International Journal on Recent and Innovation Trends in Computing and Communication
ISSN: 2321-8169 Volume: 11 Issue: 9
Article Received: *25 July 2023* ***Revised:*** *12 September 2023* ***Accepted:*** *30 September 2023*

Comparative Metabolite Extraction Protocols from Breast Cancer Mouse Lung Tissue for LC-MS/MS Analysis

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Abstract— Triple-negative breast cancer (TNBC) stands out for its heightened invasiveness, leading to distant metastasis in nearly 46% of cases, with common targets being the brain, lungs, and bones. This subtype is associated with significantly shorter median survival compared to other breast cancer types. Analyzing metabolic compounds in lung tissues affected by breast cancer metastasis provides valuable insights into biological information and regulatory processes. Despite the recognized severity of TNBC spreading to other sites, there are limited reported studies investigating metabolome information in distant organ tissues, particularly the lungs. Therefore, accurately quantifying the abundance of metabolites requires careful extraction procedures. This study aims to investigate and compare extraction protocols for lung tissue metabolites in TNBC mice using liquid chromatography with tandem mass spectrometry (LC-MS/MS). Left lung tissues were collected from mice xenografted with breast cancer. Three different extraction methods were evaluated to assess their metabolite coverage and biochemical compound classes. Our findings revealed distinct differences in metabolite compositions among the three methods. The extraction solvent comprising isopropanol, acetonitrile, and water in a 3:2:2 ratio proved most suitable for studying breast cancer metastasis to lung tissues. This extraction solvent could serve as a protocol for future studies analyzing the lung cancer metabolome in mice.

Index Terms— Metabolomics, Extraction methods, Breast cancer, Lung metastasis

I INTRODUCTION

Metabolomics represents an interdisciplinary research field in which diverse methodologies and technologies continue to develop. This field endeavors to comprehensively elucidate alterations in enzymatic and proteinaceous compound production, collectively known as the metabolome. Such insights offer a nuanced understanding of molecular processes. Widely employed in modern research, metabolomics serves as a potent tool for investigating metabolite biomarkers implicated in various pathologies, including but not limited to diabetes, asthma, and cancer, predominantly through untargeted metabolomic approaches.

Triple-negative breast cancer (TNBC) is a subtype of breast cancer associated with a significant 40% mortality rate within 5 years of diagnosis [1]. TNBC is characterized by high invasiveness, leading to distant metastasis in about 46% of cases, with common occurrences in the brain, lungs, and bones [2],[3]. Following metastasis, the median survival duration is a mere 13.3 months, coupled with a noteworthy post-surgical recurrence rate of 25% [4]. In comparison, non-TNBC patients typically face a more extended average relapse period ranging from 35 to 67 months, highlighting the relatively shorter duration of 19 to 40 months for TNBC patients [5]. Moreover, the mortality rate escalates dramatically to approximately 75% among TNBC patients within a 3-month timeframe following recurrence [6]. Although the recognized severity of TNBC spreads to other sites, few reported studies investigate metabolome information in distant organ tissues, particularly the lungs.

Nowadays, there is no standardized approach for preparing lung tissue to achieve comprehensive profiling of metabolites. There are several reported methodologies, including metabolite extraction and data acquisition. In the study of lung metabolic profiling, gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance spectroscopy (NMR), and liquid chromatography-mass spectrometry (LC-MS) are the most frequently used as analytical platforms. The LC-MS platform was chosen for this research because it is a robust analytical instrument capable of identifying, validating, and measuring various molecules, offering diverse applications across multiple research domains. In contrast to GC-MS, LC-MS analysis is more cost-effective, requires more straightforward sample preparation and offers both high-resolution and rapid

We thank Dr. Bumpenporn Sanannam, Faculty of Medicine, Thammasat University, for assistance in lung sample collection. This work was supported by Development and Promotion of Science and Technology Talents Project, Thai government scholarship (DPST), and Center of Excellence in Systems Microbiology (CESM), Department of Biochemistry, Faculty of Medicine, Chulalongkorn University.

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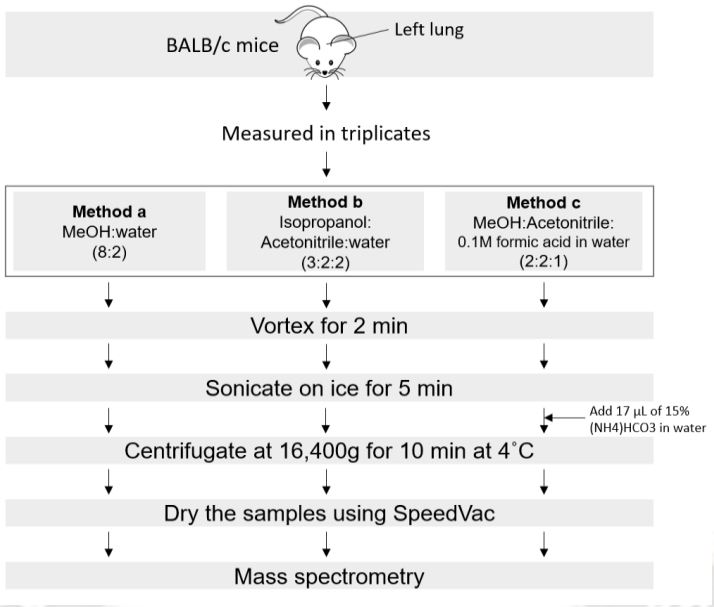

Figure 1 Overview of the experimental setup and extraction procedure. Each type of sample underwent measurement in triplicate across the various extraction protocols.

detection of analytes [7]. Moreover, the GC-MS study can only identify approximately 100 metabolites, whereas LC-MS can detect nearly 500 metabolites [8]. Additionally, LC-MS methods have shown effective separation of numerous metabolites with high efficiency in diverse samples without compromising sensitivity [9].

Several chemical solvents are used in different compositions and percentages for metabolite extraction, such as methanol/water, acetonitrile (ACN), and a mixture of isopropanol (IPA)/ACN/water [10],[11]. Most TNBC metastasis metabolomic research primarily focuses on examining blood and tumor tissue. Considering this, it is essential to examine preparation methods for the lung tissue that TNBC metastasizes to.

This study aims to investigate metabolite extraction protocols from lung tissues in TNBC mice. We opted for three distinct extraction solvents and concentrations that had been widely published and used. We analyzed the outcomes of their metabolite coverage by comparing them across the three procedures. The results of this investigation can contribute to the analysis of the lung tissue metabolome, offering insights into the functional reflections and enhancing our understanding of the impact of TNBC metastasis on mouse lungs.

II MATERIAL AND METHODS

A. Sample collection

The animal care and handling protocols received approval from the Institutional Animal Care and Use Committee (IACUC) at Chulalongkorn University Laboratory Animal Center in Bangkok, Thailand, under Protocol Review Number 2173024.

Left lung specimens were collected from three BALB/c breast cancer xenografted mice. These samples were obtained after the transplantation of 4T1 cells over 5 weeks. Subsequently, the collected specimens were rapidly snap-frozen in liquid nitrogen and preserved at -80°C until further analysis. frozen in liquid nitrogen and preserved at -80°C until further analysis.

B. Metabolite extraction solvent and sample preparation

Three extraction techniques were chosen and adapted, following established procedures for analyzing tissue metabolomes. The extraction solvents were MeOH: water in an 8:2 ratio (method a), IPA: ACN: water in a 3:2:3 ratio (method b), and MeOH: ACN: 0.1M formic acid in water in a 2:2:1 ratio (method c) [10],[11].

Samples of the left lung from each mouse were weighed at 30 mg and homogenized for 5 minutes at 30 Hz using 1.6 mm stainless steel beads. The homogenized material was then transferred into three new tubes. Metabolite extraction solvents (a, b, and c) were added to each Eppendorf (Figure 1). After vortexing and sonicating for 2 and 5 minutes, respectively, the mixture of tissue and solvent was centrifuged at 16,400g for 10 min at 4°C. The supernatants were then dried using SpeedVac for 4 h. and stored at -80˚C until utilized.

C. Liquid chromatography-mass spectrometry (LC-MS) analysis

Samples for metabolite extraction were prepared for injection into a Dionex Ultimate 3000 HPLC coupled with an Orbitrap Q Exactive Focus mass spectrometer from Thermo Fisher Scientific, Inc. In summary, 20 µg of supernatant was moved to an HPLC vial by Agilent Technologies, Inc. The separation occurred on a C18 reversed-phase column (Poroshell 120 EC-C18, 2.7 µm x 100 mm, Agilent, USA). Solvent A, composed of 0.1%

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formic acid in water, and solvent B, consisting of 0.1% formic acid in 100% acetonitrile, were used in the analytical column. Metabolites were eluted through a 5-95% gradient of solvent B at a constant flow rate of 0.25 ml/min for 45 min. The elution gradient included phases of 5% mobile phase B for 5 minutes (0.0-5.0 min), 5%-95% B for 40 minutes (5.0-45.0 min), 95%-100% B for 1 minute (45.0-46.0 min), 100% B for 9 minutes (46.0-55.0 min), 100%-5% B for 1 minute (55.0-56.0 min), and 5% B for 9 minutes (56.0-65.0 min). Electrospray ionization was executed at a voltage of 3 kV. Additional electrospray ionization parameters were set as follows: Sheath gas flow at 30 arbitrary units, auxiliary gas flow at 10 L/min, capillary temperature at 350˚C, S-lens RF level at 60, and the auxiliary gas heater temperature maintained at 300˚C. Scanning occurred in the mass range of m/z 80 to 1,000 in both MS1 and MS2 analyses.

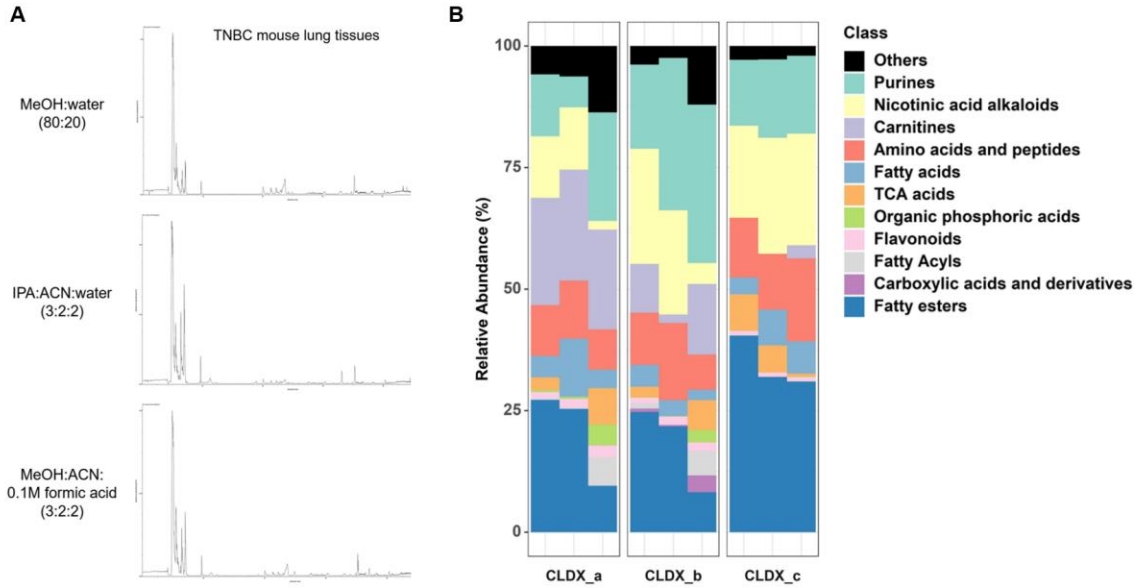

Figure 2 (A) Total ion chromatograms (TIC). Three TICs represent the lung metabolome, employing three distinct extraction solvents. (B) Relative abundance of mouse lung metabolite abundance obtained via various extraction methodologies

D. Metabolite identification, data processing, and statistical analysis

The raw MS data files in *.raw format were converted to the Analysis Base Framework (*.abf) format using the ABF Converter (https://swharden.com/software/AbfConvert/). Subsequently, these converted files were imported into MS-DIAL software for analysis, specifically using version 5.1 (http://prime.psc.riken.jp/compms/msdial/main.html).

The following MS-DIAL analysis parameters were chosen: The tolerances for MS1 and MS2 were set at 0.01 Da and 0.025 Da, respectively. The mass range of 80–1,000 Da was retained for 5–45 min. A minimum peak height of 10,000 and a mass slice width of 0.1 Da were used for peak identification. Using a mass tolerance of 0.01 Da for MS1 and 0.05 Da for MS2, along with an identification score cutoff of 75%, metabolite identification was carried out for both positive and negative mode analyses using the standards+bio+in silico databases (http://prime.psc.riken.jp/compms/msdial/main.html#MSP). The maximum tolerance for retention time was set at 0.1 minutes for peak alignment. Unidentified peaks were eliminated from the analysis. Metabolite information was omitted if the peak heights in 30% of the samples were below the mean of the blank samples. Subsequently, the remaining annotations obtained from MS2 were chosen and checked through MS-FINDER version 3.60 (http://prime.psc.riken.jp/compms/msfinder/main.html). Only metabolites with a score exceeding seven were considered. Subsequently, drugs and their corresponding metabolites were systematically excluded.

The collected intensity data was performed a log2 transformation. Before conducting a two-tailed Student's t-test for group comparisons, the normality of the distributions was assessed using the Shapiro–Wilk test. A significance level of P-value < 0.05 was used to determine statistical significance. The visualizations including statistical significance. The visualizations, including two-dimensional projections of Sparse partial least squares discriminant analysis (sPLS-DA), bar plots, volcano plotst-test for group comparisons, the normality of the distributions was assessed using the Shapiro–Wilk test. A significance level of *P*-value < 0.05 was used to determine statistical significance. The visualizations including two-dimensional projections of Sparse partial least squares discriminant analysis (sPLS-DA), bar plots, volcano plots, heatmap, and box plots, were generated by mixOmics, ggplot2, pheatmap, and ggstatsplot R packages.

III RESULTS AND DISCUSSION

This study was the first to evaluate the different extraction methods for studying breast cancer lung metastasis metabolome using LC-MS/MS. After data cleaning steps, the

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metabolome data contained 54 metabolites for 3 mice. The identified metabolites were subdivided into twenty-one main biochemical classes: amino acids and peptides, fatty acids, fatty esters, purines and pyrimidines.

A. Overall LC-MS/MS chromatogram and quantification depend on the extraction protocol

The representative total ion chromatogram (TIC) for the lung tissue extraction resulting from the three different extraction methods is shown in Figure 2A. The discrepancy distribution of chromatograms was investigated in TNBC mouse lung tissues. The number of peaks in the chromatogram among the three extraction solutions was similar.

After peak deconvolution and identification, over 130 peaks were detected in positive and negative modes before

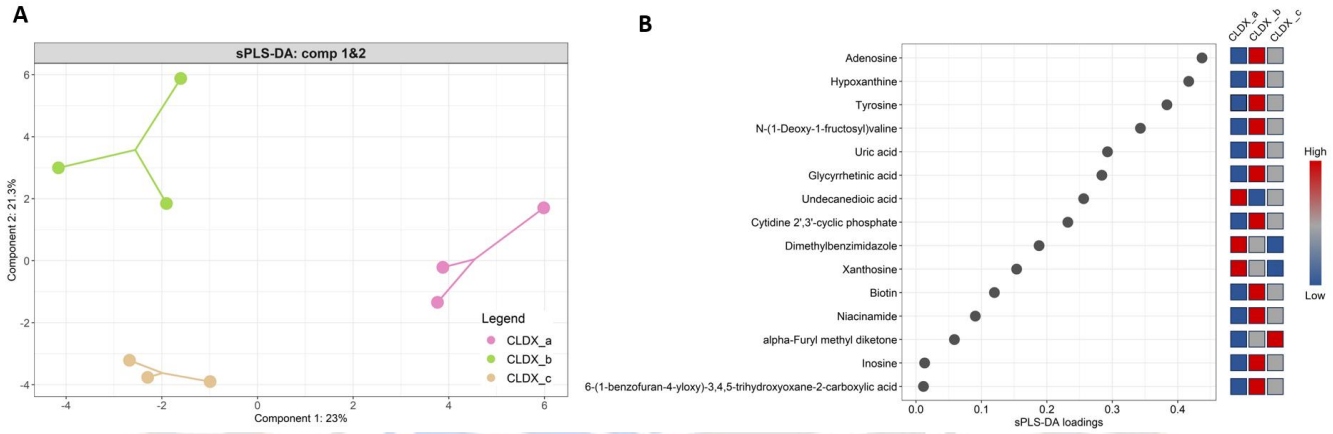

Figure 3 (A) Sparse partial least squares-discriminant analysis (sPLS-DA). The plot of the two-component sPLS-DA model exhibited clustering of stool samples based on three different extraction methods. (B) Loading plots of 15 metabolites exhibit the most significant separation among the four groups for component 1.

54 metabolites were chosen after data cleaning. Of the 54 metabolites, we classified them into twenty-one main classes. The highest abundance of metabolite classes were carnitines, purines and fatty esters following extraction with MeOH:water (8:2), IPA:ACN:water (3:2:2) and MeOH:ACN:0.1M formic acid in water (2:2:1) shown in Figure 2B.

B. Three different extraction methods show a distinct metabolic profile

Sparse partial least squares discriminant analysis (sPLS-DA) was performed to discern the metabolite profile signature linked to three distinct extraction protocols in xenografted mice. The study demonstrated a distinct separation among methods a, b, and c along PC1 (23%) and PC2 (21.3%) for mice with TNBC (Figure 3A). The loading plot for the first component of the cancer mouse group revealed adenosine, hypoxanthine, tyrosine, N-(1-Deoxy-1-fructosyl)valine, uric acid, glycyrrhetinic acid, and undecanedioic acid as significant factors contributing to the observed separation (loading score ≥ 0.25) (Figure 3B).

C. Significant metabolite profile of three different methods

Volcano plots are reported in Figure 4 using a two-tailed Student's t-test at a *P*-value lower than 0.05. The 24 metabolites were found to be significantly different between the compared methods. Among these metabolites, metabolites related to the purine class, including 5'-S-Methylthioadenosine, adenosine, guanosine, hypoxanthine, uric acid, and xanthosine, were highlighted to be a high expression in method b. At the same time, metabolites related to the amino acids and peptide class, gamma-glutamylleucine, glutamylglutamine, glutathione, N2-gamma-Glutamylglutamine, and tyrosine had high expression in methods a and b (Figure 5).

D. Purine and glutathione metabolism in cancer

Volca

Method b exhibited high expression levels in both amino acid and peptide and purine metabolite classes, which are associated with glutathione and purine metabolisms. Because purine nucleotides are essential and required for tumor cell proliferation, purines and enzymes for the de novo purine biosynthesis pathway are elevated in tumor cells [12],[13]. Purines are significant modulators of immune cell response and cytokine production via numerous receptor subtypes, which play an important role in the development of oncogenesis and tumorigenesis [14],[15].
development of oncogenesis and tumorigenesis [14],[15].
To illustrate, adenosine regulates neutrophil activity and their interactions with pathogens [16]. Mutations or insufficient adenosine deaminase, a critical enzyme for purine metabolite breakdown, may increase susceptibility to infections and autoimmunity. Additionally, adenosine deaminase activity has been utilized as a diagnostic marker for malignancies [17].

Another importance is glutathione metabolism. This process occurs only in the cytosol, where the two enzymes, including glutamate-cysteine ligase and glutathione synthetase, are found in animals. Glutamate cysteine ligase initiates the first step of the pathway that connects glutamate

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and cysteine via the gamma-carboxyl residue of cysteine to generate gamma-glutamylcysteine [18]. Numerous types of malignancies, such as ovarian, breast, and lung, regularly demonstrate higher levels of glutathione metabolism9. Changes in GSH levels in malignant cells have been indicated as necessary for tumor formation and medication resistance [19],[20].

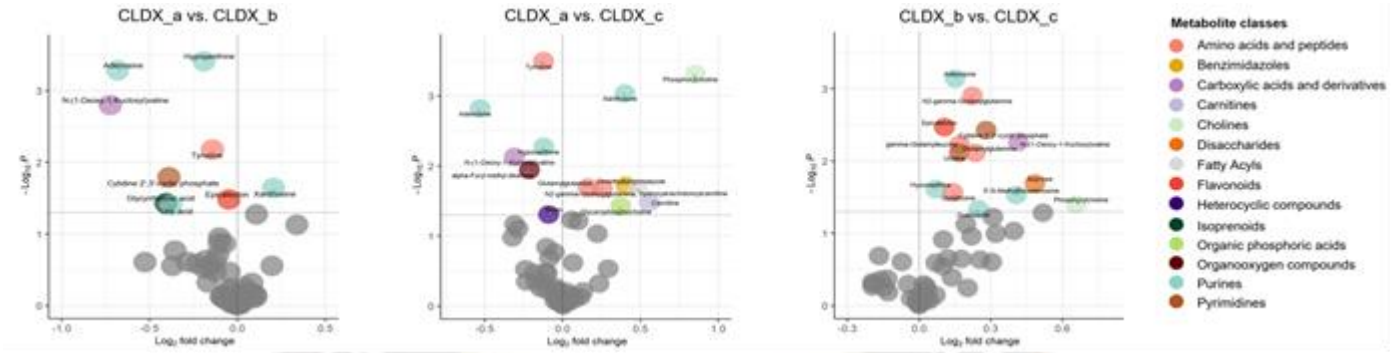

FIGURE 4 VOLCANO PLOT. EACH COLOR REPRESENTS A METABOLITE CLASS WITH UP- AND DOWN-REGULATED METABOLITES (P-VALUE < 0.05).

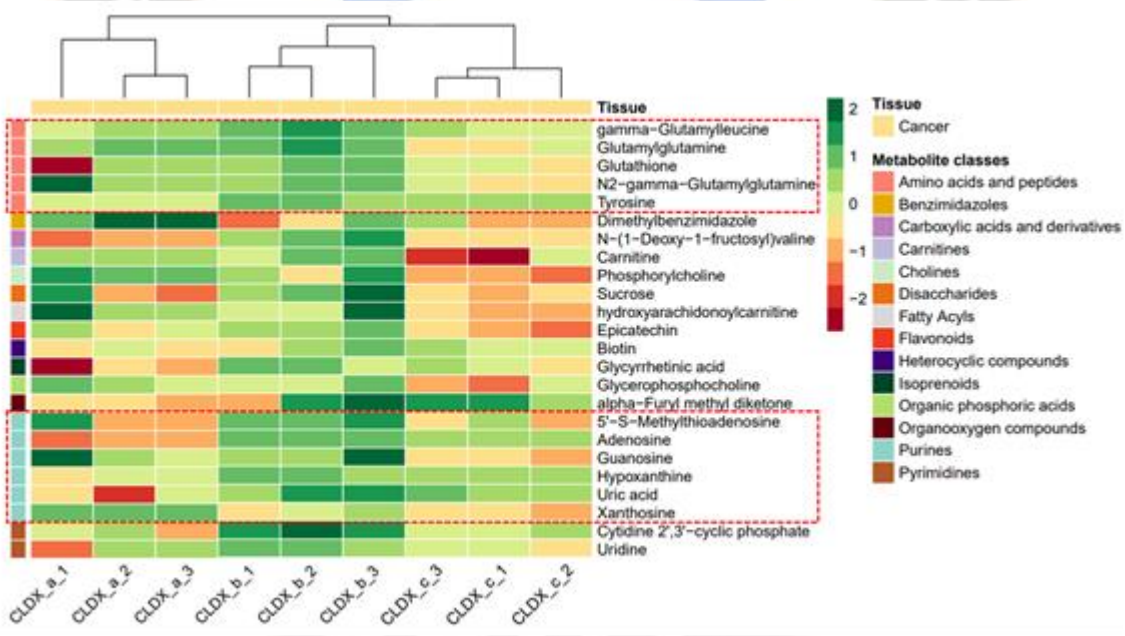

FIGURE 5 THE HEAT MAP DISPLAYS METABOLITES THAT EXHIBITED SIGNIFICANT DIFFERENCES ACROSS VARIOUS SOLUTIONS IN LUNG SAMPLES, WITH GREEN COLORS INDICATING HIGHER METABOLITE ABUNDANCE AND RED COLORS INDICATING LOWER METABOLITE ABUNDANCE.

CONCLUSION

In summary, this study investigates the extraction solvent for analyzing TNBC lung metastasis tissues of mice using LC-MS/MS. Our findings suggest that of three extraction methods, IPA: ACN: water (3:2:3) had the highest efficiency for extracting metabolite classes in purine and amino acids and peptides from lung cancer tissues. Nevertheless, future studies should explore additional solvent methods and test them on various types of organ tissues to which cancer cells metastasize.

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