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Comparison of protein precipitation methods for sample preparation prior to proteomic analysis of Chinese hamster ovary cell homogenates

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ABSTRACT

Background: Chinese hamster ovary (CHO) cells are the workhorse for obtaining recombinant proteins. Proteomic studies of these cells intend to understand cell biology and obtain more productive and robust cell lines for therapeutic protein production in the pharmaceutical industry. Because of the great importance of precipitation methods for the processing of samples in proteomics, the acetone, methanol-chloroform (M/C), and trichloroacetic acid (TCA)-acetone protocols were compared for CHO cells in terms of protein recovery, band pattern resolution, and presence on SDS-PAGE.
Results: Higher recovery and similar band profile with cellular homogenates were obtained using acetone precipitation with ultrasonic bath cycles (104.18 ± 2.67%) or NaOH addition (103.12 ± 5.74%), compared to the other two protocols tested. TCA-acetone precipitates were difficult to solubilize, which negatively influenced recovery percentage (77.91 ± 8.79%) and band presence. M/C with ultrasonic homogenization showed an intermediate recovery between the other two protocols (94.22 ± 4.86%) without affecting protein pattern on SDS-PAGE. These precipitation methods affected the recovery of low MW proteins (<15 kDa).
Conclusions: These results help in the processing of samples of CHO cells for their proteomic study by means of an easily accessible, fast protocol, with an almost complete recovery of cellular proteins and the capture of the original complexity of the cellular composition. Acetone protocol could be incorporated to sample-preparation workflows in a straightforward manner and can probably be applied to other mammalian cell lines as well.
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1. Introduction

Chinese hamster ovary (CHO) cells have been studied extensively due to their capacity to produce large amounts of recombinant proteins (RP) with the desired quality, both at laboratory and industrial scale [1,2,3,4,5]. This mammalian cell line offers several advantages over other expression systems in terms of cell culture, safety, productivity, product quality, and protein purification [6,7,8], which makes these cells an ideal platform for investigation of new proteins and production of those that have already been tested for clinical applications.

Despite the success of RP in many indications, their high costs limit the global access to this market [9,10,11]. The high costs are caused in part by the expression of many biopharmaceuticals in mammalian cells, regulatory approval for intended use, and high doses needed to achieve clinical efficacy [12,13]. In this scenario, government and private healthcare providers are reluctant to fund very expensive drug treatments. Thus, investments in strategies that increase product titer are strongly encouraged. In this sense, cell engineering of CHO cells, based on omics studies (transcriptomic, proteomic, metabolomic, and fluxomic) of cell populations subjected to different stimulus [14,15,16,17] or culture conditions [18,19,20,21], or with different intrinsic properties [4,22,23,24,25], plays an essential role. These studies, which can be reviewed elsewhere [26,27,28], are important in providing valuable targets for successful cell engineering and highlight the paramount importance of studying CHO cells through samples obtained from their homogenates.

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Although the high biotechnological value of CHO cells has largely driven their cell biology research, more studies are required to obtain insights of molecular functions that contribute to the development of more productive phenotypes. In this sense, proteomics research can provide a large amount of useful information to test new hypotheses in this area.

Throughout proteomic experimental design, sample preparation is a critical step to obtain successful and significant results. Protein precipitation is an essential part of sample preparation and has been widely incorporated into common protocols due to the important advantages it confers [29,30,31,32,33,34]. Precipitation methods have been developed and improved over the years and consist of the use of organic solvents, salts, divalent cations, complex acids, and chemico-physical properties such as pH and temperature, which are usually combined in favor of a higher efficiency [30,31,34,35,36,37]. Methanol-chloroform (M/C), acetone, and trichloroacetic acid (TCA) are among the precipitation methods most commonly used for proteomic purposes. Nevertheless, these methods should be applied to each new sample in order to choose the most appropriate one in each case, because some of their specific advantages depend on the nature of the sample, technical settings, and improvements incorporated into protocols [38,39,40,41,42,43]. Plant seeds [39], animal tissues [38,40,43], bacteria homogenates [44], human plasma [41,42], and CHO cell culture supernatants [45] are some of the samples where these common precipitation protocols have been compared. However, to the best of our knowledge, no study has previously reported the application of different precipitation protocols to the complex mixture of proteins that is the homogenate of CHO cells. Therefore, the goal of the present study is to evaluate and select the best precipitation method for CHO cell homogenates among M/C, acetone, and TCA-acetone protocols through the analysis of their performance in protein recovery and band pattern presence on SDS-PAGE. The evaluation of the percentage of protein recovered after precipitation allows us to know the minimum amount of sample to carry out a proteomic analysis and to select the highest yield method. The visualization of the universe of recovered proteins, by one-dimensional electrophoresis, allows us to know whether the methods under study precipitate a set of proteins that constitute a representative sample of the entire cell. Therefore, suitable methods are those with the highest recovery efficiency, as well as those with an SDS-PAGE banding pattern similar to that of the initial homogenate, which could be a measurable profile of the recovered proteins. Incorporation of well-characterized and well-performing methods to protein precipitation during sample processing of CHO cell homogenates for proteomics will ensure obtaining high quality results and the conception of a new hypothesis that will impact the biotechnology of the coming years.

2. Materials and methods

2.1. Cell line and culture conditions

Cell line CHO DP-12 clone #1933 ATCC® CRL-12444™ (US6025158A, 2000) which secretes a humanized monoclonal antibody against human interleukin 8 was used throughout the study. CHO cells were gradually adapted and cultured in CDM4CHO cell culture medium (Hyclone, Logan, UT, USA) and supplemented with 6 mM stable glutamine (Biowest LLC, Kansas City, MO, USA), 0.002 mg/ml isophane insulin (Humulin N, Eli Lilly, Indianapolis, IN, USA), and 200 nM methotrexate (Pfizer, New York, NY, USA) at 37°C in a 5% CO_2 atmosphere in a humidified incubator.

Inoculum for spinner flasks was expanded in 75 cm^2 T-flasks at an orbital agitation of 60 rpm (Bellco Glass, Vineland, N.J, USA). Cells were seeded at 0.25×10^6 cells/ml in duplicate in spinner flasks and agitated at 90 rpm in a magnetic stirrer (Equipar, Mexico City, Mexico). Cell concentration and viability were recorded every 24 h by cell counting in a Neubauer chamber using the trypan blue dye exclusion method.

2.2. Extraction of cellular proteins

Cells were collected in exponential phase, centrifuged at $185 \times g$ for 5 min at room temperature, and washed twice in phosphate buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Cellular proteins were extracted by solubilization of pellet at a concentration of 3.0×10^7 cells/ml in isoelectric focusing (IEF) buffer (7 M urea, 2 M thiourea, 2% [w/v] 3-[3-Cholamidopropyl dimethylammonio]-1-propanesulfonate hydrate [CHAPS], and 40 mM dithiothreitol [DTT]), supplemented with 10% (v/v) SigmaFast Protease Inhibitor Cocktail (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), prepared according the manufacturer's recommendations. Cell lysate was sonicated twice for 1 min at an amplitude of 10 μm (Soniprep 150, MSE, Heathfield, East Sussex, UK) in ice to reduce the viscosity of the sample, followed by a centrifugation at $16,000 \times g$ for 25 min at 4°C. Supernatant was stored at −20°C until its use.

2.3. Protein precipitation and recovery methods

M/C, acetone, and TCA-acetone precipitation methods, with a set of modifications, were evaluated to determine the recovery percentage and band pattern on SDS-PAGE of CHO cell homogenates. Protein solutions were prepared by diluting the clarified cell lysate in 10 mM HEPES buffer, pH 7.4, supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA) and 1.31 M sucrose, at a final protein concentration of 0.2 mg/ml. Protein precipitates were recovered by solubilization in IEF buffer, except in cases where co-solubilization agents were employed. Precipitation methods, their abbreviations and introduced modifications with respect to the reference method, all variants employed to improve protein recovery, and experimental measures to assess their performance were summarized in Table 1. All conditions were evaluated in three independent experiments, where two of them corresponded to one cell culture and the third to the other culture.

M/C precipitation was carried out as published before [34]. For TCA-acetone, a volume of 30% [w/v] TCA in acetone was added to the protein solution, mixed, and incubated at −20°C overnight. Protein pellet, obtained by centrifugation at $16,000 \times g$ for 25 min, was washed three times with 80% [v/v] acetone [46]. For acetone precipitation, previous recommendations were followed [47]. In brief, NaCl was added to protein solution at a final concentration of 100 mM, followed by addition of 4 volumes of 80% [v/v] acetone, mixing, and overnight incubation at −20°C. After centrifugation at $16,000 \times g$ for 25 min, protein precipitate was washed twice with 4 volumes of 80% [v/v] acetone. Protein precipitates were recovered by centrifugation and air-dried.

For M/C and acetone methods, the following modifications were introduced to increase protein recovery: 5 cycles of freeze–thaw/mix (FT), 10 ultrasound periods of 10 s each with an interval of 10 s between each period at 4°C in an ultrasonic bath (UB, 9354001, UL 1K58, Ney Ultrasonic) and 10 ultrasound periods of 10 s each with an interval of 10 s between each period, in ice, at an amplitude of 5 μm in an ultrasonic homogenizer (UH, Soniprep 150, MSE, Heathfield, East Sussex, UK). These modifications were applied in the presence or absence of a pre-solubilization step in 10% (v/v) of 0.2 M NaOH for 2 min before the addition of IEF buffer, as previously described [48]. In the special case of the TCA-acetone method, all M/C and acetone modifications were used, and FT cycles were incorporated to each because precipitates have been described as difficult to solubilize [40,42,43,48,49,50]. The use of reducing agents during protein precipitation has been explored before for plant tissues [46] to prevent the formation of disulfide bonds and therefore the generation of protein aggregates. The incorporation of these reducing agents could guarantee a greater recovery and minimize the loss of proteins with a tendency to form insoluble aggregates through covalent bonds. Thus, the efficacy of 2-mercaptoethanol (2-ME), a widely employed reducing agent, was tested by the addition of 0.07% (v/v) to TCA-

Table 1
Precipitation methods and their modifications. Evaluated protocols for protein precipitation and introduced modifications are describe below, as well as the experimental measures employed to assess their performance.

Precipitation method	Abbreviation	Modifications with respect to the reference method	Evaluated variants to improve protein recovery	Experimental measures
Methanol-chloroform[a]	M/C	None	1. S[d] 2. S + NaOH[e] 3. FT[f] 4. FT + NaOH 5. UB[g] 6. UB + NaOH 7. UH[h] 8. UH + NaOH	Protein recovery[j] - Protein recovery - SDS-PAGE[k]
Trichloroacetic acid-acetone[b]	TCA-acetone	- Final TCA concentration 15% - 2-ME not included in all conditions - TCA incubation extended to 16 h (overnight) - 80% acetone in wash solution - Solubilization solution was changed to IEF buffer to adapt the protocol for cell culture samples	1. S 2. S + NaOH 3. FT 4. FT + 2-ME[i] 5. FT + NaOH 6. FT + NaOH +2-ME 7. FT + UB 8. FT + UB + 2-ME 9. FT + UB + NaOH 10. FT + UB + NaOH + 2-ME 11. FT + UH 12. FT + UH + NaOH	Protein recovery - Protein recovery - SDS-PAGE
Acetone[c]	None	- 80% acetone in precipitants and wash solution - One additional washing step	1. S 2. S + NaOH 3. FT 4. FT + NaOH 5. UB 6. UB + NaOH 7. UH 8. UH + NaOH	Protein recovery - Protein recovery - SDS-PAGE Protein recovery

[a] Reference method: Wessel and Flügge [34].
[b] Reference method: Mechin et al. [46].
[c] Reference method: Crowell et al. [47]. All procedures include NaCl addition to protein samples at a final concentration of 100 mM before solvent precipitation.
[d] Standard protocol that refers to the original reference.
[e] Standard protocol with NaOH addition before solubilization step.
[f] Standard protocol with 5 cycles of freeze-thaw/mix.
[g] Standard protocol with 10 ultrasound periods of 10 s each with an interval of 10 s between each period, at 4°C (cold room) in an ultrasonic bath.
[h] Standard protocol with 10 ultrasound periods of 10 s each with an interval of 10 s between each period, in ice, at an amplitude of 5 μm in an ultrasonic homogenizer.
[i] FT protocol with addition of 0.07% (*v*/v) to TCA-acetone and acetone solutions.
[j] Quantification of percentage of recovered proteins by calculating protein mass after solubilization of precipitates; protein concentration was determined by Bradford method.
[k] Separation of recovered proteins by one dimensional electrophoresis to evaluate the representation of all cellular proteins in the obtained sample.

acetone and acetone solutions during four chosen conditions of TCA-acetone precipitation.

2.4. Determination of protein concentration

Protein concentration was determined by Bradford assay in 96-well microplates using Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendations. Bovine serum albumin (BSA) (GE Healthcare Bio-Sciences, USA) was used as standard in calibration curves in a concentration range of 0–0.5 mg/ml in water. Bradford compatibility was tested by comparing BSA curves diluted in water with those prepared in different dilutions of IEF buffer (1:5–1:40) and sucrose concentrations (0.43–1.75 M). Other chemicals were not tested because their final concentrations are far from the threshold values reported by Bio-Rad for this method (Bio-Rad, Hercules, CA, USA).

2.5. Protein band patterns on SDS-PAGE

To compare the protein patterns resulting from the three precipitation methods, the best condition, based on recovery percentage, was selected in each case and processed in duplicate by SDS-PAGE, with each replicate corresponding to a different cell culture [51]. Laemmli buffer [52] was added to 30 μg of total proteins at a final composition of 60 mM Tris–HCl pH 6.6 supplemented with 10% (v/v) glycerol, 70 mM sodium dodecyl sulfate (SDS), 2.5% (v/v) 2-ME, and 0.2 mM bromophenol blue. Samples were mixed, boiled to 95°C for 5 min, centrifuged at 8161 × *g* for 5 min, and applied to 12% SDS-polyacrylamide gels. PageRuler Prestained Protein Ladder (ThermoFisher Scientific) was used as a molecular weight (MW) marker. Resolution of protein mixtures was achieved in a SE260 Mighty Small II Deluxe Mini Vertical Protein Electrophoresis System (Hoefer, Holliston, USA), using Tris-Glycine pH 8.3 (25 mM Tris, 192 mM Glycine, 0.1% [w/v] SDS) as running buffer at 60 mA. Gels were washed with water and stained with Coomassie Brilliant Blue G 250 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 30 min in a shaker at room temperature [53]. Finally, gels were washed again with water and destained in 5% (v/v) methanol and 7.5% (v/v) acetic acid.

2.6. Image processing

GraphPad Prism v5.01 (GraphPad Software Inc., San Diego, CA, USA) was used for the construction of growth kinetics, Bradford assay curves, and bar plots of precipitation results. Acquisition and analysis of digital images of SDS-PAGE gels were performed in Gel Doc™ EZ imager using Image Lab software, v6.0.1 (Bio-Rad, Hercules, CA, USA). SDS-PAGE images were analyzed by densitometry using ImageJ software, v1.52a (National Institutes of Health, Bethesda, Maryland, USA).

2.7. Statistical analysis

All statistical analyses were performed in R language [54]. The Kuskal–Wallis test by ranks and post hoc Conover test with Bonferroni correction were used to detect significant statistical differences between the BSA standard curves in the Bradford assay and between the recovery percentages of the precipitation conditions [55,56]. Mann–Whitney U test was used to compare the different precipitation conditions of TCA-acetone with or without 2-ME.

3. Results

3.1. CHO cell cultures showed a growth phase during 72 h

During CHO cells cultures, growth phase was observed during the first four days (Fig. 1), which led to a maximum cell concentration of $2.42 \pm 0.16 \times 10^6$ cells/ml. Kinetic parameters such as specific growth rate (μ) and duplication time (t) were calculated as $0.036 \pm 0.03\ h^{-1}$ and 19.32 ± 1.52 h, respectively. Viability was higher than 95% from the beginning of the culture until day 5, after which both cell concentration and viability decreased with time, due to cell death.

3.2. Sucrose and IEF buffer could be used during quantification of protein recovery by Bradford assay

Bradford assay compatibility with sucrose and IEF buffer was evaluated prior to characterize precipitation methods due to conflicting evidence in literature regarding sucrose [57,58,59] and interactions between components present in 2-DE buffers [60]. Sucrose was added to cell homogenates before precipitation to increase protein recovery [59], and precipitates were solubilized with IEF buffer because of its properties of protein denaturation and solubilization and intrinsic compatibility with two-dimensional gel electrophoresis (2-DE) [61].

It should be noted that the threshold concentrations of urea, thiourea, CHAPS, and DTT from the IEF buffer used herein are 4, 2, 2.5, and 125 times lower, respectively, than those reported before (Table S1). Compatibility with Bradford assay was checked by comparison of standard curves of BSA prepared in water with different dilutions of IEF buffer and sucrose concentrations (Fig. S1). Minimum dilutions of 5 times and maximum dilutions of 40 times were selected for the IEF buffer, in favor of the compatibility of the components and to avoid the excessive dilution of proteins. No significant differences were observed at any sucrose concentrations or buffer dilutions compared to BSA curves in water. Accordingly, the 5-fold dilution of the IEF buffer on reconstituted samples and the highest concentration of sucrose (1.75 M) on initial samples were selected for further protein determinations.

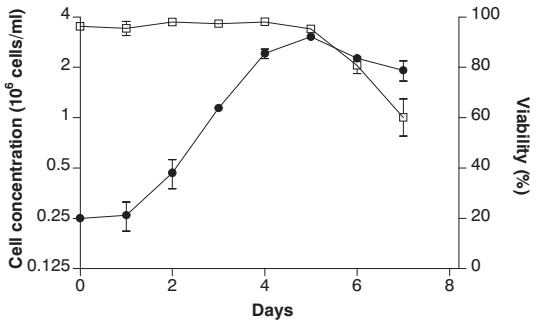

Fig. 1. Growth kinetics of Chinese hamster ovary (CHO) CRL-12444 cells. CHO cells were cultured in spinner flasks in CDM4CHO medium. Viable cell concentration (-●-) and viability (-□-) of cells over time were determined by trypan blue dye exclusion method in a Neubauer chamber.

3.3. Highest protein recovery was achieved by acetone precipitation and NaOH pre-solubilization

To select the best precipitation method for CHO cell homogenates among M/C, acetone, and TCA-acetone with regard to protein recovery, protein quantity was determined in each case by Bradford assay before and after precipitation (Fig. 2). Different letters in Fig. 2 indicated significant differences within the same panel (a–d, $p < 0.05$), according to the Kruskal–Wallis test by ranks and post hoc Conover test with Bonferroni correction. FT cycles, ultrasonic homogenization, alkali treatment of precipitates, and reducing reagents were included to increase recovery. All these modifications and the precipitation methods were summarized in Table 1.

Protocols tested without modifications were statistically different in recovery percentage ($p < 0.05$) according to Kruskal–Wallis test by ranks. To establish which treatments are different from each other and which are not, the post hoc Conover test with Bonferroni correction was carried out after the Kruskal–Wallis test. Conover test showed that acetone ($87.99 \pm 13.58\%$) was superior to TCA-acetone ($29.06 \pm 19.22\%$), and M/C ($46.09 \pm 4.92\%$) was not different from the other two methods (Fig. 2a–c). Considering modifications added to each standard protocol, they did not follow the same tendency in all methods. In case of M/C precipitation, NaOH and FT cycles showed no statistical improvement, whereas both UB and UH cycles increased protein recovery in 42% and 28%, respectively, in the absence of NaOH (Fig. 2a, $p < 0.05$). Modifications to the acetone protocol did not result in statistical differences (Fig. 2b). Pre-solubilization of precipitates with NaOH dramatically changed protein recovery after TCA-acetone precipitation, and no other modification showed an additional advantage in the presence of NaOH (Fig. 2c). A recovery increase of 41, 25, 40, and 47% was observed for the TCA-acetone standard protocol and its modifications of FT, FT + UB, and FT + UH, respectively. Inclusion of 2-ME in TCA-acetone protocol did not increase protein recovery in any of the tested conditions (Fig. 2c–d, $p < 0.05$).

Subsequently, to choose the best precipitation method for protein recovery, the conditions with the highest mean value and lowest standard deviation were selected, marked by an asterisk in Fig. 2. The three chosen protocols were the precipitation by M/C with UH cycles, acetone using the standard protocol, and TCA-acetone with FT and UH cycles without 2-ME, with the inclusion of a NaOH pre-solubilization step in the three protocols. In the case of acetone, the standard protocol with NaOH addition was considered over UB cycles due to a wider accessibility to this reagent, although no significant differences in recovery protein percentage were observed between both methods. The three selected protocols were statistically different ($p < 0.05$), and the best recovery protein percentage (Fig. 2) was achieved with acetone ($103.12 \pm 5.74\%$), followed by M/C ($94.22 \pm 4.86\%$) and TCA-acetone ($77.91 \pm 8.79\%$).

3.4. Band pattern of proteins homogenates was almost unaffected after M/C and acetone precipitation

Besides recovery efficiency, the type of proteins obtained by each precipitation method was assessed to avoid any preference towards a particular protein group and ensure a visible pattern representation of most cellular proteins in recovered samples. Separation by MW with the use of SDS-PAGE was the qualitative criterion selected. No obvious differences were observed between the proteins recovered by the top three conditions selected to compare protein recovery between protocols and of these with the non-precipitated ones (Fig. 3). However, in all the precipitated samples (Fig. 3, lanes 3-5), the two lower MW bands (around 10 kDa) could not be detected in comparison with the CHO cell lysate (Fig. 3, lane 2). The amount of some specific protein bands, with approximated MW >100 kDa and <25 kDa, recovered unevenly (indicated by white asterisks in Fig. 3). TCA-acetone presented a lower protein recovery compared with the

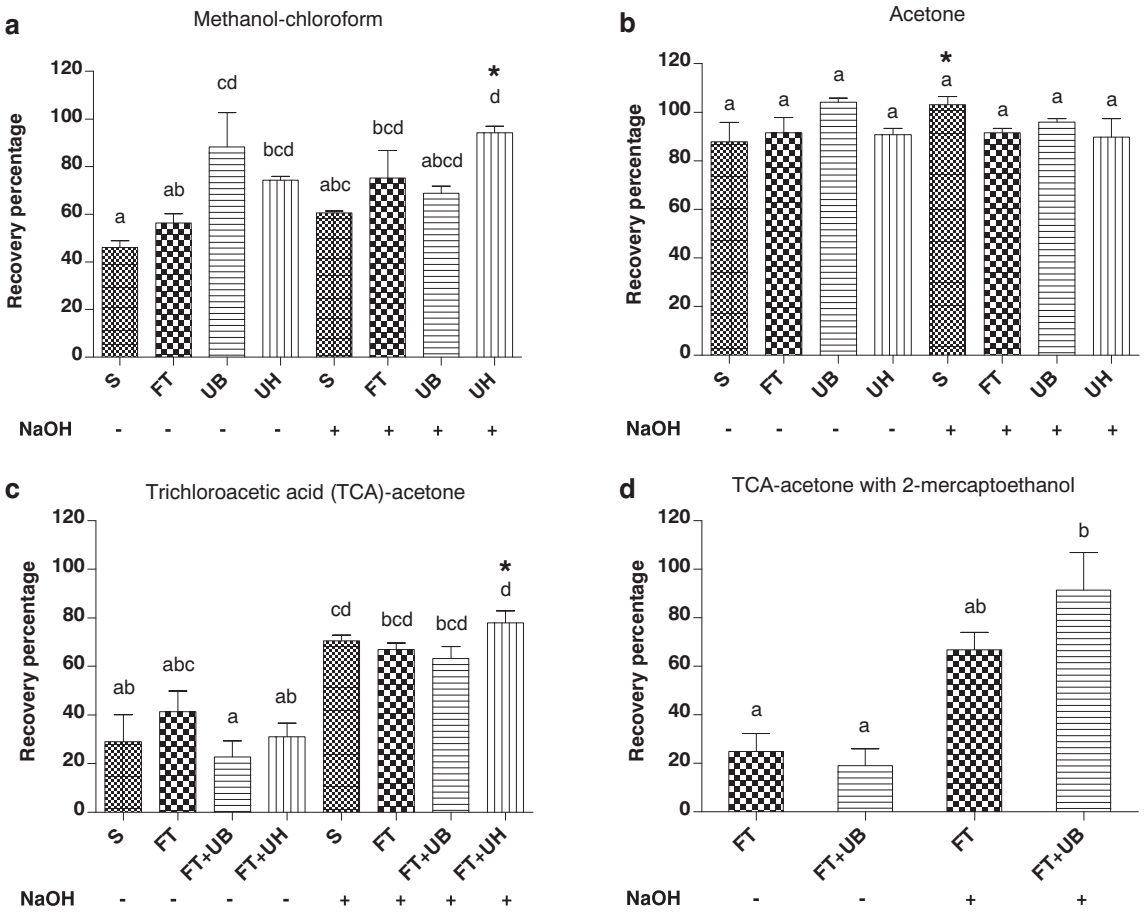

Fig. 2. Recovery efficiency of three common methods of precipitation of proteins obtained from a lysate of Chinese hamster ovary (CHO) cells. Proteins were quantified before and after precipitation with methanol-chloroform (M/C) (a), acetone (b), and trichloroacetic (TCA)-acetone (c), using only the standard protocol (S) or in conjunction with the following modifications: freeze-thaw (FT) cycles, incubation in ultrasonic bath (UB), or ultrasonic homogenizer (UH) cycles. Both standard protocols and modifications were carried out with and without NaOH addition before the precipitate was solubilized in isoelectric focusing (IEF) buffer. 2-Mercaptoethanol (2-ME) was added to TCA and acetone solutions in four precipitation conditions of TCA-acetone (d). Error bars represent the standard deviation of three biological replicates. Different letters indicate significant differences within the same panel ($p < 0.05$) according to the Kruskal–Wallis test by ranks and post hoc Conover test with Bonferroni correction. Recovery efficiencies from top condition in each method (M/C with UH cycles, acetone using the standard protocol, and TCA-acetone with FT and UH cycles without 2-ME, including in the three conditions a NaOH pre-solubilization step) were chosen as that with the highest mean value and lowest standard deviation, indicated by an asterisk, and compared in the text of manuscript according to the Kruskal–Wallis test by ranks and post hoc Conover test with Bonferroni correction.

other protocols (Fig. 3, lane 4) in line with the protein quantification (Fig. 2). M/C and acetone diverged mainly in proteins with a MW > 130 kDa, where apparently different species are enriched in each case.

For the densitometric evaluation of SDS-PAGE gels, two different quantitative analysis of these images were carried out using ImageJ software. In the first, the percentage of the total area of each lane in relation to CHO cell homogenate lane (set as 100%) was calculated to avoid any incorrect appreciation regarding the comparison of abundance of the bands between the different lanes. The qualitative image evaluation was not noticeably affected by the quantity of the total proteins in each lane, even though the maximum percentage differences were around 10% (M/C of 94.27 ± 2.46 %, TCA-acetone of 89.71 ± 6.06 % and acetone of 93.51 ± 2.10 %), in comparison with the CHO cell homogenate lane.

In the second analysis and to corroborate these results, the percentage represented by 2 bands, in a range of 10–15 kDa and marked by white arrows in Fig. 3, was calculated with respect to the total amount of proteins in each lane. While the band with the highest MW (B1) was not indicated as different between the different precipitation protocols in the qualitative evaluation, the abundance of the band with the lowest MW (B2) varied considerably. In agreement with these results, B1 abundance was not markedly affected between the precipitation conditions, while B2 diminished in the three protocols and particularly in the TCA-acetone protocol (Table S2).

4. Discussion

Biopharmaceuticals have been intensively used for many medical indications. Their importance is reflected in the number of these drugs approved, ranging from 16 during 1990–1994 to 112 in the period from 2015 to 2018 [62]. Given the increasing structural complexity of these RP and the requirements of post-translational modifications, most of them (84%) are being produced in mammalian expression systems [62]. Among them, CHO cells are the preferred host due to the advantages that they offer in terms of growth [63,64,65], productivity [3], specific culture mediums [66], safety [67], and product quality [68]. Despite the product titers achieved in CHO cell cultures (approximately 5 g/l), the price of RP continues to be high for health care systems [9,10,11]. Thus, strategies that increment RP yields are urgent and mandatory. In this sense, cell engineering of CHO cells, based on knowledge of potential protein targets obtained from proteomics, plays a fundamental role.

Protein precipitation methods play a critical role during sample preparation workflow for proteomics analysis. They are frequently incorporated at the beginning of sample preparation protocols to isolate and concentrate the proteins from cell cultures lysates [41,69,70]. Other advantages offered by these methods include, but are not limited to, inactivation of proteases to avoid protein degradation, and elimination of reagents employed during protein

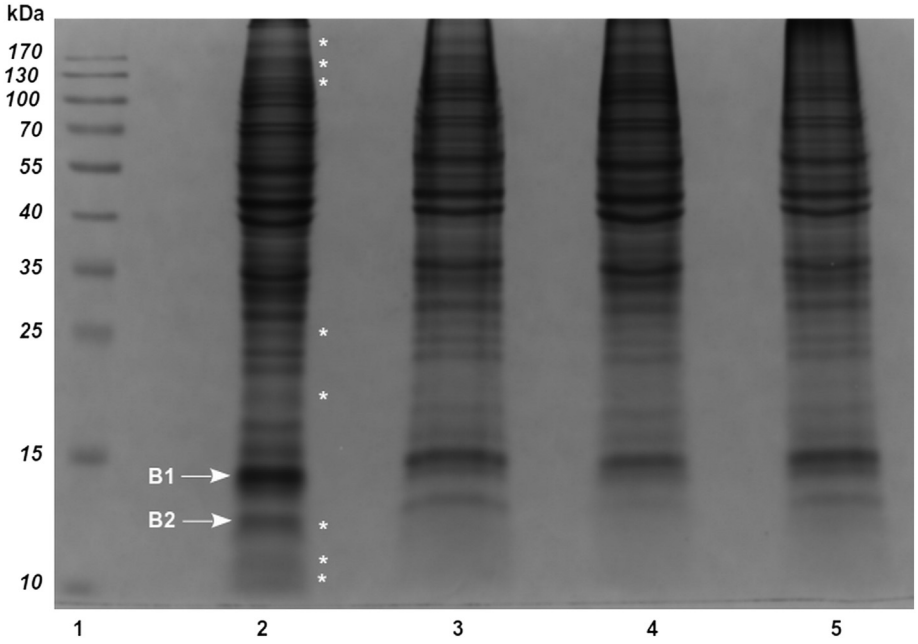

Fig. 3. SDS-PAGE of Chinese hamster ovary (CHO) cellular proteins recovered after three top precipitation methods. Proteins from a CHO cell lysate were precipitated with methanol-chloroform (M/C) with ultrasonic homogenizer (UH) cycles, acetone using the standard protocol, and trichloroacetic acid (TCA)-acetone with FT and UH cycles without 2-ME, including in the three conditions a NaOH pre-solubilization step, and then solubilized in isoelectric focusing (IEF) buffer. White asterisks indicate protein bands with different concentrations between the lanes. Lanes: 1: Protein ladder, 2: CHO cell lysate, 3: M/C, 4: TCA-acetone, 5: Acetone.

extraction (detergents, salts) and other interfering compounds (lipids, nucleic acids) present in the sample, that hamper protein recovering, two-dimensional electrophoresis, mass spectrometry analysis, and reproducibility of the results [34,46,61,71]. Thus, incorporation of protein precipitation into sample preparation protocols guarantee accurate, reproducible, and quantitative proteomics results.

However, selecting one precipitation method is commonly a challenging task because a universal method and protocol is neither available nor feasible. Taking into account the diversity of proteins present in the proteome of a certain sample and its different physico-chemical properties, the most suitable protein precipitation method must be selected from the experimental evaluation of various protocols [38,39,40,42,43,45,72]. Furthermore, each method is subject to modifications that can influence the proteins' recovery [44,47,48,49,73,74].

To the best of our knowledge, the comparison of different methods of protein precipitation for CHO cell homogenates has not been carried out so far, which prevents the expedited use of a suitable protocol for this type of sample and therefore makes side by side comparisons between different proteomic results difficult due to the lack of a homogeneous sample processing protocol. Thus, in order to choose the most suitable precipitation method for CHO cell proteomics, M/C, acetone, and TCA-acetone protocols were compared in this study with regard to recovery protein percentage and band pattern on SDS-PAGE.

Specific growth rate ($0.036 \pm 0.03\ h^{-1}$), maximum cell concentration ($2.42 \pm 0.16 \times 10^6$ cells/ml), and duration time of exponential phase (72 h) of model cells were similar to previous reports of closely related cell lines (Fig. 1) [75,76,77,78,79]. Cells were collected during the 72-hour growth phase, washed, and homogenized to obtain cell lysates for evaluation of precipitation methods. Prior to quantification of protein recovery, the compatibility of the Bradford assay with sucrose and IEF components was verified (Fig. S1). From 20 to 83 mM concentration in lysates, sucrose did not interfere in this assay, which is in agreement with Bradford [58] and Cheng et al. [59] wherein concentrations within 20–26 mM did not affect the absorbance of the dye (Table S1). Higher concentrations than those reported here should be checked because a report has communicated a 36.5% deviation from true value at 263 mM [57]. Although concentrations of components of IEF buffer are lower than those reported as compatible [60,80,81,82], it was tested because some components can interfere in a non-additive manner when mixed [60]. In this case, dilutions of 5 to 40 times did not interfere with assay, which allowed the samples to be quantified at least at a 5-fold dilution (1.4 M urea, 0.4 M thiourea, 0.4% [m/v] CHAPS, 8 mM DTT).

M/C, acetone, and TCA-acetone methods were selected due to their high precipitation performance, protease inactivation, low cost, speed, ease of execution, and elimination of common components such as detergents, chaotropic agents, and lipids [29,34,38,44,47,59,71,83], in addition to being successfully applied to a wide variety of samples [34,38,70,71,74,39,40,41,42,43,44,45,47].

When standard protocols without any modification were compared with regard to protein recovery (Fig. 2), acetone ($87.99 \pm 13.58\%$) was significantly superior to TCA-acetone ($29.06 \pm 19.22\%$) and M/C ($46.09 \pm 4.92\%$). In line with these results, high recovery after acetone precipitation has been reported before for proteins from ribosomes (98–100%) [29], rat brain (70%) [40] and homogenates, and cytosol and membranes from *Escherichia coli* (88–100%) [44]. Even though other authors did not report the percentages of recovery, acetone has been a better approach than TCA and has results very similar to M/C for animal tissues and bacteria homogenates [38,40,43,44]. In case of bronchoalveolar fluids, acetone precipitation showed the second highest recovery ($67.9 \pm 11.0\%$) after M/C ($97.9 \pm 7.0\%$) among TCA, ammonium sulfate, ethanol, and polyethylene glycol methods [70]. Successful precipitation of serum proteins, chloroplast membrane proteins [34], human stromal vascular fraction [38], bronchoalveolar fluids [70], and some rat brain structures [40] with M/C with quinoa seeds [39] and human platelets [83] with TCA, has also proven the efficacy of these methods for some samples. However, both protocols were outperformed by acetone in this study, suggesting the preferential use of acetone for protein precipitation from CHO cell homogenates.

Regarding CHO cells, only a comparison and optimization of several precipitation methods have been conducted for proteins secreted into culture medium [45]. In this case, protein recovery after solvent precipitation with acetone (≈80%), M/C (≈85–90%), or ethanol (≈80%) was superior than TCA (≈40%). However, given the extended and differential glycosylation pattern of secreted proteins [84] and

their hydrophilic nature [85] and large non-overlapping proteomics with intracellular proteins [86], the secretome could be considered as a biochemical entity different from whole cell proteome and therefore can behave differently when the same precipitation methods are employed. Valente et al. [87] reported a maximum recovery efficiency of 80% of CHO cell proteins using absolute ethanol as a precipitant and different levels of solubility-enhancing agents in the suspension solution. However, in the case of the acetone protocol optimized in this work, the recovered proteins reached around 100%.

To eliminate electrostatic repulsive forces and promote hydrophobic interactions that lead to protein precipitation in acetone protocol, NaCl was added in the present work following previous recommendations [45,47]. A possible drawback introduced during precipitation with acetone is the modification of peptides having a glycine as second amino acid in their sequences which can be confused with a proline residue in mass spectra. However, this may be overlooked because of the low frequency of this type of peptide in mammalian proteome (approximately 5%) and the selection of shot-gun strategies [88].

Some modifications to precipitation methods have been previously proposed to increase protein yield and reproducibility and detection and specificity of proteomic results for rat brain, inclusion bodies from *E. coli*, and soluble proteins from *Aspergillus oryzae* and *E. coli* [40,48,49,89]. These include FT cycles [89], sonication [40,49], and pre-solubilization with NaOH [48], which were incorporated into M/C, acetone, and TCA-acetone protocols tested in this study (Fig. 2). In the case of acetone protocol, no statistical difference was detected among the different conditions, where all of them exhibited recovery percentages above 80%. As UB cycles (104.18 ± 2.67%) or NaOH addition (103.12 ± 5.74%) recovered most proteins presented in homogenates, any of these variants of the acetone method could be used for CHO cell homogenates. For M/C, bath sonication in the absence of NaOH was significantly better than the standard method, while UH was adequate regardless of NaOH presence.

Considering that the strong binding of TCA to proteins and its precipitates is difficult to solubilize [36,40,48,49,73], FT cycles were incorporated in all the successive modifications, except in standard protocol. Unlike M/C and acetone protocols, where NaOH only showed a maximum efficiency increase of 1.34 and 1.17 times, respectively, this alkali treatment during the TCA-acetone protocol significantly augmented the recovery in almost all conditions, with a maximum increase of 2.78 times. This result could be explained by the fact that an increment in pH helps to dissociate trichloroacetate anion from proteins and aids in their solubilization [90,91]. In agreement with this, Nandakumar et al. [48] have reported a 5-fold increase in the recovery of soluble proteins from *A. oryzae* and *E. coli* by the addition of NaOH to TCA precipitates. As TCA is sometimes supplemented with reducing agents to prevent protein aggregation and aid in the precipitation process [69,72], 2-ME was tested in our protocols for improved recovery; however, no improvement was demonstrated with its use, which is why 2-ME was excluded from further experiments. This lack of enhancement by using reducing agents has been described previously for host cell proteins secreted into culture medium of CHO cells and precipitated with acetone [45].

Along with recovery percentage, any bias of some method towards a particular set of proteins should be verified for a satisfactory selection and to ensure that the recovered proteins constitute a representative sample of the cellular proteome. The MW, as determined by SDS-PAGE, was the qualitative criterion selected to address this question for the best condition of each method. The general distribution of the proteins was very similar along all samples, and only approximately nine bands were detected as differently recovered, marked by asterisks in Fig. 3. Additionally, an arrangement of bands in the high MW area can be seen for samples treated with acetone, where an aggregation of membrane proteins could have probably occurred [44,83]. Loss of intensity in various bands of samples from TCA-acetone, higher than 170 kDa and lower than 25 kDa, was noted as well, which is consistent with the lower recovery efficiency of this method. A quantitative analysis of the percentage of all proteins in each lane in relation to the sample of cell homogenate and of specific low MW bands, confirms that, with the exception of a few bands, there are no large differences in the composition of the proteins recovered with each precipitation method.

All precipitation reagents evaluated in this work belong to organic solvents, which means that their precipitating effect is mediated by lowering the dielectric constant of the solvent, and in this way, they increase the attractive forces between the protein molecules. Further, these reagents cause a dehydration effect on the proteins, which facilitates the interaction between them, mediated by hydrophobic spots on the surface [35,36]. Thus, given that precipitation depends on the interaction of proteins through electrostatic and hydrophobic forces on the surface, it is understandable that small proteins, which have a smaller exposed surface, are more difficult to precipitate using these methods [33].

Due to the special attention to organellar proteomics in recent times [92,93] and the positive effect of sucrose on protein recovery [59], the strategy presented here has the advantages of applying the same principle to samples collected from density gradients and of recovering almost all cellular proteins at initial concentrations as low as 0.2 mg/ml.

Sample preparation for proteomics begins with the extraction and solubilization of cellular proteins, followed by precipitation and a final re-solubilization step. Given that all these procedures are decisive during proteomics workflow to avoid proteins loss and that they ensure reproducibility and a representative sample, optimization of each step for each particular cell line or tissue is required. In line with this evidence, the present work of comparing protein precipitation methods is well complemented by a previous study, wherein the composition of the suspension solution has been optimized for CHO cell lysates [87]. In this case, it is recommended that, in future experiments, the use of the IEF buffer as a suspension solution be substituted by another composed of 8 M urea, ≥2% CHAPS, and ≥ 32.5 mM DTT in Tris buffer [87].

Acetone precipitation should be considered as the main method for proteomics research of CHO cells during sample-processing workflow due to its high efficiency in protein recovery and similarity with band patterns of the homogenates. Practicality, accessibility, and precipitates easy to solubilize are additional advantages of this method which if incorporated into sample preparation protocols for proteomic studies as a standard technique will provide homogeneous results to future research.

5. Conclusions

Given the high biotechnological value of CHO cells in producing recombinant proteins and the immediate need to deepen the study of its biology through a proteomic approach, acetone, M/C, and TCA-acetone methods were compared in the present study in terms of protein recovery and band pattern on SDS-PAGE to choose the most appropriate precipitation protocol for proteins from this cell line. Recovery of the M/C and TCA-acetone methods increased with ultrasonic cycles and the addition of NaOH, although the major problem in the use of TCA was the solubilization of protein precipitates. Acetone precipitation with UB cycles or NaOH addition was the recommended method as a result of its highest recovery and the similarity of band pattern with non-precipitated homogenates. Ease of use, low cost, and the processing of several samples at the same time by using acetone precipitation allow for the availability of big proteomics data from CHO cells that can pave the way for the research of their cell biology.

Conflict of interest

GE Healthcare Life Sciences provided support in cell culture media and supplies, and Dr. Paola Toledo Ibelles from Inolab Especialistas en Servicio S.A. de C.V. provided support in cell culture media.

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Supplementary material

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