

Considerations of Sample Preparation for Metabolomics Investigation

Teresa Whei-Mei Fan

Abstract

Sample preparation is the gateway to metabolomic analysis, the importance of which cannot be overemphasized. There are general rules of thumb for sample preparation that help maximize sample integrity and metabolite recovery. The wide range of variations in metabolite functional groups, polarity, sizes, and stability precludes the use of a single extraction method in metabolomic studies. Common extraction methods for polar metabolites that utilize trichloroacetic acid or aqueous acetonitrile are suitable for both nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis while others that use chloroform/methanol/water partition or boiling water may not. Control of extract pH is crucial for consistent NMR assignments and chemical derivatization-linked MS analysis. Sequential polar and lipid extractions reduce sample size requirement and provide a better coverage for direct-infusion MS analysis of lipids, possibly by removing interfering salts. Cleanup of sample extracts, such as removal of fine particles or interfering cations, is often necessary but should be limited to reduce loss of metabolites.

Key words: NMR, GC-MS, FT-ICR-MS, Trichloroacetic acid, Perchloric acid, Chloroform/methanol/water, Acetonitrile, Boiling water, Mouse liver, Human lung

1. Introduction

Sample preparation is a crucial factor in metabolomic research, although this aspect is generally not sufficiently discussed in many metabolomic publications. The quality of the analytical output and subsequent data analysis are critically dependent on the sample history and preparation methods in the first place. The nature of metabolomic analyses demands the maintenance of the integrity of a large number of metabolites ranging widely in concentrations and chemical properties. This is not a trivial task and may have considerations specific to the sample types and sizes, range of analytes, instrument platforms, etc. However, there are general

guidelines on good practices of sample preparation. These include, but are not limited to, the following:

1. Maximize sample integrity during handling, storage, and processing
2. Optimize sample sharing for different applications
3. Optimize metabolite recovery both in terms of number and concentrations of metabolites as well as reproducibility
4. Versatility for analysis by different instrument platforms

In this chapter, the general practices are presented along with examples of specific considerations.

2. Sample Integrity

Immediately following the biological experimentation, sample integrity should be maintained to prevent unwanted metabolic changes. This is commonly achieved by flash freezing in liquid N₂ (−196°C) (1–3) or in acetone/dry ice bath (−78°C, http://en.wikipedia.org/wiki/Freezing_mixture) when liquid N₂ is not available. However, samples may need to be manipulated, e.g., washing or centrifugation to remove extraneous metabolites, before flash freezing. In such cases, samples should be kept cold (e.g., 4°C) and processed as soon as possible to minimize metabolic changes. Appendices 1 and 2, respectively, provide protocols for preparing mammalian cell cultures and blood plasma, which are routinely employed in our laboratory. For harvesting adherent mammalian cells, cells can be detached by treatment with trypsin, followed by centrifugation and PBS wash to remove medium components before flash freezing in liquid N₂. Both wet and dry weights of the cell mass can be readily measured for normalizing metabolite content. However, this method could introduce metabolic artifacts due to the trypsinization procedure.

To minimize this problem, cell metabolism can be quenched rapidly with cold methanol (e.g., (4)) or acetonitrile (Fan, unpublished data), which can also be integrated with subsequent metabolite extraction using the water–chloroform partition method (cf. Appendix 1). Dilute acids (e.g., ice-cold 6% perchloric acid (PCA)) (5) have also been reported for quenching cellular metabolism, although acid-labile metabolites are lost with such method. An example comparison of the metabolite profiles of human adenocarcinoma A549 cell extracts obtained from the trypsinization and methanol quench methods is shown in Fig. 1. The ¹H nuclear magnetic resonance (NMR) profiles of the two cell extracts differed quantitatively for some of the resonances. Most notably, the peak ratio of phosphocholine (P-choline) resonance at 3.22 ppm to the

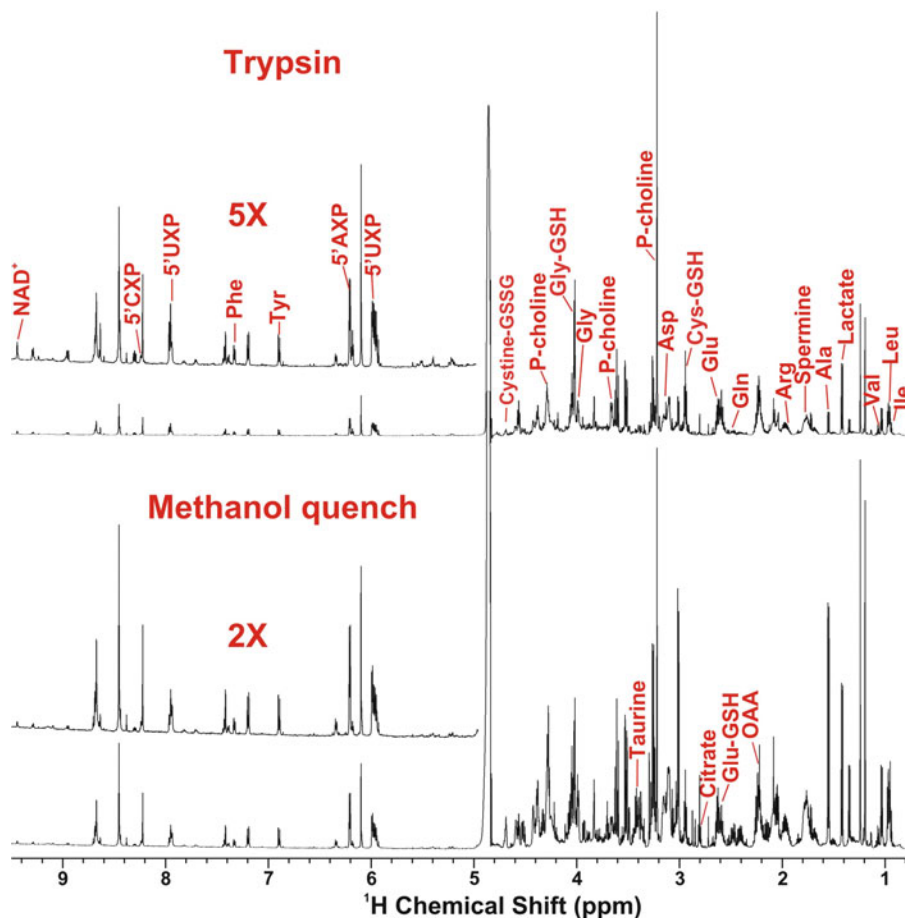


Fig. 1. Comparison of 1-D ^1H NMR profiles of human lung adenocarcinoma A549 cell extracts obtained from two cell harvest methods. The trypsinization and methanol quench methods were performed on the same batch of cells as described in Appendix 1. The ^1H NMR spectra of the two extracts were acquired at 14.1 T on a Varian Inova spectrometer system using an HCN triple-resonance cold probe. The spectral assignment was made as described in 16. The two spectral insets were overscaled relative to the largest resonance, i.e., the methyl resonance of phosphocholine (P-choline) at 3.22 ppm. *GSH* reduced glutathione; *OAA* oxaloacetate; *GSSG* oxidized glutathione; *UXP* uracil nucleotides; *AXP* adenine nucleotides; *CXP* cytosine nucleotides; *NAD* nicotine adenine dinucleotide.

rest of the resonances was much higher for the trypsinization than the methanol quench method. Marked differences in peak ratios were also evident for Ala, lactate, oxaloacetate (OAA), Gln, taurine, and NAD^+ . These distinctions could reflect some metabolic alterations caused by the trypsin treatment. On the other hand, it is nontrivial to normalize metabolite content for the direct methanol quench method, as cell weights or counts are not practical to obtain, as for the trypsinization method. Without normalization, it would be difficult to compare metabolite content of cells harvested from individual culture vessels. This is because cell mass of each vessel varies significantly, even grown under the same condition. This problem is even more pronounced in the case of time

course analysis, where cell mass is expected to change several folds over a 48-h period, depending on the cell doubling time. Thus, reliable normalization method for metabolite content needs to be carefully explored for the solvent quench method. These could include total protein or glycerol lipid analysis.

For biofluids other than blood (e.g., urine and bronchioalveolar fluids), they can be flash frozen and stored untreated at -20 to -80 °C before further processing (6, 7). Sodium azide (e.g., 0.1% w/v) can be added to biofluids to minimize bacterial contamination (7). When necessary, biofluids can be filtered through 0.22- or 0.45- μ m filters to remove particulate matter before analysis. It is also important to avoid repeated freeze–thaw cycles of biofluids by flash freezing multiple aliquots. For tissues, small samples (e.g., ≤ 5 mm diameter) can be preserved by flash freezing in liquid N₂ directly after blotting excess blood or other biofluids. To ensure fast and even freezing, it is advisable to freeze large pieces of tissue samples by freeze clamping in liquid N₂ (cf. Appendix 3). We have observed a significant buildup of lactate by leaving resected human lung tissues on ice for 15 min as opposed to freeze clamping tissues from the same source without delay (T. W.-M. Fan and A.N. Lane, unpublished result).

For microorganisms with fast metabolic rates such as *Escherichia coli*, metabolic quenching is crucial to minimizing metabolic distortion resulting from sampling procedures, such as separating cells from culture media (8). A typical method for quenching is placing cells in cold methanol at less than -20 to 50 °C, followed by centrifugation and extraction (9, 10). Fast filtration (<30 s) (11) and fast heating (12) for metabolic quenching have also been introduced. A recent paper compared the extent of metabolite loss between the cold methanol quenching and fast filtration method, and concluded that the latter method coupled with a wash solution isotonic (e.g., 2.6% NaCl) with the culture medium was suitable for recovering intracellular metabolites from different bacteria (8). However, metabolite recovery for the popular methanol quenching method can be improved by including glycerol in the quenching solution (e.g., 60% (v/v) methanol/glycerol (13) or glycerol/NaCl at 13.5 g/L (v/v) 3/2 (14)). Regardless of the quenching method employed, leakage of intracellular metabolites into the culture medium may be inevitable (15, 16), which should be taken into account in subsequent data analysis and interpretation.

3. Sample Processing

3.1. Sample Homogenization

In order to maximize and reproduce extraction efficiency, it is imperative that tissue or cell masses are homogenized into fine particles (e.g., <10 - μ m size) while maintaining their biochemical integrity. Pulverization in liquid N₂ before metabolite extraction is a method of choice. This can be achieved by manual grinding with a mortar

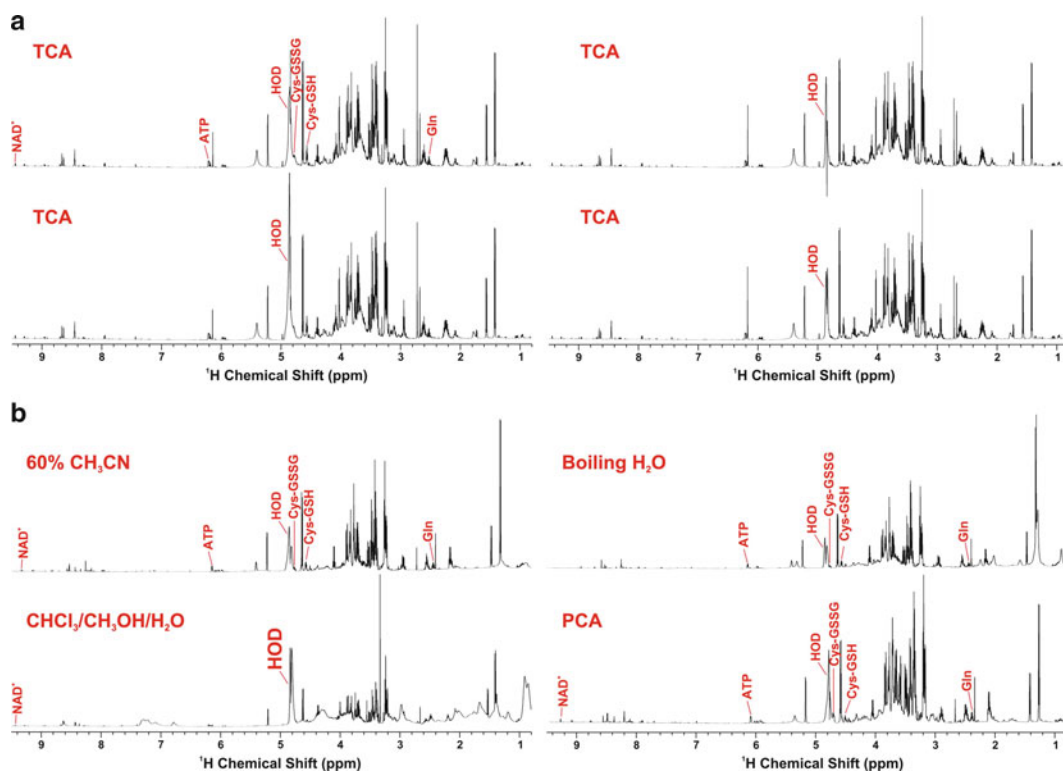


Fig. 2. 1-D ^1H NMR analysis of extracts of pulverized mouse liver using five different extraction methods. (a) illustrates four separate TCA extractions while (b) displays four other types of extractions (performed in duplicates) from the same batch of the liver sample (3.1–4.8 mg dry weight each). Reproducible NMR spectra were obtained from duplicate extraction for all five methods. The ^1H NMR spectra were acquired on a Varian Inova 600 MHz instrument (Varian, Inc., Palo Alto) using a PRESAT pulse sequence, where the HOD signal was saturated. Cys-GSH and Cys-GSSG are the cysteine residue of reduced (GSH) and oxidized (GSSG) glutathione.

and pestle equilibrated in liquid N_2 or by liquid N_2 -compatible mechanical homogenizers (cf. Appendix 8). We have obtained consistent ^1H NMR profiles of mouse liver extracts by pulverizing tissues in a liquid N_2 -based freezer mill (Retsch, Inc, Newtown, PA) (cf. Fig. 2). Tissue homogenization into frozen powder also allows the same sample to be shared for different analytical purposes, including analysis of metabolites, proteins, polysaccharides, and nucleic acids. Cell pellets can be homogenized with glass beads using a micro ball mill (e.g., MM200, Retsch, Inc.) directly in buffers or solvents that denature and precipitate proteins and other macromolecules which interfere with metabolite profiling by NMR (nuclear magnetic resonance) or MS (mass spectrometry) (see Sect. 2).

3.2. Sample Extraction

3.2.1. NMR Analysis

Extraction of Polar Metabolites

For extracting polar metabolites, various methods have been employed in the metabolomic literature (3, 8, 16–23). These methods invariably adopt acids, solvents, ultrafiltration, or heating to quench enzyme activities while minimizing the extraction of lipids and macromolecular

components, such as proteins, nucleic acids, and complex carbohydrates. In addition, to avoid major spectral interferences or excess salts, extraction solvents or buffers should be readily removed, e.g., H₂O, trichloroacetic acid (TCA), and ammonium bicarbonate by lyophilization, PCA by KOH or K₂CO₃ precipitation, and organic solvents by N₂ evaporation or vacuum drying.

In this section, five extraction methods are discussed for their compatibility with analyses by NMR and MS, which are two of the most versatile and common instrument platforms used in metabolomic research. These include extractions using ice-cold TCA and PCA, chloroform/methanol/water partition, 60% acetonitrile, and boiling water (3, 16, 17, 24–30).

The TCA (e.g., 10% w/v, minimum extraction ratio v/w 40:1) and PCA (e.g., 5% w/v, minimum extraction ratio v/w 40/1) methods have been routinely employed in our laboratory for a variety of biological samples (3, 16, 25–29). Appendix 4 lists a detailed protocol for the TCA extraction. TCA or PCA efficiently denatures and precipitates proteins and other macromolecules while the extractant itself can be removed as stated above. As the titration of PCA with potassium salts to the equivalence point (pH 7 for KOH or pH 3–3.5 for K₂CO₃) is sequential and time consuming, TCA extraction is superior in sample throughput because the removal of volatile TCA can be done batchwise by lyophilization. However, some metabolites, such as glutamine, are more prone to degradation with this method due to a more acidic end point (pH 2–2.5) of lyophilized TCA extracts. Example ¹H NMR analysis of TCA and PCA extracts of pulverized mammalian tissues is shown in Fig. 2a, b, respectively. Also illustrated in Fig. 2a is the reproducibility of the TCA extraction when the same batch of pulverized sample was extracted four times by two different operators. These strong acid agents are well suited for NMR analysis as relatively flat spectral baselines are routinely obtained, and the spectra are free of all but the smallest peptides, which are themselves of interest to metabolomic studies (e.g., glutathione).

Although efficient in removing macromolecules and lipids, acid-based extraction methods are unsuitable for extracting acid-labile metabolites, such as fructose-2,6-bisphosphate, UDP-*N*-acetylglucosamine, NAD(P)H, or dGTP. Aqueous solvent mixtures (e.g., 60% acetonitrile or 50% methanol) can be used instead. When necessary, a volatile neutral buffer (e.g., ammonium bicarbonate) can be added to buffer the pH during extraction and subsequently removed by lyophilization. Example extraction protocols using 60% acetonitrile or chloroform/methanol/water partition are described in Appendices 5 and 6, respectively. As indicated in the previous section, the solvent extraction method can be integrated with the solvent quenching method and an example protocol is given in Appendix 1.

Figure 2 illustrates the 1-D ^1H NMR analysis of the solvent-based extracts of mouse liver (Fig. 2b) in comparison with those using TCA, PCA, or boiling water. Unlike the extraction for mammalian cells (cf. Fig. 1), the aqueous solvent extraction is not as efficient in removing macromolecular interference as the acid-based extraction. This is evidenced by the presence of broad ^1H resonances in the aliphatic and aromatic regions (e.g., 0.8–1 and 7–8 ppm of Fig. 2). Such interference is most pronounced for the chloroform/methanol/water method (cf. Fig. 2b), which may be reduced by ultrafiltration (e.g., through 3.5–5 kDa MWCO membranes). As ultrafiltration membranes are typically conditioned with glycerol, the added filtration step will require an extensive water rinse of the filter in order to minimize glycerol contamination. It should also be noted that different extraction methods yield somewhat different ^1H NMR profiles, except for the TCA and PCA methods, which give comparable results. This generally precludes comparison of metabolite profiles obtained from different extraction methods.

Although commonly used for GC-MS analysis, the chloroform/methanol/water method (the solvent ratio used often varies in the literature) (17, 31) gives a distorted ^1H NMR spectrum of mouse liver extract, particularly in terms of the resonances of ATP, NAD^+ , and reduced/oxidized glutathione (GSH/GSSG), compared with those obtained from the other four extraction methods (Fig. 2). A similar problem is also evident with the CHCl_3 /methanol/water extraction of human lung tissues (32). The broad resonances present in the spectrum further interfere with the 2D total correlation spectroscopy (TOCSY) analysis (Fig. 3 and see Chap. 6 for more information about this analysis). Compared with the TOCSY spectrum of the TCA extract (Fig. 3d), the TOCSY spectrum of the CHCl_3 /methanol/water extract (Fig. 3b) exhibits a number of broad cross-peaks, which obscure many of the sharper cross-peaks arising from metabolites, such as Leu, Ile, spermine, Pro, and Arg. These interferences interfere with many of the metabolite assignments by TOCSY while jeopardizing the use of TOCSY for isotopomer determination (see Chaps. 2 and 6 (21, 33, 34)).

Broad interfering resonances and spectral distortion also occur in the boiling water extract, as shown in Fig. 2b. In addition to a reduced intensity of GSH/GSSG, Gln, and ATP resonances, those of NAD^+ are absent in the ^1H NMR spectrum of the boiling water extract. As high heat is destructive to a number of important but heat-labile metabolites, this method is not advisable for general use in metabolomic investigations.

pH Control

The solution pH is a very important parameter for sample preparation and subsequent analysis. As discussed above, modulating the pH of the extraction buffer is important for metabolite extraction. Appropriate pH is also required for derivatization in preparation for GC-MS analysis as discussed below, and in Chap. 4 by Higashi.

For NMR analysis, controlling the pH is critical because the spectral appearance of many metabolites, including amino acids, organic acids, and phosphorylated compounds, is very sensitive to pH so that their chemical shifts and/or spin coupling can vary greatly for relatively small changes in pH. Variable pH makes spectral assignment more difficult. Many NMR databases of metabolites are acquired with standards at a specific pH, such as pH 7.0, 7.4, or acidic pH, where most metabolites are in the fully protonated forms (see Chaps. 6 and 7 for further discussion of this point). It is, therefore, critical to adjust all samples to a pH value similar to that of the standard (less than ± 0.05 pH, if the pH is near the pK value) prior to NMR analysis.

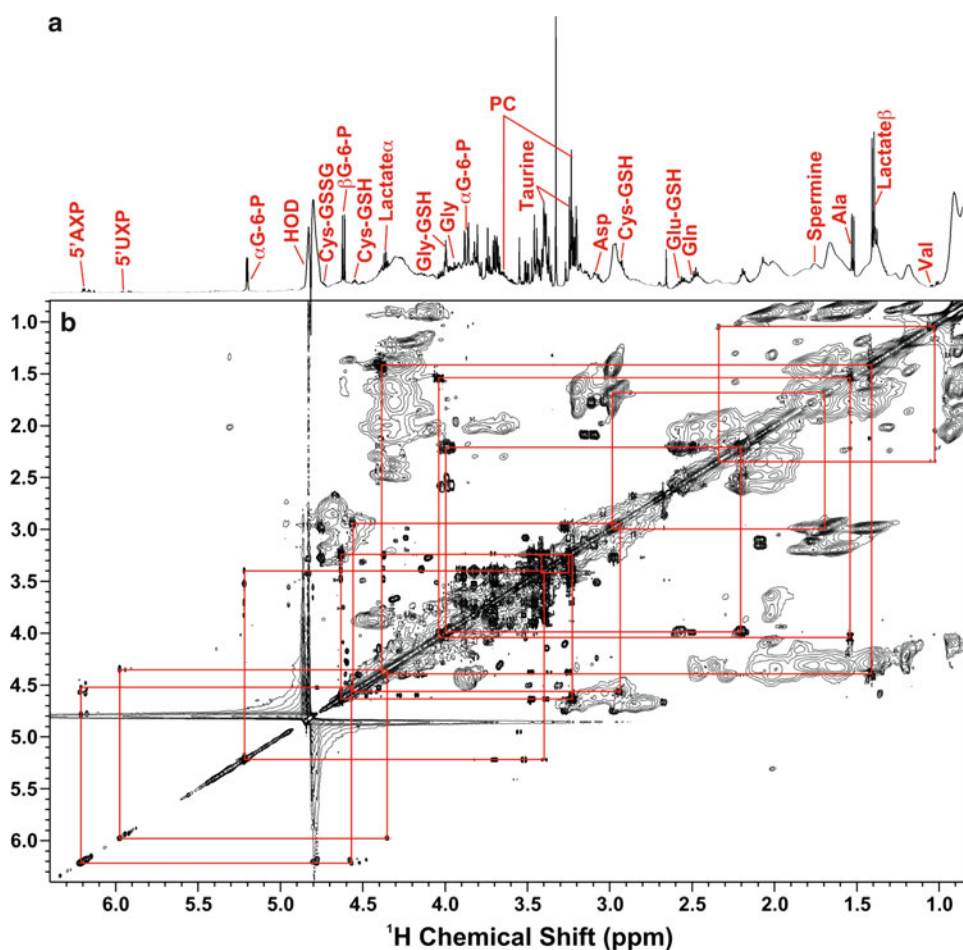


Fig. 3. 2D ^1H TOCSY analysis of TCA and chloroform/methanol/water extracts of mice liver. The extracts from Fig. 2 were analyzed on the same instrument using a standard 2D TOCSY pulse sequence. **b** and **d** are the 2D contour maps of the TCA and chloroform/methanol/water extracts, respectively, while **a** and **c** are the corresponding high-resolution 1-D ^1H NMR spectra.

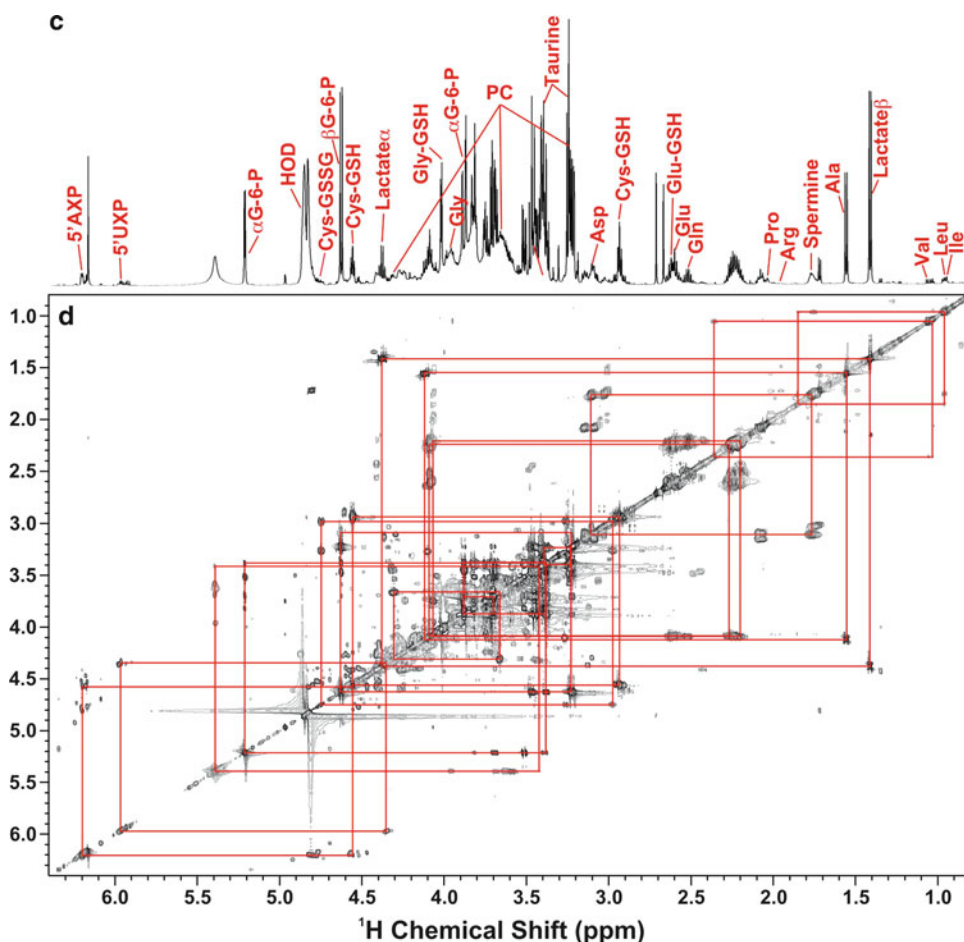


Fig. 3. (continued)

Extraction of Low-Polarity Metabolites

For extracting low-polarity metabolites, such as glycerolipids, sphingolipids, and cholesterol, methanol or chloroform/methanol (e.g., 2:1, v/v) mixtures are suitable. Methanolic extraction favors phospholipids (PLs) and sphingolipids, whereas chloroform/methanol mixtures better extract neutral lipids (35, 36). A protocol for methanol extraction is described in Appendix 6. Figure 4 illustrates the lipid profiles of methanolic extracts of human lung cancer cells and tissues, as analyzed by 2D ^1H - ^{13}C heteronuclear single quantum coherence spectroscopy (HSQC). The spectra shown are the 1-D projection along the ^{13}C dimension of the 2D data. The antioxidant butylated hydroxytoluene (BHT) was included during the extraction to protect polyunsaturated lipids from oxidation. The large peak near 56 ppm is assigned to the *N*-methyl group of the phosphocholine (P-choline) head group, which indicates the abundance of phosphatidylcholine and/or sphingolipids. Also present in the extracts are cholesterol and phosphatidylethanolamine

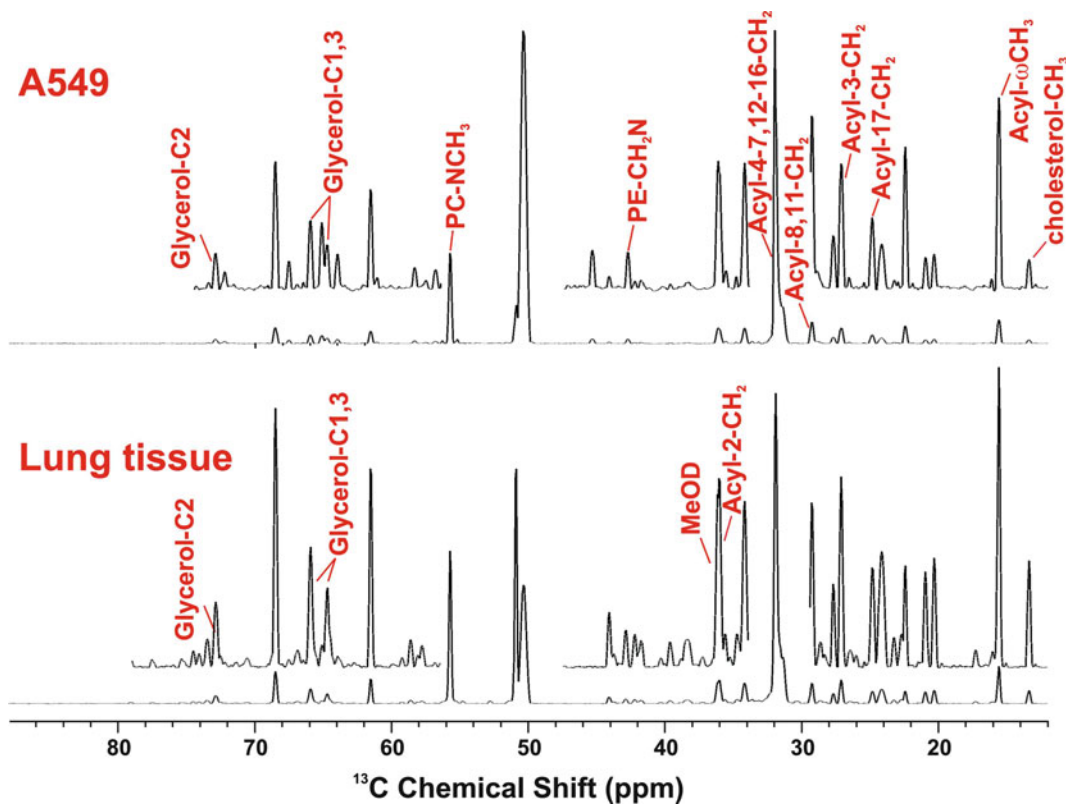


Fig. 4. HSQC analysis of methanolic extracts of human A549 cells and lung tissues. Lyophilized and pulverized human adenocarcinoma A549 cells and human lung tissue were extracted with 100% methanol in the presence of antioxidant butylated hydroxytoluene (BHT) and filtered through 0.22- μ M regenerated cellulose filter before 2D ^1H - ^{13}C HSQC NMR analysis at 14.1 T and 20°C using a standard HSQC pulse sequence. The spectra displayed are the 1-D projection spectra along the ^{13}C dimension. The inset spectra are four times magnified over the corresponding full spectra. ^{13}C chemical shift was referenced to methanol- d_6 (MeOD) at 36.07 ppm and peak assignments are made according to Fan and Lane (21).

lipids. The lipid profiles of lung cells and tissues show both similarity and differences. However, detailed lipid composition is not practical to determine with the HSQC analysis of such mixtures (see FT-ICR-MS analysis below for lipid speciation).

3.2.2. MS Analysis

The five polar extracts from Fig. 2 were also analyzed by GC-MS after derivatization with the silylation agent *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) (see Chap. 4 for discussion of MS-based methods). A comparison of the absolute content of various metabolites in these extracts as quantified by GC-MS is shown in Table 1. It is clear that the TCA extraction method performed the best overall while the CMW method was the worst in terms of the recovery of acid-resistant polar metabolites. In many cases, the recovery of the CMW method was several fold to more than an order of magnitude less than that of the TCA method for mouse liver tissues. The boiling water method was also less

Table 1**Comparison of GC-MS-analyzed metabolite profiles in extracts obtained using five different extraction methods^a**

<i>Average^b</i>	PCA	TCA	ACN ^c	Water	CMW ^c	<i>Error</i>	PCA	TCA	ACN ^c	Water	CMW ^c
Lactate	32.92	12.69	36.17	50.72	2.10	Lactate	3.38	0.03	1.82	3.06	0.11
Ala	41.07	46.37	33.67	26.52	3.18	Ala	6.33	1.83	9.80	2.85	0.26
Gly	2.05	3.13	3.96	1.60	0.59	Gly	0.24	0.19	0.46	0.06	0.07
Val	0.38	0.51	0.37	0.31	0.11	Val	0.04	0.02	0.01	0.01	0.01
Leu	0.36	0.84	0.45	0.36	0.10	Leu	0.04	0.06	0.02	0.02	0.00
Ile	0.18	0.45	0.20	0.19	0.05	Ile	0.00	0.03	0.02	0.01	0.01
Succinate	3.03	4.89	2.20	1.86	0.55	Succinate	0.55	0.09	0.47	0.12	0.03
Fumarate	0.08	0.54	0.68	0.25	0.01	Fumarate	0.01	0.04	0.17	0.02	0.01
Pro	0.29	0.42	0.35	0.25	0.09	Pro	0.02	0.01	0.04	0.04	0.02
Ser	0.17	0.52	0.28	0.20	0.07	Ser	0.03	0.00	0.05	0.01	0.01
Thr	0.48	0.89	0.59	0.43	0.23	Thr	0.06	0.03	0.11	0.03	0.02
Phe	0.18	0.31	0.24	0.16	0.05	Phe	0.01	0.00	0.04	0.01	0.01
Malate	0.45	0.77	0.10	0.19	0.09	Malate	0.07	0.02	0.02	0.01	0.02
Asp	0.57	1.00	0.53	0.07	0.21	Asp	0.06	0.06	0.05	0.02	0.01
Asn	0.19	0.66	0.46	0.25	0.08	Asn	0.01	0.04	0.04	0.03	0.01
Lys	0.03	0.29	0.42	0.09	0.05	Lys	0.00	0.03	0.06	0.01	0.05
Gln	9.56	12.34	14.61	6.00	2.94	Gln	0.65	0.10	2.11	0.39	0.46
<i>a</i> -G3P ^c	1.08	2.91	1.85	1.10	0.31	<i>a</i> -G3P ^c	0.01	0.05	0.32	0.03	0.02
Citrate	0.07	0.02	0.04	0.01	0.00	Citrate	0.02	0.00	0.00	0.00	0.00
Tyr	0.11	0.23	0.21	0.15	0.02	Tyr	0.02	0.00	0.02	0.02	0.01
Ascorbate	0.00	1.75	0.12	0.00	0.03	Ascorbate	0.00	0.19	0.04	0.00	0.00

^a The same set of extracts from Fig. 1^b Average of duplicate samples in $\mu\text{mole/g}$ tissue dry wt^c ACN 60% acetonitrile method; CMW chloroform/methanol/water partition method; *a*-G3P α -glycerol-3-phosphate

desirable than the TCA or 60% acetonitrile methods, particularly in terms of Gly, fumarate, Asp, Asn, Lys, Gln, citrate, and ascorbate. The poor yield of the antioxidant metabolites ascorbate (Table 1) and GSH (Fig. 2) in the boiled water extracts is presumably due to their sensitivity to high temperatures, which makes the boiling water method unsuitable for antioxidant determination. PCA extraction also performed poorly on selected metabolites, e.g., fumarate, Asn, Lys, and ascorbate.

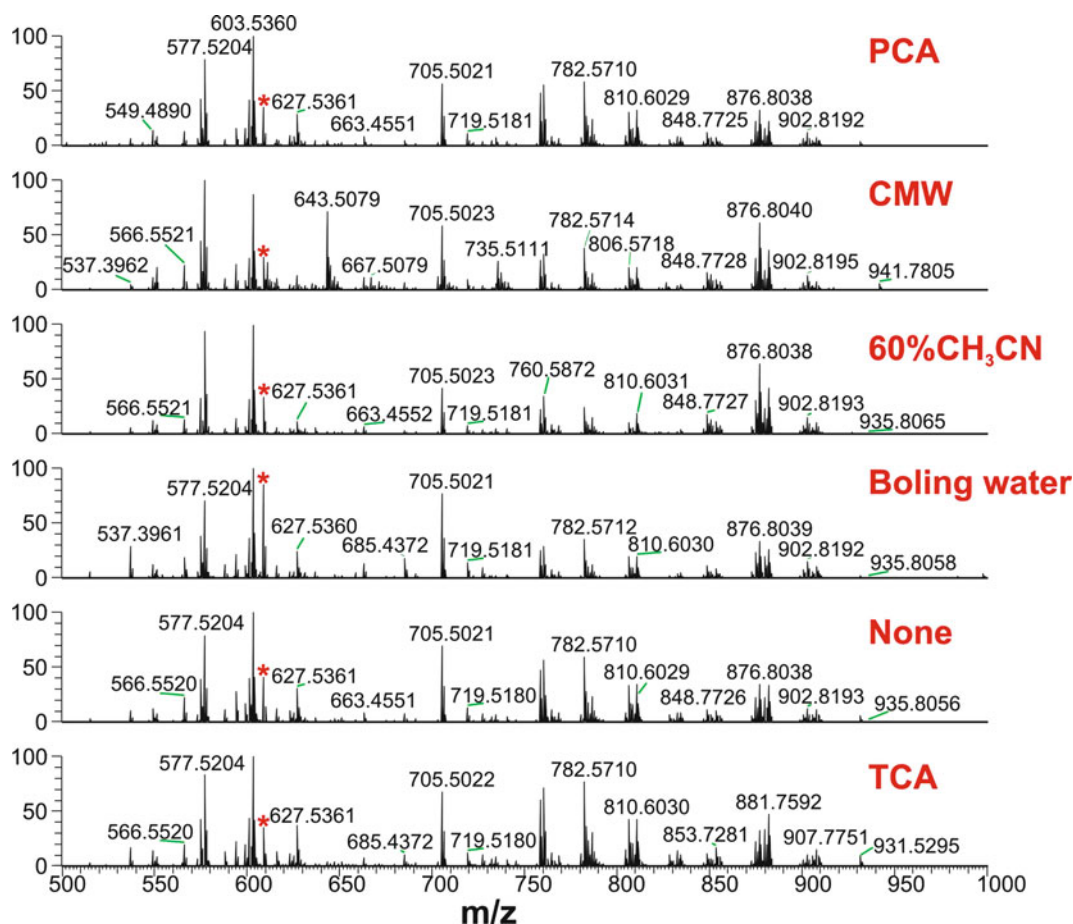


Fig. 5. FT-ICR-MS analysis of methanolic extracts of pulverized mice liver tissues. Except for the chloroform/methanol/water (CMW) extraction, the tissue residues from the other four polar extraction protocols in Fig. 2 were extracted with 100% methanol plus 1 mM BHT to recover lipid components. The PCA- and TCA-extracted residues were washed in nanopure water to remove acids and lyophilized before the methanol extraction. The CMW method simultaneously extracted lipid components in the chloroform partition. Duplicate liver samples with no prior extraction (none) were also extracted with methanol plus BHT for comparison. All lipid extracts in 500 μ L methanol were diluted 100-fold in the spray solvent mixture CH₃Cl–methanol–2-propanol (1:2:4, v/v) plus 7.5 mM ammonium acetate (all in HPLC grade) and internal calibration standard reserpine at 1 ng/ μ L (37). The diluted lipid mixtures were introduced into an LTQ-FT-ICR-MS (ThermoFinnigan) using a NanoMate device (Advion). The exact mass analysis was performed in the positive mode with a mass resolution of 200,000. The asterisk marks the mass ion arising from reserpine (m/z 609.28213).

Subsequent to the polar extraction, the tissue or cell residues can be extracted for low-polarity components using methanol or other lipophilic solvents. Such sequential extraction is highly desired when samples are limited in amounts, such as human biopsy samples. The polar extraction can also serve as a cleanup step for the lipophilic extractions. Thus, it is useful to evaluate the recovery of low-polarity components from the sequential polar-lipophilic extraction protocols described above. Figure 5 shows the ultra-high-resolution and accurate mass FT-ICR-MS profiles of the

Table 2
Summary of FT-ICR-MS analysis of PLs extracted from mouse liver tissues pre-extracted with different polar solvents

Sample	# PLs	# Assigned peaks	# False compounds	# False peaks
Blank + reserpine	4	7	0	0
PCA	39	68	1	2
CMW	35	64	1	3
60% CH ₃ CN	35	63	1	4
Boiling water	33	55	2	3
None	40	70	1	3
TCA	42	75	1	2

methanolic extracts subsequent to PCA, TCA, 60% acetonitrile, and boiling water extractions, for comparison with those obtained from the chloroform partition of the CMW extraction and from direct methanol extraction of lyophilized liver powder. All extraction procedures give an overall qualitatively similar FT-ICR-MS profile with some minor differences. For example, the CMW method yielded an additional cluster of ions in the neighborhood of m/z 643.5079 while the TCA method gave a more significant ion cluster at 931.5295. In contrary to the poor recovery of polar metabolites, the CMW method gave a better response for low-polarity metabolites (when normalized to the standard reserpine as noted by asterisk) than the other four extraction methods. Quantitatively, the boiling water method had the lowest response for all mass ions, when normalized to reserpine.

The ultrahigh mass resolution and accuracy of the FT-ICR-MS analysis allow the exact mass of hundreds to thousands of observable ions to be determined, which in turn provides the molecular formulae information. The exact mass data is also valuable for high-throughput automated assignment by matching with the theoretical mass calculated from known molecular formulae (see Chap. 4 for details). This has been developed in our laboratory and applied to the assignment of mammalian phospholipids (PLs) based on the FT-ICR-MS data of crude mammalian extracts. The algorithm used for this purpose is called PRecalculated Exact Mass Isotopomer Search Engine (PREMISE, Higashi, Fan et al., in preparation). PREMISE search was performed for the six methanolic extracts in Fig. 5 and a summary of the output is shown in Table 2. The TCA pretreatment gave the highest number of assignable mass ion peaks,

which corresponded to the highest number of identified PLs, while the boiling water pretreatment yielded the lowest number of identified PLs and assigned mass ion peaks. There were only a few falsely assigned peaks and compounds using the PREMISE algorithm. Example lipid species identified in the spectra acquired using the positive mode of FT-ICR-MS include phosphatidylcholines (e.g., C16:0/C22:4; C18:1/C20:5; C16:0/C24:0), phosphatidylethanolamines (e.g., C18:0/C20:4; C18:1/C20:5; C22:0/C20:4), sphingolipids (e.g., C18:1/C22:0; C18:1/C24:1; C18:1/C24:0), and phosphatidic acids (C20:0/C20:5; C18:0/C18:3; C18:0/C20:5).

In addition to PL assignment, PREMISE also identifies the isotopomer(s) of a given lipid species. Isotopomers are molecules with identical chemical structures but differing in isotopic composition at individual atoms (see Chap. 1 for details). For example, ^{13}C -1-alanine is an isotopomer of ^{13}C -2-alanine. Such automated assignment of isotopomers is excellently suited for FT-ICR-MS data, which routinely yield mass resolution of a neutron or electron. When coupled with ^{13}C tracer (e.g., uniformly ^{13}C -labeled glucose) study, the ability to profile ^{13}C isotopomers of various PLs is unprecedented and extremely valuable for tracing the metabolic pathways of specific lipids, which has not been possible using conventional approaches.

3.3. Sample Cleanup

For NMR analysis, extract sample cleanup mainly involves removing particulate matters and paramagnetic ions, such as Fe^{3+} , Cu^{2+} , and Mn^{2+} , that can lead to resonance line broadening or distortion. Centrifugation at a high speed (e.g., $\geq 18,000$ g) for 20–30 min helps remove insoluble materials while passage through a cation exchange resin, such as Chelex-100, is effective in removing interfering cations but time consuming (cf. TCA extract in Fig. 2). A quicker alternative is to add chelating agents, such as EDTA, to the extract to bind interfering cations. However, excess EDTA produces a large singlet at ca 3.65 ppm in the ^1H spectrum, which obscures peaks around that region. Thus, it is important to optimize the amount of added EDTA or use the commercially available perdeuterated form which is typically 98 atom% ^2H .

For GC-MS analysis, extracts are normally derivatized using silylating or alkylating agents. For example, MTBSTFA is commonly used as a derivatizing agent for polar extracts and its efficiency is best at acidic conditions in aprotic solvents. Therefore, extracts should be acidified to pH 2–3, lyophilized to remove water before reaction with MTBSTFA in acetonitrile (28). It is common to have insoluble salts in the derivatization mixture, which should be removed by centrifugation. For direct-infusion nanospray MS or LC-MS, it is common practice to filter extracts through a 0.45- or 0.22- μm membrane filter to remove fine particles. If excess salts are present, it is important to reduce the salt content by passing

through a hydrophobic column cartridge (e.g., C-18 cartridge), provided that the metabolites of interest are retained by the column material. It should be kept in mind that extensive sample cleanup can lead to distortion of metabolite profiles, which could defeat the purpose of metabolomic analysis.

In conclusion, sample preparation is the gateway to metabolomic analysis, the importance of which must be underscored. Sample preparation schemes may vary with sample nature or types of metabolites to be recovered but a given protocol should maximize sample integrity and metabolite recovery. It is also advantageous to implement sequential extraction methods that are applicable to different analytical purposes to minimize sample requirement and cleanup.

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Glossary

BHT	Butylated hydroxytoluene
CMW	Chloroform/methanol/water partition
FT-ICR-MS	Fourier transform-ion cyclotron resonance mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HSQC	Heteronuclear single quantum coherence spectroscopy
Isotopomer	Compounds of identical chemical structure but differing in isotopic composition at individual atoms
LC-MS	Liquid chromatography-mass spectrometry
MTBSTFA	<i>N</i> -methyl- <i>N</i> -(<i>tert</i> -butyldimethylsilyl)trifluoroacetamide
PCA	Perchloric acid
TCA	Trichloroacetic acid
TOCSY	Total correlation spectroscopy

Appendices

Protocol for Preparing Adherent Mammalian Cells for Metabolite Extraction

Trypsinization Method

1. When feasible, keep cells ice cold during all processing steps prior to freezing.
2. Harvest cells by trypsinization and centrifugation at $280 \times g$ for 5 min at 4°C.
3. Wash cell pellet by resuspension in excess ice-cold phosphate-buffered saline (PBS) (e.g., 10–15 mL per 10^7 cells), followed by centrifugation at $280 \times g$ for 5 min at 4°C.
4. Resuspend cell pellet in ice-cold PBS (e.g., 1 mL per 10^7 cells) and transfer cell suspension to a pre-tared microfuge tube.
5. Centrifuge cell suspension at $1,700 \times g$ (or at maximal g force without disrupting cell integrity) for 5 min at 4°C so that as much PBS as possible can be removed by pipetting with a fine-tip transfer pipette.

Note: PBS contributes salts to the sample, which can be problematic for both MS and NMR analyses.

6. Obtain wet weight of cell pellet before flash freezing in liquid N₂.
7. Store frozen cell pellet at –80°C or lyophilize and obtain dry weight before storage.

Solvent-Quenching Method

1. Place cell culture plate on ice, remove culture medium by vacuum suction, and wash cells three times with ice-cold PBS by vacuum suction.
2. In the last wash, slant plate to drain PBS and remove as much PBS as possible by vacuum suction. Keep plate on ice for all subsequent procedures.

Note: This is important to minimize salt contribution.

3. Add cold methanol (kept at –80°C) or acetonitrile (kept at –20°C) to drained plate to quench cell metabolism. Adjust solvent volume so that the plate surface is covered fully with solvent, e.g., 1 mL for 10-cm-diameter plate or 2.5 mL for 15-cm-diameter plate.
4. Scrape plate with a cell scraper to collect cell mass and transfer cell mass to a 15-ml conical polypropylene (PP) centrifuge tube.
5. Add another equal volume of cold methanol or acetonitrile to plate; scrap and collect cells as in step 4.
6. Add nanopure water to plate in a ratio of water:methanol (1:1) or water:acetonitrile (1.5:2); scrap and collect cells as in step 4.

7. Add chloroform to the cell homogenate in a ratio of CHCl_3 :water:methanol (1:2:2) or CHCl_3 :water: CH_3CN (1:1.5:2).

Note: All solvents used should be of optima or HPLC grade.

8. Mix aqueous and CHCl_3 layers rigorously in the 15-mL tube before centrifugation at $3,000\times g$ for 20 min at 4°C to separate the layers.
9. Transfer the upper aqueous and lower chloroform layers to 1.5-mL PP microfuge tube and 1.5-mL screw-cap glass vials, respectively, using a fine-tip transfer pipet for aqueous layer and PP gel-loading tip for chloroform layer.

Note: Avoid pipetting the protein/cell debris in between the aqueous and chloroform layers.

10. Dry aqueous and chloroform extracts in a vacuum centrifuge.

Protocol for Preparing Blood Plasma for Metabolite Extraction

1. Collect blood into purple-top vacutainer containing K_3 -EDTA as anticoagulant. Care should be taken to avoid lysis of the blood.

Note: EDTA is compatible with both MS and NMR analyses; for NMR, it helps remove the influence of interfering cations, such as paramagnetic Fe^{3+} and Cu^{2+} .

2. Immediately keep blood sample on ice during transport.
3. Centrifuge blood (within 30 min–1 h of collection) at $3,939 \times g$ for 15 min at 4°C .
4. Aliquot plasma into separate microfuge tubes and flash freeze in liquid N_2 .
5. Store plasma samples at -80°C until analysis.

Protocol for Freeze Clamping

1. Immerse an aluminum clamping device (cf. Fig. 6) in liquid N_2 bath to pre-equilibrate.
2. Place tissue(s) in the middle of the frozen clamp and quickly close the handles to flatten the sample(s), immediately followed by immersing in liquid N_2 to freeze.

Protocol for TCA Extraction

1. All extraction steps are carried out at 0 – 4°C (e.g., on ice).
2. Extract frozen or lyophilized powders (e.g., 30–40 mg) in microfuge tubes with 4–6:1 or 40–60:1 (v/w) volumes of 10% (w/v) ice-cold TCA, respectively. Vortex the mixture rigorously into a homogenous suspension. If homogenization is difficult by vortexing, it can be aided by a pellet pestle for microfuge tubes (cf. Fig. 7). If frozen powder is extracted, make sure that the homogenate is completely thawed before centrifugation.
3. Centrifuge the homogenate in a refrigerated centrifuge (set at 4°C) at $\geq 15,000$ rpm in a microfuge rotor for 20–30 min.
4. Recover the supernatant with a fine-tip transfer pipette and store on ice. It is important to avoid pipetting tissue powder into the supernatant.

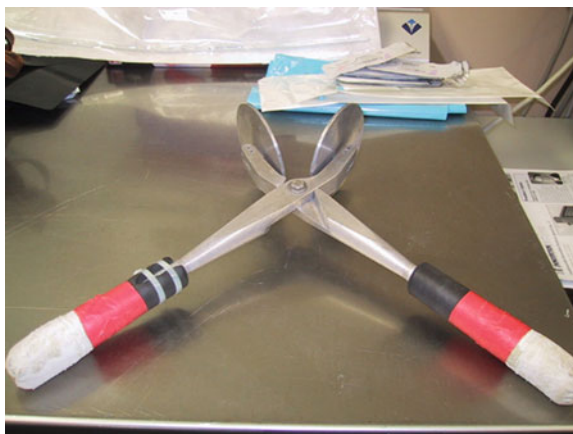


Fig. 6. Freeze clamp.



Fig. 7. Pellet pestle for microfuge tubes.

5. Extract the residue again according to Step 2 and centrifuge according to Step 3.
6. Pool supernatants from the two extractions and lyophilize using a liquid N₂ pretrap to prevent TCA vapor from reaching the vacuum pump.
7. Store lyophilized extract at -80°C until further analysis.

***Protocol for 60%
Acetonitrile Extraction***

1. Homogenize 5 mg of lyophilized tissue powder or cell mass in ≥ 0.4 mL (1:80 w/v) 60% acetonitrile (HPLC or higher solvent grade). Homogenization is achieved by rigorous vortexing or by mechanical shaking in glass beads using a micro ball mill (e.g., MM200, Retsch, Inc.).
2. Incubate the homogenate for 30 min at -80°C to facilitate precipitation of macromolecules.

3. Centrifuge the thawed sample at $\geq 15,000\ g$ for 20–30 min at 4°C.
4. Recover supernatant using a fine-tip transfer pipette into a 2-mL polypropylene microfuge tube or a 7-mL scintillation vial.
5. Repeat steps 1–3 (the -80°C step can be omitted) and pool supernatants.
6. Lyophilize extract using a liquid N_2 pretrap to prevent acetonitrile vapor from reaching the vacuum pump.
7. Store lyophilized extract at -80°C until analysis.

**Protocol for
Chloroform/Methanol/
Water Extraction**

1. Homogenize 5 mg of lyophilized tissue powder or cell mass in 0.4 mL methanol in a 2-mL microfuge tube by rigorous vortexing or by mechanical shaking in glass beads for 1 min at 30 Hz using a micro ball mill (e.g., MM200, Retsch, Inc.). *Alternatively, tissue or cell residue after polar metabolite extraction can be sequentially extracted with methanol for lipid components. For PCA- or TCA-extracted residues, remove acids with two to three times wash in nanopure water. All residues are lyophilized before lipid extraction.*
2. Incubate the homogenate for 20 min at 60°C.
3. Cool and centrifuge sample at $\geq 15,000\ g$ for 30 min at 4°C.
4. Recover supernatant using a fine-tip transfer pipette into a screw-cap glass vial.
5. Add 0.4 mL nanopure water and 0.2 mL chloroform and shake rigorously for 2 min.
6. Centrifuge the biphasic solvent mixture at $\geq 14,000\ g$ for 10 min at 4°C to separate the two phases.
7. Recover the top aqueous layer using a fine-tip transfer pipette into a 2-mL microfuge tube or a 7-mL scintillation vial. *Avoid transferring the protein pellet at the interface of the aqueous and chloroform layers.*

Note: The bottom chloroform layer can also be recovered for nonpolar metabolites.

8. Lyophilize extract using a liquid N_2 pretrap to prevent solvent vapor from reaching the vacuum pump.
9. Store lyophilized extract at -80°C until analysis.

**Protocol for Methanol
Extraction of Lipids**

1. Homogenize medium-free lyophilized cell mass or tissue powder (e.g., 1–5 mg) in 100% methanol (HPLC or MS grade) using a ratio of dry weight:methanol volume $\geq 100:1$ plus 1 mM butylated hydroxytoluene (BHT, as antioxidant).

Homogenization can be done by mechanically beating the pellet with glass beads in a micro ball mill (e.g., MM200, Retsch, Inc.) for 1 min at 30 Hz or by a pellet pestle in microfuge tubes.

2. Let the homogenate stand at room temperature in the dark for 30 min.
3. Centrifuge at $\geq 18,000 \times g$ for 10–20 min at 4°C.
4. Repeat step 1 with another aliquot of 100% methanol and centrifuge as in step 2.
5. Pool both supernatants and dry under vacuum in a Speedvac or lyophilizer with a liquid N₂ pretrap.
6. Dissolve lipid residue in methanol or other appropriate solvents and store in Teflon-faced septum screw-cap glass vials at –80°C until analysis.

Note: Lipid extract should be filtered through 0.45- μ M solvent-compatible filters (e.g., regenerated cellulose, Alltech Assoc.) prior to NMR or MS analysis.

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