Optimization of an HFIP/MTBE Fractionation Method for Sequential Extraction of Proteins, Lipids and Nucleic Acids for Systems Biology Applications.

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Introduction

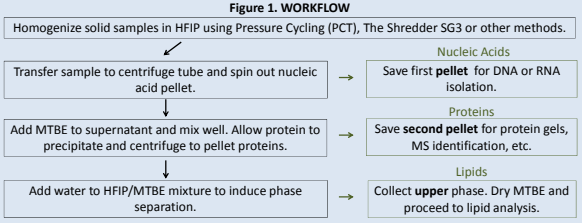
We have developed a new sample preparation technique which allows sequential isolation of proteins, nucleic acids and lipids from solid and liquid samples. The new method is based on one described in 2008 [1] that yielded good separation of proteins and lipids, but involved a lipid-partitioning solvent that was incompatible with many lipid analysis methods. Therefore, the new method described here takes advantage of the excellent protein and lipid solubilizing ability of hexafluoroisopropanol (HFIP), followed by lipid partitioning into methyl-tert-butyl ether (MTBE), a volatile solvent that is compatible with most downstream lipid analysis methods [2,3].

Initial sample homogenization is carried out in HFIP, a volatile organic solvent that has been previously shown to effectively solubilize proteins and lipids without the need for any added salts or detergents [1]. Nucleic acids, which are not soluble in HFIP, are separated by centrifugation. DNA and/or RNA can then be further purified from the nucleic acid pellet. MTBE is added to the lysate to precipitate the proteins. Water is used to induce phase separation of the lipid-containing MTBE phase from the residual HFIP/water phase. Due to its low density, the MTBE forms the upper layer after centrifugation, making lipid recovery more convenient than chloroform-based methods. This HFIP/MTBE method was previously used to extract lipids from neonatal fecal material for lipidomic profiling [4].

Initial Method Development

Solid samples, such as animal or plant tissues, must be homogenized in HFIP. Due to the highly volatile nature of this solvent, the most convenient sample homogenization methods are those that keep the samples in sealed containers. For this reason, solid tissue samples were homogenized in closed PULSE Tubes using the Shredder SG3, followed by pressure cycling in an NEP2320 Barocycler at 35,000psi.

Figure 1. WORKFLOW



Reagent Ratios: A 4x volume of MTBE relative to HFIP resulted in the best lipid partitioning into the MTBE phase, as well as the best protein yield (Table 1). Subsequent phase separation induced by the addition of water was shown to be optimal with a 3x volume of water relative to the initial HFIP volume (not shown). Lower volumes of water led to poor phase separation, while larger volumes appeared to have no added benefit. The final optimized ratio was determined to be 1:4:3 HFIP:MTBE:Water.

Table 1. Optimization of HFIP:MTBE Ratio

HFIP:MTBE Ratio	Final Protein Extract (mg/ml in ProteoSolve-IEF)	Observations
1:1	0.9	Much of the protein pellet remains undissolved
1:2	1.3	
1:3	2.4	Much of the protein pellet is dissolved
1:4	2.9	
1:5	2.9	

Protein and DNA: Various animal and plant tissues were used to validate protein and DNA extraction using the HFIP/MTBE method.

Figure 2. Genomic DNA

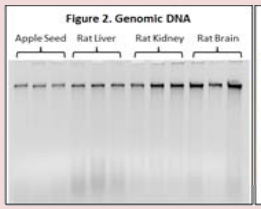
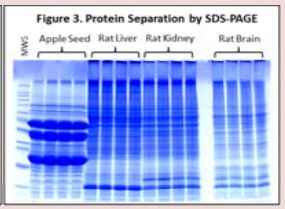


Figure 3. Protein Separation by SDS-PAGE



DNA, which is not soluble in HFIP, was purified from the insoluble pellets after the first centrifugation, and visualized on agarose gels (Figure 2). The protein pellets were solubilized directly in laemmli buffer and visualized by SDS-PAGE (Figure 3).

Lipids: Fluorescently labeled phosphatidylcholine was spiked into tissue lysates in HFIP. As expected, the fluorescent signal was found to partition almost entirely into the MTBE phase, with negligible signal detected in the HFIP phase after partitioning (Figure 4). Other fluorescently labeled lipids were also tested (Figure 5) and confirm that lipids of different classes are correctly partitioned into the MTBE. Further testing confirmed that the fluorescent lipids are recovered almost exclusively from the MTBE phase (Figure 6, n=3 per sample).

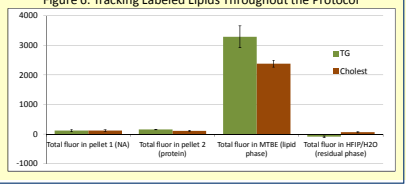
Figure 4. Labeled Phosphatidylcholine Partitioning in Tissue Lysates

	Sample:	Rat Spleen	Mouse Brain	Rat Adipose	Neonatal Human Stool	Rat Sk. Muscle	Rat Liver	Apple seed	Control
HFIP/MTBE Method	MTBE (upper) Phase	1.3×10^4	1.3×10^4	1.8×10^4	1.7×10^4	1.4×10^4	1.3×10^4	1.7×10^4	1.2×10^4
	HFIP/Water (lower) phase	0	0	$<0.07 \times 10^4$	$<0.07 \times 10^4$	0	$<0.07 \times 10^4$	$<0.07 \times 10^4$	$<0.01 \times 10^4$
Bligh-Dyer Method Control	Chloroform (lower) Phase	1.5×10^4							
	Methanol/Water (upper) Phase	0							

Figure 5. Labeled Lipids Partitioning in Blood lysate

Fluorescent Lipid:	Phosphocholine	Triglyceride (TG)	Cholesterol	Free Fatty Acids
MTBE (upper) Phase	1.4×10^4	4.1×10^4	4.5×10^4	4.1×10^4
HFIP/Water (lower) phase	0	0.3×10^4	0.2×10^4	0.4×10^4

Figure 6. Tracking Labeled Lipids Throughout the Protocol



Further lipid extraction validation was performed to demonstrate that the pool of lipids obtained by the HFIP/MTBE method is comparable to that obtained using a standard methanol/DCM extraction. Rat serum samples extracted using the two methods were compared by LC-MS (Figure 7). The results demonstrate that the two methods are essentially equivalent in the amount and type of lipids recovered. In addition, the effect of pressure cycling (PCT) was examined (Figure 8), and the results confirm that pressure cycling appears to have no deleterious effect on lipid extraction and detection, confirming that this method of sample disruption can be used to homogenize solid samples without leading to any artifactual results. SDS-PAGE of proteins extracted from the same serum samples is shown in Figure 9.

Figure 7. Serum Lipid Analysis in Positive (7A) and Negative (7B) Ionization Mode. The HFIP/MTBE method (no PCT) is compared to a standard methanol/DCM method.

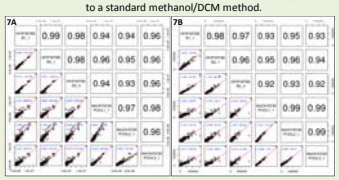
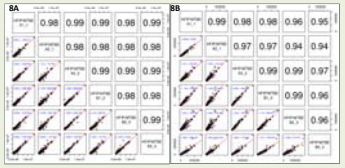


Figure 8. Serum Lipid Analysis in Positive (8A) and Negative (8B) Ionization Mode. HFIP/MTBE method performed with (A1, A2, A3) and without (B1, B2, B3) pressure cycling.

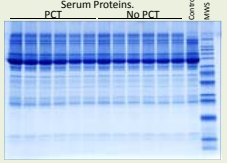


Figures 7 and 8 show scatter plots along with regression lines and coefficients of correlation. The samples (n=3 of each) were quantified using Thermo Sieve software and the correlation analysis was done in R.

Serum lipid extraction method

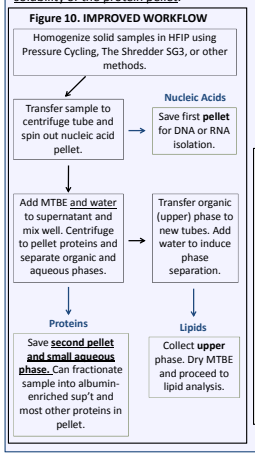
Aliquots of pooled rat serum were spiked with an internal standard mix and processed using the HFIP/MTBE method with or without pressure cycling in PCT MicroTubes as follows: To each 30µl serum aliquot, 110µl of HFIP was added and the samples were either subjected to pressure cycling (20 cycles at 35,000psi at ambient temp), or vortexed vigorously (n=6 each). The samples were transferred to centrifuge tubes and the PCT MicroTubes were rinsed with 90ul additional HFIP to ensure complete sample recovery. Proteins were precipitated by addition of 800µl MTBE (the amount of nucleic acids in this serum was too low for recovery by this method). The proteins were pelleted by centrifugation and the resulting pellets were dissolved directly in Laemmli buffer and separated by SDS-PAGE (Figure 9). After centrifugation, the supernatant was transferred to a second tube and phase separation was induced by addition of 600µl dH_2O. Phases were separated by centrifugation and the upper MTBE phase was collected and dried prior to LC-MS. Control samples extracted using methanol/DCM were from the same pool of rat serum and were extracted by conventional methods without pressure cycling.

Figure 9. SDS-PAGE Separation of Rat Serum Proteins.



Optimization for Improved Protein Recovery. During method development, we observed that the protein pellet was often difficult to dissolve, even in reagent containing urea and CHAPS detergent (ProteoSolve-IEF). Addition of a variety of detergents to the protocol did not significantly improve protein pellet solubility (data not shown). A slight method modification that involves the addition of a small amount of water to the precipitation reaction (Figure 10) was found to significantly improve protein yield by improving the solubility of the protein pellet.

Figure 10. IMPROVED WORKFLOW



The addition of water to the precipitation step at the same time as the MTBE forms a small aqueous phase below the HFIP/MTBE organic phase and contains some of the most soluble sample proteins (e.g., albumin). This has the added benefit of fractionating the sample proteins into a serum albumin-enriched supernatant fraction and a serum albumin-depleted pellet fraction (Figure 11). The total protein yield is greater than obtained using the original protocol (Figure 12), due in large part to the greater solubility of the recovered protein pellet.

Figure 11. SDS-PAGE Separation of Rat Spleen Proteins .

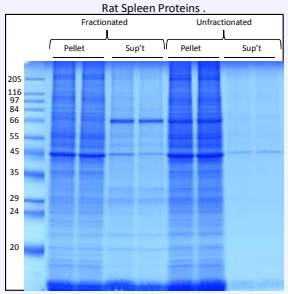


Figure 12. Protein Yield Quantification

	Soluble protein recovered in pellet (µg)	Soluble protein recovered from total supernatant after drying (µg)
Original Method (no water)	654	0
Water added (Fractionated)	607	197
Water added (Unfractionated)	855	6

Conclusions

The HFIP/MTBE method described here can be used to sequentially extract nucleic acids, proteins and lipids from solid or liquid samples for systems biology studies. The use of the volatile HFIP and MTBE solvents allows for efficient mass spectrometry-compatible protein and lipid extraction, without the use of detergents, salts or other non-volatile additives. The lipids extracted by this method are comparable to those extracted by a standard method that utilizes methanol and DCM.

References

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