Vol. 11, No. 2, 2020
ISSN 2233-4203/ e-ISSN 2093-8950
ARTICLE www.msletters.org | Mass Spectrometry Letters

Comparison of Cell Lysis Techniques via Q-TOF LC/MS

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Received April 21, 2020; Revised June 10, 2020; Accepted June 17, 2020
First published on the web June 30, 2020; DOI: 10.5478/MSL.2020.11.2.36

Abstract : Untargeted metabolomics is a useful tool for drug development focusing on novel chemotherapeutic and chemopreventative agents against cancer cells. In recent years, quadrupole time of flight liquid chromatography-mass spectrometry (Q-TOF LC/MS)-based untargeted metabolomic approaches have gained importance to evaluate the effect of these agents at the molecular level. The researchers working on cell culture studies still do not apply standardized methodologies on sample preparation for untargeted metabolomics approaches. In this study, the rough and wet lysis techniques performed on MCF-7 breast cancer cells were compared with each other via the Q-TOF LC/MS-based metabolomic approach. The C18 and hydrophilic interaction liquid chromatography (HILIC) columns were used for the separation of the metabolites in MCF-7 cell lysates. 505 peaks were detected through the HILIC column and 551 peaks were found through the C18 column for the wet lysis technique. This situation supported by the base peak chromatograms showed that the wet lysis technique allowed us to extract higher number of non-polar metabolites. Almost equal number of metabolites was found for the C18 and HILIC columns (697 peaks for the HILIC column and 695 peaks for the C18 column) when the rough lysis technique was used. However, the intensities of polar metabolites were higher for the rough lysis technique on base peak chromatograms for both the HILIC and C18 columns. Although cell lysis technique, which is the first step in the sample preparation for cell culture studies, does not cause dramatic differences in the number of the detected metabolite peaks, it affects the polar and non-polar metabolite ratio significantly. Therefore, it must be considered carefully especially for *in vitro* drug development studies.

Keywords : Cell lysis techniques, Cell membrane, Untargeted metabolomics, Q-TOF LC/MS, XCMS

Introduction

Metabolomics studies can be divided into two classes, which are targeted studies and non-targeted studies known as metabolite profiling. Targeted studies are carried out for the absolute determination of already focused metabolites while metabolite profiling is employed to compare the relative amount of metabolites affected by the environmental conditions including age, diet, and diseases.[1]

For targeted studies, the analytical procedure should be designed to have high accuracy, precision, and selectivity to determine the metabolites of interest in the samples. Data mining procedure in targeted studies is to compare the absolute amount of some metabolites known as biomarkers or the metabolites related to the subject of investigation. Data acquisition for untargeted metabolomics, on the other hand, is useful for handling the undistinguished metabolites. Therefore, the sample preparation technique and analytical methodology may lead to heterogeneity in the results on such evaluation.[2]

Metabolites differ from DNA or proteins, since the physical properties and chemical structures of metabolites are totally different within each other. The only common properties of metabolites are that their molecular masses are less than 1.5 kDa and they are organic compounds.[3]

Thus, it may be challenging to provide a unique analytical platform to determine all of the metabolites in a sample. The advantages and disadvantages of sample preparation techniques prior to liquid chromatography-mass spectrometry (LC-MS)-based metabolite profiling are currently under investigation.[4-7]

The first stage of the sample preparation in cell culture studies is the complete lysis of the cells. Complete lysis refers to the physical disruption of the cell membrane often by viral, thermal, alkaline, detergent-based, enzymatic, or osmotic methods that directly target its integrity to release the total metabolome. In molecular biology, biochemistry, and cell biology laboratories, cell cultures are subjected to lysis before purification of intracellular components such

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as organelles, nucleic acids, metabolites, and proteins.[8-10]

The aim of this study is to compare two different cell lysis methodologies with or without using detergent for sample preparation in terms of liability of the data obtained from untargeted metabolomics studies. MCF-7 breast cancer cell line was used as the sample to perform the analysis and data mining procedures. Cell lysis methodologies applied in the present study were compared to explore the effect of detergent on the recovery of polar and non-polar metabolites using quadrupole time of flight liquid chromatography-mass spectrometry (Q-TOF LC/MS) equipped with the hydrophilic interaction liquid chromatography (HILIC) and C18 columns.

Experimental

Milli-Q water supplied from Barnstead™ Water Purification Systems (Barnstead, USA) was used to prepare the solutions and mobile phases for the liquid chromatography.

Chemicals and reagents

The cells, chemicals, and reagents used for cell culture and lysis procedures are as follows: MCF-7 human breast cancer cell line (ATCC® HTB-22™, USA), complete Dulbecco's Modified Eagle Medium (DMEM) 4.5 g/L high glucose supplemented with 1% penicillin-streptomycin (10,000 U/mL), 1% L-glutamine (200 mM), and 10% heat-inactivated fetal bovine serum (HI-FBS) (Fisher Scientific, USA). The solvents and reagents to prepare the mobile phases were acetonitrile (ACN) (LC-MS grade), formic acid (for LC-MS), and ammonium formate (LC-MS grade) all purchased from Sigma-Aldrich, USA.

Equipment

The equipment used for cell culture and lysis process are as follows: Laminar Flow Cabin (Clean Air Techniek, Holland), Incubator (Panasonic MCO-18 AC-PE, Japan), Adopting light microscope (NIKON Eclipse TS100, USA), Sonicator (Sonics Vibracell VCX-750), and Vortex (Biosan V1-Plus, Litvania). The equipment used for Q-TOF LC/MS-based untargeted metabolomics are Agilent 6530 Quadruple-Time of Flight LC/MS Including ESI Source (Agilent, USA), OptimaMAX Ultracentrifuge (Beckman, USA), Refrigerated Centrifuge model 35R D78532 (Hettich Rotina, Germany), and Vacuum Centrifuge (Labconco, USA).

Software used on data mining procedure of untargeted metabolomics

The list of the software products used on data mining procedure are listed below:

R Project for Statistical Computing (Windows PC Desktop Edition) (https://www.r-project.org)

XCMS (http://xcmsonline.scripps.edu)

IPO: a tool for automated optimization of XCMS parameters (https://bioconductor.org/packages/release/bioc/html/IPO.html)[11]

Cell culture studies

MCF-7 breast cancer cells were cultured in DMEM supplemented with 1% penicillin-streptomycin (10,000 U/mL), 1% L-glutamine (200 mM), and 10% HI-FBS. The cells were preserved in a 37°C incubator under 5% CO_2.

Rough lysis procedure: lysis without detergent

Once cultured MCF-7 cells reached confluency (5×10^7 cells), the media was removed and washed twice with PBS (Fisher Scientific, USA). 3 mL 0.25% Trypsin-EDTA (Fisher Scientific, USA) was added and cells were trypsinized. The cells were resuspended in PBS and the suspension was centrifuged at $250 \times g$ for 5 min and the pellet washed with cold PBS. Following 1 s of centrifugation at $250 \times g$ for 5 min, the pellet was suspended in 1 mL equilibration buffer containing 1X protease inhibitor cocktail (Roche, Germany). By using a thin needle-syringe, the cells were shredded repeatedly, and the solution was homogenized. The homogenate was vortexed vigorously for 30 s and then transferred to an eppendorf tube preserved in ice. Straight away, the sample was subjected to sonication at 4°C using 150 W energy setting for 5 cycles of 10 s application and 50 s of pauses for further homogenization. The samples were centrifuged for 15 min at $14{,}000 \times g$ at 4°C. The supernatant was transferred to a polypropylene tube and subjected to ultracentrifugation at $100{,}100 \times g$ for 1 h at 4°C. The supernatant was collected and kept in -86°C before protein determination. The total protein content was assessed spectrophotometrically by using the BCA assay, following the manufacturer's directions (Abcam, USA).

The wet lysis procedure: detergent-based lysis

The cultured MCF-7 cells were processed as the aforementioned rough lysis approach until the step of suspension of the sample the equilibration buffer. During the wet lysis procedure, the sample was suspended in 1 mL equilibration buffer including 1X protease inhibitor cocktail 4% NP-40 and 3% Tween 20 (Sigma-Aldrich, USA), non-ionic detergents. Following partially homogenization using an eppendorf pestle; the solution was kept on ice for 20 min meanwhile vortexing it vigorously every 5 min. The subsequent homogenization was achieved by sonicating the sample at 4°C using 150 W energy setting for 5 cycles of 5 s. The sample was centrifuged for 15 min at $14{,}000 \times g$ at 4°C. The supernatant was transferred to a polypropylene tube and subjected to ultracentrifugation at $100{,}000 \times g$. The protein concentration was determined by carrying out the BCA assay and the sample was kept in -86°C before the

identification of the proteins.

Sample preparation for Q-TOF LC/MS-based metabolomics

300 μL pooled cell suspension of three replicates was added into Microcon-3kDa Centrifugal Filter Unit (Sigma Aldrich, USA) and centrifuged at 15,000 × *g* for 60 min. The extracted liquid phases were collected and evaporated under the vacuum centrifuge for 180 min at 4°C and then dissolved in 1.2 mL of ACN. Samples were divided into six vials. The first three vials were prepared in identical concentrations [40 μL sample (re-dissolved with mobile phase) + 40 μL mobile phase] and pooled to eliminate the random errors originated from sample preparation. Other three vials were prepared by serial dilution to undergo regression analysis to the peaks (vial 4: 30 μL sample + 50 μL mobile phase, vial 5: 20 μL sample + 60 μL mobile phase, and vial 6: 10 μL sample + 70 μL mobile phase). Thus, the reproducibility of the method was ensured and the unreliable peaks with the prepared regression equations based on consecutive dilution results were eliminated. The peaks not matched the criteria (R>0.90) were discarded.

Q-TOF LC/MS analysis of metabolites using the C18 column

Mass spectrometry analysis was performed by employing the Agilent 6530 LC/MS Q-TOF instrument (Agilent Technologies, USA). The C18 column (Waters X-Select C18 3.5 μM, 100 × 4.6 mm) was used as the chromatography column. Mobile phases were water and ACN, both consisting of 0.1% formic acid. Flow (0.20 mL min^{-1}) started with 90% H_2O until min 1, the ACN ratio was elevated linearly to 90% until min 12. The chromatographic conditions were kept stable between 12 and 13 min. Later, the starting conditions were retailed linearly until min 20 and a 5-min post-run was applied for further injections. The scan range for the MS device was 100-1700 *m/z*. All samples were injected into the system as duplicates in a random order. The column and the gas temperatures were adjusted to be 30°C and 350°C, respectively. The capillary voltage was 4000V and the mass spectrometer equipped with an ESI probe was operated in positive (ESI+) ion mode.

Q-TOF LC/MS analysis of metabolites using the HILIC column

The HILIC column (Agilent Zorbax HILIC Plus 1.8 μm, 50 × 2.1 mm) was used as the chromatography column. Mobile phases were 10 mM ammonium formate buffer (pH) and ACN. Flow (0.20 mL min^{-1}) was initiated with 90% ACN until min 1, the buffer ratio was escalated linearly to 90% until min 12. The chromatographic conditions were kept stable between 12 and 13 min. Later the initial conditions were re-adopted until min 20 and a 5-min post-run was applied for further injections. The scan range for the MS device was 100-1700 *m/z*. All samples were injected into the system as duplicates in a blinded order. The column/gas temperature, capillary voltage, and the probe conditions were chosen to be the same as the run conditions with the C18 column.

Results and Discussion

In this study, MCF-7 breast cancer cells were used as a cell line model to compare the results. The reason why MCF-7 was used in this study as the model was that breast cancer is the leading cause of death among women worldwide and numerous strategies to develop therapeutic or preventive agents include cell culture studies. Nowadays, it is known that genome or proteome analyses alone are not sufficient to elucidate the molecular mechanism of diseases. In contrast, a holistic evaluation that consists of metabolomics studies provide more rational and reliable data. Profiling the whole metabolome with a single injection is not an easy process since the chemical and physical properties of each metabolite are distinct. Moreover, the analytical methodologies and data mining procedures require a cautious effort to make such approaches dependable. One of the major challenges of metabolomics is proper sample preparation. Advanced analytical methodologies including Q-TOF LC/MS may be helpful for analyzing metabolites in a high precision. However, the capability of the analysis is usually related to the content of the sample which is directly correlated with the first step of the sample preparation, the lysis of the cells. The base peak chromatograms (BPC) obtained for Q-TOF LC/MS analysis of the lysates of MCF-7 cells for wet lysis and rough lysis techniques are given in Figure 1.

As it is seen from the chromatograms, the elution of the peaks for the C18 column starts within 8 min. The elution of polar metabolites makes base peaks intensities higher for rough lysis technique for the peaks between 8 and 10 min (Figure 1A and 1C). As expected, rough lysis technique allowed us to extract more polar metabolites while wet lysis technique led to the extraction of less polar components. The polar metabolites on reverse-phase HPLC eluted in a shorter retention time than the non-polar metabolites and this event could be observed directly on chromatograms.

The recently introduced LC system, termed as HILIC, provides an alternative approach to separate small polar compounds effectively on polar stationary phases.[12] For historical reasons, it has been reported that the HILIC is a variant of normal-phase liquid chromatography, but the separation mechanism used in the HILIC is more complicated than it is in the normal-phase HPLC.[13] In the literature, various studies reported the usage of HILIC on metabolomics.[14-16] The basic advantage of the HILIC over the C18 in metabolomics is its coverage of some compounds suitable for both normal and reverse-phase

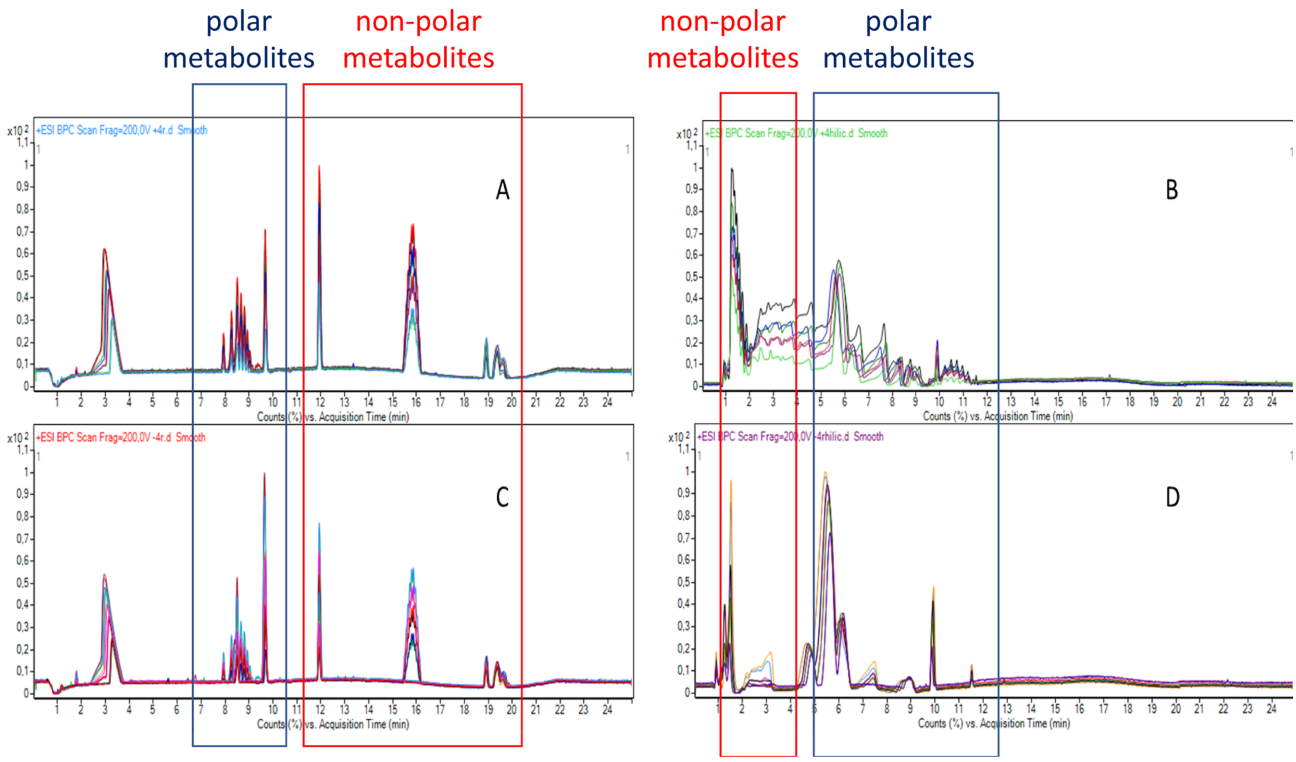

Figure 1. Base peak chromatograms for the MCF-7 samples prepared wet lysis and rough lysis techniques. A: Wet Lysis - C18 column, B: Wet Lysis- HILIC column, C: Rough Lysis- C18 column, D: Rough Lysis - HILIC column.

Table 1. Number of metabolites scored by the choice of the physical or the detergent-based lysis method.

Lysis Method	HILIC Positive Mode	C18 Positive Mode
Wet Lysis	505	551
Rough Lysis	697	695

HPLC and its potential on the analysis of polar-ionic compounds.

In the present study, data analysis was performed based on the previously described strategies.[17] The total number of the metabolite peaks detected following the wet and rough lysis techniques for the HILIC and C18 columns were found. The results are tabulated in Table 1.

As it may be observed from the results, 505 peaks were detected on the HILIC column and 551 peaks were found on the C18 column after the application of the wet lysis technique. As it was expected, the total number of the detected metabolite peaks was higher in the C18 column for this particular approach. Besides, the intensity of non-polar metabolites was relatively higher on the C18 column (Figure 1A) when compared with those obtained through the rough lysis technique (Figure 1C). The number of the detected peaks for rough lysis technique on the HILIC and C18 columns were almost identical to be 697 and 695 peaks, respectively. However, the BPCs obtained on the HILIC column for wet and rough lysis techniques could be defined as different chromatograms (Figure 1B and 1D). The intensity ratios of polar-ionic metabolites which are suitable to be separated through the mechanism on the HILIC were relatively higher with the rough lysis technique (Figure 1D) comparing to the wet lysis technique (Figure 1B) on the HILIC column. This situation was also supported by the number of the detected metabolites on the rough lysis technique (697) and the wet lysis technique (505) when the HILIC column was used.

These situations could indicate that the wet lysis technique allowed us to extract a higher number of non-polar metabolites and rough lysis technique could extract relatively polar metabolites that are more favorable to be separated through the HILIC column.

In regular, large amounts of data from high-throughput metabolomic experiments are commonly visualized using a principal component analysis (PCA) two-dimensional scores plot. the PCA graphs for the wet and rough lysis techniques on the C18 and HILIC columns are given in Figure 2.

Based on the PCA results, it could be concluded that not only the numbers of the detected peaks were different according to the lysis technique altered; but also the peaks were quantitatively changed. Thus, the PCA graphs

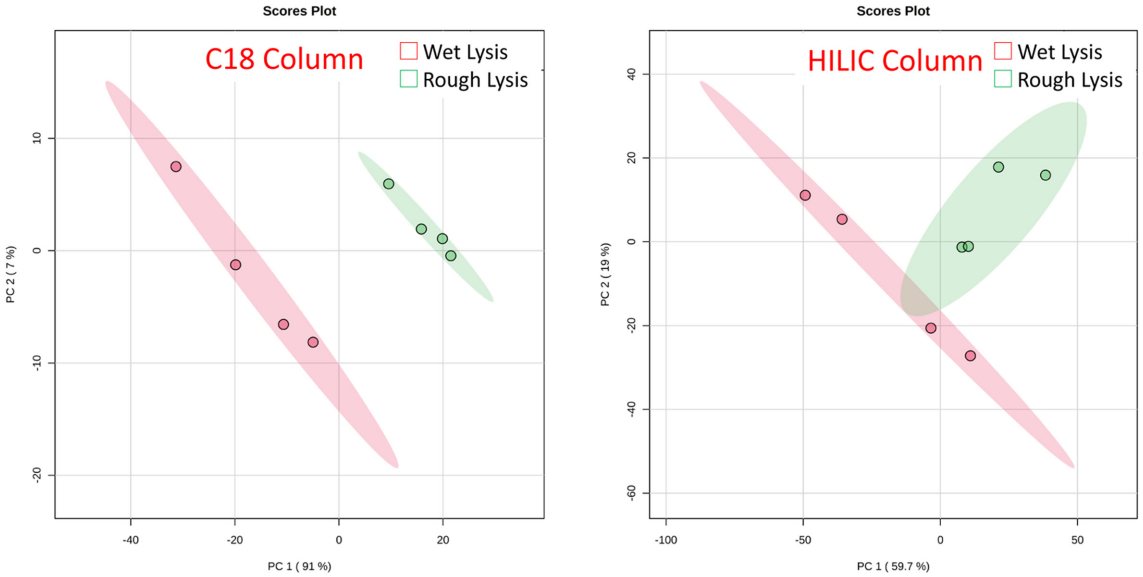

Figure 2. PCA results to present the effect of lysis techniques in metabolome level.

statistically confirmed the virtual data on BPCs.

In the present study, we focused to discriminate the two techniques "on the edge" to obtain a sharp and precise difference when it comes to evaluating metabolomics data. However, in the wet lysis procedure, the detergents used were chosen to be NP-40 and Tween 20, both non-ionic surfactants and emulsifiers, added at a final concentration of 4% and 3%, respectively. Unlike aggressive and ionic detergents, these two did not interfere with the macromolecules in the lysate, but still disintegrated the cell and nuclear membrane adequately, and most importantly, they did not fluctuate the ionic balance of proteins detected. Although the effect of some other detergents used on cell lysis might be evaluated on the metabolome level, the methodology for wet lysis used in this study is a common approach and is known to be compatible with multi-omics studies.

Conclusions

Our data showed that the researchers working on Q-TOF LC/MS-based untargeted metabolomics should carefully consider the cell lysis procedures for the *in vitro* cell culture studies. Since the lysis of the cells is the first step of the cell culture studies to launch a novel agent against cancer as well as to evaluate the effect of the components that are already established. In conclusion, the cell lysis technique is a crucial parameter affecting the results of the untargeted metabolomic approaches performed on cell cultures. The impact of some other common detergents used for the wet lysis technique and ion suppression for these detergents might be investigated individually on further studies.

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