

Video Article

Improving Infrared Spectroscopy Characterization of Soil Organic Matter with Spectral Subtractions

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Abstract

Soil organic matter (SOM) underlies numerous soil processes and functions. Fourier transform infrared (FTIR) spectroscopy detects infrared-active organic bonds that constitute the organic component of soils. However, the relatively low organic matter content of soils (commonly < 5% by mass) and absorbance overlap of mineral and organic functional groups in the mid-infrared (MIR) region (4,000–400 cm⁻¹) engenders substantial interference by dominant mineral absorbances, challenging or even preventing interpretation of spectra for SOM characterization. Spectral subtractions, a post-hoc mathematical treatment of spectra, can reduce mineral interference and enhance resolution of spectral regions corresponding to organic functional groups by mathematically removing mineral absorbances. This requires a mineral-enriched reference spectrum, which can be empirically obtained for a given soil sample by removing SOM. The mineral-enriched reference spectrum is subtracted from the original (untreated) spectrum of the soil sample to produce a spectrum representing SOM absorbances. Common SOM removal methods include high-temperature combustion ("ashing") and chemical oxidation. Selection of the SOM removal method carries two considerations: (1) the amount of SOM removed, and (2) absorbance artifacts in the mineral reference spectrum and thus the resulting subtraction spectrum. These potential issues can, and should, be identified and quantified in order to avoid fallacious or biased interpretations of spectra for organic functional group composition of SOM. Following SOM removal, the resulting mineral-enriched sample is used to collect a mineral reference spectrum. Several strategies exist to perform subtractions depending on the experimental goals and sample characteristics, most notably the determination of the subtraction factor. The resulting subtraction spectrum requires careful interpretation based on the aforementioned methodology. For many soil and other environmental samples containing substantial mineral components, subtractions have strong potential to improve FTIR spectroscopic characterization of organic matter composition.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57464/>

Introduction

Soil organic matter (SOM) is a minor constituent by mass in most soil samples but is implicated in multiple properties and processes underlying soil functions, such as nutrient cycling and carbon sequestration¹. Characterizing the composition of SOM is one of several approaches to link SOM formation and turnover with its role(s) in soil functions^{2,3}. One method of characterizing SOM composition is Fourier transform infrared (FTIR) spectroscopy, which offers detection of functional groups that constitute organic matter in soils and other environmental samples (e.g., carboxyl C-O, aliphatic C-H)⁴. However, the utility of FTIR spectroscopy for revealing SOM functional group composition is challenged by the dominant mineral component for the majority of soils (typically > 95% mass) due to strong inorganic absorbances that challenge or severely limit detection and interpretation of organic absorbances.

Spectral subtractions offer a way to improve FTIR spectroscopic characterization of organic matter in soil samples. Subtracting mineral absorbances from the soil spectrum can be used to enhance absorbances of organic functional groups of interest in the analysis of SOM composition

(Figure 1).

Advantages of spectral subtractions over standard FTIR spectroscopy (i.e., soil spectra) include:

(i) Improved resolution and interpretation of organic absorbance bands compared to normal soil spectra. Though interpretation of organic bands in soil spectra can be performed by assuming that the relative differences in absorbance are due to differences in organic functional groups,

this limits comparisons to samples with the same mineralogy and relatively high SOM content, and may be less sensitive to changes in organic bands, even those considered to be relatively mineral-free (e.g. aliphatic C-H stretch)⁵

(ii) Analysis of soils beyond high SOM samples or organic matter-enriched extracts or fractions

(iii) Highlighting changes induced by experimental treatments from mesocosm to field scales⁶

Additional applications of spectral subtractions in FTIR analysis of SOM include complementing structural and molecular characterizations (e.g., NMR spectroscopy, mass spectrometry)^{5,7}, identifying the composition of SOM removed by an extraction or destructive fractionation⁸, and fingerprinting SOM composition for forensic purposes⁹. This method is applicable to a wide variety of mineral-organic mixtures beyond soils, including sediment¹⁰, peat¹¹, and coal^{12,13}.

The potential of spectral subtractions to improve FTIR spectroscopic characterization of SOM is demonstrated using examples of organic matter removal to obtain mineral reference spectra, and then, using these mineral reference spectra, performing and evaluating ideal and non-ideal spectral subtractions. This demonstration focuses on diffuse reflectance infrared Fourier transform (DRIFT) spectra collected in the mid-infrared region (MIR, 4,000 - 400 cm⁻¹), as this is a widespread approach for the analysis of soil samples⁴.

The two example methods of SOM removal for obtaining a mineral-enriched reference spectrum are (i) high-temperature combustion ('ashing') and (ii) chemical oxidation, using dilute sodium hypochlorite (NaOCl). It should be noted that these are examples of commonly employed SOM removal methods, rather than prescriptive recommendations. Other methods of SOM removal may offer reduced mineral artifacts and/or enhanced removal rates (e.g., low-temperature ashing)¹⁴. High-temperature ashing was one of the first methods used to obtain mineral-enriched reference spectra for performing subtractions, initially for OM-enriched samples derived from soils (e.g., dissolved organic matter, litter)^{15,16} followed by its application to bulk soil samples^{17,18}. The example chemical oxidation used to remove SOM is based on the method of NaOCl oxidation described by Anderson¹⁹. This was originally developed as a pretreatment for removing organic matter in soil samples prior to X-ray diffraction (XRD) analysis, and has been investigated as a potential chemical fractionation sensitive to SOM stabilization^{20,21}. Both high-temperature removal and chemical oxidation using NaOCl can entail soil-specific artifacts and have limitations on spectral interpretation that should be considered when selecting a method of SOM removal^{14,22}.

Protocol

1. Prepare Soil for Non-treated DRIFT Spectroscopy and SOM Removal

1. Sieve the soil to < 2 mm using a stainless-steel mesh (the 'fine-earth fraction').

NOTE: This demonstration employs two soils of similar texture but a nearly 3-fold difference in total SOM content (Table 1).

2. SOM Removal by Chemical Oxidation: Example of NaOCl

1. Adjust the pH of 6% w/v NaOCl to pH 9.5 by adding 1 M HCl dropwise to the solution while mixing and measuring with a pH meter.
NOTE: Most commercial bleaches (e.g., Clorox) are suitable in quality and concentration (typically 3-7% NaOCl v/v) but will have pH > 12. As NaOCl oxidation of organic matter is pH-dependent, and pH 9.5 is recommended for its use with soil samples^{19,23}, it is necessary to adjust the pH of most commercially available bleaches.
2. Add 25 mL NaOCl (6% w/v, pH 9.5) to 4 g soil (sieved, air dried) in a 50-mL conical tube and mix by sonication (600 s, output frequency 20 kHz, power 200 W).
3. Incubate the mixture in a hot-water bath (15 min, 80 °C) to increase oxidation rate.
4. Centrifuge to obtain a clear supernatant (e.g., 15 min at 4000 × g for coarser textured soils; room temperature). Manually decant the supernatant into a waste container.
NOTE: The concentration of NaOCl in the supernatant (conservatively assuming no oxidation and thus no consumption of NaOCl) is the same as commercially available bleach for household use. Finer textured soils may require longer centrifugation time (e.g., up to an additional 15-30 min) at a given centrifuge speed (e.g., 4000 × g) to obtain a clear supernatant.
5. Repeat steps 2.3 and 2.4 twice for a total of three oxidation steps.
6. After the last oxidation step, add 20 mL deionized H₂O (dH₂O) to the soil and mix for 5 min using a horizontal shaker (120 rpm). Centrifuge for 15 min at 4000 × g and room temperature. Repeat for a total of three treatments.
7. Using a spatula and dH₂O from a squirt bottle as needed, extract and wash out the soil pellet from the bottom of the centrifuge tube into a plastic weigh boat (or another container with high surface area). Oven-dry (60 °C maximum, 48 h) to an air-dried state.
8. Once the soil sample is dried, quantify total organic carbon content by combustion-gas chromatography using a C/N analyzer²⁴. Calculate SOM removal as the difference in organic carbon concentration before and after oxidation treatment.
NOTE: Due to loss of organic matter and soil structure, soil will be prone to crusting, in particular for soils with low sand content. It may be necessary to apply gentle pressure and/or hand grinding to re-homogenize the crusted soil. Soils with inorganic carbon (i.e., carbonates) require additional steps for quantifying organic carbon by combustion-gas chromatography^{25,26}.

3. SOM Removal by High-Temperature Combustion

1. Measure ~1-2 g of soil (sieved, air dried) into a porcelain crucible using a spatula.
2. Heat at 550 °C for 3 h using a muffle furnace.
NOTE: This is an example method of SOM removal using combustion at a relatively high temperature. Refer to **Discussion** on alternative procedures (e.g., temperature).

4. DRIFT Spectroscopy

NOTE: For this example, the FTIR spectrometer software listed in the **Table of Materials** will be used.

1. Acquire spectra of untreated soil and mineral enriched reference sample (treated to remove SOM).

1. Prepare the soil samples.

1. Dilute the samples (optional).
 1. Use analytical grade KBr (or other halide salt) dried at 105 °C and stored in a desiccator to remove residual moisture. For soil samples, effective KBr dilutions can be achieved at a range of 1-33%, in contrast to < 1% for pure compounds.
 2. Mix soil and KBr for a final sample size of 100-400 mg. For example, for a 3% dilution, gently grind 12 mg of dry sample with 60 mg of KBr for 60 s with an agate mortar and pestle. Then, 'fold in' 328 mg of KBr to fully homogenize the sample.
 3. Use serial dilutions with KBr to obtain a high final dilution rate (< 1%). Perform replicate dilutions to ensure reproducibility, especially since diluted samples use 10^1 - 10^2 less soil than neat samples.
2. Grind untreated and treated soil samples to similar consistency by hand grinding and sieving (e.g., 250 µm using a 60# sieve). NOTE: Compared to hand grinding, greater consistency is facilitated by automation, in particular by ball milling. However, the relatively small amount of soil used in SOM removal (e.g., 1-3 g for ashing due to crucible volume) means that hand grinding may be more practical.

2. Collect the background spectrum.

1. Load a sample of KBr (ground in the same manner as soil samples (see 4.1.1.2) to mimic soil matrix effects) into a sample cup or plate well.
NOTE: The "background spectrum" is different from the mineral-enriched reference spectrum (see 4.1.3) used for performing subtractions. The background spectrum will be used by the software to remove atmospheric and other ambient absorbances during collection of spectra on soil samples. All software descriptions are specific to the chosen software and will need to be adapted to other software.
2. Purge the spectrometer chamber with CO₂- and H₂O-scrubbed air (via a purge gas generator) or with N₂ gas for greater consistency in collection conditions. For example, collection of spectra under ambient atmosphere may entail small fluctuations in humidity and CO₂ that can cause changes in absorbance spectra.
NOTE: Newer spectrometers may have mirrors (e.g., gold, SiC) that can potentially reduce humidity effects.
3. Collect a background spectrum using the same detector and acquisition parameter settings, including scan number, wavenumber range, and resolution, that will be used to collect spectra of samples.
 1. Open the drop-down menu for **Experiment** and select the desired experimental collection method (e.g., **acquisition mode**).
NOTE: In this example using chosen spectrometer (see the **Table of Materials**), the selected method is **iS50 Main Compartment**.
 2. Click the **Experimental Setup** icon to select spectral acquisition parameters.
 3. Under the **Collect** tab, check that the number of scans and resolution are appropriate for experimental objectives; for example, a common setting for DRIFT spectra of neat soils is 128 scans at 4 cm⁻¹ resolution. Click **Ok** to save changes.
 4. Click the **Collect Background** icon to collect a background spectrum. Save the background spectrum for use in the collection of spectra of soils (treated and untreated).

3. Acquire spectra of soil samples.

NOTE: Use the same acquisition parameters to collect background and sample (untreated soil, mineral-enriched soil) spectra. Differences among detectors in acquisition time and resolution pose trade-offs that impact collection time and spectral quality. Typical scan numbers for soil spectra range from 128-512 scans. Scan number can be decreased and replicates averaged to obtain a total target scan number. For example, two analytical replicates - the same sample loaded in two separate wells - can be collected using 64 scans each and averaged for a total of 128 scans.

1. Load the soil sample. To ensure consistent loading and minimize surface roughness, pour samples into the sample cup (or well) to the point of slightly overfilling above the lip or edge of the cup. Then, surface-smooth the soil in the cup using a flat edge (e.g., razor) such that the height of soil sample in the cup is flush with the lip of the cup.
NOTE: Due to the interaction of infrared light with a matrix such as soil in diffuse reflectance mode, sample loading can influence DRIFT spectra. Samples should not be tamped or subjected to pressure because packing density can affect absorbance. Finer particle size of samples ensures greater ease of surface smoothing (see 4.1.2.1). Depending on the spectrometer model and the sample density, the mass of sample needed to fill a sample cup will range from 300 to 600 mg. In the case of plate wells, this also depends on the well size. Plates with a greater number of wells will have smaller wells and will therefore require less sample. For example, 96-well plates commonly have a well volume of 360 µL whereas 24-well plates have a well volume of 3.4 mL.
2. Collect spectra of untreated and treated soil samples. First check that the background spectrum collected previously (see 4.1.2.3.4) is used. Click **Experimental Setup**. Under the **Collect** tab, select **Use specified background file** and load the background spectrum file. Click **Ok** to save changes. To commence spectral collection on the soil, click **Collect Sample**.
NOTE: Re-load the same sample in a different well or sample cup to collect the replicate spectra to account for scattering artifacts produced by surface roughness and by variability in matrix density.

2. Perform spectral subtractions.

NOTE: The **subtraction factor (SF)** weighs the degree to which absorbances in the mineral reference spectrum are subtracted from absorbances at the corresponding wavenumber in the spectrum of the untreated soil. For subtractions focused on improving resolution of organic absorbances to characterize SOM, it is recommended to utilize the entirety of the MIR afforded by most spectrometers (e.g., 4,000 to

650 or 400 cm^{-1} , depending on the detector). The next steps describe an empirical method for determining the SF. All software descriptions are specific to the chosen software and will need to be adapted to other software.

1. Zero out peaks by using the subtraction option of the software program to change the subtraction factor (SF) to minimize or reduce a target mineral peaks and/or mineral peaks, and/or to maximize a linear baseline¹⁴.
2. Simultaneously select the untreated and treated soil spectra and click the **Subtract** icon (top center of screen); the first spectrum selected (untreated soil) will be the spectrum from which the second spectrum (treated soil) will be subtracted.
3. Use the vertical toggle bar or arrows to increase or decrease the SF (left-hand of screen). Observe the changes in the previewed subtraction spectrum.

1. Use this iterative feature to determine an appropriate SF as described in **Representative Results**. The numerical SF value appears in the middle of the toggle bar. To adjust the range of SF values, use the **Finer** and **Coarser** buttons.

4. Click **Add** (upper right-hand of screen) to load the calculated subtraction spectrum into a window.

NOTE: Since the majority of mineral absorbances are not linear with concentration in most (if not all) soil samples, it is usually not feasible to remove all mineral peaks. It is recommended that mineral peaks considered less prone to inversion (e.g., quartz-like Si-O at 2,100-1,780 cm^{-1})¹⁴ be used as the target peak to zero-out by adjusting the SF.

5. Record and report methodological details on how the subtraction was performed with sufficient detail to allow independent calculation of the same subtraction spectrum from the untreated soil spectrum, including: (1) the wavenumber region used for subtraction, (2) the SF or range of SFs used, and (3) the (mineral) peak or region targeted for zero-ing out.

NOTE: A good test of the reliability of a subtraction is to have it performed anew by the same user and/or independently by a different user using the reported subtraction parameters.

3. Interpret the spectra.

1. Perform spectral interpretation using various resources available to analyze and interpret the resulting subtraction spectra, in particular assignments of absorbances to organic functional groups⁴.

NOTE: Other uses of subtraction spectra include multivariate analysis (e.g., principal component analysis), chemometric prediction of soil analytes²⁷, and even forensic fingerprinting⁹.

Representative Results

The method of SOM removal has practical as well as theoretical implications for the interpretation of subtraction spectra. For example, mineral alterations from high temperature ashing can manifest as losses or appearances of peaks and/or as shifted or broadened peaks in the mineral reference spectrum. These spectral artifacts are prone to occur in regions of overlap with organic bands at 1,600-900 cm^{-1} ,²² compromising interpretation of organic bands. Common changes of mineral bands following high temperature ashing ($\geq 550^\circ\text{C}$) are evident in **Figure 2** and include loss of OH peaks at 3,700-3,600 cm^{-1} , and peak losses and shifts in lattice Si-O and Al-O peaks at 1,050-800 cm^{-1} . On the other hand, SOM removal using chemical oxidation tends to preserve mineral functional groups and thus risks fewer artifacts (see **Discussion**). However, this typically comes at a cost of lower SOM removal²⁸, as evidenced for the example soils (**Table 2**). This restricts interpretation of organic functional group composition in the subtraction spectrum to less than the total SOM pool.

Chemical oxidations can be an attractive alternative to ashing for furnishing mineral backgrounds because they generally preserve mineral structure and thus avoid artifactual absorbance features in reference spectra and thus the resulting subtraction spectrum^{14,22}. For example, Soil A lost 89% of soil organic carbon (SOC) by NaOCl oxidation compared to 97% by ashing (**Table 2**) while preserving mineral absorbance features altered by ashing (**Figure 2**). On the other hand, incomplete and potentially selective removal of SOM means that NaOCl-resistant organic matter will not be characterized^{5,29,30}, requiring careful interpretation of the subtraction spectrum¹⁴. Quantifying SOM loss with each additional oxidation treatment performed can be used to identify the optimal number of oxidative treatments for SOM removal. Evaluation of the example oxidation method using NaOCl showed that SOM removal plateaued after 3 oxidation steps regardless of whether SOM content of samples was low (<2% C) or high (36% C). With each successive oxidation step, the color of the supernatant is likely to change from various shades of purple/black/orange to lighter brownish-red until the original greenish-yellow color of bleach (i.e., unreacted dilute NaOCl).

Grinding to reduce and homogenize soil particle size can improve spectral quality by reducing light scattering from large particles and inversion of strongly absorbing components³¹. Though a maximum particle size of 250-100 μm has been considered optimal to minimize such artifacts³², recent evaluations suggest that grinding beyond 2000 μm does not necessarily improve spectral quality for chemometric predictions^{33,34}. However, more finely ground soil samples can still yield enhanced (sharper) absorbance peaks and exhibit decreased absorbance variability³³, which would be expected to benefit spectral subtractions. On the other hand, some studies have found that grinding (e.g., to 200 μm) can induce more heterogeneity in particle size distribution than standard sieving (e.g., 2,000 μm)^{35,36}. The effect of this on spectral quality is likely to depend on experiment objectives (e.g., interpretation of absorbance bands, chemometric prediction of C fractions) and soil properties (e.g., texture, mineralogy)²⁹. It is recommended that effects of grinding size on subtraction spectra be assessed for a given set of soils and objectives. The treated soil sample used for the mineral reference spectrum is likely to have finer particle size than the corresponding untreated soil due to disruption of aggregates by SOM removal.

The use of neat (undiluted) soil samples is widespread in the use of DRIFT spectroscopy due to greater ease of analysis and higher throughput rate. Sample dilution is traditionally performed for high purity samples using infrared-invisible halide salts (e.g., KBr, NaBr, KI). For DRIFT spectroscopic characterization of the organic component of soils, however, halide salt dilution is less advantageous. This is because dilution of samples tends to improve resolution of absorbances for dominant (mineral) components but not necessarily those representing minor (organic) components^{5,37}. Nonetheless, there can be benefits to the dilution of soil samples, which can be empirically evaluated for each sample⁵.

To illustrate the role of the subtraction factor (SF) in performing spectral subtractions, consider the subtraction spectra obtained for soil A using the spectrum of the NaOCl oxidized soil as the reference (**Figure 3**). As the SF increases from 0 to 1, the absorbance of bands corresponding to minerals decrease, most notably mineral surface O-H at $3,618\text{ cm}^{-1}$ and Si-O at $1,880$ and 808 cm^{-1} . Concurrently, absorbance increases for bands representing organic functional groups such as aliphatic C-H stretch at $2,920\text{ cm}^{-1}$ and potentially amide C-N and N-H and/or aromatic C=C at $1,558\text{ cm}^{-1}$. With increasing scaling of the SF, however, inversions of mineral absorbances such as Si-O at $2,100 - 1,780\text{ cm}^{-1}$ emerge. As NaOCl oxidation removed 89% of total SOC in a soil with 2.2% C (**Tables 1, 2**), and given that SOM is estimated to be 58% C³⁶, a theoretical SF of 0.97 can be calculated. However, non-linearity of absorbance results in over subtraction of mineral features using this SF (**Figure 3**).

A more practical approach is to operationally determine the SF based on removal of a mineral absorbance feature by 'canceling' or zero-ing out a target peak(s). The quartz-like Si-O features at $2,100 - 1,780\text{ cm}^{-1}$ are suitable target mineral absorbances because they arise from fundamental Si-O vibrations that are less susceptible to specular reflection^{14,37}. For example, in **Figure 3**, zero-ing of quartz Si-O at $2,100 - 1,780\text{ cm}^{-1}$ suggests a SF in the range of 0.7 - 0.8 to be more suitable for reducing the dominant mineral absorbances than the *a priori* SF of 0.97 calculated from the difference in mass (*i.e.*, SOM removed) between the two soil samples.

Potential pitfalls in selecting a SF include inversion and oversubtraction, both evidenced by 'negative peaks' that resemble an upside-down peak or "W" shape¹⁴, or in the case of strong subtraction, a mirror image of the untreated soil spectrum. Inversions may not be possible to avoid altogether since these are common for Si-O at $1,150 - 800\text{ cm}^{-1}$, but can be minimized. Inversions should be noted as a region *non-grata* for spectral interpretation^{22,39} because in these regions absorbance can reflect how the subtraction was performed more so than absorbing components, especially for minor constituents such as OM.

Spectral software accompanying modern FTIR spectrometers can be useful to guide and execute subtractions. The utility of such software lies in real-time and iterative adjustment of SFs while viewing the resulting subtraction spectrum. However, the default SF suggested by spectral software should be regarded with caution because the software criteria may not be appropriate for the experiment goals and samples such as soils (*e.g.*, non-linear absorbance, inversion of mineral features). For example, the particular software used to calculate subtraction spectra in **Figure 3** (see the **Table of Materials**) suggested a SF of 1.45, based on linearization of baseline in the resulting subtraction spectrum⁴⁰, which misses the objective of zero-ing out Si-O at $2,100 - 1,780\text{ cm}^{-1}$ for interpretation of organic absorbances at $1,800 - 1,000\text{ cm}^{-1}$. It is important to note that the SF suggested by software is also influenced by the wavenumber range selected for the subtraction because the software optimizes a subtraction for the defined spectral region. Additional benefits of software include spectral processing transformations (*e.g.*, Kubelka-Munk, power functions) to improve spectral quality (*e.g.*, linear baselines) prior to performing subtractions.

The mineral reference spectrum may contain absorbance features influenced by the method of SOM removal, with consequences for performing and interpreting spectral subtractions. Comparison of differences among untreated and ashed mineral reference spectra (**Figure 2**) can help identify which of these features may be due to artifacts of SOM removal. In contrast to NaOCl oxidation, ashing produced changes in absorbance bands representing mineral functional groups, notably phyllosilicate surface and interlayer O-H ($3,700 - 3,600\text{ cm}^{-1}$), Al-OH (915 cm^{-1}), and Si-O ($796, 521\text{ cm}^{-1}$). Quartz-like Si-O bands ($2,100 - 1,780, 1,159\text{ cm}^{-1}$) did not exhibit marked changes in absorbance (intensity or wavenumber) for either treatment.

An example of over-subtraction and the risk of unidentified artifacts resulting from the SOM removal method of choice is demonstrated for ashing at 550°C in **Figure 4**. Though zero-ing out quartz-like Si-O at $2,100 - 1,780\text{ cm}^{-1}$ is achieved with an SF of 0.76, a prominent W-shaped inversion at $1,100 - 450\text{ cm}^{-1}$ suggests that interpretation of the subtraction spectrum should be limited to $> 1,200\text{ cm}^{-1}$. As the reference spectrum was obtained by high-temperature ashing, loss of mineral surface OH at $3,700 - 3,600\text{ cm}^{-1}$ (see **Figure 2**) means that these sharp peaks are an artifact that should not be a target to zero out. Attempts to do so will likely compromise other spectral regions. For example, not recognizing the feature at $3,700 - 3,600\text{ cm}^{-1}$ as an artifact could motivate the user to increase in the SF to reduce (SF = 1.24) or attempt to zero out (SF = 1.51) this artifact at the expense of over-subtracting the remaining spectral regions, including organic absorbances at $1,800 - 1,200\text{ cm}^{-1}$ (**Figure 4**).

It is also possible to over-subtract the mineral reference spectrum, such as in an attempt to enhance a specific band without considering effects on other spectral regions (**Figure 5**). While this may be justified by experimental objectives (see **Discussion**), the example in **Figure 5** of enhancing the aliphatic C-H stretch at $3,000 - 2,800\text{ cm}^{-1}$ 'at all costs' renders the remaining portion of the spectra uninterpretable, including the region corresponding to the majority of organic functional groups relevant to SOM characterization at $1,800 - 1,000\text{ cm}^{-1}$. An indicator of over-subtraction is the deepening inversion of spectral features beyond inversion-prone Si-O bands as the SF increases from 0.81 to 1.35 to 1.72. Conversely, the incomplete removal of these target mineral bands means that the SF is likely insufficient, as evidence for the subtraction performed with an SF of 0.32. A SF of 0.81 ideally zero-outs out the target Si-O features at $2,100 - 1,780\text{ cm}^{-1}$ while minimizing inversions.

Comparison of subtraction spectra calculated from reference spectra obtained by high-temperature ashing and chemical oxidation indicate common features enhanced by subtraction at $1,750 - 1,350\text{ cm}^{-1}$ ascribable to organic functional groups, but also exhibit differences due to alteration of minerals and potentially the amount of SOM removed (**Figure 6**). Note that a similar SF was obtained for subtractions using a mineral reference obtained by ashing (SF = 0.76, **Figure 4**) versus NaOCl oxidation (SF = 0.81, **Figure 5**). For a given method of SOM removal, differences are visually evident between the subtraction spectra of high and low organic matter soils that are less visible or absent in the untreated soil spectra (**Figure 6**), corresponding to aliphatic C-H (stretch) at $2,916\text{ cm}^{-1}$, and ketone and amide C=O with possible contributions of conjugated C=C at $1,647\text{ cm}^{-1}$, amide C-N and N-H at $1,568\text{ cm}^{-1}$, aliphatic C-H (bend) at $1,427\text{ cm}^{-1}$, and phenol and/or carboxyl C-O at $1,275\text{ cm}^{-1}$. It is essential that subtraction spectra be understood as an operational method to assess relative changes in absorbances that may be assigned to organic functional groups with varying certainty depending on sample type and absorbance range (due to potentially overlapping bands)⁴.

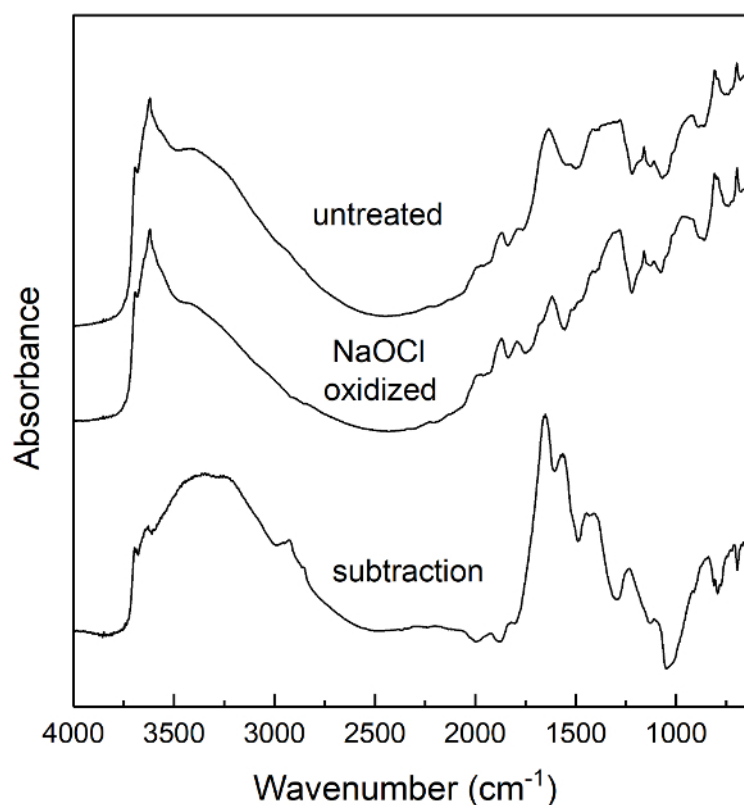


Figure 1. Illustration of spectral subtraction to enhance organic bands using a background spectrum of the same soil following SOM removal by chemical oxidation (NaOCl). DRIFT spectra were collected without KBr dilution of the soil sample. The soil is a Mollic Xerofluvent under agricultural use in the Sacramento Valley of California (22 mg C g⁻¹ soil). Subtraction spectra were calculated with a subtraction factor of 1.0. [Please click here to view a larger version of this figure.](#)

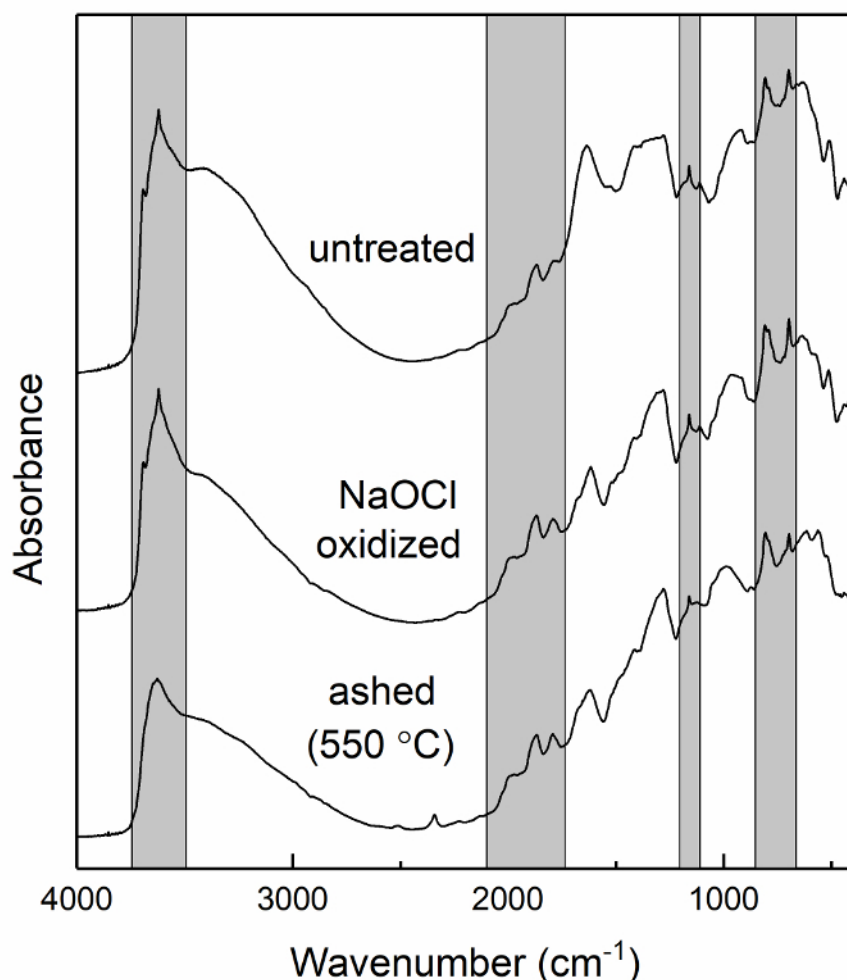


Figure 2. Comparison of mineral-enriched background or reference spectra for performing spectral subtractions obtained by different SOM removal methods. Comparisons of treated and untreated soils are shown for a surface horizon (A_p) of a Mollic Xerofluvent formed on mixed alluvium in California, USA (22 mg C g^{-1}). Spectra correspond to the original soil without treatment (top), followed by soils following SOM removal treatments of chemical oxidation (NaOCl) (middle) and high-temperature combustion (550°C) (bottom). DRIFT spectra were collected on soil samples without KBr dilution. Grey boxes highlight mineral absorbance bands characteristic of soil spectra, centered at 3620 cm^{-1} , 1870 cm^{-1} and 800 cm^{-1} . [Please click here to view a larger version of this figure.](#)

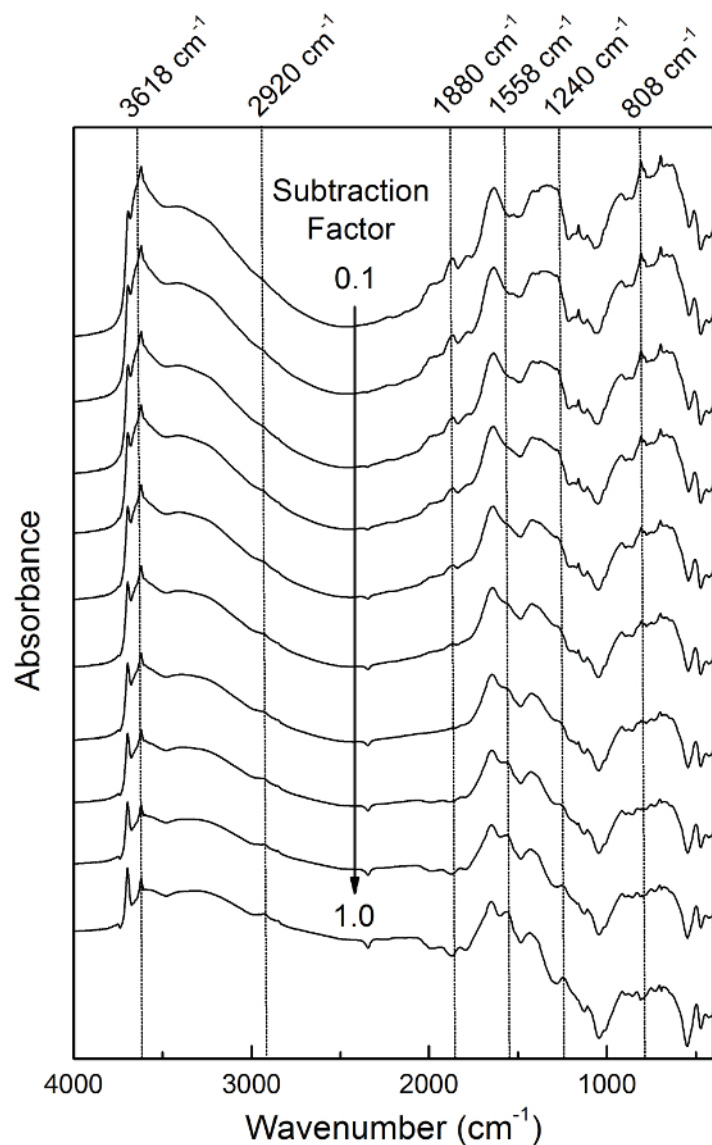


Figure 3. Example of increasing the subtraction factor (SF) at 0.1 intervals from 0.1 (minimal subtraction) to 1.0 (equal subtraction of original soil spectrum and reference spectrum). The SF weights the degree to which the reference spectrum (soil A after heating at 550 °C for 3 h) is subtracted from the original (untreated) soil spectrum (soil A). As the subtraction factor decreases, absorbance bands corresponding to minerals increase (3,618 cm^{-1} , 1,880 cm^{-1} , 808 cm^{-1}), and bands representing organics decrease (2,920 cm^{-1} , 1,558 cm^{-1} , 1,240 cm^{-1}). DRIFT spectra were collected on soil samples without KBr dilution. [Please click here to view a larger version of this figure.](#)

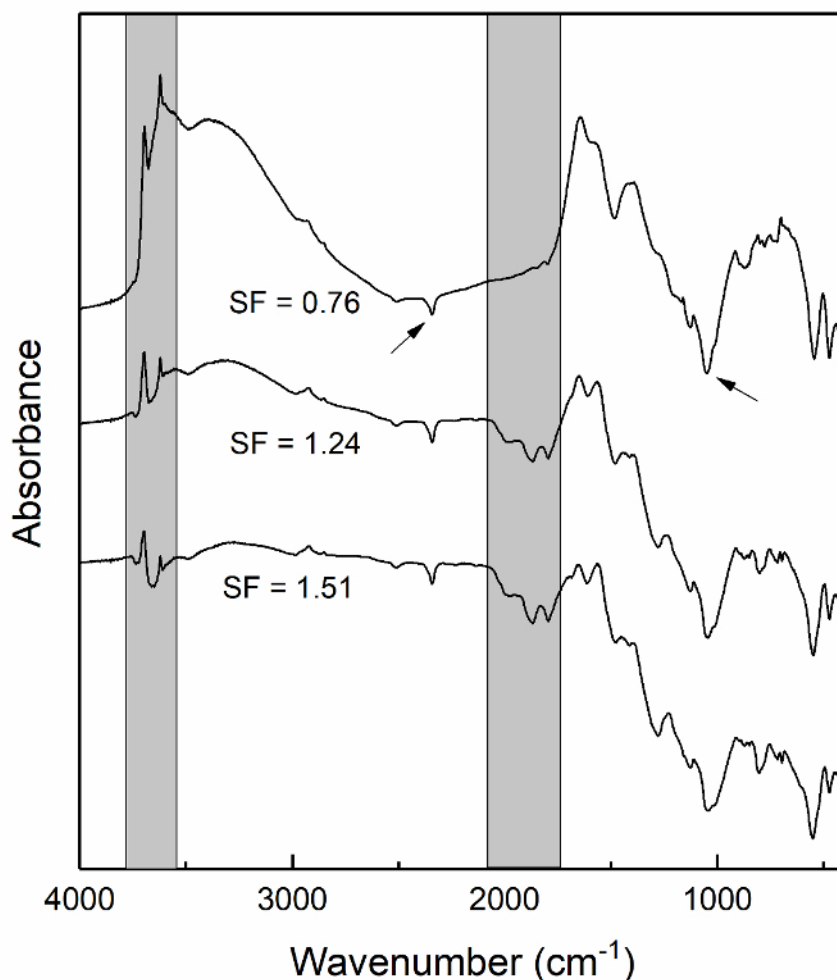


Figure 4. Examples of over-subtraction and the risk of unidentified artifacts resulting from the SOM removal method (ashing at 550 °C). Even with zero-ing out quartz-like Si-O at 2,100-1,780 cm^{-1} (gray box) (SF = 0.76), a prominent W-shaped inversion at 1100 - 450 cm^{-1} (arrows) suggests interpretation of the subtraction spectrum should be limited to $>1200 \text{ cm}^{-1}$. Since the reference spectrum was obtained by high-temperature ashing (550 °C), loss of mineral surface OH at 3,700 - 3600 cm^{-1} (gray box) (see **Figure 2**) indicates that this peak is a likely an artifact. Not recognizing the feature at 3,700-3,600 cm^{-1} as an artifact can mislead the user to increase the SF to reduce (SF = 1.24) or zero out (SF = 1.51) this spectral feature at the expense of oversubtracting the remaining spectral regions, including organic absorbances at 1,800-1,200 cm^{-1} . Additional artifacts from ashing include a feature at 2300 cm^{-1} (arrow). DRIFT spectra were collected on soil samples without KBr dilution. [Please click here to view a larger version of this figure.](#)

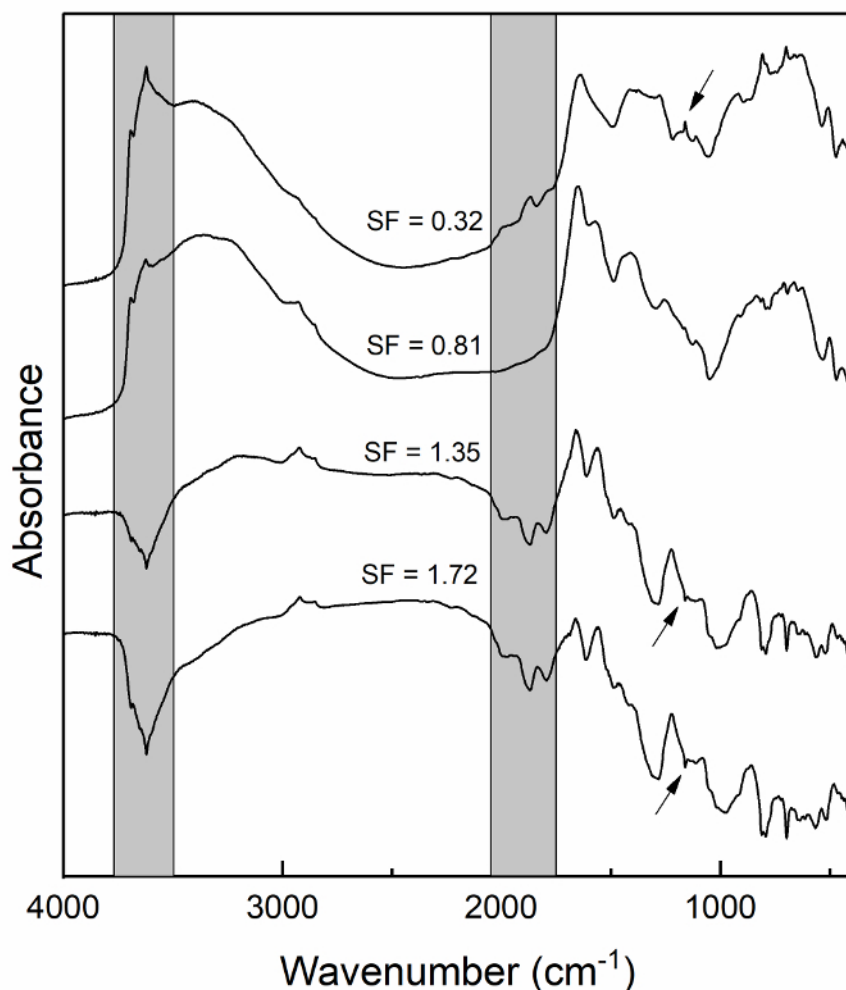


Figure 5. Examples of ideal and non-ideal subtraction spectra using a mineral reference obtained by NaOCl oxidation. Insufficient subtraction (low SF = 0.32) means that prominent mineral absorbances (grey boxes) are still present. Increasing the SF to zero out quartz-like SiO at 2,100-1,780 cm^{-1} (SF = 0.81) strikes a balance among the criteria of removing this mineral absorbance as a proxy for mineral overlap at 1,800-1,000 cm^{-1} , minimizing inversions of Si-O <1,100 cm^{-1} , and maintaining a linear baseline. In an attempt to further enhance the aliphatic C-H-stretch at 3,000-2,800 cm^{-1} , the SF may be increased (to 1.35 or 1.72), but this results in oversubtraction of varying degrees. Spectra were collected on neat (no KBr dilution) samples by DRIFT spectroscopy. [Please click here to view a larger version of this figure.](#)

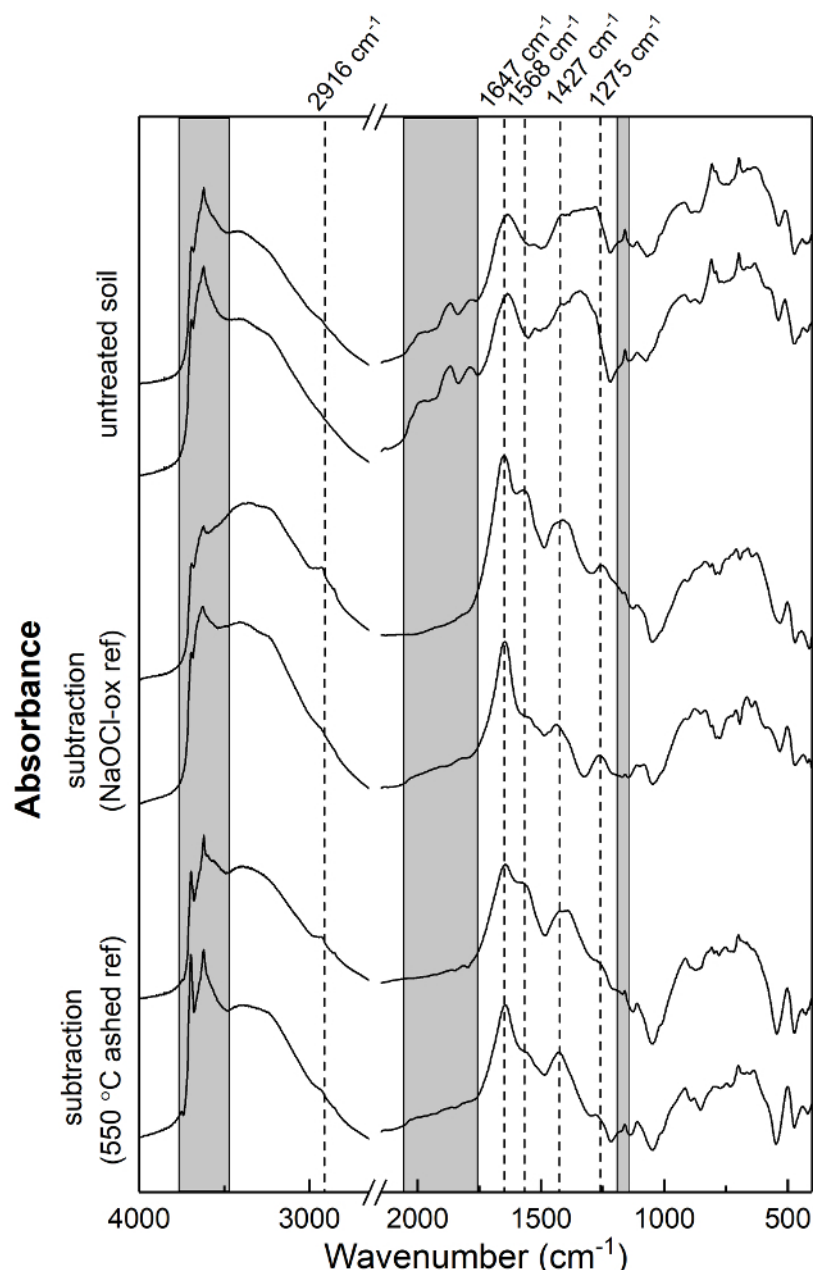


Figure 6. Paired comparison of DRIFT spectra of high (top) and low (bottom) organic matter soils showcasing the potential of spectral subtractions to improve interpretation of organic absorbances compared to the original soil spectra. Differences in absorbances corresponding to mineral (grey box) and organic (dashed line) absorbance bands are evident between high and low SOM soils and/or among the three set of spectra: spectra of the original (untreated) soils, and spectra obtained by subtracting a mineral reference from the untreated soil spectrum, using a mineral-enriched soil for the mineral reference spectrum obtained by thermal combustion ('ashing') or chemical oxidation. Organic absorbances that differ between high and low SOM samples may be ascribed to aliphatic C-H (2,916 cm^{-1}), ketone and/or amide C=O with possible contributions of conjugated C=C (1,647 cm^{-1}), amide C-N and N-H and/or possible aromatic C=C (1,568 cm^{-1}), aliphatic C-H (1,427 cm^{-1}), and carboxyl and/or phenol C-O (1,275 cm^{-1}). Soils are from the A_p horizon of Entisols formed from mixed alluvium in the Sacramento Valley (California, USA). Spectra were collected on neat (no KBr dilution) samples. [Please click here to view a larger version of this figure.](#)

Soil	SOM content	SOC (mg g ⁻¹)	C:N	pH (1:2, water)	clay (mg g ⁻¹)	sand (mg g ⁻¹)
A	High	22	9.4	6.5	166	133
B	Low	7	9.3	6.7	126	451

SOC, soil organic carbon; C:N, carbon to nitrogen ratio

Table 1. Properties of soils used to demonstrate methods for spectral subtraction to improve characterization of SOM using DRIFT spectroscopy. Soils are of similar mineralogy but exhibit differences in organic matter concentration. Soils were sampled from the A_p horizon of Entisols formed on mixed alluvium in the Sacramento Valley of California, USA.

Soil	OM content	SOC (mg g ⁻¹)	SOC loss (%)	
			NaOCl-ox	ashed (500 °C)
A	High	22	-89.1	-96.5
B	Low	7	-82.5	-97.2

SOC, soil organic carbon; NaOCl-ox, sodium hypochlorite oxidized

Table 2. Example of varying removal rates of SOM by combustion versus chemical oxidation. Soil samples treated to remove organic matter are used to acquire a mineral-enriched reference spectrum for performing spectral subtractions to improve characterization of SOM functional group composition using DRIFT spectroscopy.

Discussion

The method of removing SOM carries two considerations: 1) the amount of SOM removed, and 2) absorbance artifacts in the resulting mineral reference spectrum. It is fortunately possible—and arguably necessary—to identify and quantify these issues in order to avoid biased interpretations of SOM composition from the resulting subtraction spectrum. Ideally, spectral subtractions would employ a mineral-only reference spectrum to yield a spectrum of ‘pure’ SOM. In reality, the resulting subtraction spectrum exhibits absorbances corresponding to SOM that are *enhanced* relative to the original (untreated) soil spectrum. This is because non-linear absorbance of mineral components in soil samples prevents complete subtraction of all mineral absorbances. Artifactual mineral absorbances and/or incomplete removal of SOM limit interpretation of specific absorbance features or interpretation of the total SOM pool, respectively.

As evidenced by the examples shown here, the two common methods for removing SOM to obtain mineral reference spectra (thermal combustion or ashing, and chemical oxidation) tend to engender a trade-off between the degree of SOM removal and spectral artifacts. These have distinct consequences for the interpretation of subtraction spectra. For the same reason that it is used in the loss on ignition (LOI) method to estimate SOM content, high-temperature ashing (≥ 350 °C) removes nearly all SOM but is likely to alter crystalline mineral structures and thus produce changes in mineral absorbance bands. As illustrated here, these will manifest as artifacts in the subtraction spectrum if spectral contributions from minerals before and after ashing differ. The thermal sensitivity of minerals, which can be mineral-specific, forces a compromise on the utility of spectral subtractions calculated with reference spectra obtained by ashing, especially at high temperatures (e.g., 550 °C). Mineral alterations specific to mineral type and ashing temperature include dehydroxylation, interlayer collapse and dehydration^{41,42,43}, and have been documented in spectra of soils of diverse mineralogy^{10,28,44,45}.

Information on the mineralogy of a soil sample can be used beforehand to determine an ashing temperature that avoids or minimizes mineral alteration (e.g., interlayer collapse of kaolinite at or above 350 °C). Mineral artifacts can be identified and anticipated by subjecting a soil sample to ashing at varying temperatures and comparing the resulting spectra with the spectra of untreated soil and pure mineral standards^{10,46}. One may also constrain sample set mineralogy in order to maintain subtraction artifacts that result from ashing constant across soil samples. Finally, lower ashing temperatures (e.g., 350 °C) can be used to enhance some organic bands^{10,47}. Similar to chemical oxidations, the use of lower temperatures generally comes at a cost of lower²⁸ (Table 2) and potentially selective¹⁰ SOM removal.

Chemical oxidations are in general less likely to cause mineral alteration, in particular for phyllosilicates, but may impact other mineral fractions such as iron (hydr)oxides. If known, the mineralogy of soil samples should be considered when selecting an oxidant. For example, sodium hypochlorite can remove relatively high amounts of SOM with minimal or no dissolution of mineral oxides, in contrast to other oxidizing agents (e.g., hydrogen peroxide)^{48,49,50,51}. These effects are expected to depend on mineralogy and/or SOM (e.g., a high proportion of mineral-bound organic matter may be more resistant to some but not other oxidations).

The reason why zero-ing out target mineral bands is a better strategy than relying on software-calculated or *a priori* SFs is that these may need to be adjusted if target mineral bands are not fully removed. As this is common for mineral bands due to high phyllosilicate concentrations in soil samples and thus non-linear absorbance in spectra (e.g., Si-O stretch at 1100-950 cm⁻¹,²² especially for the widespread practice of analyzing neat samples (no halide salt dilution), the adjustment of the SF should be explored to minimize mineral bands as per experimental objectives.

Spectral subtractions offer a marked improvement on the ability of soil spectra to be used to interpret organic functional groups that compose SOM by improving the interpretability of organic absorbances in regions with overlapping mineral absorbances. Even organic bands though to be relatively mineral-free can benefit from spectral subtractions. For example, the aliphatic C-H stretch at 3000-2800 cm⁻¹ is considered to be the least mineral-confounded organic band in soil spectra²², but its resolution can still be markedly improved with spectral subtractions⁵². Challenges to interpretation of FTIR spectra of a chemically complex sample such as SOM should not be conflated with the limitation of the method or

its modifications (e.g., subtractions) because such complexity is an inherent attribute of SOM, which also challenges other spectroscopic approaches (e.g., nuclear magnetic resonance spectroscopy)^{1,5}.

Spectral subtractions offer a unique strategy to address constraints inherent to spectroscopic analysis of SOM arising from mineral dominance of soil samples, and with strong complementarity to other methods with their own distinct limitations^{5,14}. For example, a separate approach to improve FTIR spectroscopy analysis of SOM is the pre-treatment of soils with hydrofluoric acid (HF) to remove a majority of the mineral component. Since demineralization of soil samples is routine for NMR spectroscopic analysis, the same HF-treated soils are often also analyzed by FTIR spectroscopy. However, demineralization with HF can engender significant losses of SOM that compromise its utility as a stand-alone approach^{53,54}. For example, nearly one-third of SOM of soil A (**Tables 1 and 2**) was removed during HF treatment⁵.

It is important to note that the utility of subtractions for analysis of soils is to enhance potential organic bands of interest at a particular wavenumber(s) rather than yielding a complete subtraction of all interfering mineral absorbances^{55,56,57,58}. For this reason, subtractions may be more or less suited for particular spectral regions. Nguyen *et al.*⁴ recognized that subtractions were likely to be most useful to enhance organic bands at 1800-1600 cm⁻¹, which Reeves III²² diagnosed as a spectral region for which accurate subtractions were possible even with artifacts from reference spectra obtained by high-temperature ashing. Mineral Si-O absorbances in this region subtract accurately, in contrast to other regions (e.g., 1,000-400 cm⁻¹) due to non-linear absorbance and inversions of mineral bands such as Si-O at 1,050-980 cm⁻¹^{37,59}.

A potentially fruitful area of future work is to systematically address soil properties expected to entail sample-specific artifacts introduced by the method of SOM removal. The main challenge to spectral subtractions is the difficulty of obtaining a mineral reference spectrum that has near or full SOM removal yet minimal or no artifacts from SOM removal. Current methods of high-temperature ashing and chemical oxidation engender a trade-off between these two objectives. Identifying mineralogy-specific artifacts detectable in FTIR spectra across a range of SOM removal conditions and soil types, as well as pure mineral standards, would be a first step toward establishing soil-specific recommendations²². Alternative SOM removal methods have yet to be systematically investigated. Promising removal methods include low-temperature ashing (often performed under low pressure) and for chemical oxidations, hydrogen peroxide and sodium peroxodisulphate¹⁴.

Spectra of soils demineralized using HF can be used to compare subtraction spectra obtained by various methods. HF can be used to dissolve and remove a majority of the mineral component of soil samples, yielding an SOM-enriched sample. Since the spectrum of an HF-treated soil sample should in theory be similar to a subtraction spectrum of the same soil using a mineral reference spectrum obtained by full removal of SOM, comparing the spectra of SOM obtained by HF demineralization versus subtraction could be used to cross-validate absorbance bands, identify artifacts and increase confidence in spectral assignments⁵, in particular in the regions prone to subtraction artifacts via inversion (< 1,200 cm⁻¹). However, incomplete and/or selective removal of mineral and organic components during HF treatment means that spectra of HF-treated soils may not offer an 'objective' spectrum of SOM, only an operational comparison which, not unlike spectral subtractions, must be carefully considered in light of potential artifacts.

Disclosures

The authors have nothing to disclose.

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