acid lipid polymer and incubated at 4 °C with gentle agitation overnight. The next day, after centrifugation at 100,000 *g* for 1 hour at 4 °C, the supernatant was mixed with 10 mM $MgCl_2$ and 20 mM β-Decyl Maltoside (DM), incubated for 4 hours at 4 °C, then transferred to pre-equilibrated Talon resin (Clontech) and incubated, while rocking, overnight at 4 °C. The column was then drained and washed once with lysis buffer containing 20 mM DM. This was followed by several washes with buffer containing 20 mM Tris-HCl pH 7.5/ 5 mM imidazole/ 150 mM KCl/ 5 mM DM as well as the previous buffer with 0.5 M NaCl. Each of these washes was collected for SDS PAGE analysis. The protein was then eluted from the resin with 6 CV of lysis buffer containing 100 mM imidazole and

5 mM DM. Fractions containing K-K were pooled and their concentrations determined by their A_{280} measurement. Some of this purified protein was then cleaved with caspase-3 to remove the N-terminal His-tag. To achieve cleavage, 6 µg of caspase-3 was added to 350 µg of the protein, which was then incubated at 4 °C for 6 hours. Successful His-tag cleavage was accessed by the denaturing LC-MS method described in the main text.

Coq10$^{N\Delta30}$ preparation and purification. The His-SUMO-tagged Coq10$^{N\Delta30}$ was expressed in transformed *E. coli* BL21-(DE3) cells (Invitrogen). The *E. coli* culture was grown in LB with 50 µg/mL kanamycin at 37 °C to an OD_{600} of 0.6-0.8 before the expression was induced with 1 mM IPTG at 16 °C for 18 hours. The cells were harvested by centrifugation at 4,500 *g* for 10 min at 4 °C and lysed with a M-110P microfluidizer (Microfluidics) in lysis buffer consisting of 50 mM NaH_2PO_4, pH 8.0/ 500 mM NaCl/ 10 mM imidazole/ 10% glycerol/ 1 mM DTT/ 0.1% Triton X-100/ 1 mM phenylmethylsulfonyl fluoride (PMSF)/ Roche Complete EDTA-free protease inhibitor (Sigma-Aldrich). The crude lysate was centrifuged at 10,000 *g* for 45 min at 4 °C and the collected soluble fraction was incubated with pre-equilibrated Ni-NTA resins (Qiagen) for 2 hours at 4 °C. To remove impurities, Ni-NTA resin was step washed three times each with 2 CV of wash buffer containing 20 mM or 35 mM imidazole in 50 mM NaH_2PO_4, pH 8.0/ 300 mM NaCl/ 10% glycerol/ 1 mM DTT/ 0.1% Triton X-100, and the protein was eluted five times with 1.5 CV of wash buffer containing 200 mM imidazole. The protein eluates were pooled and concentrated using a 10 kDa Amicon (Millipore). Recombinant His-tagged SUMO protease, also known as Ulp1, was added to the concentrated eluate (approximately 400 µg of His-tagged Ulp1 was added to every 10 mL of concentrated eluate) to cleave the His-SUMO moiety during overnight dialysis against 50 mM NaH_2PO_4 pH 8.0/ 300 mM NaCl/ 10% glycerol/ 1 mM DTT/ 0.1% Triton X-100 at 4 °C using a 10,000 MWCO Slide-A-Lyzer dialysis cassette (Thermo). After overnight dialysis, the protein inside the dialysis cassette was removed and allowed to incubate with pre-equilibrated Ni-NTA resins for 2 hours at 4 °C to remove His-tagged Ulp1 protease, His-SUMO moiety (released after Ulp1 cleavage), and any uncleaved His-SUMO-tagged Coq10$^{N\Delta30}$ fusion protein, thereby leaving cleaved Coq10$^{N\Delta30}$ in the flow through. The Ni-NTA resins were washed four times each with 2 CV of wash buffer without imidazole. The combined flow through and washes were concentrated using a 10 kDa Amicon and further purified by gel filtration chromatography on a Superdex 75 Increase column (GE Healthcare) to remove any residual contaminants and to buffer exchange into 10 mM Tris, pH 7.0/ 10 mM NaCl/ 0.5 mM Tris (2-carboxyethyl)-phosphine hydrochloride (TCEP)/ 3% 2-methyl-2,4-pentanediol (MPD)/ 0.1% n-dodecyl β-D-maltoside (DDM).

AmtB preparation and purification. AmtB was purified as described in Reid. et. al. 2017. Anal. Chem.

Proteolytic digestion of bR P50A. Chymotrypsin digestion conditions were as follows: 20 µg of bR P50A was incubated at ambient temperature in 150 mM Tris, pH 7.5/ 8 M urea/ 40 mM hydroxylamine for 30 minutes before being diluted with water to a final concentration of 37.5 mM Tris pH 7.5/ 2 M urea/ 10 mM hydroxylamine. The digestion was brought to a total volume of 30 µL with deionized water after the addition of 1 µL of chymotrypsin. The digestion was then incubated overnight at 37 °C, after which 30 µL of 0.1% formic acid (FA) was added, followed by 25 mM tris (2-carboxyethyl) phosphine (TCEP) for an incubation period of 15 minutes at 37 °C prior to LC-MS/MS analysis. Following digestion with chymotrypsin and prior to acidification/ TCEP reduction, some of the material was also digested with Glu-C by adding 1 µg of Glu-C and incubating again at 37 °C overnight. This digestion reaction was also analyzed by LC-MS/MS.

For bR P50A, both chymotrypsin and chymotrypsin/ Glu-C digests were analyzed to yield N- and C-terminal information. The digests were analyzed by LC-MS/MS using an Agilent 1260 capillary HPLC linked to a Thermo Q-Exactive MS (Thermo Scientific; Waltham, MA). The HPLC utilized a ZORBAX 300SB-C18 5 µm, 250 x 0.5 mm column (Agilent Technologies; part# 5064-8266). For peptide separations, mobile phases A and B consisted of 0.1% FA in H_2O and 0.1% FA in acetonitrile, respectively. Peptides were separated at 25 µl/min at 30 °C using the following gradient: 10 minutes at 1% B, up to 55% B over 85 minutes, up to 97% B over 5 minutes, isocratic at 97% B for 5 minutes, and

then down to 1% B over 10 minutes. bR was analyzed by LC-MS/MS using a full MS scan of *m/z* [350-2000] at 70K resolution (FWHM), followed by high resolution HCD scans of the top 10 most abundant precursors at 17.5K resolution. A spray voltage of 3.5 kV, S-Lens RF level of 50, isolation width of 2.0 Da, collision energy (CE) of 27 and a 10 second dynamic exclusion were used.

Proteolytic digestion of Coq10. Glu-C digestion conditions were as follows: 5 µL of Coq10 (~10 µg) was incubated at 37 °C overnight in 10 µL of water and 5 µL of 150 mM Tris pH 7.5/ 8 M urea/ 40 mM hydroxylamine (final buffer composition of 37.5 mM Tris pH 7.5/ 2 M urea/ 10 mM hydroxylamine). To this, 0.5 µg of Glu-C was added prior to incubation. The digest was acidified by adding 20 µL of 0.1% TFA prior to LC-MS analysis. The digest was analyzed on a Thermo OrbiTrap Velos MS (Thermo Scientific; Waltham, MA) with the EASY Spray nanospray ionization source. An integrated 75 µm C18 column/emitter assembly (Thermo P/N ES 800; pepMap RSLC C18 3µm, 100 Å, 75 µm x 15 cm) was used at a spray voltage of 1.9 kV. Coq10 was analyzed using a full MS scan from *m/z* [300-2000] at 30K resolution, followed by low resolution CID of the top 10 most abundant precursor ions in the LTQ.

Alternative chromatography conditions. Both a Waters C4 and an Agilent cyano column were thoroughly investigated using an Agilent 1200 series in line with a Waters Synapt G2 ion mobility Q-ToF MS. AqpZ eluted off the C4 column during both 15 and 10-minute gradients using H_2O with 0.1% FA as mobile phase A and either acetonitrile or isopropanol (with 0.1% FA) as mobile phase B. However, after investigation of multiple 5-minute gradient configurations, no AqpZ was observed to elute. Due to these results, C4 column chemistry was abandoned, however, with further testing of various mobile phases it is possible that a 5-minute gradient could be achieved.

A cyano column was also investigated (custom Agilent column) with the following specifications: 2.1 x 50 mm, 300 Å pore size and 1.8 µm particle size. This column was investigated with various 15, 10 and 5-minute gradients employing H_2O with 1% FA as mobile phase A and acetonitrile with 1% FA as mobile phase B. Each of these gradients were investigated at both 40 and 65 °C, and for this column chemistry, less baseline fluctuation was observed at 40 °C. Each of the gradients tested succeeded in eluting AqpZ. However, this column required a higher flow rate of 750 µL/min to achieve chromatography with low background. Additionally, carry over was consistently observed, even after two post protein blank injections. For this reason, TFA at 0.1% v/v was added to both mobile phases to improve chromatography. However, even with the addition of TFA, the chromatography improved only slightly. Additionally, with the high flow rate still necessary to elute AqpZ within the 5-minute gradient described in the main text, method development was moved to a UPLC compatible C3 column for further development.

Longer 9 and 7-minute methods were both explored on the C3 column, and both eluted several of the membrane proteins investigated employing H_2O with 0.1% TFA/0.1% FA as mobile phase A and 90% *n*-propanol with 0.1% TFA/0.1% FA as mobile phase B. These methods also provided more separation of the protein and DDM as compared to the final 5-minute method. Both of the following 7-minute methods were successful in protein elution and chromatographic separation of the protein from DDM. The first gradient was held at 20% B starting from 0-2 minutes, then ramped over the next 2 minutes to 72% B, slowly ramped to 90% B at 4.5 minutes then brought to 20% B ending at 7 minutes. The other 7-minute method was also held at 20% B starting from 0-2 minutes, then ramped to 90 %B at 3 minutes and held there for 0.5 minutes. After which, the % B was ramped down to 50% B at 5 minutes then down to 20% B at 7 minutes. The 9 minute gradient was held at 20% B from 0-3 minutes then ramped as described for the first 7 minute method and held at 20% B from 7-9 minutes.

If analysis of a membrane protein on the 5-minute gradient detailed in the main text does not provide a chromatographically separated protein peak the authors suggest several options as starting points for troubleshooting. The first would be to lengthen the gradient to one of the 7-minute methods described above. Both methods successfully eluted each of the membrane proteins investigated in this manuscript and provided chromatographic separation from detergents and other low MW species present in the

samples. Additionally, the same 5-minute method described in the main text can be explored at varying starting %B. As was observed for Coq10 and K-K, while good chromatography is achievable at multiple starting percentages, some membrane proteins seem to elute optimally at a slightly lower % B.

Xevo Q-ToF Tune Settings and Pressures. The Q-ToF MS was operated at the following pressures (mbar): backing 2.24, collision cell 1.17 e -2 and ToF 5.87 e -7. For all injections the capilary voltage was 3 kV, sampling cone voltage was either 25, 40, 60, or 75 V (depending on the size of the monomeric protein), source and desolvation temperatures were 80 and 300 °C, respectively. The cone and desolvation gases were maintained at 20 and 800 L/hr, respectively. The detector voltage was 1950 V and 6 V of collision energy was applied during each injection.

MaxEnt parameters were set as follows with iterations to convergence: output mass ranges (dependent on the theoretical monomeric protein mass); 1 Da/channel resolution; minimum intensity ratio left and right at 80%; width at half height for uniform Gaussian model from 0.3–1.1 Da (construct dependent). This deconvolution spectrum was then area centered for the final mass measurement.

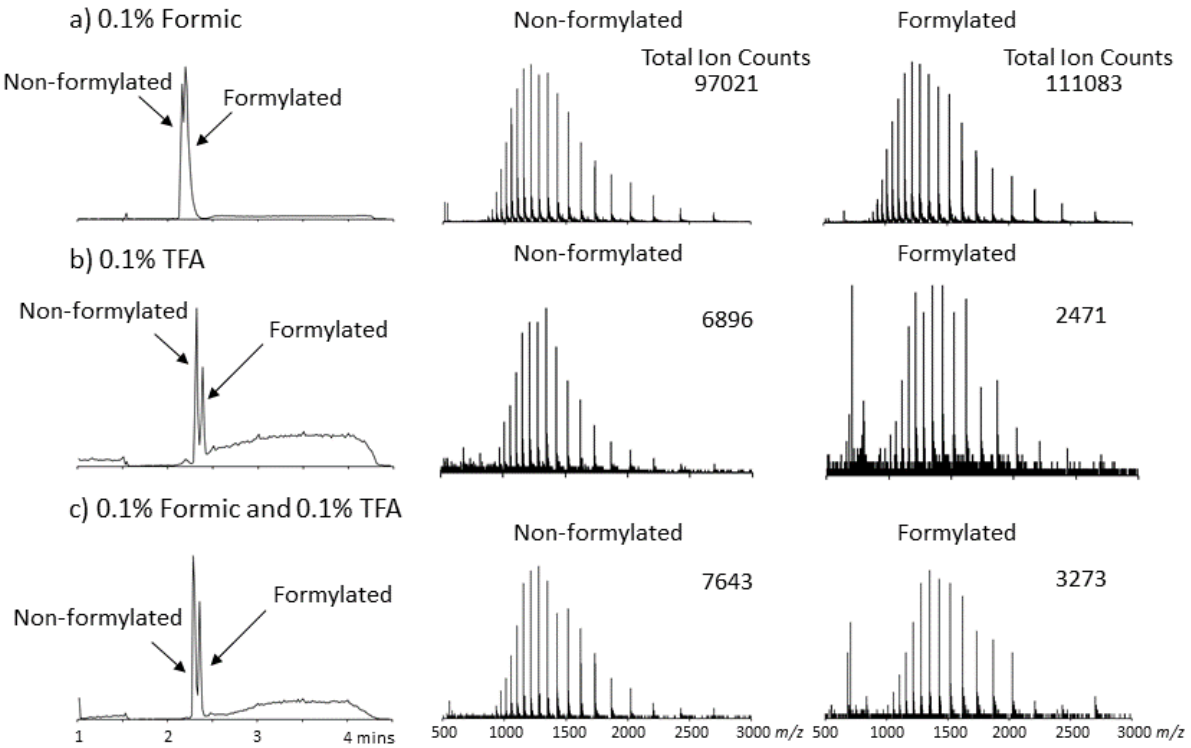

Figure S1. Comparison of WT AqpZ chromatograms and spectra obtained on the BioResolve Polyphenol column with 0.1% FA (a); 0.1% TFA (b) and 0.1% TFA/0.1% FA mix (c) as additives to both mobile phase A (H_2O) and B (90% *n*-propanol). Protein peaks are marked in each chromatogram by a black arrow. Numbers in the top right of each spectra represent the total ion counts obtained from area centered, deconvoluted data. The y-axis for the chromatograms and spectra represent intensity with arbitrary units.

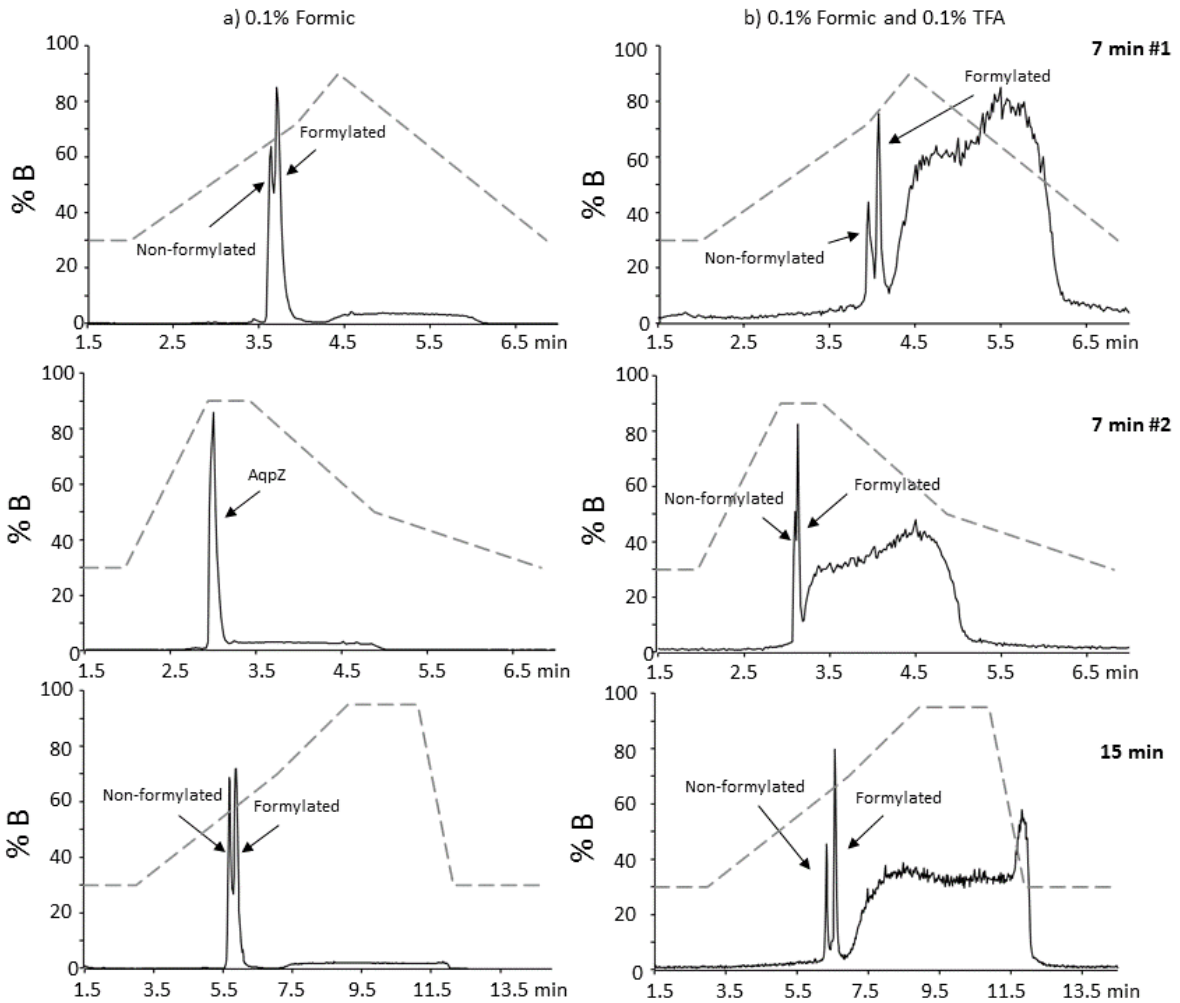

Figure S2. Comparison of three gradients for the separation of non-formylated and formylated WT AqpZ. Profiles of % B (dashed lines) are overlaid on each reconstructed ion chromatogram (RIC). Both 7-minute methods are detailed in the *Supporting Information*. As shown, improved separation is consistently observed with the mix of 0.1% formic/ 0.1% TFA, regardless of gradient length, as compared to 0.1% formic acid.

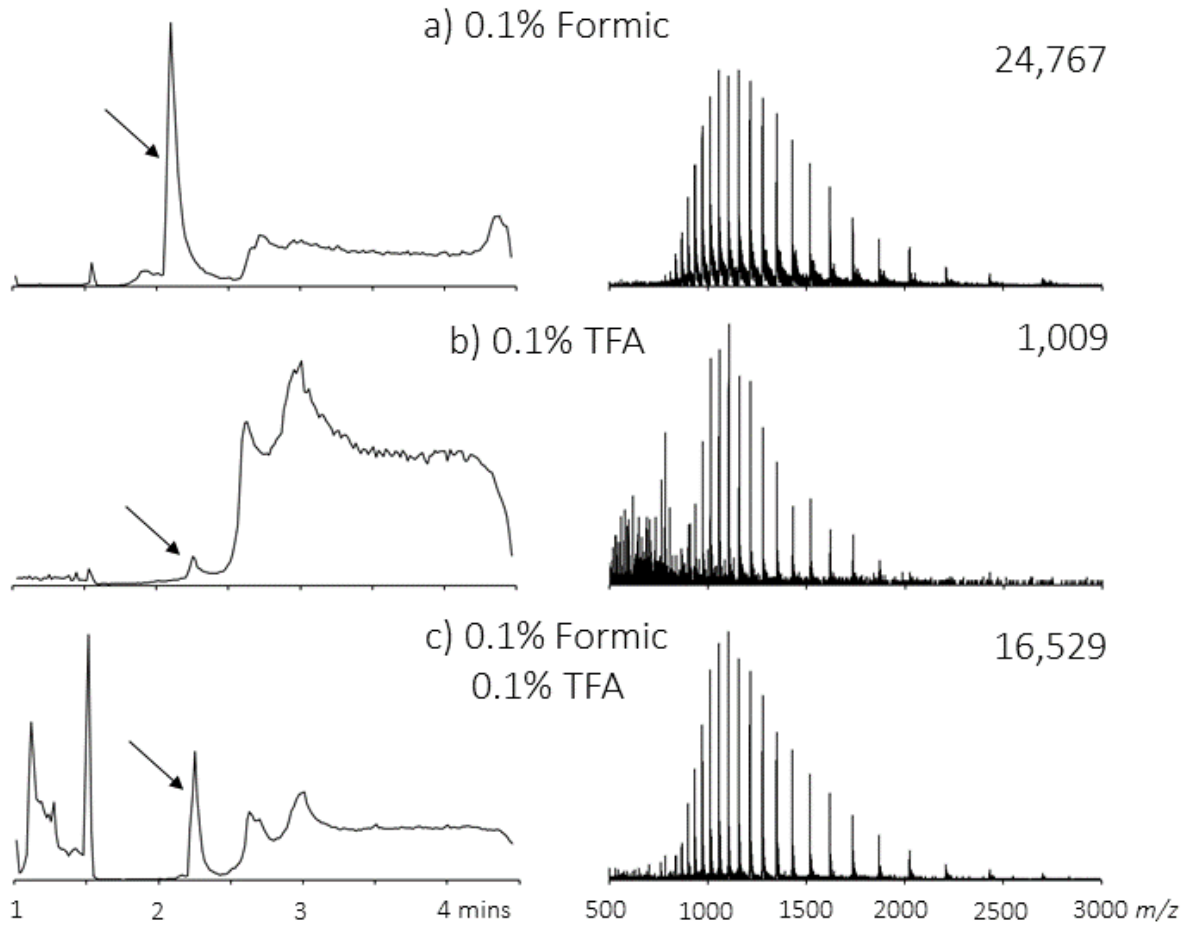

Figure S3. Comparison of WT AqpZ chromatograms and spectra obtained on the ZORBAX 300SB C3 column with 0.1% FA (a); 0.1% TFA (b) and 0.1% TFA/0.1% FA mix (c) as additives to both mobile phase A (H_2O) and B (90% n-propanol). Protein peaks are marked in each chromatogram by a black arrow. Numbers in the top right of each spectra represent the total ion counts obtained from area centered, deconvoluted data. The y-axis for the chromatograms and spectra represent intensity with arbitrary units.

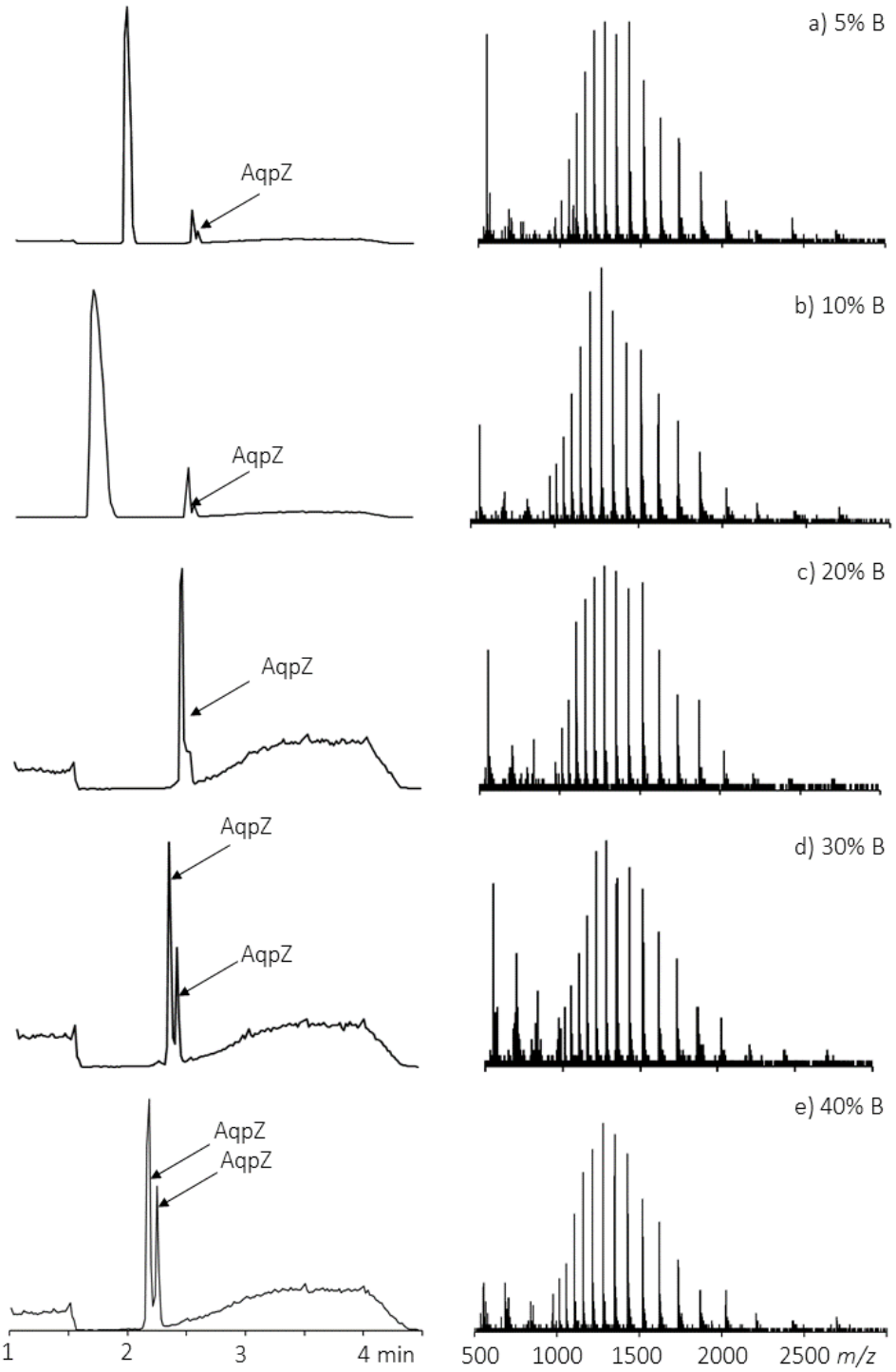

Figure S4. Determination of optimal starting %B with WT AqpZ at (a) 5%, (b) 10%, (c) 20%, (d) 30% and (e) 40% starting %B. Optimal starting concentration was determined from chromatographic separation of non-formylated and formylated protein. Spectra shown are a combination of both the non-formylated and formylated AqpZ as for 5, 10 and 20% runs the chromatographic separation between the two species was difficult to discern. The y-axis for the chromatograms and spectra represent intensity with arbitrary units.

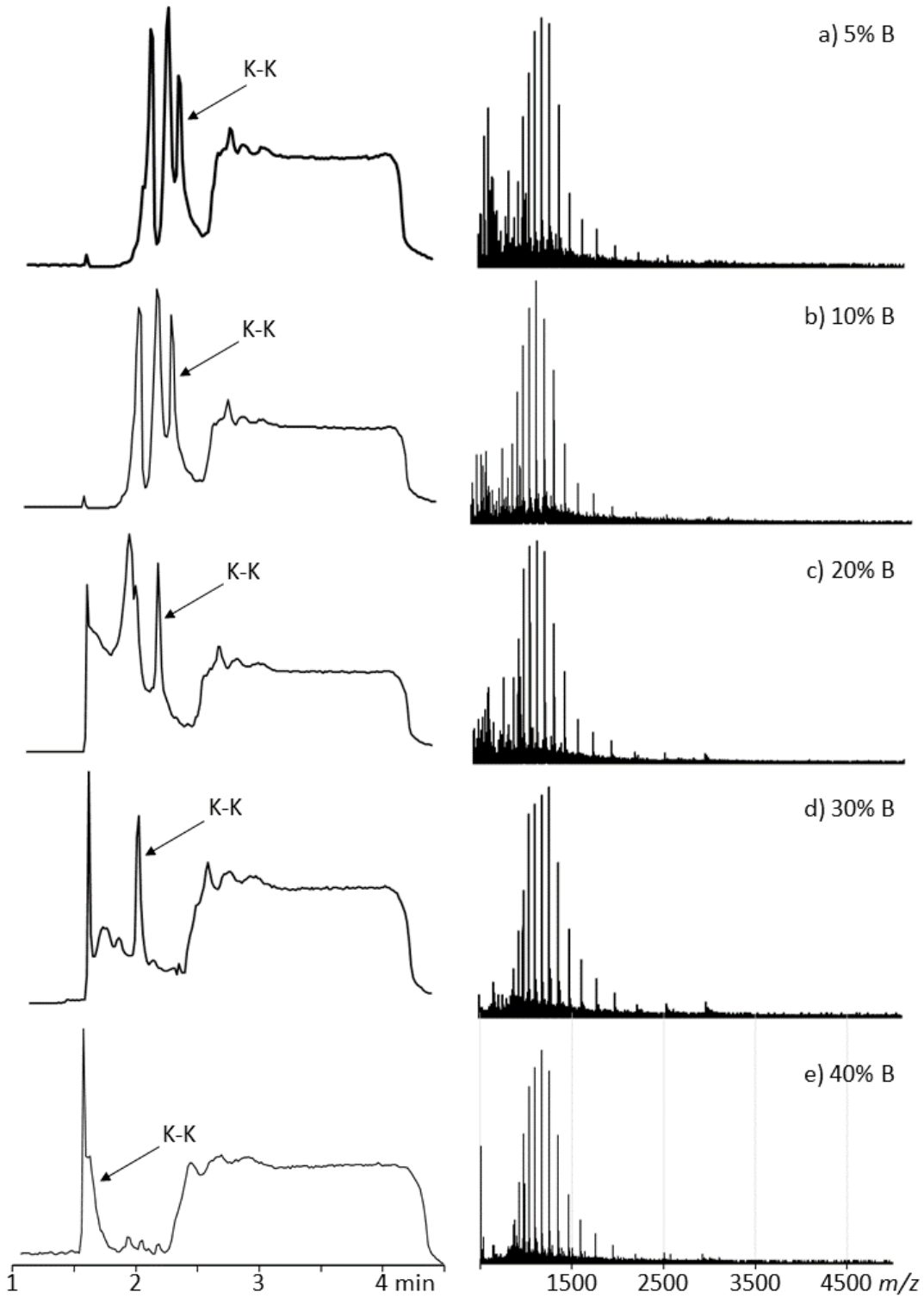

Figure S5. Determination of optimal starting %B with K-K on ZORBAX 300SB C3 column at (a) 5%, (b) 10%, (c) 20%, (d) 30% and (e) 40% starting %B. Optimal starting concentration was determined from both chromatographic separation and lowest signal for low *m/z* species. The y-axis for the chromatograms and spectra represent intensity with arbitrary units.

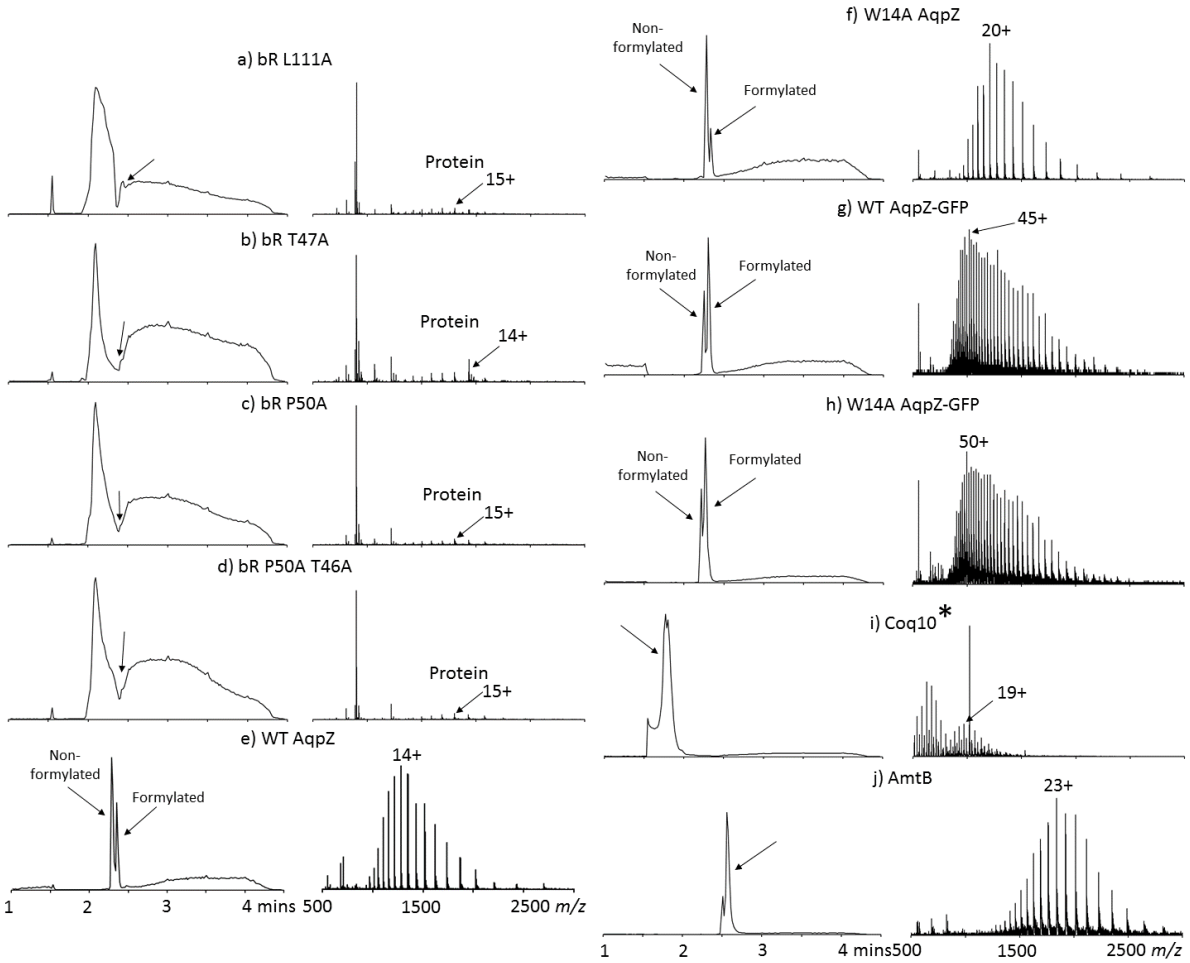

Figure S6. Representative chromatograms and spectra obtained with the BioResolve Polyphenol column from (a) bR L111A; (b) bR T47A; (c) bR P50A; (d) bR P50A T46A; (e) WT AqpZ; (f) W14A AqpZ; (g) WT AqpZ-GFP; (h) W14A AqpZ-GFP; (i) Coq10; (j) AmtB-MBP and (l) AmtB. Protein signal in the chromatograms are marked with a black arrow. The * denotes that the Coq10 chromatographic and MS spectrum data were obtained at 25% B starting as opposed to 30 % B. The most intense charge state is labeled in each MS spectrum. For bR mutant samples, the protein peak is chromatographically unresolved. Monitoring the protein by UV absorbance shows the retention time at about 2.4 minutes for all mutants. The y-axis for the chromatograms and spectra represent intensity with arbitrary units.

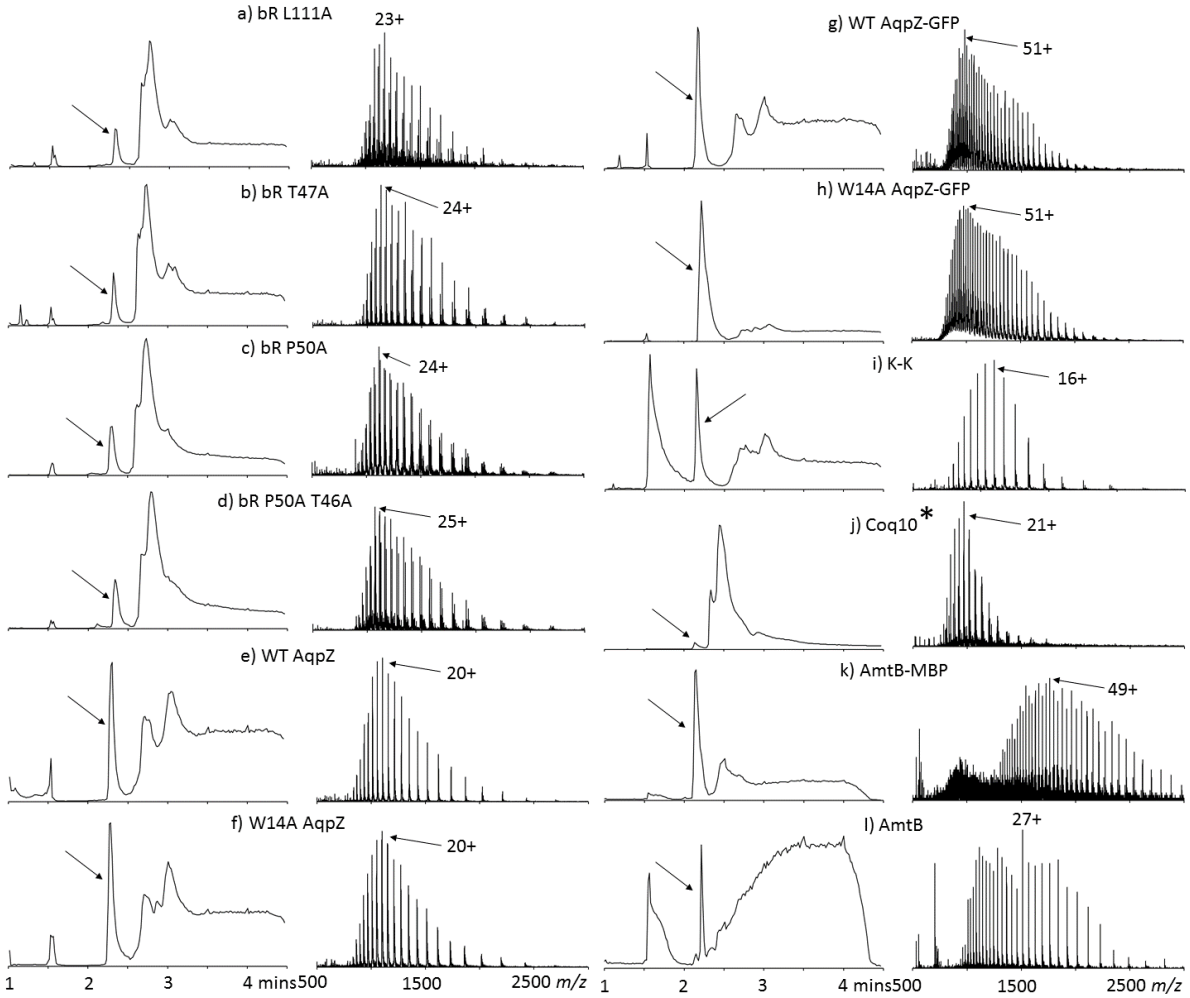

Figure S7. Representative chromatograms and spectra obtained with the ZORBAX 300SB C3 from (a) bR L111A; (b) bR T47A; (c) bR P50A; (d) bR P50A T46A; (e) WT AqpZ; (f) W14A AqpZ; (g) WT AqpZ-GFP; (h) W14A AqpZ-GFP; (i) K-K; (j) Coq10; (k) AmtB-MBP and (l) AmtB. Protein signal in the chromatograms are marked with an arrow. The * denotes that the Coq10 chromatographic and MS spectrum data were obtained at 20% B starting as opposed to 30% B. The most intense charge state is labeled in each MS spectrum. The y-axis for the chromatograms and spectra represent intensity with arbitrary units.

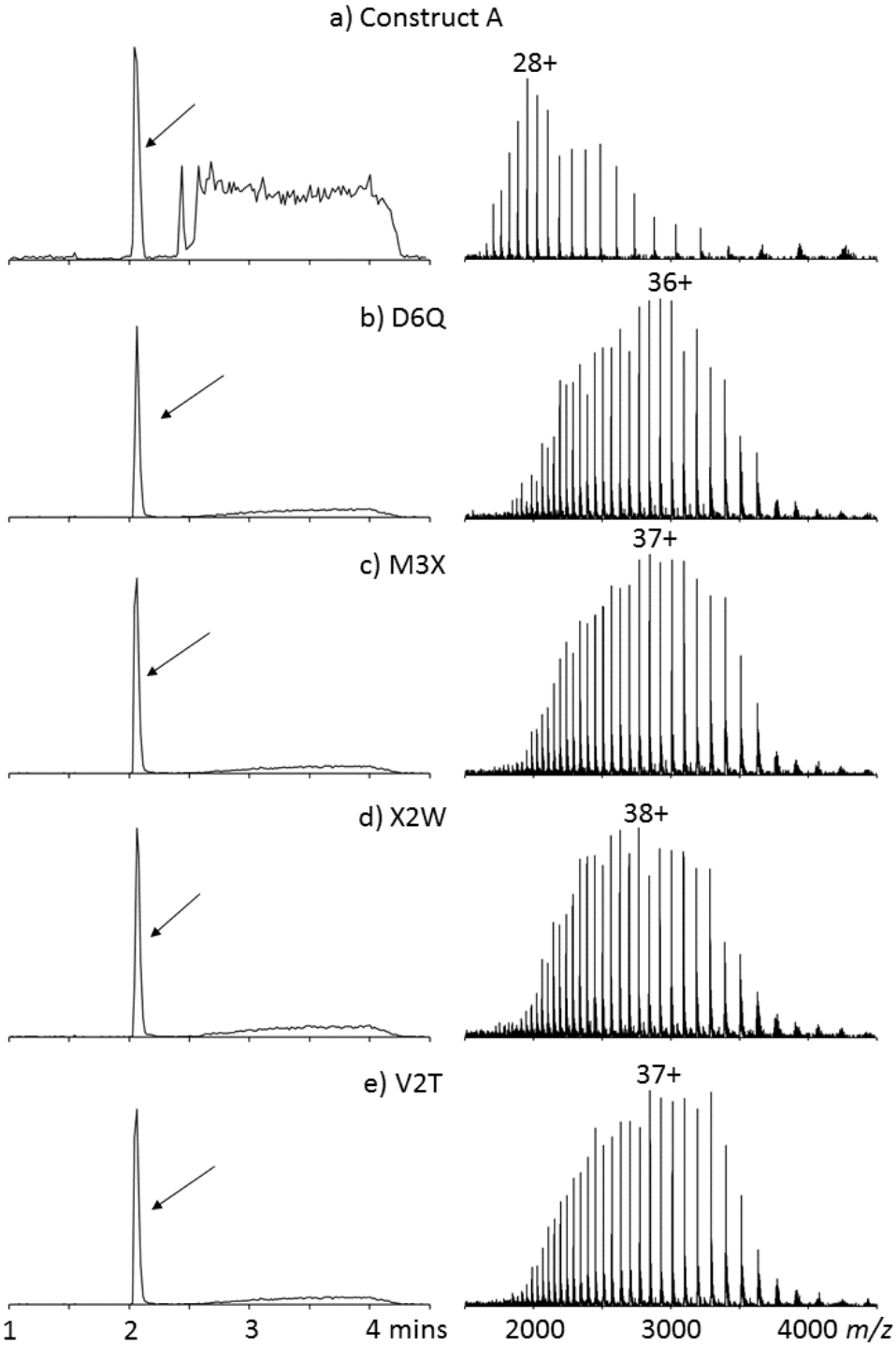

Figure S8. Representative RICs and spectra for five of the bispecific antibody constructs analyzed (a) construct A; (b) D6Q; (c) M3X; (d) X2W; and (e) V2T. The most intense charge state is labeled in each MS spectrum. The y-axis for the chromatograms and spectra represent intensity with arbitrary units.

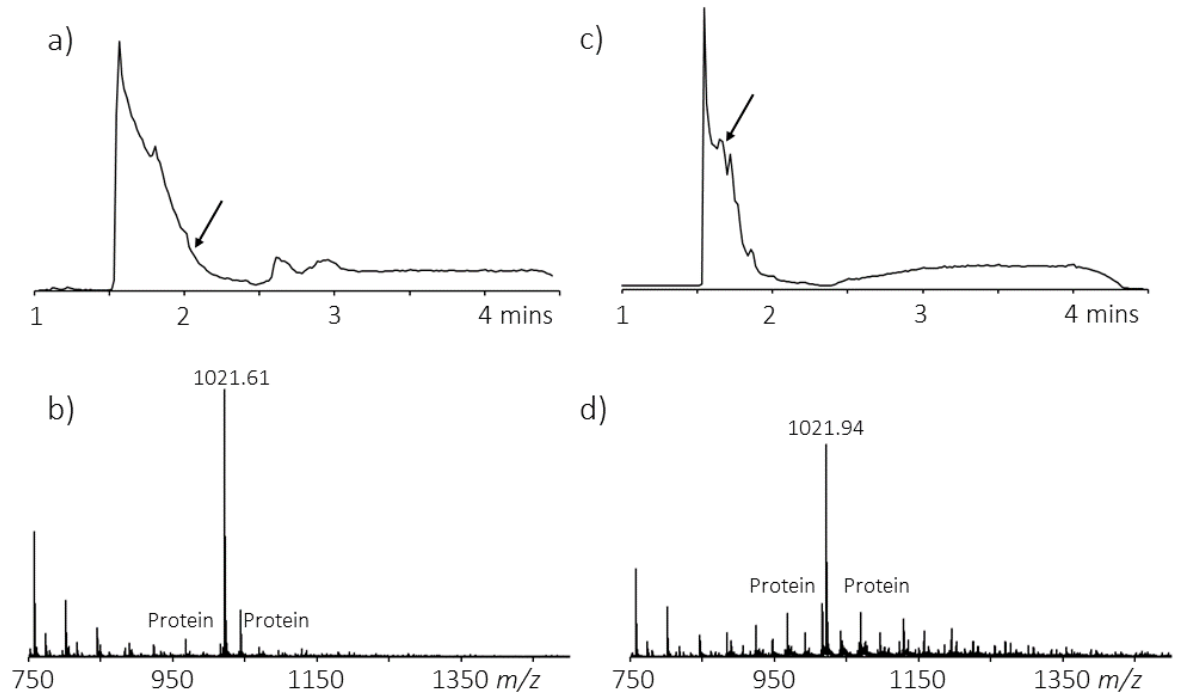

Figure S9. C3 chromatography (a) and spectrum (b) and polyphenol chromatography (c) and spectrum (d) obtained for Coq10 at 30 %B. Coq10 elutes as a shoulder of the first peak (denoted by an arrow) and the protein signal is heavily suppressed by the signal at 1021.61 *m/z*. Both the clipped and unclipped species were observed in the deconvoluted spectrum and provided average masses of 18,306.77 ± 0.22 Da and 20,302.38 ± 0.05 Da, respectively. The mass accuracy obtained from triplicate injections at 30 %B was Δ 0.66 and 0.23 Da for the clipped and unclipped species, respectively. Protein signal suppression was less on the polyphenol column and again, both the clipped and unclipped species were observed in the deconvoluted spectrum. The species provided average masses of 18,313.67 ± 0.31 Da and 20,310.11 ± 0.10 Da with mass accuracy of Δ +6.2 and +7.5 Da for the clipped and unclipped species, respectively. The y-axis for the chromatograms and spectra represent intensity with arbitrary units.

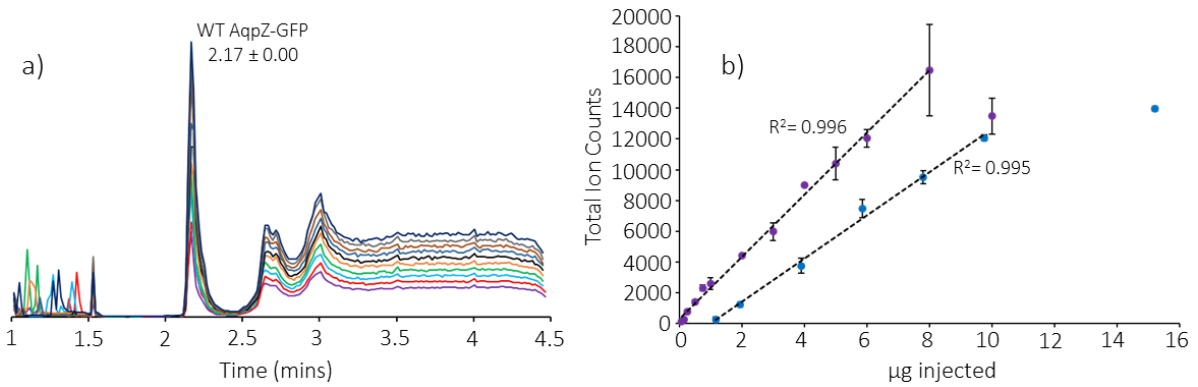

Figure S10. Reproducibility and limit of detection experiments on the ZORBAX 300SB C3 column. Reproducibility tests (a) with WT AqpZ-GFP at 1.6 µg/injection. Each run is represented with a different color and shows an overall reproducibility of retention time of 2.17 ± 0.00 minutes. Limit of detection studies (b) with bR P50A T46A (blue) and K-K (purple), injection amounts based on A_{280} readings ranged from 0.2 to 15 µg and 0.075 to 10 µg for bR P50A T46A and K-K, respectively. Outliers for bR P50A T46A existed at 0.2 and 15 µg and for K-K at 10 µg's. Total ion counts on the y-axis were obtained from MaxEnt deconvolution of the LC integrated peak area. The y-axis for the chromatograms represent intensity with arbitrary units.

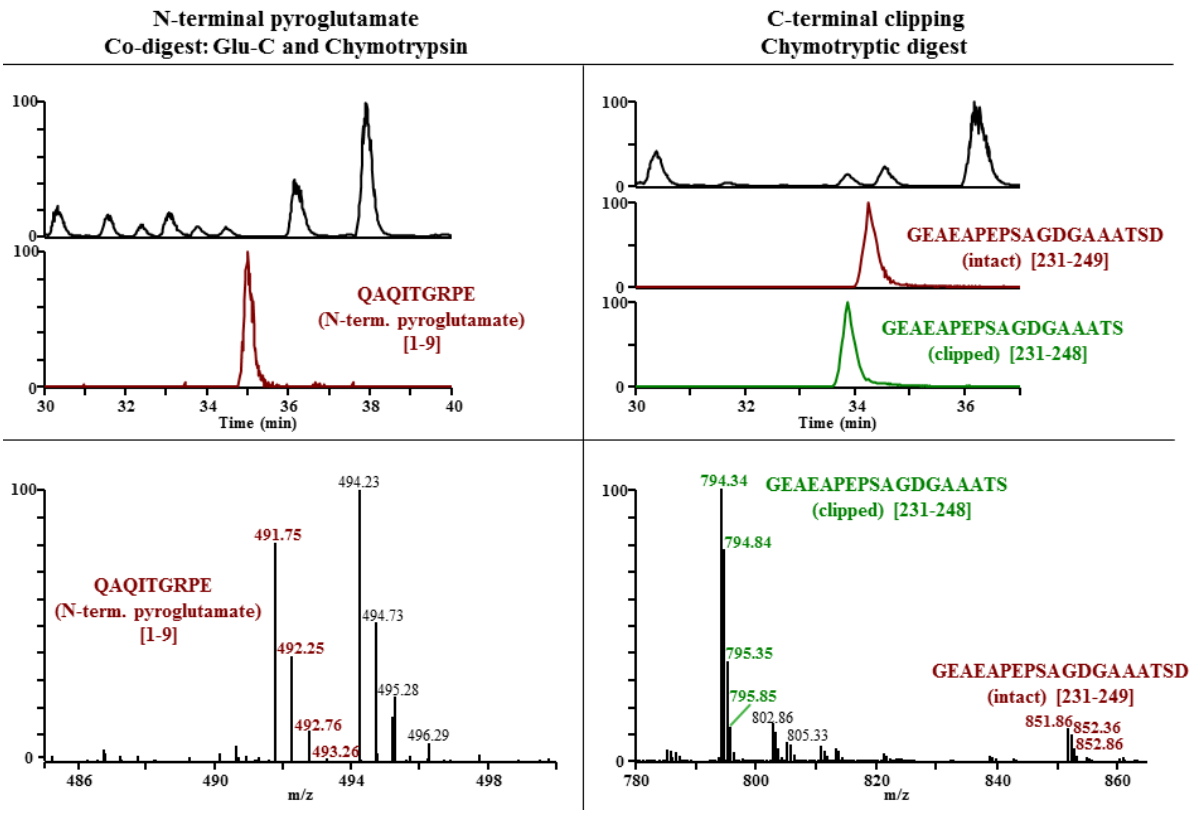

Figure S11. Chymotrypsin and chymotrypsin/ Glu-C digestion of bR P50A revealed both N- and C-terminal modification. LC-MS/MS of a chymotrypsin/ Glu-C digest (*left panel*) shows the presence of pyroglutamate on the N-terminus. XIC signal of N-terminal peptide [top], full MS signal of same peptide [bottom]. LC-MS/MS of a chymotrypsin digest (*right panel*) shows two C-terminal peptide species: intact and one clipped missing the C-terminal aspartate. XIC signal of both C-terminal species [top]. The relative intensities of the two species show that loss of the aspartate had occurred in roughly 85% of this construct [bottom].

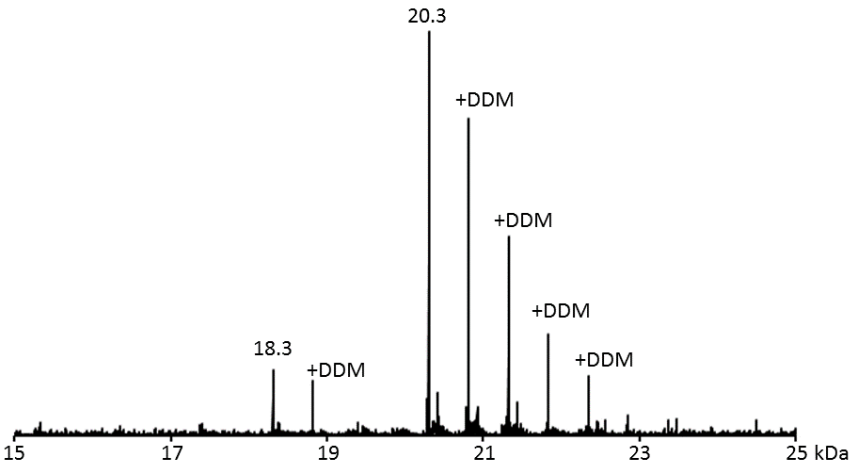

Figure S12. Deconvoluted spectrum of Coq10 showing the presence of a clipped sequence and the intact sequence at 18,307.04 Da and 20,302.13 Da, respectively. Both species display DDM adduction, more pronounced on the intact construct. Each DDM adduct corresponds to a mass addition of +510 Da. Coq10 is the only protein construct for which detergent adducts were observed upon spectral deconvolution. The y-axis for the spectrum represent intensity with arbitrary units.

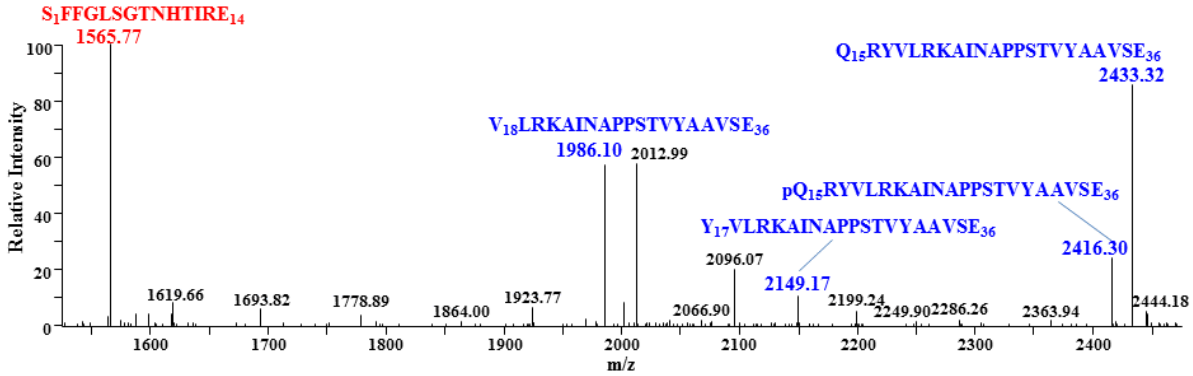

Figure S13. Deconvoluted full MS data from the Glu-C digest of Coq10 revealed two major N-terminal peptides (1565.77; S_1 – E_{14} and 1986.10; V_{18}- E_{36}). The confirmation of N-terminal cleavage was provided by the appearance of several truncated forms of the expected Glu-C derived Q_{15}- E_{36} peptide that could only be observed if N-terminal clipping was present. These peptides are V_{18}-E_{36} (1986.10) and Y_{17}- E_{36} (2149.17 *m/z*).