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)

Joeri Kaal ^{a,b,*} and Timothy R. Filley ^c

^a Institute for Heritage Science (Incipit), Spanish National Research Council (CSIC), Santiago de Compostela, Spain.

^b Departamento de Edafoloxía e Química Agrícola, Fac. Biología, Universidade de Santiago de Compostela, Santiago de Compostela, Spain.

^c Department of Earth and Atmospheric Sciences and the Purdue Climate Change Research Center, Purdue University, West Lafayette, IN, USA. E-mail: filley@purdue.edu.

* Corresponding author: E-mail: joeri@samage.net.

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 (*BnCA*, with *n* = number of carboxylic groups) and naphthalene analogues (*NnCA*) 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 *BnCA* and *NnCA* 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 ¹³C-labeled tetramethylammonium hydroxide (¹³C-TMAH) to oak-derived black C samples isolated from a colluvial soil profile dating to 50–8100 cal BP. The results showed that the *BnCA* and *NnCA* 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 *BnCA* (excluding B1CA) and *NnCA* increased with age of the black C ($P < 0.05$) from ca. 0.05 to 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.

Keywords: aged black C, biochar, THM-GC-MS, ¹³C-labeled TMAH, biomarkers

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 CO₂) [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.

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 (*BnCA*, with *n* carboxylic acid groups). Supported by intense resonances of carboxylic groups by solid-state ¹³C cross nuclear magnetic resonance (NMR), the *BnCA* were proposed to originate from oxidized black C moieties [27]. Other materials in which the *BnCA* 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 *BnCA* when applied to soil and sedimentary organic matter. More specifically, *BnCA* were produced upon oxidation by KMnO₄ [31-33], RuO₄ [34], CuO [35-36], NaBO₃ [37-38] and HNO₃ [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 *BnCA* and naphthalene carboxylic acids (*NnCA*) [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 *BnCA* in THM-GC-MS as having an artificial origin as well. In order to use *BnCA* and *NnCA* 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 *BnCA* or *NnCA*. Using ^{13}C labelled TMAH in the THM-GC-MS of aged black C the first pathway for artificial *BnCA* 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 *BnCA* and *NnCA* 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 (*BnCA* and *NnCA*), (2) to determine whether the carboxylic groups of the *BnCA* and *NnCA* are native or artificial and (3) to assess trends in oxidation patterns during the 8100 yrs period of storage in soil.

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, $243 \text{ (average)} \pm 30 \text{ (standard deviation)} \mu\text{g}$ 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 BnCA follow the equation $^{12}\text{C}_6 + (\text{H}_6-n) + (^{12}\text{COO})_n + (^{13}\text{CH}_3)_n$, 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") BnCA when using ^{13}C -TMAH the M^+ is one amu heavier for each carboxylic group than the analogous fragment ion from BnCA with native carboxylic groups. For both native and artificial BnCA, the base ion is produced upon elimination of the $-\text{O}^{13}\text{CH}_3$ moiety and therefore equals M^+-32 .

GC/MS response factors could not be determined for individual BnCA and NnCA because several substituted BnCA and NnCA 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 *BnCA*. We estimated the concentrations of the *BnCA* and *NnCA*, 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 *BnCA*) by a THM-GC-MS method using a Pyroprobe 5000 (CDS Analytical) and unlabelled TMAH to investigate production of *BnCA* and *NnCA* 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 *BnCA* or *NnCA* upon THM-GC-MS. THM-GC-MS of individual *BnCA* 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 alkanolic 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 *BnCA*, *NnCA* and lignocellulose derivatives (di- and trimethoxybenzenes). The *BnCA* and *NnCA* 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 *BnCA* and *NnCA* compounds with native carboxylic groups is presented in Table 2, which includes the masses of the ions when unlabelled TMAH would be used. Figure 1 shows the partial ion chromatograms of the native *BnCA* of selected samples (S1, S4 and S7).

3.3 Distinguishing native and artificial *BnCA* and *NnCA*

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 BnCA (i.e. methyl BnCA and hydroxy-BnCA) and NnCA 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 that artificial formation of BnCA and NnCA is negligible and that hydroxy-BnCA (B2CA, B3CA and B4CA), C₁-BnCA and NnCA 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 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].

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 BnCA (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 NnCA 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 NnCA reflect oxidized *polyaromatic* groups, structures not found in lignin or tannins, which implies that these compounds are useful markers of oxidized Black C. Furthermore, the NnCA 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 NnCA can provide evidence of oxidized black C in samples where non-pyrogenic components might interfere with the BnCA. Several authors reported the presence of NnCA 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 NnCA is kerogen from immature coals [62-63], albeit that NnCA were also detected among the THM products of macromolecular insoluble organic matter from various meteorites [29].

The sum of the BnCA and NnCA that are useful markers of oxidized Black C (B2CA, B3CA, B4CA, C₁-B2CA, C₁-B3CA, C₁-B4CA, hydroxy-B2CA, hydroxy-B3CA and NnCA) 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 NnCA (from 1 to 8 %; $P < 0.01$), while a decrease is observed for B2CA (from 35 to 15 %; $P < 0.05$) and hydroxy-BnCA (from 30 to 15 %; $P < 0.01$). The ratio of NnCA to BnCA 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 BnCA are the most abundant products, 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 BnCA and NnCA 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 BnCA and NnCA are the result of early 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 BnCA 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 *BnCA* and *NnCA* production during the THM reaction. The oxidized black C can be recognized from *BnCA* (B2CA-B4CA), methyl-*BnCA* (B2CA-B4CA), hydroxy-*BnCA* (B2CA-B3CA) and *NnCA*, 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 *BnCA*, methyl-*BnCA*, hydroxy-*BnCA* and *NnCA*, 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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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

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

Compound (isomers)* ¹	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

*¹Numbers 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).

Figure captions

Figure 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.

Figure 2: Partial ion chromatograms of unsubstituted benzene carboxylic acids (BnCA, 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 BnCA.

Figure 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-BnCA, f) hydroxy-BnCA, g) NnCA, h) sum all BnCA and NnCA.

Figure 4: a) Age-dependent relative proportions of the unsubstituted benzene carboxylic acids (BnCA with n carboxylic groups) and total naphthalene carboxylic acids (NnCA), b) NnCA/BnCA 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 x2).

Supplementary information S1: analysis of standard B_nCA, PAHs and alkylarenes