Thermal separation coupled with elemental and isotopic analysis: A method for soil carbon characterisation

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Abstract

A new analytical approach (Thermally-Based Separation, TBS) for the determination of inorganic carbon (IC) and organic carbon (OC) pools in alluvial soils has been tested. The method does not involve any chemical pre-treatment of samples and relies on different thermal stabilities of IC and OC pools, which can be accurately determined with the use of an EA-IRMS analytical system. Elemental (C wt%) and isotopic ($\delta^{13}C$ ‰) carbon data were used to check the reliability of the TBS method by calculating the mass balances using the measured IC and OC fractions and total carbon (TC).

TBS was applied to 7 soil samples from the Padanian alluvial plain (NE Italy) that were collected from different depths and characterised by textural/geochemical heterogeneity. The method allowed for a mean carbon elemental recovery of 99% (SD = 2%) and mean isotopic deviation ($\Delta^{13}C$) between theoretical ($\delta^{13}C_{OC}\, X_{OC} + \delta^{13}C_{IC}\, X_{IC})/(X_{OC} + X_{IC})$ and measured $\delta^{13}C_{TC}$ of 0.2‰ (SD = 0.3 ‰).

A comparison of TBS with other conventional methods for carbon speciation provides insights into the possible effects of sample acidification on the soil organic pools. The results suggest a higher robustness of TBS compared to conventional methods in the determination of organic and inorganic carbon pools in soils. This is because TBS bypasses any possible fractionation derived from the hydrolysis of soil organic matter and therefore accurately determines the carbon isotopic composition of the OC and IC fractions. TBS appears to be more robust than conventional methods, and is independent from the IC/OC ratio of the samples, from the unpredictable complexity of soil organic matter and from the arbitrary application of the acidification routine. On this basis, TBS represents the most promising approach for a correct and complete characterisation of soil carbon pools.

Keywords: soil carbon; thermal separation; organic vs. inorganic pools; stable isotopes

Introduction

Soils are vital for agriculture and ~~for~~ the functioning of the Earth's natural systems. They represent an essential planetary resource both in terms of food security and as a carbon stock (Sanderman et al., 2017). Unfortunately, conventional agriculture and on-going climatic changes are threatening this important resource, and their negative effects on soil properties deserve detailed investigation. In fact, ~~the~~ agricultur~~e~~al management strongly contributes to greenhouse gas~~es~~ (GHG) emission~~s~~ in the atmosphere (Lal, 2004; Johnson et al., 2007). Soil ecosystems partly offset GHG emission~~s~~ by sequestering C in ~~different~~ both organic and inorganic pools (Egan et al., 2018), some of which could keep C 'locked' for decades or even ~~for~~ centuries (Sollins et al., 2009). Moreover, the characterisation of carbon pools (organic and inorganic) is of fundamental importance ~~for~~ in the study of soil fertility, organic matter turnover and ~~its~~ ecological functionality. ~~For these reasons~~Therefore, ~~the~~ determination of the total C (TC) ~~and~~ in both organic and inorganic fractions is a ~~daily~~ continuous task~~, and~~ that ~~needs~~ requires ~~the set-up of~~ rapid and precise analytical protocols ~~which for~~provides thorough soil ~~investigation~~analysis.

~~The total C (~~TC~~)~~ determination by dry combustion is straightforward, ~~while~~whereas ~~the~~ separation and analysis of ~~the~~ inorganic and organic carbon (IC and OC) fractions ~~are~~ is more complex, time-consuming, costly, and usually require~~s~~ the use of dangerous chemicals. Various physico-chemical pre-treatments of the sample have been proposed for the analysis of distinct carbon fractions in different environmental matrices (Walkley and Black 1934; Froelich 1980; Cachiers et al. 1989; Caughey and Barcelona 1994; Serrano et al. 2008; Meyer et al. 2013). Unfortunately, all of these analytical protocols invariably require acidification of the sample to remove ~~the~~ IC prior to the determination of the OC fraction, either when measured directly by dry combustion (Verardo et al. 1990; Bisutti et al. 2004; Phillips et al. 2011) or ~~by~~ when using colorimetric techniques (Soon and Abboud 1991) after wet chemical oxidation (e.g., Walkley and Black 1934). ~~This~~The ~~approach~~use ~~of,~~ ~~employing~~ acidification pre-treatment, although very popular, is highly contentious. Several studies have investigated the effects of ~~distinct~~ acidification methods and acid types on different environmental matrices and found~~,~~ ~~indicating~~ that the procedures can ~~variously~~ lead to C losses, in turn resulting in nonlinear, sometimes significant, deviations in elemental and isotopic values (e.g., Serrano et al. 2008; Brodie et al. 2011 and the reference~~s~~ therein; Schlacher and Connolly 2014 and the reference~~s~~ therein).

Alternatively, ~~the measure the~~ OC fraction measurement can be based on thermal methods (thermogravimetry – TG; Differential Scanning Calorimetry – DSC; and Infrared Gas Analysis - IRGA) that exploit ~~its~~ distinctive oxidation temperature~~s~~ (~~e.g.~~ e.g., Boyle, 2004; Lopez-Capel et al., 2005a; Leifeld 2007; Hsieh and Bugna 2008; Fernández et al., 2012; Pallasser et al., 2013; Edmonson et al., 2015). Accordingly, the OC fraction is measured ~~in the~~within a temperature interval between 200 and 500 °C, whereas calcite, dolomite and other common soil carbonate minerals break down at higher temperature~~s~~, above 650 °C (Cuthbert and Rowland, 1947; Manning et al., 2005; Pallasser et al., 2013). The IC fraction of soil samples can also be determined ~~by~~ using dry combustion, following the preliminary thermal oxidation of OM method (Rabenhorst 1988, Dorodnikov et al., 2007). To satisfy the pressing demand for an analytical protocol that is precise, fast and cost effective, thermal separation prior to elemental and isotope determination has been investigated to quantify and

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78 characterise distinct soil carbon pools. Thermal separation and isotopic analysis have been coupled,
79 providing a powerful tool for the discrimination of the distinct carbon pools in heterogeneous
80 environmental materials, such as soils (Lopez-Capel et al. 2005b; 2006; Bisutti et al., 2007; Manning
81 et al. 2005, 2008; Natali and Bianchini, 2015). The carbon isotopic composition ($^{13}C/^{12}C$) represents
82 a very useful tracer for the discrimination of ~~the~~ organic and inorganic soil compounds, since the
83 former are distinctly ^{13}C- depleted with respect to the latter (Cerling, 1984; Bird and Pousai, 1997).

84 In this paper, we further explored the potential of a thermal separation method to selectively extract
85 the different soil organic and inorganic fractions on the basis of their distinctive thermal stability.
86 This therm~~ally~~ally -based separation (TBS) is very efficient, especially if an automated elemental
87 analyser (EA) is coupled with an IRMS device; in this case, the quantitative estimate of the various
88 carbon pools can be cross-checked and validated ~~by~~ with a C (%)-$\delta^{13}C$(‰) mass- -balance calculation
89 between the ~~total~~ TC values ~~(TC)~~ and those of the OC and IC fractions. In particular, the thermal
90 separation of carbon pools has been tested on alluvial soils from the Padanian plain, which are
91 characterised by ~~a~~ large elemental (TC) and isotopic ($\delta^{13}C_{TC}$) variability; ~~and~~ the results, compared
92 with those obtained ~~by~~ with the application of conventional sample acidification pre-treatment, have
93 been critically discussed. The aim of this study ~~is~~ was to convince the reader that TBS represents the
94 most promising approach for a correct and complete characterisation of soil carbon pools.

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Materials and methods

97 *Overview of ~~the~~ TBS methodology*

98 TBS is an analytical approach that involves the use of EA-IRMS and a muffle furnace ~~for~~ in the
99 thermal separation and analysis (elemental and isotopic) of the OC and IC fractions (Fig. 1). The
100 method exploits the separation of different carbon fractions on the basis of their different temperature
101 stabilities, which are compound specific. The OC fraction has a lower ~~destabilization~~ destabilisation
102 temperature ~~respect to~~ compared to IC and can be separated, and analysed ~~by~~ with EA-IRMS, setting
103 the combustion module (EA) at a temperature between 450 and 550°C. On the other hand, pre-heating
104 the samples ~~by~~ with a muffle furnace in the same temperature interval induces ~~the~~ complete oxidation
105 of the OC, leaving only the IC fraction, ~~that~~ which can be subsequently analysed by EA-IRMS setting
106 the combustion module at 950°C. The choice of ~~the temperature of~~ separation temperature depends
107 on the nature of the OC and IC fractions and can be critical in case of overlap of the respective thermal
108 stability fields. Therefore, the method ~~has to~~ must be calibrated, for any type of matrix, to
109 investigat~~eing~~ the 450-550°C interval to define the ~~more~~ most appropriate temperatures that allow~~ing~~
110 ~~the~~ correct separation and analysis of the OC and IC fractions. Thi~~se~~ methodology has been
111 successfully applied to a set of complex environmental matrices (see Natali and Bianchini, 2015),
112 and the specific application to soil samples has been tested in this work.

113 ~~The e~~Elemental and isotopic carbon composition of the different carbon pools ~~have been carried~~
114 ~~out,~~ were implemented at the Department of Physics and Earth Sciences of the University of Ferrara,
115 ~~by the use of~~ using an Elementar Vario Micro Cube EA in line with an ISOPRIME 100 IRMS
116 operating in continuous-flow mode. The system allows variation~~s~~ of the combustion module
117 temperature ~~of~~ up to 1050°C; this, theoretically, permits extraction of different components ~~having~~
118 with distinctive ~~destabilization~~ destabilisation temperatures ~~and~~ to analyse the respective elemental

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119 and isotopic composition. Powdered samples are introduced in tin capsules that are wrapped and
120 weighed; these capsules, ~~that~~ which allow loading ~~of~~ up to 40 mg of sample, are subsequently
121 introduced in~~to~~ the Vario Micro Cube autosampler to be analysed. Flash combustion takes place in a
122 sealed quartz tube filled with copper oxide grains (padded with corundum balls and quartz wool)
123 ~~which~~ that acts as catalyst, in excess of high purity (6 grade purity) O_2 gas. The formed CO_2 gas,
124 carried by dry He (5-grade purity), pass~~es~~ through a water-trap filled with Sicapent®-, ensuring the
125 complete removal of moisture~~;~~, ~~and~~ then, it is quantitatively determined on a thermo-conductivity
126 detector (TCD) and finally reaches the IRMS compartment for the determination of carbon isotopic
127 ratios. The detection of the distinct isotopic masses of the sample are compared to those of reference
128 CO_2 (5 grade purity) gas, which has been calibrated using a series of reference materials, in turn
129 calibrated against IAEA international standards, such as the limestone JLs-1 (Kusaka and Nakano,
130 2014), the peach leaves NIST SRM1547 (Dutta et al., 2006), the Carrara Marble (calibrated at the
131 Institute of Geoscience and Georesources of the National Council of Researches of Pisa), and the
132 synthetic ~~sulfanilamide~~sulphanilamide provided by Isoprime Ltd. Mass peaks were recalculated as
133 isotopic ratios by the Ion Vantage software package. The elemental precision, estimated by repeated
134 standard analyses, and accuracy, estimated by the comparison between reference and measured
135 values, were ~~in~~ on the order of 5% of the absolute measured value. The ~~u~~Uncertaint~~yies~~, increased
136 for contents approaching the detection limit (0.001 wt %). The ~~c~~Carbon isotope ratios ~~are~~ were
137 expressed in the standard (δ) notation in per mil (‰), relative to the international Vienna Pee Dee
138 Belemnite (V-PDB) isotope standard (Gonfiantini et al., 1995). The $\delta^{13}C$ values were ~~characterized~~
139 characterised ~~by~~ with an average standard deviation of ±0.1‰, defined by repeated analyses of the
140 above--mentioned standards.

141 *Investigated samples*

142 The soils of the easternmost Padanian Plain (N Italy) developed from alluvial (and deltaic) deposits
143 (Bianchini et al., 2012; 2013; Natali and Bianchini, 2017). They are characterised by ~~a~~ limited profile
144 development, in which the lack of soil maturity is related to young depositional age (Holocene),
145 fluvial reworking and extensive agricultural activities (ploughing). The samples considered in this
146 study were selected from a sample set previously studied by Di Giuseppe et al. (2014) that provided
147 a complete geochemical characterisation. They include 4 samples (AR6A, AR16A, AR19A, AR34A)
148 collected at ~~the~~ a depth of 20-30 cm, corresponding to the plough horizon and 3 samples (AR32B,
149 AR34B, AR41B) collected at ~~the~~ a depth of 90-110 cm, corresponding to layers unaffected by
150 agricultural activities. On the basis of the available data, the selected samples are representative of
151 the whole population and show wide textural (sand 5-32, silt 18-52, clay 25-76 %)- and geochemical
152 (SiO_2 46.9-52.5, Al_2O_3 12.9-21.1, CaO 1.0-11.7 wt%) variability (Table 1).

153 *TC, OC and IC measurement by TBS*

154 The elemental and isotopic composition~~s~~ of the Total Carbon (TC), Organic Carbon (OC), and
155 Inorganic Carbon (IC), have been carried out by refining the method proposed by Natali and
156 Bianchini (2015). According to this analytical protocol:

157 - TC is measured by EA-IRMS, with a sample combustion at 950°C;

158 - OC is measured by EA-IRMS, with a sample combustion set at a lower temperature (tests at 450,
159 500 and 550°C);

160 - IC is measured by EA-IRMS, with combustion at 950°C ~~on~~ in samples where organic matter has
161 been previously removed by combustion in a muffle furnace (tests at 450, 500 and 550°C for 1~~2~~2h
162 h); the relative gravimetric loss (LOI) ~~is~~ was also determined ~~in order to~~to correct the elemental
163 concentration of the IC fraction.

164 ~~As concerns the~~When measur~~ing~~e ~~of the~~ OC, the sample is exposed to ~~the~~ a defined temperature until
165 the full oxidation of SOM is achieved. In practice, the sample is introduced once in the combustion
166 tube and multiple analytical EA-IRMS runs are executed until CO_2 ~~totally~~ vanishes entirely, ~~i.e.~~i.e.,
167 the detected C signal on the EA drops ~~toward~~ to typical blank values. The C contribution of the first
168 analytical run to the whole OC varies ~~being~~ from 74-85% at 450°C,- 83-90% at 500°C and 86-92%
169 at 550°C, in turn indicating that the C (wt%) extracted ~~by~~ during successive analytical runs decreases
170 at increasing temperature~~s~~. The combustion kinetics ~~is~~ are therefore dependent ~~by~~ on the defined
171 temperature, and the number of analytical runs necessary to measure the whole OC of the studied soil
172 samples varies from 3 (generally at higher temperature~~s~~) to 5 (generally at lower temperature~~s~~). In
173 other words, the ~~philosophy of the~~analytical approach ~~to the~~of OC quantification ~~is~~ aims to perform
174 multiple analytical runs on the same sample at the target temperature and to sum all of the
175 contributions of stripped CO_2 ~~having~~ that have a similar "organic" (~~i.e.~~ i.e., $\delta^{13}C_{OC}$ generally lower
176 than -15‰; Bird and Pousai, 1997) isotopic signature.

177 ~~As concerns the~~For the IC fraction, the C elemental analysis of the samples ~~has to~~must be corrected
178 for ~~the~~ weight loss, which occur~~r~~~~s~~ed during the thermal pre-treatment; this correction obviously has
179 a great effect, especially ~~in~~ on the IC determination of organic-rich soil matrices (weight loss close to
180 100 %), but is also fundamental in clay-rich soils, which can be affected by appreciable weight loss
181 due to mineral dewatering (e.g., Boyle, 2004).

182 The resulting wt% and $\delta^{13}C$ (‰) of the OC and IC fractions ~~allow~~ support a mass balance ~~to~~
183 calculat~~ion of~~e a theoretical TC fingerprint, which is compared with ~~that~~ the directly measured
184 values($\delta^{13}C_{TC\ Measured}$):~~.~~

185 $$\delta^{13}C_{TC\ Theoretical} = (\delta^{13}C_{OC}*X_{OC} + \delta^{13}C_{IC}*X_{IC})/(X_{OC}+X_{IC})$$

186 where X_{OC} and X_{IC} represent the organic and inorganic fractions, respectively.

187 The difference between the theoretical and measured bulk isotopic ratios, expressed as $\Delta^{13}C$,
188 complement~~s~~ the elemental carbon recovery and ~~is~~ are used to cross-check the reliability of the
189 method, as follows:

190 $$\Delta^{13}C = \delta^{13}C_{TC\ Measured} - \delta^{13}C_{TC\ Theoretical}$$

191 *OC and IC determination by acid pre-treatment*

192 The elemental and isotopic composition of the OC fraction ~~has been~~was measured by EA-IRMS
193 analysis (at a combustion temperature of 950°C); this was also conducted ~~on~~ on samples where
194 carbonates ~~have been~~were previously removed by pre-treatment~~s~~ with hydrochloric acid (HCl for
195 Trace Analysis 37%). Two sample pre-treatment procedures ~~have been~~were tested: acid fumigation
196 (~~e.g.~~ e.g., Harris et al., 2001) and acid wash-rinse (~~e.g.~~ e.g., Midwood and Boutton, 1998). Acid
197 fumigation was ~~has been performed~~for 24 h on ca. 20 mg powdered sample contained in a silver

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capsule, whereas ~~an~~ an acid wash-rinse ~~has been~~was performed ~~by~~ adding HCl drops on~~to~~ ca. 100 mg
powdered sample in glass beakers until the end of effervescence, followed by repeated washing (5
times) with ~~deionized~~ deionised water (Millipore water 18.2 MΩ). In both acid treatments, the
samples were previously wetted ~~by~~ with a solution of HCl 1% (Walthert et al., 2010) to avoid violent
effervescence in the acid wash-rinse and to promote acid permeation in the acid fumigation procedure
(Yamamuro and Kayanne 1995; Harris et al. 2001). The IC elemental and isotopic composition ~~has~~
~~been~~were determined ~~by~~ using the difference and mass balance calculation, respectively, between TC
and OC, measured on the acid pre-treated samples.

Results

TC determination

The total carbon concentration (TC wt%) and the relative isotopic composition ($\delta^{13}C_{TC}$ ‰) are
reported in Table 1. The TC varie~~d~~s between 1.60 and 3.40 wt%, whereas $\delta^{13}C_{TC}$ range~~d~~s between -
7.4 and -25.2 ‰. Notably, the ~~more~~ most negative isotopic value ~~is~~ was recorded in the finer sample
(AR41B), characterised by the lowest CaO/Al_2O_3, whereas the samples characterised by a less
negative $\delta^{13}C_{TC}$ value (~~e.g.~~ e.g., AR34B) ~~display~~ exhibited the highest CaO/Al_2O_3.

OC determination

The results of the OC measure~~ment~~ are ~~summarized~~ summarised in Table 2 and fully reported in
Supplementary Table 1. The lowest and highest OC content~~s were~~is measured in sample~~s~~ AR34B
and AR6A, respectively, varying between 0.66 and 2.11 using the acid rinse method, and between
0.83 and 2.31 using the acid fumigation method. The results are compared in Fig. 2, which indicates
that the acid rinse method leaves a systematically lower amount of C with respect to the acid
fumigation method in the residua of investigated soil samples. This demonstrates that the acid rinse
method is excessively aggressive and removes ~~part~~ a portion (8-20%) of the organic carbon. The
isotopic ratio associated with acid rinse residua (from -23.0‰ in AR34B to -26.3‰ in AR34A) is
slightly more negative (up to 0.25‰) than that recorded in the respective fumigated samples (from -
22.7‰ to -26.3‰), suggesting that the acid labile SOM compounds are ^{13}C-enriched.

~~The~~ A measure of the OC fraction was carried out by TBS at 450, 500 and 550°C, with the aim of
investigating the thermal behaviour of the organic carbon contained in the studied soils, ~~and to~~ and
~~compare~~ comparing the TBS results with those obtained by acid pretreated samples (Table 1). The
amount of OC extracted by TBS increase~~d~~s with combustion temperature, varying from 0.81-0.84 in
sample AR34B to 1.86-2.23 in sample AR6A. In particular, the OC measured by TBS at ~~the~~ a
temperature of 500°C yielde~~d~~s the best fit relative~~ly~~ to that obtained on acid fumigated soil samples
with average recovery [(OC_{TBS500}/OC_{fum})*100] of ~ 99% (SD=5 %).

The OC isotopic composition obtained by TBS ~~shows~~ exhibited ~~a~~ general ^{13}C-enrichment with
increasing combustion temperature (ca. 1 ‰ on average), ~~showing~~ exhibiting the least negative values
in sample AR32B (from -21.5‰ at 450°C to -20.2‰ at 550°C) and the most negative values in sample
AR34A (from -25.9‰ at 450°C to -25.2‰ at 550°C), suggesting that the thermally labile organic
compounds ~~are~~ were ^{13}C-depleted with respect to ~~those~~ the thermally recalcitrant samples. The carbon
isotopic ratios obtained by TBS at 500°C ~~show~~ indicate that the δ^{13}C values were in reasonable

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237 agreement with those obtained by EA-IRMS ~~on~~ in the fumigated samples, with an average
238 discrepancy ($\delta^{13}C_{TBS500}$ - $\delta^{13}C_{OCfum}$) of 1.1‰ (SD=1.1‰).

239 *IC determination*

240 The results of the IC calculation obtained by mass balance using the measured TC and OC fractions
241 in the acid pre-treated samples (Table 3) ~~are~~ were generally inconsistent, as they were variably
242 affected by the carbon losses described above. The IC varie~~s~~d between 0.1 (AR16A) and 2.45
243 (AR34B) wt% ~~by~~ in the acid rinse method, and between 0.07 (AR41B) and 2.28 (AR34B) ~~by~~ in the
244 acid fumigation method.

245 As concerns the IC measured by TBS at 450, 500 and 550°C, the lowest values ~~are~~ were recorded in
246 ~~sample~~ AR16A sample (0.14-0.11 wt%), and the highest values were found in ~~samples~~ AR34B (2.16-
247 2.23 wt%)~~.~~ and AR16A (2.35-2.18 wt_%)~~.~~ samples. In general, the results (Fig. 3) ~~are characterised~~
248 ~~by~~indicate a slight decrease in C (wt%) with increasing pre-heating temperature, which is systematic
249 in *A* soils. The isotopic values recorded in the samples pre-heated at 450°C ~~are~~ were the most negative,
250 ~~showing~~ indicating signatures reflecting a variable contribution by persistent organic compounds
251 (values down to -22.2‰ in AR41B). A consistent ^{13}C-enrichment in the IC isotopic composition ~~is~~
252 was recorded in samples pre-heated at higher temperature~~s~~, with a $\delta^{13}C_{IC}$ that tend~~ed~~s to become
253 uniform at temperatures above 500°C. In any case, we suggest a pre-heating -temperature of 550°C
254 for the most reliable elemental and isotopic IC determination~~, because~~ because it represents a
255 temperature above the ~~destabilization~~ destabilisation of thermally recalcitrant organic compounds
256 (with the exception of black carbon forms such as soot and graphite; Leifield, 2007)~~;~~, ~~but~~ below this
257 temperature, there was ~~destabilization~~ destabilisation of common soil carbonates, ~~that~~ which ~~are~~ were
258 dominated by $CaCO_3$. This temperature has to be refined in the presence of a significant amount of
259 secondary oxalate/carbonate phases, which have ~~a~~ more complex thermal behaviou~~r~~, with phase
260 transition ~~around 5~~approximately 500-550°C (Alkac and Atalay, 2008; Plante et al., 2009; Manning
261 et al., 2005). From this point of view, critical samples that deserve further investigation~~s~~ are those
262 ~~characterized~~ characterised by decidedly negative $\delta^{13}C_{IC}$ values (~~e.g.~~ e.g., samples AR41B and
263 AR16A), which are significantly different from those typical of primary "sedimentary" carbonates
264 (δ^{13}C approaching 0‰; Cerling, 1984).

265 *Reliability of the TBS methodology applied to soil samples*

266 The reliability of the TBS application to soil samples ~~is~~ was demonstrated on the basis of elemental
267 and isotopic recovery obtained by mass balance calculation involving TC, OC and IC data (Table 4).
268 Worth noting, unlike other studies, in the current research, we directly measure~~d~~ the TC, OC and IC
269 ~~by~~ with the same analytical technique~~s~~ (EA-IRMS). The best fit between the elemental and isotopic
270 composition of TC and that derived by the combination of OC and IC fraction~~s~~ measured at different
271 temperatures ~~is~~ was obtained for OC at 500°C and for IC at 550°C. The results of this reliability check
272 are shown in Fig. 4, where the TC, OC and IC measure~~ments~~d ~~at~~ in the ~~above cited~~aforementioned
273 conditions, together with the results of mass balance calculation~~s,~~ are reported. The carbon elemental
274 recovery varie~~d~~y from 98% (AR34B, AR41B) to 102% (AR16A), with a mean of 99% (SD=2%),
275 whereas the isotopic mass balance ~~shows~~exhibited a deviation (Δ^{13}C) from the measured $\delta^{13}C_{TC}$,
276 varying from -0.8‰ (AR41B) to 0.1‰ (AR32B, AR6A), with an absolute mean of 0.2‰ (SD = 0.3
277 ‰).

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Discussion

Critical Evaluation of the carbon speciation methodologies in soil samples

The TBS method represents a tool for ~~the~~ rapid and precise determination of the distinct carbon pools in soil samples. The proposed method allows the EA-IRMS user to measure the TC, IC and OC fractions of soils and to ~~check~~ determine the reliability of ~~the determination~~evaluation ~~through~~ based on the $\Delta^{13}C$ parameter. Another notable advantage of the TBS method is the absence of sample handling and processing by acid pre-treatments for removing carbonates, ~~that~~ which requires sample-tailored procedures for ~~a~~ correct application. Acid pre-treatments could result in overestimation/underestimation of OC due to incomplete removal of carbonates/loss of OM, and impl~~ies~~y significant ~~losses of time~~time consumption, even for processing small sample sets. Further inconsistencies arise in methods where IC is calculated ~~by~~based on the differences between measured TC and OC~~, because~~ because no cross-check on the above cited OC under/over estimations can be carried out. The OC determination by TBS performed at 500°C (at which the method gives the best C recovery) –generally shows $\delta^{13}C_{OC}$ values that are less negative than those obtained on their respective acid pre-treated soils, and this difference is more pronounced on *B* samples. As suggested by some authors, the application of acid pre-treatment may induce shifts in the isotopic value up to – 2‰~~,~~ (Kennedy et al., 2005; Komada et al., 2008), which is probably due to the loss of acid-labile OM moieties (~~e.g.~~ e.g., carbohydrates and amino acids), which are relatively enriched in ^{13}C compared with lipids and non-hydrolysable residue. Artefacts are mainly induced by rinses that cause a loss of acid-labile SOM compounds (~~e.g.~~ e.g., Wang et al. 1998; Hwang and Druffel, 2003, Fernandes and Krull, 2008), and they are minimised by acid fumigation methods. However, as already noted by Komada et al. (2008) and Brodie et al. (2011), acid fumigation treatment can also induce small and selective losses of acid-labile OC, ultimately leading to significant shifts in isotopic values. Unfortunately, the magnitude of the carbon isotopic shift produced by acid pre-treatments is strictly dependent ~~by~~ on the SOM composition, and is therefore unpredictable without preliminary ~~investigations~~analysis. TBS results have been preferentially compared with those carried out by EA-IRMS after acid fumigation since, as discussed above, this pre-treatment induces smaller artefacts.

The TBS methodology used in the OC determination, over the 450 to 550°C interval, provides further elucidation ~~on~~of the SOM content (and quality) based on the selective extraction of thermally labile (extracted at 450°C) and labile+recalcitrant (extracted at 500-550°C) organic carbon pools. The ~~r~~Results indicate that, in general, thermally labile organic pools are associated with lighter isotopic composition and that ~~a~~progressive ^{13}C-enrichment is probably associated with ~~the~~an increasing contribution by recalcitrant compounds. This is in agreement with recent ~~investigations~~research that ~~revealed~~found that thermally labile aliphatic compounds (~~destabilized~~destabilised ~~around 3~~approximately 300-350°C) are ca. 3‰ ^{13}C-depleted with respect to the more refractory aromatic compounds that decompose at higher temperature~~s~~ (400-450°C, Manning et al., 2005; Lopez-Capel et al., 2006; 2008; De La Rosa et al., 2008; Araya et al., 2017).

The ^{13}C-enrichment that characterises the OC fraction of *B* (90-110 cm depth), with respect to *A* (20-30 cm depth) samples, is a commonly~~-~~ observed phenomenon along soil profiles (~~e.g.~~ e.g., Natelhoffer and Fry, 1988). It can be ~~due~~a result of ~~to~~several processes, such as the preferential

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319 ~~stabilization~~ stabilisation of ^{13}C-enrich~~mented~~ (polysaccharides and amino acids) and the preferential
320 decomposition of ^{13}C-depleted (lipids and lignin) compounds, or it could result from ~~to~~ SOM
321 decomposition by microbial activity (Rumpel and Kögel-Knaber, 2011 and the references therein).
322 Similar effects have been observed by (Lopez-Capel et al., 2006), ~~which~~ who reported a progressive
323 homogenisation of isotopic composition of coexisting SOM compounds toward ^{13}C-enriched values,
324 as a result of fungal degradation. From the TBS results, we cannot strictly ascribe this effect to one
325 of the a~~fore~~~~bove~~-mentioned process~~es~~, but we observed that two of the three *B* investigated soils ~~give~~
326 provided the highest isotopic differences in~~respect to~~ the acid fumigation method ($\delta^{13}C_{TBS500}$ -
327 $\delta^{13}C_{OCfum}$ up to 3.5‰), confirming that acid hydrolysis mainly affects ^{13}C-enriched SOM compounds.
328 Several authors have suggested that ~~the~~ soil texture is another important factor controlling the
329 increase~~s~~ in $\delta^{13}C_{OC}$ values along the soil~~s~~ profile. They inferred that ~~the~~ ^{13}C-enrichment is a direct
330 effect of SOM decomposition, which is limited by the presence of clays that increase the spatial
331 inaccessibility of SOM within soil aggregates and the interaction with mineral surfaces (Von Lützow
332 et al., 2006; Gunina and Kuzyakov, 2014, Guillaume et al., 2015). Coherently, we observe an inverse
333 correlation between the $\delta^{13}C_{OC}$ values obtained by TBS and the clay content in the investigated *B*
334 soils. It is n~~N~~oteworthy that~~,~~ the discrepancies between the $\delta^{13}C_{OC}$ values obtained by TBS and those
335 ~~by~~ from EA-IRMS ~~on~~ for the respective fumigated soils decreased with ~~the~~ clay content. This
336 suggests that acid fumigation pre-treatment particularly affects the SOM composition of soils with a
337 coarser grain size.

338 Overall, the results suggest that TBS is a reliable methodology for the isotopic characterisation of
339 OC, since it avoids unpredictable fractionations of SOM induced by acid pre-treatment.

340 The application of the TBS methodology to the measure~~ment~~ of the IC fraction reveals that pre-
341 heating at 450°C for 12 h ~~doesn't~~ does not ~~allow the~~ complete~~ly~~ ~~removal~~ remove ~~of~~ SOM from the
342 investigated soil samples, whereas at 550°C, TBS ~~results show~~indicated a general agreement with the
343 calculated C-δ^{13}C values after acid fumigation. The~~se~~ variable discrepancies ~~observed~~ in the IC
344 isotopic values of some samples are plausibly related to the incorrect isotopic determination of the
345 OC fraction on acid fumigated samples. Other inconsistencies could be related to the significant
346 presence of black carbon in the soil samples, ~~for the determination of~~for which, further TBS
347 refinement is required (~~e.g.~~ e.g., Emdondson et al., 2015). In any case, the very good Δ^{13}C values
348 obtained in the reliability test of ~~the~~ TBS (Table 4) allow us to declare that the methodology is able
349 to measure all carbon contained in the investigated soils without any significant loss, also identifying
350 the presence of additional (pedogenic and/or black carbon) contributions to sedimentary carbonates
351 in the IC fraction by the $\delta^{13}C_{IC}$ value.

352

Conclusions

354 The TBS methodology, based on EA-IRMS data, represents a suitable tool for ~~the~~ carbon speciation
355 in soils and sediments. The OC and IC fractions appear to be correctly measured, as demonstrated by
356 ~~the~~ a comparison of the results with those obtained by conventional techniques involving acid pre-
357 treatments, as well as ~~by~~ those using mass balance calculation (~~including~~ with both elemental and
358 isotopic data). As concerns the determination of the OC fraction, the SOM investigation through the
359 450-550°C temperature interval allows the possible identification of the distinct organic pools, from

thermally labile to more recalcitrant, in relation to their diverse isotopic composition. Future investigations research based on TBS results will could potentially relate the distinct isotopic compositions to SOM characterised by diverse stages of maturity.

As concern tThe IC fraction in, TBS appears to be more reliable than other conventional techniques, considering that the isotopic variation obtained on from the tested soils is less scattered with respect to that obtained by conventional methods. The robustness of the TBS IC determination is due to its direct measurement, avoiding any artefact derived by from the incorrect determination of the OC.

In order toTo carry out a correctly measurement of the OC and IC fractions by with the TBS methodology in soils and sediments, we recommend combustion at 500°C for the former, and with thermal pre-treatment at 550°C, followed by combustion at 950°C for the latter. This represents the best compromise in order tothat excludes the contribution of pedogenic minerals (oxalates/secondary metastable carbonates) to the OC, which are included in the IC fraction, together with the most thermally ally recalcitrant black carbon components. The latter are represented by subordinate mineralized mineralised carbon forms such as graphite, and by anthropogenic soot, that which is abundant only in the contaminated soils of urban environments.

The developed TBS method developed is of particular interest becauseconsidering that it bridges the EA-IRMS analytical approach with other compound specific thermally ally based methods that are exploringexplore the composition of SOM and its evolution by with physico-chemical and biological processes

Table Captions:

Table 1 – Geochemical (Di Giuseppe et al., 2014), textural and total carbon elemental (TC) and isotopic ($\delta^{13}C_{TC}$)- composition of the investigated soils.

Table 2 – Carbon elemental (OC) and isotopic ($\delta^{13}C_{OC}$) composition of the organic fraction of investigated soil samples. The OC fraction is was measured after using "conventional" (acid pre-treatment) and by TBS (tested at 450, 500 and 550°C) methodologiesmethods. The reported values are the averages of two coherent replicate analyses (see the extended dataset in Supplementary Table 2).

Table 3 – Carbon elemental (IC) and isotopic ($\delta^{13}C_{IC}$) composition of the inorganic fraction of the investigated soils. The IC fraction is calculated for "conventional" (acid pre-treatment) and measured

10

397 by TBS (tested at 450, 500 and 550°C) ~~methodologies~~methods. Replicates were available only for a
398 few samples~~,~~. ~~show~~ indicating a precision comparable to that obtained ~~on~~ in standards.

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399 **Table 4 –** Carbon elemental (TC $_{Theoretical}$) and isotopic ($\delta^{13}C_{TC\ Theoretical}$) composition calculated by
400 mass balance using the measured OC (at 500°C) and IC (at 550°C) fractions obtained by TBS ~~on~~ for
401 the investigated soils. The reliability of the methodology is expressed by $C_{recovery}$ (%) and $\Delta^{13}C$ (‰)
402 parameters~~,~~, resulting from ~~the~~ a comparison between theoretical and measured TC values. See the
403 text for further details.

404

405 **Supplementary Table 1 –** EA-IRMS results ~~for~~ from the analysis of the total carbon (TC) and
406 organic carbon (OC) ~~carbon~~ fractions of the investigated soils. The OC fraction ~~is~~ was measured ~~after~~
407 with "conventional" acid pre-treatment and ~~by~~ using TBS.

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