Acetone Precipitation Effectively Removes SDS and Results in the Detection of Low Abundance Proteins

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ABSTRACT

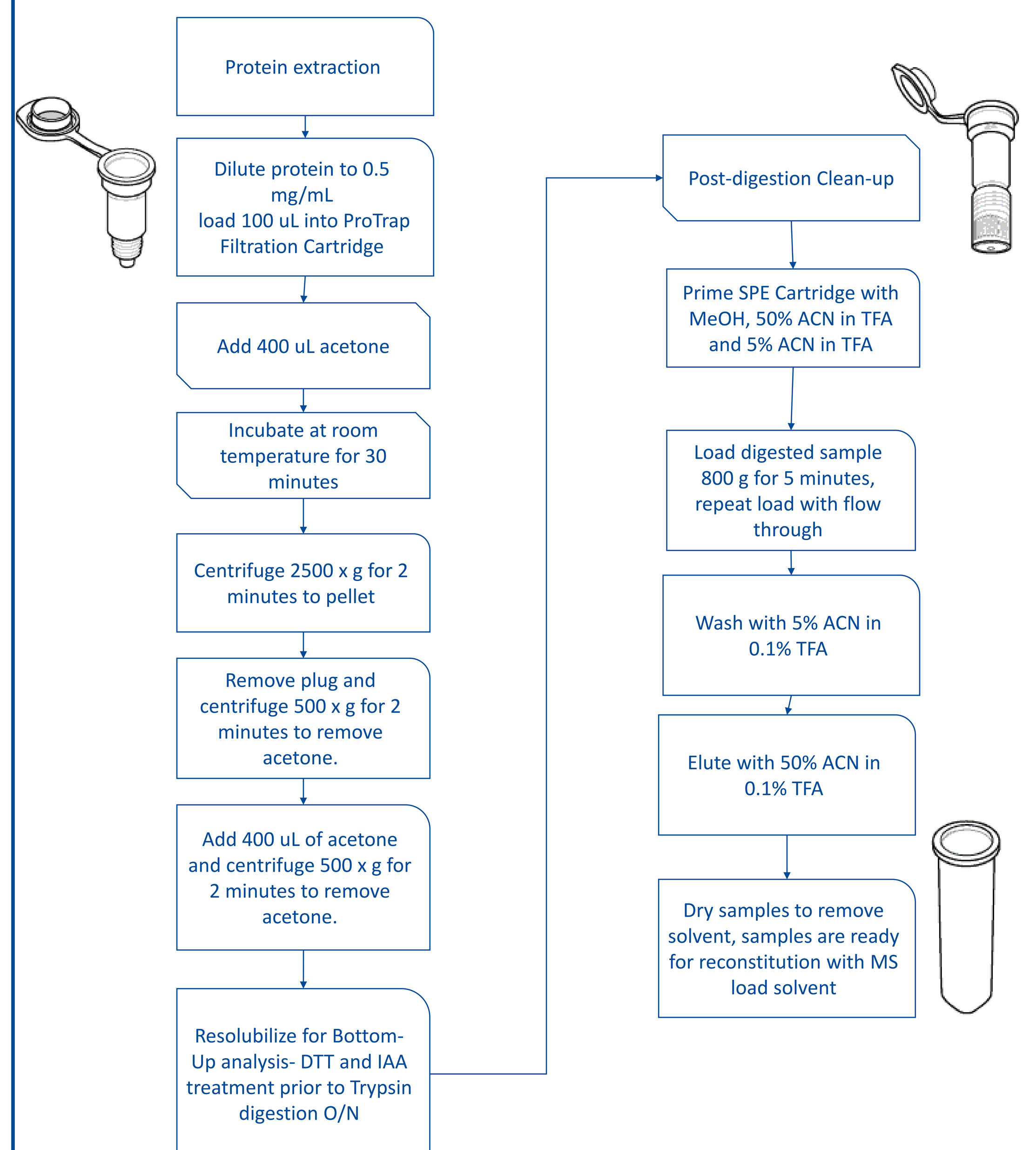
Introduction
Sodium dodecyl sulphate is a commonly used detergent for protein extraction, particularly for membrane proteins. Adequate removal of the anionic detergent, to levels below 100 ppm, or preferably lower, has been a source of frustration for mass spectrometry users for some time. Precipitation has been used in the past but has a reputation for inconsistent performance. Other methods have been suggested including dialysis and buffer exchange, such as FASP (centrifugal-aided), as well as ion exchange, electrophoresis, and detergent affinity matrices. Many of these strategies have drawbacks including loss of proteins, incomplete detergent removal, and a high cost per use. Standardization of the precipitation process can result in the minimization of sample loss and improvement in reproducibility.

Methods
A standard protein mix of beta-galactosidase, cytochrome c and enolase-1 or yeast extract were precipitated in the presence and absence of 2% SDS with 50 mM Tris pH8.0 and 50 mM NaCl. 50 µg samples were precipitated with four volumes of acetone in the ProTrap XG, digested after reduction and alkylation, then desalted using the integrated reverse-phase SPE Cartridge. Samples were subjected to analysis on an Orbitrap Fusion Lumos Tribrid mass spectrometer using an EASY-nLC and 60 (standard) or 90 (yeast) minute gradient. Residual SDS was determined using the MBAS assay.
The effect of increased NaCl on acetone precipitation was explored by precipitating BSA in the presence of 0.5 to 5% SDS. Residual SDS was determined using the MBAS assay.

Preliminary data
Residual SDS levels were 30.03 +/- 13.99 and 15.12 +/- 4.16 ppm for standard protein mix and yeast samples, respectively. 150 ng of each mixture was analyzed by mass spectrometry. All three proteins were positively identified in both test conditions (+/- SDS). The proteins precipitated in the presence of 2% SDS resulted in more uniform peptide detection compared to those precipitated in the absence of SDS. Interestingly, and somewhat expected, the presence of SDS during the full extraction process of a yeast sample, resulted in more proteins identified, and also identified additional lower molecular weight proteins. Though the full yeast proteome was not detected (6000+ proteins), analysis conditions were not intended to detect all possible proteins. Reproducibility between replicates was adequate.
To further determine limits of the salt effect, 50 µg samples of BSA were precipitated in the presence of 0.5 to 5% SDS with variable NaCl concentrations, increasing up to 300 mM NaCl. At higher SDS concentrations and NaCl at 300 mM, increased variability in SDS removal was observed. Optimal results were obtained at 2% or lower SDS in the presence of 150 mM or lower NaCl. The use of the ProTrap XG decreases the variability observed during precipitation. The inclusion of a precise amount of NaCl to standardize the ionic strength of the starting sample is a key element to decreasing variability.

Novel aspect
Precipitation can be successfully used to reproducibly and reliably remove detergent contamination before mass spectrometric analysis.

PRECIPITATION WORKFLOW IN THE PROTRAP XG



RESULTS – SDS CONTENT INFLUENCES PRECIPITATION EFFICIENCY

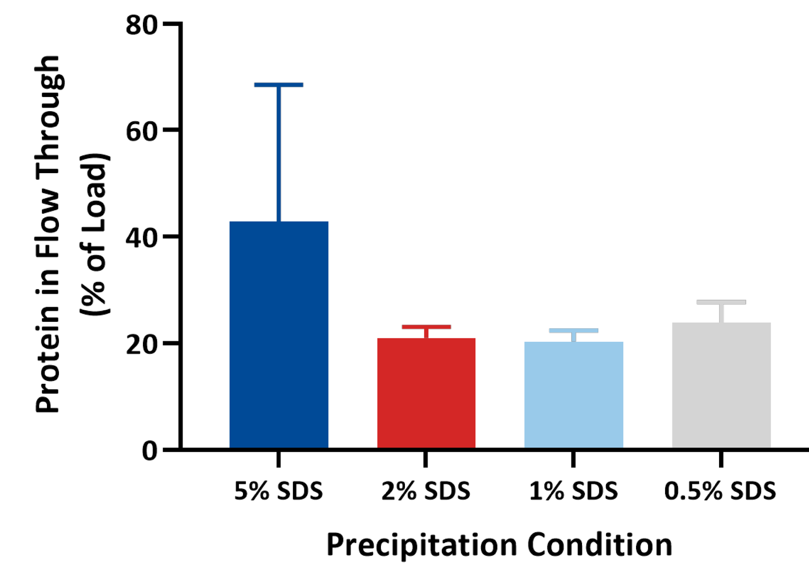


Figure 1: Bovine liver proteins were extracted in 5% were diluted to give final SDS concentrations of 5%, 2%, 1% and 0.5% SDS and the amount of protein in the flow through measured by BCA assay (Pierce). In the representative data above, all samples were precipitated in the presence of 50 mM Tris pH 8.0/50 mM NaCl. Proteins in the flow through consisted primarily of low molecular weight proteins, those under 10 kDa..

RESULTS – SDS CAN BE REMOVED TO ALLOWABLE LEVELS BY PRECIPITATION

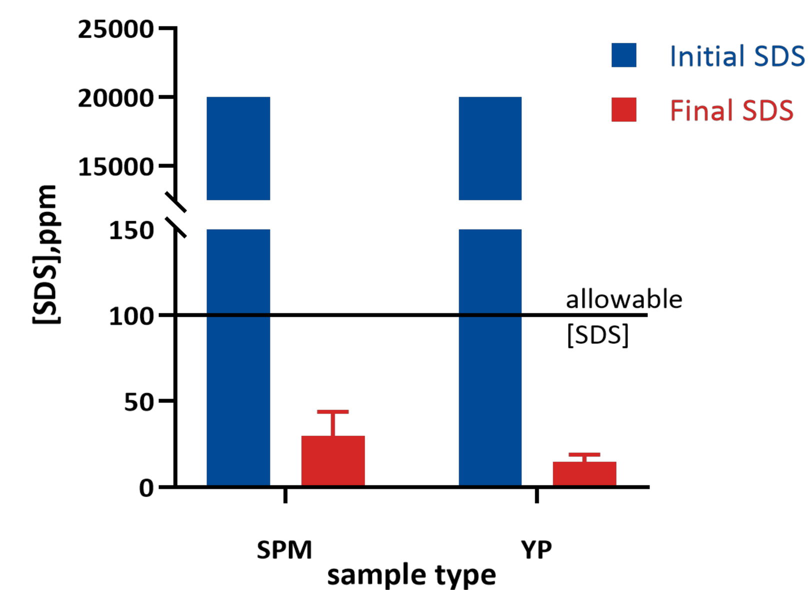


Figure 2: A mixture of 3 commercially available proteins were mixed in the following proportions: 50 µg of β-galactosidase, 50 ng of cytochrome C and 5 ng of enolase-1. 50µg of the mixture was precipitated in the presence of 2% SDS in 50 mM Tris pH 8.0 and 50 mM NaCl using the ProTrap XG. 4 volumes of acetone was added, and the mixture was incubated at 30 minutes at room temperature. The acetone was removed by centrifugation through the filter, the pellet was washed with acetone, then resolubilized in 8 M urea with 10 mM DTT (30 minutes at 37°C. Iodoacetamide was added to 50 mM final concentration and incubated for 15 minutes in the dark at room temperature. The urea was diluted by the addition of 50 mM Tris pH 8.0 and the sample digested with Trypsin (100:1 protein to enzyme) overnight at 37°C. The resulting peptides were cleaned up by passing over the ProTrap XG SPE cartridge, eluting bound peptides with 50 % ACN in 0.1% TFA. Similarly, yeast proteins were extracted by mechanical lysis by mortar and pestle, grinding in 2S SDS in 50 mM Tris pH8.0/50 mM NaCl. A sample consisting of 50 µg in 100 µL was subjected to the same process as the purified proteins. Residual SDS was measured using the methylene blue active substances (MBAS) assay. Final SDS concentration reported was normalized to the starting protein concentration. The MBAS assay standard curve illustrated excellent linearity at 50 ppm SDS and below allowing for quantitation in the desired range.

RESULTS - PRECIPITATION ALLOWED FOR DETECTION OF 4 ORDERS OF MAGNITUDE

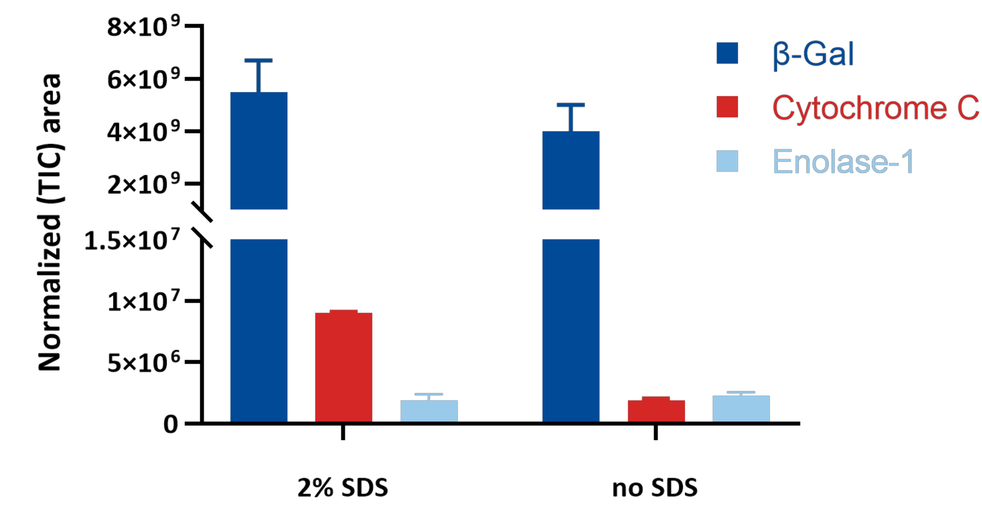


Figure 3: Three commercially available proteins were mixed in the following proportions: 50 µg of β-galactosidase, 50 ng of cytochrome C and 5 ng of enolase-1. 50µg of the mixture was precipitated in the presence of 2% SDS in 50 mM Tris pH 8.0 and 50 mM NaCl using the ProTrap XG. 4 volumes of acetone was added, and the mixture was incubated at 30 minutes at room temperature. The acetone was removed by centrifugation through the filter, the pellet was washed with acetone, then resolubilized in 8 M urea with 10 mM DTT (30 minutes at 37°C. Iodoacetamide was added to 50 mM final concentration and incubated for 15 minutes in the dark at room temperature. The urea was diluted by the addition of 50 mM Tris pH 8.0 and the sample digested with Trypsin (100:1 protein to enzyme) overnight at 37°C. The resulting peptides were cleaned up by passing over the ProTrap XG SPE cartridge, eluting bound peptides with 50 % ACN in 0.1% TFA. The samples were dried prior to sending to SPARC BioCentre for analysis. In parallel, a sample of the protein mix, not containing SDS, was subjected the same reduction, alkylation and digestion procedure, then cleaned-up on the ProTrap SPE, before being dried prior to shipment. The samples were solubilized in loading solvent and 150 ng was injected onto a Orbitrap™ Fusion Lumos Tribrid mass spectrometer using an EASY-nLC. The resulting peptides were searched at BSI using PEAKS Online v1.6 against a combined database of the 3 species involved

All three proteins were detected in approximately the proportion present in the mix, and good coverage of each protein was observed.

RESULTS – SDS EXTRACTS MORE PROTEIN THAN MECHANICAL LYSIS ALONE

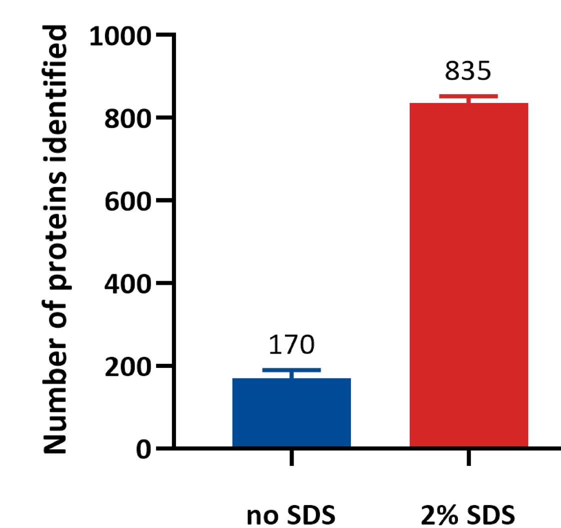
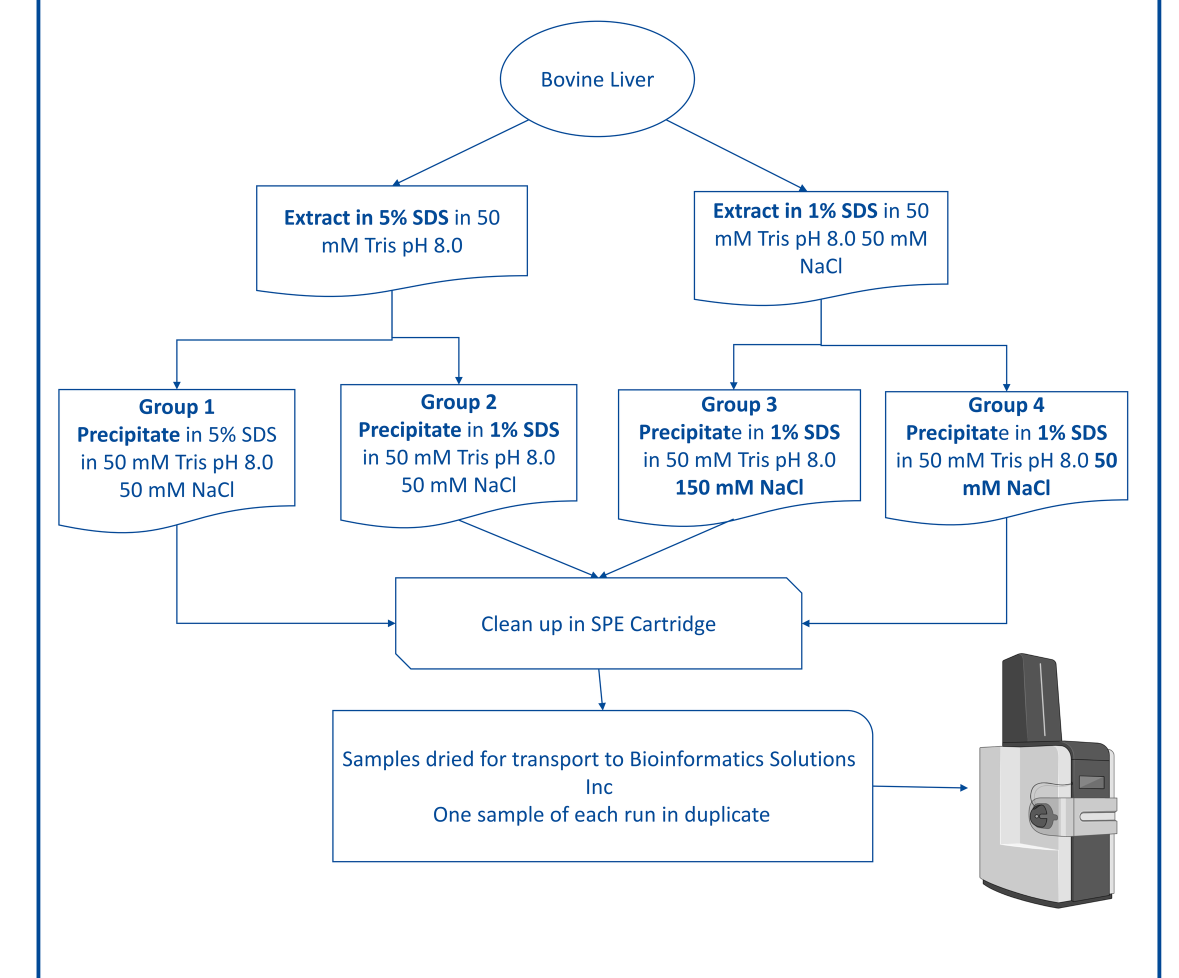


Figure 4: *Saccharomyces cerevisiae* was grown to A600 of 1.0 in YPD broth. The resulting cell pellet was divided in half and subjected to mechanical lysis by grinding in liquid nitrogen in the presence of either 50 mM Tris pH 8.0/ 50 mM NaCl or 2% SDS in 50 mM Tris pH 8.0/50 mM NaCl. 50 µg of each lysate condition was precipitated by the addition off 4 volumes of acetone, and the mixture was incubated at 30 minutes at room temperature. The acetone was removed by centrifugation through the filter, the pellet was washed with acetone, then resolubilized in 8 M urea with 10 mM DTT (30 minutes at 37°C. Iodoacetamide was added to 50 mM final concentration and incubated for 15 minutes in the dark at room temperature. The urea was diluted by the addition of 50 mM Tris pH 8.0 and the sample digested with Trypsin (100:1 protein to enzyme) overnight at 37°C. The resulting peptides were cleaned up by passing over the ProTrap XG SPE cartridge, eluting bound peptides with 50 % ACN in 0.1% TFA. The samples were dried prior to sending to SPARC BioCentre for analysis. The samples were solubilized in loading solvent and 150 ng was injected onto a Orbitrap™ Fusion Lumos Tribrid mass spectrometer using an EASY-nLC and ran with a 90-minute gradient.

The resulting peptides were searched at BSI using PEAKS Online v1.6 against the *S. cerevisiae* database.

As expected, the inclusion of SDS improved protein extraction.

WORKFLOW for EXTRACTION CONDITION DIFFERENCES



RESULTS – SDS CONTENT DURING PRECIPITATION AFFECTS VARIABILITY OF PROTEINS IDENTIFIED

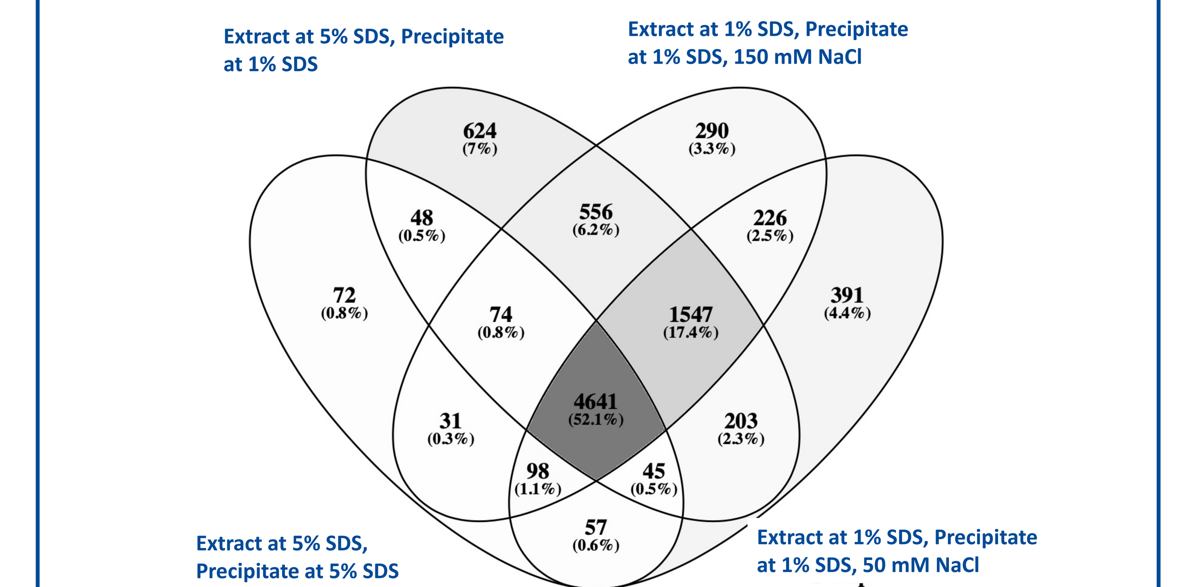


Figure 5: A sample of bovine liver was extracted by a motorized pestle in either 5% SDS in 50 mM Tris pH 8.0 or in 1% SDS in 50 mM Tris pH 8.0/50 mM NaCl. Prior to precipitation in the ProTrap XG, 50 µg samples were adjusted to 0.5 mg/mL in 5% SDS, 50 mM Tris pH 8.0 50 mM NaCl, 1% SDS in 50 mM Tris pH 8.0 50 mM NaCl or 1% SDS in 50 mM Tris pH 150 mM NaCl as noted above. 100 µL samples were precipitated as outlined in Figure 3. Three replicates at each precipitation condition were prepared and forwarded to Bioinformatics Solutions in Waterloo, Canada. The samples were solubilized in loading solvent and 150 ng was injected onto a tims TOF Pro. The resulting peptides were searched at BSI using PEAKS Online v1.6 against a bovine protein database.

Proteins found in common for each replicate set were compared across the groups. Variability in the number of peptides found and the proteins identified for group 1- extracted and precipitated at 5% SDS was noted. Good overlap was observed across the 4 groups, though approximately 17% of the proteins identified in groups 1-3 were not found in group 4. Variability in precipitation efficiency at 5% SDS can be mitigated by dilution of the SDS to 1%, and more robust precipitation at 1% SDS is seen with the addition of 150 mM NaCl.

CONCLUSIONS

Precipitation can be successfully used to reproducibly and reliably remove detergent contamination before mass spectrometric analysis.
Precipitation at 5% SDS can negatively impact both protein recovery and protein identification, this is mitigated by dilution to 1% SDS.

ACKNOWLEDGEMENTS

- Yeast and Standard Protein Mix mass spectrometry analysis was performed by SPARC BioCentre (Molecular Analysis), The Hospital for Sick Children, Toronto, ON, Canada.
- Bovine Liver extract samples mass spectrometry analysis was performed by Bioinformatics Solutions Inc Waterloo, ON, Canada. Further data analysis of the yeast and Standard Protein mix data was performed by Bioinformatics Solutions Inc Waterloo, ON, Canada.

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