LIMITED DRYING AND ITS EFFECT ON PEPTIDE RECOVERY RATES

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Abstract: Protein and/or peptide loss is an undesired but inevitable side effect of purification procedures. We compared three different containers, three different peptide standards and a representative complex proteome digest, and show that a partial drying could improve sample recovery from a glass surface and standard polypropylene tubes. Further, we show that the partial drying minimizes differences between the low-binding and standard polypropylene tubes.

Key Words: proteomics, digest, sample preparation

INTRODUCTION

Peptide-based analyses often employ techniques that concentrate small-volume samples to achieve the desired concentration or to remove volatiles such as methanol or acetonitrile. Lyophilization, vacuum evaporation or evaporation under a stream of gaseous nitrogen are the most common methods to reduce the sample volume. However, each of these methods presents a different obstacle. The evaporation in a centrifugal vacuum concentrator which is often the method of choice in proteomics does not require a sample freezing and vacuum effectively limits oxidations. However, the dried sample has a high potential for adsorption to the wall of a container containing the sample. Here, we compare the peptide recovery rates from three different containers and show that limited drying may significantly improve the yield.

MATERIAL AND METHODS

Plant material

Leaf blades of barley (cv. Sebastian) were homogenized (Retsch Mill MM400), aliquoted and stored at -80 °C.

Protein standards

Lyophilized ovalbumin and albumin (>97% purity) were purchased from Sigma Aldrich and dissolved (8 M urea, 50 mM ammonium bicarbonate) to the final concentration of 1 mg/ml. Aliquots corresponding to 500 µg were diluted and digested overnight with an immobilized trypsin (Promega) as described previously (e.g. Skalák et al. 2016).

Protein extraction

Total protein extracts were prepared by acetone/TCA/phenol extraction (Černý et al. 2014, Novák et al. 2015) from app. 300 mg of ground tissue. The resulting protein pellets were solubilized and digested with an immobilized trypsin (Promega) overnight.

Protein desalting

Samples were desalted on C18 SPE (Agilent) and dried (Speed-vac system, Thermo).

Peptide content determination

Peptide concentration was determined with a modified BCA colorimetric assay (Pierce Quantitative Colorimetric Peptide Assay kit; Tecan Spectra Rainbow microplate reader) and a commercial peptide standard included within this kit.

LC-MS analysis

Protein analysis was performed as described previously (e.g. Baldrianová et al. 2015). Briefly, tryptic digests analyzed by nanoflow C18 reverse-phase liquid chromatography using a 15 cm column (Zorbax, Agilent), a Dionex Ultimate 3000 RSLC nano-UPLC system (Thermo) and a UHR maXis impact q-TOF mass spectrometer (Bruker). Peptides were eluted with a 120-min, 4% to 40% acetonitrile gradient and spectra were acquired at 2 Hz (MS) and 10 to 20 Hz (MS/MS) using an intensity-dependent mode with a total cycle time of 7 s.

Protein identification

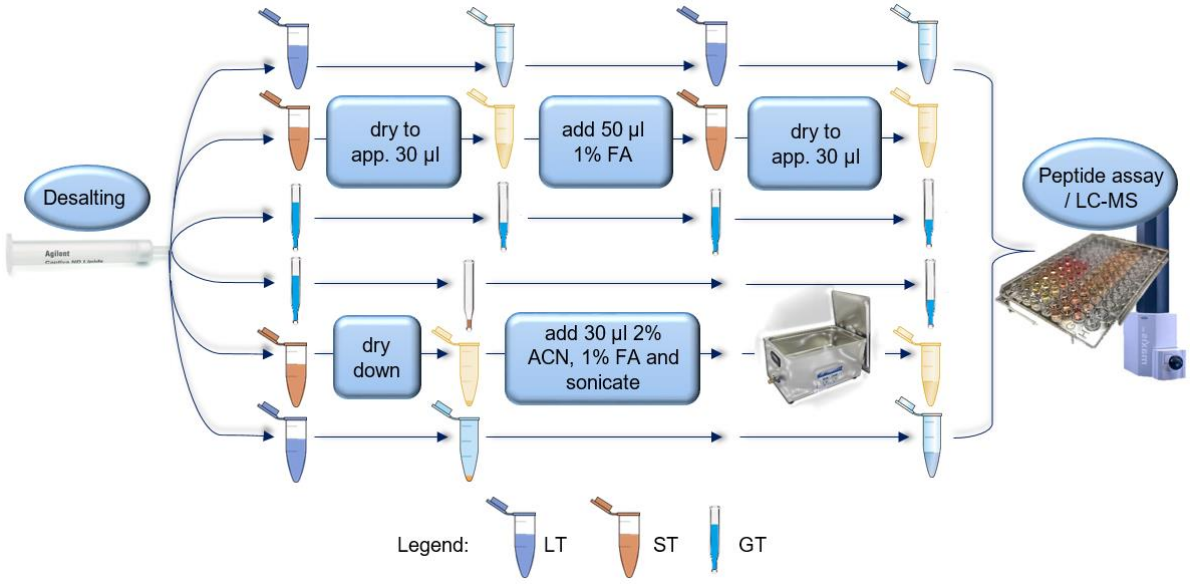
The measured spectra were extracted by Bruker's Data Analysis 4.1 and processed as described previously (e.g. Cerna et al. 2016) with minor modifications. In brief, spectra were recalibrated by Preview (www.proteinmetrics.com) and were searched against barley (6/2016) protein sequence database by Sequest HT with the following parameters: Enzyme - trypsin, max two missed cleavage sites; Mass tolerance - 35 ppm (MS) and 0.1 Da (MS/MS); Modifications - up to three dynamic modifications including Met oxidation, Asn/Gln deamidation, Lys methylation, N-terminal acetylation, Ser/Thr/Tyr phosphorylation. Data were processed and visualized by ProteomeDiscoverer 2.2 (Thermo).

RESULTS AND DISCUSSION

In silico trypsin digestion

Routine protein digestion protocols employ an in-gel digestion (e.g. Dobrá et al. 2014) or an in-sol digestion (e.g. Černý et al. 2013). Prior the MS analysis, the resulting peptides have to be extracted, desalted and concentrated. These steps are prone to an extensive sample loss. Hydrophilic peptides are lost during the washing steps, shorter and more volatile peptides during the drying, and large and hydrophobic peptides precipitate or may stick to the surface of the container.

Figure 1 Analysis of peptide loss in three different containers.



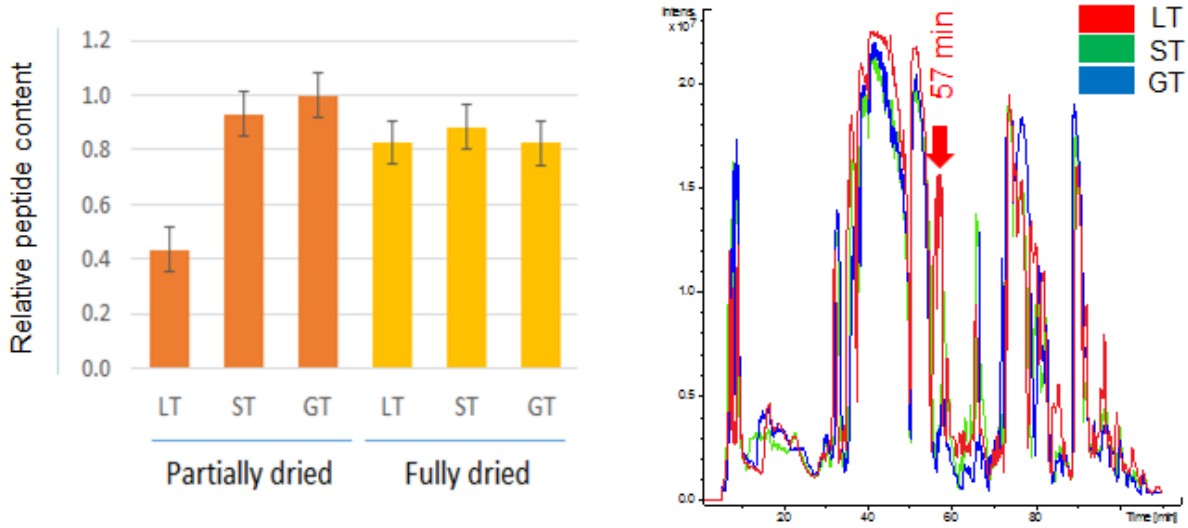
Here, we followed the last step in the procedure and compared sample loss in three different containers: a low-binding tube designed to decrease protein/peptide interactions with the tube surface (LT; LoBind Tube, Eppendorf), standard polypropylene 0.5 ml lab tube (ST) and glass insert (GT). Samples of protein standards and barley leaf total protein extracts were desalted on C18 SPE columns (Agilent) and eluted with a three-step polarity gradient (150 µl methanol, 150 µl acetonitrile, 150 µl acetone). Each sample was divided into six equal fractions and processed as indicated (Figure 1). For each experiment, three samples were dried completely, reconstituted in app. 30 µl solution of 2% acetonitrile in 1% formic acid and sonicated for 10 min. In parallel, three samples were dried to app. 30 µl, mixed with 50 µl 1% formic acid in ultrapure water (LC-MS grade, Sigma-Aldrich) and dried again to app. 30 µl.

Partial drying improves peptide yield

Previous studies have found that the peptide-surface interaction depends on the peptide concentration and its primary sequence, and the materials and solutions used in the sample preparation (Hoofnagle et al. 2016). The surface adsorption is not easy to compensate because the additives that could improve the peptide recovery (e.g. surfactants dimethyl sulfoxide or Triton X-100) are not compatible with an LC-MS analysis (Suelter and Deluca 1983, Midwoud et al. 2007). To compare the peptide recovery rates from three different containers in fully- and partially-dried samples, we employed a colorimetric assay and a label-free LC-MS analysis (Figure 2). We did not find any significant difference in the absolute peptide content of fully dried samples of albumin (BSA), ovalbumin (OVA) or a commercial peptide standard. However, we note that the qualitative results from the LT tubes were slightly better which was reflected in the LC-MS base peak chromatograms (e.g. a peak at 57.0 min; Figure 2B). Amines tend to adsorb to glassware via its positively charged chain (Broek et al. 2008) but our results indicate that this binding is either reversible or that the glass insert surface capacity is lower compared to that of polypropylene tubes. The partial sample drying improved the peptide yields in ST and GT but not in the LT samples. The LT polypropylene tube features a protein-repelling surface that prevents a protein adsorption and thus should have the highest peptide recovery rate. However, it seems that this surface is increasing the sample loss during the repeated evaporation steps.

Figure 2 Peptide recovery rates in a high concentration low-complexity peptide sample. Effects of drying and container material; (A) The means and standard deviations of total PSMs count in BSA and OVA samples; (B) Representative base peak chromatograms of fully dried peptide samples.

A) *B)*



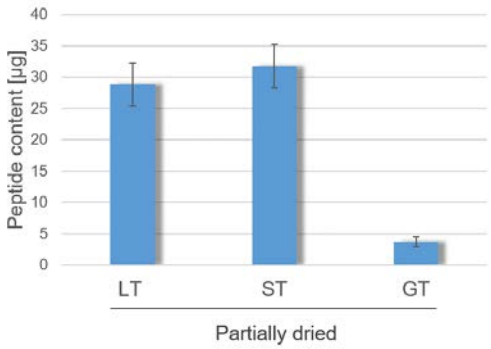
Complications with a complex sample

Our results indicated that it would be beneficial to modify the sample preparation technique and employ a method of a partial drying. To further support these results, we analyzed a complex proteome sample obtained from barley leaf blades. In these experiments, the total protein input for the digestion step was decreased by app. 90% to obtain samples of a high complexity and a low concentration. We have analyzed three complete experimental replicates (Figure 3). In contrast to the previous assays with standard digests, the testing with a complex barley proteome digest showed a much lower recovery rate from the glass surface, indicating more than 80% higher sample loss than in polypropylene tubes. The difference between the total yields in the LT and ST sets was not statistically significant ($p<0.05$). We believe that the extensive sample loss in GT is related to peptide-surface interactions and that the benefits of a glass surface observed earlier (Figure 2) are revealed only once the surface is saturated. This seems to be well in line with the reported lower binding capacity of the wetted solid surface area (John et al. 2004).

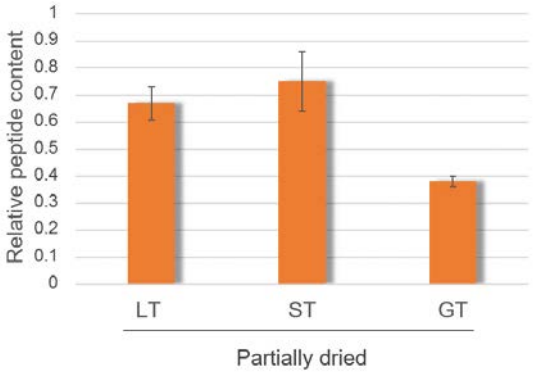
We have also found a surprising difference between quantitative data from the peptide assay (Figure 3A) and the PSMs-based total protein content approximation (Figure 3B). We did not see this difference with the high-abundant standard digests and we believe that this overestimation in the LC-MS quantitation originates in a disproportional sample loss and a depletion of some of the high abundant peptides. In accordance, we have identified 5% more proteins in the GT samples but we did not detect HORVU0Hr1G036870.1 and HORVU6Hr1G016850.1 that in polypropylene tubes represent over 2% and 1% of the total PSMs, respectively.

Figure 3 Peptide recovery in a complex barley proteome digest in partially dried samples. (A) Peptide content determined by the peptide assay (B) Relative peptide content from the total PSMs count; The means and standard deviations of three independent replicates.

A)



B)



CONCLUSION

Our results showed that a limited drying could improve the peptide sample yield. We demonstrated that the benefits of a modified low-binding surface are minimized during the drying and that inexpensive polypropylene tubes have similar or higher peptide recovery rates. Our analyses also imply that a glass surface could improve the protein identification by a limited depletion of abundant proteins. However, the extensive sample loss and a certain level of unpredictability will limit the applicability of this depletion technique.

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