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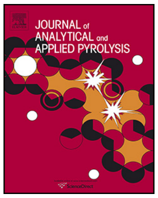

Novel molecular proxies for inferring pyrogenic black carbon oxidation state using thermally assisted hydrolysis and methylation (THM-GC–MS) with ^{13}C-labeled tetramethylammonium hydroxide (TMAH)

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ABSTRACT

Analytical techniques to assess the degree of alteration of pyrogenic black carbon (C) in soils and sediments are needed to gauge the role of environment and time in the dynamics of this stable carbon form. The benzenecarboxylic acids (B*n*CA, with *n* = number of carboxylic groups) and naphthalene analogues (N*n*CA) released by thermally assisted hydrolysis and methylation (THM) of black C have the potential to yield information about black C oxidation state but it remains unclear to what extent the carboxylic groups in B*n*CA and N*n*CA are native to the sample or form as analytical artefacts during the THM reaction. In an effort to clarify the source of analytical products and investigate long term black C oxidation in a soil environment we applied THM gas chromatography mass spectrometry (THM-GC–MS) using ^{13}C-labeled tetramethylammonium hydroxide (^{13}C-TMAH) to oak-derived black C samples isolated from a colluvial soil profile dating to 50–8100 cal BP. The results showed that the B*n*CA and N*n*CA originate from the black C and are potentially usable as oxidation proxies, with the exception of B1CA and hydroxyl-B1CA which have a significant source in lignin. The cumulative abundance of B*n*CA (excluding B1CA) and N*n*CA increased with age of the black C ($P < 0.05$) from ca. 0.05-0.20 mg/100 mg organic C. Furthermore, the ratios B3CA/B2CA and (B3CA + B4CA)/B3CA increased with age, and are proposed as novel proxies of black C oxidation state. The results support previous studies showing that carboxyl functionalities dominate over hydroxyl and that molecular markers of lignin are selectively depleted within 1000 yrs after wildfire. The accumulation of hydroxyl and especially carboxyl functional groups on black C structures seems to be a gradual process that continued at least until the end of the studied ageing window of 8100 yrs.

1. Introduction

The increased awareness of the role of pyrogenic black C in the global C cycle [1–4] and its application to agricultural soils as biochar [5–7] call for a deeper understanding of the changes in its molecular composition induced by microbial or abiotic degradation [8–11]. Black C degradation is often studied from a mass balance perspective (loss rate as CO_2) [12] but also by determination of changes in its chemical composition, mostly surface oxidation processes, using spectroscopic and molecular tools [11,13–16]. Although laboratory experiments simulating environmental oxidation of black C are important for mechanistic inferences of possible decomposition pathways, long term (*i.e.* millennia) *in-situ* decomposition of black C in soil provides for a more realistic and potentially harsher integrated abiotic/biotic decay [16,17]. Hence, much of the detailed knowledge concerning the molecular changes to black C following long-term ageing rely upon analysis of ancient black C retrieved from soil and sediment with an intense palaeofire regime, in particular the anthropogenic dark earths, such as the *Terra Preta de Índio* in the Amazon basin [18], *Terra Preta Australis* in Australia [19], Māori garden soils in New Zealand [20], Chernozems of Central Europe and Russia [21,22] or Atlantic rankers in NW Spain [23], the subject of the present study.

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The Atlantic rankers [24], or Haplic Umbrisols (humic/alumic) [25], are thick dark-coloured acidic colluvial soils that accumulated during the Holocene. These soils contain large amounts of black C in the microaggregated fabric but also abundant larger particles (>2 mm), which has been used for Holocene fire regime reconstructions [26]. Previous studies using thermally assisted hydrolysis and methylation coupled to gas chromatography and mass spectrometry (THM-GC–MS) with unlabelled tetramethylammonium hydroxide (TMAH) for derivatization, on 700–7200 yrs old black C, revealed benzene carboxylic acids (B*n*CA, with *n* carboxylic acid groups). Supported by intense resonances of carboxylic groups by solid-state ^{13}C cross nuclear magnetic resonance (NMR), the B*n*CA were proposed to originate from oxidized black C moieties [27]. Other materials in which the B*n*CA have been interpreted as methylation products of aromatic acids include black crusts on historical buildings [28], insoluble organic matter in meteorites [29] and immature kerogen [30].

Despite of its potential for assessing black C oxidation, THM-GC–MS is rarely used for that purpose. This can probably be largely explained by the controversy on results from wet-chemical degradation techniques, many of which show prolific production of B*n*CA when applied to soil and sedimentary organic matter. More specifically, B*n*CA were produced upon oxidation by $KMnO_4$ [31–33], RuO_4 [34], CuO [35,36], $NaBO_3$ [37,38] and HNO_3 [39,40]. Many of these oxidation agents, and in a particular the harsh ones, are capable of disrupting C—C bonds in polycyclic aromatic hydrocarbons (PAH) followed by carboxylation, artificially creating B*n*CA and naphthalene carboxylic acids (N*n*CA) [39,41]. For THM-GC–MS, reactions other than hydrolysis, transesterification, and methylation do occur, such as cleavage in C—C interflavanoid bonds in tannins [42] and oxidation of alkyl side chains on fresh lignin (lacking carboxylic groups) to B1CA [43]. These results created a common view of the B*n*CA in THM-GC–MS as having an artificial origin as well. In order to use B*n*CA and N*n*CA abundances and relative proportions as proxies of black C oxidation, the importance of artificial carboxylation needs to be addressed.

There are two possible pathways of artificial carboxylation upon TMAH-THM. The first pathway involves the interaction of aromatic radicals that form during thermal alteration of aromatic compounds [44] and the CH_3 radicals from the methyl donor TMAH [45,46]. After substitution of C—H by C—CH_3 groups, the alkyl group is oxidized to form the B*n*CA or N*n*CA. Using ^{13}C labelled TMAH in the THM-GC–MS of aged black C the first pathway for artificial B*n*CA can be identified as the added CH_3 will contain a ^{13}C isotope label which is quantified in the mass spectral analysis, similar to distinguishing methoxyl and methylated hydroxyl groups from lignin and tannin or fungal and bacterial demethylated lignin in the THM-GC–MS processes [47–49]. The second pathway could proceed *via* an oxidative ring opening of polycyclic aromatic hydrocarbons (PAH) and subsequent oxidation of the alkyl groups, or oxidation of alkyl groups in alkylarenes in the THM procedure, *i.e.* similar to the aforementioned effects of other chemical oxidation agents [39,41]. This second pathway cannot be identified by use of ^{13}C TMAH because the oxidized C atom is native to the sample, yet its relevance can be assessed by THM-GC–MS (labelled or unlabelled TMAH) of commercial PAH model compounds. As far as we know THM-GC–MS using TMAH of PAH and alkylarenes compounds have not been reported.

In the present study we address the methodological question related to original source or artificial production of B*n*CA and N*n*CA in black C by investigating aged black C fragments from a colluvial soil profile in NW Spain using THM-GC–MS with ^{13}C TMAH. The depth/age model of this soil showed that the black C formed between 50 and 8100 yrs cal BP [50]. This soil was investigated by anthracological means which allows limiting the analysis to oak-derived black C, and consequently circumvent one important source of heterogeneity, *i.e.* botanical origin of the wood [51]. The main objectives are (1) to identify the aromatic carboxylic acids produced upon THM-GC–MS of aged black C (B*n*CA and N*n*CA), (2) to determine whether the carboxylic groups of the B*n*CA and N*n*CA are native or artificial and (3) to assess trends in oxidation patterns during the 8100 yrs period of storage in soil.

Table 1
Sample codes, depth interval from PRD-4 record (Campo Lameiro, NW Spain), estimated age and C content.

Code	depth (cm)	Estimated age (yr)	organic C (% of dry weight)
S1	0–5	50	0.597
S2	15–20	400	0.463
S3	30–35	1000	0.552
S4	65–70	3300	0.534
S5	95–100	4700	0.504
S6	120–125	6300	0.500
S7	145–150	8100	0.404

2. Methods and materials

A soil column of a 2.35 m thick Atlantic ranker from the Archaeological Rock Art Park in Campo Lameiro (NW Spain) was obtained from a mechanical trench and cut in 5 cm slices (47 samples). This soil is referred to as soil Paredes-IV (PRD-4) and was subject of a detailed anthracological study [50]. The black C, isolated by wet sieving over a 2 mm sieve, was identified as deciduous oak (*Quercus robur*). Seven samples were selected on the basis of age and availability of oak-derived black C (Table 1). The samples consisted of ca. 10–50 charcoal particles which were ground to a powder, dried at 35 °C and analyzed by THM-GC–MS without further treatment. Organic C contents of the black C, obtained dry-combustion elemental analysis, are given in Table 1.

THM-GC–MS with ^{13}C-labeled TMAH was performed according to Filley et al. [52] with modifications by Filley et al. [53] and Klotzbücher et al. [54]. Two analytical replicates were performed for each sample. Briefly, 243 (average) ± 30 (standard deviation) μg of powdered black C was weighed into Pt buckets containing 200 ng eicosane as internal standard, and amended with 3.5 μl aliquots of ^{13}C TMAH (25% in water). The buckets were placed in the sample holder on top of the pyrolysis unit (Pyr-4a, Shimadzu Corp., Kyoto, Japan) and kept there under a He flow for 15 min at room temperature to maximize the contact between samples and TMAH solution. Then, the buckets were dropped into the pyrolyzer maintained at 350 °C. Compounds were analysed with a Shimadzu GC17A–QP5050A GC–MS system. The GC was equipped with a 30 m (length), 0.25 mm (i.d.) and 0.25 μm (film thickness) fused silica column (SPB-1, Supelco, Bellefonte, USA), and operated in 1:10 split mode with the inlet at 320 °C. The oven was initially held at 60 °C (1 min), raised to 140 °C at 10 °C min^{-1} and then to the final temperature of 300 °C at a rate of 6 °C min^{-1}, which was maintained for 20 min. The MS scanned in the m/z 40–550 range.

The molecular formulae of the native B*n*CA follow the equation $^{12}C_6 + (H_6\text{-}n) + (^{12}COO)_n + (^{13}CH_3)_n$, where ^{12}C represents the natural abundance of C stable isotope in plant tissue and ^{13}C is the 99% ^{13}C content derived from the ^{13}C-labeled TMAH, and the molecular mass and molecular ion M^+ can be calculated from $72 + (6 - n) + (44n) + (16n) = 78 + 59n$. To identify the analytical artefact of an artificially produced ("^{13}C-enriched") B*n*CA when using ^{13}C TMAH the M^+ is one u heavier for each carboxylic group than the analogous fragment ion from B*n*CA with native carboxylic groups. For both native and artificial B*n*CA, the base ion is produced upon elimination of the $-O^{13}CH_3$ moiety and therefore equals M^+-32.

GC/MS response factors could not be determined for individual B*n*CA and N*n*CA because several substituted B*n*CA and N*n*CA are not available commercially and for B2CA, B3CA and B4CA quantitative derivatization was not achieved. Nevertheless, the analysis of permethylated standards confirmed the retention times and mass spectral patterns of the B*n*CA. We estimated the concentrations of the B*n*CA and N*n*CA, in mg/100 mg organic C (OC), by dividing the peak area of the ion fragments used for quantification by that of the eicosane internal standard (*m*/*z* 57 and 71).

Statistically significant linear correlations ($P < 0.05$) between variables are reported as Pearson product-moment correlation coefficients (r) and corresponding *P* values. Statistically significant differences between values and the population mean (of the same variable) were tested using the one-sample *t* test.

We also analyzed a series of standard compounds (PAHs, alkylarenes and B*n*CA) by a THM-GC–MS method using a Pyroprobe 5000 (CDS Analytical) and unlabelled TMAH to investigate production of B*n*CA and N*n*CA by oxidation of native alkyl or aromatic carbon (pathway 2). The methodology involved is described in Supplementary information S1.

3. Results and discussion

3.1. Standard PAH and alkylarene compound

The standard PAHs (Supelco PAH set) and alkylarenes (1,2,4,5-tetramethylbenzene and 1,2-dimethylnaphthalene, Sigma Aldrich) did not produce B*n*CA or N*n*CA upon THM-GC–MS. THM-GC–MS of individual B*n*CA standards permethylated successfully and were identified as their corresponding methyl esters (Supplementary information S1). These results show artificial carboxylation by TMAH-THM of native C groups (either by direct methyl group oxidation or aromatic ring opening followed by oxidation) is unlikely. In particular the fact that acenaphthene, which has a much higher reactivity than PAHs lacking a methylene group [55] did not produce N2CA confirms that the TMAH-THM reaction is not strong enough for artificial oxidation: acenaphthene is oxidized to N2CA in aqueous dichromate or acid-catalyzed UV radiation through nucleophilic attack of the active methylene group [39,41].

3.2. ^{13}C TMAH-THM-GC–MS products of aged black C

The THM chromatograms were dominated by a set of methyl esters of short-chain alkanoic acids (FAMEs) and, in case of the relatively recent black C (sample S1), long-chain FAMEs and diacids (DAMEs) as well (not shown). These compounds correspond to either thermally altered aliphatic sources or aliphatic biopolymers incorporated after burial. These hydrolyzable and low molecular weight (“free”) constituents compose only a minor proportion of the total C of the sample, but overrepresented in the THM process because they are easily liberated and the majority of the C is present as polyaromatic clusters that are not released at the applied THM reaction temperature of 350 °C [51]. This explains why, contrary to Kaal et al. [27] who applied a higher THM temperature of 600 °C, the monocyclic and polycyclic aromatic hydrocarbons (MAHs/PAHs) without O-containing functional groups (benzene, toluene, naphthalene, etc.) were below the detection limit. The FAMEs and DAMEs do not interfere in the assessment of oxidation patterns of aromatic moieties in black C and are ignored in the following sections.

The other compounds identified were the B*n*CA, N*n*CA and lignocellulose derivatives (di- and trimethoxybenzenes). The B*n*CA and N*n*CA detected were (1) unsubstituted B1CA–B4CA (and B5CA tentatively), (2) methyl-B1CA–B3CA, (3) hydroxy-B1CA–B3CA and (4) N1CA–N3CA. The list of M^+ and base ions of the 29 B*n*CA and N*n*CA compounds with native carboxylic groups is presented in Table 2, which includes the masses of the ions when unlabelled TMAH would be used. Fig. 1 shows the partial ion chromatograms of the native B*n*CA of selected samples (S1, S4 and S7).

3.3. Distinguishing native and artificial B*n*CA and N*n*CA

For B1CA, the $M^+–O^{13}CH_3$ base peak with a native carboxylic group (*m*/*z* 105) accounts for 90.6 ± 2.3% of the sum of the native and artificial B1CA (*m*/*z* 105 and 106) (not shown). The remaining 9.4 ± 2.3% is significantly different (one-sample *t* test: $P < 0.005$) from 7.3% (the percentage of the naturally-occurring ^{13}C in the C skeleton), which may be indicative of a small contribution of TMAH-derived C and thus artificial carboxylation, but traces of co-eluting compounds may also account for this small difference. Furthermore, lignin and other polyphenols such as tannins generate significant amounts of B1CA [56,57] upon THM and there is a possibility that artificial carboxylation of lignin occurs on a native C atom, *i.e.* the α-carbon atom of the propanoid side chain [58,59]. Therefore, B1CA should be omitted from black C oxidation pattern studies.

Of the B2CA (Fig. 2a), only traces of artificially-produced B2CA with excess ^{13}C (*m*/*z* 198 and 166) were found, *i.e.* 1.2 ± 0.3%, 1.8 ± 0.9% and 1.9 ± 0.8% for the 1,2-B2CA, 1,3-B2CA and 1,4-B2CA, respectively, which implies that the vast majority if not all of the carboxylic groups of the B2CA are native to the samples. At a retention time of 10.9 min a lignin derivative producing *m*/*z* 164 and 165 is found in the youngest sample (S1), indicating potential confounding issues in B2CA peak assignment due to coelution when lignin residues are present.

The peaks of the first (1,2,3-B3CA) and second (1,2,4-B3CA) isomers of B3CA (*m*/*z* 223 and 255) are dominated by base peak *m*/*z* 223 (99.9 ± 0.1% and 99.8 ± 0.1% of total B3CA, respectively) and thus derived from native carboxylic groups (Fig. 2b). A minor *m*/*z* 226 at the same RT is more likely to originate from natural abundance ^{13}C in the black C, than from artificial carboxylation. To the contrary, the third isomer of native B3CA (1,3,5-B3CA) coincides with a peak of *m*/*z* 226 and 258 due to partial co-elution with a dimethoxy-B2CA. Hence, the estimated proportion of native 1,3,5-B2CA (35.1 ± 34.5%) is underestimated, particularly in the younger samples where this lignin products is more abundant (S1 = 82%, S2 = 90%, S3 = 36%, other samples ca. 10%). Nonetheless, the 1,3,5-B3CA can be used because it has different M^+ and base peaks than dimethoxy-B2CA, even if unlabelled TMAH is applied.

Based on mass spectral patterns, artificial production of B4CA were insignificant (Fig. 2c): the base peak of the first and third isomers were not detected, whereas for the second isomer the *m*/*z* 282 base peak was accompanied by only traces of *m*/*z* 286 (0.5 ± 0.1%), indicating that these compounds are reliable markers of oxidized aromatic moieties. Similarly, B5CA was not accompanied by artificial B5CA (not shown) but this compound was scarce and tentatively identified, and therefore not used as marker.

The mass spectral patterns of the carboxyl groups of the substituted B*n*CA (*i.e.* methyl B*n*CA and hydroxy-B*n*CA) and N*n*CA suggested that these compounds were derived from black C as well (not shown), with the exception of *p*-hydroxy-B1CA which is also a lignin product [56]. It is concluded that artificial formation of B*n*CA and N*n*CA is negligible and that hydroxy-B*n*CA (B2CA, B3CA and B4CA), C_1-B*n*CA and N*n*CA can be traced back to naturally oxidized aromatic moieties, whereas the B1CA and hydroxy-B1CA should be disregarded for their source in plant polyphenols.

3.4. Effects of ageing on Black C oxidation patterns

A series of lignin products, *i.e.* 3,4-dimethoxybenzoic acid methyl ester (from G6), 3,4,5-trimethoxy benzoic acid methyl ester (S6), 3,4,5-trimethoxybenzaldehyde (S4) and 3,4-dimethoxyphenylpropenoic acid methyl ester (G18), were more

Table 2
Mass spectrometric properties of the benzene and naphthalene carboxylic acid methyl esters examined.

Compound (isomers)[a]	M^+ ^{13}C-TMAH	Base peak ^{13}C-TMAH	*M^+ ^{12}C-TMAH*	*Base peak ^{12}C-TMAH*
B1CA (1)	137	105	*136*	*105*
B2CA (3)	196	164	*194*	*163*
B3CA (3)	255	223	*252*	*221*
B4CA (3)	314	282	*310*	*279*
B5CA (1)	373	341	*368*	*337*
B6CA (0)	432	400	*426*	*395*
C1-B1CA (0)	151	119	*150*	*119*
C1-B2CA (1)	210	178	*208*	*177*
C1-B3CA (1)	269	237	*266*	*235*
C1-B4CA (2)	328	296	*324*	*293*
N1CA (1)	187	155	*186*	*155*
N2CA (3)	246	214	*244*	*213*
N3CA (4)	305	273	*303*	*271*
hydroxy-B1CA (1)	168	136	*166*	*135*
hydroxy-B2CA (3)	227	195	*224*	*193*
hydroxy-B3CA (3)	286	254	*282*	*251*

[a] Numbers within brackets refer to the number of isomers detected of each compound (0 means that the compound was not detected, included for the sake of completeness).

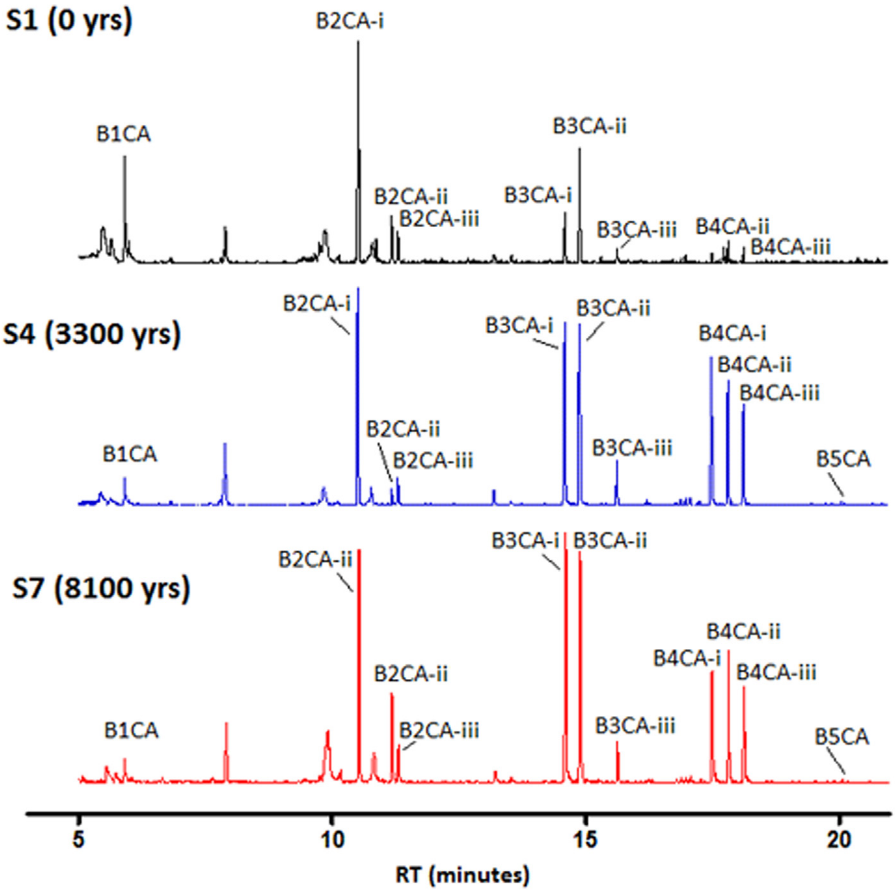

Fig. 1. Partial ion chromatograms (m/z 105 + 164 + 223 + 282 + 341 + 400) using the base ions of the unsubstituted benzene carboxylic acids (BnCA, with n methylated carboxylic groups) of samples S1, S3 and S7. Letters indicate isomer numbers (*e.g.* B3CA-iii for benzene-1,3,5-tricarboxylic acid trimethyl ester). RT = retention time.

abundant in samples S1 and S2 (0.041 ± 0.030 mg/100 mg) than in older samples (0.003 ± 0.002 mg/100 mg OC) (Fig. 3a). This negative age trend ($P < 0.05$) confirms earlier studies on much larger sample set from this same soil analyzed by pyrolysis-GC–MS, indicating that some black C fragments contain lignin in either incompletely charred cores [51] or as absorbed dissolved organic matter (DOM) that is progressively degraded over time and therefore absent by the age of S3 (1000 yrs). Use of ^{13}C TMAH allowed for differentiation of unambiguous lignin products (with native methoxylic groups) and demethylated lignin or perhaps tannin derivatives (*e.g.* [47]). This suggests that the analysis of black C using ^{13}C-labelled TMAH is useful to determine lignin demethylation during a fire. Finally, the estimated concentration of B1CA is 0.012 ± 0.006 mg/100 mg OC, with no clear trends with age (not shown). As aforementioned, this compound should not be used for the assessment of black C oxidation patterns as it is not specific to Black C [56].

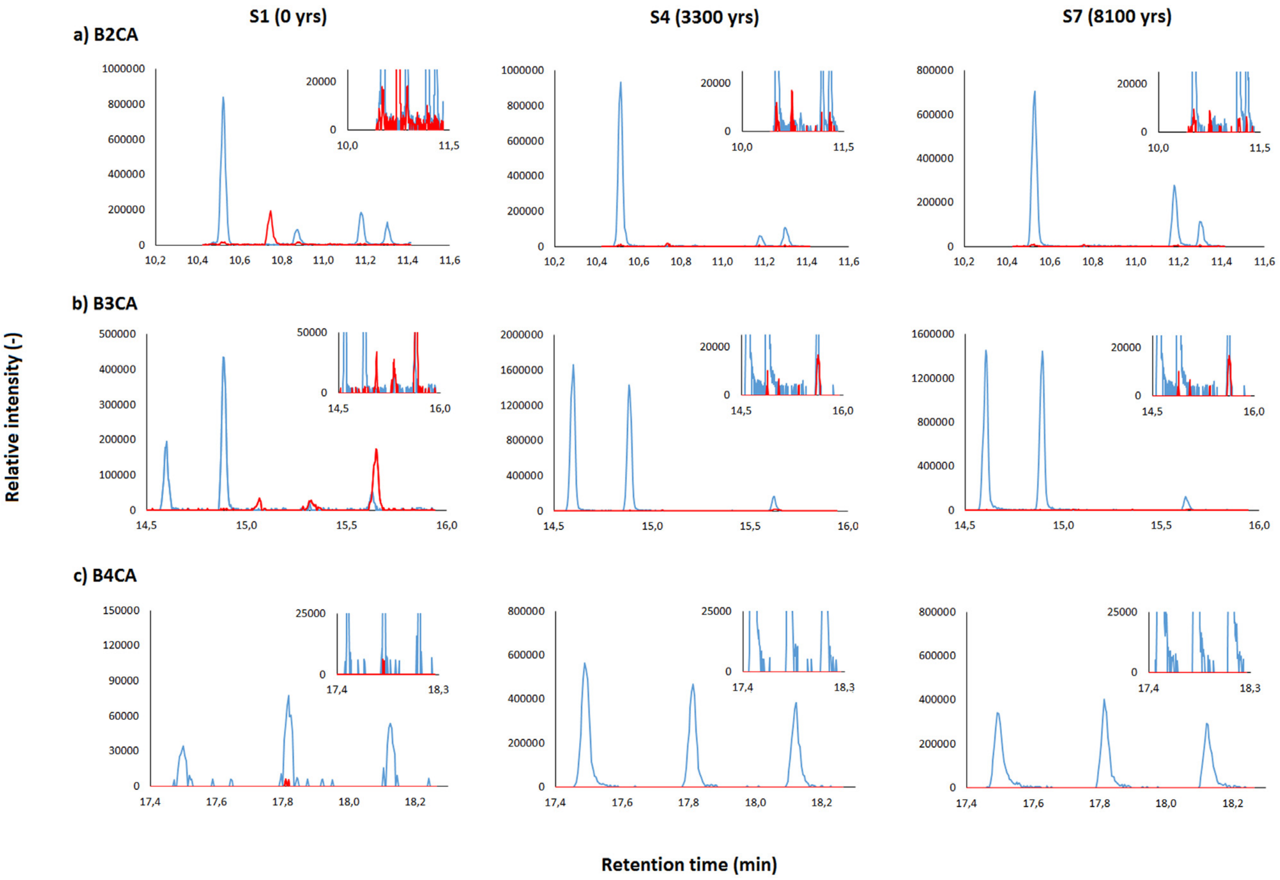

Fig. 2. Partial ion chromatograms of unsubstituted benzene carboxylic acids (B*n*CA, with *n*-carboxylic groups), of samples S1, S4 and S7. Blue lines represent the intensities of the base ions of BnCA with native carboxylic groups, whereas red lines represent base ions of BnCA from artificial carboxylation). (a) B2CA (native: *m*/*z* 164, artificial, *m*/*z* 166), (b) B3CA (native: *m*/*z* 223, artificial, *m*/*z* 226), (c) B4CA (native: *m*/*z* 282, artificial, *m*/*z* 286). Insets show details of artificial B*n*CA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The concentration of B2CA homologs (0.045 ± 0.021 mg/100 mg OC), most of which corresponds to 1,2-B2CA, do not trend with age of the black C (Fig. 3b). The concentration profiles of the B3CA (Fig. 3c) confirm that 1,2,3- and 1,2,4-B3CA are much more abundant than 1,3,5-B3CA. The concentrations of B3CA (0.119 ± 0.041 mg/100 mg) and B4CA (0.041 ± 0.014 mg/100 mg OC; Fig. 3d), which have very similar concentration profiles, increase with age ($P < 0.05$). The sum of methyl B*n*CA (0.007 ± 0.003 mg/100 mg OC), derivatized hydroxy-B2CA (0.018 ± 0.006 mg/100 mg OC) and hydroxy −B3CA (0.020 ± 0.008 mg/100 mg OC), which had hitherto not been mentioned as black C oxidation products, do not increase significantly with depth (Fig. 3e/f).

Of the N*n*CA released by THM, the N2CA were more abundant (0.010 ± 0.001 mg/100 mg OC) than N1CA (0.001 ± 0.001 mg/100 mg OC) or N3CA (0.003 ± 0.003 mg/100 mg OC) (Fig. 3g). The N1CA ($P < 0.01$) and N2CA ($P < 0.05$) increase significantly with age. The N*n*CA reflect oxidized *poly*aromatic groups, structures not found in lignin or tannins, which implies that these compounds are useful markers of oxidized Black C. Furthermore, the N*n*CA have very low abundances in the youngest sample, and the increase in concentration with age suggests that these compounds can be linked to the gradual opening of the polycondensed aromatic backbone upon long-term storage. In more difficult sample matrices than the black C fragments used here, such as soils and sediments, the N*n*CA can provide evidence of oxidized black C in samples where non-pyrogenic components might interfere with the B*n*CA. Several authors reported the presence of N*n*CA among the THM products of Black C-rich soils and sediments [60,61], but their usefulness as markers of oxidized Black C had not been discussed. In environmental samples, the only relevant potentially interfering source of N*n*CA is kerogen from immature coals [62,63], albeit that N*n*CA were also detected among the THM products of macromolecular insoluble organic matter from various meteorites [29].

The sum of the B*n*CA and N*n*CA that are useful markers of oxidized Black C (B2CA, B3CA, B4CA, C_1-B2CA, C_1-B3CA, C_1-B4CA, hydroxy-B2CA, hydroxy-B3CA and N*n*CA) is 0.10 ± 0.05 mg/100 mg OC (Fig. 3h) and increases significantly with age of the black C ($P < 0.05$) during the 8000 yrs of storage in this particular soil environment. Despite of the analysis of well-defined oak-derived black C fragments, there is significant scatter from the main depth trend. This may be explained by differences in maximum temperature during the wildfires or pedogenetic processes that influence the extent of oxidation of the black C [64–66].

Apart from estimated concentrations profiles, the relative proportions of the oxidized black C products reveal additional information on black C oxidation processes (Fig. 4a) that may be more sensitive as oxidation markers than absolute concentrations. With increasing age, we observe an increase in B3CA (from 20 to 50%; $P < 0.01$), B4CA (from 5 to 20%, albeit that this trend is not significant) and N*n*CA (from 1 to 8%; $P < 0.01$), while a decrease is observed for B2CA (from 35 to 15%; $P < 0.05$) and hydroxy-B*n*CA (from 30 to 15%; $P < 0.01$). The ratio of N*n*CA to B*n*CA increases with age ($P < 0.01$) (Fig. 4b) and probably reflects changes in the condensed aromatic backbone of the black C, thereby increasing the proportion of THM-released polyaromatic groups. However, as the unsubstituted B*n*CA are the most abundant prod-

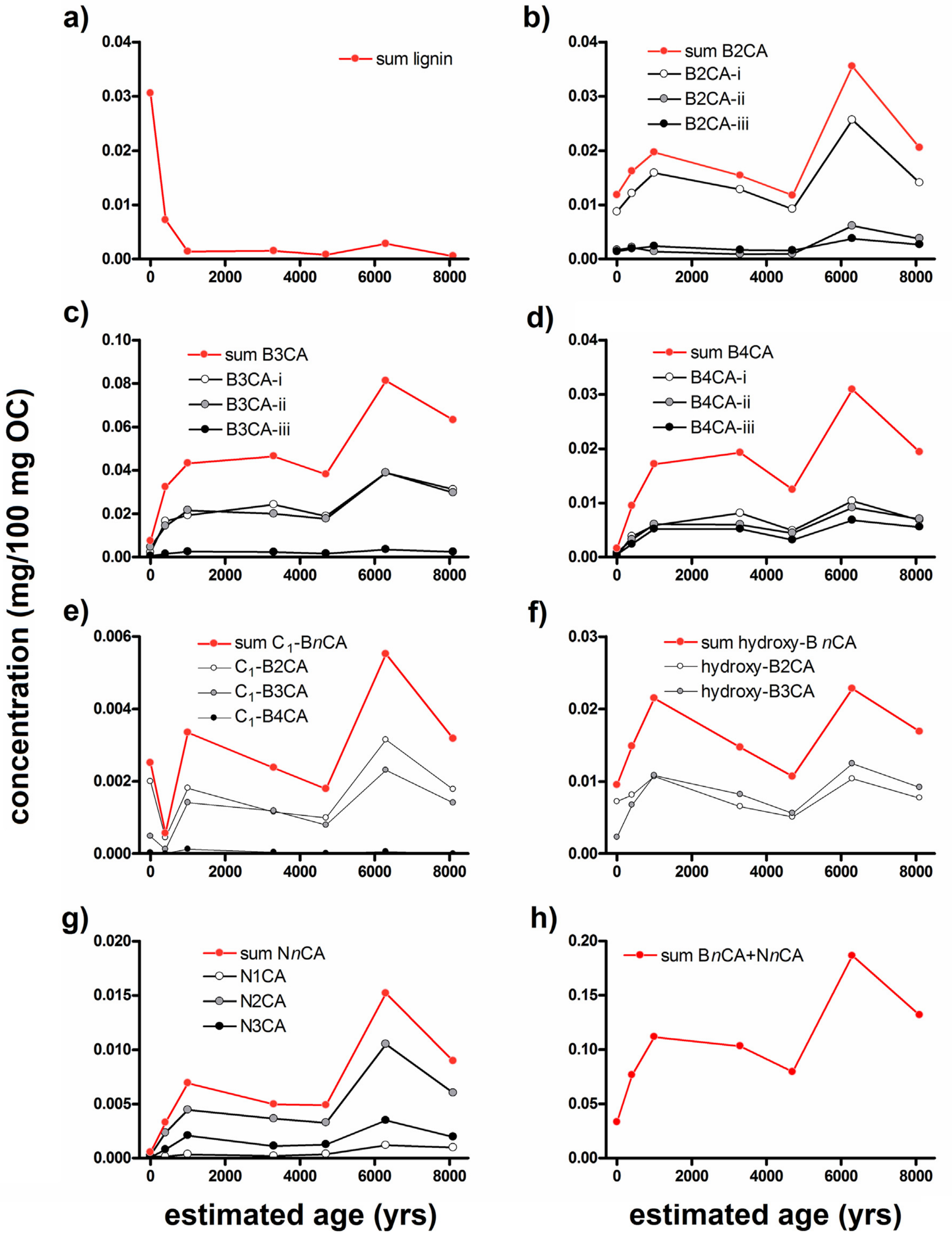

Fig. 3. Concentrations of THM-GC–MS products and groups of products: (a) sum of all lignin-derived products, (b) B2CA, (c) B3CA, (d) B4CA, (e) methyl-B*n*CA, f) hydroxy-B*n*CA, (g) N*n*CA, (h) sum all B*n*CA and N*n*CA.

ucts, these compounds are probably the best-suited for assessing the degree of oxidation of Black C, especially if less severely oxidized Black C samples are concerned. The ratios of B3CA/B2CA and (B4CA + B3CA)/B2CA are proposed as proxies of the degree of Black C oxidation. Both ratios increase significantly with age of the black C ($P < 0.01$) (Fig. 4c). Additionally, the ratio of B3CA/hydroxy-B2CA ($P < 0.01$) and B4CA/hydroxy-B3CA ($P < 0.01$) increase consistently with age as well (Fig. 4d), and suggest that even though hydroxyl groups develop during ageing, carboxylation mechanisms prevail.

This study demonstrates the viability of B*n*CA and N*n*CA as markers of black C oxidation in samples consisting predominantly of black C and lays the foundations for assessing black C oxidation after soil amendment as a biochar or upon its passage through the different compartments of the global C cycle [2,10,67]. As far as we know, the only possibly interfering materials of B2CA, B3CA and B4CA upon THM, are (1) kerogens in the lignite to early subbituminous coal rank [30,62] and (2) humic substances isolates [68,69]. With regard to kerogens, the B*n*CA and N*n*CA are the result of early

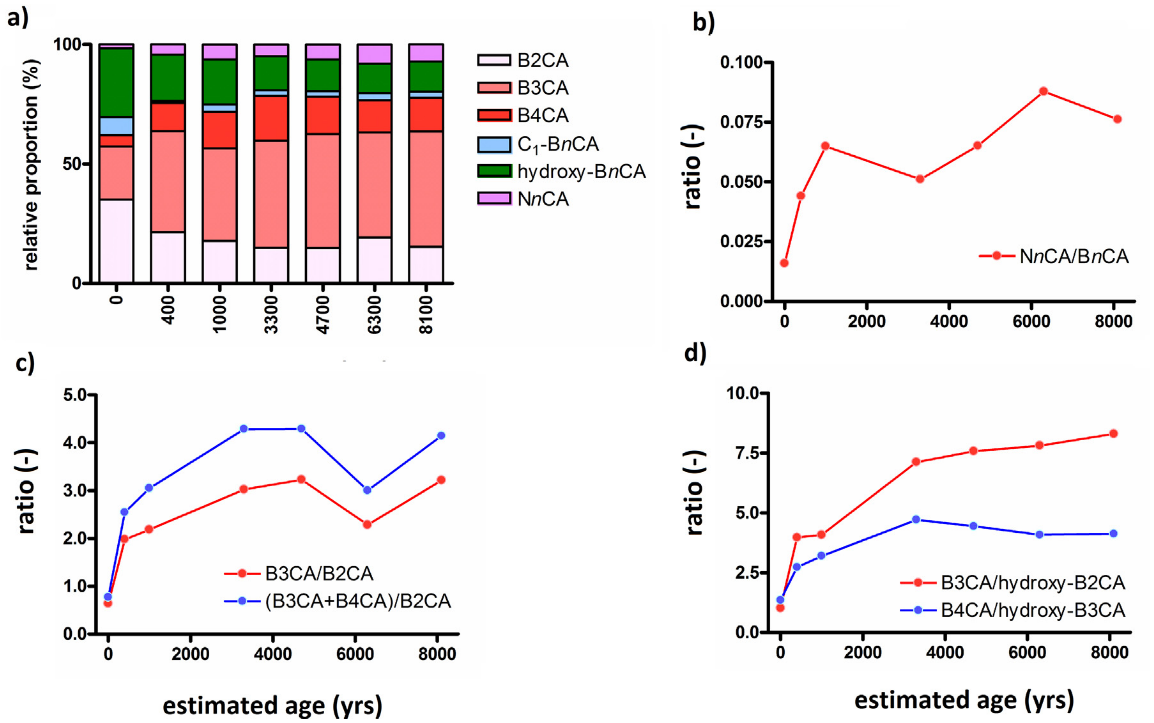

Fig. 4. (a) Age-dependent relative proportions of the unsubstituted benzene carboxylic acids (B*n*CA with *n* carboxylic groups) and total naphthalene carboxylic acids (N*n*CA), (b) N*n*CA/B*n*CA ratio, (c) B3CA/B2CA ratio (red line) and (B3CA + B2CA)/B2CA ratio (blue line), (d) B3CA/hydroxy-B2CA ratio (red line) and B4CA/hydroxyl-B3CA ratio (blue line, exaggeration ×2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

diagenetic oxidation, which seems to mimic thermal alteration and subsequent oxidation during wildfires. Regarding the humic substances; humic acid and fulvic acid extracts may contain black C and these samples are therefore not suitable for assessment of interference potential. In fact, the demonstrated presence of polyfunctionalized aromatic moieties in aged black C is a legitimate formation pathway of highly aromatic (Type 1) humic acids [59], and would explain why black C becomes increasingly hard to distinguish from the alleged humified non-pyrogenic sources [70–76]. Nevertheless, until this issue is resolved, in the THM-GC–MS analysis of environmental samples, interpretation of the B*n*CA should be made with caution.

4. Conclusions

For the first time, the use of labelled ^{13}C TMAH on aged black C samples allowed to discard the possibility of a significant amount of artificial B*n*CA and N*n*CA production during the THM reaction. The oxidized black C can be recognized from B*n*CA (B2CA-B4CA), methyl-B*n*CA (B2CA-B4CA), hydroxy-B*n*CA (B2CA-B3CA) and N*n*CA, altogether 27 markers. It appeared that with age, the oxidation patterns becomes more intense and proxies to reflect this process are proposed. It is concluded that THM-GC–MS is a promising tool for recognizing black C oxidation degree. A definite improvement of the method would be to create calibration mixtures of commercially available B*n*CA, methyl-B*n*CA, hydroxy-B*n*CA and N*n*CA, methylated with unlabelled or ^{13}C-labelled TMAH, to improve the quantification of the oxidized black C moieties. The data show that the technique is also useful to identify incompletely charred lignin in the charcoal.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jaap.2016.07.015.

References

[1] E.D. Goldberg, Black Carbon in the Environment, John Wiley and Sons, New York, 1985.
[2] C.A. Masiello, New directions in black carbon organic geochemistry, Mar. Chem. 92 (2004) 201–213.
[3] C.M. Preston, M.W.I. Schmidt, Black (pyrogenic) carbon: a synthesis of current knowledge and uncertainties with special consideration of boreal regions, Biogeosciences 3 (2006) 397–420.
[4] C. Santín, S.H. Doerr, E.S. Kane, C.A. Masiello, M. Ohlson, J.M. de la Rosa, C.M. Preston, T. Dittmar, Towards a global assessment of pyrogenic carbon from vegetation fires, Global Change Biol. 22 (2016) 76–91.
[5] J. Lehmann, J. Gaunt, M. Rondon, Bio-char sequestration in terrestrial ecosystems – a review, Mitig. Adapt. Strat. Global Change 11 (2006) 395–419.
[6] R.S. Kookana, A.K. Sarmah, L. van Zwieten, E. Krull, B. Singh, Biochar application to soil: agronomic and environmental benefits and unintended consequences, Adv. Agron. 112 (2011) 103–143.
[7] S. Jeffery, F.G.A. Verheijen, M. van der Velde, A.C. Bastos, A quantitative review of the effects of biochar application to soils on crop productivity using meta-analysis, Agric. Ecosyst. Environ. 144 (2011) 175–187.
[8] U. Hamer, B. Marschner, S. Brodowski, W. Amelung, Interactive priming of black carbon and glucose mineralisation, Org. Geochem. 35 (2004) 823–830.
[9] C.-H. Cheng, J. Lehmann, J.A. Thies, S.D. Burton, M.H. Engelhard, Oxidation of black carbon by biotic and abiotic processes, Org. Geochem. 37 (2006) 1477–1488.

[10] W.C. Hockaday, A.M. Grannas, S. Kim, P.G. Hatcher, Direct molecular evidence for the degradation and mobility of black carbon in soils from ultra high-resolution mass spectral analysis of dissolved organic matter from a fire-impacted forest soil, Org. Geochem. 37 (2006) 501–510.
[11] C. Gibson, T.D. Berry, R. Wang, J.A. Spencer, C.T. Johnston, Y. Jiang, J.A. Bird, T.R. Filley, Weathering of pyrogenic organic matter induces fungal oxidative enzyme response in single culture inoculation experiments, Org. Geochem. 92 (2016) 32–41.
[12] A.R. Zimmerman, Abiotic and microbial oxidation of laboratory-produced Black Carbon (Biochar), Environ. Sci. Technol. 44 (2010) 1295–1301.
[13] C.-H. Cheng, J. Lehmann, M.H. Engelhard, Natural oxidation of black carbon in soils: changes in molecular form and surface charge along a climosequence, Geochim. Cosmochim. Acta 72 (2008) 1598–1610.
[14] S.D. Joseph, M. Camps-Arbestain, Y. Lin, P. Munroe, C.H. Chia, J. Hook, L. van Zwieten, S. Kimber, D.A. Cowie, B.P. Singh, J. Lehmann, N. Foidl, R.J. Smernik, J.E. Amonette, An investigation into the reactions of biochar in soil, Aust. J. Soil Res. 48 (2010) 501–515.
[15] R. Calvelo Pereira, M. Camps Arbestain, M. Vazquez Sueiro, J.A. Maciá-Agulló, Assessment of the surface chemistry of wood-derived biochars using wet chemistry, Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy, Soil Res. 53 (2015) 753–762.
[16] K. Wiedner, D. Fischer, S. Walther, I. Criscuoli, F. Favilli, O. Nelle, B. Glaser, Acceleration of biochar surface oxidation during composting? J. Agric. Food Chem. 63 (2015) 3830–3837.
[17] B. Singh, Y. Fang, B.C.C. Cowie, L. Thomsen, NEXAFS and XPS characterisation of carbon functional groups of fresh and aged biochars, Org. Geochem. 77 (2014) 1–10.
[18] B. Glaser, J.J. Birk, State of the scientific knowledge on properties and genesis of anthropogenic dark earths in Central Amazonia (terra preta de Índio), Geochim. Cosmochim. Acta 82 (2012) 39–51.
[19] A.E. Downie, L. van Zwieten, R.J. Smernik, S. Morris, P.R. Munroe, Terra Preta Australis: reassessing the carbon storage capacity of temperate soils, Agr. Ecosyst. Environ. 140 (2011) 137–147.
[20] R. Calvelo Pereira, M. Camps Arbestain, J. Kaal, M. Vázquez Sueiro, M. Sevilla, J. Hindmarsh, Detailed carbon chemistry in charcoals from pre-European Maori gardens of New Zealand as a tool for understanding biochar stability in soils, Eur. J. Soil Sci. 65 (2014) 83–95.
[21] A. Rodionov, W. Amelung, L. Haumaier, I. Urusevskaja, W. Zech, Black carbon in the zonal steppe soils of Russia, J. Plant Nutr. Soil Sci. 169 (2006) 363–369.
[22] E. Eckmeier, R. Gerlach, E. Gehrt, M.W.I. Schmidt, Pedogenesis of chernozems in Central Europe – a review, Geoderma 139 (2007) 288–299.
[23] J. Kaal, A. Martínez Cortizas, K.G.J. Nierop, P. Buurman, A detailed pyrolysis-GC/MS analysis of a black carbon-rich acidic colluvial soil (Atlantic ranker) from NW Spain, Appl. Geochem. 23 (2008) 2395–2405.
[24] T. Carballas, P. Duchaufour, F. Jacquin, Évolution de la matière organique des rankers, Bull. Ec. Natl. Super. Agron. Nancy 9 (1967) 20–28.
[25] International Union of Soil Sciences – ISRIC World Soil Information – FAO, World Reference Base for Soil Resources 2006. A Framework for International Classification, Correlation and Communication. World Soil Resources Reports 103 (2006), Food and Agriculture Organization of the United Nations, Rome.
[26] Y. Carrión, J. Kaal, J.A. López-Sáez, L. López-Merino, A. Martínez Cortizas, Holocene vegetation changes in NW Iberia revealed by anthracological and palynological records from a colluvial soil, Holocene 20 (2010) 53–66.
[27] J. Kaal, S. Brodowski, J.A. Baldock, K.G.J. Nierop, A. Martínez Cortizas, Characterisation of aged black carbon using pyrolysis-GC/MS, thermally assisted hydrolysis and methylation (THM), direct and cross polarisation ^{13}C nuclear magnetic resonance (DP/CP NMR) and the benzenepolycarboxylic acid (BPCA) method, Org. Geochem. 39 (2008) 1415–1426.
[28] M. Gaviño, B. Hermosin, V. Vergès-Belmin, W. Nowik, C. Sáiz-Jiménez, Composition of the black crusts from the Saint Denis Basilica France, as revealed by gas chromatography-mass spectrometry, J. Sep. Sci. 27 (2004) 513–523.
[29] L. Remusat, S. Derenne, F. Robert, H. Knicker, New pyrolytic and spectroscopic data on Orgueil and Murchison insoluble organic matter: a different origin than soluble? Geochim. Cosmochim. Acta 69 (2005) 3919–3932.
[30] J.C. del Río, F.J. Gonzalez-Vila, F. Martin, T. Verdejo, Characterization of humic acids from low-rank coals by ^{13}C NMR and pyrolysis-methylation. Formation of benzenecarboxylic acid moieties during the coalification process, Org. Geochem. 22 (1994) 885–891.
[31] S.U. Khan, M. Schnitzer, Permanganate oxidation of humic acids fulvic acids, and humins extracted from Ah horizons of a Black Chernozem, a Black Solod and a Black Solonetz soil, Can. J. Soil Sci. 52 (1972) 43–51.
[32] K. Matsuda, M. Schnitzer, The permanganate oxidation of humic acids extracted from acid soils, Soil Sci. 114 (1972) 185–193.
[33] G. Almendros, F.J. González Vila, F. Martín, Room temperature alkaline permanganate oxidation of representative humic acids, Soil Biol. Biochem. 21 (1989) 481–486.
[34] H. Yoshioka, R. Ishiwatari, An improved ruthenium tetroxide oxidation of marine and lacustrine kerogens: possible origin of low molecular weight acids and benzenecarboxylic acids, Org. Geochem. 36 (2005) 83–94.
[35] M. Schnitzer, E. Vendette, Chemistry of humic substances extracted from an Arctic soil, Can. J. Soil Sci. 55 (1975) 93–103.
[36] A.F. Dickens, J.A. Gudeman, Y. Gélinas, J.A. Baldock, W. Tinner, F.S. Hu, J.I. Hedges, Sources and distribution of CuO-derived benzene carboxylic acids in soils and sediments, Org. Geochem. 38 (2007) 1256–1276.
[37] G. Almendros, J. Sanz, A structural study of alkyl polymers in soil after perborate degradation of humin, Geoderma 53 (1992) 79–95.
[38] P. Tinoco, G. Almendros, J. Sanz, R. González-Vázquez, F.J. González-Vila, Molecular descriptors of the effect of fire on continental Mediterranean soils under pine forest, Org. Geochem. 37 (2006) 1995–2018.
[39] R. Hayatsu, R.E. Winans, R.G. Scott, L.P. Moore, M.H. Studier, Oxidative degradation studies of coal and solvent-refined coal. Organic Chemistry of Coal, ACS Symp. Ser. 71 (1978) 108–125, http://dx.doi.org/10.1021/bk-1978-0071.ch007.
[40] B. Glaser, L. Haumaier, G. Guggenberger, W. Zech, Black carbon in soils: the use of benzenecarboxylic acids as specific markers, Org. Geochem. 29 (1998) 811–819.
[41] R.S. Tipson, Oxidation of polycyclic, aromatic hydrocarbons: a review of the literature, National Bureau of Standard, NBS Monograph, 87, (1965) pp. 62.
[42] F. Shadkami, R. Helleur, Recent applications in analytical thermochemolysis, J. Anal. Appl. Pyrol. 89 (2010) 2–16.
[43] P.G. Hatcher, R.D. Minard, Comment on the origin of benzenecarboxylic acids in pyrolysis methylation studies, Org. Geochem. 23 (1995) 991–994.
[44] I.C. Lewis, L.S. Singer, Electron spin resonance of stable aromatic radical intermediates in pyrolysis, Carbon 7 (1969) 93–99.
[45] L. Braier, Anti-benzene compounds. Conversion of benzene to toluene by methyl donors, in man, Rev. Bras. Pesqui. Med. Biol. 10 (1977) 319–323.
[46] S. Derenne, F. Robert, Model of molecular structure of the insoluble organic matter isolated from Murchison meteorite, Meteorit. Planet. Sci. 45 (2010) 1461–1476.
[47] T.R. Filley, P.G. Hatcher, W.C. Shortle, R.T. Praseuth, The application of ^{13}C-labeled tetramethylammonium hydroxide (^{13}C-TMAH) thermochemolysis to the study of fungal degradation of wood, Org. Geochem. 31 (2000) 181–198.
[48] T.R. Filley, Assessment of fungal wood decay by lignin analysis using tetramethylammonium hydroxide (TMAH) and ^{13}C-labeled TMAH thermochemolysis, In: B. Goodell, D.D., Nicholas, T.P. Schultz (Eds.), Wood Deterioration and Preservation: Advances in Our Changing World, ACS Symposium Series 845 (2003) 119–139.
[49] K.G.J. Nierop, T.R. Filley, Assessment of lignin and (poly-)phenol transformations in oak (*Quercus robur*) dominated soils by ^{13}C-TMAH thermochemolysis, Org. Geochem. 38 (2007) 551–565.
[50] J. Kaal, Y. Carrión Marco, E. Asouti, M. Martín Seijo, A. Martínez Cortizas, M. Costa Casáis, F. Criado Boado, Long-term deforestation in NW Spain: linking the Holocene fire history to vegetation change and human activities, Quat. Sci. Rev. 20 (2011) 161–175.
[51] J. Kaal, A. Martínez Cortizas, K.G.J. Nierop, Characterisation of aged charcoal using a coil probe pyrolysis-GC/MS method optimised for black carbon, J. Anal. Appl. Pyrol. 85 (2009) 408–416.
[52] T.R. Filley, R.D. Minard, P.G. Hatcher, Tetramethylammonium hydroxide (TMAH) thermochemolysis: proposed mechanisms based upon the application of ^{13}C-labeled TMAH to a synthetic model lignin dimer, Org. Geochem. 30 (1999) 607–621.
[53] T.R. Filley, K.G.J. Nierop, Y. Wang, The contribution of polyhydroxyl aromatic compounds to tetramethylammonium hydroxide lignin-based proxies, Org. Geochem. 37 (2006) 711–727.
[54] T. Klotzbücher, T.R. Filley, K. Kaiser, K. Kalbitz, A study of lignin degradation in leaf and needle litter using ^{13}C-labelled tetramethylammonium hydroxide (TMAH) thermochemolysis: comparison with CuO oxidation and van Soest methods, Org. Geochem. 42 (2011) 1271–1278.
[55] E. Clar, The Aromatic Sextet, J. Wiley, New York, 1972.
[56] F. Martín, J.C. del Río, F.J. González-Vila, T. Verdejo, Thermally assisted hydrolysis and alkylation of lignins in the presence of tetra-alkylammonium hydroxides, J. Anal. Appl. Pyrol. 35 (1995) 1–13.
[57] K.G.J. Nierop, C.M. Preston, J. Kaal, Thermally assisted hydrolysis and methylation of purified tannins from plants, Anal. Chem. 77 (2005) 5604–5614.
[58] P.G. Hatcher, M.A. Nanny, R.D. Minard, S.C. Dible, D.M. Carson, Comparison of two thermochemolytic methods for the analysis of lignin in decomposing wood: the CuO oxidation method and the method of thermochemolysis with TMAH, Org. Geochem. 23 (1995) 881–888.
[59] B. Chefetz, M.J. Salloum, A.P. Deshmukh, P.G. Hatcher, Structural components of humic acids as determined by chemical modifications and carbon-13 NMR pyrolysis-, and thermochemolysis-gas chromatography/mass spectrometry, Soil Sci. Soc. Am. J. 66 (2002) 1159–1171.
[60] K. Quénéa, S. Derenne, F.J. González-Vila, A. Mariotti, J.-N. Rouzaud, C. Largeau, Study of the composition of the macromolecular refractory fraction from an acidic sandy forest soil (Landes de Gascogne, France) using chemical degradation and electron microscopy, Org. Geochem. 36 (2005) 1151–1162.
[61] J.M. de La Rosa, J.A. González-Pérez, P.G. Hatcher, H. Knicker, F.J. González-Vila, Determination of refractory organic matter in marine sediments by chemical oxidation, analytical pyrolysis and solid-state ^{13}C nuclear magnetic resonance spectroscopy, Eur. J. Soil Sci. 59 (2008) 430–438.
[62] G. Almendros, F.J. González-Vila, F. Martín, J. Sanz, C. Álvarez-Ramis, Appraisal of pyrolytic techniques on different forms of organic matter from a Cretaceous basement in Central Spain, Org. Geochem. 28 (1998) 613–623.
[63] F.J. González-Vila, A. Amblès, J.C. del Río, L. Grasset, Characterisation and differentiation of kerogens by pyrolytic and chemical degradation techniques, J. Anal. Appl. Pyrol. 58–59 (2011) 315–328.

[64] C.-H. Cheng, J. Lehmann, J.A. Thies, S.D. Burton, M.H. Engelhard, Oxidation of black carbon by biotic and abiotic processes, Org. Geochem. 37 (2006) 1477–1488.

[65] C.-H. Cheng, J. Lehmann, Ageing of black carbon along a temperature gradient, Chemosphere 75 (2009) 1021–1027.

[66] A.R. Zimmerman, B. Gao, M.-Y. Ahn, Positive and negative carbon mineralization priming effects among a variety of biochar-amended soils, Soil Biol. Biochem. 43 (2011) 1169–1179.

[67] T. Dittmar, J. Paeng, A heat-induced molecular signature in marine dissolved organic matter, Nat. Geosci. 2 (2009) 175–179.

[68] F. Martín, F.J. González-Vila, J.C. del Río, T. Verdejo, Pyrolysis derivatization of humic substances 1. Pyrolysis of fulvic acids in the presence of tetramethylammonium hydroxide, J. Anal. Appl. Pyrol. 28 (1994) 71–80.

[69] F. Martín, G. Almendros, F.J. González-Vila, T. Verdejo, Experimental reappraisal of flash pyrolysis and low-temperature thermally assisted hydrolysis and methylation using tetramethylammonium hydroxide for the molecular characterization of humic acids, J. Anal. Appl. Pyrol. 61 (2001) 133–145.

[70] H. Shindo, Elementary composition humus composition, and decomposition in soil of charred grassland plants, Soil Sci. Plant Nutr. 37 (1991) 651–657.

[71] L. Haumaier, W. Zech, Black carbon – possible source of highly aromatic compounds of soil humic acids, Org. Geochem. 23 (1995) 191–196.

[72] J.O. Skjemstad, P. Clarke, J.A. Taylor, J.M. Oades, S.G. McClure, The chemistry and nature of protected carbon in soil, Aust. J. Soil Res. 34 (1996) 251–271.

[73] A. Golchin, P. Clarke, J.A. Baldock, T. Higashi, J.O. Skjemstad, J.M. Oades, The effects of vegetation and burning on the chemical composition of soil organic matter in a volcanic ash soil as shown by ^{13}C NMR spectroscopy. I. Whole soil and humic acid fraction, Geoderma 76 (1997) 155–174.

[74] M.W.I. Schmidt, J.O. Skjemstad, E. Gehrt, I. Kögel-Knabner, Charred organic carbon in German chernozemic soils, Eur. J. Soil Sci. 50 (1999) 351–365.

[75] R.W. Kramer, E.B. Kujawinski, P.G. Hatcher, Identification of black carbon derived structures in a volcanic ash soil humic acid by Fourier transform ion cyclotron resonance mass spectrometry, Environ. Sci. Technol. 38 (2004) 3387–3395.

[76] P.L. Ascough, M.I. Bird, S.M. Francis, T. Lebl, Alkali extraction of archaeological and geological charcoal: evidence for diagenetic degradation and formation of humic acids, J. Archaeol. Sci. 38 (2011) 69–78.