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# Utilizing Soil Density Fractionation to Separate Distinct Soil Carbon Pools

**Derek Pierson**[*,1,2], **Kate Lajtha**[*,1], **Hayley Peter-Contesse**[*,1], **Amy Mayedo**[*,1]

[1] Department of Crop and Soil Sciences, Oregon State University [2] USDA Forest Service

[*] These authors contributed equally

**Corresponding Author**

**Derek Pierson**
derek.pierson@usda.gov

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## Abstract

Soil organic matter (SOM) is a complicated mixture of different compounds that span the range from free, partially degraded plant components to more microbially altered compounds held in the soil aggregates to highly processed microbial by-products with strong associations with reactive soil minerals. Soil scientists have struggled to find ways to separate soil into fractions that are easily measurable and useful for soil carbon (C) modeling. Fractionating soil based on density is increasingly being used, and it is easy to perform and yields C pools based on the degree of association between the SOM and different minerals; thus, soil density fractionation can help to characterize the SOM and identify SOM stabilization mechanisms. However, the reported soil density fractionation protocols vary significantly, making the results from different studies and ecosystems hard to compare. Here, we describe a robust density fractionation procedure that separates particulate and mineral-associated organic matter and explain the benefits and drawbacks of separating soil into two, three, or more density fractions. Such fractions often differ in their chemical and mineral composition, turnover time, and degree of microbial processing, as well as the degree of mineral stabilization.

## Introduction

Soil is the largest store of terrestrial carbon (C), containing upward of 1,500 Pg of C in the top 1 m and almost double that amount in deeper levels globally, thus meaning soil contains more C than plant biomass and the atmosphere combined[1]. Soil organic matter (SOM) retains water and soil nutrients and is essential for plant productivity and the function of the terrestrial ecosystem. Despite global recognition of the importance of adequate SOM stocks for soil health and agricultural productivity, soil C stocks have been substantially depleted due to unsustainable forest and agricultural management, landscape change, and climate warming[2,3]. Increased interest in restoring soil health and in using soil C retention as a key player in natural climate solutions has led to efforts to understand the factors that

control soil C sequestration and stabilization in diverse environments[4,5].

Soil organic matter (SOM) is a complicated mixture of different compounds that span the range from free, partially degraded plant components to more microbially altered compounds held in the soil aggregates (defined here as a material formed by the combination of separate units or items) to highly processed microbial by-products with strong associations with reactive soil minerals[6]. In cases where it is impractical to identify the full suite of individual compounds in the SOM, investigators often focus on identifying a smaller number of functional pools of C that exist as physical realities and that vary by turnover rates, general chemical composition, and the degree of stabilization with the mineral components of the soil[1,7]. In order for pools to be critically interpreted and modeled, it is essential that the separated pools be small in number, be directly measurable rather than just theoretical, and exhibit clear differences in composition and reactivity[8].

Many different techniques, both chemical and physical, have been employed to isolate meaningful pools of soil C, and these are well summarized by von Lützow et al.[9] and Poeplau et al.[10]. Chemical extraction techniques aim to isolate specific pools, such as C associated with either poorly crystalline or crystalline Fe and Al[11]. Organic solvents have been used to extract specific compounds such as lipids[12], and either the hydrolysis or oxidation of SOM has been used as a measure of a labile pool of C[13,14]. However, none of these extraction methods categorize all the pools of C into measurable or modellable fractions. The physical fractionation of soil categorizes all soil C into pools based on size and assumes that the decomposition of plant debris results in fragmentation and increasingly smaller particles. Although size alone cannot separate free plant debris from mineral-associated SOM[15], quantifying these two pools is critical for the understanding of soil C stabilization due to common spatial, physical, and biogeochemical differences in formation and turnover[16].

The fractionation of soil C based on density is increasingly being used, and it is easy to perform and identifies different pools of C based on the degree of association with different minerals[17,18,19]; thus, soil density fractionation can help elucidate differing soil C stabilization mechanisms. The primary requirement for soil to be fractionated is the ability to fully disperse the organic and mineral particles. Once dispersed, degraded organic matter that is relatively free of minerals floats in solutions lighter than ~1.85 g/cm$^3$, while minerals typically fall in the range of 2-4.5 g/cm$^3$, although iron oxides may have densities up to 5.3 g/cm$^3$. The light or free particulate fraction tends to have shorter a turnover time (unless there is significant contamination by charcoal) and has been shown to be highly responsive to cultivation and other disturbances. The heavy (>1.85 g/cm$^3$) or mineral-associated fraction often has a longer turnover time due to the resistance to microbially mediated decomposition gained when organic molecules bind with reactive mineral surfaces. However, the heavy fraction may saturate (i.e., reach an upper limit for mineral complexation capacity), while the light fraction can theoretically accumulate almost indefinitely. Thus, understanding the physical distribution of organic matter in pools of mineral-associated versus particulate organic matter helps to elucidate which ecosystems can be managed for efficient carbon sequestration and how different systems will respond to climate change and shifting patterns of anthropogenic disturbance[20].

While the use of density fractionation using solutions of sodium polytungstate at different densities has increased greatly in the last decade, the techniques and protocols vary

significantly, making the results from different studies and different ecosystems hard to compare. Although a density of 1.85 g/cm$^3$ has been shown to recover the greatest amount of free light fraction with minimal inclusion of mineral-associated organic matter (MAOM)[17], many studies have used densities ranging from 1.65-2.0 g/cm$^3$. While most studies have fractionated soils into just two pools (a light fraction and a heavy fraction, hereafter LF and HF), other studies have used multiple densities to further refine the heavy fraction into pools that differ by the minerals that they are associated with, the relative ratio of minerals to organic coating, or the degree of aggregation (e.g., Sollins et al.[17], Sollins et al.[18], Hatton et al.[21], Lajtha et al.[22], Yeasmin et al.[23], Wagai et al.[24], Volk et al.[25]). In addition, more complex fractionation procedures have been suggested that combine both size and density separation, resulting in a larger number of pools (e.g., Yonekura et al.[26], Virto et al.[27], Moni et al.[15], Poeplau et al.[10]) but also more room for error, both in the methodology and in relation to the pool size. Further, authors have also used sonication at varied intensities and times in an effort to disperse aggregates and MAOM from mineral surfaces[28,29,30].

Here, we describe a robust density fractionation procedure that identifies, first, two unique pools of soil carbon (LF and HF, or POM and MAOM), and we offer both the techniques and the arguments to further separate the HF pool into additional fractions that differ based on their mineralogy, degree of organic coating, or aggregation. The fractions identified here have been shown to differ in terms of their chemical composition, turnover time, degree of microbial processing, and degree of mineral stabilization[18,19].

The following procedure separates bulk soil into particulate organic matter (POM) and mineral-associated organic matter (MAOM) by mixing a known quantity of soil in a solution with a specific density. The efficacy of the procedure is measured by the combined recovery of soil mass and carbon relative to the initial soil sample mass and C content. A dense solution is achieved by dissolving sodium polytungstate (SPT) in deionized water. The soil is initially mixed with the dense SPT solution and agitated to thoroughly mix and disperse the soil aggregates. Centrifugation is then used to separate the soil materials that either float (light fraction) or sink (heavy fraction) in the solution. The mixing, isolation, recovery, and washing steps are repeated multiple times to ensure the separation of the light and heavy fractions, along with the removal of SPT from the material. Finally, the soil fractions are dried, weighed, and analyzed for C content. The fractionated material may be used for subsequent procedures and analyses.

## Protocol

### 1. Making stock solutions of sodium polytungstate (SPT)

CAUTION: SPT is an irritant and is harmful if swallowed or inhaled. It is toxic to aquatic organisms; avoid its release into the environment.

1. To make 1 L of SPT solution with a density of 1.85 g/cm$^3$, dissolve 1,051 g of crystalized SPT in approximately 600 mL of deionized distilled (DDI) water. Stir the solution until the SPT has fully dissolved, approximately for 15 min, and then bring the solution volume to 1 L with DDI.
   NOTE: Carbon recovery using a solution density <1.85 g/cm$^3$ may under-recruit light fraction carbon derived from particulate organic matter[17,18], thus misrepresenting the quantity of carbon in the sample. Thus, an SPT solution

density of 1.85 g/cm$^3$ is suggested[8,17] in order to be more inclusive of carbon associated with particulate organic matter for a typical soil sample (i.e., most sand, silt, and clay loams with C content <10 %).

2. To make 1 L of SPT solution with a density of 2.40 g/cm$^3$, dissolve 1,803 g of solid SPT in approximately 500 mL of DDI water. Stir the solution until the SPT has fully dissolved, and then bring the solution volume to 1 L with DDI.

   NOTE: Beyond the potential use for soil fractionation, a solution with a density greater than 1.85 g/cm$^3$ is often required for the adjustment of the SPT solution at later steps in the protocol (see step 3.2). If an extra 2.40 g/cm$^3$ solution is leftover, the solution may be diluted to 1.85 g/cm$^3$ with deionized water and used for soil fractionation.

3. Prior to use in fractionation, analyze the SPT for C and N content. Perform this analysis by using a solid or liquid elemental analyzer (example methods: ISO 10694:1995, ISO 20236:2018).

   1. Perform a 1:100 dilution of the solution from step 1.1 for the liquid elemental analyzers to reduce the deterioration of the elemental scrubbers and catalysts. The tolerance for C and N contamination in the SPT solution will depend on the sample and the subsequent uses of the soil fractions. Typically, an SPT solution with a C and N content <1 ppm and <0.1 ppm, respectively, is considered suitable for use, as solutions such as this present minimal capacity for altering the much larger soil C and N pools.

## 2. Dissolution of soil in SPT

1. Add 50 g of soil that is air-dried and sieved to 2 mm to a 250 mL conical polypropylene centrifuge tube. Record the mass to at least four significant figures. Do not use oven-dried soil as this may increase the soluble carbon due to heat-induced cell lysis[31].

   NOTE: Field moist soil may be used[31], but further adjustment is required in the later steps to maintain the target density of the SPT solution. Sieving the soil material to 2 mm is recommended to remove large material that may skew the fractionation results, such as rocks and woody debris.

   1. Adjust the soil mass to ensure an adequate mass of each fraction is recovered to avoid significant error in the quantification. The most common reason for mass adjustment is low POM content (e.g., <2% of the total soil mass). For such soils, provide additional soil mass to accurately quantify the POM recovery. Overall, it is acceptable to adjust the soil mass for each individual sample, since changing the sample mass will not alter the proportion of POM to MAOM. However, it is often useful to use a consistent mass to aid the balancing of the centrifuge.

   2. Treat soils rich in carbonates to remove inorganic carbonates prior to fractionation[32].

2. Add 50 mL of 1.85 g/cm$^3$ density SPT to the centrifuge tube, and replace the lid tightly. As with the soil amounts, adjust the SPT volume as needed. In POM-rich surface soils (e.g., many temperate forest soils), use a larger ratio of soil to SPT (e.g., 30 g of soil to 60 mL of SPT) to achieve adequate separation of the light and heavy fraction materials.

3. Shake the tube vigorously by hand for ~60 s to break up non-water-stable aggregates. The forceful collision of the soil aggregates with the side walls of the centrifuge tube is desired, meaning simply vortexing the solution may be insufficient.

4. Secure the tube to a platform shaker. Often, placing the tube on its side aids in soil dispersion by increasing the sloshing force of the solution and reducing the standing height of the soil layer. Take care that the tube is tightly sealed, and shake for 2 h at 40-120 rpm. Periodically remove the tube from the shaker and shake vigorously by hand to increase the agitation of the denser aggregated material.

## 3. Performing a coarse soil fractionation

1. Remove the tube from the shaker. Equalize the centrifuge tube masses by carefully adding additional SPT solution to reach a consistent mass across the set of tubes to be centrifuged, ensuring to shake vigorously by hand for 30 s after adding the SPT solution. Centrifuge for 10 min at 3,000 x *g* in a swinging bucket centrifuge.

2. Before aspirating the sample, test the density of the supernatant by drawing off 5 mL of the solution with a pipette and checking the mass on a balance. Adjust the SPT density as necessary to achieve the desired density. Shake and centrifuge again if a solution density adjustment was performed.

3. Attach a 1 L sidearm flask to a vacuum pump. Place a 110 mm glass fiber filter (0.7 µm pore size) in a 12 cm internal diameter (ID) porcelain Buchner funnel. Seal the funnel carefully using a conical rubber gasket onto the sidearm flask.

   NOTE: The glass fiber filters should be pre-washed in a drying oven at 150 °C and rinsed with DDI before use.

4. Set up one additional 1 L sidearm flask attached to the vacuum. Place a rubber stopper in the top of the flask with a ~0.5 m protruding length of tubing attached for aspiration.

   NOTE: It may be helpful to attach a plastic tip (such as a 5 mL disposable pipet tip, with the end clipped off at an angle) to the end of the aspiration tubing to improve the control of the suction during aspiration (see **Figure 1**).

5. Gently aspirate the supernatant and suspended material that has settled at the top layer of the solution along the sides of the centrifuge tube, being careful not to touch the tip of the aspiration tube to the pelleted soil surface underneath.

   NOTE: If any soil pellet material (heavy fraction) is mistakenly aspirated along with the suspended (light fraction) material, the fractionation procedure should be repeated. If unnoticed, such an error will result in a heavier than expected light fraction mass with a lower than expected C content, which may be evident through the data analysis of samples with similar soil properties.

   1. To clean the aspiration tube between samples, plunge the tip of the tube quickly (e.g., submerge for 0.1 s) in DDI water, and draw ~5 mL of DDI water through the line with the vacuum pump on. Repeat until all the material has been flushed from the vacuum tube.

   2. Remove the rubber stopper and aspiration tube attachment from the sidearm flask, and pour the contents into the top of the Buchner funnel with the vacuum pump on.

3. Rinse the flask with DDI water, swirl, and pour the flask contents into the Buchner funnel. Repeat until all the residue adhered to the sides of the flask is removed.

6. Add 50 mL of SPT to the centrifuge tube, and shake vigorously by hand for 60 s (or use a shaker table if the soil does not rapidly disperse), making sure to break up the hard pellet at the bottom of the tube so that all the residue is resuspended. Centrifuge for 10 min at 3,000 x *g*.

7. Repeat step 3.5. Pour the flask contents into the same Buchner funnel as used in step 3.5.2.

8. Add 50 mL of SPT to the centrifuge tube, and shake vigorously by hand, making sure to break up the hard pellet at the bottom of the tube. Centrifuge for 10 min at 3,000 x *g*.

9. Repeat step 3.5. Pour the flask contents into the same Buchner funnel as used in step 3.5.2.

## 4. Additional density separation(s) using higher-density SPT

NOTE: If performing more than one additional density fraction, the subsequent fractionations must be performed in order of increasing density. Here, steps for isolating using 1.85-2.4 g/cm$^3$ and >2.4 g/cm$^3$ density SPT are shown.

1. Add 50 mL of 2.4 g/cm$^3$ SPT to the centrifuge tube containing the >1.85 g/cm$^3$ soil material from step 3. Shake vigorously by hand (>60 s), making sure to break up the hard pellet at the bottom of the tube. Centrifuge for 10 min at 3,000 x *g*.

2. Before aspirating the sample, test the density of the supernatant by drawing off 5 mL of the solution with a pipette and checking the mass on a balance. Adjust the SPT density as necessary to achieve the desired density. Shake and centrifuge again if a solution density adjustment was performed.

3. Repeat step 3 using a 2.4 g/cm$^3$ SPT solution in place of the 1.85 g/cm$^3$ SPT solution used previously. At the end of step 3, the material isolated in the Buchner funnel will have a density between 1.85-2.4 g/cm$^3$, while the material remaining in the centrifuge tube will have a density >2.4 g/cm$^3$.

## 5. Washing the SPT from the heavy and light fraction samples

NOTE: The following washing steps must be performed for all the fractionated material. If the SPT solution is not completely rinsed from the material, the corresponding fraction weights will be inaccurate.

1. Add 50 mL of DDI water to the centrifuge tube with the heavy fraction material, and shake vigorously by hand (60 s), making sure to break up the hard pellet at the bottom of the tube. Centrifuge for 10 min at 3,000 x *g*.

2. Aspirate as in step 3.5. At this point, all the light fraction material should have been removed. Dispose of the clear aspirate in a waste bucket instead of adding it to the filter funnel.

3. Repeat steps 5.1-5.2 twice. Before finally aspirating the solution in the tube, use a transfer pipette to draw off 25 mL of the supernatant, and check the density by dividing the solution weight by the volume to ensure that the SPT has been adequately removed from the sample. If the density is <1.01 g/mL, proceed to the next step. If the density is 1.01 g/mL or greater, perform additional water washes as above until the density is less than 1.01 g/mL.

4. To ensure the complete removal of the SPT from the light fraction, fill each Buchner funnel with DDI water, and filter the contents through glass fiber filters. Once the water has filtered through completely, repeat this twice more. If the soil is high in organic matter, filtration may take up to 48 h.

### 6. Collection of the heavy fraction material

1. Carefully scrape the soil from the centrifuge tube into a clean, labeled glass beaker or jar. Pour enough DDI water into the tube to loosen the remaining soil; replace the cap and shake, and then add the slurry to the glass container. Rinse all the remaining soil from the centrifuge tube, and transfer into the glass container using deionized water.
2. Place the glass container in a drying oven set between 40-60 °C. Dry until a constant dry weight is reached, typically for 24-72 h.

### 7. Collection of the light fraction material

1. Turn off the vacuum pump, and remove the funnel from the sidearm flask. Holding the funnel horizontally over a labeled glass beaker or jar, gently rinse the particles from the filter using a DDI water wash bottle.
   NOTE: It may be necessary to gently scrape the filter using a spatula and to rinse both sides of the filter to remove all the residue.
2. Place the glass container in the drying oven set between 40-60 °C. Dry until a constant dry weight is reached, typically for 24-72 h.

### 8. Weighing the dry mass of the fractionated material

1. Gently scrape all the dried material from each container into a plastic weigh boat. Record the mass up to the fourth decimal place. Place the sample into a labeled storage vial or bag.
2. Repeat for all the dried samples.

### 9. Data collection and analysis for total organic carbon

1. Follow the analysis procedures in accordance with the instrument to be used for the analysis of the elemental C content (e.g., ISO 10694:1995).
   NOTE: Grinding the dried fraction material into a fine powder is a common practice to ensure the homogeneity of the fractionated sample before elemental analysis.
2. Ensure that the cumulative mass of all the fractions is equal to at least ~90% of the original soil sample mass. If the losses of material are >10%, additional replicate fractionations are recommended.
3. Quantify the cumulative recovery of soil organic carbon (SOC) in the fractions. Losses of SOC may not correlate perfectly with mass loss due to the disproportionate loss of fraction material and the loss of dissolved organic carbon. Yet, losses of SOC should also be <10 % of the initial SOC in the soil sample.

## Representative Results

Soil density fractionation is ideally suited for investigating how soils differ in their particulate and mineral-associated organic matter content. Separating the SOC into these two distinct pools provides an avenue to elucidate the changes in soil C content and stabilization dynamics that may otherwise be

unclear when observing trends in bulk soil C content. The further separation of the heavy material (density >1.85 g/cm$^3$) provides additional insight into the changes and trends in soil C stabilization but increases the complexity of the procedure and the associated interpretation and is associated with additional costs. Nonetheless, the fractionation of the soil into three or more density pools may elucidate complex trends and chemical differences in soil C pools. As with any soil fractionation procedure, the separation of these soil C pools is imperfect, and the potential influence of such errors and the assumptions of the method should be recognized when reporting the results. Finally, practitioners should be aware of the variety of soil fractionation methods that exist and their unique strengths and weaknesses (see reviews and comparisons provided by von Lützow et al.[9] and Poeplau et al.[10]). Many of these soil fractionation methods are not mutually exclusive and may be appropriately combined to improve or validate analyses of soil C dynamics.

Choosing the number of density fractions to be used is the critical first step before beginning the fractionation procedure. While multiple pools can always be mathematically combined to produce a single light and a single heavy fraction to compare results to other studies, fractionating the heavy fraction into more than one pool adds significant time and expense. Pierson et al.[33] quantified the change in SOC across three density pools following a long-term detrital manipulation study. When combining the two heavier pools (**Figure 2**), the effects of the detrital treatments were distinct between the light (<1.85 g/cm$^3$) and heavy (>1.85 g/cm$^3$) fractions, especially relative to the effects observed from the bulk SOC content. By performing the additional density fractionation at 2.40 g/cm$^3$ (**Figure 3**), it could be further determined that the treatment effects on MAOM were predominantly confined to the higher-density material (>2.4 g/cm$^3$). Finally, the reported C:N content of the bulk soil relative to the density fractionated pools (**Figure 4**) presents a clear demonstration of the effectiveness of the density fractionation method for separating plant-based particulate material from mineral matter with relatively low C:N content.

After 50 years of detrital manipulations, Lajtha et al.[22] used six sequential density fractionations to closely examine the detrital influences on SOC stabilization and destabilization (**Figure 5**). By isolating seven SOC pools, the authors were able to observe a greater nuance in the POM and MAOM response following litter addition and removal treatments. Pools with densities <2.20 g/cm$^3$ were more responsive to the treatment, in agreement with the long-standing expectation of a positive relationship between litter input and SOC accumulation. However, the response of SOC pools with densities >2.20 g/cm$^3$ was less pronounced, and specifically for the litter removal treatment, an opposite, negative relationship was identified between the inputs and the SOC (i.e., the inputs were reduced, but the SOC content of the fraction increased). By performing subsequent analyses of $^{14}$C, the contributions and losses of more recent C inputs for each pool were ascertained, providing mechanistic insight into the detrital control of the formation and destabilization pathways for MAOM.

Combining isotopic analysis with sequential density fractionation provides additional avenues to investigate the intricacies of SOM dynamics. Yet, investigators should take care to consider the influence of mineralogy on the density fractionation results. Unique mineral structure and reactivity cause inherent differences in particle density distributions between minerals. As an example, Sollins et al.[18] examined the isotopic and chemical characteristics of multiple density pools in four forested soils with disparate parent materials

and morphologies (**Figure 6**). The contrasting mineralogy between the four soils contributed heavily to the observed differences in $^{13}C$, $^{14}C$, and $^{15}N$ for each density fraction. Without the consideration of mineralogy, such results may be misinterpreted with respect to SOC formation and stabilization dynamics. Finally, returning to the practicality of performing additional density separations, little additional information was gained by Sollins et al.[18] from the analysis of six density pools as opposed to only three (**Figure 6**).

Helbling et al.[31] determined the effect of seasonality on the light fraction content of forested soils, as well as the effect of soil drying treatment on the loss of C to the soluble pool (**Figure 7**). Two significant results emerged from this work. First, while oven-drying the soil yielded significantly greater dissolved organic C loss to the SPT solution, the amount of C lost was insignificant. Secondly, there did not appear to be any seasonality to the light fraction C pool, meaning that the soil sample collection timing did not influence the fractionation results. However, the results may be expected to differ across soils and environments given the differences in POM stocks and decomposition rates.

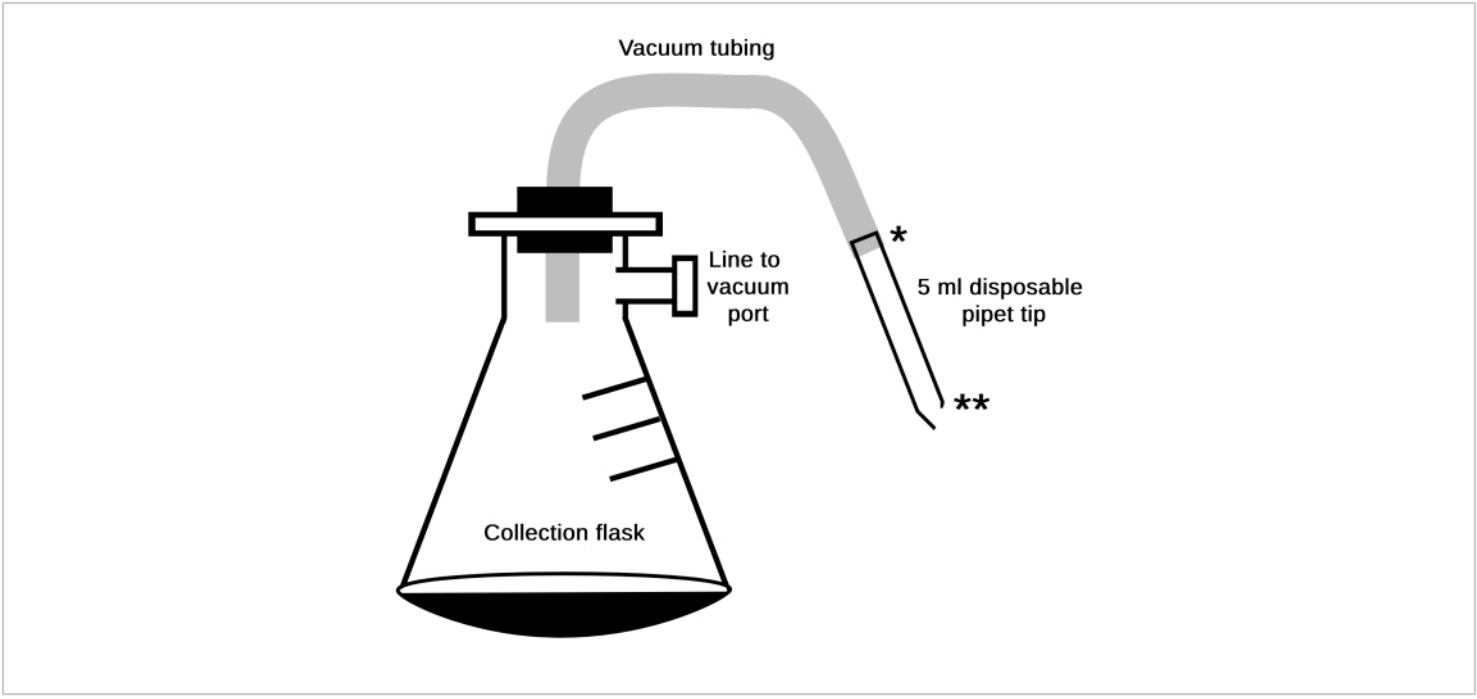


**Figure 1: Light fraction aspiration apparatus.** Schematic of the vacuum apparatus for the aspiration of the light fraction. *Parafilm may be used to secure and seal the junction between the pipet tip and the vacuum tube. **Cutting the pipet tip at an angle may be useful to increase the size of the tip opening, as well as for applying close suction to the side walls of the centrifuge tube. Please click here to view a larger version of this figure.

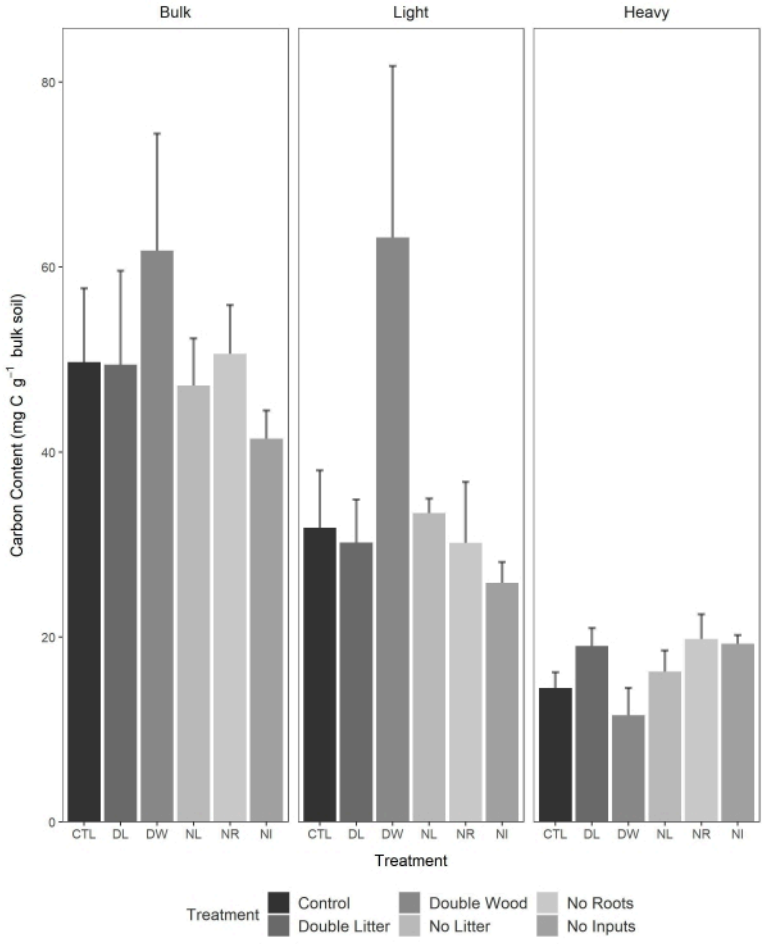


**Figure 2: Comparison of the carbon content for bulk, light, and heavy fraction soil.** Soil was collected from the Detrital Input and Removal Treatment (DIRT) plots in the H.J. Andrews Experimental Forest after 20 years of treatment (n = 3). The mineral-associated C for the root removal treatments (NR, NI) was significantly increased, contrasting with the observed effects on bulk C content. Further, the fractionated results show that the increase in bulk C from the double wood (DW) treatment was derived from an increase in the light fraction C. The error bars represent the standard error. This figure has been modified from Pierson et al.[33]. Please click here to view a larger version of this figure.

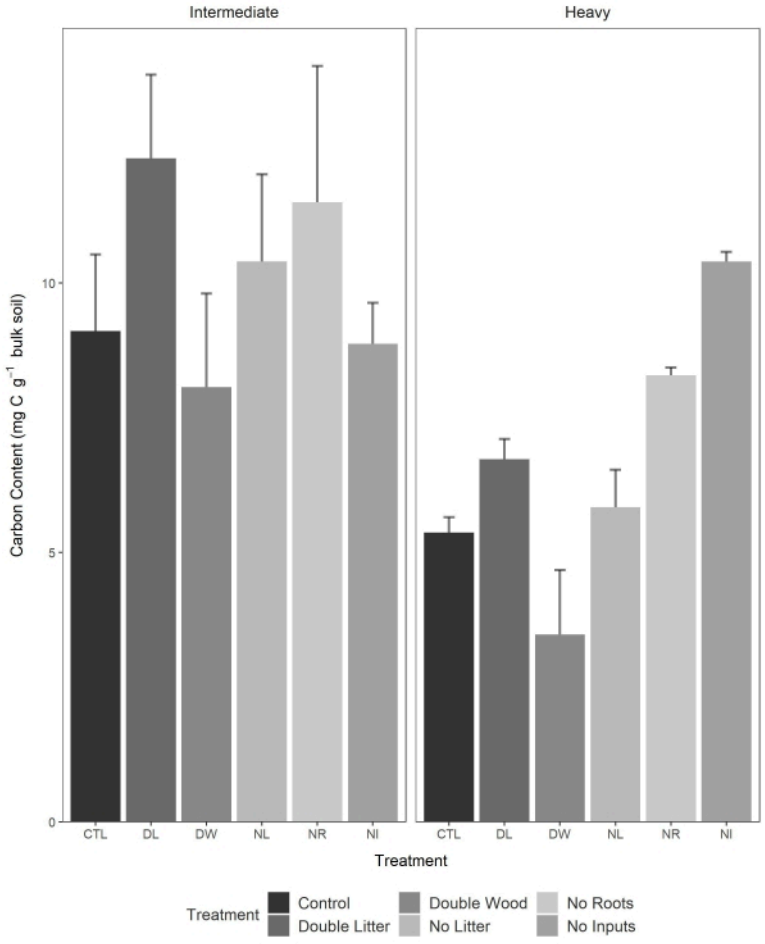


**Figure 3: Comparison of the intermediate and heavy fraction pools.** Soil was collected from the Detrital Input and Removal Treatment (DIRT) plots in the H.J. Andrews Experimental Forest after 20 years of treatment (n = 3). The results demonstrate the findings from the isolation of an intermediate fraction (1.85-2.40 g/cm$^3$) and a heavier fraction (>2.40 g/cm$^3$). The intermediate fraction C content showed greater variability, and no treatment effects were significant. The error bars represent the standard error. This figure has been modified from Pierson et al.[33]. Please click here to view a larger version of this figure.

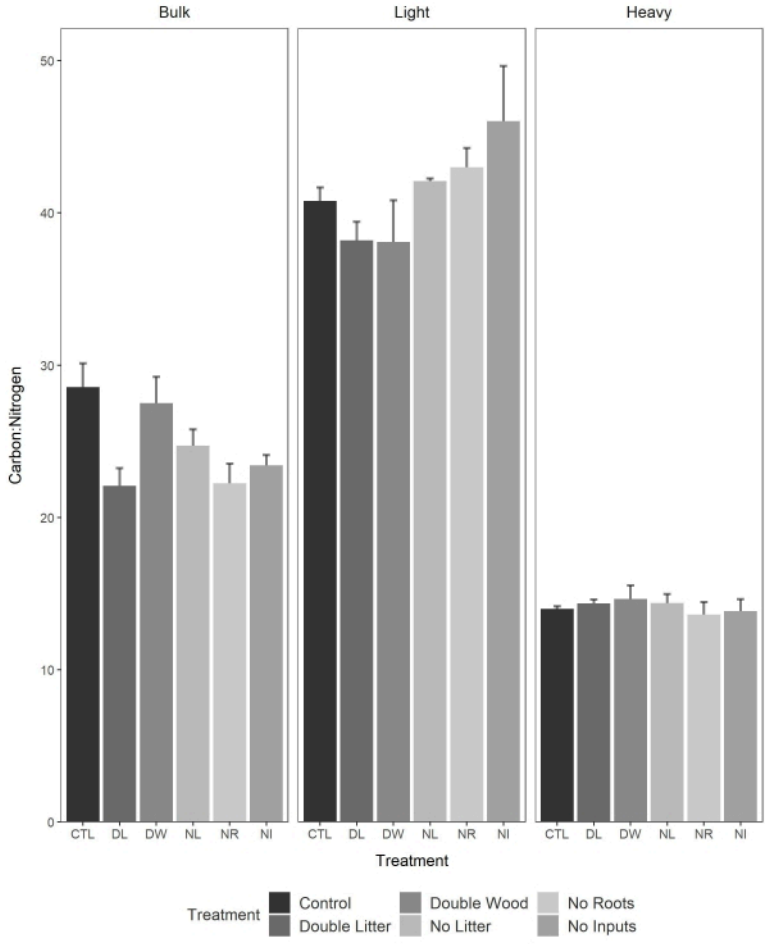


**Figure 4: Carbon to nitrogen ratios for bulk, light, and heavy fraction soil.** Soil was collected from the Detrital Input and Removal Treatment (DIRT) plots in the H.J. Andrews Experimental Forest after 20 years of treatment (n = 3). Given the high C:N content of plant tissue relative to soil minerals, the observed difference in C:N content between the light and heavy fractions clearly demonstrates the capability of soil density fractionation to separate particulate organic matter from bulk soil. The error bars represent the standard error. This figure has been modified from Pierson et al.[33]. Please click here to view a larger version of this figure.

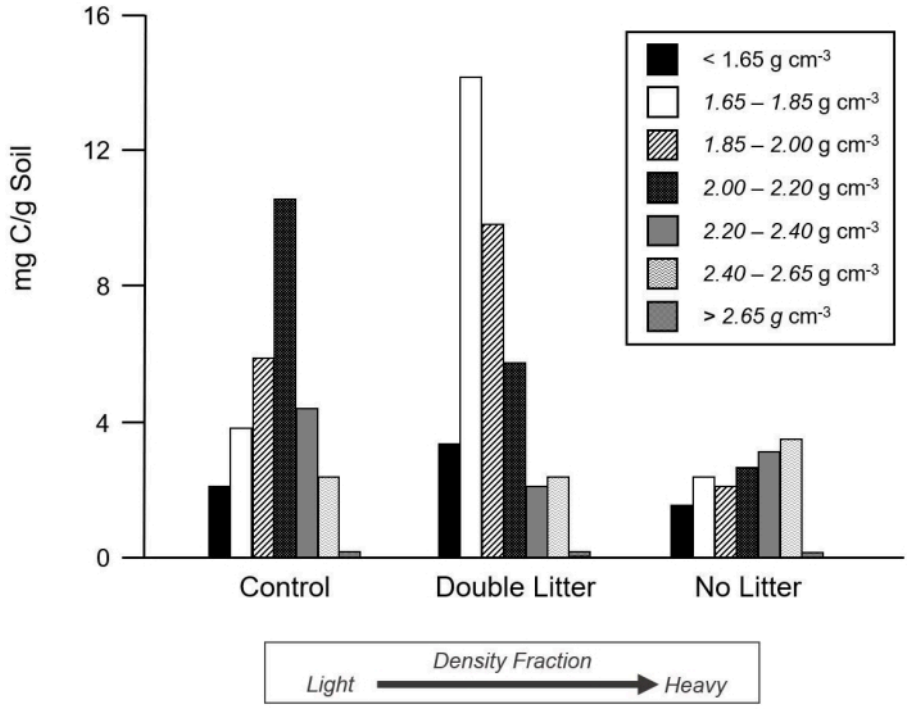


**Figure 5: Sequential density fractionation of soil following 50 years of detrital manipulations (n = 4).** Separating the soil into seven density fractions provided insight into the nature of the C loading on soil minerals. The double litter treatment, which increased soil C, led to the C loading of mineral material in the 2.00-2.40 g/cm$^3$ fraction, as shown by the change in the fraction C concentrations relative to the control. The losses of soil C from the no litter treatment were greatest in the soil fractions with densities between 1.85-2.20 g/cm$^3$. The error bars represent the standard error. This figure has been modified from Lajtha et al.[22]. Please click here to view a larger version of this figure.

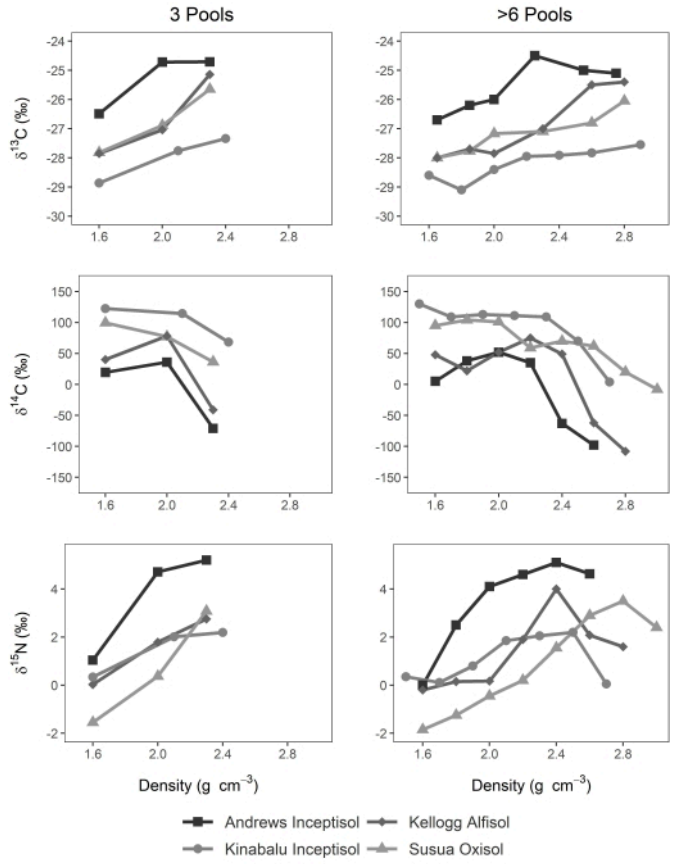


**Figure 6: Effects of mineralogy on soil density pools.** Soils with unique morphology were collected from four forested sites. The isotopic analysis demonstrates how soil mineralogy may influence biogeochemical properties across soil density pools. Further, in this instance, the analysis of three density pools, as opposed to six or more, largely captured the trends within and between the different isotopic signatures. This figure has been modified from Sollins et al.[18]. The original data and graphs are shown in the column with more than six pools; the data were recalculated and displayed to demonstrate the results for only three pools. Please click here to view a larger version of this figure.

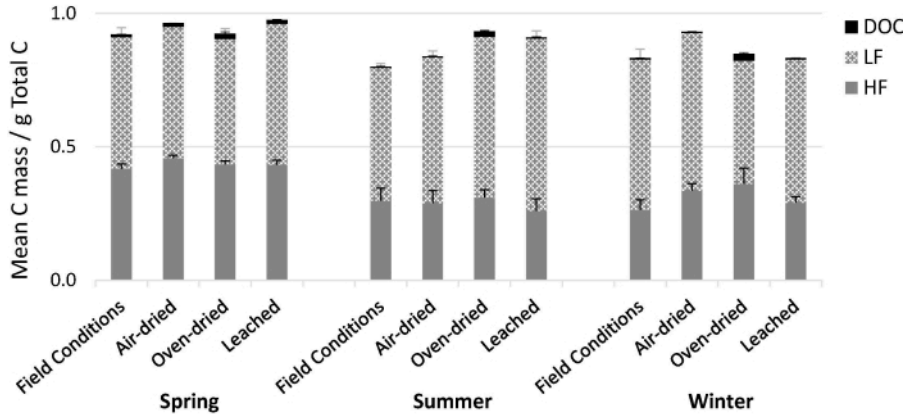


**Figure 7: Sample collection and preparation effects on soil density fractionation.** Helbling et al.[31] found that oven-dried soil often yields greater dissolved organic carbon relative to air-dried, field moist, and leached soil. Across sample collection seasons, the proportion of light to heavy fraction mass was not significantly different. This figure has been modified from Helbling et al.[31]. The error bars represent the standard error. Please click here to view a larger version of this figure.

## Discussion

Throughout the soil density fractionation protocol, there are a few specific procedures that must be monitored closely to help reduce error in the separation and analysis of the soil fractions. A critical step in the soil density fractionation procedure is to repeatedly verify the density of the SPT solution. Moisture in the soil sample will often dilute the SPT solution, thus lowering the density of the SPT. Therefore, the researcher must always ensure that complete separation of the light and heavy solutions has been achieved following centrifugation. If the fractions do not adequately separate, more of the SPT solution should be added, or the mass of the soil should be reduced. Sandy soils separate quickly, while finely textured soils, such as Oxisols in particular, may remain cloudy for a long time during centrifugation due to a high suspended load of fine particles. When the solutions appear cloudy after centrifugation, either the centrifugation time or speed should be increased. As an alternative, an estimate of the C loss from the suspended sediment may be determined by analyzing the C content of the aspirated solution.

Determining the quality of sodium polytungstate (SPT) to purchase depends on the analyses that will be performed after fractionation. Kramer et al.[34] found that commercial SPT may be enriched in high $^{15}N$ ammonium and, thus, may significantly alter the $^{15}N$ signature of soil fractions. Thus, a high-purity grade SPT (e.g., SPT-0) should be used to ensure minimal C and N contamination for studies in which the isotopic signatures of soils will be measured. However, SPT with a purity grade that is one step lower (e.g., SPT-1) often has minimal N and C enrichment and is less expensive, thus providing a more economical option when isotopes will not be measured.

To avoid significant error, care must be taken to remove all the light fraction from the solution, which often sticks to the side walls of the centrifuge tubes during aspiration and to

the funnel during the subsequent filtration steps. Excess light fraction remaining in the heavy fraction material will result in a low estimate of the light fraction C while simultaneously over-estimating the C content of the heavy fraction. A close examination of the final data may help to identify such errors when fractionating a series of samples with similar soil properties.

The loss of dissolved organic carbon (DOC) to solution is typically small, usually <5% of the total soil C, and cannot be avoided (**Figure 7**)[31]. However, the losses of DOC can be far greater when fractionating soils with high soluble C pools, such as those found in some desert environments[35]. In such instances, the water-extractable DOC pool should also be quantified. Typically, errors stemming from soil mass loss, especially of the light fraction, are far greater than the errors caused by DOC loss.

The adsorption of polytungstate to the soil sample is a chemical possibility, and the extent to which such chemical exchanges occur is currently unknown. Further, the binding affinity of polytungstate is expected to vary across soils with different chemical properties. Currently, the correlation between soil mass loss and SOC loss at the end of the procedure provides a straightforward and logistically feasible form of assurance that any mass gains from polytungstate exchange are negligible for the quantification of the SOC in the fractionated material. If the cumulative mass of the soil fraction is greater than the initial sample mass, or if the mass losses are starkly less than the SOC losses, then the samples may absorb the polytungstate. Additional wash steps should first be performed to attempt to resolve such an issue. If the absorption of polytungstate in the fractionated material persists, additional elemental analysis may be required to verify and correct for an increase in the tungsten content of the fractionated material. Notably, such issues involving the inability to rinse out the polytungstate from the soil material are uncommon.

Although density fractionation ideally separates out the free particulate matter (POM) from the mineral-associated organic matter (MAOM), the presence of water-stable aggregates complicates the interpretation of the density fractionation results. Silt and clay may form strong associations and bind with organic matter, and soil biota, roots, and fungal hyphae can form macroaggregates that help protect organic compounds from microbial decomposition. This aggregate-protected organic matter, often referred to as occluded organic matter, is not MAOM but will be recovered in the heavy fraction (i.e., >1.85 g/cm$^3$). The inclusion of occluded POM is likely to be most impactful on the results and interpretation of the intermediate density fractions. While fractions at a density over ~2.40 g/cm$^3$ are likely to contain organic-poor minerals devoid of occluded organic material and material with a density less than 1.85 g/cm$^3$ is assumed to be mineral-free organic matter, intermediate fractions can be mixes of organic-rich heavy minerals, aggregates, and organic-poor light minerals. To date, no common consensus or pervasive method has emerged for the interpretation of differences in C found in the intermediate fraction material. When reporting such information, we suggest acknowledging the potential influence of occluded organic matter and mineralogy on the results.

Various chemical and physical techniques have been employed to disperse aggregates to facilitate the release of occluded POM, with ultrasonic energy representing the most commonly used method. Unfortunately, there is no one sonication energy level that can cause complete dispersion across all aggregates, as aggregate strength

and binding mechanisms vary widely over both soil types and aggregate size classes[36]. Amelung and Zech[36] found that microaggregates (20-250 µm) required more ultrasonic energy to disperse than larger macroaggregates but also found that particulate organic matter was disrupted at these higher energies. Further, sequential sonication with increasing intensity continues to yield free occluded organic matter[37], again suggesting that there is no single pool of occluded organic matter and that, at higher sonication levels, much of the separated light fraction material could be a colloidal artifact. Kaiser and Guggenberger[30] also demonstrated the potential for sonication to alter the density distribution of the mixtures of the light fraction organic matter with different minerals. While incorporating ultrasonic dispersion techniques during or after soil density fractionation provides unique opportunities to disperse and isolate SOM pools, these studies warrant the consideration of the dispersion efficacy and the destruction of the POM and mineral structures.

The most prevalent alternative method for separating soil C into easily measurable pools is size fractionation. Size fractionation is quick and low-cost relative to density fractionation and may provide similar insights into SOM dynamics given the correlation between clay content and MAOM. Indeed, Poeplau et al.[10] found no significant difference in SOM turnover rates for C pools separated by size and density fractionation across three different soils. However, particulate organic matter (POM) with a size equivalent to or smaller than clay is common, meaning size fractionation methods alone are not capable of accurately separating POM from MAOM. The incorporation of POM in fine size fractions can, thus, lead to errors in the elemental and organic chemical analysis in certain soils with a significant amount of fine particulate material[15]. If a need exists to quantify the C content of mineral material at a specific particle size (e.g., sand, silt, clay), the two methods may be combined by performing a single density fractionation followed by the size fractionation of the heavy fraction material.

## Disclosures

The authors have nothing to disclose.

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