**The Rapid Denaturing Organic Digestion Method for Targeted Protein Identification and Characterization**

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## Abstract

Bottom-up mass spectrometry-based protein analysis methods employing protease digestion are routinely used to identify and characterize proteins, with high specificity and sensitivity. Method performance is generally measured by sequence coverage capability and the total number of characteristic peptides identified, when compared to predicted databases. Limitations to commonly used solvent-based digestion methods currently employed include long digest times (18-24 h or more), leading to protease autolysis which also precludes automation, decreases sensitivity, and increases both intra- and inter-day performance variability. This report describes the development and validation of a simple, 5 min tryptic Denaturing Organic Digestion (DOD) method for use with tandem mass spectrometry in bottom-up protein identification and characterization. It has been evaluated across select protein toxins and diagnostic clinical protein targets substantially improving digestion performance when compared to other solution-based and enzyme immobilized methods. The method was compared to two currently used bottom-up methods, the 24 h Filter Aided Sample Prep (FASP) and Flash Digest (1 h and 4h) methods. Single proteins used here to compare the methods included ricin light chain, ricin heavy chain, ricin holotoxin, serotype A Clostridium botulinum toxin, Staphylococcus enterotoxin B, ribonuclease A, and thyroglobulin. In tests, across the proteins investigated, the 5 min DOD digestion method resulted in sequence coverages ranging from 55-100%, with relative high reproducibility and precision; results were better than or equal to FASP method results and were greatly enhanced when compared to Flash method results. Importantly, DOD method intra- and inter-day precision was much improved as compared to results for both FASP and Flash digestions. These data indicated that the DOD method, when compared to the FASP and Flash Digest methods, dramatically reduced digestion time while maintaining or improving the ability to detect and characterize targeted proteins, and reduced analytical variability for tryptic digestion resulting in markedly faster and more precise analyses.

## Introduction

Protein identification and quantification is a challenging task. The most commonly used mass spectrometry-based protein analysis approach, bottom-up protein analysis, does not have a requirement for high resolution mass spectrometry, usually requires a much shorter analysis time, and has historically been much more sensitive than top-down approaches. Because the mass range of many of the mass spectrometers used is limited, bottom-up methods initially employ proteolytic digestion in order to produce characteristic peptides, unique to the protein targeted, and amenable to analysis by these instruments. To identify the peptides, their masses or their tandem mass spectra are then compared to sequence database or annotated peptide spectral library predictions. The peptide data is then compiled to predict the sequence of the parent protein. These methods, however, have generally been limited by an inability to provide sufficient sequence coverage to fully characterize targeted proteins and are limited in their ability to fully characterize PTM maps and point mutations located in digestion fragments that are not easily ionized. In addition, bottom-up methods have had relatively poor reproducibility, often attributed to the tendency for protease autolysis during lengthy digestion times, especially at high relative protease concentrations, and inconsistent target analyte recoveries inherent with lengthy multi-step processes. To improve sequence coverage, multi-protease strategies have been devised which have proven to dramatically improve results[1,2,3], but the additional processes can reduce sensitivity, make results more variable due to compounded inherent recovery losses, and dramatically increase analytical time by increasing sample preparation time from hours to days.

The most common protease used in targeted protein analysis is trypsin, which cleaves the amide bond on the C-terminal side of lysine and arginine residues, except when they are immediately followed by a proline. Most previously published benchtop tryptic digestion methods require 18-24 h digestion times[4]. Trypsin autolysis, directly correlated with trypsin concentration and digestion time, is the primary factor contributing to inter- and intra-day variability[5,6]. Recent descriptions of methods employing on-column and bead surface immobilization of protease[7] have dramatically reduced digestion times, improved method reproducibility and almost eliminated autolysis. These methods, however, often provide limited sequence coverage as compared to in-solution methods, likely due to steric hindrance issues. Another limitation to column-immobilized protease digestion is the inability to multiplex sample preparation.

Previously, the effects of organic modifiers in solvent systems or elevated temperatures have been used to speed protein digestion, improve digestion reproducibility, provide increased sequence coverage for protein characterization, and to identify conditions under which the protein folding could be characterized. Data demonstrated that trypsin proteolytic activity was maintained across a relatively wide temperature range in aqueous environments and in a variety of organically modified solvent systems, and that organic solvents denatured proteins, exposing otherwise protected domains. In addition, work had been conducted using either a combination of low organic solvent concentration and temperatures up to 40°C or higher organic solvent concentrations alone.

In 1987, Fink et al.[8] investigated the denaturation or unfolding of carboxymethylated, disulfide-reduced ribonuclease A in methanol-water cryosolvent systems. The goal was to identify cryosolvent conditions under which the protein was stabilized, in multiple partially folded intermediate forms, in order to examine the thermodynamics, kinetics, and structural aspects of protein folding mechanisms. They monitored the degree of exposure or burial of tyrosine residues to measure the degree of protein unfolding. The results demonstrated that there was a positive correlation between increased unfolding and both methanol (MeOH) concentration and temperature and that unfolding increased even more with increases in both MeOH content and temperature. In the course of their studies, they discovered that with MeOH as co-solvent, protein unfolding was 100% reversible up to a 60% MeOH concentration (v:v), over a

temperature range of -40 to 70 °C. Ultimately, the authors selected a 50% (v:v) concentration with which to conduct further studies.

In 1988, Welinder et al.[9] studied the activity and specificity of eight proteases when digesting reduced and carboxymethylated ribonuclease using organic solvent-modified systems. The authors evaluated MeOH, ethanol, isopropanol, and acetonitrile (AcN) as organic modifiers, and digests were analyzed by amino acid sequencing, using commercially available sequencers; results were compared to aqueous digestion. Experiments were conducted to determine if digestion could be performed directly in the chromatographic mobile phases in which samples were collected following protein isolation. The authors stated that at least partial trypsin activity was maintained in solvents containing up to 40% organic (v:v), but digestion efficiency was only evaluated at 22 and 37 ℃, using 2 and 18 h digestion times. The authors concluded that trypsin activity was reduced with organic solvent modification; activity was most reduced using AcN, and at 37 ℃, proteolysis was even more reduced.

In 2000, Park et al.[10] showed that thermal protein denaturation prior to digestion generally increased the digestion efficiency of soluble proteins otherwise resistant to proteolytic digestion in aqueous solvents, and similar results were demonstrated by the same group with mixtures of proteins[11]. Though it improved the capability to digest resistant soluble proteins, thermal denaturation alone did not result in an improved ability to digest membrane proteins.

Subsequently, Russell et al.[12] investigated the use of organic-modified solvent systems with proteolytic digestion for use in high through-put bottom-up peptide mapping. Their goal was to reduce digestion time in order to more rapidly characterize proteins. Investigated proteins included rabbit phosphorylase, bovine serum albumin, bovine hemoglobin, rabbit aldolase, chicken ovalbumin, rabbit carbonic anhydrase, horse myoglobin, horse cytochrome C, chicken lysozyme and bovine ubiquitin. With MeOH, acetone, 2-propanol, or AcN addition, digestion rate dramatically increased, the sequence coverage for the proteins also increased, and proteins resistant to proteolysis under aqueous conditions could be digested to yield sequence data. Though digestion times were dramatically improved with no need for detergents or other chemical denaturants, the increases in digestion efficiency observed across the eleven proteins investigated were generally relatively modest.

Proc et al.[13] evaluated the effects of chaotropic agents, surfactants and two organic solvents on trypsin digestion efficiency. They used a method employing isotopically labelled characteristic peptides as internal standards in order to quantitatively measure the "absolute amounts" of peptides produced. Forty-five clinically important plasma proteins were digested. Results were compared to those from a common denaturing digestion method using sodium deoxycholate. Solvents evaluated were MeOH and AcN. Digestion was conducted at 37°C using 40% and 20% AcN and MeOH (v:v), respectively, with digestion times ranging from 0.5 to 23 h. The organic solvent was added to the digestion solution following reduction and alkylation and just prior to the addition of trypsin, to "prevent protein precipitation". The proteins were divided into three groups based on the results; "rapidly digested", "moderately digested" and "resistant to digestion". For most proteins, digestion "signal" peaked at 4 h and plateaued, though for a few proteins, digestion continued throughout the 23 h incubation without plateau. They also observed that after 4 h, the digestion signal for some proteins decreased. With AcN as solvent, though digestion did occur, it was significantly reduced compared to the sodium deoxycholate denaturation method; the authors said of the AcN results, "in our experiments, a significant reduction in digestion efficiency was observed for all 45 analytes in the presence of 40% v/v acetonitrile, even though no protein precipitation was observed. Moderate digestion of some proteins, such as haptoglobin however, was observed when using AcN denaturation."; MeOH results were similar.

We investigated the hitherto unexplored combined use of high solvent concentrations (consisting of 40-100% AcN (v:v)), along with "high" temperatures (ranging from 40-80°C), to digest select single

protein toxins and diagnostically important proteins, for bottom up protein analysis, using liquid chromatography/tandem mass spectrometry detection and identification, as part of an effort to develop a rapid diagnostic method for targeted proteins. Proteins investigated included the toxins ricin, botulinum neurotoxin serotype A (BotNT-A) and staphylococcus enterotoxin B (SEB).

Ricin holotoxin (RH), a lectin found in castor beans, was initially investigated as a "proof of concept" experiment. RH is a protein heterodimer composed of an A and B chain (RAC and RBC, respectively) linked through a disulfide bond. It is a ribosome-inactivating protein that disrupts cellular protein synthesis[14,15]. It has significant clinical toxicity, is highly stable, and there are no effective therapies. BotNT-A is one in a family of toxins produced by gram negative anaerobic bacteria in the genus Clostridium[32]. BotNTs are the most toxic substances known to man and, like ricin, potential weapons of terror and mass destruction[16,17,18]; BotNT-A is one of four clinically significant serotypes. Like RH, BotNTs are heterodimers consisting of heavy (HC) and light chains (LC) linked by disulfide bridges. SEB is an enterotoxin produced by the gram-positive bacterium Staphylococcus aureus. It induces severe diarrhea and nausea, is a superantigen that can lead to severe gastroenteritis[19], and is a cause of toxic shock syndrome. It is also quite stable, even capable of withstanding boiling in aqueous solution at 100 ºC for several minutes. Due to the ease with which it can be produced, its stability, and its capability for causing human morbidity and mortality, it is also considered a potential weapon of terror and mass destruction[20]. For the aforementioned reasons, it was important to develop rapid, sensitive assays for these protein toxins.

After the initial successes in tests with toxins, the high solvent/high temperature digestion method was used to digest two clinically important diagnostic proteins, thyroglobulin (TG) and ribonuclease A (RNAse A) for which routinely used clinical digestion methods have lacked sensitivity, specificity, and reproducibility, largely due to digestion method inefficiency and lack of reproducibility. TG, a ~660 kDa glycoprotein homodimer, is produced mainly by the thyroid, is involved in the synthesis of thyroxine and triiodothyronine, and acts as a storage site for thyroid hormone and iodine[21]. Detection and quantification of TG in the clinical laboratory can be problematic especially for patients that express anti-TG antibodies (ATAs) which can interfere with the immunoaffinity methods commonly used[22]. Since successful long-term patient monitoring using these methods can be difficult, the American Thyroid Association has emphasized the need for development of sensitive TG methods, not affected by ATAs, in clinical samples. RNAse A, a relatively small protein (124 residues, ~13.7 kDa), is a very stabile endoribonuclease typically prepared in the lab for analysis by boiling for 30 min to remove deoxyribonuclease [23]. Blood levels have been used in combination with other biomarkers to diagnose a variety of cancers and infectious diseases[41]. Current, largely activity-based indirect methods, as with TG, are relatively long and have lacked the specificity, sensitivity and reproducibility needed.

We describe the development and validation of the simple, 5-minute rapid proteolytic Denaturing Organic Digestion method (DOD), using trypsin, that was compared briefly to a 100% AcN digestion solvent method and more rigorously to the Filter Aided Sample Prep (FASP) and Flash methods, two commonly used proteolytic digest methods used in MS-based protein analysis. FASP is a tryptic digest method developed to generate peptides from crude cell lysates for subsequent MS analyses. It employs a detergent to disrupt cells exposing cellular proteins to proteolytic digestion. Critical method steps occur in a filter using 8 M urea, hence the name. Method advantages include that genomic material is removed from samples prior to digestion, samples are not subjected to precipitation, the method can be used successfully with samples containing strong detergents at relatively high concentrations, samples containing a relatively wide range of protein concentrations can be prepared using a single filter device, and targeted protein concentrations are kept relatively high[24,25,26]. The Flash Digest method employs immunoaffinity-immobilized protease. Advantages include very rapid digestion times resulting from a

very high enzyme-to-target protein concentration ratio, steric hindrance of protease autolysis resulting in good reproducibility, and the ability to reuse enzyme[27,28].

The optimized DOD method employed a digestion solvent composed of a high relative amount of AcN (60% v:v) and a high relative (60 °C) incubation temperature. In this study, method digestion efficacies were compared for select toxins and two clinically previously difficult to digest diagnostic proteins by measuring and comparing the sequence coverage and number of characteristic peptides identified using each method. Samples containing single proteins or protein cocktails were digested and analyzed and results compared.

## Methods

### FASP 24 h Digestion Method

Samples were processed by the FASP digestion method according to the protocol described by Wizniewski et al.[29]. Briefly, proteins were introduced onto a Microcon YM-10 (Millipore, Cat. No. number 42407) filter and reduced with 20 mM DTT (prepared fresh daily) for 1 h at 60 ºC followed by alkylation with 55 mM iodoacetamide for 45 min at room temperature. Samples were digested overnight at 37 ºC using 0.02 µg/µL trypsin prepared in 50 mM ammonium bicarbonate. Post-digestion, the samples were vacuum-centrifuged to dryness for 40 min and stored at -20 ºC until analysis. Prior to nanoLC/MSMS analysis, samples were suspended in 100 µL of buffer consisting of 95% LC/MS-grade water/5% acetonitrile with 0.5% formic acid.

### Flash Digestion Method

Samples were digested according to the method described by Griffiths et al.[30]. To each Flash Digest tube was added a 150 µL aliquot of Flash Digest buffer and 50 µL of 50 mM triethyl ammonium bicarbonate containing 20 µg of each target protein. Samples were placed in an Eppendorf ThermoMixer C equipped with a 96-well PCR plate shaker with a heated lid (pre-heated to 70ºC) and digested for 1 h constantly stirred at 1400 RPM. Digestion was terminated by adding 400 µL of 0.1 % TFA with agitation. Samples were allowed to stand for 40 minutes at 37 ºC followed by filtration through 3kD and 10kD membranes to remove particulates. Each supernatant was transferred to an autosampler vial and diluted to the desired target concentration with LC/MSMS buffer prior to nanoLC/MSMS analysis.

### Denaturing Organic Digestion (DOD) and 100% AcN Methods

Sample digestion using the optimized DOD and 100% (v:v) AcN methods was performed as follows. A master mix was formulated consisting of 10-70 µg of targeted protein, 22 µL of 1.0 M DTT (final concentration 20 mM), 660 µL of AcN and 418 µL LC/MS grade water (60% AcN ((v:v)) for the DOD method or 1078 µL of 100% (100% AcN method) for a total sample volume of 1100 µL. A 1 µg trypsin/µL LC/MS-grade water solution was prepared fresh daily. Aliquots (50 µL or 450-3180 ng of protein) of the master mix were transferred to separate 1.5 mL Eppendorf snap-cap tubes. Digestion was initiated by adding trypsin at a 1:3 molar ratio to target protein(s). Vial caps were affixed, and samples were incubated at 60 ºC at 700 rpm in an Eppendorf ThermoMixer C equipped with a heated lid for five minutes (During method optimization, digestion times and temperatures were varied from 5-60 min and 40-80 ºC, respectively. Our first digestion attempts were conducted using an Eppendorf ThermoMixer C equipped *without* a heated lid and failed; results improved markedly after procuring a heated lid.). Digestion was terminated by adding 1 µL of 50% TFA to each tube. Samples were vacuum-dried for 30 minutes in a Speedvac under medium heat and stored at -20 ºC until analysis. Prior to nanoLC/MSMS analysis, samples were suspended in 50 µL LC/MSMS buffer consisting of 95% LC/MS-grade water/5% AcN with 0.5% formic acid.

## Results

**Ricin**

Since previous studies have shown that protease digestion efficiency for peptides and proteins with known potential cleavage sites appears to be limited by steric hindrance resulting from protein tertiary structure[31], we reasoned that a simple treatment of RH with AcN, an organic solvent routinely employed to crudely "crash" proteins out of aqueous solutions through tertiary structure disruption, might expose potential cleavage sites providing trypsin more access thereby improving bottom-up sequence coverage. Following DTT reduction, RH was digested using the 24 h FASP, 1 and 4 h Flash, and 100% AcN method, digesting for 1 and 4 h. Samples were digested in quintuplicate. The mean number of characteristic peptides identified for the FASP, 1 and 4 h Flash and 1 and 4 h 100% AcN methods were 29, 7, 6, 12 and 9, respectively, and mean sequence coverages for the methods were 72, 11, 9, 14 and 13%, respectively.

Subsequently, RAC and RBC were digested using solvents containing 50-80% AcN (v:v), incubated for 15-60 min at 40-70 ºC; quadruplicate biological replicates were digested under each condition. Both sequence coverage and number of unique peptides identified significantly improved when compared to results from previous 24 h FASP and 1 and 4 h Flash experiments. Optimal results were observed when employing 60-70% AcN (v:v) at 60-70ºC (Figure 1 illustrates 15 min digestion results). The data also suggested that AcN content was the principal variable affecting digestion across this temperature range and that digestion could be conducted in as little as 15 min without affecting performance.

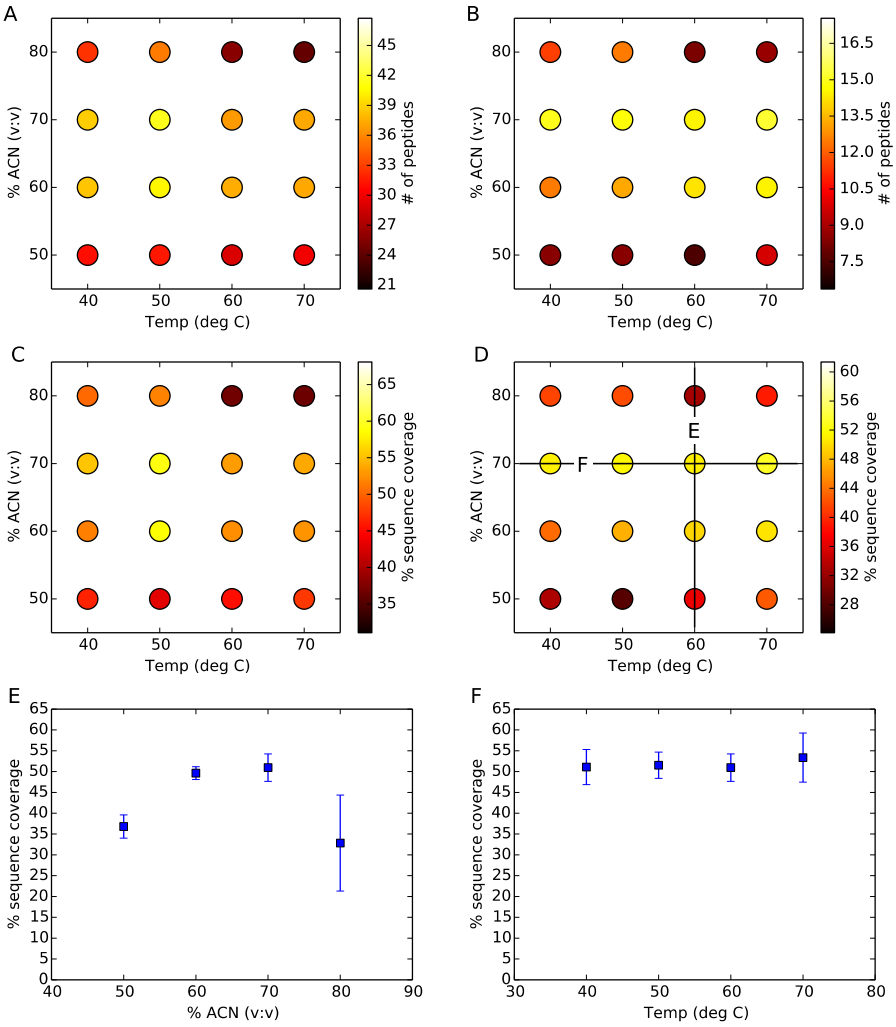

Fig. 1: Number of characteristic peptides identified and sequence coverages from 15 min digestion of RAC and RBC varying temperature and AcN concentration; proteins were not reduced or alkylated in this experiment. Panels A and B illustrate AcN-assisted 15 min digestion of RAC and RBC, with respect to number of unique peptides identified. Panels C and D illustrate % sequence coverage results for RAC and RBC, respectively. Panels E and F show transects through (D) to illustrate the shape of the surface as a function of AcN concentration (E) and temperature (F). Error bars illustrate the standard deviation from 4 biological replicates.

To confirm that DTT reduction could be performed in 60% AcN, we reduced and digested RH, RAC and RBC in the solvent. Results are illustrated in Figure 2. Across the proteins, substantial improvements in sequence coverage and the number of characteristic peptides identified were observed with disulfide reduction. Not surprisingly, the number of characteristic peptides improved mostly with RBC, in which four of the five holotoxin disulfide bonds lie. Additionally, across the proteins, precision improved with disulfide reduction, especially for sequence coverage.

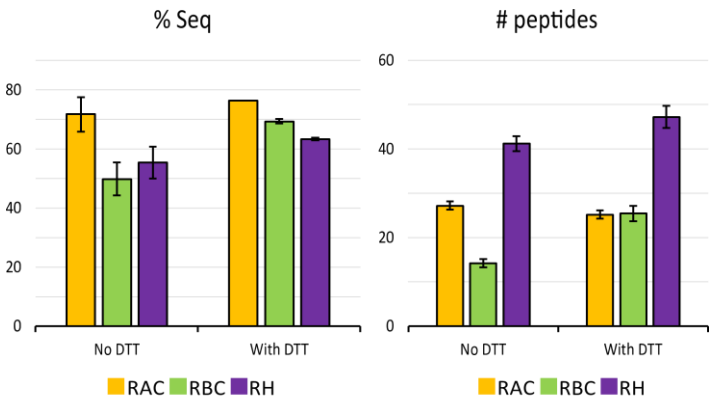


Fig. 2. DOD method sequence coverages and number of characteristic peptides identified for four replicate digestions each of Ricin-A chain ☐, Ricin-B chain ☐ and ricin holotoxin ☐ with and without 0.5 M DTT.

Subsequently, separate RAC, RBC, and RH samples were reduced and digested in quintuplicate using the DOD method employing 15, 30, and 60 min digestions. Data were compared to that from 24 h FASP digests collected previously, as shown in Figure 3 - results for RAC and RBC from 60 min DOD digestion were comparable and are not shown. The DOD method, for all digestion times, showed consistently increased numbers of characteristic peptides detected and much better precision across replicates, as compared to those attained with the FASP method.

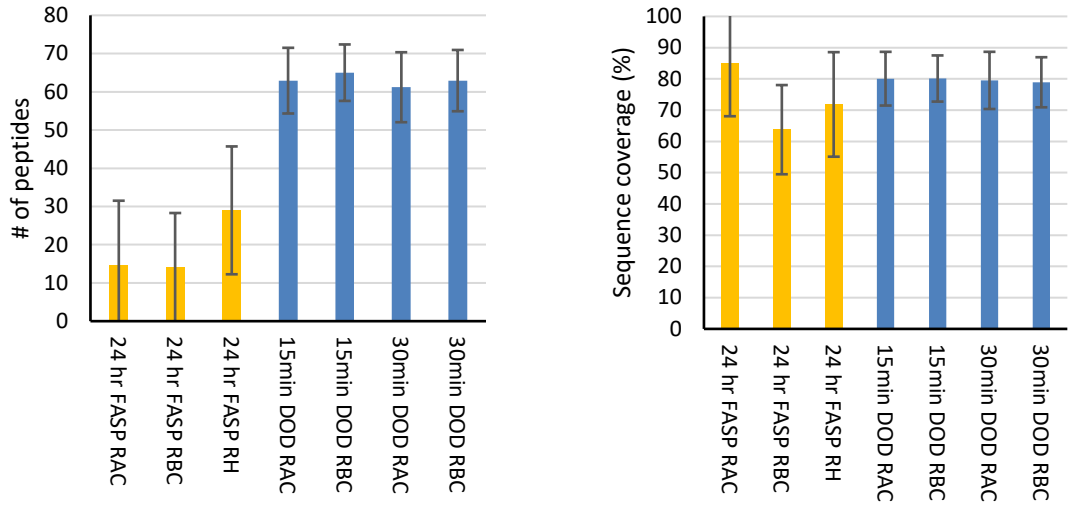


Fig. 3: Mean number of peptides identified (left) and sequence coverage (right) for RAC, RBC and RH, respectively, using the 24 h FASP and 15 and 30 min DOD digestion methods. Results are mean data across five biological replicates with variability expressed as %CV (error bars).

The mean number of characteristic peptides identified and mean sequence coverages for the 1 and 4 h Flash, 24 h FASP, 1 and 4 h Flash, 1 and 4 h 100% AcN methods collected from the initial experiment and the results for the 15 min DOD method are illustrated in Figure 4. Specific sequence coverages for RH using the 24 h FASP and the 15 min DOD methods are illustrated in Figure 1 of the supplemental information. Compared to the 24 h FASP method, the DOD method provided equivalent sequence coverages for RAC, RBC and better results for RH, with a substantial increase in the number of characteristic peptides identified. Compared to the Flash method, the % sequence coverage and number of characteristic peptides identified improved dramatically. Additionally, intra-day data variability across replicates improved as compared to that for the FASP or Flash methods.

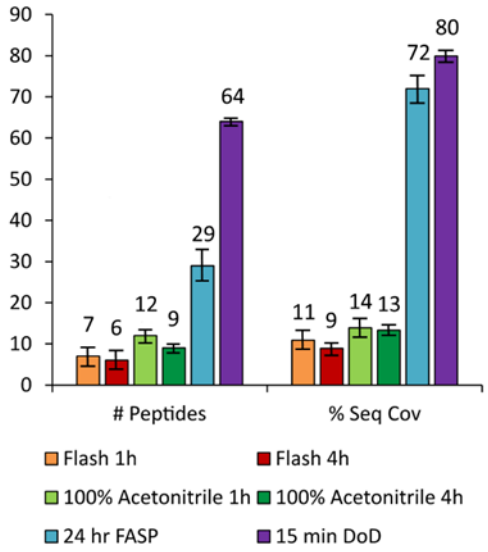


Fig. 4: Number of RH peptides identified and sequence coverages for 1 and 4 h Flash, 24 h FASP, 1 and 4 h 100% AcN and the new 15 min DOD digestion methods with disulfide reduction.

## Toxin Cocktail

DOD method performance for a protein cocktail containing RH, BotNT-A, and SEB was then evaluated. A 1 mL sample containing 22 µg/mL each of RH and BotNT-A, and 4 µg/mL of SEB was reduced and digested in quintuplicate using the FASP, Flash and DOD methods. Results confirmed our preliminary results using RAC, RBC and RH. Using the DOD method, sequence coverages for all three toxins were comparable to those attained with the 24 h FASP method, but there was a >20% increase in detected peptides for BotNT-A, and a >25% increase in detected peptides for Ricin. The DOD method produced only slight increases for SEB, but SEB was well digested with both the FASP and Flash methods. The number of characteristic peptides identified and percent sequence coverages for RH and BotNT-A using the 1 h Flash method were substantially lower (data not illustrated).

## Digestion Time Optimization

Remarkably, a digestion time course study of RH, BotNT-A and SEB in a cocktail comparing the FASP method to the DOD method using digestion times ranging from 5-60 minutes, demonstrated that 5-minute digestion produced equivalent results to 60 min digestion. With the DOD method, across digestion times the number of peptides identified and percent sequence coverages obtained were consistent down to as little as 5 minutes, with comparable precision (Figure 5).

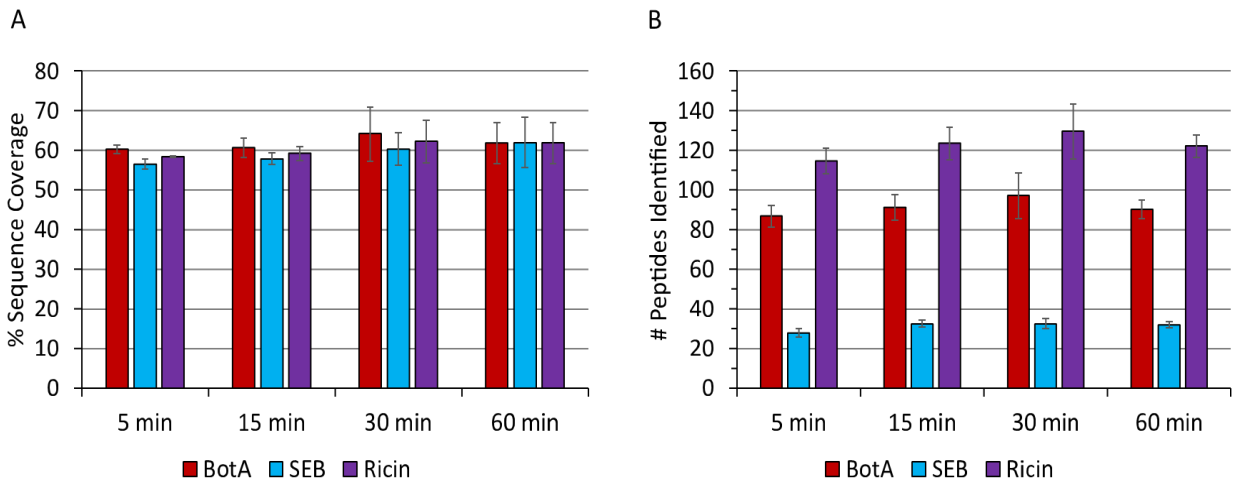


Fig 5: Time course study comparing DOD digestion time to sequence coverage (A) and number of characteristic peptides identified (B) for BotNT-A ■, SEB ■ and ricin ■ in a cocktail of the three proteins at a concentration of 22 µg/mL for BotNT-A and SEB, and 4.4 µg/mL for ricin.

## Thyroglobulin and Ribonuclease A

Using 5, 15, 30 and 60 min digestions, samples in quintuplicate containing 1 µg each of TG and RNAse A were digested and analyzed as technical duplicates (total of 40 samples). Method precision was very high both within and across digestion times. Between 260-280 characteristic peptides were identified for TG and sequence coverages ranged from 50-55%, with no significant variability in the data across digestion times. For RNAse A, the number of characteristic peptides identified per sample ranged from 35-45 with no significant difference within and across digestion times, and sequence coverages for all 40 samples were 100%. Sequence coverages and number of peptides identified under each condition for TG and RNAse A are illustrated in Figure 6 and Table 1. Sequence coverage results were so consistent across

digestion times that we believe digestion time could likely be reduced even further without affecting method capability for these proteins.

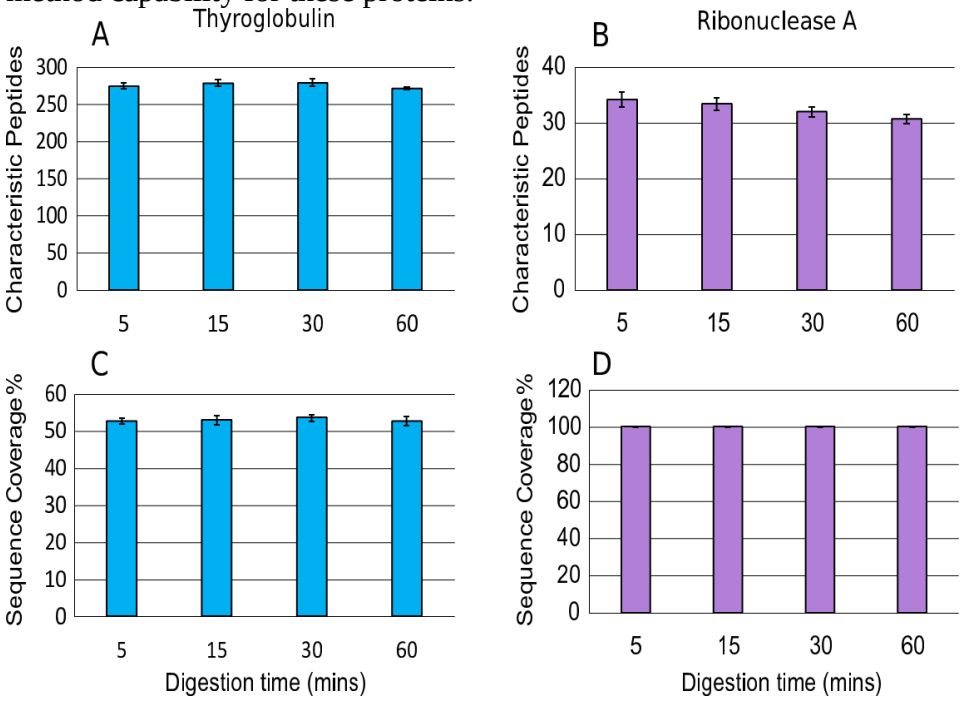


Fig. 6. Number of characteristic peptides and sequence coverages for TG and RNAse A over different digestion times using the DOD method. Mean data with error bars representing %CV for quintuplicate biological replicates analyzed as technical duplicates is illustrated.

| Digestion time | 5 min | 15 min | 30 min | 60 min |
|---|---|---|---|---|
| TG peptides | 273 | 279 | 280 | 271 |
| TG sequence coverage (%) | 53 | 54 | 54 | 53 |
| RNAse A peptides | 35 | 35.0 | 34 | 33 |
| RNAse A sequence coverage (%) | 100 | 100 | 100 | 100 |

Table 1. Peptides identified and sequence coverage for TG and RNAse A using the optimized DOD method. The table lists mean data for characteristic tryptic peptides identified and the sequence coverage for each protein from technical duplicates digesting quintuplicate biological replicates and analyzing 1 µg of each protein.

The effect of pH and temperature on DOD method digestion efficacy was further investigated by digesting quadruplicate biological replicates containing 1 µg each of TG and RNAse A in a cocktail under neutral pH conditions and under acidic conditions at 60 and 25ºC. Digestion was also compared employing trypsin-to-protein ratios of 1:5 and 1:1. Little difference was observed across different trypsin-to-protein ratios (data not shown) indicating that a stoichiometric excess of trypsin was efficacious even at a trypsin-to-protein ratio of 1:5. Method performance was optimal at 60ºC and neutral pH. The greatest numbers of characteristic peptides identified and greatest sequence coverage were produced at 60ºC under neutral pH conditions. Though the samples consisted of only spiked proteins containing no clinical matrix, sequence coverage was extremely good for these two proteins using the DOD method. The method worked at room temperature with somewhat less efficiency and with no detectable digestion occurring under acidic conditions.

## Discussion and Conclusion

As described, a new rapid, in-solution trypsin digestion method was successfully developed, optimized and validated for a select list of toxins and standard clinical diagnostic proteins with a proteolytic digestion time of as little as 5 min. Digesting spiked protein samples containing no environmental or clinical matrix, the DOD method produced sequence coverage results and sensitivities that were equivalent to or often greatly exceeded those provided by the 24 h FASP method across all samples, and dramatically exceeded those provided by the Flash 1 and 4 h digestion methods. In addition, compared to the FASP method, intra-day method reproducibility was in most cases substantially improved. The DOD method substantially reduced analysis time compared to traditional methods, which can require hours to more than a day to sufficiently digest targeted proteins. In addition, in most cases, the DOD method provided equal or better sequence coverages than the methods to which it was compared.

For targeted analysis of single or multiple proteins, the DOD method, while greatly reducing digestion times, appeared to provide an increase in the number of characteristic peptides identified, increased sequence coverages for many, and there was a marked increase in intra- and inter-day precision.

Prior to method use for specific applications however, digestion capabilities need to be verified with spiked samples containing the sample-specific environmental or biomedical matrix. With its comparatively short digestion time, consistent capability for identifying relative high numbers of characteristic tryptic peptides and proteins, and its consistently high sequence coverage capabilities when compared to other bottom-up methods, the DOD method could dramatically affect not only targeted analysis of toxins and other peptides/proteins of interest but could also be used to great advantage in general proteomics to rapidly identify biomarkers of disease, including the identification of microorganisms and viruses of interest. Along with increases in analytical performance, time and cost savings in diagnostic and research laboratories could be substantial and current processes simplified. In addition, the demonstrated improvements in precision inherent with this method could enhance the absolute quantification of characteristic peptides and their parent proteins.

To date, we have also performed bottom-up protein analysis with the DOD tryptic digestion method employing it to characterize other target proteins for which characteristic peptide identification and resulting sequence coverage from currently used longer digestion methods is relatively poor and to characterize the proteomes of two complex systems. Using a study design similar to that described in this report, initial results with select complex samples were compared to results from other digestion methods currently in use. These results were very promising and will be forthcoming in a second report.

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