A Rapid Visual Indicator for Surfactant Abundance (VISA) in MS-Based Membrane and General Proteomics Applications

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ABSTRACT:

The existence of surfactants in proteomics samples can severely reduce enzymatic digestion efficiency, liquid chromatography (LC) separation efficiency, column lifetime and mass spectrometry (MS) sensitivity. Although various techniques are able to remove surfactants, surfactants may occasionally be retained in samples due to variations in sample preparation method or personal skill. Evaluation of surfactant residue in a sample, however, usually requires additional instrument and is time consuming. In this study, a simple and rapid visual indicator for surfactant abundance (VISA) was developed. By detecting a visible surfactant pellet in the solution, this assay was able to detect surfactant residue in aqueous solutions within 5 min. Without the need of additional equipment such as mass spectrometer, every user can perform the test in their laboratory before sending the sample to the MS facility. The detection limit for the commonly used surfactants, Triton X-114 and SDS, was about 0.0005% and 0.0002%, respectively. The VISA was successfully applied to evaluate the efficiency of removal of surfactants in Triton X-114 extracted membrane proteins using tube-gel. With the combination of Triton X-114 extraction and tube-gel protocol, a study of spermatozoa membrane proteome identified about 252 proteins of which about 78% were classified as membrane proteins. The co-existence of protein and surfactant did not affect the VISA sensitivity, suggesting that this indicator is suitable for proteomics applications. The VISA also has potential for the detection of other surfactants and can be applied to other surfactant removing protocols.

INTRODUCTION

Surfactants are commonly used in protein chemistry. Because of their amphiphile nature, they are widely used for the dissolution, extraction and denaturing of membrane proteins in aqueous solution.[1-3] As membrane proteins participate in many important biological processes, including cell-cell interactions, molecular trafficking and signal transduction,[4-5] the need for comprehensive analysis of membrane proteome is continuously increasing.[6-13] MS-based proteomics has rapidly become the major approach for the global analysis of membrane proteins because of its high throughput, high sensitivity and high identification accuracy.[14-17] However, the use of surfactants for membrane protein extraction and dissolution in MS-based proteomics approaches can produce several challenges. This is because the surfactants may affect protease activity thus reducing the sequence coverage for protein identification.[18] Trace surfactant residues can also result in poor LC separation which is due to the adsorptive interaction of surfactants with analytes and the stationary phase.[19] In terms of MS detection, the presence of surfactants can interfere with analyte signals owing to ion suppression effect. In addition, the formation of surfactant ion clusters can result in difficult interpretation of mass spectra.[20-23] Therefore, in order to obtain sensitive and high quality analysis results using MS-based proteomics approaches, surfactants have to be removed from the sample prior to the MS or LC-MS analysis.

In proteomics applications, numerous strategies can be applied to remove surfactants from protein samples before performing proteolytic digestion. These strategies include protein precipitation, dialysis and chromatographic methods.[23-24] However, the removal of the surfactants from the protein sample may reduce the

membrane protein solubility and recovery.[23] Recently, a simple protocol, tube-gel digestion, was developed to digest the hydrophobic proteins.[18] The tube-gel protocol allows the application of a variety of surfactants to dissolve and unfold the protein to facilitate trypsin digestion. By this method, proteins are dissolved in the concentrate surfactant solution and then the final protein solution is incorporated into a polyacrylamide gel matrix. Before performing the proteolytic digestion, surfactants can be removed from the gel matrix by solvent wash while the proteins still remain in the gel matrix. This method allows hydrophobic proteins to be successfully digested and analyzed. A wide variety of surfactants and sample preparation methods can be adapted to this protocol. However, the surfactant removal efficiency may vary according to different surfactants, gel size and sample preparation methods. Thus, surfactant may occasionally be retained with proteins after the gel wash, finally co-extracted with digested peptide and result in poor proteomic analysis results. However, more extensive gel wash may cause sample loss and reduce identification sensitivity. With these concerns in mind, it is better to monitor surfactant residue during the gel wash before performing the in-gel digestion. The evaluation of surfactant residue, however, is usually time-consuming and may require additional equipment such as MS.[25-27] However, not all of the laboratory can directly access the MS to test the surfactant residue during the wash procedure and the surfactant can easily contaminate the MS and damage the LC column. Thus, an ease of use assay is required to detect the surfactant residue during the proteomics sample preparation.

In this study, a simple and fast visual indicator for surfactant abundance (VISA) is introduced. This method can help optimize the wash procedure for surfactant removal using the gel-based approach and can help avoid sample loss due to over washing of the gel. In addition to the gel based approach, this method was also

demonstrated to be feasible for the in-solution based approach. The performance and limitations of this indicator are discussed.

EXPERIMENTAL SECTION

Materials

The α-cyano-4-hydroxycinnamic acid (CHCA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), silver nitrate, methyl methylmercaptomethyl sulfoxide (MMTS), N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium bicarbonate (ABC), formaldehyde, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), glucose, sodium dithionite and Triton X-114 (TX-114) were purchased from Sigma (St. Louis, MO). Coomassie Brilliant Blue G was also purchased from Sigma and 1 mg of powder was dissolved in 1 ml deionized water followed by centrifugation at 6000 × g to remove the precipitation. Trypsin (modified, sequencing grade) was from Promega (Madison, WI). Tris (Base), Tris-HCl, sodium carbonate, sodium dodecyl sulfate (SDS) were purchased from J.T Baker (Phillipsburg, NJ). Tryptic enolase was purchased from Waters (Milford, MA). Acetone, methanol, acetonitrile and trifluoroacetic acid (TFA) were analytical grade and were purchased from Merck (Darmstadt, Germany). For LC-MS analysis, the acetonitrile (ACN) with 0.1% formic acid [7] and water with 0.1% formic acid (LC-MS grade, J. T. Baker, Phillipsburg, NJ) were used as the mobile phase. Acryamide and Coomassie Blue staining solution were obtained from BioRad (Hercules, CA). Deionized water (18.1 MΩ·cm resistivity) from a Milli-Q system (Millipore, Bedford, MA) was used throughout this work.

Peptide Mass Fingerprinting

To investigate the influence of surfactants on MALDI analysis, tryptic enolase

(12.5 fmole) mixed with various concentrations of surfactants were analyzed by peptide mass fingerprinting (PMF) using MALDI-MS. The MALDI-MS spectra were obtained with a 4800 MALDI-TOF/TOF (Applied Biosystems, Houston, TX) equipped with Nd-YAG laser (355 nm). The matrix (α-CHCA, saturated solution in 75% ACN containing 0.1% TFA) was mixed with equal volume of tryptic enolase, spotted on the sample plate and dried in air. The spectra were recorded in the reflector mode using acceleration voltage of +20 kV and 200 ns delay time. For PMF analysis, the peak list of MS spectra was extracted by Data Explore v.4.5 (Applied Biosystems) software. The extracted peak list was searched against a UniProtKB/Swiss-Prot (release 57.11) using MASCOT v.2.2.04 (Matrix Science, London). For the search parameter, the peptide mass tolerance was 50 ppm, with allowance for one missed cleavage made from the trypsin digest, variable modifications of carbamidomethyl (C) and oxidation (M) and the significant threshold was $P < 0.05$.

nanoLC-ESI-Q-TOF MS/MS Analysis

LC-MS/MS was performed with a nanoflow LC system (nanoACQUITY UPLC, Waters, Millford, MA) coupled to a hybrid Q-TOF mass spectrometer (Synapt HDMS, Waters, Manchester, U.K.). The sample was injected into a trap column (Symmetry C18, 5 μm, 180 μm × 20 mm, Waters, Milford, MA), and separated online with a reverse-phase (BEH C18, 1.7 μm, 75 μm × 250 mm, Waters, Milford, MA) at the flow rate of 300 nl/min using a 50 min 10-80% acetonitrile/water gradient for tryptic enolase and 120 min 15-90% acetonitrile/water gradient for membrane proteomics analysis. The acquisition method was set to one full MS scan (400-1600 m/z) with 0.6 sec scan time and switched to three 1.2 sec product ion scans (MS/MS) when a

target ion arrived on intensity of greater than 20 counts. In addition, the charge state was set to +2 and +3. For the MASCOT search, the data was first deisotoped, centroided and converted to a pkl file by the ProteinLynx v2.2.5. The search parameters of MASCOT for peptide and MS/MS mass tolerance were ±0.3Da and ±0.3Da, respectively with allowance for one missed cleavage in the trypsin digest and variable modifications of carbamidomethyl (C) and oxidation (M). Peptides were considered as identified if their MASCOT individual ion score was higher than the MASCOT score 30 ($P < 0.001$).

Silver Staining

After gel electrophoresis, the gel was fixed for 60 min with a fixing solution (50% (v/v) ethanol, 10% (v/v) acetic acid, and 0.05% (v/v) formaldehyde). The gel was then washed with 30% ethanol solution for 20 min. This procedure was repeated a further two times. Afterward, the gel was incubated for another 2 min in a sensitizing solution (0.02% sodium dithionite). Then the gel was washed three times with water for 5 min and incubated for 20 min at room temperature in the staining solution (0.2% $AgNO_3$, 0.001% formaldehyde). The gel was then washed twice with 50 ml of water for 1 min and incubated twice for 5 min with the developing solution (6% sodium carbonate and 0.074% formaldehyde solution dissolved in water). When the protein bands became visible, the gel was incubated for 10 min in a stopping solution (50% methanol, 10% glacial acetic acid) and then rinsed with 50 ml of water three times for 5 min. Finally the stained gel image was acquired by a flatbed scanner (4990 photo, Epson, CA).

Preparation of Mouse Spermatozoa

The HM medium (0.12 mM NaCl, 2 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 0.36 mM NaH_2PO_4, 15 mM $NaHCO_3$, 10 mM HEPES, 5.6 mM glucose and 1.1 mM sodium pyruvate) was adjusted to pH = 7.3-7.4 by aeration with humidified air/CO_2 (19:1 v/v) in a 37°C incubator overnight. Adult male mice (age = 12-16 wks, ICR) were sacrificed by cervical dislocation, and the epididymides were removed and immersed in the HM medium. Spermatozoa were extruded from the distal portion of the epididymides at 37°C for 10 min. The cells were gently filtered through two layers of nylon gauze, washed in three volumes of the same medium, and collected by 600 × g centrifugation for 10 min at room temperature. The spermatozoa were then resuspended and centrifuged twice. [28]

Extraction and Digestion of Membrane Proteins

The sperm membrane proteins were extracted by TX-114 partition [1]. The 50 μg of mouse spermatozoa was washed twice with Tris-buffered saline (TBS, 20 mM Tris-HCl and 150 mM NaCl, pH 7.6) and centrifuged at 600 × g. After centrifugation, the sperm pellet was mixed with 1 mL of extraction reagent (1.7% of TX-114 in TBS) and incubated at 4°C for 60 min with mild vortex. The solution was then centrifuged at 10,000 × g, 4°C for 20 min to separate tissue debris and the supernatant. The supernatant was placed into a new vial and incubated at 37°C for 3 min. Then supernatant was centrifuged by a 10,000 × g for 3 min at room temperature. The upper aqueous layer was carefully removed and the bottom surfactant phase was resuspended with 1 ml of cold TBS at 4°C. The same extraction procedure was repeated once, thus the membrane proteins of spermatozoa

were extracted to the surfactant phase. To remove the surfactants and digest the protein, the modified tube-gel digestion protocol was applied. For the tube-gel digestion, the proteins in the surfactant phase were chemically reduced by 5 mM TCEP at 37°C for 30 min, alkylated with 2 mM MMTS at room temperature in the dark for 30 min and the diluted with water to the volume of 50 μL. The solutions of 17.5 μl acrylamide (40%, 29:1), 2.45 μl 10% ammonium persulfate and 1.05 μl TEMED were sequentially added to the protein solution. After 30 min incubation at room temperature, the acrylamide was polymerized and the protein was embedded in the gel. The tube-gel was cut into small pieces and washed with 25 mM ABC (pH 8.2) containing 50% ACN for 15 min four times. To test the surfactant residue in the wash solution, the wash solution was first dried in a centrifugal concentrator (miVac Duo Concentrator, Genevac, NY) and then the VISA test was conducted. After the gel wash and VISA test, the gel pieces were dehydrated with 100% acetonitrile and digested with trypsin (1:100 trypsin to protein ratio in weight) in 25 mM ABC at 37°C overnight. After digestion, the tryptic peptides were extracted from the gel using 25 mM ABC, 0.02% TFA, 0.02% TFA in 50% ACN, and 100% ACN sequentially. All of the extracted solutions were combined and concentrated using a centrifugal concentrator and analyzed with LC-MS/MS.

In-Solution Protein Digestion of the Total Lysate of Jurkat T-Cell

The cells were washed twice with PBS and the lysis buffer (0.05 % SDS, 6M Urea, 5mM EDTA and 50 mM HEPES at pH=8.3) was added to the cells. The cells were subjected to the sonication 3 times for 10 sec each with 30 sec interval between sonications. The lysate was centrifuged at 10,000xg and the pellet was discarded and about 100 μg extracted protein was reduced with 5 mM TCEP for 30 min at 37 ^{o}C

and followed by addition of 5 mM IAA for 2 hours at 25 ^{o}C. The protein mixture was precipitated with the addition of -20^{o}C acetone for 2 h. The protein pellet was recovered by the 16000xg for 30 min and washed with -20^{o}C acetone to remove the residue SDS. After the acetone wash, the protein pellet was resuspened with TEABC (pH=8.0) and the trypsin was added to the protein mixture at an enzyme-to-substrate ratio of 1:50 (w/w). After 16 h tryptic digestion at 37 ^{o}C, the tryptic peptides were reduced and alkylated as describe above, and more trypsin 1:100 (w/w) was added. After 3 h incubation, the resulting tryptic peptides were analyzed by the LC-MS/MS.

RESULTS AND DISCUSSION

Impact of Surfactants on Mass Spectrometry

In this study, two commonly used surfactants, TX-114 and SDS were studied. Triton X-114, a non-ionic surfactant, forms a homogenous solution below its cloud point (20~22^{o}C), but separates into an aqueous phase and a surfactant-rich phase above the cloud point, thereby rapidly and simply separating hydrophobic and hydrophilic macromolecules.[1,29] TX-114 is widely used for the enrichment and purification of membrane proteins.[30-31] This strategy has been demonstrated to be an efficient way to purify membrane proteins with high specificity and recovery.[32] SDS is the most commonly used surfactant in protein chemistry. It is used in cell lysis, SDS-PAGE, protein extraction, dissolution and denaturation and other procedures.[33-35]

To test the impact of the surfactant residue on peptide mass fingerprinting (PMF), a sample of tryptic enolase with TX-114 and SDS were analyzed by MALDI-TOF MS. Table 1 summarizes the PMF search result for enolase peptides with different TX-114 and SDS concentrations. As shown in Table 1, enolase could not be characterized by PMF when the concentration of TX-114 was $\geq$ 0.005% or the concentration of SDS was $\geq$ 0.01%. For PMF, the sequence coverage of enolase was > 40% when the TX-114 and SDS concentrations were $\leq$ 0.0005% and 0.005%, respectively.

The less tolerance for TX-114 in the MALDI-TOF analysis was because TX-114 has a repeated unit of polyethylene glycol which can be easily ionized by forming cluster ions and is mostly distributed in the m/z range of 400-1200 Th, which is

located in the peptide m/z range. [21] The production of surfactant clusters can make the MS spectrum more complicated and can suppress the peptide ionization. Besides, the lower critical micelle concentration (CMC) of TX-114 (0.011% at 25°C)[36] in comparison with the SDS (0.23% at 25°C),[37] shows that TX-114 forms micelles more easily which may affect the co-crystallization process of the sample with the MALDI matrix. Poor co-crystallization of the sample and matrix has been found to be a major factor contributing to low MALDI ionization efficiency.[38]

In addition to the MALDI-TOF, a more commonly used strategy for protein identification is LC-MS/MS. To study the influence of surfactants on LC-MS/MS, 25 fmole tryptic enolase samples with different concentrations of TX-114 and SDS were analyzed by LC-MS/MS. Table 2 shows three enolase peptides that were selected to evaluate the impacts of surfactants on the chromatographic retention time, separation resolution and sensitivity. The impact of the TX-114 on LC-MS/MS was insignificant when TX-114 was lower than 0.0005%. In the tryptic enolase with 0.005% TX-114, the peptide signals were only 35-72% of those in the surfactant free sample. This was due to the ionization suppression of peptides by the coeluted TX-114. There were three major elution time windows in the mass chromatogram in which a significant TX-114 spectrum could be observed (Supporting Information Figure S1). The first elution time window was overlapped with the elution times of tryptic enolase, in which the peptide signal was suppressed by the coeluted TX-114. The second and third windows were located in the high organic gradient (≥95% ACN) due to the tight binding of TX-114 . The tight binding of TX-114 may reduce the trapping capacity and efficiency of the LC column. In contrast to the TX-114, SDS had a more significant impact on the separation retention time, resolution and sensitivity . As shown in Table 2, for the 0.005% SDS tryptic enolase, the

retention times were 5-10 min longer and the chromatographic peak width was about 3-fold wider than the surfactant free enolase. For the tryptic enolase with 0.001% SDS, the retention times were 1-4 min longer the peptides signals were only 4% of the surfactant free sample. However, for the enolase with 0.0002% SDS, both of the retention time delay and peak broadening became insignificant and the peak intensity was 38%-73% of the surfactant free enolase.

In general, the performance of LC separation and the MS signal can be affected by the existence of surfactant. This phenomenon occurs because surfactants have a high capability to interact with proteins and peptides, as well as the stationary phase.[19] This interaction will produce more hydrophobic and overall similar polarity species during the chromatographic separation and thus result in retention time delay, poor resolution and sensitivity. Poor reproducible retention time and sensitivity can make label-free quantification more of a challenge. Due to the tight binding between surfactants and the stationary phase, the LC column usually needs to be washed for a long period of time or replaced to eliminate the interference of surfactants.

Development of the VISA for Detection of TX-114

In this study, a new protocol, VISA, was developed for the detection of TX-114 residue. Because of the low cloud point, TX-114 can easily aggregate to form a pellet after a brief centrifuge at room temperature. The TX-114 aggregated pellet can be used as an indication of the TX-114 residue. However, as TX-114 is colorless, the small surfactant pellet produced at low TX-114 concentrations cannot be easily observed. The lowest concentration of TX-114 necessary for the visual detection of the TX-114 pellet was found to be ~0.02%. Therefore, Coomassie Blue

was added, thus staining the TX-114 pellet and allowing it to be easily observed (Figure 1A). As Coomassie Blue is hydrophobic it can be partitioned into the surfactant pellet. As shown in Figure 1B, with the addition of Coomassie Blue, the detection limit of TX-114 can be improved from 0.02% to 0.005%. This sensitivity is similar to the analysis of TX-series compounds using CEC-ESI-MS.[26]. With the use of VISA, the major challenge to further improving the detection limit of TX-114 is that the cloud point significantly increases with the decrease of the TX-114 concentration; this results in difficulty in obtaining pellet aggregation at room temperature. Because the cloud point is affected by ionic strength,[39] several ABC concentrations were added into the TX-114 solution to decrease the cloud point at low TX-114 concentrations. With the addition of 200 mM ABC buffer to the solution, the detection limit of TX-114 can be improved from 0.005% to 0.0005% (Figure 1C). Because the size of the blue pellet is directly proportional to TX-114 quantity, it is also possible to evaluate the quantity of TX-114 directly by the pellet size.

As outlined above, the VISA uses high ionic strength to facilitate the detergent aggregation by lowering the cloud point, and hydrophobic dye to visualize the aggregated surfactant to evaluate the quantity of TX-114 washed out during the gel wash step of the tube-gel protocol. As shown in Figure 2, gel pieces were washed with 50% ACN/H_2O. After the gel wash, the final wash solution (1 ml) was transferred to a new vial and dried by the centrifugal concentration. Then the $VISA_{114}$ reagent, which contained Coomassie Blue and 200 mM ABC, was added to the vial. If a blue pellet is detected after the addition of $VISA_{114}$ reagent and brief centrifugation, it indicates that the TX-114 concentration is higher than 0.0005%, and the gel pieces should be washed more extensively (as shown in Figure 2A). For the

aqueous wash solution with 200 mM ABC, the $VISA_{114}$ can be directly used and the time for total analysis can be as little as 5 min.

Evaluation of TX-114 Removal Efficiency Using Tube-Gel

To optimize the tube-gel protocol for the TX-114 extracted membrane protein, the VISA protocol was applied. Four vials of 50 µl TX-114 pellet were polymerized with polyacryamide to produce four 70 µl tube-gels. The four gels were sliced into 32, 16, 8 and 4 pieces respectively and washed with 25 mM ABC buffer (4^oC) for 6 min. Examination of the first wash solution using VISA showed that the gel cut into 32 pieces had the best TX-114 removal efficiency with ~40% of the TX-114 extracted into the wash solution. (Figure 3A). The TX-114 removal efficiency of ACN/H_2O (50/50) was significantly better than that of the aqueous 25 mM ABC buffer since 70% of TX-114 was extracted into the wash solution with the gel cut into 32 pieces (Figure 3B).

Although a smaller gel size proved more efficient for surfactant removal, the sample loss can be more significant during the gel wash. To evaluate the sample loss during the wash steps, equal quantities of Jurkat cell lysates were incorporated into four tube-gels and they were sliced into 4, 8, 16 and 32 pieces, respectively. After washing the gel with ACN/H_2O (50/50) four times, the gel pieces were further ground into smaller particles in a microcentrifuge tube and mixed with the SDS-PAGE sample buffer (250 mM Tris-base, 5% SDS, 50% glycerol, 5% ß-mercaptoethnol and 0.02% bromophenol blue) and loaded into the SDS-PAGE. As shown in Figure 4, the 32 gel pieces had the lowest protein recovery. To

minimize sample loss without compromising wash efficiency, for a 70 μl tube gel, cutting into 16 slices is recommended.

Application of a Combination of Tube- Gel and $VISA_{114}$ to Extracted Spermatozoa Membrane Protein

TX-114 phase partition is considered an efficient method for the extraction of spermatozoa membrane proteins with high specificity and recovery. First the tube-gel protocol was used to remove the excess TX-114 before performing the tryptic digestion. Briefly, the TX-114 extracted epidermal mouse sperm membrane proteins were fixed in polyacryamide gel and then the gel was sliced in 16 pieces and washed with 50% ACN/H_2O solution. To understand how extensive the gel wash applied before performing the tryptic digestion should be, the VISA was used to monitor the surfactant residue in the tube-gel protocol. As shown Figure 5A, most of the TX-114 in the tube-gel was washed out of the gel in the first wash. After the third wash, a TX-114 pellet was observed after the addition of $VISA_{114}$ reagent. This indicated that an additional wash was required to remove the TX-114 more completely from the gel. After the fourth wash with 50% ACN/H_2O solution, no pellet was observed in the VISA test, which indicated that the surfactant concentration was lower than 0.0005%. The extracted proteins in the tube-gel were further digested and analyzed by the LC-MS/MS. The LC-MS/MS result was further searched against the mouse database from the International Protein Index consortium (database version 3.48, 55308 entries).[23] In this study, the result showed that 252 proteins were identified by MASCOT with $P < 0.05$ (Supporting Information Table S1) and the false discovery rate (FDR) evaluated by the decoy database was 4.10%. About 78% and 52% of the identified proteins were classified as membrane associated

and integral membrane proteins, respectively, using gene ontology (GO) consortium (Figure 5B). This experiment demonstrated that the use of VISA can help to optimize the surfactant removal efficiency in the tube-gel protocol. It was concluded that in addition to the original tube-gel protocol using three replicated washes,[18] additional wash step is required to remove the TX-114 more completely.

Detection of SDS Using VISA Protocol

In addition to the analysis of TX-114, the VISA is also capable of detecting other surfactants**.** Currently, the most common surfactant used for the dissolution and denaturing of proteins is SDS. Unlike TX-114, SDS cannot easily aggregate in solution. Thus, it is not possible to monitor it directly by the formation of the SDS pellet. Because the cloud point of TX-114 is elevated by the presence of SDS, the TX-114 pellet can be "dissolved" by the addition of SDS. As shown in Figure 6A, the stained pellet of 0.01% TX-114 was dissolved when the concentration of SDS was higher than 0.0002%, which is the detection limit for SDS analysis. Based on this strategy, a more generic VISA approach can be used for the detection of SDS or other surfactants. We have found that the minimum SDS concentration required to dissolve the TX-114 pellet is dependent on the concentration needed for the formation of TX-114 pellet. In Figure 6B, a linear correlation (r^2 = 0.9565) was observed between the TX-114 concentration and the minimum SDS concentration which can dissolve the TX-114 pellet completely. As illustrated in Figure 2B, the VISA can be applied to the tube-gel or in-solution digestion when the SDS was used to dissolve the protein.

For the in-solution digestion, although there are several approaches avoiding use of strong surfactant such as SDS during the digestion, however, these method might not suitable for large scale protein identification especially when highly complex protein mixture such as whole cell lysates are applied.[40] Because the strong surfactants are commonly used for prepare total lysate or dissolution of membrane protein. These surfactants must be removed by C18, IEX, or precipitation before the protein identification or derivatization process such as methylation.[40] In this study, the VISA was applied to monitor the surfactant removal efficiency using acetone precipitation. The total protein in Jurkat cell was extracted by 0.1% SDS in 50 mM HEPS buffer (pH=8.3). After reduction and alkylation reaction, the 100 μg total protein was precipitated by addition of 10-fold volume -20^{o}C acetone followed by cold acetone washes. As shown in Figure 7, significant SDS residue in the supernatant and the first wash solution can be detected by the VISA. After second cold acetone washes, no surfactant residue was detected by the VISA, which indicated that the surfactant concentration was lower than the 0.0002%. With the use of two cold acetone washes, the protein pellet was dissolved in TEABC followed by tryptic digestion for 16 hours. After 16 hours, the reduction and alkylation reactions were performed and the more trypsin was added for the complete digestion. The digested peptide was analyzed by LC-MS. About 372 proteins can be identified by MASCOT with the significant threshold $P < 0.05$ (Supporting Information Table S2). In addition, no significant SDS signal and peptide chromatographic peak broadening was observed, which suggest the SDS was removed by the acetone precipitation.

CONCLUSIONS

The VISA approach was developed to detect surfactant residue in proteomic samples. For MALDI-MS analysis, the tolerance of TX-114 and SDS was determined to be 0.001% and 0.005%, respectively. However, for LC-MS analysis, the tolerance of TX-114 and SDS was 0.001% and 0.0002%. The detection limits of VISA for TX-114 were 0.0005% and SDS were 0.0002% which was approximately equal to the tolerance for MS analysis. For monitoring TX-114 residue during the tube-gel protocol, the removal efficiency using 25 mM ABC buffer was shown to be gel-size dependent. The use of ACN/H_2O has better wash efficiency for TX-114. Protein recovery was also found to be gel-sized dependent, thus a compromised gel size and wash procedure can be determined with the assistance of VISA. VISA cannot only detect TX-114 and SDS but has the potential to detect other surfactants. Moreover, this method can also be applied to other surfactant-removed methods, such as organic solvent precipitation .

ACKNOWLEDGMENT

This work was financially supported by the National Council of Science.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Figure Legends

Table 1: Peptide mass fingerprinting analysis of tryptic enolase in the presence of different concentrations of TX-114 and SDS

Table 2: LC-MS/MS analysis of tryptic enolase in the presence of different concentrations of TX-114 and SDS Three enolase peptides were selected to evaluate the effect of the surfactant on the chromatographic retention time, peak height and peak width.

Figure 1. Observation of surfactant pellet formation for (A) 0.025% TX-114 in 25 mM ABC and 25 mM ABC with Coomassie Blue (B) different concentrations of TX-114 ranging from 0.1% to 0.005% with Coomassie Blue in 25 mM ABC (C) 0.0005% TX-114 with Coomassie Blue in 25 mM ABC and 200 mM ABC. The experiment was performed at room temperature (25°C).

Figure 2. Schemes of visual indicators for surfactant abundance (VISA) for the detection of (A) TX-114 (B) SDS.

Figure 3. VISA test for TX-114 extraction efficiency in the first wash solution. The 70 μL tube-gels were sliced in to 32, 16, 8 and 4 pieces, washed with (A) 25 mM ABC (B) 50% ACN and tested by the VISA test.

Figure 4. Effect of gel slice number on protein recovery. Four 70 μL tube-gel samples containing about 5 μg Jurkat membrane protein were sliced into 4,

8, 16 and 32 pieces respectively and each of the samples was washed with 50% ACN four times. After the gel wash, the gel pieces from each of the samples were ground and loaded into the SDS-PAGE.

Figure 5. Analysis of TX-114 extracted mouse spermatozoa membrane protein using tube-gel protocol. Spermatozoa membrane proteins were extracted from 50 μg using 1.4% TX-114 and embedded into the tube-gel. (A) The VISA was applied to monitor the TX-114 washed out from four replicated 50% ACN washes. (B) The proteins identifies were categorized according to their subcellular localization using geneontology (GO).

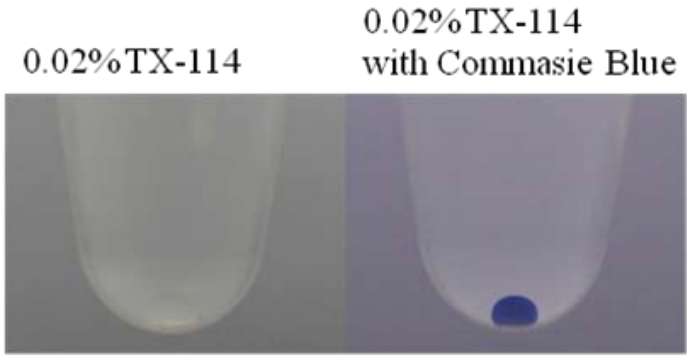
Figure 6. Detection of SDS using VISA. (A) A pellet of 0.01% TX-114 was dissolved with 0.0002% SDS. (B) Correlation of the TX-114 concentration and SDS concentration required to completely dissolve the TX-114 pellet.

Figure 7. Monitoring of the SDS residue during acetone precipitation. The test was performed by comparing the (A) VISA reagent only with the addition of VISA reagent to the (B) precipitation supernatant and (C) wash solutions. The acetone was evaporated before performing the VISA test.

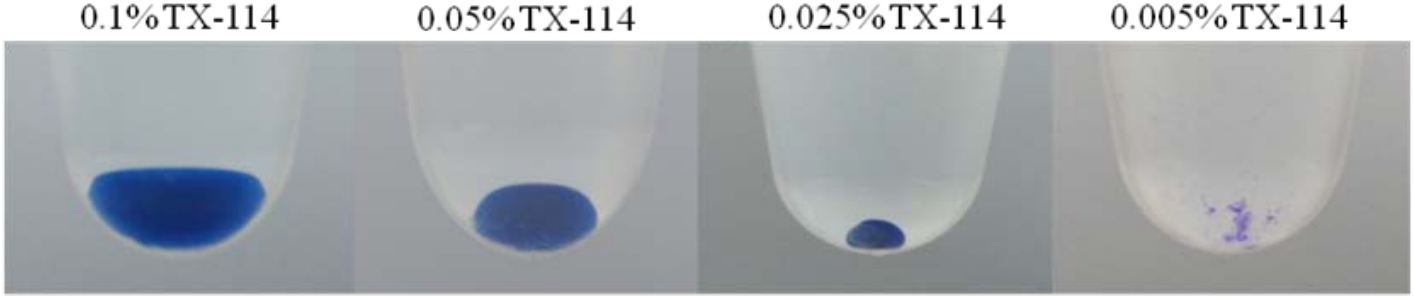
	Concentration	Score	Sequence Coverage
no surfactant	0%	87	45%
TX-114	0.1%	n.d.	n.d.
	0.01%	n.d.	n.d.
	0.005%	n.d.	n.d.
	0.001%	70	39%
	0.0005%	72	45%
	0.0002%	75	49%
SDS	0.1%	n.d.	n.d.
	0.01%	n.d.	n.d.
	0.005%	70	43%
	0.001%	72	48%
	0.0005%	74	47%
	0.0002%	78	49%

			Mass Chromatography		
		Peptide (m/z)	Retention Time	Peak Height	Peak Width
No Surfactant	0%	614.31	16.55	304	0.09
		789.91	21.74	396	0.12
		878.48	19.37	397	0.10
TX-114	0.005%	614.31	16.74	105	0.13
		789.91	21.79	286	0.16
		878.48	19.50	220	0.14
	0.001%	614.31	16.70	269	0.10
		789.91	21.77	352	0.13
		878.48	19.48	374	0.10
	0.0005%	614.31	16.72	293	0.09
		789.91	21.76	342	0.13
		878.48	19.47	364	0.10
	0.0002%	614.31	16.68	380	0.09
		789.91	21.75	378	0.12
		878.48	19.39	386	0.09
SDS	0.005%	614.31	27.04	11	0.28
		789.91	27.33	11	0.40
		878.48	25.87	7	0.39
	0.001%	614.31	20.80	12	0.94
		789.91	22.93	9	0.60
		878.48	20.93	8	0.78
	0.0005%	614.31	18.14	29	1.23
		789.91	22.09	135	0.33
		878.48	19.72	111	0.35
	0.0002%	614.31	16.71	117	0.11
		789.91	21.87	279	0.12
		878.48	19.55	292	0.09

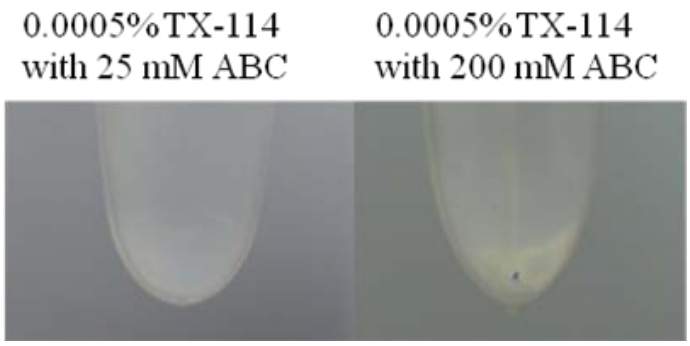
(A)

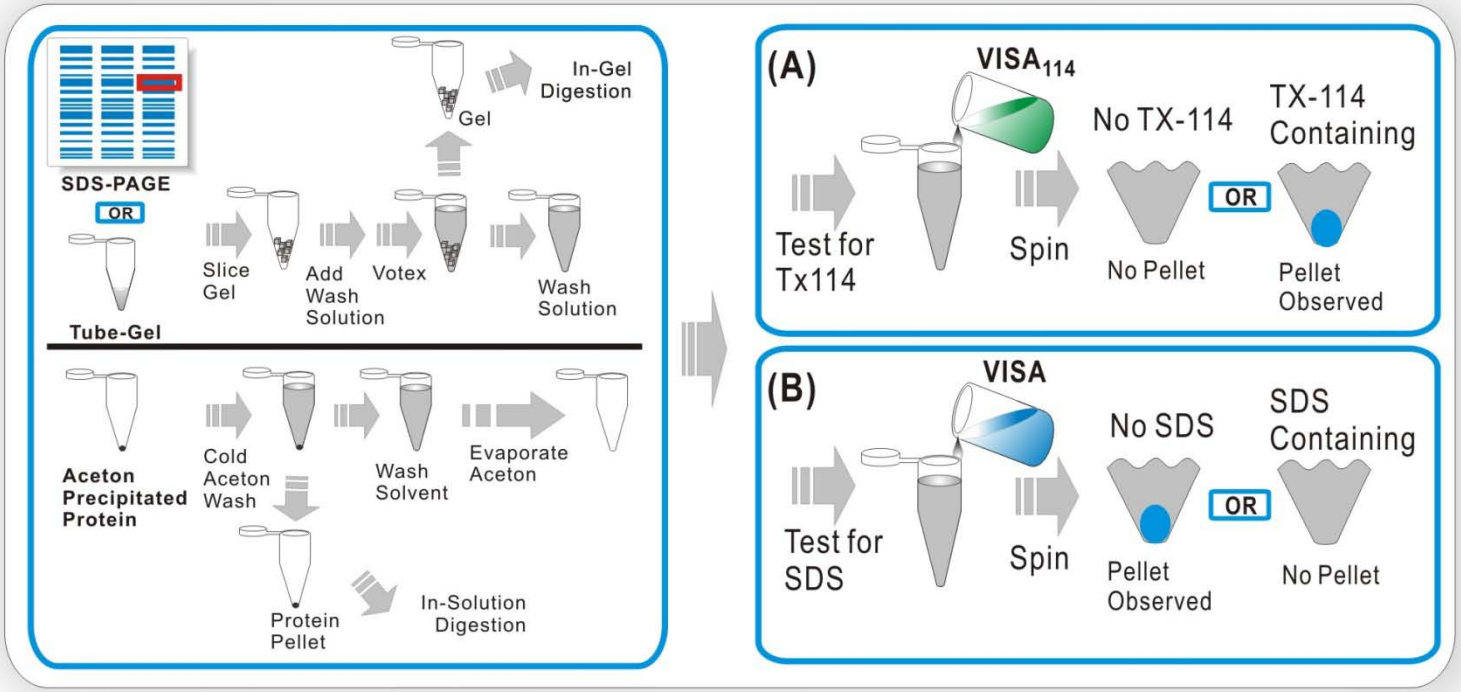

0.02%TX-114
0.02%TX-114
with Commasie Blue

(B)

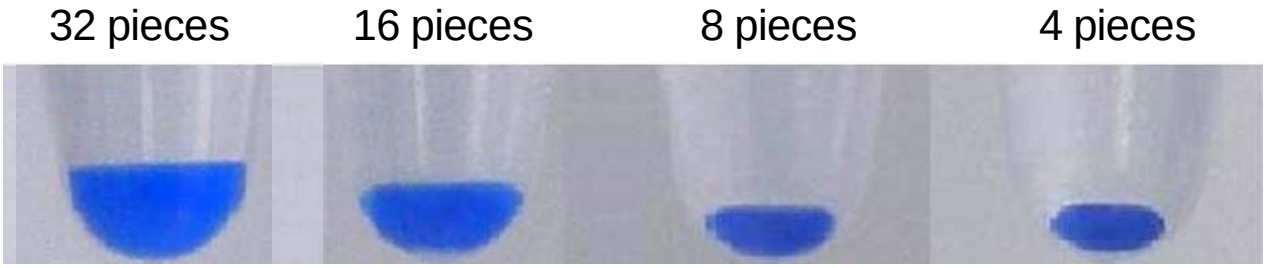

0.1%TX-114
0.05%TX-114
0.025%TX-114
0.005%TX-114

(C)

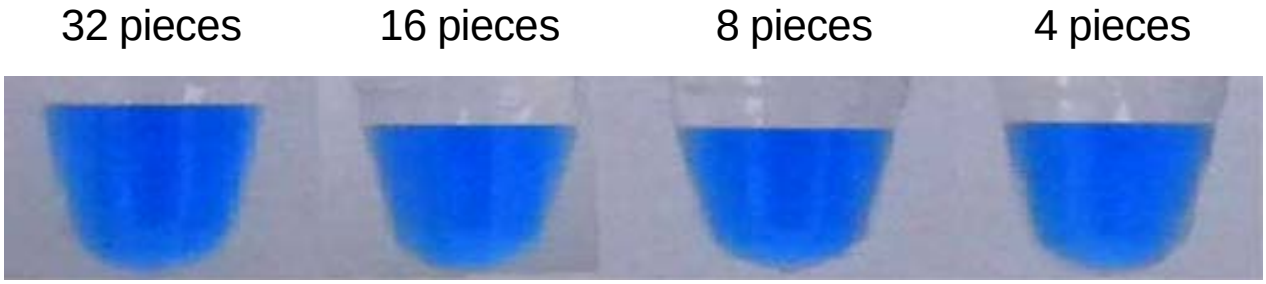

0.0005%TX-114
with 25 mM ABC
0.0005%TX-114
with 200 mM ABC

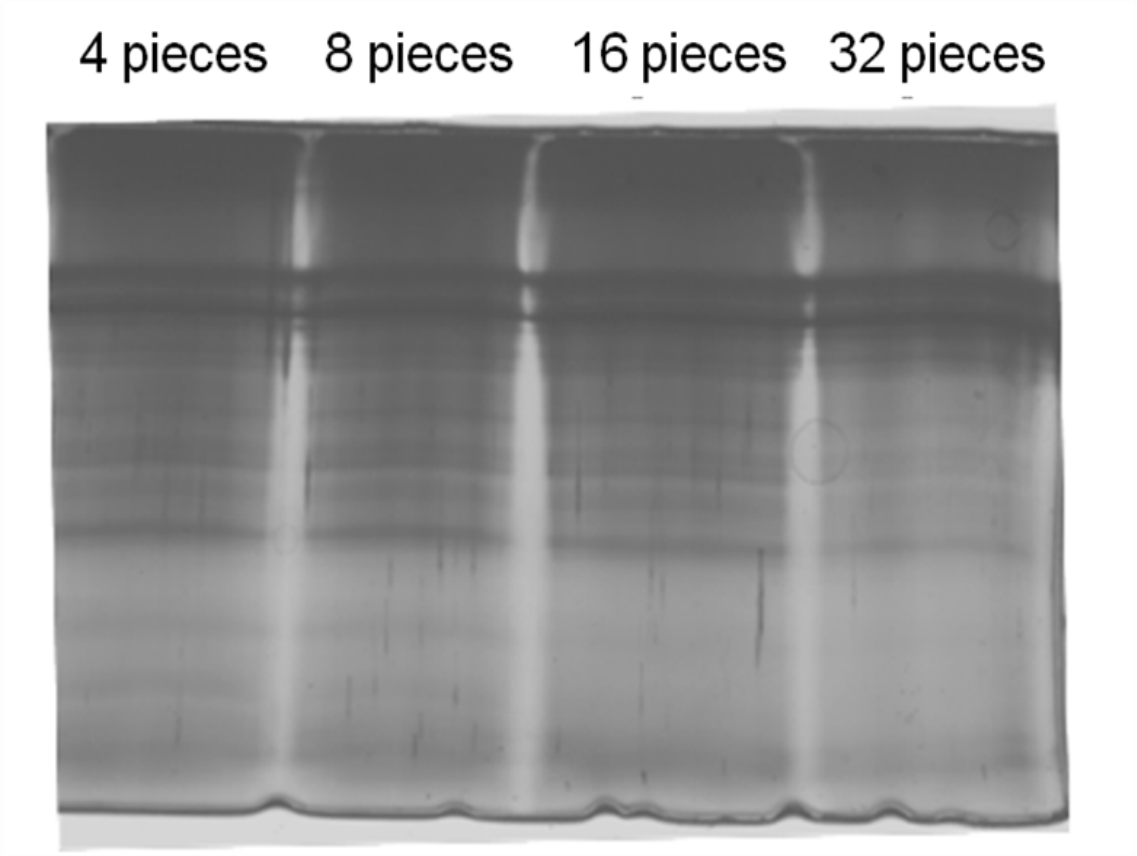

SDS-PAGE
OR
Tube-Gel
Gel
In-Gel Digestion
Slice Gel
Add Wash Solution
Votex
Wash Solution
Aceton Precipitated Protein
Cold Aceton Wash
Wash Solvent
Evaporate Aceton
Protein Pellet
In-Solution Digestion
(A)
VISA114
Test for Tx114
Spin
No TX-114
No Pellet
OR
TX-114 Containing
Pellet Observed
(B)
VISA
Test for SDS
Spin
No SDS
Pellet Observed
OR
SDS Containing
No Pellet

(A)

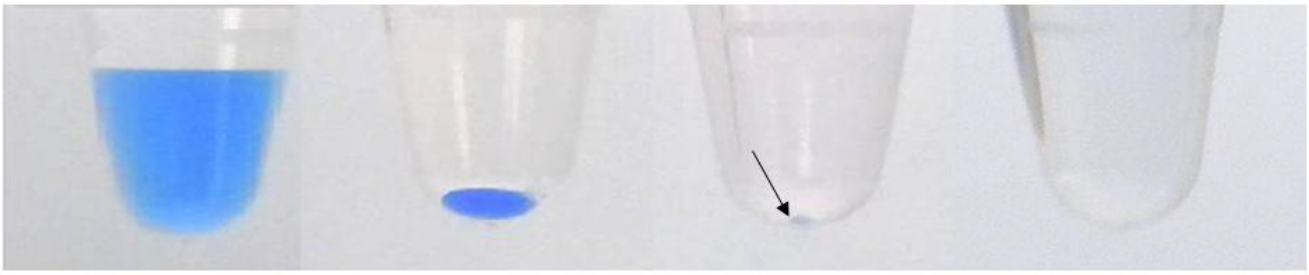

32 pieces
16 pieces
8 pieces
4 pieces

(B)


32 pieces
16 pieces
8 pieces
4 pieces

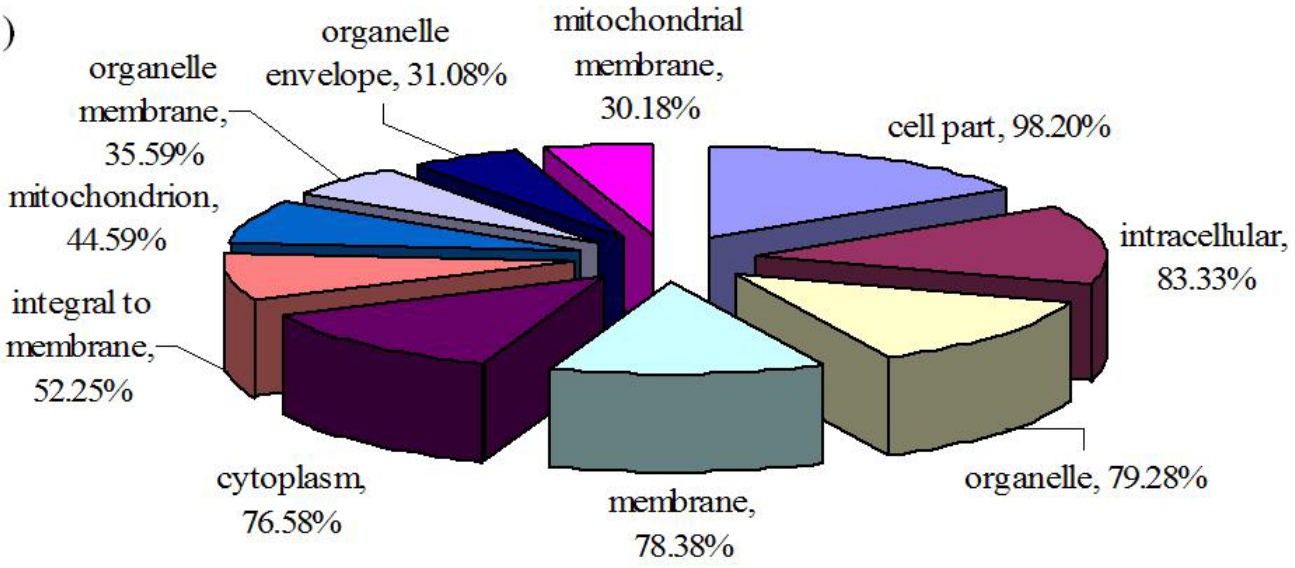

4 pieces
8 pieces
16 pieces
32 pieces

(A)

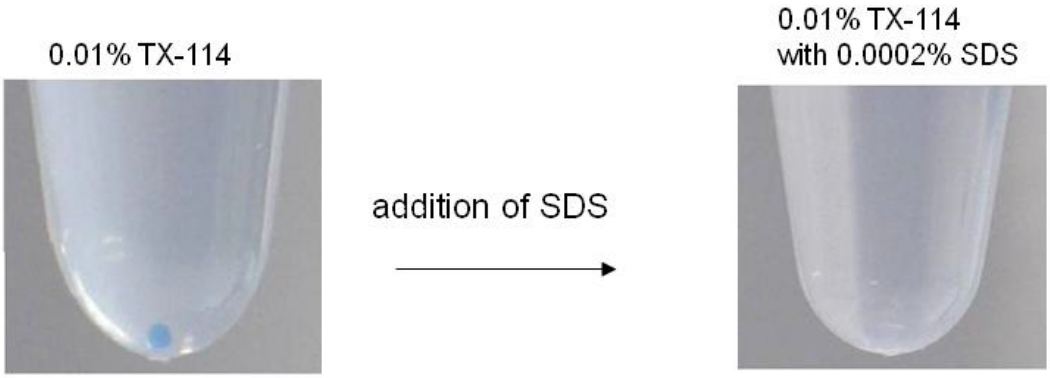


1st wash with ACN/H_2O | 2nd wash with ACN/H_2O | 3rd wash with ACN/H_2O | 4th wash with ACN/H_2O

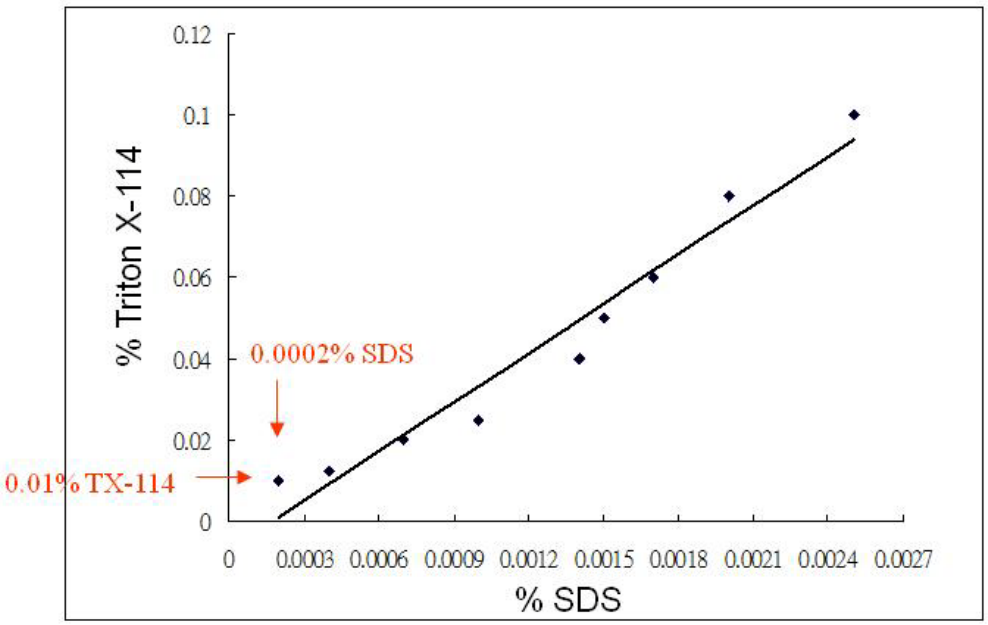
(B)



(A)


0.01% TX-114
addition of SDS
0.01% TX-114
with 0.0002% SDS

(B)


0.12
0.1
0.08
0.06
0.04
0.02
0
% Triton X-114
0.0002% SDS
0.01% TX-114
0
0.0003
0.0006
0.0009
0.0012
0.0015
0.0018
0.0021
0.0024
0.0027
% SDS

(A)

VISA Solution
(No Surfactant)

(B)

Precipitation
Supernatant

(C)

1st Cold
Acetone Wash
Supernatant

2nd Cold
Acetone Wash
Supernatant

REFERENCES

(1) Bordier, C. *J. Biol. Chem.* **1981**, *256*, 1604-1607.

(2) Almgren, M. *Biochim. Biophys. Acta* **2000**, *1508*, 146-163.

(3) Garavito, R. M.; Ferguson-Miller, S. *J. Biol. Chem.* **2001**, *276*, 32403-32406.

(4) Meredith, J. E., Jr.; Schwartz, M. A. *Trends Cell Biol* **1997**, *7*, 146-150.

(5) Martin, K. H.; Slack, J. K.; Boerner, S. A.; Martin, C. C.; Parsons, J. T. *Science* **2002**, *296*, 1652-1653.

(6) Baruthio, F.; Quadroni, M.; Ruegg, C.; Mariotti, A. *Proteomics* **2008**, *8*, 4733-4747.

(7) Kreir, M.; Farre, C.; Beckler, M.; George, M.; Fertig, N. *Lab. Chip.* **2008**, *8*, 587-595.

(8) Zhang, X.; Scalf, M.; Westphall, M. S.; Smith, L. M. *Anal. Chem.* **2008**, *80*, 2590-2598.

(9) Behan, A. T.; Byrne, C.; Dunn, M. J.; Cagney, G.; Cotter, D. R. *Mol. Psychiatry* **2009**, *14*, 601-613.

(10) Kittanakom, S.; Chuk, M.; Wong, V.; Snyder, J.; Edmonds, D.; Lydakis, A.; Zhang, Z.; Auerbach, D.; Stagljar, I. *Methods. Mol. Biol.* **2009**, *548*, 247-271.

(11) Liang, B.; Arora, A.; Tamm, L. K. *Biochim. Biophys. Acta* **2009,** *1798*, 68-76.

(12) Taylor, J. D.; Linman, M. J.; Wilkop, T.; Cheng, Q. *Anal. Chem.* **2009**, *81*, 1146-1153.

(13) Wang, Q.; He, J.; Meng, L.; Liu, Y.; Pu, H.; Ji, J. *J. Proteom. Res.* **2010,** *9*, 22-29.

(14) Redeby, T.; Emmer, A. *Anal. Bioanal. Chem.* **2005**, *381*, 225-232.

(15) Carlsohn, E.; Nystrom, J.; Karlsson, H.; Svennerholm, A. M.; Nilsson, C. L. *J. Proteome. Res.* **2006**, *5*, 3197-3204.

(16) Zhang, N.; Chen, R.; Young, N.; Wishart, D.; Winter, P.; Weiner, J. H.; Li, L. *Proteomics* **2007**, *7*, 484-493.

(17) Lee, H. J.; Kwon, M. S.; Lee, E. Y.; Cho, S. Y.; Paik, Y. K. *Proteomics* **2008**, *8*, 2168-2177.

(18) Lu, X.; Zhu, H. *Mol. Cell Proteomics* **2005**, *4*, 1948-1958.

(19) Vissers, J. P.; Chervet, J. P.; Salzmann, J. P. *J. Mass Spectrom.* **1996**, *31*, 1021-1027.

(20) Loo, R. R.; Dales, N.; Andrews, P. C. *Protein Sci.* **1994**, *3*, 1975-1983.

(21) Zhang, N.; Li, L. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 889-896.

(22) Han, P.; Chen, C. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 1137-1145.
(23) Nagaraj, N.; Lu, A.; Mann, M.; Wisniewski, J. R. *J. Proteom. Res.* **2008**, *7*, 5028-5032.
(24) Maja, P.; Ann, W.; Kaj, B.; Pia, D. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 344-349.
(25) Moody, C. A.; Kwan, W. C.; Martin, J. W.; Muir, D. C. G.; Mabury, S. A. *Anal. Chem.* **2001**, *73*, 2200-2206.
(26) Norton, D.; Shamsi, S. A. *Anal. Chem.* **2007**, *79*, 9459-9470.
(27) Li, X.; Brownawell, B. J. *Anal. Chem.* **2009**, *81*, 7926-7935.
(28) Huang, Y. H.; Chu, S. T.; Chen, Y. H. *Biol. Reprod.* **2000,** *63*, 1562-1566.
(29) Kittelberger, R.; Hansen, M. F.; Hilbink, F.; de Lisle, G. W.; Cloeckaert, A. *J. Microbiol. Methods* **1995**, *24*, 81-92.
(30) Lee, R. P.; Doughty, S. W.; Ashman, K.; Walker, J. *J. Chromatogr. A* **1996**, *737*, 273-279.
(31) Kaczmarski, W.; Wisniewski, K. E.; Golabek, A.; Kaczmarski, A.; Kida, E.; Michalewski, M. *Mol. Genet. Metab.* **1999**, *66*, 261-264.
(32) Sanchez-Ferrer, A.; Perez-Gilabert, M.; Nunez, E.; Bru, R.; Garcia-Carmona, F. *J. Chromatogr. A* **1994**, *668*, 75-83.
(33) Molloy, M. P.; Herbert, B. R.; Walsh, B. J.; Tyler, M. I.; Traini, M.; Sanchez, J. C.; Hochstrasser, D. F.; Williams, K. L.; Gooley, A. A. *Electrophoresis* **1998**, *19*, 837-44.
(34) Cordwell, S. J.; Nouwens, A. S.; Verrills, N. M.; Basseal, D. J.; Walsh, B. J. *Electrophoresis* **2000**, *21*, 1094-1103.
(35) Fountoulakis, M.; Takacs, B. *Electrophoresis* **2001**, *22*, 1593-1602.
(36) Banerjee, P.; Dasgupta, A.; Chromy, B. A.; Dawson, G. *Arch. Biochem. Biophys.* **1993**, *305*, 68-77.
(37) Love, L. J. C.; Habarta, J. G.; Dorsey, J. G. *Anal. Chem.* **1984**, *56*, 1132A-1148A.
(38) Amado, F. M. L.; Santana-Marques, M. G.; Ferrer-Correia, A. J.; Tomer, K. B. *Anal. Chem.* **1997**, *69*, 1102-1106.
(39) Gu, T.; Galera-Gomez, P. A. *Colloids and Surf. A: Physicochem. Eng. Aspects* **1995**, *104*, 307-312.
(40) Kim, S. C.; Chen, Y.; Mirza, S.; Xu, Y.; Lee, J.; Liu, P.; Zhao, Y. *J. Proteom. Res.* **2006**, *5*, 3446-3452.