



Minimizing technical variation during sample preparation prior to label-free quantitative mass spectrometry



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ARTICLE INFO

Article history:

Received 17 March 2015

Received in revised form

1 July 2015

Accepted 13 August 2015

Available online 22 August 2015

Keywords:

Sample preparation

SDC

HDMS^E

ABSTRACT

Sample preparation is the crucial starting point to obtain high-quality mass spectrometry data and can be divided into two main steps in a bottom-up proteomics approach: cell/tissue lysis with or without detergents and a(n) (in-solution) digest comprising denaturation, reduction, alkylation, and digesting of the proteins. Here, some important considerations, among others, are that the reagents used for sample preparation can inhibit the digestion enzyme (e.g., 0.1% sodium dodecyl sulfate [SDS] and 0.5 M guanidine HCl), give rise to ion suppression (e.g., polyethylene glycol [PEG]), be incompatible with liquid chromatography–tandem mass spectrometry (LC–MS/MS) (e.g., SDS), and can induce additional modifications (e.g., urea). Taken together, all of these irreproducible effects are gradually becoming a problem when label-free quantitation of the samples is envisioned such as during the increasingly popular high-definition mass spectrometry (HDMS^E) and sequential window acquisition of all theoretical fragment ion spectra (SWATH) data-independent acquisition strategies. Here, we describe the detailed validation of a reproducible method with sufficient protein yield for sample preparation without any known LC–MS/MS interfering substances by using 1% sodium deoxycholate (SDC) during both cell lysis and in-solution digest.

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Sample preparation is a critical step toward high-quality liquid chromatography–tandem mass spectrometry (LC–MS/MS) data in proteomics. In addition, not only protein identification but also reproducibility between samples becomes very important when using label-free strategies such as high-definition mass spectrometry (HDMS^E) and sequential window acquisition of all theoretical

fragment ion spectra (SWATH) data-independent acquisition [1]. When starting from intact cells for a proteome analysis, sample preparation most often involves the use of a surfactant to increase the protein recovery during cell lysis. Commonly used surfactants for cell lysis, prior to mass spectrometry (MS), are Triton X, NP-40, sodium dodecyl sulfate (SDS), sodium deoxycholate (SDC), and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). NP-40 and Triton X are non-ionic, non-denaturing surfactants that are chosen for mild cell lysis when conservation of native biological structure is required. SDS and SDC, on the other hand, are ionic and denaturing surfactants that can disrupt cell membranes and can cause protein denaturation by breaking protein–protein interactions. The zwitterionic and non-denaturing surfactant CHAPS disrupts protein aggregates and is most often used for two-dimensional gel electrophoresis instead of ionic surfactants [2]. An important disadvantage of the use of Triton X and NP-40 is that these surfactants are composed of polyethylene glycol (PEG) structures [3]. PEG, a hydrophobic agent, can give rise to ion suppression at the ion source of a mass spectrometer and can be

Abbreviations: LC–MS/MS, liquid chromatography–tandem mass spectrometry; HDMS^E, high-definition mass spectrometry; SWATH, sequential window acquisition of all theoretical fragment ion spectra; MS, mass spectrometry; SDS, sodium dodecyl sulfate; SDC, sodium deoxycholate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PEG, polyethylene glycol; FASP, filter-aided sample preparation; DMEM, Dulbecco's modified Eagle's medium; TeABC, triethylammonium bicarbonate; DTT, dithiothreitol; TCEP, tris(2-carboxyethyl)phosphine; MMTS, methyl methanethiosulfonate; IAM, iodoacetamide; TFA, trifluoroacetic acid; trypsin–EDTA, trypsin–ethylenediaminetetraacetic acid; AP, acid precipitation; PT, two-phase solvent extraction; RSD, relative standard deviation; iTRAQ, isobaric tags for relative and absolute quantitation.

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observed in the mass spectrum as repeating elements of 44 Da [4,5]. For this reason, removal of PEG is required, resulting in sample loss and (possible) loss of reproducibility in the case of label-free quantitative analysis. SDS also has disadvantages in that (i) it denatures enzymes such as trypsin, leading to impaired digestion; (ii) it is liquid chromatography (LC) incompatible; and (iii) it causes ion suppression. Despite these unwanted side effects, SDS is still used on a regular basis for cell lysis and digestion prior to MS. Although SDS can be removed after digestion by filter-aided sample preparation (FASP) as described by Wisniewski and coworkers and Shevchenko and coworkers [6,7], it has been reported that this time-consuming method was not able to deplete all SDS, still causing LC–MS problems [7,8]. In addition, reproducible results, which are crucial for label-free quantitation during, for example, HDMS^E or SWATH, are difficult to obtain with the FASP protocol [9]. CHAPS equally is MS incompatible (ion suppression), and sample cleanup must be performed by, for example, C18 Zip-Tips [10]. Finally, SDC also needs to be removed prior to MS, but this can be done by either acid precipitation or two-phase solvent extraction after digesting. After these removal steps, no LC–MS/MS interference is detected [11,12]; however, at least for two-phase solvent extraction, higher variability in the peptide and protein identification rate has been described in comparison with acid precipitation [13]. For this study, a detergent with denaturant characteristics (SDC and SDS) was chosen for addition to the cell lysis buffer because one can expect that a loss in the native conformation of proteins will lead to more protein identifications in a bottom-up proteomics approach.

After cell lysis, extracted proteins are cleaved into peptides by means of a digesting enzyme, mostly trypsin. The addition of a denaturant in this step will keep hydrophobic proteins in solution and denature proteins, making the cleavage by a digesting enzyme more efficient. The effect of different denaturants on protein denaturation and solubilization during digestion has been studied extensively, and denaturants can be grouped as surfactants (SDS/SDC), chaotropic agents ([thio]urea), and solvents (methanol/acetone/nitrile). Because of the above-mentioned problems with SDS, different companies have developed MS-compatible surfactants by (i) making them easily removable after digestion by acid precipitation before MS analysis (RapiGest, PPS Silent Surfactant, and Protease Max) or (ii) ensuring that the surfactant did not coelute with the peptides on a C18 reverse column LC system (Invitrosol) [14–17]. Although these surfactants are able to improve the digest efficiency of different proteins in comparison with no addition of any denaturant, they are expensive relative to SDS or SDC. In 2007, Masuda and coworkers [18] compared 27 additives, analyzing the effect on the solubilization of a membrane fraction derived from both *Escherichia coli* and HeLa cells prior to digestion. SDS gave the best result on protein yield determined with bicinchoninic acid assay, followed by RapiGest and SDC [11,18]. Proc and coworkers compared 14 different digesting protocols on their efficiency to digest soluble human plasma proteins. In particular, proteins resistant to digestion (e.g., myoglobin) showed better digestion efficiency with SDS and SDC in 4, 9, or 16 h digesting time compared with urea or combinations of methanol with SDC or trifluoroethanol. They were the first to also consider reproducibility, which scored best in SDC, whereas the lowest reproducibility was observed in urea, which indicates once more the advantage of the use of SDC in HDMS and SWATH above others [19]. Finally, Leon and coworkers analyzed the digestion efficiency of an in-solution digest of 1% RapiGest, 8 M urea, or 5% SDC for denaturation and solubilization of proteins (denaturants were diluted when trypsin was added to the sample). SDC in general scored best for peptide/protein identifications and protein sequence coverage [13].

Taken together, these reports indicate that the low-cost denaturants, SDS and SDC, seem to be the best additives to be used for cell lysate in combination with subsequent digestion. In this study, we focused for the first time on the effect of using these reagents throughout the whole protocol, starting from cell lysis all the way to the final peptide samples. We focused specifically on the reproducibility of these approaches without substantial loss in protein identification to ensure their compatibility with emerging label-free quantitation strategies such as HDMS^E and SWATH. A reproducible protocol was accomplished here by means of using one type of buffer and detergent throughout the whole sample preparation protocol (from cell lysis to MS analysis).

Materials and methods

Materials

All products were purchased from Life Technologies (Carlsbad, CA, USA) unless stated otherwise.

Cell culture of cell lines (RAJI, HeLa, and THP1)

Two suspension cell lines (RAJI and THP1) and one adherent cell line (HeLa) were cultured to confluence in a T175 flask (37 °C, 5% CO₂) using medium composed of basal medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Dulbecco's modified Eagle's medium (DMEM), DMEM/F12, and RPMI 1640 medium were used as basal media for RAJI, HeLa, and THP1 cultures, respectively.

Cell lysis

After cell counting with a hemocytometer, 4 million cells were washed two times with 1× phosphate-buffered saline and were subsequently lysed in a Eppendorf protein LoBind tube with 50 mM triethylammonium bicarbonate (TeABC; Sigma–Aldrich, St. Louis, MO, USA) supplemented with 100 U of benzonase nuclease (Sigma–Aldrich) and 1× Halt Protease and Phosphatase Inhibitor Cocktail (Perbio Science, Erembodegem, Belgium) whether or not in combination with a denaturant (4% SDS [MP Biomedicals, Illkirch, France]; 1, 4, or 10% SDC [Sigma–Aldrich]).

Cells were vortexed and subsequently sonicated for 10 min on ice. After centrifugation (10 min at 14,000 rpm), the supernatant was used for further analysis.

Analysis of protein concentration

Protein concentration was determined by means of absorbance at 280 nm with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Rockford, IL, USA). NanoDrop was used because no Coomassie results could be obtained due to incompatibility with SDC and SDS.

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Differences were evaluated by a Student's *t*-test. A *P*-value < 0.05 was considered statistically significant.

Trypsin digest

First, compatibility of 1% SDC with other digesting reagents was analyzed. Compatibility of 1% SDC with a digestion reagent was defined when no white precipitation in a blank sample (50 or 500 mM TeABC, 1 mM CaCl₂ [Sigma–Aldrich], and 5% acetonitrile [BioSolve]) was observed. Different reducing agents (10 mM dithiothreitol [DTT] vs. 5 mM tris(2-carboxyethyl)phosphine [TCEP];

Sigma–Aldrich]) and alkylating agents (10 mM methyl methanethiosulfonate [MMTS; Sigma–Aldrich] vs. 20 mM iodoacetamide (IAM; Sigma–Aldrich]) were added to a blank sample and visually checked for precipitation.

THP1 cell lysate was digested overnight at 37 °C in 500 mM TeABC, 1% SDC (w/v), 1 mM CaCl₂, 5% acetonitrile, and trypsin/lysC (25:1 protein/enzyme ratio; Promega, Madison, WI, USA) after reduction with 10 mM DTT for 60 min at 60 °C and alkylation with 10 mM MMTS for 10 min at room temperature.

SDC removal by means of acid precipitation or two-phase solvent extraction

Removal of SDC after a trypsin digest from THP1 cell lysates with 1% SDC was obtained by acid precipitation (pH 2.0) with 2% (v/v) trifluoroacetic acid (TFA; Sigma–Aldrich) or by two-phase solvent extraction with ethyl acetate (1:1) followed by the addition of 2% (v/v) TFA. After centrifugation, the supernatant of the acid precipitation and the aqueous phase (lower phase) in the two-phase system contained the peptides and were transferred to another Eppendorf tube. The precipitates of acid precipitation and organic phase (after vacuum evaporation) in the two-phase system were washed with 3 × 0.5% TFA. All samples were dried afterward. Each removal protocol was performed on five replicas.

LC–HDMS^E

After digestion, dried peptides were dissolved in H₂O with 0.1% formic acid. Peptides were separated on a NanoACQUITY system (Waters, Manchester, UK) with direct injection on a NanoACQUITY UPLC column (1.7 μm BEH130, 100 μm × 100 mm C18) at a flow rate of 300 nL/min. The column temperature was maintained at 35 °C. The LC gradient (1–40% B in 60 min, followed by 7 min on 85% B) was obtained by a combination of mobile phase A (H₂O + 0.1% formic acid + 3% dimethyl sulfoxide) and mobile phase B (acetonitrile + 0.1% formic acid). All samples were analyzed by HDMS^E with an in-house optimized collision energy look-up table (ultrahigh-resolution mass spectrometry [20]) on a Synapt G2Si instrument (Waters). Therefore, ion mobility-dependent collision energy profiles (look-up table) in the transfer region are assigned to each individual ion mobility separation cycle across the full ion mobility separation range. The ion mobility separation wave height was set to 40 V. All analyses were performed in resolution mode with a scan time of 0.8 s. Mass accuracy was maintained using a lock spray with GluFib (*m/z* 785.8426, 100 fmol) and leucine enkephalin (*m/z* 556.2771, 200 pg) with a flow rate of 0.5 μL/min. Traveling wave velocity was ramped from 1200 to 400 m/s over the full ion mobility separation cycle. Wave heights in the trap and transfer were both set to 4 V, and wave velocities were set to 311 and 190 m/s, respectively. In low- and high-energy MS mode, the collision energy was set to 4 eV in the trap region.

HDMS^E data analysis

Uniform optimal processing parameters (low energy, high energy, and intensity) for HDMS^E analysis were first determined with Protein Lynx Global Server Threshold Inspector and the data was subsequently analyzed with Progenesis 2.0 software (Waters). First, retention time correction between samples needed to be performed. This was accomplished by the alignment of each sample run to a homemade-quality control sample run, created by generating an equal mixture of all samples. Subsequently, peak picking was performed and data were filtered by charge state (only 2–4+ features were held for analysis). Next, normalization was performed to all proteins. After processing, the data were searched

against a human databank with methylthio (C) as fixed modification and deamidation (NQ) and oxidation (M) as variable modifications. The enzyme specificity was set to trypsin with a maximum of 1 missed cleavage. The false discovery rate was set to 4%, corresponding to a peptide score threshold in our search environment of ±5.4. Two peptides were required to identify a protein.

Cell lysate analysis: addition of no detergent in comparison with addition of 1% SDC

Possible protein/peptide differences between a cell lysate with or without 1% SDC were analyzed with LC–HDMS^E. In short, the same amount (μL) of both types of THP1 cell lysates (*n* = 3 per condition [1% SDC/no detergent]) was digested as described in the “Trypsin digest” section above. Each sample was digested in duplicate (6 samples/condition). SDC was removed by acid precipitation as described in the “SDC removal by means of acid precipitation or two-phase solvent extraction” section above. No pellet wash was performed. Peptides were analyzed with LC–HDMS^E (see “LC–HDMS^E” section above). Normalization was performed against all proteins. Data analysis was performed with Protein Lynx Global Server. Only common proteins (in two or more replicas per condition) were retained to define differences in protein/peptide identifications in both conditions.

Possible differences in abundances between the same proteins and peptides were identified with Progenesis 2.0 software. An independent *t*-test with false discovery rate correction was performed with Excel. A *Q*-value ≤ 0.001 was considered statistically significant. All significant peptides/proteins were further analyzed on possible differences in their hydrophobicity (GRAVY) by means of ProtParam software [21].

Results

Cell lysis: need for a detergent

SDS and SDC are two of the most used detergents for cell lysis. To validate their added value in terms of yield and reproducibility, protein concentration was determined in triplicate by a NanoDrop assay because colorimetric assays are incompatible with detergents. Cell lysis without any detergent, with different concentrations of SDC (1, 4, or 10%), and with 4% SDS (based on FASP protocol [6]) were compared after cell lysis in two suspension cell lines (RAJI and THP1) and one adherent cell line (HeLa) to coordinate any cell line-specific effects. Indeed, a significant increase in protein yield in both suspension cell lines (THP1 and RAJI) for even the lowest concentration of SDC (1%) could be found (Fig. 1). Compared with 1%, the addition of 4 or 10% SDC had no added value in increasing the protein content. When comparing 1% SDC with 4% SDS (FASP protocol), one could observe a higher protein yield with the use of 1% SDC in comparison with 4% SDS (*P*-value < 0.05 in RAJI, *P*-value = 0.06 in THP1 cell lysate) (Fig. 1). We concluded that 1% SDC could be used as detergent during cell lysis and can be used as a substitute for 4% SDS.

For the adherent HeLa cell line, 0.25% trypsin–ethylene diaminetetraacetic acid (trypsin–EDTA) was used to detach cells prior to cell lysis. However, the addition of 1% SDC gave no significantly higher protein yield compared with no detergent in this cell line; in multiple experiments, the addition of 1% SDC gave rise to equal or higher protein yield compared with no detergent. This larger variability in protein yield between experiments might be due to differential clustering of cells after detachment from the culture plate, which can interfere with subsequent protein extraction efficiency. Therefore, direct cell lysis of adherent cells without the detachment with 0.25%

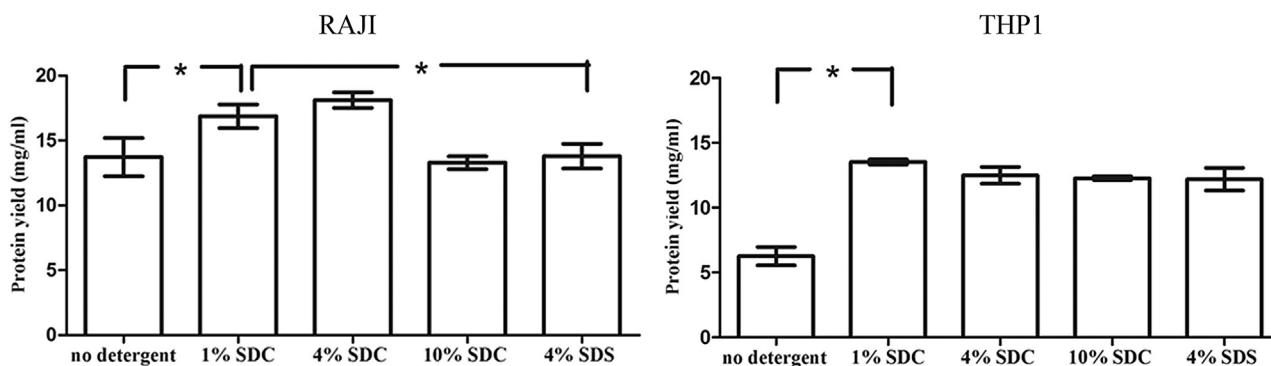


Fig. 1. Overview optimization cell lysis protocol for RAJI (left) and THP1 (right) cells. One representative of each experiment is shown: NanoDrop results concerning the effect of SDC and SDS on cell lysis in two cell lines. *Significant difference (P -value < 0.05) between no detergent and 1% SDC or 1% SDC and 4% SDS.

trypsin–EDTA was analyzed as well. Interestingly, when cells were directly lysed from the washed plate using the different amounts of detergent, more reproducible results were obtained compared with cell lysis after cell detachment with trypsin. Importantly, protein yield after direct cell lysis in 1% SDC was comparable to the “standard” procedure with the 0.25% trypsin–EDTA step.

Trypsin digest optimization of a cell extract with SDC

Compatibility of SDC with chemicals needed for trypsin digest

During the subsequent steps of the digest, we noticed that precipitation occurs by the addition of some reagents. Because SDC precipitates in an acid environment, it is important to avoid fluctuations in the buffer pH. Thus, we tested the use of TCEP or DTT as reducing agent as well as IAM or MMTS as alkylating reagent in a 50- or 500-mM TeABC buffer environment. SDC precipitation occurred when either TCEP or MMTS was added to the blank sample (in 50 mM TeABC, 1 mM CaCl_2 and 5% acetonitrile). Indeed, these solutions have pH values of 1.0 and 4.0, respectively. However, precipitation could be avoided for all tested reducing and alkylating agents when using 500 mM TeABC. Thus, increasing the buffer capacity is strongly recommended when using SDC for sample preparation.

Of note, using 500 mM TeABC instead of 50 mM TeABC also during cell lysis would greatly increase the simplicity of the protocol, resulting in better reproducibility. Although it is known that osmolarity can theoretically have an influence on cell lysis [2], in our hands no significant difference in protein yield could be observed in any of the cell lines tested above when using 50 or 500 mM TeABC. In conclusion, we recommend the use of 500 mM TeABC in both cell lysis and in-solution digest.

SDC removal by means of acid precipitation or two-phase solvent extraction

After the tryptic digest, the SDC needs to be removed. Using the THP1 and RAJI cell lines, we compared the use of acid precipitation (AP) and two-phase solvent extraction (PT) in terms of protein and peptide identification efficiency. As can be seen in Fig. 2 for the THP1 cell line, no significant differences were detected in the numbers of proteins (white bars) and peptides (gray bars) between PT and AP. Yet, the use of AP produced a more reproducible list of identifications (see [Supplementary Table 1](#) in online supplementary material). Of note, when washing the pellet, as suggested by Lin and coworkers, a small number of proteins/peptides could be identified in the pellet wash of both PT and AP [12]. However, no

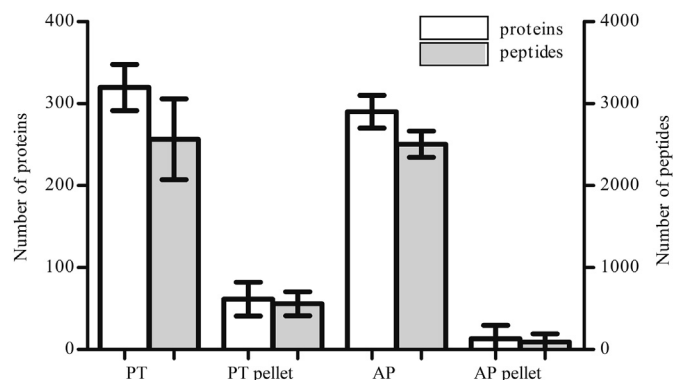


Fig. 2. SDC removal by means of two-phase solvent extraction (PT) or acid precipitation (AP) for THP1 cell lysates. The need for a pellet wash was also taken under consideration (PT pellet and AP pellet). The numbers of proteins (left axis, white bars) and peptides (right axis, gray bars) of the different conditions are presented. Each removal protocol was performed on five samples.

new peptide identifications could be detected in these pellet washes, and thus we discarded this additional step.

Proteomic analysis of cell lysates obtained with or without 1% SDC

In a final analysis, identical amounts of THP1 cell lysates with and without 1% SDC were analyzed with HDMS^E. Indeed, only small normalization factors were calculated when normalization was done against all proteins. Surprisingly, the same number of protein/peptide identifications in both conditions was observed (with ~83% of all identified proteins common between both conditions). The identification efficiency (% annotation) was also the same in both conditions. However, in the SDC samples, additional unidentified precursor masses (10%, charge 2–4+) were found at the peptide level.

By defining the reproducibility at the level of feature intensity, we can directly define the technical variability that would actually interfere with each of the features present in a sample. Within the different replicas ($n = 6$) in THP1 cell lysates with and without 1% SDC, therefore, we calculated the relative standard deviation (RSD) of all features (Fig. 3). RSD was determined by dividing the standard deviation by the mean of the normalized abundances of all replicas/condition for all features separately. Out of these data, a frequency plot was made. As can be seen in Fig. 3, more than 60% of all features had an RSD lower than 20% in a cell lysate with 1% SDC (gray bars). In contrast, the same RSD was achieved for $\pm 38\%$ of all features for a cell lysate with no detergent (white bars), indicating a

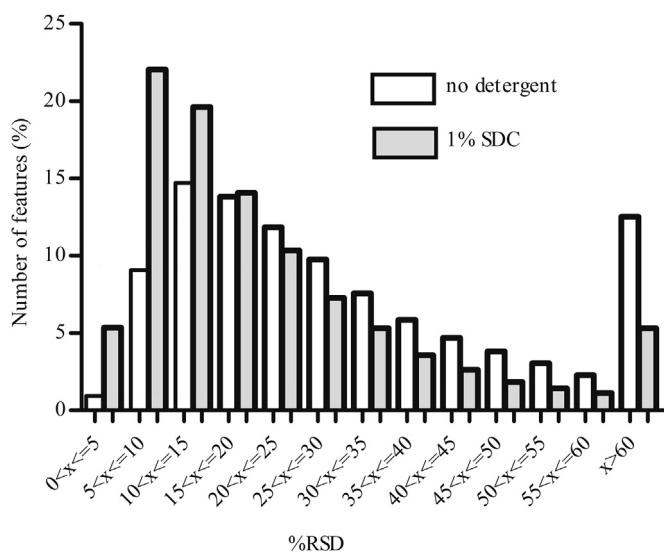


Fig. 3. Frequency plot considering reproducibility. The x-axis represents the relative standard deviation (RSD: standard deviation normalized abundances/mean normalized abundances of all replicas/condition $\times 100$), and the y-axis represents the number of features with this RSD (%). Cell lysates with 1% SDC (gray bars) have higher reproducibility in comparison with cell lysates without 1% SDC (no detergent, white bars).

higher reproducibility when using 1% SDC. Although protein identifications is a metric that is used in most protocol optimizations, the above results illustrate that this actually shows only a limited picture.

Discussion

The majority of studies focusing on optimizing sample preparation use the number of peptide or protein identifications as the metric of validation. However, reproducibility in sample preparation is of main importance when using label-free quantification approaches such as HDMS^E and SWATH [1]. This reproducibility during sample preparation can be obtained only by using a protocol with a minimum of steps. Here, we have presented a reproducible protocol by using a single buffer and a single detergent throughout the entire protocol.

Tris–HCl, a commonly used buffer, was not the first choice because of its reported ion suppression effect, the formation of Tris–protein adduct ions, and its incompatibility with iTRAQ (isobaric tags for relative and absolute quantitation) (contains primary amines) [22]. TeABC, on the other hand, can be evaporated by means of vacuum drying and is compatible with iTRAQ analysis. Therefore, TeABC is widely used as digesting buffer (pH 8.0). Because of its suitability during in-solution digestion, this buffer is also the first choice for cell lysis. In the literature, the concentration of TeABC in digest protocols is not uniform (concentration between 50 and 500 mM). However, we observed precipitation of SDC when MMTS and TCEP were added to an in-solution digest containing 50 mM TeABC. Because the change in osmolarity does not change cell lysis efficiency, here we argue for the use of 500 mM TeABC where no precipitation was observed. Of note, because of the known side reactions with IAM (N alkylation and O alkylation), MMTS was used as alkylation agent in an in-solution digest [23].

Next, the use of detergents for cell lysis was validated. A detergent was added to the cell lysis buffer for several reasons: (i) solubilization of hydrophobic proteins or membranes and (ii) denaturation of proteins (breaking protein–protein interactions)

[2]. Several detergents are available, with each one having its own advantages and disadvantages. In our experiment, SDC and SDS were chosen because of their denaturant characteristics, low cost, and promising results based on digest optimizations [6,11]. Different concentrations of SDC (1, 4, and 10%) were compared with 4% SDS (amount used during FASP [6]) and no detergent addition. In general, one could observe a reproducible and significant higher protein yield with 1% SDC in comparison with no detergent or 4% SDS addition. Therefore, SDC can be considered as an alternative for SDS for cell lysis. Next, we showed that higher reproducibility between experiments is obtained in adherent cell lines when using direct cell lysis instead of first using 0.25% trypsin–EDTA for detaching the cells. Therefore, direct cell lysis is recommended for use in HDMS^E and SWATH data-independent acquisition strategies.

After cell lysis, a trypsin digest is carried out to obtain peptides that can be analyzed in LC–MS/MS. As for cell lysis itself, different denaturants can be added to promote the unfolding of proteins. We reasoned, however, that using the same buffer as for the cell lysis would benefit reproducibility. Indeed, one must keep in mind that the addition of denaturants needs to be done with care: (i) inhibition of trypsin activity (0.1% SDS, 4 M urea, and 50% methanol will lead to trypsin activities of 20, 71, and 31%, respectively) [14]; (ii) incompatibility with LC–MS/MS (SDS gives rise to ion suppression) [11]; and (iii) introduction of modifications (high temperature will convert [thio]urea to cyanate, resulting in carbamylation of lysine, arginine, and N termini) [6]. The 1% SDC was chosen as denaturant during digestion because of the already reported promising results and its LC–MS/MS compatibility [11–13,18].

Removal of SDC can be achieved by AP with TFA or by PT by means of 1:1 ethyl acetate with TFA. In short, the addition of TFA (= AP) leads to SDC precipitation because SDC is insoluble in an acid and aqueous environment. During PT, SDC solubilizes in the organic solvent (ethyl acetate) while the peptides remain in the aqueous part. Masuda and coworkers found that more peptides ($\pm 32\%$)/proteins ($\pm 37\%$), in particular hydrophobic peptides/proteins, could be identified in an *E. coli* membrane fraction by means of PT compared with AP. They hypothesized that hydrophobic proteins will precipitate with SDC when using AP [18]. In contrast, Lin and coworkers found that more hydrophilic and hydrophobic peptides and proteins (± 11 – 12%) could be identified in rat liver membrane with AP in comparison with PT [12]. In both articles, no information is available concerning reproducibility of the comparison between AP and PT [12,18]. Leon and coworkers compared the reproducibility of PT and AP in a rat liver mitochondrial sample; no difference was found between AP and PT at the protein level. In contrast, more peptides (11.51%) were found in PT in comparison with AP, resulting in slightly higher protein coverage for PT. Higher reproducibility was observed in AP [13]. Our comparison led to the conclusion that no difference between protein or peptide numbers could be observed between AP and PT. A slightly higher reproducibility appeared to be present with AP. These results are generally a confirmation of the results as described in Leon and coworkers [13].

In a final experiment, the impact of the addition of 1% SDC during cell lysis of THP1 cells on protein identification and quantification was examined with HDMS^E. The same proteins and peptides were identified with and without SDC. Increased coverage will undoubtedly be obtained by using a longer LC gradient. Higher reproducibility was observed in a cell lysate with 1% SDC (lower % RSD) than without SDC, which is a major advantage when using HDMS^E and SWATH data-independent acquisition strategies.

In conclusion, a sample preparation protocol has been presented here with good reproducibility and protein yield by using the same buffer (500 mM TeABC) and same detergent (1% SDC) starting from cell lysis to HDMS^E analysis.

Conclusion

We have demonstrated that the addition of 1% SDC to a cell lysis buffer resulted in a higher and more reproducible protein yield in comparison with no detergent addition in three different cell lines, making it the most recommended method for HDMS^E and SWATH data-independent acquisition strategies.

Acknowledgments

This research was (partially) financed by GOA project 01G01112 (“Pathways to pluripotency and differentiation in embryos and embryonic stem cells”).

Appendix A. Supplementary material

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ab.2015.08.018>

References

- [1] Y. Levin, Label-free quantification of proteins using data-independent acquisition, in: C.E. Eyers, S.J. Gaskell (Eds.), *Quantitative Proteomics (New Developments in Mass Spectrometry)*, Royal Society of Chemistry, 2014, pp. 175–183.
- [2] A. Bodzon-Kulakowska, A. Bierzynska-Krzysik, T. Dylag, A. Drabik, P. Suder, M. Noga, J. Jarzebinska, J. Silberring, Methods for samples preparation in proteomic research, *J. Chromatogr. B* 849 (2007) 1–31.
- [3] C. Klammt, D. Schwarz, K. Fendler, W. Haase, V. Dotsch, F. Bernhard, Evaluation of detergents for the soluble expression of α -helical and β -barrel-type integral membrane proteins by a preparative scale individual cell-free expression system, *FEBS J.* 272 (2005) 6024–6038.
- [4] X.Y. Xu, H. Mei, S.Y. Wang, O. Zhou, G.F. Wang, L. Broske, A. Pena, W.A. Korfmacher, A study of common discovery dosing formulation components and their potential for causing time-dependent matrix effects in high-performance liquid chromatography tandem mass spectrometry assays, *Rapid Commun. Mass Spectrom.* 19 (2005) 2643–2650.
- [5] D.C. Chamrad, G. Koerting, J. Gobom, H. Thiele, J. Klose, H.E. Meyer, M. Bluggel, Interpretation of mass spectrometry data for high-throughput proteomics, *Anal. Bioanal. Chem.* 376 (2003) 1014–1022.
- [6] J.R. Wisniewski, A. Zougman, N. Nagaraj, M. Mann, Universal sample preparation method for proteome analysis, *Nat. Methods* 6 (2009) 359–360.
- [7] G. Shevchenko, S. Musunuri, M. Wetterhall, J. Bergquist, Comparison of extraction methods for the comprehensive analysis of mouse brain proteome using shotgun-based mass spectrometry, *J. Proteome Res.* 11 (2012) 2441–2451.
- [8] H.K. Hustoft, L. Reubsaet, T. Greibrokk, E. Lundanes, H. Malerod, Critical assessment of accelerating trypsin methods, *J. Pharm. Biomed.* 56 (2011) 1069–1078.
- [9] J. Vowinckel, F. Capuano, K. Campbell, M.J. Deery, K.S. Lilley, M. Ralser, The beauty of being (label)-free: sample preparation methods for SWATH-MS and next-generation targeted proteomics, *F1000Res.* 2 (2013) 272, <http://dx.doi.org/10.12688/f1000research.2-272.v2>.
- [10] N. Zhang, L. Li, Effects of common surfactants on protein digestion and matrix-assisted laser desorption/ionization mass spectrometric analysis of the digested peptides using two-layer sample preparation, *Rapid Commun. Mass Spectrom.* 18 (2004) 889–896.
- [11] Y. Lin, J. Zhou, D. Bi, P. Chen, X.C. Wang, S.P. Liang, Sodium-deoxycholate-assisted tryptic digestion and identification of proteolytically resistant proteins, *Anal. Biochem.* 377 (2008) 259–266.
- [12] Y. Lin, Y. Liu, J.J. Li, Y. Zhao, Q.Z. He, W.J. Han, P. Chen, X.C. Wang, S.P. Liang, Evaluation and optimization of removal of an acid-insoluble surfactant for shotgun analysis of membrane proteome, *Electrophoresis* 31 (2010) 2705–2713.
- [13] I.R. Leon, V. Schwammle, O.N. Jensen, R.R. Sprenger, Quantitative assessment of in-solution digestion efficiency identifies optimal protocols for unbiased protein analysis, *Mol. Cell. Proteomics* 12 (2013) 2992–3005.
- [14] Y.Q. Yu, M. Gilar, P.J. Lee, E.S.P. Bouvier, J.C. Gebler, Enzyme-friendly, mass spectrometry-compatible surfactant for in-solution enzymatic digestion of proteins, *Anal. Chem.* 75 (2003) 6023–6028.
- [15] Y. Kawashima, N. Takahashi, M. Satoh, T. Saito, S. Kado, F. Nomura, H. Matsumoto, Y. Kodera, Enhanced recovery of lyophilized peptides in shotgun proteomics by using an LC-ESI-MS compatible surfactant, *Proteomics* 13 (2013) 751–755.
- [16] E.I. Chen, D. Cociorva, J.L. Norris, J.R. Yates, Optimization of mass spectrometry-compatible surfactants for shotgun proteomics, *J. Proteome Res.* 6 (2007) 2529–2538.
- [17] D. Winter, H. Steen, Optimization of cell lysis and protein digestion protocols for the analysis of HeLa S3 cells by LC-MS/MS, *Proteomics* 11 (2011) 4726–4730.
- [18] T. Masuda, M. Tomita, Y. Ishihama, Phase transfer surfactant-aided trypsin digestion for membrane proteome analysis, *J. Proteome Res.* 7 (2008) 731–740.
- [19] J.L. Proc, M.A. Kuzyk, D.B. Hardie, J. Yang, D.S. Smith, A.M. Jackson, C.E. Parker, C.H. Borchers, A quantitative study of the effects of chaotropic agents, surfactants, and solvents on the digestion efficiency of human plasma proteins by trypsin, *J. Proteome Res.* 9 (2010) 5422–5437.
- [20] U. Distler, J. Kuharev, P. Navarro, Y. Levin, H. Schild, S. Tenzer, Drift time-specific collision energies enable deep-coverage data-independent acquisition proteomics, *Nat. Methods* 11 (2014) 167–170.
- [21] E. Gasteiger, C. Hoogland, A. Gattiker, S. Duvaud, M.R. Wilkins, R.D. Appel, A. Bairoch, Protein identification and analysis tools on the ExPASy server, in: J.M. Walker (Ed.), *The Proteomics Protocol Handbook*, Humana, Totowa, NJ, 2005, pp. 571–607.
- [22] I.F. Shieh, C.Y. Lee, J. Shiea, Eliminating the interferences from Tris buffer and SDS in protein analysis by fused-droplet electrospray ionization mass spectrometry, *J. Proteome Res.* 4 (2005) 606–612.
- [23] E.S. Boja, H.M. Fales, Overalkylation of a protein digest with iodoacetamide, *Anal. Chem.* 73 (2001) 3576–3582.