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Optimization of harvesting, extraction, and analytical protocols for UPLC-ESI-MS-based metabolomic analysis of adherent mammalian cancer cells

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Abstract

In this study, a liquid chromatography mass spectrometry (LC/MS)-based metabolomics protocol was optimized for quenching, harvesting, and extraction of metabolites from the human pancreatic cancer cell line Panc-1. Trypsin/ethylenediaminetetraacetic acid (EDTA) treatment and cell scraping in water were compared for sample harvesting. Four different extraction methods were compared to investigate the efficiency of intracellular metabolite extraction, including pure acetonitrile, methanol, methanol/chloroform/H₂O, and methanol/chloroform/acetonitrile. The separation efficiencies of hydrophilic interaction chromatography (HILIC) and reversed-phase liquid chromatography (RPLC) with UPLC-QTOF-MS were also evaluated. Global metabolomics profiles were compared; the number of total detected features and the recovery and relative extraction efficiencies of target metabolites were assessed. Trypsin/EDTA treatment caused

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substantial metabolite leakage proving it inadequate for metabolomics studies. Direct scraping after flash quenching with liquid nitrogen was chosen to harvest Panc-1 cells which allowed for samples to be stored before extraction. Methanol/chloroform/H₂O was chosen as the optimal extraction solvent to recover the highest number of intracellular features with the best reproducibility. HILIC had better resolution for intracellular metabolites of Panc-1 cells. This optimized method therefore provides high sensitivity and reproducibility for a variety of cellular metabolites and can be applicable to further LC/MS-based global metabolomics study on Panc-1 cell lines and possibly other cancer cell lines with similar chemical and physical properties.

Keywords

Metabolomics; Sample preparation; Metabolite extraction; Panc-1 cell line; HILIC

Introduction

Metabolism is either directly or indirectly involved in every aspect of cell function. Cellular metabolomics can uncover information reflecting changes to biochemical reactions, metabolic pathways, and processes occurring within the cells [1]. Thus, metabolomic analysis of cells has emerged as an important approach for studying cellular biochemistry. Metabolic activity of cells, in particular central carbon metabolism, fatty acid, and lipid metabolism, have been shown to play an important role in cancer progression and drug response [2]. Therefore, characterization of cancer cell-specific metabolomic signatures could be of great value in the early diagnosis of cancer as well as in following therapeutic response [3]. Pancreatic cancer is one of the most aggressive and lethal cancers and is the fourth leading cause of cancer-related deaths worldwide [4]. The human pancreatic cancer cell line Panc-1 has been used extensively as a cell culture model to study advanced stage of pancreatic cancer. The analysis of metabolic profiles of Panc-1 cells using mass spectrometry (MS)-based metabolomics could provide important information on the biochemical phenotypes and indicate molecular mechanisms of cancer progression, response, and resistance to therapy. However, it is important to ensure reproducibility when carrying out metabolomics experiments to glean biologically meaningful information. Therefore, an optimized and standardized protocol for efficient quenching, harvesting, and extraction of cells is highly warranted and will aid in the metabolomics study of pancreatic cancer.

To prepare cells for metabolomics investigations, quenching and harvesting are aimed to inactivate intracellular enzymes and halt metabolism as rapidly as possible to avoid metabolite degradation and ex vivo alteration of the sample composition since a number of metabolic reactions occur in seconds [5]. This would ensure that the metabolomic profile is “frozen” and representative of normal living cells. After cell quenching and harvesting, metabolite extraction is crucial and often the rate-limiting step in metabolomics. Since the polarity of intracellular metabolites varies widely, optimization of the extraction and analytical methodology is extremely important to recover and detect as many metabolites as possible. Moreover, sample preparation should be highly reproducible, robust, and fast to allow high-throughput studies [6].

Although several studies on sample preparation for metabolomics analysis of yeast and bacteria have been reported [5, 7–9], these findings may not be fully applicable to adherent mammalian cells because of substantial differences in eukaryotic and prokaryotic cells. Consequently, some metabolomic studies of adherent mammalian cells have been undertaken, as summarized in recent papers [10, 11]. These studies report a wide variety of rinsing, quenching, and extraction methods for different cell types including human breast

cancer cell MCF7 [11, 12], MDA-MB-468 [12], clonal β -cell INS-1 [10], CHO [13], and the colon adenocarcinoma cell line SW480 [6]. Since there are significant chemical and physical differences between different types of cancer cells, these reported methods may not be directly applicable to study Panc-1 cells. Furthermore, due to the large variety of metabolites contained within cells, with wide-ranging chemical stability, solubility, and polarity of metabolites, it is necessary to optimize the sample preparation protocol for each cell line. To date, there have been no reports thoroughly evaluating the robustness and efficiency of determining the harvesting and extraction protocols for global metabolomic profiling of Panc-1 cells.

Therefore, the objective of this study was to propose a strategy for reliable and reproducible harvesting and extraction method for liquid chromatography mass spectrometry (LC/MS)-based metabolomics study of Panc-1 cells. In this study, the effect of trypsin/EDTA-based cell detachment was compared with cell scraping for harvesting while the effects of several extraction solvents on metabolomic signature were compared to optimize metabolite extraction protocol for Panc-1 cells. In addition, hydrophilic interaction liquid chromatography (HILIC) and reverse-phase chromatography was compared for efficiency of metabolite separation and quantitation.

Materials and methods

Chemicals

All authentic compounds were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Dulbecco's modified Eagle's medium (DMEM), penicillin sodium, and streptomycin sulfate solution for cell culture were purchased from Mediatech (Manassas, VA). Fetal bovine serum (FBS) was purchased from Lonza (Walkersville, MD). The BCA Protein Assay Kit was from Thermo Scientific (Rockford, IL). Trypsin/EDTA (0.25 % trypsin/2.21 mM EDTA in HBSS) was from Mediatech Inc. (Manassas, VA). All the other chemicals and solvents were of the highest grade commercially available.

Cell culture

Human pancreatic cancer cells Panc-1 was purchased from the American Type Culture Collection (Manassas, VA). Panc-1 cells were grown in DMEM containing 10 % FBS, 100 U/mL of penicillin sodium, and 100 μ g/mL of streptomycin sulfate at 37 °C in a humidified atmosphere of 5 % CO₂. The cells were subcultured in 10 cm polystyrene dishes at a density of 5×10^4 cells/dish and were grown to approximately 90 % confluence for metabolomics analysis.

Cell samples harvesting

For sample collection, cells were detached using either trypsin solution or a rubber tipped cell scraper (BD Biosciences, Bedford, MA). To quench the cells with liquid nitrogen and harvest the cells by scraping, the growth medium was removed and cells were rapidly rinsed twice by gently dispensing 5.0 mL isotonic saline (37 °C) to the cell surface, and then 1 mL ice cold water was added into the dish. The dish was flash frozen in liquid nitrogen, the cells were detached using a cell scraper, and the cell suspensions were transferred into 1.5 mL tubes.

For trypsin/EDTA-treated sample collection, the cells were washed once with 4 mL PBS and 2-mL trypsin/EDTA solution was added to the dish. After incubation at 37 °C for 2–3 min and tapping of the dishes, 2-mL medium was added to dilute the trypsin/EDTA and the media containing the detached cells was transferred into a falcon tube. Cells harvested from different dishes were combined in one tube. The cell suspension was centrifuged at $125 \times g$

for 4 min at 4 °C; the media was removed; and the cell pellet was washed twice with 5-mL ice-cold water to remove extracellular metabolites. After the final wash step, cells were suspended in water (1 mL water/dish) and transferred into 1.5-mL Eppendorf tubes.

All the cell suspensions were lysed by two cycles of freeze–thaw (fresh frozen in liquid nitrogen and thawed at 37 °C for 10 min), followed by 30 s of sonication on ice. Protein concentrations were measured by BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) for normalization. The cell suspensions were stored at –80 °C until analysis. Cell samples generated either by trypsin/EDTA detachment or scraping were extracted using the most optimal method below to compare the intracellular metabolites.

Extraction protocols

Pooled samples from cell suspensions generated by trypsin/EDTA and scraping were used for testing each extraction procedure. Complete lysis of all cell compartments was achieved, and protein concentration of each sample was measured by the methods described above. Four different extraction methods were investigated on the Panc-1 cells to compare the efficiency of intracellular metabolite extractions. Five replicates were performed for each method.

For the methanol (MeOH) and acetonitrile (ACN) extraction procedure, 900 μ L chilled (–20 °C) MeOH or ACN (containing 2.5 μ M α -aminopimelic acid or 5 μ M chlorpropamide as internal standards for HILIC and reversed-phase liquid chromatography (RPLC) modes, respectively) was added to 300 μ L cell suspension. The mixture was vigorously vortexed and centrifuged at 14,000 $\times g$ for 15 min at 4 °C to precipitate proteins and particulates. The supernatants were transferred to a fresh glass tube and dried under nitrogen flow. The residuals were resuspended in 200 μ L 70 % ACN/water or 35 % ACN/water for HILIC and RPLC modes, respectively, centrifuged at 14,000 $\times g$ for 5 min at 4 °C. Finally, 5 μ L of the supernatant was injected for UPLC-ESI-QTOF-MS analysis.

For the MeOH/chloroform/H₂O extraction, 900 μ L of chilled extraction solvent 75 % MeOH/chloroform (MC; 90:10 (v/v))-containing internal standard (2.5 μ M α -aminopimelic acid or 5 μ M chlorpropamide as internal standards for HILIC and RPLC modes, respectively) was added into 300 μ L of cell suspension followed by extraction as described above. This method was adapted from Lorenz et al. [10].

For the MeOH/chloroform/ACN extraction, a dual-phase extraction with slight modifications of that reported [12], was adopted. Briefly, 450- μ L chilled MeOH (–20 °C) was added to 300 μ L of cell suspension, vortexed followed by addition of 450- μ L chilled chloroform. The mixtures were vortexed and centrifuged at 14,000 $\times g$ for 15 min at 4 °C. The two phases were carefully transferred to separate fresh collection tubes. Chilled ACN-containing (450 μ L) internal standard (2.5 μ M α -aminopimelic acid for HILIC mode and 5 μ M chlorpropamide for RPLC mode) was added to each tube, vortexed, and incubated at –20 °C for 15 min. The tubes were centrifuged at 14,000 $\times g$ for 15 min at 4 °C; the supernatant was transferred to collection tubes and dried under nitrogen flow. The residuals were reconstituted for HILIC and RPLC modes as mentioned above.

HILIC and RPLC UPLC-QTOF-MS analysis of intracellular metabolites

Traditional RPLC method provides efficient separation of nonpolar metabolites whereas the HILIC method helps to separate highly polar metabolites. These two methods are complementary and, together, can provide enhanced coverage of metabolites [14]. Therefore, extracted cell samples were analyzed in both HILIC and RPLC modes to compare the recovery, coverage, separation, and sensitivity of the metabolomic protocols.

For the RPLC mode, samples were injected onto a 50×2.1-mm ACQUITY® 1.7 μm BEH C18 column (Waters Corp, Milford, MA). Metabolite separation was achieved using a gradient of mobile phases A and B containing 0.1 % formic acid, respectively, in water and ACN on an ACQUITY® UPLC system (Waters Corp, Milford, MA). The flow rate was kept at 0.5 mL/min during a 10-min run with the following gradient: 98 % A for 0.5 min to 20 % B at 4.0 min to 95 % B at 8 min. The column was washed with 98 % B for 1 min then equilibrated with 98 % A before subsequent injections.

For the HILIC mode, chromatography was performed using an ACQUITY® BEH Amide (1.7 μm, 50×2.1 mm) column (Waters Corp, Milford, MA) using an ACQUITY® UPLC (Waters Corp, Milford, MA). Separation was achieved using a gradient of mobile phases C and D containing 10 mM ammonium acetate in 10 % ACN (pH= 9.0) and 90 % ACN (pH=9.0), respectively. The flow rate was kept at 0.4 mL/min during a 12-min run with the following gradient: 99 % D for 0.5 min to 60 % D at 6.0 min to 20 % D at 8 min. The gradient was held at 20 % D for 1 min then returned to 99 % D for 2 min for column equilibration before subsequent injections.

MS spectrometry was performed on a Waters® Xevo G2™ QTOF-MS system operating both in positive (ESI⁺) and negative (ESI⁻) ionization modes. The capillary voltage and cone voltage were set to 3,000 and 30 V, respectively. Source and desolvation temperatures were set at 150 and 550 °C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (850 L/h), and argon was used as collision gas. Mass accuracy was maintained on both systems using Waters LockSpray™ technology. Sulfadimethoxine was used as the lock mass to maintain mass calibration in real time. For MS scanning, data were acquired in centroid mode from 50 to 850 *m/z* and for MS/MS fragmentation of target ions the collision energy was ramped from 5 to 40 eV. To avoid artifacts resulting from sample injection order, all samples were randomized. The quality controls were generated by pooling 5 μL of each sample. Standard samples were also made containing target compounds with known concentrations. Blank samples were 70 % ACN/water or 35 % ACN/water. Quality control samples (pooled, standards, and blanks) were injected intermittently during the run to monitor the stability of LC/MS/MS system and the response of target compounds. Mass chromatograms and mass spectral data were acquired using MassLynx software (Waters Corp.).

A set of metabolites representing various metabolic pathways and metabolites classes was selected for targeted analysis of metabolite recovery, separation and detection using aforementioned methods. The presence of these metabolites in cell extracts was confirmed by comparison of retention times and MS/MS fragmentation patterns with authentic standards. The relative abundance of these metabolites was calculated from integrated peak areas extracted from the ion chromatogram (with 20 ppm window) as internal standard-normalized response.

Multivariate data analysis

The mass spectral data were deconvoluted and integrated to generate a multivariate data matrix containing integrated peak area for detected features (unique mass-retention time pair) generated by peak picking, alignment, and deisotoping using MarkerLynx® (Waters Corp, Milford, MA). The raw data were transformed into a multivariate matrix containing aligned peak areas with matched mass-to-charge ratios (*m/z*) and retention times. The data were normalized to both protein concentration and peak area of the internal standards (chlorpropamide for RPLC mode which appeared at a retention time of 5.3 min, 275.026 [M-H]⁻ and 277.041 [M+H]⁺; aminopimelic acid for HILIC mode which appeared at a retention time of 4.9 min, 174.076 [M-H]⁻ and 176.092 [M+H]⁺). The Pareto-scaled data matrix was analyzed by SIMCA-P+12 software (Umetrics, Kinnelon, NJ). The overall

differences in metabolomic signatures obtained from different protocols were examined using unsupervised principal components analysis (PCA).

Statistical analysis

All values were expressed as mean $\pm$ SD. Statistical analysis was performed by Student's *t* test using Prism 5 (GraphPad Software Inc., San Diego, CA). Difference was considered as significant if the probability (*P* value) was less than 0.05 (*P*<0.05).

Results and discussion

Due to the heterogeneity of the intracellular metabolome and the inherent limitation of analytical platforms, development of unbiased, standardized protocols is highly challenging. Changing one parameter of the protocol may change the response of many groups of metabolites. In this study, trypsin/EDTA cell detachment and cell scraping in water were compared for harvesting Panc-1 cells for metabolomics analysis. Trypsinized and scraped cell-pooled samples were then extracted using four different extraction protocols, including pure ACN, MeOH, MeOH/chloroform/H₂O, and MeOH/chloroform/ACN. Moreover, the separation of HILIC and RPLC modes were evaluated. The metabolic profiles were compared and relative response of 20 selected metabolites involved in Panc-1 cell metabolic pathways was compared under different strategies. The optimized strategy is convenient, efficient, and provides good sensitivity and reproducibility for a variety of Panc-1 cellular metabolites. The strategy for cell metabolomics was further validated and applied successfully in several cell lines including a special microRNA overexpression Panc-1, pancreatic epithelial, HEK293, and HEK293 cells with K-ras overexpression (data not shown).

Efficiency of extraction methods

Usually, different extraction methods are tailored to analyze specific classes of metabolites involved in a number of important metabolic pathways such as the citrate acid (TCA) cycle, glycolysis, amino acid metabolism, fatty acid metabolism, and glutamine metabolism. Several different extraction methods have been recommended for different types of adhered cell lines ranging from cold MeOH, MeOH/water, chloroform, MeOH/chloroform/water, ACN, hot ethanol or MeOH, perchloric acid, and potassium hydroxide extraction [10, 12, 15–19]. To date, there has been no report on the effectiveness of different metabolite extraction methods for Panc-1 cells, which will be of great significance in studying cellular metabolism in pancreatic tumors.

In an effort to define the most efficient extraction method for global metabolite profiling of Panc-1 cells, four different extraction methods were assessed. Cells harvested by using either scraping or trypsinization were combined for extraction study. Maximum lysis of cell compartments was achieved by two shock freeze-thaw cycles and ultrasonic treatment allowing release of intracellular metabolites. The extraction solvents used included pure ACN and MeOH for monophasic extraction, 75 % 9:1 MC, and MeOH/chloroform/ACN (MCA) for a biphasic extraction procedure. It was reported that acidic or alkaline conditions cause chemical modifications that could result in nonphysiological profiles, and the use of heat in some of the extraction methods may cause the loss of heat labile metabolites and a build-up of degradation products [5, 13]. Therefore, acidic (such as perchloric acid), alkali (such as potassium hydroxide), and hot conditions were not used in the current study.

For comparison of variability of the extraction methods on overall metabolomic profiles, a PCA was performed after stepwise normalization of the area of each feature by the area of internal standard and the total protein concentration for respective sample. PCA of the data obtained from HILIC-ESI⁺/ESI⁻-MS and RPLC-ESI⁺/ESI⁻-MS showed clear segregation of

samples according to extraction methods (Fig. 1). Such segregation patterns in the PCA model also indicate that the extraction methods influence metabolomic profiles of cellular extracts. The proximity of the replicate samples ($n=5$) in the scatter plots reflects the reproducibility of the extraction procedures. The PCA scatter plots showed that the samples representing metabolites extracted by MC had the smallest sample-to-sample variation under all HILIC and RPLC modes thus indicating this method had the best robustness. The samples extracted using MeOH had similar metabolic profiles to those extracted with MC, but the variability in positive ionization mode seemed to be larger and one sample was an outlier. It was also apparent that the other two extraction methods, ACN and MCA, had similarly high sample-to-sample variation under all analytical modes. These findings indicate that MC extraction is the most suitable procedure for obtaining a robust and reproducible metabolic profile of Panc-1 cells.

In order to obtain a qualitative evaluation of the extraction protocols, detection of features (see Table S1 in the Electronic supplementary material (ESM)), relative response (Fig. 2), and relative extraction efficiency (Table 1) of targeted metabolites from the various extraction methods were further compared. Under positive or negative HILIC and RPLC modes, a total of 4,993, 5,025, 5,053, and 4,960 molecular features were obtained for ACN, MeOH, MC, and MCA extraction methods, respectively. No significant difference was found among the number of detected features in these four extraction methods under different LC modes. However, the MC method had the highest number of features under most of the analytical conditions and the MCA method the lowest. Twenty metabolites representing diverse classes and belonging to a number of important metabolic pathways such as the TCA cycle, glycolysis, amino acid metabolism, fatty acid metabolism, glutamine metabolism, and glutathione metabolism were monitored to evaluate extraction efficiencies across metabolite classes and pathways. Relative responses of the selected metabolites showed that MeOH and MC extractions were generally the most effective for most of the metabolites (Fig. 2). MC extraction resulted in preferential relative response of metabolites such as adenosine, guanine, hypoxanthine, creatine, glutamate, inosine, and uridine. Relative response of these compounds by MeOH extraction was comparable to the MC method. To profile the extraction efficiencies, the relative extraction efficiency of 20 metabolites involved in Panc-1 metabolism pathways were examined. The data are presented as extraction efficiency relative to pure ACN set at 100 % (Table 1). The overall recovery of the MCA extraction for most of the analytes was much lower in comparison to the MeOH and MC methods, although it did have preferential recovery of several metabolites such as GSSG. Again, MC extraction resulted in the highest total average extraction efficiency (168.3 %) and MeOH method had comparably good overall efficiency (149.5 %) for most of the analytes. These results are consistent with a previous metabolite extraction study which found methanolic solutions to be suitable extraction solvents for INS-1 cells [10].

Comparison of extraction methods showed that each provided advantages and disadvantages for different metabolite classes as was expected due to their very different chemical and physical properties such as structure and polarity. Thus, the overall intracellular metabolite profiling may be limited by the extraction method [13]. A desirable extraction method would enable recovery of the broadest range of metabolites with highest possible extraction efficiency. The results in this study indicate that the MC extraction is the most suitable extraction method for extraction of broadest range of metabolites for highly reproducible metabolic profile of Panc-1 cells. Overall, MC extraction was an adequate extraction method for Panc-1 cells due to the overall good extraction efficiency, the higher yields of metabolites and the good reproducibility with smaller variability.

Evaluation on the harvesting approaches

Trypsinization and scraping are two widely used methods to harvest adherently growing cells for metabolomics studies. Although several cell metabolomic studies have been reported using trypsinization to harvest cell samples [6, 20, 21], the influence of trypsinization on metabolomic composition and recovery has not been investigated. Some cell preparation procedures use cold organic solvents to simultaneously quench metabolism, harvest cells and initiate metabolite extraction. However, the combination of all steps impedes the determination of cell count or protein concentration, a parameter often used to normalize data generated in cell culture systems [6]. Furthermore, previous studies have demonstrated that metabolite leakage occurs during quenching of metabolism with organic solvents [5, 22]. In the current scraping protocol, a three-step process consisting of an initial saline rinse to remove contaminating media residue was used, followed by liquid nitrogen quenching to stop all metabolic activity, and finally scraping of the cells in water. In the trypsinization protocol, the cell pellets were also rinsed and then suspended in water. This approach of separating quenching and extraction allowed the measurement of protein concentration. Additionally, postponing of extraction allows samples to be stored and extracted more conveniently as well as leaving room for adopting complex and lengthier extraction protocols before extraction with more flexibility to analyze samples whenever the instruments are available.

PCA of data obtained from HILIC-ESI⁺/ESI⁻-MS and RPLC-ESI⁺/ESI⁻-MS resulted in clear segregation of cell samples harvested by scraping or trypsinization (see Fig. S1 in the ESM), indicating that these two harvesting methods influence metabolomic profiles of cellular extracts. It is not surprising to find from the PCA scatter plots that the samples representing metabolites harvested by the trypsinization had smaller sample-to-sample variation than those produced by the manually scraping method, thus indicating that the trypsinization method had a better robustness. However, the scores loadings plots revealed that a number of ions were significantly depleted in the trypsinized samples. A possible reason is that trypsin can cleave cell adhesion proteins and at the same time cause changes in cell morphology, which of course will make the difference in cell metabolic profiles. The incubation time with trypsin/EDTA was ~3 min to completely detach the cells from dish, which meant that metabolic activities were not quenched during this time. This maybe another reason that causes significant differences in metabolomic profiling of scraping and trypsinization samples.

To further evaluate the difference in profiles caused by scraping or trypsinization, the relative response of 20 selected metabolites were compared. The heatmap in Fig. 3 clearly shows that most of the relative response of the 20 selected metabolites normalized to internal standard and protein concentration were obviously lower in the cells harvested by trypsinization thus indicating substantial metabolite leakage during trypsin treatment. Fig. 4 shows the fold change decrease of Panc-1 metabolite peak area with trypsinization versus scrapping collection analyzed by HILIC and RPLC modes. The response of the scrapping collection was set at 100 % as a reference. In the HILIC mode, the relative response of most of the target metabolites was significantly decreased by 2- to 8-fold, except for adenosine that showed a 2-fold increase with the trypsinization protocol. Others also previously reported that trypsinization treatment caused metabolite leakage in SW480 cells [6]. However, it should be noted that the effect of trypsin on the cellular metabolite leakage and corresponding cell metabolomics profiles most likely depends on the cell type. In the current study, Panc-1 cells were incubated with trypsin for about 3 min to detach them from the dish. For other cell lines that only need a very short incubation time (less than 1 min), it might result in lower degree of metabolite leakage.

These observations indicate that scraping cells is the most suitable harvesting method for Panc-1 cells to obtain highest recovery of a diverse class of metabolites.

Comparison of separation and sensitivity under HILIC and RPLC Modes

RPLC is by far the most widely applied method for analysis of small molecules. Although RPLC can be used for various applications, its main drawback is that very polar and hydrophilic compounds are not or insufficiently retained [23]. Polar metabolites elute in the void volume and suffer from ion suppression from co-eluting ions that may lead to errors in estimation of the actual intracellular concentration. HILIC provides an alternative approach to effectively separate small polar compounds using polar stationary phases. They can provide more abundant information on metabolites for global metabolomics studies. HILIC is now increasingly being used to analyze urine, serum, and cellular samples for global metabolomics studies [24–28]. Since intracellular metabolites are dominated by small polar molecules, most of the intracellular metabolites that elute close to the solvent front in the RPLC system could potentially be profiled using HILIC. Thus, separation of target metabolites was compared using both HILIC and RPLC.

The number of detected features analyzed by positive or negative mode HILIC and RPLC is summarized (see Table S1 in the ESM). HILIC had significantly larger number of molecular features than RPLC irrespective of the harvesting and extraction protocols employed. It can be seen that response under HILIC mode was stronger than that of RPLC mode. It was also reported that HILIC mode can generally produce a greater response than the RPLC in the ESI-MS detector due to the volatile nature of HILIC mobile phases [29], which may explain why there is a greater response. Under the same conditions, the higher the proportion of organic solvents present in the mobile phase, the better MS response can be observed [25]. Since a high content of organic solvent was used in HILIC, a much higher response could be achieved. From Fig. 5a (positive mode), b (negative mode), it can be seen that more detected peaks appeared under positive and negative HILIC modes, which indicates a better separation of metabolites. It can also be observed in Fig. 5c (positive mode) and Fig. 5d (negative mode) that target metabolites that were not retained by RPLC and eluted in the void volume with very early and close retention time at 0.3–0.4 min were well separated by HILIC under both positive and negative modes. Comparison of retention times of target metabolites under RPLC and HILIC modes was further summarized in Table 2. As expected, polar molecules showed a much better separation in HILIC mode, whereas hydrophobic molecules such as LPC and LPE were better separated by RPLC.

These findings indicate that HILIC can generally produce a better separation and response than RPLC, which is consistent with previous reports [14, 25, 27, 29, 30]. Although a combination of both modes should ideally be adopted, HILIC method performs better in separating a wider range of metabolites and will contribute to enriching the knowledge about the cellular metabolome of Panc-1 cells.

Conclusions

This study is the first report, comparing several protocols for quenching, harvesting, and efficient extraction and separation of metabolites from Panc-1 cells. The results show that trypsinization caused substantial metabolite leakage compared with cell scraping. Based on the overall performance, 75 % 9:1 MC extraction was found to be the most efficient and reproducible method for extraction of Panc-1 cells. HILIC mode provided better separation of metabolites than RPLC mode, thus enhancing Panc-1 cell metabolome coverage. Taken together, these results suggest that cell scraping followed by 75 % 9:1 MC extraction and HILIC-based chromatography can constitute a robust, efficient, and reproducible method for metabolomic study of Panc-1 cells using UPLC-ESI-MS platform.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. PCA of deconvoluted metabolomic signatures obtained in HILIC-ESI⁺ (A) and HILIC-ESI⁻ (B) spectra and RPLC-ESI⁺ (C) and RPLC-ESI⁻ (D) spectra of intracellular metabolites extracted by using acetonitrile (*inverted triangles*), MeOH (*circles*), MeOH/chloroform/H₂O (*squares*), and MeOH/chloroform/acetonitrile (*diamonds*) extraction methods. The area of each feature (*m/z* and retention time pair) was normalized by the area of internal standard and protein concentration

Fig. 2.

Relative abundance of 20 metabolites representing different metabolite classes and metabolic pathways in different extraction methods under HILIC-ESI⁺ (A), HILIC-ESI⁻ (B), RPLC-ESI⁺ (C), and RPLC-ESI⁻ (D) modes of analyses. Each bar represents the average of five independent extractions normalized to the protein concentration and the internal standard. Error bars represent standard deviations

Fig. 3. Heatmap analysis of the log-transformed relative abundance of targeted metabolites with trypsinization versus scrapping collection analyzed by HILIC (**A**) and RPLC (**B**). Each value represents the peak area of each sample normalized to the internal standard and protein concentration

Fig. 4.

Decrease in Panc-1 metabolite due to trypsinization (presented as fold change with respect to scraping) as shown by analysis in HILIC (**A**) and RPLC (**B**) modes. Each *bar* represents the average of five independent extractions normalized to the protein concentration and the internal standard. *Error bars* represent standard deviations

Fig. 5. Typical total ion chromatograms separated on positive (**A**) or negative (**B**) HILIC and RPLC modes. Improved separation of polar target metabolites through HILIC separation under positive (**C**) or negative (**D**) ionization modes

Table 1

Relative extraction efficiency (in percent) for different extraction methods of pooled cell suspensions harvested using trypsinization or scraping

Metabolites	HILIC mode				RPLC mode			
	ACN	MeOH	MC	MCA	ACN	MeOH	MC	MCA
Positive mode								
Adenosine	100.0±21.3	231.1±21.0	400.8±71.0	80.9±5.1	100.0±11.1	125.5±23.0	217.1±91.3	60.7±10.4
Guanine	100.0±24.9	234.1±39.9	205.8±52.5	20.7±5.6	100.0±9.4	108.8±2.6	120.6±6.6	75.1±5.4
Leucine	100.0±61.5	231.2±49.8	240.8±69.8	127.1±21.7	100.0±8.4	97.2±8.4	105.3±17.4	102.2±4.9
GSH	100.0±4.3	115.1±4.4	120.9±11.6	52.6±12.5	100.0±6.3	111.6±9.8	127.1±27.5	113.0±37.3
GSSG	100.0±33.2	171.1±8.9	211.7±41.8	412.7±303.9	100.0±10.7	76.9±33.3	84.3±28.5	312.2±345.2
AMP	100.0±12.9	87.2±2.1	105.4±13.2	100.9±25.6	100.0±11.5	106.4±7.6	110.5±3.9	134.8±9.6
Hypoxanthine	100.0±23.0	269.6±7.5	202.1±21.5	72.2±9.4	100.0±19.6	113.1±4.6	96.8±7.4	64.2±8.1
Camitine	100.0±22.4	171.0±14.9	205.4±3.1	152.5±7.0	100.0±7.9	97.8±4.7	98.5±3.6	108.6±4.6
Creatine	100.0±20.8	197.9±1.5	174.3±6.1	133.9±24.8	100.0±8.4	105.3±17.4	97.2±8.4	102.2±4.9
LPC (14:0)	100.0±33.1	315.0±61.4	380.5±17.6	106.1±13.6	100.0±4.4	99.1±14.1	87.3±10.4	45.0±6.5
Negative mode								
Uridine	100.0±17.6	110.6±7.3	98.2±23.2	76.7±8.0	100.0±45.4	153.6±45.1	77.3±37.4	65.2±5.2
Inosine	100.0±10.2	113.9±10.8	130.7±6.6	72.1±6.2	100.0±7.1	112.9±24.1	126.1±9.5	60.3±7.9
Ornithine	100.0±31.3	78.4±30.9	163.2±30.4	61.5±12.4	100.0±9.8	102.5±6.2	116.0±6.4	63.4±5.6
Glucose	100.0±9.6	98.4±5.0	99.1±9.2	123.9±23.0	100.0±27.0	116.4±6.6	119.4±3.6	99.2±23.0
Citrate	100.0±13.9	82.8±14.6	75.2±14.7	83.3±31.6	100.0±12.9	85.4±29.9	98.9±4.6	144.3±9.6
Malate	100.0±22.1	96.0±19.5	172.4±5.6	75.5±14.1	100.0±11.3	98.0±5.1	110.1±5.1	86.3±6.3
Glutamate	100.0±4.2	102.6±5.1	116.7±7.0	70.5±14.0	100.0±6.1	94.4±2.7	107.1±3.2	98.1±4.8
Glutamine	100.0±1.2	93.0±2.1	96.3±3.4	86.7±1.7	100.0±3.1	96.7±11.8	105.1±5.8	104.4±1.6
Taurine	100.0±5.9	106.9±1.7	102.3±5.1	76.2±5.3	100.0±4.5	96.5±4.6	94.8±6.3	95.6±3.9
LPE (20:4)	100.0±10.2	83.5±11.8	64.7±4.7	61.8±9.2	100.0±63.5	63.3±10.2	58.7±3.9	67.8±19.5
Average	100.0	149.5	168.3	102.4	100.0	103.1	107.9	100.1
Minimum	100.0	78.4	64.7	20.7	100.0	63.3	58.7	45.0
Maximum	100.0	315.0	400.8	412.7	100.0	153.6	217.1	312.2

Relative response of each metabolite was normalized by the internal standard and protein concentration ($n=5$). Extraction using acetonitrile (ACN) was set as 100 % as reference. Data are given as mean ±SD. Values set in italics were the highest among the four extraction methods

Table 2

Comparison of retention time of target metabolites under RPLC and HILIC mode

Compounds	Experimental ion mass (<i>m/z</i>)	RT in HILIC ⁺ (min)	RT in HILIC ⁻ (min)	RT in RP ⁺ (min)	RT in RP ⁻ (min)
Adenosine	268.1041+	1.06		0.33	
Hypoxanthine	137.0459+	1.13		0.33	
Guanine	152.0568+	1.88		0.32	
L-leucine	132.1021+	2.70		0.28	
Propionylcarnitine	218.1387+	3.02		0.34	
Acetyl carnitine	204.1232+	3.44		0.30	
L-carnitine	162.1126+	3.96		0.29	
Phosphoglyceric acid	186.9955+	3.55		0.32	
L-serine	106.0503+	4.48		0.29	
L-glutathione reduced (GSH)	308.0916+	5.01		0.32	
AMP	348.0709+	5.36		0.32	
L-lysine	147.1134+	6.14		0.30	
L-glutathione oxidized (GSSG)	613.1598+	6.37		0.32	
Creatine	132.0773+	3.96		0.28	
LPC (14:0)	468.3077+	0.52		6.45	
Uridine	243.0618-		1.08		0.31
Inosine	267.0725-		1.81		0.31
L-lactic acid	89.0239-		2.24		0.27
Methionine	148.0431-		3.11		0.30
Trypsin	180.0667-		3.51		0.33
L-glutamine	145.0610-		4.37		0.30
L-glucose	179.0556-		4.43		0.28
L-glutamic acid	146.0453-		4.88		0.31
UDP-GalNAC	606.0736-		5.40		0.46
L-lysine	145.0976-		6.14		0.26
Malic acid	133.0136-		4.94		0.31
Citric acid	191.0192-		6.02		0.33
Ornithine	131.0821-		4.52		0.31
Taurine	124.0070-		3.29		0.39

Compounds	Experimental ion mass (<i>m/z</i>)	RT in HILIC ⁺ (min)	RT in HILIC ⁻ (min)	RT in RP ⁺ (min)	RT in RP ⁻ (min)
LPE (20:4)	500.2789–		0.49		6.65