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# Evaluation of a microplate spectrophotometer for soil organic carbon determination in south-central Idaho

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## Abstract

Determination of soil organic carbon (SOC) is highly desirable for assessing fertility and carbon sequestration; however, numerous methods of determination warrant study of method agreement. Recently, a novel method was developed following dichromate oxidation using a microplate spectrophotometer. This novel method was compared with (a) total C by dry combustion − soil inorganic carbon ($DC_{\text{w/o pretreatment}}$–pressure calcimater [Pcal]); (b) traditional Walkley–Black titration (WBTIT) and (c) loss on ignition ($LOI_{360^{\circ}C}$) in calcareous soils of south-central Idaho ($n = 75$) in conjunction with North American Proficiency Testing program soils ($n = 10$). A two-way ANOVA was fit with soils as a blocking factor to identify any difference between methods; means were separated using Tukey's honestly significant difference test ( = .05). Additional comparisons were made for all soils ($n = 85$) and for soils in the lower 75th percentile of SOC determined by WBTIT ($n = 56$) using regression analysis. Only the WBTIT and $LOI_{360^{\circ}C}$ methods were statistically equivalent nevertheless there was high agreement (Lin's concordance coefficients > .90) between all methods ($n = 85$). Under low SOC soils ($n = 56$) the agreement between all methods decreased, but the Walkley–Black spectrophotometric method (WBSPEC) method fit other methods comparatively well $r^2 = .71$, .74, and .78 for $LOI_{360^{\circ}C}$, $DC_{\text{w/o pretreatment}}$–Pcal, and WBTIT respectively. The WBSPEC method provided estimates of SOC between the methods currently used in the region while reducing hazardous waste generation over traditional WBTIT and sample handling over $LOI_{360^{\circ}C}$ and $DC_{\text{w/o pretreatment}}$–Pcal methods, positioning it as a sensible option for SOC determination in low SOC calcareous soils of south-central Idaho.

## 1 | INTRODUCTION

Successful soil tests should be representative, reproduceable, and prone to limited error with additional considerations for ease of execution, waste production, and cost. The applicability of a soil test may vary by region. For example, Mehlich-1, Mehlich-3, Olsen, and Bray phosphorous tests

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Abbreviations: DC, dry combustion; DI, deionized; Lin's c, Lin's concordance correlation coefficients; LOI, loss on ignition; NAPT, North American Proficiency Testing program; Pcal, Pressure calcimeter; SIC, soil inorganic C; SOC, soil organic C; SOM, soil organic matter; WBSPEC, Walkley–Black spectrophotometric method; WBTIT, Walkley–Black titration vary in effectiveness in different regions depending on soil properties (Dari, Rogers, Leytem, & Schroeder, 2019; Mallarino, 1995). Soil organic matter (SOM) is only a fraction of the solid state of soil but influences a myriad of soil properties making its determination highly desirable. The direct determination of SOM is performed quantitatively using loss on ignition (LOI) after pretreatment with hydrofluoric (HF) and hydrochloric (HCl) acid to remove hydrated mineral species (Nelson & Sommers, 1982; Rather, 1917). Semi-quantitative SOM determination is made through either $H_2O_2$ oxidation, LOI without pretreatment to remove hydrated mineral species, or by indirect estimation from soil organic carbon (SOC) content. Soil organic matter and SOC are at times incorrectly equated as the same. Soil organic matter is approximately 50% SOC by mass and is approximated by applying the van Bemmelen correction factor (1.724) to determined organic C contents (van Bemmelen, 1890). The correction factor of 1.724 is the reciprocal of 58% organic C content and was applied in van Bemmelen's accredited study (van Bemmelen, 1890). Other research has challenged the accuracy of the van Bemmelen factor (Broadbent, 1953; Pribyl, 2010); nevertheless, it remains in use today. It has been suggested that SOC be measured and reported due to its connection with SOM and because SOC can be quantified through a variety of methods (Nelson & Sommers, 1982). Methods of SOC determination should be considered in terms of their strengths, limitations, and suitability for soils of various regions.

Measurement of SOC is performed using three techniques: (a) direct measurement of SOC following wet or dry combustion where soil inorganic C (SIC) has been removed by pretreatment (Allison, 1960; Bremner, 1949; Piper, 1942), (b) direct measurement of SOC through oxidation by dichromate via titration or colorimetry (Graham, 1948; Walkey & Black, 1934), or (c) indirect measurement of SOC by pairing soil total C measurement through wet or dry combustion without pretreatment with SIC determination via gravimetric mass loss or volumetric displacement where total soil C − SIC = SOC (U.S. Salinity Lab, 1954; Wagner, Hanson, Olness, & Voorhees, 1998). Pretreatment of soil with acid complicates the procedure for SOC determination by wet or dry combustion reducing sample throughput. Additionally, pretreatment with sulfurous acid may not fully remove carbonates in highly calcareous soils due to the precipitation of calcium sulfite (Fernandes & Krull, 2008; Schmidt, Smernik, & McBeath, 2012). Methods that utilize dichromate often result in substantial hazardous waste generation. In soils where SIC is negligible, total soil C can be considered equivalent to SOC and determined by high temperature dry combustion and thermal conductivity detection without pretreatment with acid (Tabatabai & Bremner, 1970). While convenient, this method is erroneous in soils containing inorganic carbon. The presence of carbonates, frequently as calcite, dolomite, and siderite, confound the dry combustion method when pretreatment is omitted due to their thermal decomposition at 700, 600, and 500 °C, respectively (Gallagher & Warne, 1981; Kohobhange, Manoratne, Pitawala, & Rajapakse, 2019; McIntosh, Sharp, & Wilburn, 1990). Fortunately, the presence of soil carbonates can be corroborated by effervescence after acid addition or even predicted by soil pH as carbonates buffer pH in alkaline soils (Bache, 1984).

Core Ideas

- Soil carbonates begin to accumulate at a pH of 7.4 in soils of south-central Idaho.
- Currently used SOC methods did not produce equivalent SOC values despite high correlation.
- The WBSPEC method performed satisfactorily, especially in soils with low (<13.11 g $kg^{-1}$ ) SOC.

Alternatively, SOC can be converted from SOM values measured by mass loss after oxidation at temperatures below the thermal decomposition point of carbonaceous minerals using muffle furnaces (Davies, 1974; Storer, 1984). Loss of structural water may still occur along with minor incidences of incomplete combustion (Ranney, 1969). The conversion of SOM to SOC is achieved using the reciprocal of the van Bemmelen factor or a more appropriate conversion that has been experimentally determined. This method is commonly referred to as LOI. Anecdotally, the LOI method is subject to increased risk of human error as best practice requires rapid handling of the sample after heating. Sample crucibles must be moved from muffle furnaces directly into desiccators to cool, which can only viably be done by hand with metal tongs. The additional time and effort from a lack of automation may limit the LOI method in some laboratories.

The paired determination of total soil C and SIC should be considered in soils containing inorganic C as it is dependent on two direct and simple measurements. Total soil C is quantified by dry combustion and thermal conductivity detection without the need for pretreatment. Quantification of SIC is done gravimetrically and volumetrically. In the gravimetric determination, soil is exposed to HCl, and the mass loss is proportional to $CO_2$ evolved from the sample (U.S. Salinity Lab, 1954). In the volumetric determination, soil is exposed to HCl inside a sealed reaction vessel, and the increased pressure is proportional to SIC content (Sherrod, Dunn, Peterson, & Kolberg, 2002; Wagner et al., 1998). Although probably the most preferred method of SOC determination in soils containing inorganic C sources, it is not without potential drawbacks. For example, the gravimetric determination of SIC is prone to error from incomplete effervescence (U.S. Salinity

Lab, 1954). The volumetric determination of SIC calls for two different reaction vessel volumes based on the unknown SIC contents; screening of soils must be completed if the SIC is not known (Sherrod et al., 2002). Minor leaks from the reaction vessel are undetectable during analysis. Additionally, this method of SOC determination is a function of two independent measurements, and the propagation of error must be considered. The resulting SOC measurement error is equal to the square root of the sum of squares from independent variables $[(a)^2 = (b)^2 + (c)^2]$ and may be higher than a single measurement (Harvard, 2013; Hogan, 2006). Similar concern on pairing total soil C and SIC determination was voiced by Schmidt et al. (2012), who reviewed SOC methodology in calcareous Australian soils, and Bisutti, Hilke, and Raessler (2004), who provided a review of current organic carbon methodology.

Determination of SOC using dichromate has been extensively used for some time due to its simplicity as a direct measurement of SOC and because it can be used without specialized laboratory equipment. Nevertheless, the traditional titrimetric dichromate method produces a large volume of hazardous chromium waste ( 250 ml per sample) and is also criticized for incomplete oxidation of SOC without external heating (Allison, 1960; Walkley & Black, 1934). For these reasons, the method is commonly scaled down by reducing soil and reagent volume, and a correction factor of 1.33 is applied when external heating is omitted. More appropriate correction factors can be experimentally derived using wet or dry combustion methods (Walkley & Black, 1934). Another consideration is that the dichromate method is subject to interference from chloride, ferrous iron, and manganese dioxide in soil (Walkley, 1947). Modifications have been made to the dichromate method including external heating in a block digestor to complete SOC oxidation (Mebius, 1960) or alternatively calculating SOC using spectrophotometry of dichromate (Carolan, 1948) or trivalent chromium (Debolt, 1974; Graham, 1948; Sims & Haby, 1971). Recently, SOC has been determined by spectrophotometry in commercial microplate readers (Souza, Morais, Matsushige, & Rosa, 2016); however, the sample size was low (n = 9) and aimed at optimization of the procedure. Despite this, the spectrophotometric method proposed by Souza et al. (2016) drastically reduces hazardous waste generation and human error of end point detection relative to the titrimetric variant. A more extensive evaluation of this microplate spectrophotometric method is warranted given its potential to reduce waste generation where dichromate methods are utilized.

Soils of south-central Idaho are of national agricultural significance. Idaho produces 33% of all U.S. potatoes (Solanum tuberosum L.), 20% of all U.S. sugar beets (Beta vulgaris L.), and 70% of hybrid sweet corn (Zea mays L.) seed globally (Idaho State Department of Agriculture, n.d.). Growing interest in soil health may increase producer demand for SOC testing in this region. Soils of the region are low in SOC (typically <15 g $kg^{-1}$) and calcareous in nature, thus posing challenging conditions for accurate determination of SOC. To circumvent carbonate contents, commercial laboratories use dichromate oxidation methods or low temperature LOI and convert SOM to SOC using a correction factor. In some cases, dry combustion without pretreatment has been paired with volumetric determination of SIC to measure SOC. In select research requests, SOC has been determined by dry combustion, including pretreatment with sulfurous acid to remove carbonates, but is not used extensively. Therefore, the objectives of this study were (a) to determine carbonate influence on total soil C; (b) to compare SOC values obtained by the commonly utilized methods of SOC determination; (c) to compare the commonly utilized methods of SOC determination for low SOC soils; and (d) to compare SOC values obtained by the Walkley–Black spectrophotometric method (WBSPEC) using a microplate spectrophotometer with three other commonly utilized methods of SOC determination in soils from south-central Idaho.

## 2 | MATERIALS AND METHODS

### 2.1 | Site selection and soil sampling

Selected sites represent soil series of agricultural significance within south-central Idaho, with preference given to soil series representing larger geographical areas. In this study, south-central Idaho refers to the arable land within the Snake River plain. Soils of south-central Idaho are primarily Aridisols formed from loess that overlays volcanic basalt. Some lacustrine pockets originating from the flooding of historic Lake Bonneville are present. The climate of the region is Köppen-Geiger classified BsK (Peel, Finlayson, & McMahon, 2007), annual average precipitation is 24 cm, and average annual temperature is 9.6 ˚C. Most agriculture is irrigation-dependent, but some dryland grain production occurs. Soil samples were collected in part (n = 41) from Rogers, Schroeder, Rashed, and Roberts (2018) and Dari et al. (2019). In brief, soils from Rogers et al. (2018) and Dari et al. (2019) were collected to a depth of 30 cm by bucket auger with four subsamples taken at each site. Additional soil samples were collected from a variety of locations throughout south-central Idaho. The remainder of samples were taken to a depth of 15 cm by soil probe with four subsamples at each site (n = 34). Standard soils (n = 10) were included from the North American Proficiency Testing Program (NAPT) to assess the accuracy of the SOC methodologies (Table 1); thus, the total sample number was n = 85.

TABLE 1 Methods of soil organic content determination used, abbreviation, and reference matter

| Soil organic carbon method | Abbreviation | Reference(s) |
|---|---|---|
| Loss on ignition at 360 °C | $\text{LOI}_{360^\circ\text{C}}$ | Storer, 1984; Miller et al., 2013 |
| Walkley–Black titrimetric variant | WBTIT | Walkley & Black, 1934 |
| Walkley–Black spectrophotometric variant | WBSPEC | Walkley & Black, 1934; Souza et al., 2016 |
| Dry combustion$_{\text{w/o pretreatment}}$–Pressure calcimeter | DC$_{\text{w/o pretreatment}}$–Pcal | Sherrod et al., 2002; Wagner et al., 1998 |

## 2.2 | Ancillary analysis

Soil series of each sample was determined based on existing mapping data from the Natural Resources Conservation Service (Soil Survey Staff, 2019). Particle size analysis was completed using a hydrometer method (NAPT S-14.10) adapted from Gee and Bauder (1986). Soil pH was determined in a 1:1 soil/deionized (DI) water solution using NAPT S-2.20 (Miller et al., 2013). Soil carbonates were quantified by volumetric displacement utilizing the pressure calcimeter method (Sherrod et al., 2002; Wagner et al., 1998). Soil series, taxonomic class, particle size fractions, pH, and carbonate content for all samples can be found in the Supplemental Material (S1).

## 2.3 | Walkley–Black titrimetric method

The Walkley–Black titrimetric variant (WBTIT) used here was modified from Walkley and Black (1934) to reduce sample and reagent size by half. Briefly, 0.5 g of soil was weighed into 250-ml Erlenmeyer flasks, 5 ml of 0.167 M potassium dichromate was added to each flask, and 10 ml of concentrated sulfuric acid was added to each flask. Samples were gently swirled and left to rest for 30 min. After rest, 100 ml DI water was added to each flask and four drops of ferroin indicator were added before each sample was titrated with 0.5 M ferrous sulfate. End point determination was made on a lighted stir plate. The standard correction factor of 1.33 was applied to account for incomplete oxidation of SOC.

## 2.4 | Walkley–Black spectrophotometric method

The Walkley–Black spectrophotometric method is also dependent on the oxidation of SOC by dichromate as in the titrimetric variant. However, the quantification of reduced chromium is made through ultraviolet–visible light absorbance using a spectrophotometer. The present study followed the procedure of Souza et al. (2016) with a minor increase in soil volume as SOC tends to be low in soils of south-central Idaho. A brief description of the method follows. Sequentially, 0.25 g soil, 4 ml of 0.167 M potassium dichromate, and 8 ml of concentrated sulfuric acid were added to each 50 ml Erlenmeyer flask. Samples were gently swirled and left to rest for 30 min before 12 ml DI water was added to each flask. The flasks were gently swirled an additional time and left to settle overnight. After settling, 0.25 ml was decanted from each sample into 96-well flat bottom plates (Costar #3596, Corning Inc.) and run in a microplate spectrophotometer. The microplate spectrophotometer was set to endpoint detection in absorbance mode at 590 nm, and pass speed was set to normal (Biotek powerwave 340 plate reader, Biotek Instruments Inc.). The color of each sample is related to organic C through a standard curve that is generated by adding increasing amounts of ferrous ammonium sulfate to reduce dichromate in otherwise identical standard flasks (S2). This method of SOC determination is also subject to incomplete oxidation of SOC (Souza et al., 2016); therefore, the standard correction factor of 1.33 was applied.

## 2.5 | Loss on ignition

The presence of carbonates in soils of south-central Idaho required the use of a low temperature (360 °C) LOI procedure (NAPT S-9.20; Miller et al., 2013). This method, hereafter $\text{LOI}_{360^\circ\text{C}}$, used a digitally programable muffle furnace for the heating sequence. First, 10 g soil was added to preweighed crucibles and placed into a drying oven for 2 h at 105 °C to remove any remaining soil water. Samples were transferred directly to a desiccator and cooled under vacuum for 1 h before being reweighed. Samples were then placed in a muffle furnace (Sentrotech ST-1200-242424, Sentro Tech) and heated for 2 h after the temperature reached 360 °C. The furnace was allowed to cool to a holding temperature of 105 °C before samples were transferred directly to a desiccator. The desiccator was placed under vacuum, and samples were allowed to cool for 1 h before reweighing. Organic matter was calculated by mass loss using Equation 1:

$$\%\mathrm{OM} = \frac{\text{wt soil\&crucible, ovendry (105 C)} - \text{wt soil\&crucible, postcombustion (360 C)}}{\text{wt soil\&crucible, ovendry (105 C)} - \text{wt crucible}} \times 100 \quad (1)$$

The determined SOM content was converted to SOC based on the method of Miller et al. (2013). Soil organic C determined by the WBTIT method was fit against SOM obtained by $\mathrm{LOI}_{360^{\circ}\mathrm{C}}$, and the resulting fit equation was used to convert SOM to SOC in the $\mathrm{LOI}_{360^{\circ}\mathrm{C}}$ method. This regression is typically done for only a subset of soils. The NAPT soils were chosen as the subset for the regression as they cover a wide range of SOC contents and because the SOC determined by the WBTIT method can be validated by the reported values of the NAPT program. The conversion equation produced by NAPT soils and applied in this study, along with the conversion equation produced from all south-central Idaho soils, is available as Supplemental Material (S3). It is advised that laboratories using the $\mathrm{LOI}_{360^{\circ}\mathrm{C}}$ method for SOC determination in south-central Idaho update their conversion equation to that obtained with all south-central Idaho soils if conversion equations are not routinely updated.

## 2.6 | Dry Combustion $_{\text{w/o pretreatment}}$ –Pressure Calcimeter

Dry combustion without pretreatment was paired with volumetric determination of SIC using the modified pressure calcimeter method (Sherrod et al., 2002). This method, hereafter referred to as $\mathrm{DC}_{\text{w/o pretreatment}}$–Pcal, relies on paired determination of total soil C and SIC where SOC = total soil C − SIC. Here, a variant of NAPT S-13.30 was used in conjunction with NAPT S-9.30 (Miller et al., 2013). Total soil C was determined by thermal conductivity detection after dry combustion at 950 ˚C in an automated CN analyzer (Flash 1112 EA CN Analyzer, Thermo Fisher Scientific). Soil inorganic C was determined using the pressure calcimeter method. Sequentially, 1 g soil was placed into pressure vessels, and 2-ml vials containing 2 ml of 6 M HCl and 3% ferrous chloride by weight were placed upright in each vessel. Vessels were sealed and shaken to create a reaction between HCl and soil carbonates. The reaction was allowed to proceed for 2 h before the seal was perforated by a needle connected to a pressure transducer. Voltage readings were converted to SIC values by a standard curve of increasing carbonate contents. Carbonate was converted to inorganic C stoichiometrically and subtracted from total C content obtained by dry combustion to yield SOC.

## 2.7 | Statistics

All soils were analyzed in duplicate and the mean value used thereafter. Converted SOM values for NAPT soils (n = 10) by $\mathrm{LOI}_{360^{\circ}\mathrm{C}}$ and WBTIT in the present study were compared with values reported by the NAPT for LOI and WBTIT methods using Welch's two sample t-test. A Pearson correlation table was constructed (n = 75) to assess the influence of pH and carbonate contents on the methods of SOC determination and the influence of carbonate content on total soil C. The significance of correlation was calculated using the Hmisc package (Harrell & Dupont, 2019) in R (R Core Team, 2019). A linear regression model was fit (n = 75) between total soil C and carbonate contents to visualize the influence of carbonate content on total soil C. Linear regression models (n = 75 and n = 56) were fit between WBTIT SOC and total soil C to assess the degree to which total soil C is indicative of SOC in south-central Idaho soils. The model was fit with a reduced number of samples (n = 56) to further visualize this relationship on soils with low SOC contents. Soils defined as having low SOC contents were those within the lower 75th percentile of SOC values determined by the WBTIT method ($<13.11\ \mathrm{g\ kg^{-1}}$, n = 56).

It was possible to calculate the pH at which carbonates began to accumulate in south-central Idaho soils. A piecewise regression was fit (n = 75) between soil pH and carbonate content to determine a critical point in pH below which carbonates were absent and total soil C could be considered analogous to SOC. The model parameters were optimized using nonlinear least squares, and the break point was directly determined (Ryan & Porth, 2007). Segment convergence at the breakpoint was achieved by allowing the slope of the left-hand line to be a function of the breakpoint and other model parameters. Soil organic C values were arcsinh-transformed to better satisfy assumptions of normality and homogeneity of variance before a two-way ANOVA was performed fitting SOC methodology and soil as a blocking factor in R (R Core Team, 2019). Tukey's honestly significant difference approach for all possible comparisons was used to separate SOC method means. The values of SOC between all possible combinations of methods used in this study were compared using regression analysis for all soils (n = 85) and for soils with low SOC contents ($<13.11\ \mathrm{g\ kg^{-1}}$, n = 56). A slope <1 indicates overestimation of y by x while a slope >1 suggests underestimation of y by x. A slope approaching 1 is ideal, although the y-intercept must be considered as passing through the y axis above or below the plot origin is indicative of bias. To that end, Bland–Altman estimates of fixed bias and Lin's concordance correlation coefficients (Lin's c) were calculated between all possible combinations of methods to assess method agreement. Bland–Altman plots are primarily used to identify the agreement between two measurement techniques; the mean between techniques is plotted against the difference between techniques for n samples. This permits identification of any systematic differences and provides an estimate of fixed bias between the measurement techniques (Bland & Altman, 1986). Lin's c succinctly represents the agreement between two continuous measurements by combining measures of accuracy and precision into one coefficient; Lin's c ranges from −1 to 1 with perfect agreement at 1 (Lin, 1989). The significance of Bland–Altman fixed bias was determined by Student's t test on the difference between measures. Regressions comparing each sampling method with the WBSPEC method were plotted to aid interpretation. Bland–Altman plots were plotted for comparisons between each sampling method and the WBSPEC method using the "blandr" package in R (Datta, 2017). Lin's c was calculated using the "DescTools" package in R (Signorell et al., 2020). Where applicable, analyses were considered significant at the = .05 level.

## 3 | RESULTS AND DISCUSSION

### 3.1 | Soil properties

Soils selected for study were representative of soils of south-central Idaho. Soils were alkaline in nature, and mean soil pH was 8.0, ranging from 4.8 to 8.7 (Figure 1). Soil texture was also representative of soil surveys of the study region; the most frequently observed texture was silt loam, and the mean silt size particle fraction was 538 g $kg^{-1}$. Soils with higher sand content were also represented with 24 samples exceeding a sand-size particle fraction of 400 g $kg^{-1}$. Carbonate content of soils was considerable with a mean of 59 g $kg^{-1}$ and spanning from below detection limit to 325 g $kg^{-1}$ (Figure 1). A comparable mean value of SIC (53 g $kg^{-1}$) was reported in another study of this region (Entry, Sojka, & Shewmaker, 2004). Elsewhere, carbonate contents were assessed on loess deposits in this region, and a higher mean carbonate content (162 g $kg^{-1}$) was reported relative to the present study (Lewis & Fosberg, 1982). The present study adequately represents coarse alkaline soils containing carbonates; however, future comparison is warranted on more finely textured soils of a more moderate pH. The NAPT reports SOM content by LOI at 1000 ˚C and WBTIT methods; these certified values were compared with $LOI_{360^\circ C}$ and WBTIT methods used in the present study. Converted SOM content from $LOI_{360^\circ C}$ and WBTIT methods were not significantly different from the NAPT values for the 10 NAPT soils included in this study ($p = .93$ and .94, respectively). A value index for all methods of SOC determination and total soil C is provided as Supplemental Material (S4) along with basic soil property data (S1).

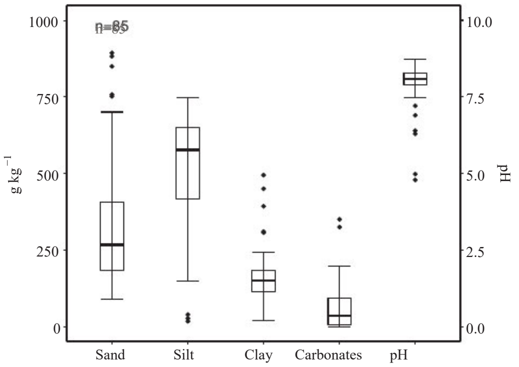

FIGURE 1 Quantile plots of basic soil properties. (sand, silt, or clay) soil particle size analysis; (carbonates) soil carbonate content determined by pressure calcimeter; (pH) soil pH determined in 1:1 soil/water mixture

### 3.2 | Soil carbonate influence on SOC detection

Total soil C was determined by dry combustion at 950 ˚C without pretreatment to remove carbonates as a step in the $DC_{w/o\ pretreatment}$–Pcal method, which is representative of SOC determination when total soil C is presumed equivalent to SOC. Predominantly, this assumption is not practicable in soils of south-central Idaho as soil carbonates had a strong positive significant correlation with total soil C ($r = .82$, $p < .0001$, Figure 2, left, and Table 2). Regressions fit between WBTIT SOC and total soil C demonstrate this. Total soil C is a poor predictor of SOC for south-central Idaho soils, especially when SOC contents are low ($r^2 = .52$ and .16, Figure 2, middle and right). All methods of SOC determination had a significant positive correlation to soil carbonate (Table 2). An examination of Cook's distance of the regressions determined that this was the result of a highly influential data point that, if removed, eliminated the significance of any correlation. The $LOI_{360^\circ C}$ and WBTIT methods had a significant negative correlation with soil pH while the $DC_{w/o\ pretreatment}$–Pcal and WBSPEC methods were not significantly correlated with soil pH ($p = .108$ and .075, Table 2). Here, the soil pH was related to SOC content presumably because biological activity is optimal

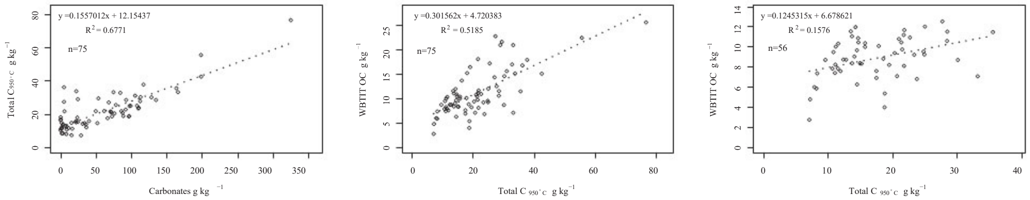

FIGURE 2 (left) Soil carbonate influence on total soil C determination of south-central Idaho soils by dry combustion without pretreatment. Soil organic C (SOC) determined using the Walkley–Black titrimetric method compared with total soil C analysis using dry combustion without pre-treatment; (middle) all soils from south-central Idaho, and (right) soils within the lower 75th percentile of organic carbon values (<13.11 g $kg^{-1}$) determined by the Walkley–Black titrimetric method. The dotted line indicates the model fit. Total $C_{950^{\circ}C}$, total soil C determined by dry combustion; carbonates, soil carbonate content determined by pressure calcimeter; WBTIT, SOC determined by the Walkley–Black titrimetric method

TABLE 2 Pearson correlation coefficients for soil properties determined for south-central Idaho soils

| | Carbonates | Total $C_{950^{\circ}C}$ | $DC_{w/o\ pretreatment}$–Pcal | $LOI_{360^{\circ}C}$ | WBSPEC | WBTIT |
|---|---|---|---|---|---|---|
| pH | 0.37** | −0.11 | −0.19 | −0.33** | −0.21 | −0.32** |
| Carbonates | | 0.82*** | 0.32** | 0.24* | 0.32** | 0.28* |
| Total $C_{950^{\circ}C}$ | | | 0.80*** | 0.68*** | 0.73*** | 0.72*** |
| $DC_{w/o\ pretreatment}$–Pcal | | | | 0.87*** | 0.88*** | 0.89*** |
| $LOI_{360^{\circ}C}$ | | | | | 0.94*** | 0.95*** |
| WBSPEC | | | | | | 0.96*** |

Note. Total $C_{950^{\circ}C}$, total soil C determined by dry combustion; carbonates, soil carbonate content determined by pressure calcimeter; WBTIT, soil organic C (SOC) determined by the Walkley–Black titrimetric method; LOI $OC_{360^{\circ}C}$, SOC determined by the loss on ignition at 360 ˚C method; $DC_{w/o\ pretreatment}$–Pcal, SOC determined by dry combustion without pretreatment paired with determination of inorganic C by pressure calcimeter; WBSPEC, SOC determined by the Walkley–Black spectrophotometric method employing a microplate spectrophotometer.

*,** , *** Significance at $p < .05$, .01, and .001 respectively. Significance of correlation was performed in R (Hmisc package).

near neutral pH values and decreases with increasing or decreasing pH.

Carbonates accumulate in arid regions due to the dissolution of calcium rich minerals within soil parent material. There is often insufficient rainfall to leach base-forming cations from the soil, and this is exacerbated by shallow irrigation (Entry et al., 2004). Carbonate buffers soil pH in these regions as carbonate will react with water to form bicarbonate, releasing hydroxyl ions, and bicarbonate reacts with calcium to form calcium carbonate. It is then feasible to predict the pH at which soils begin accumulating carbonates by regressing soil carbonates against soil pH. Here, soil carbonate and pH fit a piecewise regression, and a breakpoint pH of 7.4 was determined by nonlinear least squares estimation (Figure 3, Ryan & Porth, 2007). This is in line with the results of Dari et al. (2019), who independently identified a break point of 7.5 in a subset (n = 41) of soils used in the present study, and Rogovska, Blackmer, and Mallarino (2007), who identified a break point of 7.5 in Iowa soils. Ebeling, Bundy, Kittell, and Ebeling (2008) plotted inorganic C against pH in alkaline Wisconsin soils; however, a break point was not directly specified. In the present study, all soils with detectable carbonate contents were above the break point pH of 7.4; however, several soils above a pH of 7.4 lacked substantial carbonate content (Figure 3). Although limited data were collected below

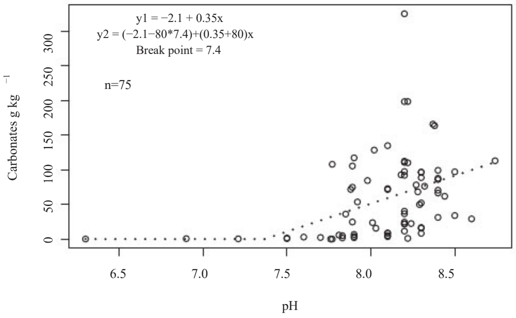

FIGURE 3 Soil carbonate content as influenced by pH for soils of south-central Idaho. The segmented regression was fit using nonlinear least squares, convergence at the break point (7.4) was achieved by allowing the slope of the left-hand line to be a function of the breakpoint and other model parameters. Carbonates: Soil carbonate content determined by pressure calcimeter; pH: soil pH determined in 1:1 soil water mixture

the break point, this suggests that when pH is <7.4, total soil C can be presumed equal to SOC in south-central Idaho soils.

## 3.3 | Comparing soil organic carbon testing methodology

A two-way ANOVA fitting SOC methodology with soil as a blocking factor identified significant differences ($p < .001$) between methodologies. Tukey's honestly significant difference test indicated the WBTIT and $LOI_{360^{\circ}C}$ methods were not significantly different ($p_{adj} = .287$). Presumably this was because $LOI_{360^{\circ}C}$ SOM values were converted to SOC by applying the equation obtained by regressing SOM of NAPT soils with SOC values from the WBTIT method (S3). All other SOC methods were identified as statistically different (all $p_{adj} < .01$), accentuating the need to report methodology concomitantly with SOC values. Direct comparisons of SOC values between the $DC_{w/o\ pretreatment}$–Pcal and WBTIT or $LOI_{360^{\circ}C}$ methods will necessitate the use of a correction factor as they do not produce the same values. Nevertheless, each SOC method had a strong positive correlation with all other methods of SOC determination (all $p < .001$, Table 2). The degree of correlation between methods was similar to, but slightly less than, that obtained by Soon and Abboud (1991) in a comparable study of SOC methods. Presently, regressions fit between all SOC methodologies were significant ($p < .001$); however, all fit statistics decreased when evaluating only soils with low SOC (<13.11 g $kg^{-1}$, n = 56, Table 3). Lin's c was lower under all method comparisons when only low SOC soils were considered, signifying inferior method agreement at low SOC contents. Difficulty in measurement of numerically low SOC values was expressed in similar studies (Conyers, Poile, Oates, Waters, & Chan, 2011; Soon & Abboud, 1991; Wang, Wang, & Zhang, 2012).

Understandably, the $LOI_{360^{\circ}C}$ and WBTIT methodologies were the most equivalent; under all soils the intercept (0.07) was near the origin and the slope (0.98) near 1 (Table 3). Corroborating this, Lin's c (.98) indicated exceptional agreement with no significant indication of fixed bias (Tables 3 and 4). A similar fit (slope = 0.95, intercept = 0.44) was reported when the WBTIT method was compared with LOI at 375 °C on 31 calcareous soils of northwest China (Wang et al., 2012). When the present study made this comparison in low SOC soils, there was no indication of bias; however, Lin's c decreased (.80), which indicates increased scatter around the regression.

The $DC_{w/o\ pretreatment}$–Pcal method identified more SOC than all other methodologies as the slopes for all comparisons were numerically higher than 1 with intercepts near the origin and positive estimates of fixed bias (n = 85, Tables 3 and 4). This was markedly different than the reported slope (0.96) between the $DC_{w/o\ pretreatment}$–Pcal and WBTIT methods from Sherrod et al. (2002). It was not stated if a correction factor was used for the WBTIT method in the Sherrod study; however, the standard WBTIT correction value for incomplete oxidation (1.33) may need to be modified for south-central Idaho if the $DC_{w/o\ pretreatment}$–Pcal method is interpreted as the reference method. Elsewhere, when a correction factor of 1.3 was applied to the WBTIT method on calcareous Mediterranean soils, better agreement (slope = 1.017 and intercept = 0.256) was obtained with the $DC_{w/o\ pretreatment}$–Pcal method (Santi et al., 2006). Presently, when evaluating only low SOC soils, the $DC_{w/o\ pretreatment}$–Pcal method also measured numerically higher SOC than all other methods, as evident by intercepts >0 and a positive fixed bias (n = 56, Tables 3 and 4).

The WBSPEC method resulted in SOC recovery between the WBTIT and $DC_{w/o\ pretreatment}$–Pcal methods, as indicated by slopes above and below 1 in addition to significant positive and negative bias with each method, respectively (n = 85, Tables 3 and 4). This was likely due to the higher ratio of sulfuric acid to soil mass in the WBSPEC procedure, which is advantageous for both additional heat release and SOC oxidation relative to the standard WBTIT method (Chan, Bowman, & Oates, 2001; Souza et al., 2016). Indeed, Walinga, Kithome, Novozamsky, Houba, and van der Lee (2008) compared a typical Walkley–Black spectrophotometric variant to the standard WBTIT method after identical oxidation regimes with external heating and reported a slope and intercept of 1.01 and −0.01, respectively. Another comparison can be made with data (n = 9) from Souza et al. (2016) between WBTIT and WBSPEC methods; the slope (0.99) and intercept (0.19) indicated better agreement than in the current study, although sample size was much lower, and the WBTIT method was modified to increase sulfuric acid addition (Figure 4, top left). Despite some difference in SOC recovery in the current study, Lin's c was >.90 for comparisons of each method with the WBSPEC method (n = 85, Table 3). Systematic increasing positive bias was indicated by the funnel shape and position of the limits of agreement in the Bland–Altman plots comparing WBSPEC and WBTIT methods (Figure 5, top and bottom, left). Positive bias was also present between WBSPEC and $LOI_{360^{\circ}C}$ methods, although data points were more scattered around the regression at lower SOC contents (Table 4 and Figure 4, top and bottom, middle). Bias increased with increasing SOC contents between WBSPEC and $DC_{w/o\ pretreatment}$–Pcal methods, although the direction of increase was less defined (Figure 5, top and bottom, right). Regardless, all systematic bias was largely diminished in the low SOC soil comparison as the differences between means had limited directionality (Figure 5, bottom). When comparing Lin's c between the three common methods of SOC determination used in south-central Idaho, it is notable that none have exceptionally high agreement when only low SOC soils are considered (Table 3). Moreover, Lin's c calculated between the WBSPEC and other

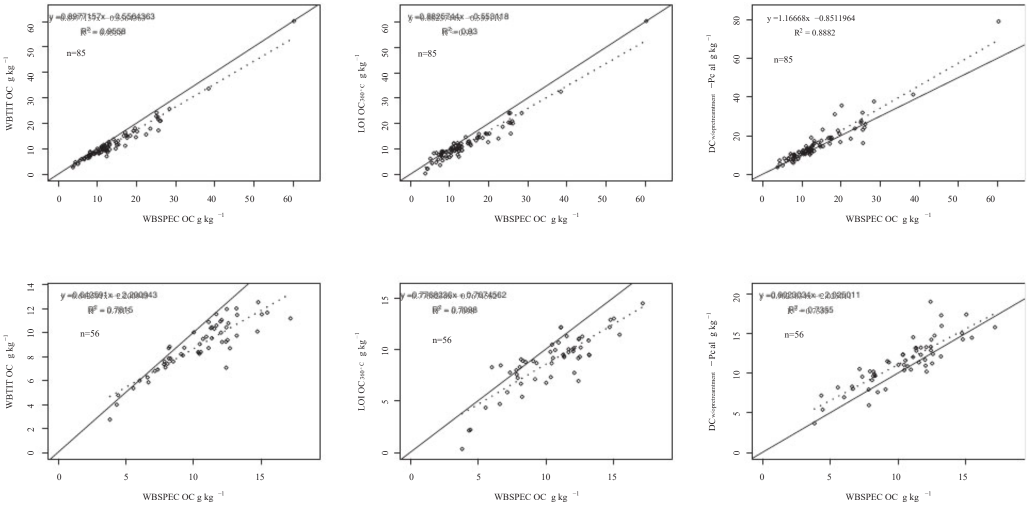

FIGURE 4 (Top row) Spectrophotometric Walkley–Black soil organic carbon (SOC) compared with other methods of organic C determination for all soils, n = 85. (Bottom row) Spectrophotometric Walkley–Black SOC compared with other methods of organic C determination for the lower 75th percentile of SOC values ($<13.11$ g kg$^{-1}$) determined by the Walkley–Black titrimetric method of south-central Idaho soils, n = 56. For all graphs, the solid line indicates a slope of 1 from the plot origin, and the dotted line indicates the model fit. WBTIT, SOC determined by the Walkley–Black titrimetric method; LOI OC$_{360^\circ C}$, SOC determined by the loss on ignition at 360 °C method; DC$_{w/o\ pretreatment}$–Pcal, SOC determined by dry combustion without pretreatment paired with determination of inorganic C by pressure calcimeter; WBSPEC, SOC determined by the Walkley–Black spectrophotometric method employing a microplate spectrophotometer

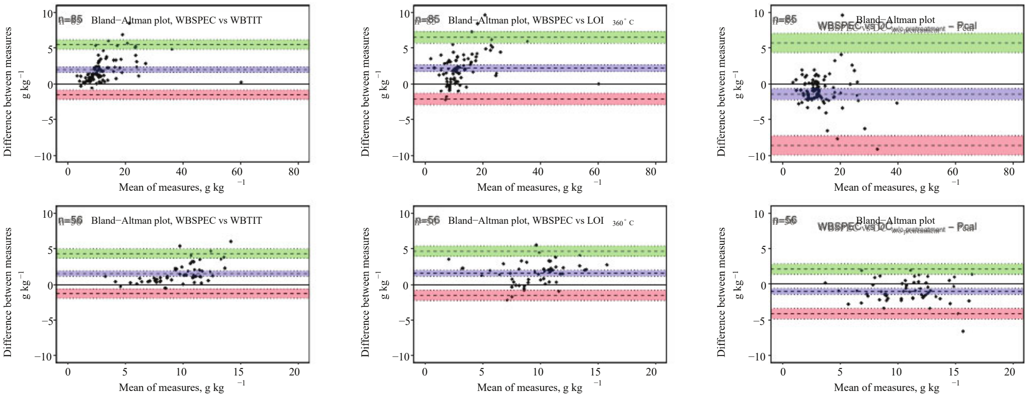

FIGURE 5 Bland–Altman plots constructed for spectrophotometric Walkley–Black soil organic carbon (SOC) and other methods of SOC determination for all soils, n = 85 (top row), and for the lower 75th percentile of SOC values ($<13.11$ g kg$^{-1}$) determined by the Walkley–Black titrimetric method of south-central Idaho soils, n = 56 (bottom row). The dotted green, blue, and red lines represent estimates of the upper limit of agreement, fixed bias, and lower limit of agreement with the shaded regions indicating 95% confidence intervals, respectively. Plots were created using the "blandr" package in R. WBTIT, soil organic carbon (SOC) determined by the Walkley–Black titrimetric method; LOI OC$_{360^\circ C}$, SOC determined by the loss on ignition at 360 °C method; DC$_{w/o\ pretreatment}$–Pcal, SOC determined by dry combustion without pretreatment paired with determination of inorganic C by pressure calcimeter; WBSPEC, SOC determined by the Walkley–Black spectrophotometric method employing a microplate spectrophotometer

TABLE 3 Regression coefficients for the methods of soil organic C (SOC) determination for all soils, n = 85, and the 75th percentile of SOC content, determined by the Walkley–Black titration, for soils of south-central Idaho, n = 56. Lin's concordance correlation coefficients (Lin's c) were calculated from the regressions using the "DescTools" package in R

| | Intercept | | Slope | | | |
|---|---|---|---|---|---|---|
| | Estimate | SE | Estimate | SE | $R^2$ | Lin's c |
| All soils, n = 85 | | | | | | |
| LOI$_{360^{\circ}\mathrm{C}}$ x DC$_{\text{w/o pretreatment}}$–Pcal | 0.99 | 0.49 | 0.70 | 0.03 | .89 | 0.83 |
| LOI$_{360^{\circ}\mathrm{C}}$ x WBSPEC | −0.55 | 0.43 | 0.88 | 0.03 | .93 | 0.92 |
| LOI$_{360^{\circ}\mathrm{C}}$ x WBTIT | 0.07 | 0.31 | 0.98 | 0.02 | .96 | 0.98 |
| DC$_{\text{w/o pretreatment}}$–Pcal x LOI$_{360^{\circ}\mathrm{C}}$ | 0.41 | 0.68 | 1.28 | 0.05 | .89 | 0.83 |
| DC$_{\text{w/o pretreatment}}$–Pcal x WBSPEC | −0.85 | 0.73 | 1.17 | 0.05 | .89 | 0.91 |
| DC$_{\text{w/o pretreatment}}$–Pcal x WBTIT | 0.00 | 0.61 | 1.29 | 0.04 | .91 | 0.85 |
| WBSPEC x LOI$_{360^{\circ}\mathrm{C}}$ | 1.56 | 0.44 | 1.05 | 0.03 | .93 | 0.92 |
| WBSPEC x DC$_{\text{w/o pretreatment}}$–Pcal | 2.21 | 0.55 | 0.76 | 0.03 | .89 | 0.91 |
| WBSPEC x WBTIT | 1.21 | 0.35 | 1.06 | 0.03 | .96 | 0.94 |
| WBTIT x LOI$_{360^{\circ}\mathrm{C}}$ | 0.40 | 0.31 | 0.98 | 0.02 | .96 | 0.98 |
| WBTIT x DC$_{\text{w/o pretreatment}}$–Pcal | 1.03 | 0.44 | 0.71 | 0.02 | .91 | 0.85 |
| WBTIT x WBSPEC | −0.56 | 0.34 | 0.90 | 0.02 | .96 | 0.94 |
| 75th percentile, n = 56 | | | | | | |
| LOI$_{360^{\circ}\mathrm{C}}$ x DC$_{\text{w/o pretreatment}}$–Pcal | 1.80 | 1.00 | 0.62 | 0.08 | .50 | 0.50 |
| LOI$_{360^{\circ}\mathrm{C}}$ x WBSPEC | 0.77 | 0.73 | 0.78 | 0.07 | .71 | 0.73 |
| LOI$_{360^{\circ}\mathrm{C}}$ x WBTIT | −0.44 | 0.89 | 1.04 | 0.10 | .68 | 0.80 |
| DC$_{\text{w/o pretreatment}}$–Pcal x LOI$_{360^{\circ}\mathrm{C}}$ | 4.30 | 1.02 | 0.80 | 0.11 | .50 | 0.50 |
| DC$_{\text{w/o pretreatment}}$–Pcal x WBSPEC | 2.03 | 0.79 | 0.90 | 0.07 | .74 | 0.81 |
| DC$_{\text{w/o pretreatment}}$–Pcal x WBTIT | 1.62 | 1.16 | 1.10 | 0.13 | .58 | 0.48 |
| WBSPEC x LOI$_{360^{\circ}\mathrm{C}}$ | 2.31 | 0.73 | 0.91 | 0.08 | .71 | 0.73 |
| WBSPEC x DC$_{\text{w/o pretreatment}}$–Pcal | 1.09 | 0.78 | 0.82 | 0.07 | .74 | 0.81 |
| WBSPEC x WBTIT | −0.41 | 0.80 | 1.22 | 0.09 | .78 | 0.71 |
| WBTIT x LOI$_{360^{\circ}\mathrm{C}}$ | 3.13 | 0.56 | 0.65 | 0.06 | .68 | 0.80 |
| WBTIT x DC$_{\text{w/o pretreatment}}$–Pcal | 2.88 | 0.72 | 0.53 | 0.06 | .58 | 0.48 |
| WBTIT x WBSPEC | 2.20 | 0.50 | 0.64 | 0.05 | .78 | 0.71 |

Note. WBTIT, SOC determined by the Walkley–Black titrimetric method; LOI OC$_{360^{\circ}\mathrm{C}}$, SOC determined by the loss on ignition at 360 ˚C method; DC$_{\text{w/o pretreatment}}$–Pcal, SOC determined by dry combustion without pretreatment paired with determination of inorganic C by pressure calcimeter; WBSPEC, SOC determined by the Walkley–Black spectrophotometric method employing a microplate spectrophotometer.

SOC methods was at least as favorable as many other inter-method comparisons for low SOC soils.

As a result, the WBSPEC method appears to be a reasonable option for SOC determination in soils of south-central Idaho and could be considered in other low SOC calcareous soils. Other subjective benefits of the WBSPEC method include a reduction in sample handling relative to LOI$_{360^{\circ}\mathrm{C}}$ and DC$_{\text{w/o pretreatment}}$–Pcal methods and a considerable reduction (89%) in hazardous waste generation relative to the typical WBTIT method. Using a spectrophotometric variant after dichromate oxidation removes the need for technicians to make endpoint determinations in titrimetric variants, thereby removing human bias. Additionally, the integration of a microplate spectrophotometer increases processing speed relative to traditional benchtop spectrophotometers. New techniques are being developed that measure SOC and SIC from one sample aliquot by thermogravimetrics without hazardous waste production or excessive handling; however, their use may be limited to research applications as sample throughput is low (Pallasser, 2013).

TABLE 4 Bland–Altman estimates of fixed bias for all soils, n = 85, and the lower 75th percentile of soil organic C (SOC) content determined by the Walkley–Black titration (<13.11 g kg$^{-1}$) for soils of south-central Idaho, n = 56. Student's t tests were performed on the differences between measures. Estimates of fixed bias and their confidence intervals were obtained using the "blandr" package in R

| | Bland–Altman fixed bias | | | |
|---|---|---|---|---|
| | Estimate | P, Student's t test | Lower 95% C.I. | Upper 95% C.I. |
| All soils, n = 85 | | | | |
| LOI$_{360^{\circ}C}$ x DC$_{\text{w/o pretreatment}}$–Pcal | −3.66 | <.001 | −4.51 | −2.81 |
| LOI$_{360^{\circ}C}$ x WBSPEC | −2.18 | <.001 | −2.67 | −1.71 |
| LOI$_{360^{\circ}C}$ x WBTIT | −0.21 | .207 | −0.53 | 0.11 |
| DC$_{\text{w/o pretreatment}}$–Pcal x LOI$_{360^{\circ}C}$ | 3.66 | <.001 | 2.81 | 4.51 |
| DC$_{\text{w/o pretreatment}}$–Pcal x WBSPEC | 1.47 | <.001 | 0.68 | 2.26 |
| DC$_{\text{w/o pretreatment}}$–Pcal x WBTIT | 3.45 | <.001 | 2.66 | 4.25 |
| WBSPEC x LOI$_{360^{\circ}C}$ | 2.19 | <.001 | 1.71 | 2.67 |
| WBSPEC x DC$_{\text{w/o pretreatment}}$–Pcal | −1.47 | <.001 | −2.26 | −0.68 |
| WBSPEC x WBTIT | 1.98 | <.001 | 1.60 | 2.37 |
| WBTIT x LOI$_{360^{\circ}C}$ | 0.21 | .207 | −0.12 | 0.53 |
| WBTIT x DC$_{\text{w/o pretreatment}}$–Pcal | −3.45 | <.001 | −4.25 | −2.66 |
| WBTIT x WBSPEC | −1.98 | <.001 | −2.37 | −1.60 |
| 75th percentile, n = 56 | | | | |
| LOI$_{360^{\circ}C}$ x DC$_{\text{w/o pretreatment}}$–Pcal | −2.56 | <.001 | −3.16 | −1.96 |
| LOI$_{360^{\circ}C}$ x WBSPEC | −1.55 | <.001 | −1.97 | −1.12 |
| LOI$_{360^{\circ}C}$ x WBTIT | −0.04 | .835 | −0.45 | 0.36 |
| DC$_{\text{w/o pretreatment}}$–Pcal x LOI$_{360^{\circ}C}$ | 2.56 | <.001 | 1.96 | 3.16 |
| DC$_{\text{w/o pretreatment}}$–Pcal x WBSPEC | 1.01 | <.001 | 0.59 | 1.44 |
| DC$_{\text{w/o pretreatment}}$–Pcal x WBTIT | 2.52 | <.001 | 1.98 | 3.05 |
| WBSPEC x LOI$_{360^{\circ}C}$ | 1.55 | <.001 | 1.12 | 1.97 |
| WBSPEC x DC$_{\text{w/o pretreatment}}$–Pcal | −1.01 | <.001 | −1.44 | −0.59 |
| WBSPEC x WBTIT | 1.50 | <.001 | 1.12 | 1.89 |
| WBTIT x LOI$_{360^{\circ}C}$ | 0.04 | .835 | −0.36 | 0.45 |
| WBTIT x DC$_{\text{w/o pretreatment}}$–Pcal | −2.52 | <.001 | −3.05 | −1.98 |
| WBTIT x WBSPEC | −1.50 | <.001 | −1.89 | −1.12 |

Note. WBTIT, SOC determined by the Walkley–Black titrimetric method; LOI OC$_{360^{\circ}C}$, SOC determined by the loss on ignition at 360 ˚C method; DC$_{\text{w/o pretreatment}}$–Pcal, SOC determined by dry combustion without pretreatment paired with determination of inorganic C by pressure calcimeter; WBSPEC, SOC determined by the Walkley–Black spectrophotometric method employing a microplate spectrophotometer.

## 4 | CONCLUSIONS

Four methods of SOC determination were compared for soils of south-central Idaho, including a more expansive test of the recently developed Walkley–Black dichromate oxidation method utilizing a microplate spectrophotometer (WBSPEC). The presence of considerable soil carbonate contents was verified, signifying total soil C cannot be presumed equivalent to SOC in soils of this region. Soil carbonate contents were fit as a function of soil pH, yielding a piecewise regression with a breakpoint of 7.4 determined by nonlinear least squares estimation. In soils of south-central Idaho, when pH is below 7.4, total soil C can be presumed equivalent to SOC; otherwise, removal of or compensation for SIC is required when determining SOC via dry combustion. Only the LOI$_{360^{\circ}C}$ and WBTIT methods were not significantly different; SOC was highest when determined by the DC$_{\text{w/o pretreatment}}$–Pcal method followed by the WBSPEC and LOI$_{360^{\circ}C}$ or WBTIT methods. Despite lacking a 1:1 relationship, SOC method agreement was high when all soils (n = 85) were considered. Reducing the analysis to low SOC soils (n = 56) decreased agreement; however, agreement between the WBSPEC method and the three currently used methods of SOC determination was frequently as high as other inter-method comparisons. This, coupled with tangential benefits of decreased hazardous waste production and reduced sample handling, positions the WBSPEC method as a sensible option for SOC determination in south-central Idaho.

## DATA AVAILABILITY STATEMENT

In support of open access to research data, the data for this publication were deposited in the Dryad digital data repository at the following link: https://doi.org/10.5061/dryad.95/69p8hr.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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