

# Liquid Chromatography-Mass Spectrometry Metabolic and Lipidomic Sample Preparation Workflow for Suspension-Cultured Mammalian Cells using Jurkat T lymphocyte Cells

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## Abstract

Metabolomics is the comprehensive study of metabolism as it pertains to an organism or biological system. Lipidomics, a subset discipline of metabolomics, encompasses the study of cellular lipid functions: including pathways, networks, and interactions. The abundance of metabolites and lipids, along with their contribution to health and disease, makes metabolomics a valuable tool for biomarker research. Disease biomarker identification requires a reproducible, sensitive, and accurate analytical platform. Although transcriptomic and proteomic areas have well-established protocols for sample preparation and data processing, the metabolomics field is still developing comparable standardized conventions. Furthermore, of the few comparative LC-MS metabolomic studies that have been applied to mammalian cell cultures, most are targeted to adherent cell lines. The purpose of this work was to optimize a sample preparation workflow for the cellular metabolomic analysis of suspension-cultured mammalian cells using commercially available Jurkat T lymphocytes as a model system. The current investigation evaluated commonly used sample preparation techniques for reproducibility, accuracy, and applicability to untargeted biomarker discovery. Results show ammoniated cell rinsing solutions to be an effective means to remove extracellular components present in the media without causing ion suppression or affecting the integrity of the cellular membrane. Additionally, a novel workflow was designed to allow for the combined analysis of metabolites and lipids from mammalian suspension cells from a single cell pellet. The Folch lipid extraction protocol was coupled to an 80% MeOH metabolite isolation to ensure high extraction efficiency for phospholipids and triacylglycerides. While the workflow was tailored to cells in suspension, it could also be applied to adherent cell lines.

**Keywords:** Sample preparation; Metabolomics; Lipidomics; Mammalian suspension cells

**Abbreviations:** LC-MS: Liquid Chromatography-Mass Spectrometry; MeOH: Methanol; MTBE: Methyl Tert-Butyl Ether; ISTD: Internal Standard; CHCl<sub>3</sub>: Chloroform; H<sub>2</sub>O: Water; IPA: Isopropanol; TG: Triacylglyceride; LPC: Lysophosphatidylcholine; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PS: Phosphatidylserine; PG: Phosphatidylglycerol; BD: Bligh-Dyer; ABD: Acidified Bligh-Dyer

## Introduction

Metabolomics is the comprehensive study of metabolism as it pertains to an organism or living biological system in response to a stimulus, a pathophysiological condition, or genetic modification [1-5]. Lipidomics, is a subset discipline that encompasses the study of cellular lipid pathways, networks, functions, and interactions. Cellular metabolomics has evolved into an important technique for analyzing the biochemical response of metabolites and lipids within representative cell cultures [6]. Metabolites and lipids, although chemically and physically diverse (e.g., molecular weight, polarity, acidity, stability, and function), represent the naturally occurring biological events within a cell and the phenotypic state of a biological system [1-3,5,7-9]. Metabolism, regardless of the sample type, is an essential component of cellular function as it encompasses the up-stream interactions between the genome, transcriptome, and proteome [4,5,10]. Examination of involved endogenous metabolites and lipids provides insight into the affected metabolic pathways in biological processes and the fine detailed information of disease perturbations [2,4-6,10].

Mass spectrometry has developed into an analytical tool of choice in metabolomics due to increasing instrument speeds, wider dynamic

signal ranges, and enhanced quantitative capabilities [11]. Coupling mass spectrometry with chromatographic separation methods such as liquid chromatography has allowed for reliable identification of low-level metabolites and lipids in complex mixtures [1,3,11,12].

Metabolomic approaches for biomarker discovery can include targeted or untargeted studies. The latter focuses on a comprehensive or global analysis of primary and secondary metabolites and/or lipids, whereas targeted studies analyze predefined selections of compounds originating from a specific metabolic pathway to better understand a biological event [3,9,12]. Sample preparation is the first step in ensuring reproducibility and accuracy for untargeted biomarker discovery. All samples undergoing mass spectrometric analysis should be prepared in an unbiased and reproducible manner that maintains the integrity of intracellular compounds [7]. Each 'omics' field (e.g., genomics, proteomics, metabolomics, and lipidomics) has its own unique considerations in sample preparation [13]. Because metabolomics is a relatively new discipline, standard protocols for sample preparation

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of suspension-cultured mammalian cells such as those available for proteomics and genomics analyses have not been fully vetted [2,5,13]. Additionally, cellular metabolomics protocols should be tailored for specific mammalian cell lines (adherent or suspension cells) [1,2,7,14].

Lipids must be extracted to investigate changes in composition influenced by cell growth, differentiation, and intracellular signaling processes [8]. Historically, the Folch lipid extraction method (2:1 chloroform/methanol mixture) was established to isolate brain lipids. This method along with the Bligh-Dyer lipid extraction method (1:2 chloroform/methanol mixture) are two of the most common extraction systems to recover lipids in the chloroform layer [8]. Both methods can be applied to cells. Previous research has shown other extraction methods such as a single-phase butanol, isopropanol/hexane mixture, or isopropanol/chloroform mixture to exhibit high extraction efficiency for cholesterol, but a low recovery for triacylglycerides. The chloroform/methanol mixture systems, compared to those previously listed, have high extraction efficiencies for phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), and triacylglyceride (TG) groups [8]. Without comparative studies, it is difficult to evaluate sample-dependent procedures found in the literature for lipid recovery of a wide range of compounds [6].

Thus, this work presents a comparative evaluation of select mammalian cell sample preparation methods and lipid extraction procedures using relative LC-MS quantification of exogenous standards and endogenous compounds. These findings are used to develop an efficient and reproducible sample preparation workflow to measure cellular metabolite and lipid concentrations in an unbiased manner [15]. Because the metabolite and lipid contents are often analyzed from separate aliquots of a cell suspension, a novel workflow was designed to allow for the combined analysis of metabolites and lipids from a single cell pellet.

## Materials and Methods

### Materials

Roswell Park Memorial Institute (RPMI) 1640 medium (10-043-CV), fetal bovine serum (FBS), phosphate buffered saline (PBS), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 1M) were purchased from Corning, Inc. Sodium pyruvate (100 mM), ammonium formate, sodium chloride, exogenous triacylglyceride (TG 15:0/15:0/15:0 and TG 17:0/17:0/17:0) lipid standards, and D-glucose were purchased from Sigma-Aldrich. Exogenous lysophosphatidylcholine (LPC 17:0 and LPC 19:0), phosphatidylcholine (PC 17:0/17:0 and PC 19:0/19:0), phosphatidylethanolamine (PE 15:0/15:0 and PE 17:0/17:0), phosphatidylserine (PS 14:0/14:0 and PS 17:0/17:0), and phosphatidylglycerol (PG 14:0/14:0 and PG 17:0/17:0) lipid standards were purchased from Avanti Polar Lipids. All lipid standards were diluted prior to analysis in 1:2 (v/v) chloroform:methanol (CHCl<sub>3</sub>:MeOH) and a working 100 ppm standard mix was then prepared by diluting the stock solution with the same solvent mixture. The following isotopic standards were purchased from C/D/N isotopes: creatine-D3 H<sub>2</sub>O (methyl-d<sub>3</sub>), D-leucine-D10, L-tryptophan-2,3,3-D3, and caffeine-D3 (1-methyl-d<sub>3</sub>). Ammonium acetate and all analytical grade solvents (formic acid, chloroform, methyl-tert butyl ether, and methanol) were purchased from Fisher-Scientific. All mobile phase solvents were Fisher Optima LC/MS grade (acetonitrile, isopropanol, and water).

### Cell culture

Jurkat cells (Clone E6-1) were purchased as a frozen cell culture

from ATCC (Manassas, VA) and grown to confluence according to the manufacturer's recommendations. Jurkat cells were maintained as a continuous cell culture ( $1 \times 10^5$  to  $1 \times 10^6$  viable cells/mL) in sterile RPMI 1640 medium supplemented with 10% FBS, 5 mM D-glucose, 10 mM HEPES, and 1 mM sodium pyruvate. Cells were maintained at 37°C in an atmosphere containing 5% CO<sub>2</sub>. Cell viability was determined by the trypan blue exclusion test before reaching confluency and prior to metabolite/lipid extraction. Once confluent but still in the logarithmic growth phase, triplicate cell pellets were washed 3x with one of the ice cold rinsing solvents (0.3% ammonium acetate, 0.3% ammonium formate, 0.9% NaCl, 1 M PBS, and 100 mM HEPES), and centrifuged at 311 x g for 5 min. The supernatant was discarded and the cell pellet was dried under nitrogen. The dried cell pellet was stored at -80°C until extraction. Samples were kept on ice from collection to metabolite extraction to prevent metabolite turnover or a change in the metabolite profile.

### Protein precipitation for metabolite isolation

A 15 ppm exogenous, isotopically labeled metabolite standard mix (creatine-D3; D-leucine-D10; L-tryptophan-2,3,3-D3; and caffeine-D3) was spiked into cell pellets (9 μL) washed with one of the four rinsing solutions. The extraction buffer (ice cold 80% methanol, 2 mL) was added to the cell pellet. The suspension was incubated on ice for 5 min and centrifuged at 311 x g for 5 min. The supernatant was removed using a pipette, transferred to a new eppendorf tube, and then dried under nitrogen. Samples were reconstituted in 30 μL of 0.1% formic acid in water, vortexed briefly, and centrifuged at 311 x g for 5 min at 4°C to remove any insoluble components. The supernatant was transferred to an LC vial with a glass insert (Fisher Scientific) and then used for metabolomic analysis.

### Lipid extraction from Jurkat T-lymphocyte cells

Pelleted Jurkat cells were suspended in 0.3% ammonium formate. Twelve aliquots, each containing  $1.5 \times 10^7$  cells in 600 μL were placed into 15 mL conical tubes. Three aliquots for each of the four solvent extraction volumes were extracted according to either the Folch [16], Bligh & Dyer [17], acidified Bligh & Dyer methods, or Matyash [7,18]. An exogenous lipid standard mix (15 ppm) containing 12 lipids was spiked (15 μL) into each aliquot pre-extraction. The relative abundance of the lipid standards was quantified by comparing the integrated peak area of each compound.

[Folch method]: Chloroform:methanol (2:1, v/v) was used according to the original Folch procedure allowing for a CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O ratio of 8:4:3 (v/v). Briefly, ice-cold methanol (0.5 mL) and chloroform (1 mL) were added directly to the cell pellet. The suspension was vortexed occasionally to bring about physical mixing and incubated on ice for 20-40 min. After the addition of water (0.38 mL), used to separate the aqueous and organic layer, the suspension was incubated on ice for an additional 10 min. Samples were centrifuged at 311 x g for 5 min at 4°C. The lower phase (organic) layer was transferred to a new tube. The aqueous layer was re-extracted with 2:1 chloroform/methanol. The chloroform layers were combined for analysis. Samples were then dried under nitrogen and reconstituted in 50 μL of isopropanol (IPA) and transferred to an LC vial with glass insert.

[Bligh-Dyer (BD)]: Chloroform/methanol (1:2, v/v) was used according to the original procedure. The cell suspension was extracted by the addition of the chloroform/methanol mixture (1.5 mL of a 1:2, v/v), vortexed, and incubated on ice for 20 min. The mixture was washed with water (0.47 mL), vortexed, and centrifuged. The organic layer was collected and the aqueous layer was re-extracted with 1:1

chloroform/methanol (2 mL). The chloroform layers were combined for analysis. Samples were then dried under nitrogen and reconstituted in 50  $\mu$ L of IPA.

[Acidified Bligh-Dyer (ABD)]: The cell suspension was extracted using the same procedure as the Bligh-Dyer, but with 25  $\mu$ L of 3 M HCl added after the chloroform addition.

[Matyash method]: The cell suspension was extracted according to the procedure found in literature [18]. To each cell pellet, methanol (0.5 mL) and MTBE (1.7 mL) were added. The cell suspension was incubated at room temperature on an orbital shaker for 1 hr. After the addition of water to account for a final ratio of MTBE:MeOH:H<sub>2</sub>O (10:3:2.5, v/v/v), the mixture was incubated at room temperature again for 10 min, and centrifuged at 311 x g for 10 min. The organic (upper) layer was collected and the aqueous layer was re-extracted with 10:3:2.5 (v/v/v) MTBE:MeOH:H<sub>2</sub>O. The MTBE layers were combined for analysis. Samples were then dried under nitrogen and reconstituted in 50  $\mu$ L of IPA.

### Analysis of metabolite and lipid extracts by UHPLC-MS

Mass spectra were acquired on a Thermo Scientific Q Exactive orbitrap equipped with a heated electrospray ionization (HESI II) probe in positive ion mode. All mass spectra were acquired using background ions from diisooctyl phthalate ( $m/z$  391.2842) and polysiloxanes ( $m/z$  371.1012, 445.1200) as lock masses to ensure mass accuracy. HESI and mass spectrometric parameters were as follows: spray voltage: 3.3 kV, sheath gas and auxiliary nitrogen pressures: 30 and 5 arbitrary units respectively, capillary and heater temperatures: 300 and 350°C, and S-lens RF level: 35. Full scan mode data were collected in profile mode from  $m/z$  100-1500, corresponding to the mass range of most expected cellular lipids and  $m/z$  70-1000 for metabolites. External calibration was applied before each run to allow for LC-HRMS analysis at 70,000 resolution ( $m/z$  200).

An UHPLC system (Thermo Dionex UltiMate 3000 RS) was coupled to the Q Exactive orbitrap for the chromatographic separation of metabolites and lipids present in Jurkat T cells. The autosampler temperature was maintained at 4°C for all experiments.

An ACE Excel 2 C18-PFP column (2.1  $\times$  100 mm, 2  $\mu$ m particle size) maintained at 35°C was used for all metabolomic studies. The injection volume was 5  $\mu$ L with a mobile phase flow rate of 350  $\mu$ L/min. The gradient program consisted of mobile phase A [0.1% formic acid in water] and mobile phase B [ACN modified with 0.1% formic acid] at 0-1 min hold at 0% B, a linear ramp to 65% B at 11 min, a hold at 65% B until 13 min, a linear ramp to 95% B at 18 min, and a hold at 95% B until 20 min. The total run time was 22 minutes, including a 2 min equilibration. A quality control containing exogenous metabolite standards spiked into the reconstitution solvent was injected over the course of a batch (7-9 samples) to evaluate the mass accuracy (less than 0.5 ppm error) and instrument variability across the run.

A Supelco Analytical Titan C18 column (2.1  $\times$  75 mm, 1.9  $\mu$ m particle size) maintained at 30°C was used for all lipidomic studies. The injection volume was 2  $\mu$ L with a mobile phase flow rate of 500  $\mu$ L/min. The gradient program consisted of mobile phase C [60:40 acetonitrile/water] and mobile phase D [90:10 isopropanol/acetonitrile], each containing 10 mM ammonium formate and 0.1% formic acid. The gradient included 32% D at 0 min, 40% D at 1 min, a hold at 40% D until 1.5 min, 45% D at 4 min, 50% D at 5 min, 60% D at 8 min, 70% D at 11 min, and 80% D at 14 min. The total run time was 21 min, including a 3 min equilibration. A quality control containing exogenous

lipid standards spiked into the reconstitution solvent was injected over the course of a batch (7-9 samples) to evaluate the mass accuracy (less than 0.8 ppm error) and instrument variability across the run.

### Data processing and statistical analysis

All LC-MS data were collected and initially processed by the Thermo Xcalibur Workstation software (version 2.2.44). Data processing was performed with Thermo Scientific's SIEVE 2.1, XCMS R Script [19-21], and MetaboAnalyst 3.0 online [22,23]. Proteowizard's MS Convert (version 3.0.5759) was used to centroid and convert Thermo (.raw) files into mzXML data formats for XCMS R processing. The background was subtracted from all acquired LC-MS raw data files using a random blank chromatogram from the same batch of analysis in the SIEVE program. Peaks that showed S/N ratios greater than 10 were selected as the detected compounds to be used in further analysis. P-values <0.05 were considered statistically significant. Compounds were searched against the Human Metabolome Database (HMDB), LipidMAPS, and the METLIN database for putative identifications.

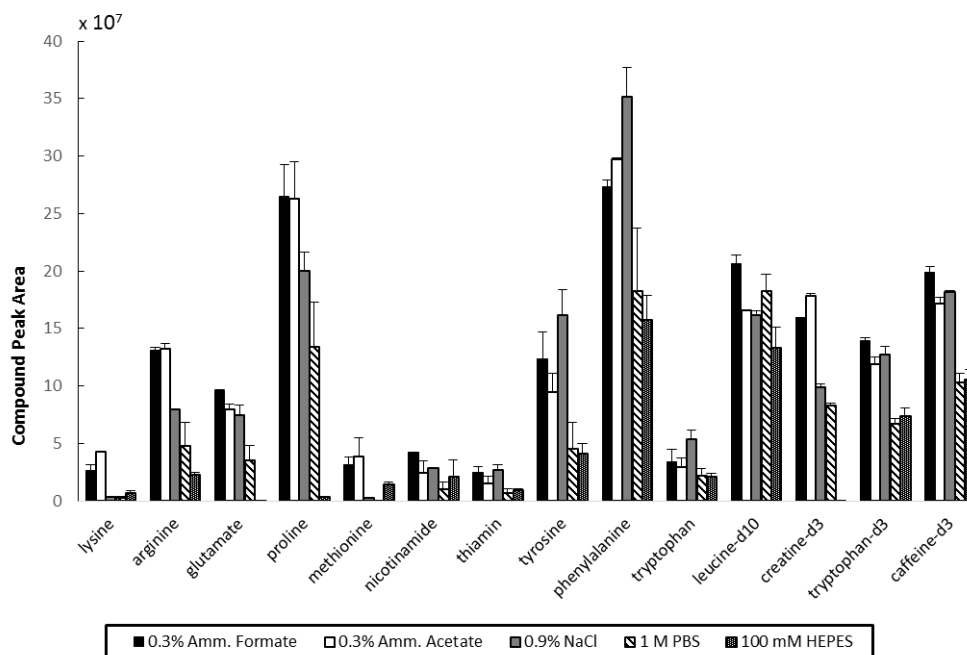
## Results

### Rinsing solvent for media removal from suspension cells

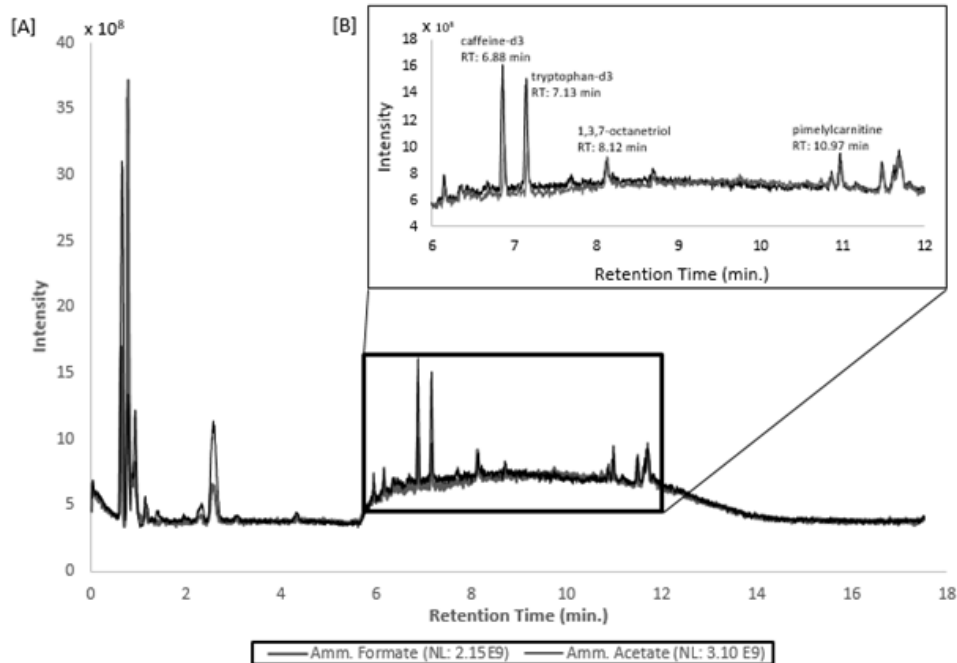
While over 20 metabolites were analyzed for the metabolite response to the rinsing solution, ten endogenous metabolites were chosen for retention time elution, peak area order of magnitude similarity, and metabolite class variety in positive ionization mode. Four exogenous metabolites were also chosen to compare this response. The entire list of metabolites analyzed in this study can be found in the supporting information. Dietmair et al. evaluated the compatibility of various quenching solutions for the metabolic analysis of CHO cells [2]. Cells quenched or rinsed with ice cold 0.9% NaCl effectively stopped metabolism and resulted in a higher concentration of ATP compared to ADP and AMP levels. Rinsing cell pellets with HEPES resulted in decreased peak areas for all endogenous metabolites and the exogenous leucine-D10 internal standard (Figure 1). For many endogenous compounds, the HEPES rinse substantially reduced the peak areas, resulting in the complete absence of some compounds. Additionally, the PBS rinse caused lower peak areas compared to the 0.9% NaCl standard washing solution. The ammoniated buffer rinsing solutions (ammonium formate and ammonium acetate) provided the highest compound peak areas for many endogenous species. While compounds such as guanine and thiamin did not show a significant difference between the rinsing solutions, other metabolites (proline, lysine, and phenylalanine) were greatly affected; oftentimes showing complete absence in the presence of HEPES. The amm. formate rinsed metabolites resulted in a similar variance percentage compared to ammonium acetate (2.0 %CV, 2.4 %CV respectively) for the internal standards. The chromatographic profile showed that the majority of the variation between an ammonium acetate and ammonium formate rinsing solution for early eluting compounds occurred between 0.5-2.5 min (Figure 2).

### Lipid extraction solvent system for untargeted lipidomics

In contrast to the solvent systems containing MeOH and CHCl<sub>3</sub>, (Folch, Bligh-Dyer, and acidified Bligh-Dyer), the Matyash method resulted in the top layer being the organic (methyl tert-butyl ether) layer. This aided in the isolation of the organic layer for lipid analysis. The Folch and Matyash lipid extractions provided the highest integrated peak area for most lipid classes (Figure 3). This trend held true for many endogenous compounds. However, the Matyash method required more time than the other methods with an additional 1 hour incubation



**Figure 1:** Peak area of endogenous metabolites and spiked internal standard metabolite mix for each rinsing solution (0.3% ammonium formate, 0.3% ammonium acetate, 0.9% NaCl, 1 M PBS, 100 mM HEPES). The error bars correspond to the standard deviation of the mean for the triplicate samples from each rinsing solution.

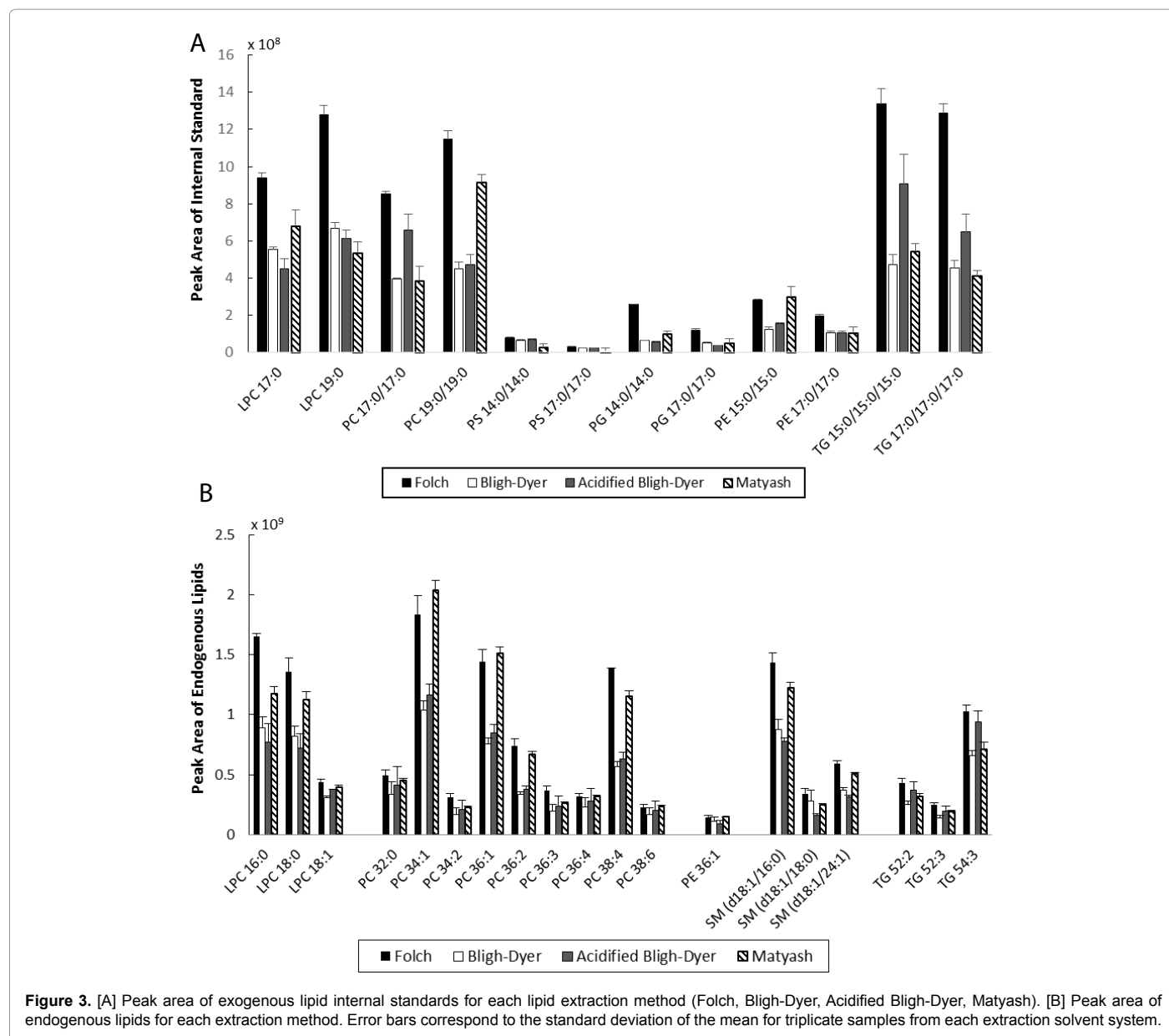


**Figure 2:** Chromatogram overlay showing the ion intensity of metabolites. [A] Total Ion Chromatogram for Jurkat cells rinsed with either 0.3% ammonium formate or 0.3% ammonium acetate. [B] Total Ion Chromatogram for 6-8 min. shows an increased peak intensity for the caffeine-d3 and tryptophan-d3 internal standards for cells rinsed with 0.3% ammonium formate. Retention time reproducibility for internal standards, caffeine-d3 and tryptophan-d3 was 0.3 seconds over the entire chromatographic run. Peak Area reproducibility for internal standards, caffeine-d3 and tryptophan-d3 was 3.6 %CV and 5.8 %CV.

step on an orbital shaker. Jurkat cells extracted with the Folch solvent system also resulted in the lowest amount of variance (0.05-8.3 %CV). The TG lipid classes showed the highest extraction recovery and lowest variance with the Folch method (2.6-3.9 %CV). The LPCs increase in

extraction efficiency for longer tail lengths regardless of the extraction solvent system. The extraction efficiencies and lower peak areas of the PG and PS lipid classes were similar for each extraction method due to poor electrospray ionization of these lipid classes in positive ion





Cell Line	Rinse	Solvent System	Ref.
GS-CHO, LB01	60% methanol w/ 0.85% (w/v) amm. bicarbonate	100% MeOH	[30]
Super-CHO, clone C2.8 SPF	0.9% (w/v) NaCl	50% ACN	[2]
CHO, DG44 line	150 mM NaCl	2:1 (v/v) MeOH:CHCl <sub>3</sub>	[31]
Cord blood lymphocytes	None	100% ACN	[32]
Embryonic kidney, HEK 293	0.9% (w/v) NaCl	50% ACN	[33]
T lymphoblastic leukemia, Jurkat	PBS	80% MeOH	[28]
Acute myeloid leukemia	None	80% MeOH	[29]

**Table 1:** Summary of Metabolite Extraction Systems for Suspension-Cultured Mammalian Cells.

mode. Larger quantities of protein precipitation were observed with the Matyash and Folch method (visual observation).

## Discussion

Standard procedures for cell washing from mammalian cell culture media are vital because culture medium is often rich with amino acids and anions (e.g., Cl<sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, PO<sub>4</sub><sup>3-</sup>) that can mask low intracellular

concentrations within cells [2,6,24,25]. Cell rinsing solutions for mass spectrometric analysis of suspension-cultured mammalian cells (Table 1) are inconsistent in the literature and have not been optimized for electrospray ionization [1,2,24,25].

As described in previous studies, [26], phosphate and sulfate buffers, salts, and detergents can be preferentially ionized over analytes,

causing ion suppression. The use of HEPES and PBS in the rinsing step resulted in reduced peak areas and ion intensities for a majority of the metabolites observed (Figure 1). LC-MS cell analysis not only requires the removal of inferences, but also that the cell remains intact during the washing step to ensure integrity and reproducibility of the molecular information [24]. Pure organic solvents containing methanol, ethanol and/or acetonitrile are unsuitable for cell washing due to cell lysing by disruption of the cellular membrane, leading to leakage of intracellular metabolites [2,25]. Even hypo-osmotic solutions such as deionized water can cause many cell types to rupture after a brief (10 sec) exposure [24]. Therefore, an isotonic cell washing solution is desired that will not only remove components in the medium at physiological pH, but also minimize cellular damage to preserve molecular information.

This work has shown that ammonium acetate or ammonium formate can be used as suitable suspension cell rinsing solutions. Ammoniated rinsing buffers are volatile solutions that do not leave salt crystals post-drying. Additionally, previous research has shown that ammonium acetate solutions as concentrated as 150 mM do not affect the integrity of the cellular membrane or the number of viable cells [24]. However, ammonium formate was preferentially chosen as the rinsing solution for the untargeted metabolomics workflow proposed in this work. Ammonium formate is already present in the mobile phase for lipid analysis, is compatible with the electrospray process, and resulted in slightly higher peak area for many metabolites.

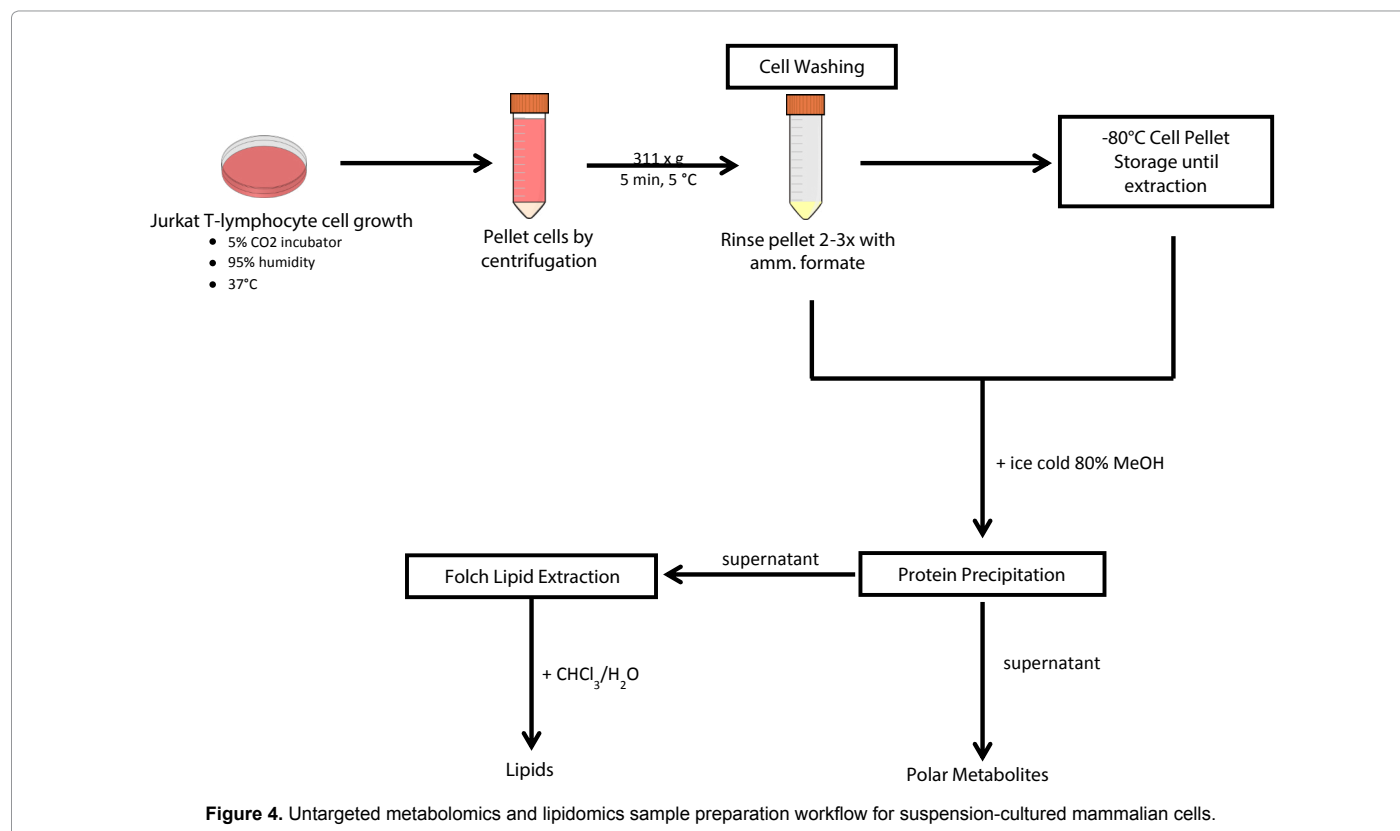
As with the cellular rinsing solution, standardized protocols for suspension cell metabolite and lipid extractions by LC-MS are not readily available in literature [1,2,14]. This can be attributed to the solvents or steps used for extraction biasing the absence or concentration of biomolecules. Extraction methods are sample-dependent and should

be optimized for quantitative coverage of a wide range of compounds [2]. Additionally, mammalian cell extraction methods should consider leakage of intracellular compounds due to the lack of a cell wall and interconversion of biomolecules during a series of steps [1,2]. For the metabolite isolation experiments detailed in this work, an 80% ice cold MeOH extraction was applied to cell samples for its high reproducibility and comprehensive extraction of metabolites. This extraction method quantitatively extracts metabolites without any chemical or physical degradation [2,6,27]. Additionally, the 80% MeOH extraction has been used for the majority of the studies involving the metabolic analysis of lymphocytes [28,29].

In summary, a sample preparation workflow was developed for suspension mammalian cells using the Jurkat cells to standardize a rinsing solution and metabolite/lipid extraction protocol that ensures optimum cell integrity and global coverage of biomolecules (Figure 4). The advantage of incorporating the Folch lipid extraction for the lipidomic analysis of suspension cells not only includes its ability to efficiently extract phospholipids and triacylglycerides, but because it can easily be coupled to an 80% MeOH metabolite isolation step in a workflow. This allows metabolites and lipids to be analyzed from the same sample. The supernatant from the MeOH metabolite isolation step is split into two aliquots. The first aliquot can be analyzed directly by LC-MS using chromatographic conditions for metabolites and the remaining aliquot can be mixed with chloroform and water to give a 8:4:3 (v/v/v)  $\text{CHCl}_3$ :MeOH:H<sub>2</sub>O ratio for lipid analysis.

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**Figure 4.** Untargeted metabolomics and lipidomics sample preparation workflow for suspension-cultured mammalian cells.

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