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TITLE:

Improving Infrared Spectroscopy Characterization of Soil Organic Matter with Spectral Subtractions

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SUMMARY:

SOM underlies many soil functions and processes, but its characterization by FTIR spectroscopy is often challenged by mineral interferences. The described method can increase the utility of SOM analysis by FTIR spectroscopy by subtracting mineral interferences in soil spectra using empirically obtained mineral reference spectra.

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.

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 sequestration[1]. 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 scales[6]

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 fractionation[8], and fingerprinting SOM composition for forensic purposes[9]. This method is applicable to a wide variety of mineral-organic mixtures beyond soils, including sediment[10], peat[11], 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 samples[4].

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 Anderson[19]. 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

1.1. 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

2.1. 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.

2.2. 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).

2.3. Incubate the mixture in a hot-water bath (15 min, 80 °C) to increase oxidation rate.

2.4. 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.

2.5. Repeat steps 2.3 and 2.4 twice for a total of three oxidation steps.

2.6. After the last oxidation step, add 20 mL deionized H_2O (dH_2O) 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.

2.7. Using a spatula and dH_2O 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.

2.8. Once the soil sample is dried, quantify total organic carbon content by combustion-gas chromatography using a C/N analyzer[24]. 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

3.1. Measure ~1–2 g of soil (sieved, air dried) into a porcelain crucible using a spatula.

3.2. 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.

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

4.1.1. Prepare the soil samples.

4.1.1.1. Dilute the samples (optional).

4.1.1.1.1. 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.

4.1.1.1.2. 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.

4.1.1.1.3. 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.

4.1.1.2. 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.

4.1.2. Collect the background spectrum.

4.1.2.1. 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.

4.1.2.2. Purge the spectrometer chamber with CO_2- and H_2O-scrubbed air (via a purge gas generator) or with N_2 gas for greater consistency in collection conditions. For example, collection of spectra under ambient atmosphere may entail small fluctuations in humidity and CO_2 that can cause changes in absorbance spectra.

Note: Newer spectrometers may have mirrors (*e.g.*, gold, SiC) that can potentially reduce humidity effects.

4.1.2.3. 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.

4.1.2.3.1. 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**.

4.1.2.3.2. Click the **Experimental Setup** icon to select spectral acquisition parameters.

4.1.2.3.3. 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.

4.1.2.3.4. 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).

4.1.3. 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.

4.1.3.1. 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.

4.1.3.2. 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.

4.2. 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.

4.2.1. 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 baseline[14].

4.2.2. 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.

4.2.3. 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.

4.2.3.1. 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.

4.2.4. 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.

4.2.5. 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.

4.3. Interpret the spectra.

4.3.1. Perform spectral interpretation using various resources available to analyze and interpret the resulting subtraction spectra, in particular assignments of absorbances to organic functional groups[4].

Note: Other uses of subtraction spectra include multivariate analysis (*e.g.*, principal component analysis), chemometric prediction of soil analytes[27], and even forensic fingerprinting[9].

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 (≥ 550 °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 removal[28], 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 spectrum[14]. 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 components[31]. Though a maximum particle size of 250–100 µm has been considered optimal to minimize such artifacts[32], 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 variability[33], 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 sample[5].

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 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 cm^{-1} and potentially amide C-N and N-H and/or aromatic C=C at 1,558 cm^{-1}. With increasing scaling of the SF, however, inversions of mineral absorbances such as Si-O at 2100 – 1780 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% C[38], 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 2100 – 1780 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 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" shape[14], 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 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 spectrum[40], which misses the objective of zero-ing out Si-O at 2100–1780 cm^{-1} for interpretation of organic absorbances at 1,800–1,000 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 cm^{-1}), Al-OH (915 cm^{-1}), and Si-O (796, 521 cm^{-1}). Quartz-like Si-O bands (2,100 –1,780, 1,159 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 cm^{-1} is achieved with an SF of 0.76, a prominent W-shaped inversion at 1,100–450 cm^{-1} suggests that interpretation of the subtraction spectrum should be limited to > 1,200 cm^{-1}. As the reference spectrum was obtained by high-temperature ashing, loss of mineral surface OH at 3,700–3,600 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 3700–3600 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 1800 – 1200 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 3000 – 2800 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 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 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 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 2916 cm^{-1}, and ketone and amide C=O with possible contributions of conjugated C=C at 1,647 cm^{-1}, amide C-N and N-H at 1,568 cm^{-1}, aliphatic C-H (bend) at 1,427 cm^{-1}, and phenol and/or carboxyl C-O at 1,275 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 certainly depending on sample type and absorbance range (due to potentially overlapping bands)[4].

FIGURE AND TABLE LEGENDS:

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.

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}.

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.

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 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.

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.

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.

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.

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 quantity 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[41-43], 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 lower[28] (**Table 2**) and potentially selective[10] 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)[48-51]. 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 spectra[22], but its resolution can still be markedly improved with spectral subtractions[52]. 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 treatment[5].

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[55-58]. 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 III [22] 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 recommendations[22]. 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 peroxodisulphate[14].

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 assignments[5], 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.

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The authors have nothing to disclose.

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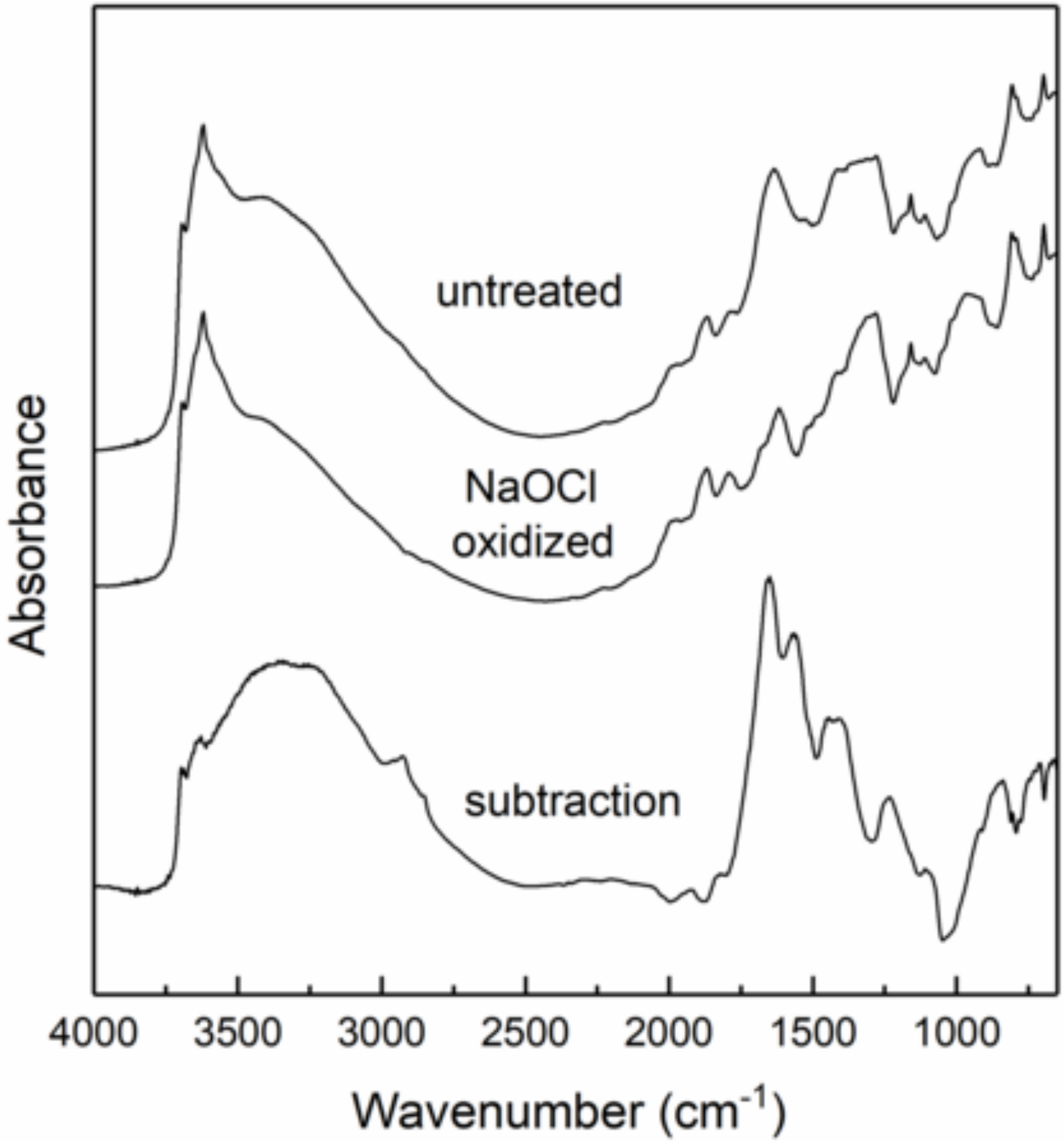
untreated
NaOCl
oxidized
subtraction
Absorbance
4000
3500
3000
2500
2000
1500
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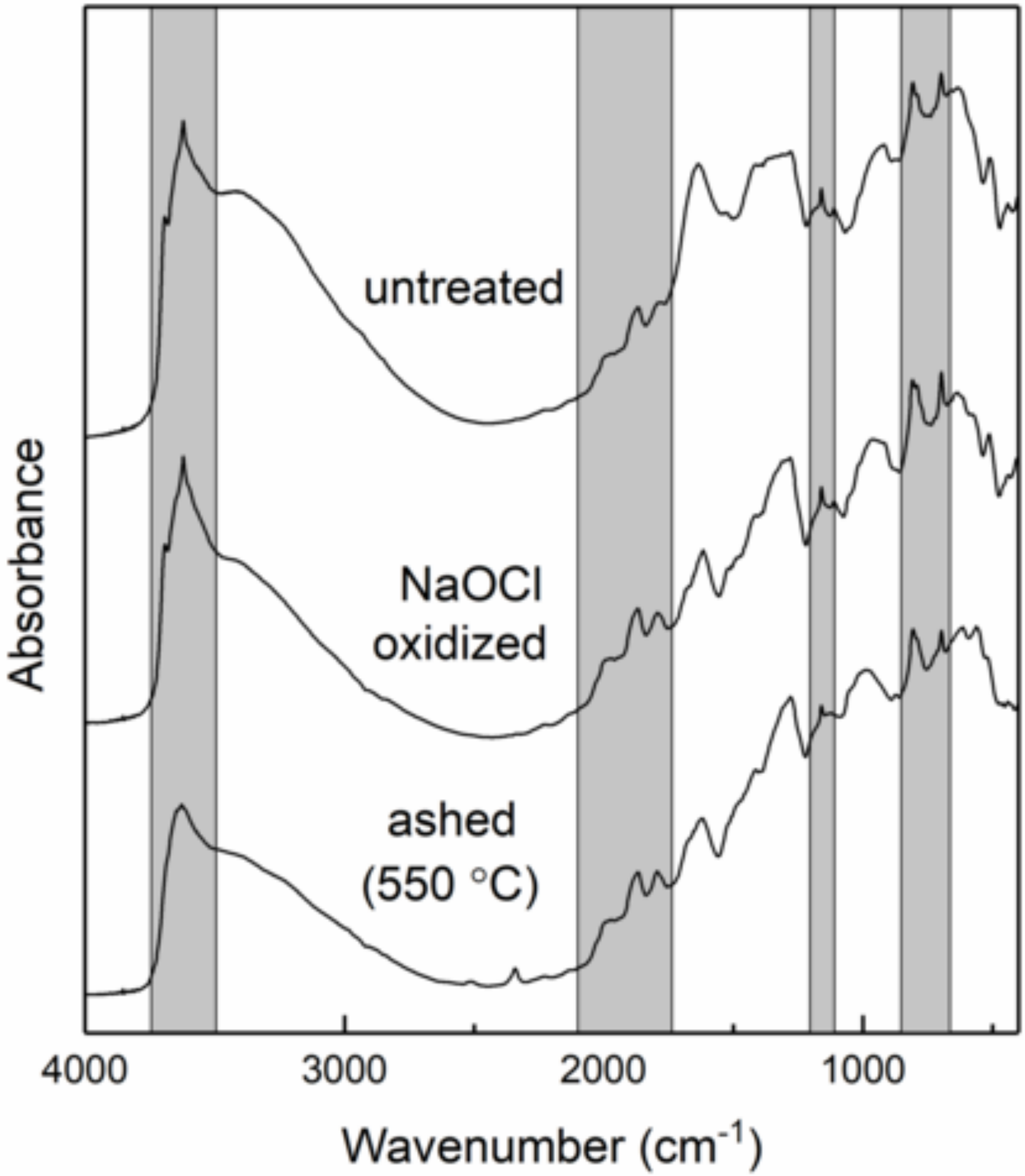
Wavenumber (cm-1)


Absorbance
untreated
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oxidized
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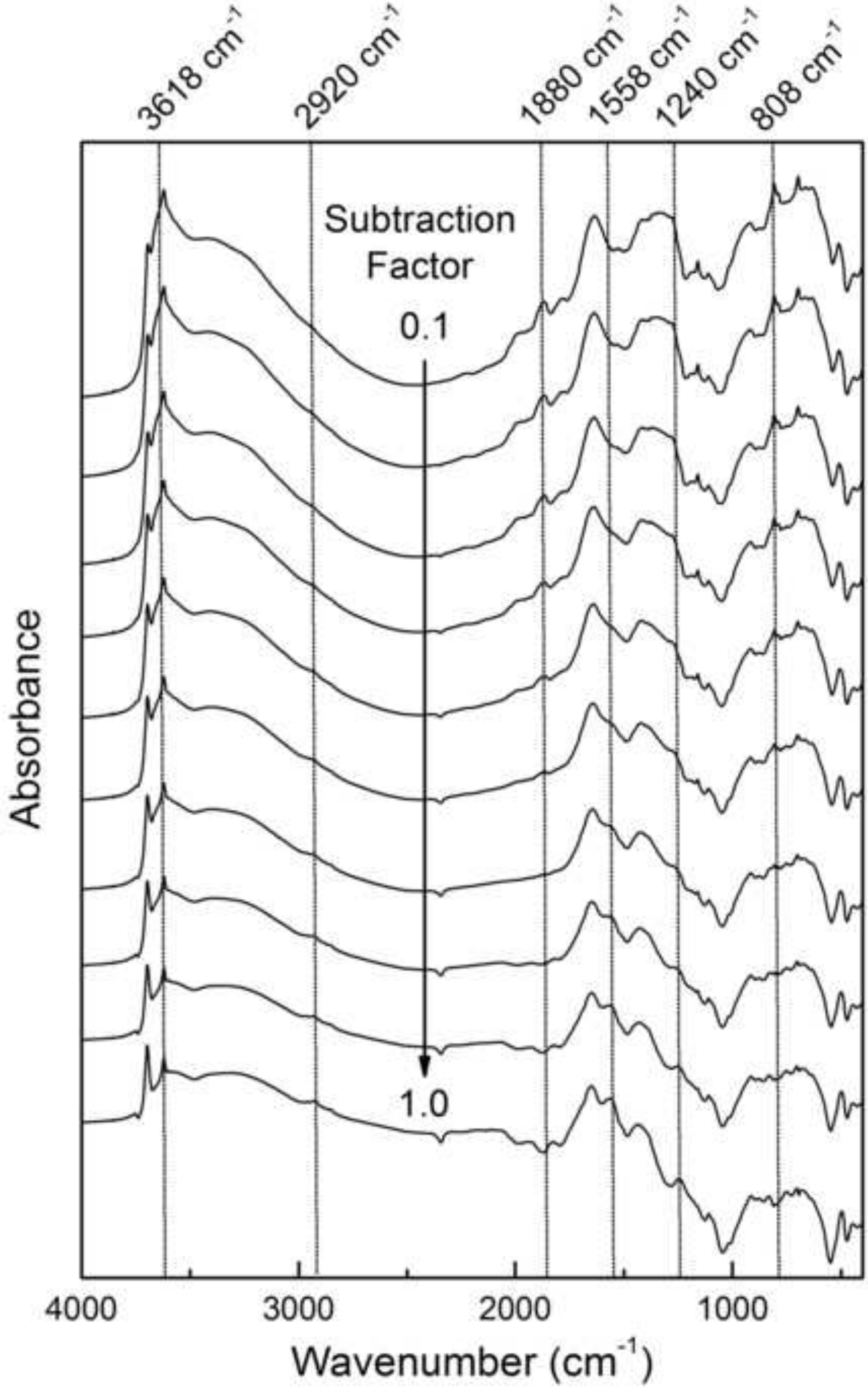
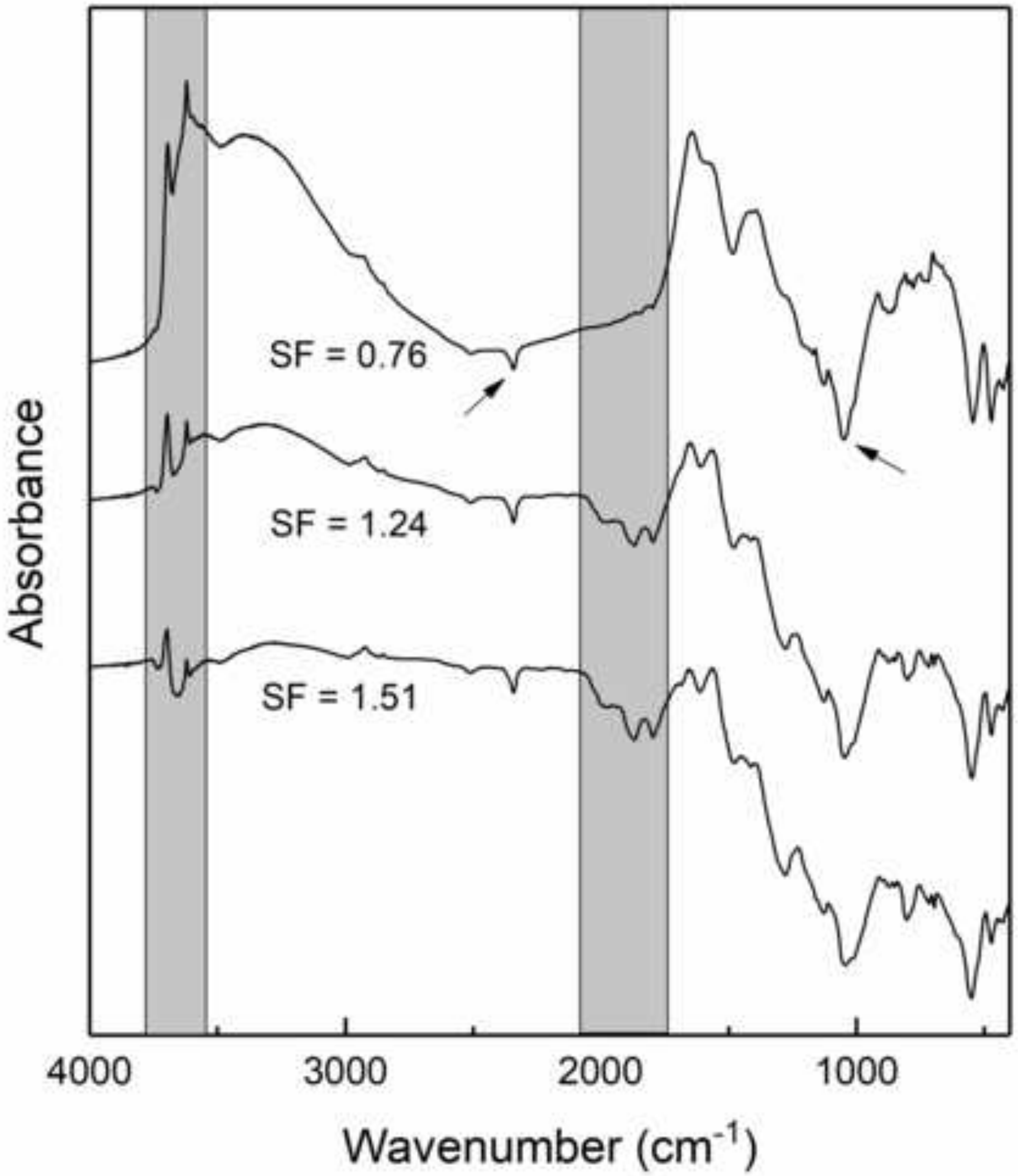
(550 °C)
4000
3000
2000
1000
Wavenumber (cm-1)

Figure 3


3618 cm⁻¹
2920 cm⁻¹
1880 cm⁻¹
1558 cm⁻¹
1240 cm⁻¹
808 cm⁻¹
Subtraction Factor
0.1
1.0
Absorbance
4000
3000
2000
1000
Wavenumber (cm⁻¹)


Absorbance
SF = 0.76
SF = 1.24
SF = 1.51
4000
3000
2000
1000
Wavenumber (cm-1)

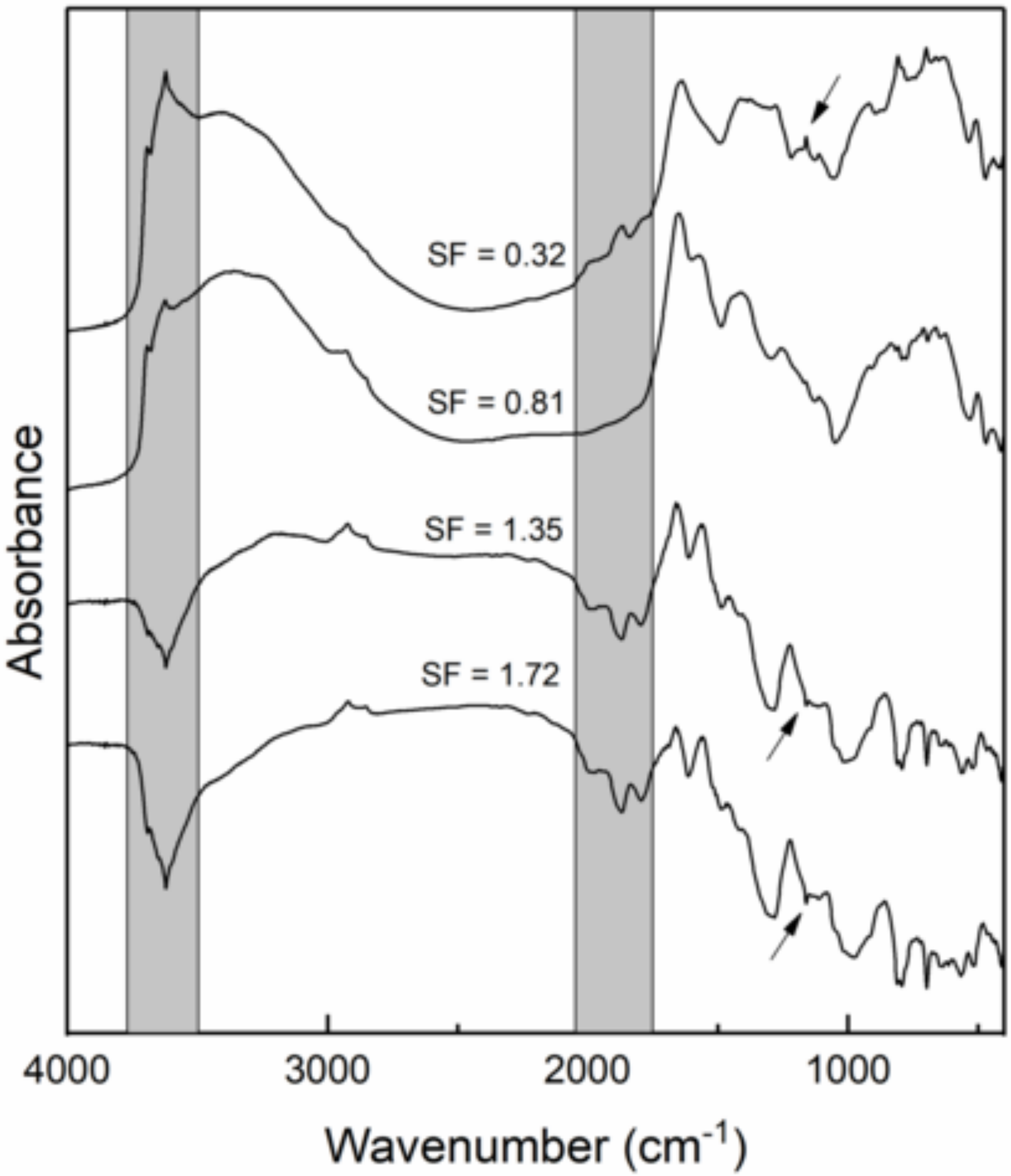

SF = 0.32
SF = 0.81
SF = 1.35
SF = 1.72
Absorbance
4000
3000
2000
1000
Wavenumber (cm^{-1})

Figure 6

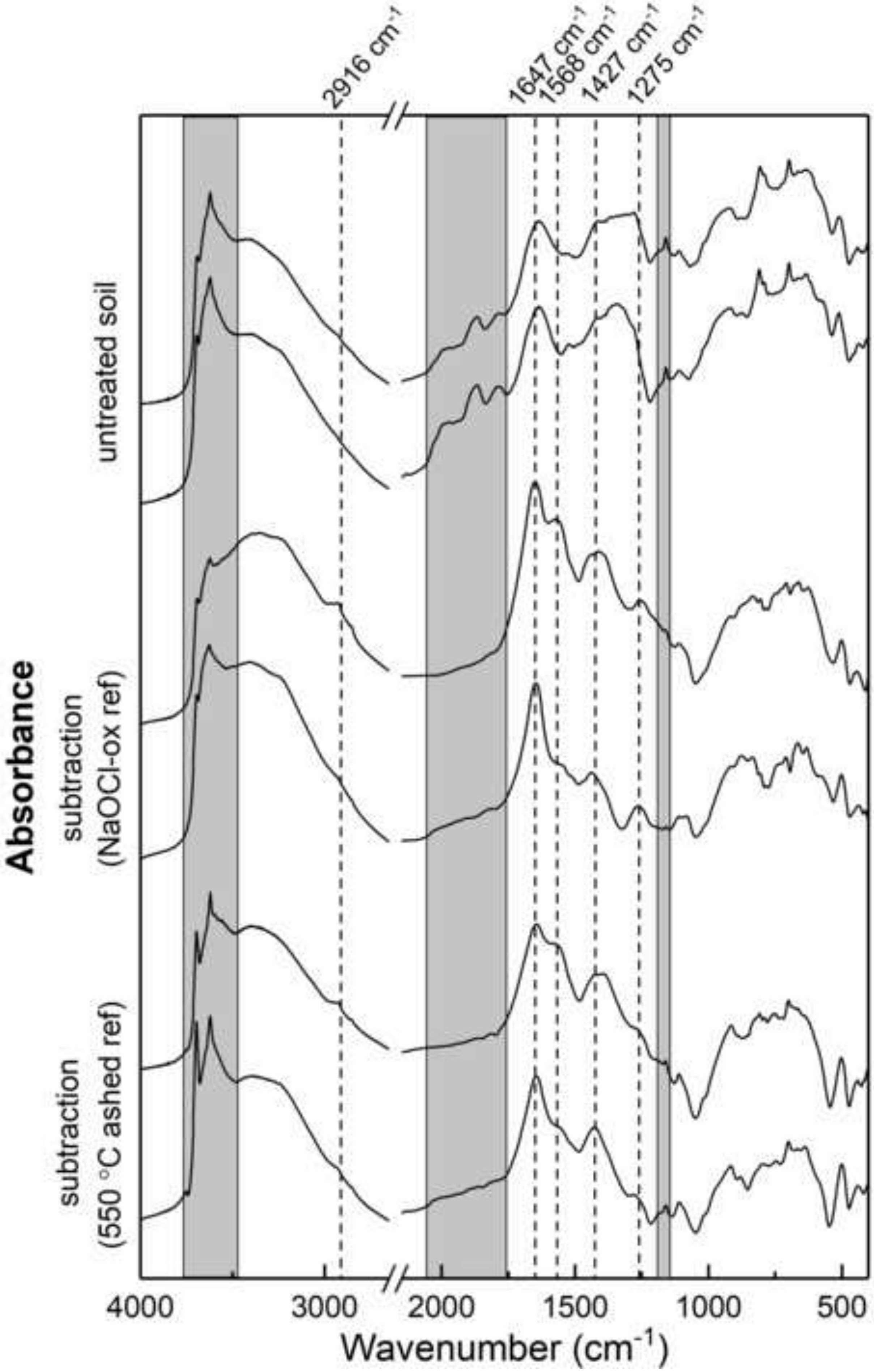

2916 cm-1
1647 cm-1
1568 cm-1
1427 cm-1
1275 cm-1
Absorbance
untreated soil
subtraction (NaOCl-ox ref)
subtraction (550 °C ashed ref)
4000
3000
2000
1500
1000
500
Wavenumber (cm-1)

Table 1

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 2

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

Name of Material/ Equipment	Company	Catalog Number
Nicolet iS50 spectrometer	Thermo Fisher Scientific	912A0760
EasiDiff	Pike Technologies	042-1040
OMNIC	Thermo Fisher Scientific	INQSOF018
6% v/v sodium hypochlorite	Clorox	n/a
Type 47900 Furnace	VWR International	30609-748
VWR Gooch Crucibles, Porcelain	VWR International	89038-038
VWR Tube 50 mL Sterile CS500	VWR International	89004-364
Forced air oven	VWR International	89511-414
VersaStar pH meter	Fisher Scientific	13 645 573

Comments/Description
infrared spectrometer used to collect spectra
high throughput sample holder
software used to perform subtractions
generic store-bought bleach for oxidative removal of soil organic matter
muffle furnace for ashing soils to removal soil organic matter
crucibles for ashing
for sodium hypochlorite
for drying soils after oxidation and water washes
for measuring pH of oxidation solution

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Author(s): **Andrew J Margenot, Sanjai J Parikh, Francisco J Calderón**

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Article Title: **Improving infrared spectroscopy characterization of soil organic matter with spectral subtractions**

Signature: Andrew Margenot

Date: 1**3 October 2017**

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Response to Revisions
57464_R1
Improving Infrared Spectroscopy Characterization of Soil Organic Matter with Spectral Subtractions

Dear Editor,

Please find below detailing of revisions made in response to editorial comments. Please note that in additional to the changes noted below, as recommended by *JoVE* online instructions we have used this as an opportunity to correct the manuscript for additional clarity in grammar and spelling.

1. The manuscript has been modified. Please read it carefully and revise if necessary. Enclosed please find the top copy. Please do not change the current format/font.

The current format and font has not been changed. Revisions have been made as indicated below.

2. Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Enclosed please find the iThenticate report. Please revise lines 280-282, 591-593, and the Figure Legend of Figure 1.

These and other sections have been rewritten with original language.

3. Please avoid sectioning the Introduction and Discussion sections.

No sections were used.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

5. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

7. For steps that involve software, please make sure to provide all the details such as "click this", "select that", "observe this", etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including *buttons clicked, inputs, screenshots, etc. This is the level of detail we're looking for. Please keep in mind that software steps without a graphical user interface cannot be filmed.*

8. We cannot film the steps that are not fully described. Please do not highlight those steps which actions are only explained in the external references. Please keep those steps in the protocol but not highlighted. Please attention that your manuscript, after acceptance, will be the source for our script writers for the video production.

9. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

All personal pronouns have now been removed.

10. Protocol: 2.7: Oven-dry for how long?

This has been specified as follows:

"Oven-dry (60 °C maximum, 24-48 hours or as needed) to an air-dried state."

11. Protocol: 4.1.2: How to collect a background spectrum? Please clearly describe the actions.

This protocol step (4.1.2) is the umbrella step for the ensuing specific steps on how to collect the background spectrum. Details on how to collect a background spectrum are provide in the 4.1.2.x steps. Please advise if this should be modified.

12. Protocol: 4.1.2.1: How is that done?

Since this is meant to a literal repetition of the loading technique described in detail for 4.1.1.3, this step is referenced as follows:

"4.1.2.1. Load a sample of KBr ground in the same manner as soil samples (see 4.1.1.3) to mimic soil matrix effects."

13. Protocol: 4.1.2.2 and 4.1.2.2: Please use the imperative tense for all the sentences.

These have been rewritten in the imperative tense as follows:

"4.1.2.2. Collect background spectra and samples under the same ambient conditions. If so, check changes in humidity and temperature mean that backgrounds and/or re-collect the background spectrum."

14. Protocol: 4.2.1: Please clearly describe all the actions in the imperative tense. Please avoid using any personal pronouns in the protocol steps. Please move the discussion to the Discussion.

These have been rewritten in the imperative tense and personal pronouns have been removed:

"1.1.1. Subtraction factor: 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 original soil spectrum. For subtractions focused on improving resolution of organic absorbances to characterize SOM, it is recommended to

> utilize the entirety of the MIR collected by most spectrometers (*e.g.*, 4000 to 650 or 400 cm^{-1}, depending on the detector)."

15. Protocol: 4.2.2: Please clearly describe all the actions. How to use the software? Please include all the buttons clicked. Please move the discussion to the Discussion.

We disagree with the recommendation to include details on the software used. This is because the procedure in question concerns spectral subtractions, which includes the pre-treatment for SOM removal and the adjustment of a subtraction factor. Whether this is used with a particular software program is arbitrary. Moreover, inclusion of software-specific steps could confuse readers who do not have this software, as FTIR softwares are highly manufacturer-dependent. We therefore believe the details specific to a software are best left out of the present method. To accommodate this, highlighting of this step has been removed so that the software-specific details of background spectra and sample spectra collection will not be in the video.

16. Protocol: 4.2.2: Note: Please move this Note to the Discussion.

This note has been removed since it is not relevant to the Discussion, but rather an observation that was originally thought to be of use to the reader by providing additional context.

17. Protocol: 4.3: Please clearly describe all the actions in the imperative tense. Please move the discussion to the Discussion.
18. Protocol: 4.3: Please clearly describe all the actions in the imperative tense. Please move the discussion to the Discussion.

The actions have been rewritten in the imperative tense and the point intended to provide context has been moved to the Discussion, beginning at line 340:

> "Since there is variability in OM and mineral types, as well as spectral artifacts from the method of packing, spectra of soils even with similar mineralogy and SOM content and removal may require slightly adjustment of the SF. If this is this case, Report the range of SF values used should be reported. If the same SF is applied across the spectral set, provide a justification. Such information is helpful to interpret results."

19. If possible, please shorten the length of the titles in the Figure Legends.

This has been done for Table 1 and Figures 1 and 6.

20. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please list all the materials, equipment, instrument, and software used in your work.

The table has been updated accordingly.

Response to Reviews
JoVE57464

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3. Please define all abbreviations before use.

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5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

6. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. The Protocol steps should contain only 2-3 actions per step and a maximum of 4 sentences per step.

8. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

9. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

10. Please leave a blank line between all protocol steps as well as Notes.

11. Protocol: 1: How to prepare the soil? In which container? Using which instrument? Please clearly describe all steps.

12. Protocol: 1.1: Sieving how? Using what? Which equipment? Which container?

13. Protocol: 2.1: How exactly is the pH adjusted? How much of each component is used? How they are mixed? Please describe all the step clearly. Please only mention the actions. Please move the discussions to the Discussion section.

14. Protocol: 2.2: "Combine" how? In which container?

15. Protocol: 2.4: Centrifuge at which temperature? Room temperature? Please use "x g" for the centrifuge force. "Pour off" how? With what?

16. Protocol: 2.4: Note 1: How much longer approximately? How to determine the time?

17. Protocol: 2.4: Notes 2-4 can be moved to Discussion.

18. Protocol: 2.5: Repeat what? 2.4? or 2.3 and 2.4?

19. Protocol: 2.6: Please describe the step clearly.

20. Protocol: 2.7: Please define ddH2O first. How is that done? How much water? Air-dry for how long? Or oven-dry for how long?

21. Protocol: 2.8: How is the quantification done? How is the Analyzer used? Please clearly describe the steps or refer to appropriate references or protocols.

22. Protocol: 3.1: "Place" using what?

23. Protocol: 4.1.1.1: Please use the imperative tense. Please move the discussion to the Discussion section.

24. Protocol: 4.2.2.2: Please clearly describe the steps. Please move the discussion to the Discussion.

25. Protocol: 4.1.1.3: Please describe the steps clearly using the imperative tense. "Collect replicate spectra" how? Please move the discussion to the Discussion.

26. Protocol: 4.1.2, 4.1.3, 4.2.1, 4.2.2, 4.3, 4.4: Please describe the steps clearly using the imperative tense. Please include all the actions. Please ensure you answer the "how" question, i.e., how is the step performed? Please move the discussion to the Discussion.

27. Protocol: For steps that involve software or analyzing devices, please make sure to provide all the details such as "click this", "select that", "observe this", etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. This is the level of detail we're looking for. Please keep in mind that software steps without a graphical user interface cannot be filmed.

28. Please avoid using sub-sections in the "Representative Results" and "Discussion".

29. Figure 1: Please add the unit to each axis.
This change has been made.

30. Figures 2, 4, 5, 6: Please describe the shadow regions.
This change has been made in the figure captions.

31. Figure 6: Please leave a white space between the value and the unit, i.e., "500 °C" instead of "500°C".
This change has been made.

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33. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please list all the materials, equipment, instrument, and software used in your work.

34. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.
This change has been made.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript describes spectral subtraction method for improving soil organic matter composition analysis by FTIR spectroscopy.
Overall it is a well put together manuscript which provides the step to step procedure of spectral subtraction method for SOM characterization by FTIR analysis and its interpretation. There is one general area which should be clarified before the paper is published.

-NaOCl oxidation can remove relatively labile OM leaving stable OM and minerals intact in the soil sample. This incident might also be reflected in the FTIR spectra which possibly could introduce terrible artifacts in the subtracted SOM spectra (Yeasmin et al., 2017 Geoderma). Anyone should take this phenomenon into consideration during interpreting the SOM spectra which are subtracted by NaOCl oxidized sample spectra. On the other hand, in some cases aliphatic carbon groups which are generally expected to be oxidized can be intact after NaOCl

treatment (Yeasmin et al., 2017 GCA). This could be due to the presence of large amounts of fresh plant materials containing substantial amounts of oxidation resistant fatty acids, waxes (aliphatics) compounds (Von Lu¨tzow et al., 2007). Thus, its quite difficult to use this NaOCl oxidation procedure for this spectral subtraction purpose without knowing the sample itself. The author has already mentioned some this fact very briefly. I think this fact need little bit more clearance in the manuscript.

We appreciate the constructive feedback. We agree that more detail could be added on this issue, and have accordingly incorporated the following at L283-298:

> "Chemical oxidations can be an attractive alternative to ashing for supplying mineral backgrounds because they generally preserve mineral structure and thus avoid artifactual absorbance features in reference spectra and the resulting subtraction spectrum[14,22]. For example, Soil A lost 89% of SOC by NaOCl oxidation compared to 97% by ashing (Table 2) while preserving mineral absorbance features altered by ashing (Fig. 2). On the other hand, incomplete and potentially selective removal of SOM means that NaOCl-resistant OM will not be characterized[5,36,37], requiring careful interpretation of the subtraction spectrum[14]."

An additional point has been added the beginning of the Discussion at L258-268:

> "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 quantity these issues in order to avoid biased interpretations on SOM composition from the subtraction spectrum. Ideally, spectral subtractions would employ a mineral-only reference spectrum to yield a spectrum of SOM. In reality, the resulting subtraction spectrum exhibits absorbances corresponding to SOM that are enhanced relative to the original soil spectrum. This is because non-linear absorbance of mineral components in soil samples prevents complete subtraction of all mineral absorbances. Additionally, artifactual mineral absorbance and/or in incomplete removal of SOM (e.g., NaOCl oxidation) and thus its enhancement in subtraction spectra limit interpretations of specific absorbance features or interpretation of the total SOM pool."

Reviewer #2:

Manuscript Summary:

This manuscript discusses a method for providing enhanced soil organic matter spectra in the presence of major soil minerals. This has been an interesting topic for many years and this paper continues the discussion.

Major Concerns:

The first issue is to do with enhancing the organic matter signal by oxidation. The paper discusses this aspect clearly and fairly thoroughly except that it fails to consider some alternative oxidizing agents and lower temperature oxidation. The second issue is to do with spectral subtraction. This is also well covered except that it does not consider or try to deal with the non-linear spectral response of the DRIFT method.

The Introduction and Discussion address drawbacks of the general methods of SOM removal (thermal combustion and wet chemical oxidation) as well as the specific examples of these two removal methods. To further emphasize the issues with this removal methods, we have made following changes have been made to the end of the Introduction (L95-109):

> “The two example methods of SOM removal for obtaining a mineral-enriched reference spectrum are (1) high-temperature combustion (‘ashing’) and (2) chemical oxidation, using dilute sodium hypochlorite (NaOCl)~~ as an example oxidant~~. It should be noted that these are examples of commonly SOM removal methods, rather than prescriptive recommendations. Given artifacts of combustion and oxidation methods are increasingly quantified and alternative SOM removal methods are evaluated (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 application to soils[17,18]. The ~~use of~~demonstrated chemical oxidation to remove SOM is based on the method of NaOCl oxidation described by Anderson[19]. This was originally developed for minimizing OM content of soils prior to X-ray diffraction (XRD) analyses, and has been investigated as a potential chemical fractionation sensitive to SOM stabilization[20,2]1. 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 removal1[4,22].”

Additional changes to address this issue in the Introduction and Discussion sections have made in response to specific reviewer comments (please see below).

Minor Concerns:
Protocols:
Part 3.2. High temperature oxidation. Why was 550C chosen? It is well known that clay minerals such as kaolinite decompose at this temperature. Lower temperature for prolonged times (~350C for overnight heating) mostly avoids mineralogical changes. The longer ignition time helps remove most of the OM even though 350C is below the ignition temperature of soil OM.

We discuss the selection of 550 °C in the Introduction at L95-100:

> “The two example methods of SOM removal for obtaining a mineral-enriched reference spectrum are (1) high-temperature combustion (‘ashing’) and (2) chemical oxidation, using dilute sodium hypochlorite (NaOCl)~~ as an example oxidant~~. Differences in 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)[1,2] followed by application to soils[3,4].”

As indicated in the Introduction, 550 °C is an example of a potential method of SOM removal, rather than a prescriptive recommendation. Issues with these example SOM removal methods are reviewed and alternatives discussed in greater detail in the Introduction and Discussion sections.

To further clarify that the two SOM removal methods are meant to be examples, the following changes have been made to the end of the Introduction (L95-109):

> “The two example methods of SOM removal for obtaining a mineral-enriched reference spectrum are (1) high-temperature combustion (‘ashing’) and (2) chemical oxidation, using dilute sodium hypochlorite (NaOCl)~~ as an example oxidant~~. It should be stressed that these are examples of commonly SOM removal methods, rather than prescriptive recommendations. Given artifacts of combustion and oxidation methods are increasingly quantified and alternative SOM removal methods are evaluated (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 application to soils[17,18]. The ~~use of~~demonstrated chemical oxidation to remove SOM is based on the method of NaOCl oxidation described by Anderson[19]. This was originally developed for minimizing OM content of soils prior to X-ray diffraction (XRD) analyses, and has

> been investigated as a potential chemical fractionation sensitive to SOM stabilization[20,2]1. 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 removal1[4,22]."

You don't say anything about checking first the mineralogy of the sample to be heated. Some samples cannot be subjected to heating because they decompose even at low temperatures (~120C) such as AlOOH and FeOOH minerals.

The important issue of mineral artifacts due to thermal combustion for SOM removal is addressed at L466-475.

> "Knowledge of a soil sample's mineralogy can also be used *a priori* to determine a maximum ashing temperature that avoids mineral alteration (e.g., interlayer collapse of kaolinite at 350 °C). Mineral artifacts can also 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[5,6]. One may also constrain sample set mineralogy in order to maintain subtraction artifacts of ashing constant across samples. Finally, lower ashing temperatures (e.g., 350 °C) can be used to enhance some organic bands[5,7]. Similar to chemical oxidations, the use of lower temperatures generally comes at a cost of lower SOM removal[8], as evidenced for the example soils (Table 2) and may engender selective loss of functional groups[5]."

This discussion notes the potential of low-temperature ashing to also incur mineral (and organic) artifacts.

We agree that low-temperature ashing is a less artifact-prone method of SOM removal. This important point has been further stressed at L537-538:

> "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 peroxodisulphate[21]."

Additionally, the potential of low-temperature ashing as an alternative SOM removal method has been added to the Introduction (L97-100):

> "The two ~~example~~ methods of SOM removal for obtaining a mineral-enriched reference spectrum are (1) high-temperature combustion ('ashing') and (2) chemical oxidation, using dilute sodium hypochlorite (NaOCl)~~as an example oxidant.~~ It should be stressed that these are examples of commonly SOM removal methods, rather than prescriptive recommendations. Other methods of SOM removal methods may offer reduced mineral artifacts and/or enhanced removal rates (e.g., low-temperature ashing) [14].

L178: "grinding beyond 2000 μm does not necessarily improve spectral quality for chemometric predictions". I dispute this. Published work shows an improvement of about 0.1 R-square units for prediction modelling by fine grinding to <0.1 mm.

The statement in question is a summary of a particular study that finds no significant benefit to grinding beyond this particle size— at least for specific soils and experimental objectives of these studies. We sought to be cautious and did not recommend against grinding beyond a maximum particle size of 2000 μm. For example, we write that grinding does not "necessarily" improve spectral quality. Our intention was to connote that for some soils and/or experimental objectives it may not be a beneficial soil pre-treatment.

To clarify this, we have modified the section at l79-189 as follows:

> "Note: Though a maximum particle size of 250 – 100 µm has been considered ideal because the aperture of most FTIR spectrometers is 1000 µm [26], recent evaluations suggest that grinding beyond 2000 µm does not necessarily improve spectral quality for chemometric predictions [27,28]. ~~However,~~ More finely ground soil samples tend to yielde~~d~~ enhanced (sharper) absorbance peaks and exhibited decreased absorbance variability [27], 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., 2000 µm)[29,30]. The effect of this on spectral quality is likely to depend on experiment objectives (e.g., interpretation of absorbance bands, chemometric prediction of soil C) and soil types[29]. It is therefore recommended that grinding size be assessed for particular set of soils and objectives."

L203: These days, mirror type of background referenced are used, such as stainless-steel, gold or SiC. These reduce the problems of contamination and humidity effects on KBr.

We have added this clarification at L214-217:

> "For example, collection of spectra under ambient atmosphere may entail small fluctuations in humidity and CO_2 that can cause changes in absorbance spectra. Newer spectrometers may have mirrors (e.g., gold, SiC) that can potentially reduce humidity effects."

Part 4. You do not discuss linearizing the DRIFT spectral responses by applying a non-linear transform such as the Kubelka Munk or a power function. This may help balance the relative different subtraction factors for OM and mineralogy.

We thank the reviewer for this suggestion; it has been added as a possibility in the Discussion section at L327-329:

> "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 softwares include spectral processing transformations (e.g., Kubelka-Munk, power functions) to improve spectral quality (e.g., linear baselines) prior to performing subtractions."

L266: SOM removal. I guess that the different SOM oxidation by chemical versus ignition is similar to the comparison between the dichromate oxidation with Walkely-Black and Leco ignition. In the chemical method, some organic species such as soil char material is not easily chemically oxidized.

The Discussion describes incomplete removal of SOM by NaOCl oxidation (or other removal methods, including low-temperature ashing) as a key trade-off of this method. This has been further clarified at L283-289:

> "Chemical oxidations can be an attractive alternative to ashing for supplying mineral backgrounds because they generally preserve mineral structure and thus avoid artifactual absorbance features in reference spectra and the resulting subtraction spectrum[14,22]. For example, Soil A lost 89% of SOC by NaOCl oxidation compared to 97% by ashing (Table 2) while preserving mineral absorbance features altered by ashing (Fig. 2). On the other hand, incomplete and potentially selective removal of SOM means that NaOCl-resistant OM will not be characterized[5,36,37], requiring careful interpretation of the subtraction spectrum[14]."

Additionally, the opening Discussion paragraph has been modified to underscore this limitation of spectral subtractions, at L258-268:

> “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 quantity these issues in order to avoid biased interpretations on SOM composition from the subtraction spectrum. Ideally, spectral subtractions would employ a mineral-only reference spectrum to yield a spectrum of SOM. In reality, the resulting subtraction spectrum exhibits absorbances corresponding to SOM that are enhanced relative to the original soil spectrum. This is because non-linear absorbance of mineral components in soil samples prevents complete subtraction of all mineral absorbances. Additionally, artifactual mineral absorbance and/or in incomplete removal of SOM (e.g., NaOCl oxidation) and thus its enhancement in subtraction spectra limit interpretations of specific absorbance features or interpretation of the total SOM pool.”

You need to stress the importance of detailed knowledge of the interpretation of MIR soil spectra. Failure in this regard may easily lead to inappropriate subtraction and thus complete miss-interpretation.

We have clarified the overall limitation of spectral subtractions (semi-quantitative, operational) and need for careful interpretation of absorbances to infer relative changes in organic functional group composition of SOM, at L374-377:

> “It is essential that subtraction spectra be understood as an operational method to assess relative changes in absorbances that can be assigned to organic functional groups with varying certainly depending on sample type and absorbance range (due to potential overlapping bands)[4].”

References

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