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## Research Article

# Chemical Underpinning of the Tea Bag Index: An Examination of the Decomposition of Tea Leaves

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Decomposition is a key flux of terrestrial carbon to the atmosphere. Therefore, gaining a better understanding of how plant litter decomposes in soil, and what governs this process, is vital for global climate models. The Tea Bag Index (TBI) was introduced by Keuskamp et al. (2013) as a novel method for measuring litter decomposition rate and stabilisation. The TBI uses two types of tea bags representing fast (green tea) and slow (rooibos tea) decomposition substrates as standardised litter bags. To date, the TBI method has been used in over 2000 locations across the globe. However, before now, there has been no information on how the composition of the tea leaves change during incubation. These data are crucial in determining the validity of the use of the TBI method globally, to ensure the tea leaves decompose in a way that is representative of so-called “native” litters. To provide chemical underpinning of the TBI method, a laboratory incubation of the tea bags was conducted with destructive sampling at 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, and 91 d. The incubated tea was analysed for total C and N. In addition, C was characterised as alkyl, O-alkyl, aromatic, or carbonyl C using solid-state <sup>13</sup>C nuclear magnetic resonance spectroscopy with cross-polarization and magic angle spinning (CPMAS NMR). The results suggest that changes in carbon in both tea types are comparable to other litter studies, with a net decrease in total C and relative proportion of O-alkyl C fraction, which contains carbohydrates and cellulose. We conclude that the decomposition of tea leaves in the bags used in the TBI is representative of other litters.

## 1. Introduction

Soil organic carbon, or SOC, is the largest terrestrial C sink, containing approximately 2344 Gt C [1]. The soil-atmosphere flux in C is primarily driven by the microbial mineralization of soil organic matter (SOM) consisting of a continuum of progressively decomposing residues of mainly plant origin [2]. Therefore, gaining a better understanding of how plant litter decomposes, and what governs this process, is vital for global climate models that seek to integrate C cycle responses and feedbacks under environmental change scenarios.

One of the most routine methods to quantify decomposition of SOM by the soil decomposer community is with field- or lab-based decomposition studies which often use litter bags [3]. However, preparing uniform litter bags that allow comparison between studies is difficult. Keuskamp

et al. [4] have suggested the Tea Bag Index (TBI) as a standardised alternative for estimating decomposition rates of SOM and have released a call for contributions to a global map of decomposition in their Teatime4science campaign (<http://www.teatime4science.org/>). In brief, the method uses commercially available tea bags and exploits the differential in decomposition between green tea from the dried leaves of *Camellia sinensis* (fast decomposing) and rooibos tea from the dried leaves of *Aspalathus linearis* (slow decomposing) over 90 days. Mass loss during incubation is used to generate a TBI describing a litter decomposition rate constant ( $k$ ) and a factor quantifying the extent of litter stabilisation ( $S$ ).  $k$  is estimated assuming exponential decay from the mass of rooibos tea remaining at  $t=90$  days.  $S$  is defined as the deviation of the actual decomposed fraction (in the green tea) compared to the fraction that should be decomposed

based on its chemical lability, and is calculated from the ratio between the fraction of green tea that is actually decomposed after 90 days and the fraction of green tea that is hydrolysable ( $H_g$ ; operationally defined as the combined total of nonpolar extractive C, water soluble C, and acid soluble C). The magnitude of  $S$  can therefore be interpreted as a quantification of the inhibiting effect of environmental conditions on the decomposition of the labile fraction [4], which might include the contribution of secondary synthesis reactions in stabilising the labile fraction (and its partial decomposition products) against eventual mineralization.

To date, the TBI has been used in upwards of 2000 locations across the globe (<http://www.teatime4science.org>). In addition, at the time of writing, there have been 71 publications citing Keuskamp et al. [4], according to Web of Science. However, it is uncertain whether tea leaves, as a litter, are representative of the decomposition of “native” litter inputs to soil. Firstly, the parameters obtained from the TBI are derived from the decomposition of a material that has undergone a degree of processing during manufacture and therefore may not behave in a similar manner to unprocessed leaf litters. Rooibos tea leaves, for example, could be subjected to oxidation processes during manufacture, which aids in the breakdown of cell walls in the leaf, compared to unprotected litters. This may influence the abundance of structures associated with the cuticle of the leaf. Secondly, in order to calculate the stabilisation factor ( $S$ ) of the tea, it is also assumed that the chemically hydrolysable fraction of the tea,  $H$ , is the only fraction of the tea that is decomposable.  $H$  has been previously determined by Keuskamp et al. [4] through sequential extraction and comprises nonpolar extractives (e.g., fats and waxes) and water soluble (e.g., simple sugars) and acid soluble (e.g., cellulose) components. The chemically nonhydrolysable component is defined as the material that is acid insoluble, such as lignin, and is assumed to be resistant to decomposition. However, there is considerable evidence suggesting that the persistence of polymeric constituents of plant residues in soil cannot be predicted based solely on the chemical structure [5, 6]. In particular, studies have found that residues with high lignin and long-chain aliphatic content, and thus traditionally thought to have chemical recalcitrance, are actually broken down rather quickly, with selective preservation only important during the primary stages of decomposition [3, 7, 8]. Furthermore, available evidence from mineral soils does not support the formation of persistent materials (namely, “humic” molecules with large molecular size) through secondary synthesis [9] that might be one explanation for the stabilised material accounted for in the calculated  $S$  factor in the TBI. In addition, compounds traditionally considered to be labile (i.e., those that are hydrolysable) have been shown to persist in soil, albeit through stabilising interactions with soil mineralogy and soil structure [5].

To date, published chemical analysis of both tea types has only been conducted before incubation [4] and therefore the assumptions made regarding (i) whether decomposition behaviour of the processed teas resembles

that of unprocessed “natural” litter and (ii) the nature of the C fractions that are being exploited by microorganisms during incubation have not been well supported by evidence.

In light of the increasing popularity of the TBI globally, this work aims to determine and quantify the C fractions in the teas used in the TBI across their 90-day degradation and compare this to similar studies using different litters. This will (i) provide a chemical understanding of degradation of green and rooibos tea and (ii) test the assumptions made in the calculation of decomposition rate and stabilisation factor in the TBI. This will thus enable a chemical underpinning of the TBI as a method for gaining uniform decomposition data across ecosystems. Whilst the original characterisation of the tea by Keuskamp et al. [4] involved sequential C extraction of organic compounds with Soxhlet equipment, this investigation uses solid-state  $^{13}\text{C}$  nuclear magnetic resonance spectroscopy with cross-polarization and magic angle spinning (CPMAS NMR) to provide a less destructive and more representative analysis of OM characteristics.

## 2. Methods

**2.1. Laboratory Incubations.** To create a time sequence of degradation, laboratory incubations of oven-dried ( $70^\circ\text{C}$ ) preweighed tea bags were conducted in pots. A total of 78 5" diameter plant pots were filled with 500 g field fresh 2 mm sieved soil. Soil was obtained from an unplanted sandy loam (pH 6.5, OM 7.29%) from Wisley, UK ( $51.323428^\circ\text{N}$ ,  $-0.474392^\circ\text{W}$ ). Each pot contained three green tea and three rooibos tea bags, each from three different production batches. Pots were incubated, in the dark at  $20^\circ\text{C}$ , on top of saturated sand.

Destructive harvests of triplicate pots were taken at 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, and 91 d (234 tea bags in total). Tea bags were oven-dried ( $70^\circ\text{C}$ ), adhered soil particles were gently removed by hand, and the bag was split open before weighing.

In 2017, the fabric of the tea bags was changed from the nylon woven bag described by Keuskamp et al. [4] to nonwoven polyethylene terephthalate (PET), while the tea leaves remained the same. In response to this, the map currently being created in the Teatime4science campaign is based solely on the original nylon mesh bags. Therefore, this paper has also focused on the original nylon mesh bags.

**2.2. Chemical Analysis.** To ensure there was sufficient material for analyses, replicate tea bags from the same production batch were bulked together and homogenised into one sample, resulting in one sample of green tea and one sample of rooibos tea for each of the three batch numbers, each week, for chemical analysis. Due to logistical constraints, chemical analysis was conducted on two out of the three batches, and the means of these two are presented here.

Samples were ground to 0.2 mm and analysed for total C and N on a Thermo Scientific Flash 2000 CHNS/O Analyser, and  $^{13}\text{C}$  spectra were also obtained using CPMAS NMR spectroscopy.

**2.2.1. Nuclear Magnetic Resonance Spectroscopy.**  $^{13}\text{C}$ PMAS NMR was conducted using a spin rate of 10,000 Hz. Analysis used a 500 MHz Bruker Ultrashield with a pulse power of 67.6 kHz. The spectrometer was retuned between each sample, and each sample was run for 1024 scans with a 500  $\mu\text{s}$  contact time and a recycle delay of 2 seconds. CPMAS NMR spectra were integrated according to the following chemical shift regions: (a) alkyl C, (b) O-alkyl C, (c) aromatic C, and (d) carbonyl C. More details of these regions can be found in Table 1.

These regions for integration were selected as they are commonly used in the literature [10–13]. Integrals reported here are the areas under the defined spectral region, expressed as a proportion of the area under the whole spectra. Integration was conducted using Bruker Topspin version 3.2.

**2.3. Decomposition Indexes.** In addition to the change in relative proportions of each of the chemical shift regions listed above, two indexes to quantify the degree of litter decomposition were calculated. These indicators were (i) the alkyl C/O-alkyl C, or AC/OAC, ratio (0–50/50–110 ppm) and (ii) the carbohydrate C/methoxyl C, or CC/MC, ratio (70–75 ppm/52–57 ppm). These ratios have been used in the literature [14] as robust indicators of the level of decomposition of leaf litters, by comparing the relative abundance of components traditionally thought as labile (O-alkyl C and carbohydrate C) against more recalcitrant components (alkyl C and methoxyl C of lignin).

**2.4. Statistical Analysis.** One-way ANOVA and Tukey's post hoc testing were conducted in Minitab 17, using a number of days of incubation as the main factor, to determine significant differences ( $p < 0.05$ ).

### 3. Results

**3.1. Mass Loss and Decomposition Rate Constant.** Figure 1 shows the decline in the relative mass in the green and rooibos tea over the course of the incubation (in relation to the initial mass at 0 d). The mass of the green tea decline was biphasic with a rapid decline to 14 d followed by a slower rate to 91 d, while the rooibos tea declines at a slower rate over the whole incubation period. Values for  $S$  and  $k$  (based on median mass losses of replicates) after 91 d were 0.032 and 0.038, respectively.

**3.2. C and N Contents.** Figure 2 presents the C and N contents in the green and rooibos tea bags (as a total mass in the tea bag), over time. The mass loss of C (Figure 2(a)) is more prominent in the green tea than the rooibos tea. The total mass loss of C in green and rooibos tea is strongly correlated to the mass proportion of tea remaining in the bag ( $r^2 = 0.99$  for green tea and 0.97 for rooibos tea).

N content in the green tea (Figure 2(b)) paralleled the mass loss ( $r^2 = 0.95$ ) but not in the rooibos tea ( $r^2 = 0.01$ ), which shows a small increase in N content over time. The C:

N ratio (Figure 2(c)) in the rooibos tea decreased significantly with incubation time ( $p < 0.05$ ).

**3.3. Nuclear Magnetic Resonance Spectroscopy.** Of the four chemical shift regions designated in this study (described in Table 1), only the O-alkyl region saw a significant reduction, in both tea types, after the 91 d incubation (Table 2). Although the relative contribution of aromatic C or carbonyl C, in either tea type, is not statistically different between 0 d and after 91 d incubation, there are some noteworthy observations to be made in these two regions (Figure 3). Subtle peaks in the aromatic region in the rooibos tea at 115, 130, 145, and 153 ppm, which persist over the incubation period, are all associated with C atoms that occur in lignin monomers [15, 16]. Peaks at 145 and 155 ppm are also attributed to tannins [17], and the green tea has significant peaks in these regions initially that quickly diminish in the first 21 d. In the green tea, lignin peaks at ~130 and 153 ppm also persist over the incubation period. There was also a peak persisting in both teas in the carbonyl region at ~172 ppm. This peak can be assigned to carboxyl C of hemicellulose or amides [18].

For green tea, all of the peaks in the O-alkyl C region that show reductions over the incubation period have been associated with units of carbohydrates (72 ppm), polysaccharides (105 ppm), and anomeric C (96 ppm) of carbohydrates [14, 16, 19]. The peak most often associated with carbohydrates in the literature (72 ppm), although reduced, did not disappear completely. Similarly to rooibos tea, a reduction in carbohydrate signals at 72 ppm and cellulose at 65 and 87 ppm [20] was shown, as well as polysaccharides at 105 ppm [19].

**3.4. Decomposition Indexes.** Figure 4 shows the decomposition indexes for the green and rooibos tea over time. The AC/OAC (Figure 4(a)) ratio increased with incubation time in the green tea, with a ratio at 91 d significantly higher ( $p \leq 0.05$ ) than that at 0 d. The difference in the AC/OAC ratio in the rooibos tea, between 0 d and 91 d, was not significant, despite the fact that there is a significant reduction in the total mass of rooibos tea between 0 d and 91 d (Figure 1).

The CC/MC ratio decreases significantly between 0 d and 91 d over time for both tea types (Figure 4(b)), with a steep decline in the green tea in the first 14 d and the rooibos being more gradual over time. Unlike the AC/OAC ratio, the CC/MC ratio in both tea types changed significantly between 0 d and 91 d ( $p < 0.05$ ). The peaks at ~53 ppm demonstrated a greater relative increase during the incubation period, in the green tea, and this peak is associated with methoxyl C found in lignin. This is also the case for the rooibos tea (Figure 4(b)), with the peak at ~73 ppm showing a large reduction and the peak at ~56 ppm persisting over the incubation period.

### 4. Discussion

For TBI data to be informative, decomposition in the tea leaves needs to be typical of other plant litters. The TBI

TABLE 1: Chemical shift regions for integration of CPMAS NMR spectra.

C functional group	Chemical shift (ppm)	Main classes of compounds included [10]; [11]	Fraction from sequential C extraction conducted by Keuskamp et al. [4]
Alkyl C	0–50	Side chains in amino acids, structures derived from cutins and suberins, aliphatic compounds, lipids, waxes	Nonpolar extractives (NPE), e.g., fats and waxes
O-Alkyl C	50–110	O-substituted C in alcohols and ethers including cellulose, hemicelluloses, and other polysaccharides, carbohydrates, lignin propyl side chains	Water solubles (WS), e.g., simple sugars Acid solubles (AS), e.g., cellulose Acid insoluble (AIS), e.g., lignin
Aromatic C	110–160	Condensed tannins, hydrolysable tannins, phenyl-propylene subunits of lignin	Acid insoluble (AIS), e.g., lignin
Carbonyl C	160–200	Peptides, carboxylic C in hydrolysable tannins and resins, secondary amide C in proteins	

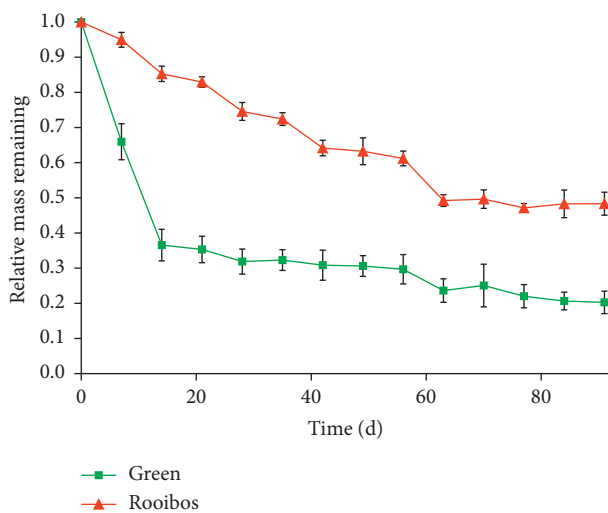


FIGURE 1: Relative mass remaining of the initial mass green tea and rooibos tea, as measured in laboratory incubation at 20°C (0 d–91 d). Error bars represent standard deviation ( $n = 3$ ).

calculations are also based on green tea leaves going through both phases of biphasic decomposition and reaching a plateau after 90 days incubation, whereas rooibos tea should still be decomposing.

Litter decomposition goes through two phases with a fast initial loss of soluble components through leaching into the soil, followed by a more gradual mass loss due to microbial decomposition and mineralization of cellulose and other compounds [21, 22]. The mass of the green tea decline was biphasic with rapid decline to 14 d, and loss of soluble tannins, followed by a slower rate to 91 d, while the mass of rooibos tea declined at a slow rate over the whole incubation period. This was also observed in initial investigations by Keuskamp et al. [4] although decline in the rooibos tea appears to be more rapid in the data presented in this paper. The mass remaining as a proportion of the initial mass after the 91 d incubation was 0.20 and 0.48 for green and rooibos tea, respectively (Figure 1) which is within the decomposable fraction value of the teas provided by Keuskamp et al. [4]. Values for  $S$  (0.032 based on median mass losses) were consistent with the lab incubations conducted at 15°C by Keuskamp et al. [4]. The value for  $k$  however (0.038 based on

median mass losses) was higher than that observed by Keuskamp et al. [4] at both 15 and 20°C, indicating a more rapid decline in the rooibos tea in this incubation.

The C:N ratio in both green and rooibos tea decreased significantly with incubation time (Figure 2(c)), which is consistent with a number of decomposition studies using a variety of litters [14, 23, 24] and is a result of C being mineralized and lost more rapidly than N. With an ample supply of N in the green tea (C:N ratio ~12), decomposers have been able to metabolise the tea C whilst satisfying their N demand from the N also contained within the tea. For the rooibos tea to be decomposed (C:N ratio ~60), decomposing microbes will have drawn in N from the surrounding environment. This exogenous N is incorporated into the decomposer microbial biomass and results in a net increase in N in the decomposing litter [25]. This process accounts for the temporal maintenance of absolute N during the incubation period (Figure 2(b)) and the greater relative decrease in the C:N ratio in the rooibos tea (Figure 2(c)). This may explain the higher observed  $k$  than those of Keuskamp et al. [4] if the soil they used had a lower N content than the soils used in this investigation.

Lignin peaks persisting in these samples support assumptions made by Keuskamp et al. [4]; i.e., in the calculation of  $S$ , it is assumed that the biochemically recalcitrant fraction is material that is nonhydrolysable. Lignin, a highly aromatic polymer with a disordered structure, is traditionally thought to be selectively preserved in soil [3, 26]. Conversely, some studies found that high lignin content amendments, thought of as chemically recalcitrant, were broken down quickly to release nutrients within the soil, and selective preservation was only important during the initial stages of decomposition of organic inputs [3, 7].

For green tea, despite the relative mass remaining reaching a plateau at the end of this incubation period, the peak most often associated with carbohydrates (72 ppm) persists. This suggests that some carbohydrates remain in the green tea that the microbial community cannot exploit. The peak that persists in the green tea at ~62 ppm has also been associated with carbohydrate C [10, 16, 20]. This carbohydrate could be microbially derived compounds which will have a similar composition to the original litter [3]. This persistence of traditionally classified labile C highlights the

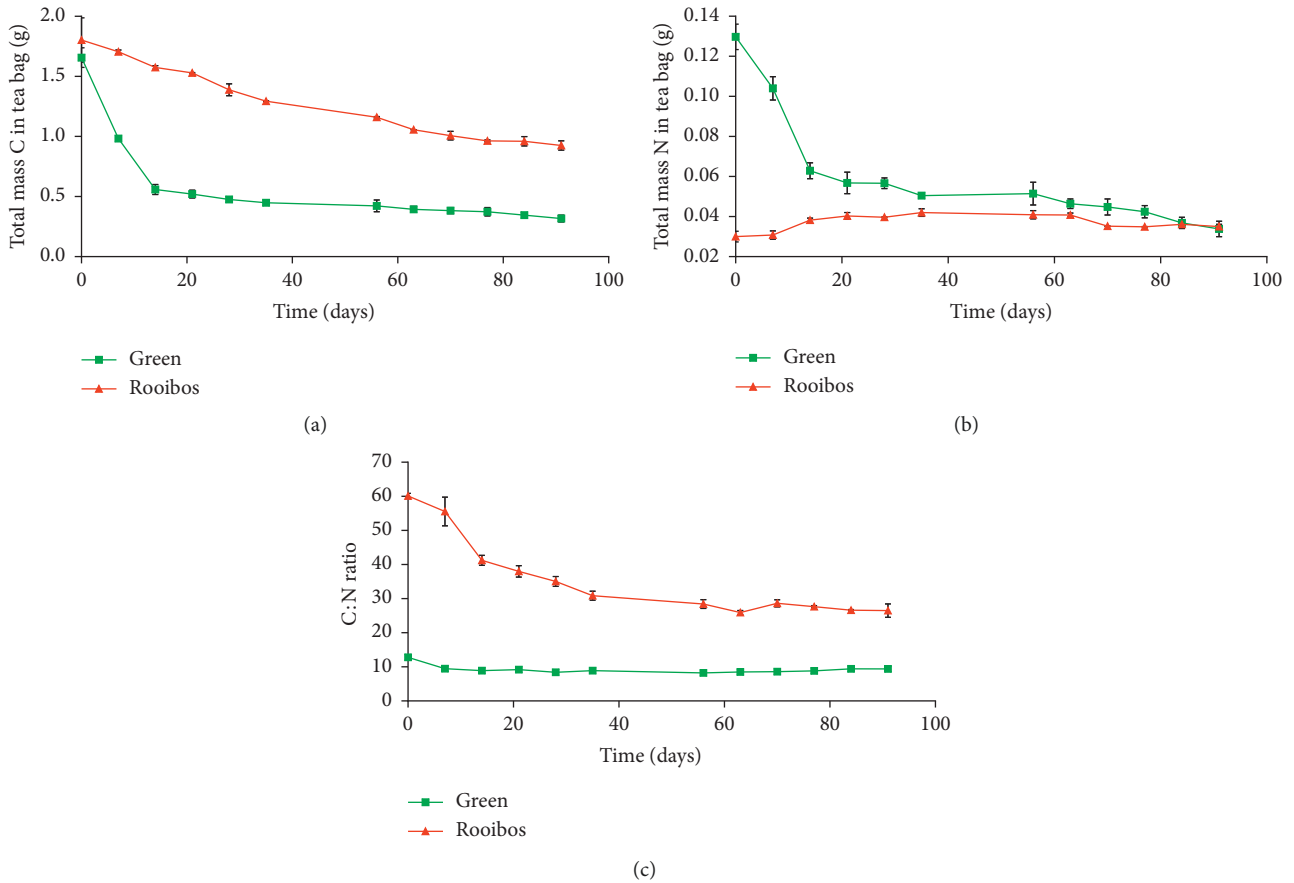


FIGURE 2: Effect of incubation time on (a) mean total C and (b) mean total N contents on an absolute mass basis and (c) mean C:N ratio in green and rooibos tea bags over 91 d. Error bars signify standard deviation ( $n=2$ ).

TABLE 2: CPMAS NMR integrals of green and rooibos tea preincubation (mean  $\pm$  standard deviation ( $n=2$ )).

Chemical shift region	Mean integral			
	Green		Rooibos	
	0 d	91 d	0 d	91 d
Alkyl C	0.230 $\pm$ 0.032b	0.405 $\pm$ 0.007a	0.152 $\pm$ 0.044b	0.237 $\pm$ 0.019b
O-Alkyl C	0.570 $\pm$ 0.003b	0.415 $\pm$ 0.030c	0.714 $\pm$ 0.018a	0.597 $\pm$ 0.031b
Aromatic C	0.146 $\pm$ 0.020a	0.108 $\pm$ 0.007a	0.102 $\pm$ 0.017a	0.123 $\pm$ 0.002a
Carbonyl C	0.054 $\pm$ 0.009a	0.072 $\pm$ 0.016a	0.032 $\pm$ 0.009a	0.044 $\pm$ 0.009a

Values within rows that share a letter are not significantly different according to ANOVA ( $p < 0.05$ ).

need for the determination of a stabilisation factor,  $S$ , as a step in the TBI calculations presented by Keuskamp et al. [4].  $S$  accounts for the fact that compounds traditionally thought of as labile can be stabilised during decomposition, rendering them resistant to decay. The fraction of O-alkyl C (50–110 ppm) in both tea types saw a significant reduction. This is consistent with a number of decomposition studies [12, 14, 23, 27].

The alkyl region persisted in both tea types, as observed in leaf litter studies [28, 29]. Alkyl C occurs in a number of structures, such as waxes and lipids, which require specific enzymes to decompose them [27], and are therefore potentially more resistant to decay. These are also important compounds to be present in tea leaves for green tea production, as they help protect the plant against

biotic and abiotic stresses [28, 30]. It has also been suggested that the alkyl region is also indicative of the presence of microbial metabolites that are produced during decomposition, further adding to this region's accumulation in litter [20]. This may have implications for the assumption that nonpolar extractives (i.e., fats and waxes) comprise part of the hydrolysable fraction in the TBI calculations. The CPMAS NMR data presented here suggest that these components are persisting in the tea leaves and may be more resistant than assumed when defining the hydrolysable fraction as labile for the calculation of  $S$ .

An increase in AC/OAC ratio during decomposition has been observed in a number of studies due to the decomposition of labile carbohydrates and selective

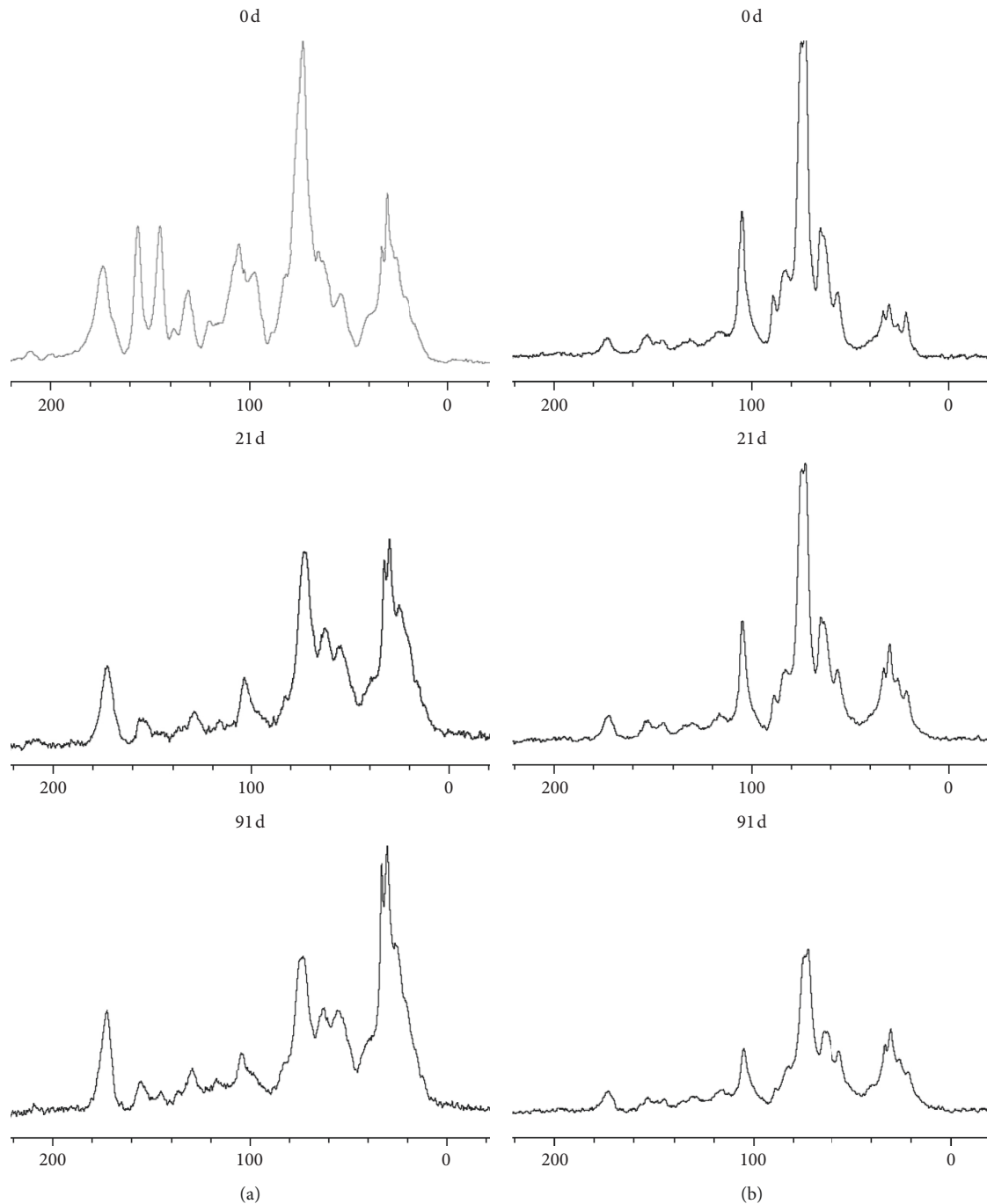


FIGURE 3:  $^{13}\text{C}$  CPMAS spectra of (a) green and (b) rooibos tea at 0, 21, and 91 d of incubation.

preservation of more recalcitrant waxes, in addition to subsequent build-up of secondary metabolites [14, 23, 31]. Both tea types see an increase in the AC/OAC ratio over the incubation period indicating that they decompose in a similar manner as other litters. There is a possibility that the AC/OAC ratio is not sensitive enough to reflect the level of decomposition in the rooibos tea because this ratio uses large chemical shift regions, which comprise a number of peaks, some of which reduce in intensity and some increase or maintain at the same level within this

operational window. Therefore, an index that focuses on individual peaks, such as the CC/MC ratio, may be more informative.

To date, there have not been as many studies in the literature that use the CC/MC ratio as a decomposition index [14, 31], compared to those that use the AC/OAC ratio. Our observed significant decrease in the CC/MC ratio with increasing decomposition is in accordance with Bonanomi et al. [14] who reported that the CC/MC ratio had the highest correlation to the decomposition rate.



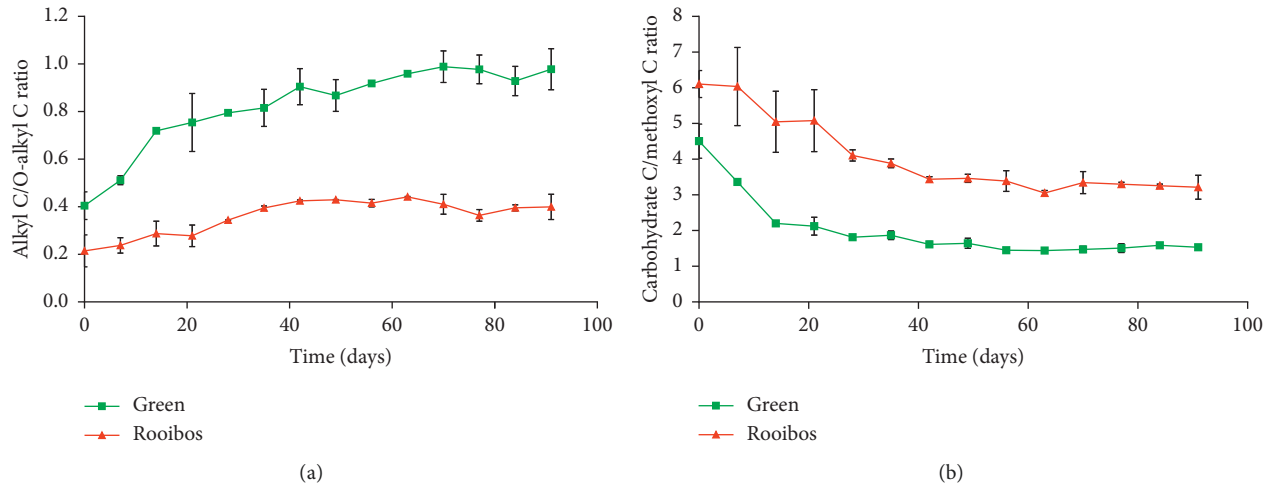


FIGURE 4: Effect of incubation time on decomposition indexes of green and rooibos tea: (a) alkyl C/O-alkyl C ratio and (b) carbohydrate/methoxyl C ratio error bars for standard deviation ( $n = 2$ ).

## 5. Conclusions

For TBI data to be informative, decomposition in the tea leaves needs to be typical of other plant litters and the results presented here suggest this is the case. However, this study has also highlighted some key considerations required when interpreting TBI data. Firstly, the use of this method in N-limited systems may affect results, as decomposition of the rooibos tea (C:N c. 60) requires an external input of N for the material to be broken down.

Secondly, the TBI method assumes that the hydrolysable fraction ( $H$ ) is labile and that any chemical constituents that are not hydrolysable (AIS) are recalcitrant. CPMAS NMR data have shown that water (WS) and acid soluble (AS) organic compounds, such as simple sugars and cellulose, are being depleted in both tea types. However, peaks associated with these compounds do not disappear completely over the incubation period. This highlights the importance of the fact that Keuskamp et al. [4] have recognised that parts of the labile fraction stabilise in the material and there is a need for calculating  $S$ , particularly as hydrolysable nonpolar extractives, such as fats and waxes, appear to be persisting in the tea bags.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

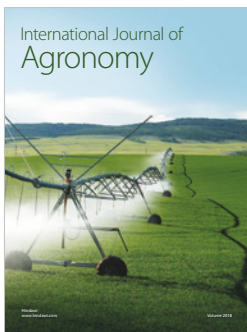
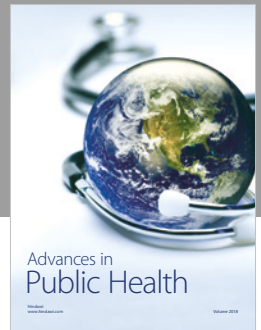
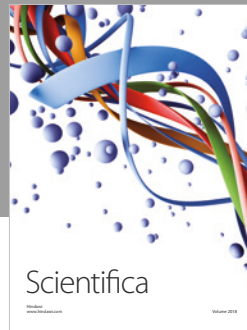
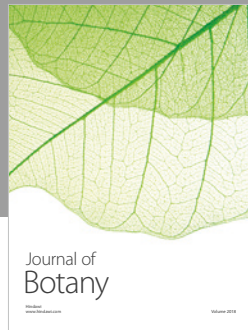
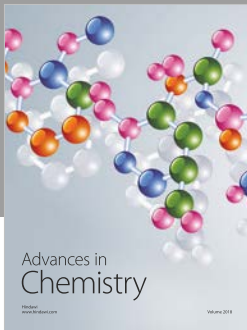
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