Structural features of humic acids from a sedimentary sequence in the Guadiana estuary
(Portugal-Spain border)

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Abstract

The structural characteristics of humic acids (HAs) from two different depths of a sedimentary sequence representing the last 13 kyr in the valley of Guadiana river estuary (SW Portugal/Spain border) have been approached using a combination of spectroscopic techniques, wet chemical degradation methods (sequential oxidation with sodium persulfate followed by KMnO₄, and oxidation with RuO₄) and analytical pyrolysis–gas chromatography/mass spectrometry (Py-GC/MS) in the presence and absence of tetramethylammonium hydroxide (TMAH). The aim was to obtain complementary information on the sources and alteration of the organic matter (OM) provided by a previous study on the vertical distribution of terrestrial and phytoplankton biogeochemical markers in the sequence. Both the FT-IR (Fourier transformed infrared) and ¹³C-NMR (nuclear magnetic resonance) spectra showed a close similarity with the structural characteristics of the HA samples. NMR signals in the alkyl region (0-45ppm) as well as FT-IR band patterns typical for methoxyl-substituted aromatic rings pointed to the presence of an important aliphatic domain, as well as to lignin derived compounds. This finding was confirmed using analytical pyrolysis. In addition, the main TMAH thermochemolysis products were typical lignin derived methoxyphenols with both guaicyl and syringyl nuclei. The detection of methoxyphenol units with three to six carbon atom (C₃ to C₆) side chains suggests that lignin and possibly suberin were only partially degraded. Compounds arising from proteins and polysaccharides were also detected, although in lesser and varying amount. The major products from persulfate oxidation were series of *n*-alkanes (C₁₆ -C₃₃ with clear odd/even

predominance) and *n*-fatty acids, both saturated (C₁₀–C₂₆ with strong even/odd predominance) and unsaturated, which may arise from the above aliphatic biomacromolecules. The major products from permanganate oxidation of the persulfate residue were α , ω -diacids (C₆–C₁₁) originating from oxidation of the ether bonds linking the building blocks constituting the core of the HA structure. Aromatic compounds (phenols, methoxy- dimethoxybenzene carboxylic acid and benzene di-, tri-, tetra- and pentacarboxylic acids), most probably derived from the aromatic backbone of the HAs, that may also include lignin moieties as well as other polyphenols (flavonoids and tannins) were also detected. The RuO₄ oxidation also released series of *n*-alkanes (C₁₆–C₃₃), linear saturated fatty acids (C₁₀–C₂₈) and α , ω -diacids (C₇–C₂₅), as well as traces of benzene polycarboxylic acids. Regarding the usefulness of the various techniques used, they provide complementary information. Indeed, spectroscopic techniques and analytical pyrolysis provide information on the backbone of the HAs, and on their origin, whereas the oxidative degradations provide different information on the structural features of the HA, particularly the nature of the linking between the building blocks. In general, the data support the idea that the HAs still contain information about the signature of aliphatic and aromatic biomacromolecules contributing to the OM deposited. The presence of lignin-derived residues suggests a large input from terrestrial carbon throughout the core.

Keywords: Holocene, sedimentary humic acids, lignin, FTIR and NMR spectroscopy, oxidative chemical degradations, pyrolytic techniques.

1. Introduction

Humic substances (HSs) are the transformation products of organic compounds in organised structures typical of organisms (e.g. carbohydrates, proteins and lipids) into randomly polymerized, heterogeneous, fairly stable and extremely complex refractory organic matter (OM). They occur ubiquitously in water, soil and sediments (Schnitzer and Khan, 1972; Aiken et al., 1985; Frimmel et al., 2002; Ghabbour and Davies, 2004). The formation of HSs can be considered the end of the diagenesis process in recent and fossil OM deposits. The need for studying them in recent sediments has been recognized in relation to the elucidation of biogeochemical processes in sediments, petroleum and coal, as well as to the ecology around sediments (Bordovskii, 1965;

69 Ishiwatari, 1971, 1992; Nissenbaun and Kaplan, 1972; Povoledo et al., 1975; Huc,
70 1988; Calace et al., 1999, 2004).

71 These previous studies demonstrated that the composition and structural features of HSs
72 may vary along a sedimentary column because of the influence of several factors, such
73 as source and amount of OM input, grain size composition, redox conditions,
74 bioturbation, etc.. It is therefore thought that HSs may preserve valuable information
75 about the evolutionary history of particular sediments in response to climatic and/or
76 environmental factors. In fact, an important proportion of the organic compounds
77 deposited in sediments (lipids in particular) over periods of time, thereby embracing
78 significant alteration in the environment, can be incorporated and preserved within the
79 poorly defined structure of these macromolecular materials (del Rio et al., 1989).

80 This work is part of a wider study of the diagenetic processes in SW Portugal/Spain
81 border estuarine areas, based on the molecular characterisation of the different forms of
82 OM deposited in the area of the Guadiana river estuary. In a previous study, the vertical
83 distribution of lipid markers was described along a 53 m core representing a record of
84 the last 13 kyr (González-Vila et al., 2003). It complemented previous sedimentological
85 and palynological proxies, revealing information about vegetation history, OM
86 diagenesis and possible anthropogenic interference, as well as climatic and
87 environmental changes (mainly derived from sea level changes) during the Holocene.

88 In order to provide complementary information about the source and alteration of the
89 sedimentary OM, we present herethe results obtained from the analysis of HAs isolated
90 from different depths in the Guadiana core. Our approach was to use a combination of
91 complementary analytical tools that included spectroscopic non-destructive techniques
92 (FT-IR and ^{13}C -NMR), wet chemical degradation methods (sequential oxidation with
93 sodium persulfate ($\text{Na}_2\text{S}_2\text{O}_8$), followed by KMnO_4 , and RuO_4 , oxidation) and, to detect
94 important constituents and biomarkers which could remain "invisible" under chemolysis
95 or spectroscopic techniques, Py-GC-MS in the presence and absence of TMAH was also
96 used.

2. Materials and methods

2.1. Sampling

The HAs were isolated from two different depth sections of a borehole (CM5) drilled to ca. 53 m near the confluence of the Beliche and Guadiana rivers and located in the intertidal zone of the latter. As HA structural characterisation using spectroscopic and degradative techniques performed in duplicate requires a minimum of 500 mg, the HA samples could only be isolated by using core sections of 1 m. The sections chosen were between 14 and 15 m (HA-14), and 38 and 39 m (HA-38), selected as representative of two well differentiated depositional environments, as revealed by previous biomarker analyses (González-Vila et al., 2003).

The geological and chronological setting of the core has been described previously (González-Vila et al., 2003). Guadiana is a major river on the Iberian Peninsula; its total length is 730 km, the last 200 km of which forms a natural border between Portugal and Spain. In this section, the river bed is divided into Upper Carboniferous schists and greywackes and follows a N–S path defined during the Quaternary (Morales, 1997).

The resistant character and faulting of the basement rocks contributed to the narrow and deep pattern of the river valley incision during marine lowstands. Boreholes and seismic profiles indicate that the palaeo-valley was 600 m wide and 70 m below mean sea level, ca 7 km inland from the mouth (Boski et al., 2002). These characteristics distinguish the Guadiana estuary from the others in the Guadalquivir basin, which are cut into soft non consolidated Plio-Pleistocene sediments in the form of broad and shallow structures (Borrego et al., 1999).

Bulk geochemical characteristics of the core have been described previously. In short, total organic carbon (TOC) content varies through the core from 0.7 to 2.3%; downcore the C/N ratio ranges between 8 and 16, indicating the occurrence of estuarine mixing of marine and terrestrially derived OM inputs, with seasonal variation being a consequence of a balance between fluvial and tidal influence; a decrease in the atomic C/S ratio in the lower sections of the core (> 10 m) may be considered as an indication of a strengthening of sulfate reduction, probably related to a transfer of larger quantities of reactive OM to the anoxic zone (Gonzalez-Vila et al., 2003 and references therein).

2.2. Isolation and purification of HAs

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132 The HSs were extracted (x 5) from lipid-free sediment. Lipids were removed from the
133 sediment with Soxhlet extraction using CH_2Cl_2 :MeOH (2:1). Lipid-free samples were
134 extracted with a mixture 0.1 M $\text{Na}_4\text{P}_2\text{O}_7$ and 0.1 M NaOH and the dark brown
135 supernatant (total humic extract) was precipitated by adjusting the pH to 2 with 12 M
136 HCl, de-ashed with 1 M HCl–HF at room temperature for 12 h, redissolved in 0.5 M
137 NaOH and centrifuged at 43500 g. The residue was discarded, and the brown sodium
138 humate supernatant was reprecipitated with 12M HCl and dialysed in cellophane bags
139 to remove the salts introduced during the extraction procedure. The HA was then freeze
140 dried and kept for further chemical characterisation.

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142 2.3. Elemental analysis

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144 The elemental composition was determined with a Carlo-Erba EA-1108-CHNS
145 microanalyzer. Oxygen concentration was calculated by difference. All results (Table 1)
146 were calculated on an ash-free basis. Ash content was determined by ignition at 550°C
147 for 5 h.

148

149 2.4. Spectroscopy

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151 FT-IR spectra were acquired with a Bruker IFS28 spectrophotometer using KBr pellets
152 (2.00 mg sample in 200 mg KBr). The spectra were acquired by accumulating 100
153 interferograms at 2 cm^{-1} resolution. The spectra in digital format were subjected to a
154 procedure for resolution enhancement based on the subtraction of the raw spectrum
155 from a positive multiple of its second derivative (Rosenfeld and Kak, 1982). The
156 intensity (absorbance) of the major peaks was measured and the values were normalized
157 by dividing them by the peak band intensity at 1510 cm^{-1} . This band is quite
158 characteristic for skeletal ring vibrations in heterogeneous macromolecular material
159 such as lignins or humic substances and its intensity is considered to be little influenced
160 by aliphatic lignocellulose constituents (Marton and Sparks, 1967; Dupuis and Jambu,
161 1969; Almendros et al., 1991). The values for the various ratios are shown in Table 2.
162 Solid state ^{13}C NMR analysis was performed using a Bruker MSL 100 spectrometer
163 (2.3 Tesla), operating at a ^{13}C resonance frequency of 25.1 MHz and using a Bruker
164 double-bearing probe with 7 mm outer diameter, phase-stabilized zirconium dioxide

rotors. The cross polarization (CP) magic angle spinning (MAS) technique was applied at 6.8 kHz. A ramped ^1H pulse during contact time (1 ms) was used in order to circumvent Hartmann–Hahn mismatches (Peersen et al., 1993). About 50000 scans were accumulated using a pulse delay of 250 ms. Prior to Fourier transformation, a line broadening between 25 and 125 KHz was applied to determine the relative C distribution. The ^{13}C chemical shift scale is referenced to tetramethylsilane (0 ppm) and adjusted with glycine (176.04 ppm) as external standard. For quantification, the spectra were divided into different chemical shift regions according to Knicker and Lüdemann (1995). The relative C distribution was determined by integrating the signal intensity in different chemical shift regions with an integration routine supplied with the instrument software. A correction for spinning side band signal intensity of aromatic C (160–110 ppm) and carboxyl C (220–160 ppm) was made according to Knicker et al. (2005).

2.5. Py-GC/MS

Samples (ca. 1 mg) were analysed using Curie-point pyrolysis with a Horizon Instruments device attached to a Varian Saturn 2000 GC/MS system. Samples were flash heated on ferromagnetic wires at 610°C for 5s. The interface of the Py unit was set to 250°C and the GC oven temperature was programmed from 50 to 100°C at 32°C min⁻¹ and then to 320°C at 6°C min⁻¹. The injection port, attached to a liquid CO₂ cryogenic unit, was adjusted from -30°C (1 min) to 300°C at 20°C min⁻¹. A fused-silica column (25 m × 0.32 mm × 0.4 µm) coated with CPSil 5CB (J&W Scientific) was used. Mass spectra were acquired at 70 eV ionising energy. For better detection of lignin residues that might be preserved in the sediment, thermochemolysis in the presence of TMAH was performed; ca. 0.5 mg of finely powdered sample (agate mortar) was placed in a 2ml glass ampoule and 100 µL of a solution of 5 ml of 25% TMAH in CH₃OH was added (Martín et al., 2001). The CH₃OH was evaporated to dryness under vacuum and the residue analyzed using Py-GC/MS under the same conditions as above. Assignment of individual compounds was achieved using single ion monitoring for different homologous series, low resolution mass spectrometry and comparison with published (Ralph and Hatfield, 1991) and stored (NIST and Wiley libraries) data.

2.6. Chemical degradation

The HAs was submitted to two oxidative treatments, using sodium persulfate followed by KMnO_4 (Almendros et al., 1987) and RuO_4 (González-Vila et al., 1994a; Reiss et al. 1997). In each case, 150–200 mg of HA were treated.

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2.6.1. Sequential oxidation

Sequential degradation was performed in order to get additional information on the speciation status of structural components within different organizational levels in HAs. The samples were heated at 140 °C for 2 h in a Teflon digestion bomb with 25 ml of a 25% wt solution of $\text{Na}_2\text{S}_2\text{O}_8$ in water. After cooling, the reaction mixture was centrifuged (15 min at 3000 rpm), and the pellets were re-suspended in 50 ml water and re-centrifuged (x 3; Martín et al., 1981, 1983). The residue was oxidized with KMnO_4 by adding 0.5 g Na_2CO_3 , 25 ml water and 35 ml 0.25 M KMnO_4 at a rate of 3 ml.min⁻¹. After 2 h at room temperature with stirring, the reaction mixture was acidified to pH 3 and the residual KMnO_4 was reduced with $\text{Na}_2\text{S}_2\text{O}_5$ (Matsuda and Schnitzer, 1971; Almendros et al., 1989). The combined digests obtained after the first and second oxidation were extracted with ethyl acetate, dried under reduced pressure, suspended in CH_3OH and methylated with trimethylsilyldiazomethane (Hashimoto et al., 1981). The methylated samples were analysed using a Hewlett Packard HP-GCD system equipped with a 30 m column coated with DB5 (0.32 mm i.d., 0.5 µm film thickness). The GC oven was programmed from 100 to 300°C at 6°C min⁻¹ and the mass spectrometer operated at 70 eV (range m/z 45–450 with a cycle time of 0.7 s). Peak identification was carried out by matching with mass spectra stored in the computer libraries and by comparison with data in the bibliography.

2.6.2. RuO_4 oxidation.

This was performed by adding to the HA 5 ml CCl_4 , 10 ml distilled water, 5 ml CH_3CN , 2.5 g NaIO_4 and 25 mg RuO_4 . After vigorous stirring at room temperature for 4 h, the reaction was quenched by addition of 10 ml isopropanol. The reaction mixture was filtered after addition of 200 ml CH_2Cl_2 . The solid residue was washed with 200 ml water and the aqueous filtrate extracted with CH_2Cl_2 . The extracts were combined, concentrated with a rotary evaporator and methylated with trimethylsilyldiazomethane. The methylated extract was analysed using the above GC/MS conditions.

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3. Results and discussion

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232 3.1. Elemental analysis

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234 The results of the elemental analysis (C, H, N, O, S) of the HAs expressed on a dry ash
235 free weight, as well as C/H, S/C, O/C and C/N ratios, are presented in Table 1. The
236 different values and ratios are within the typical ones reported for HAs from recent
237 sediments (Povoledo et al., 1975; Ishiwatari, 1992), also included in Table 1 for
238 comparison. The HA isolated from the deeper section (HA-38) has higher C, H and N
239 contents than that isolated from the upper one (HA-14), whereas the latter contains
240 higher O and S contents. There is a positive correlation between O content and
241 functionality, since O is mostly bound in acidic carboxylic and phenolic groups
242 (Schnitzer and Khan, 1972). The S content of HA-38 is on the higher side of the range
243 (0–2%) given by Yariv and Cross (1979) for geological colloidal systems. Meanwhile,
244 the S content of the HA-14 is very high, suggesting the presence of reducing conditions
245 in the depositional environment, favouring the idea that elemental S and H₂S can be
246 incorporated into the organic framework (Casagrande et al., 1980; Dellwing et al.,
247 1998).

248 The H/C ratio is believed to be a measure of the degree of unsaturation and aromaticity
249 of HSs (Stevenson, 1994). The lower H/C ratio for HA-14 is indicative of a greater
250 degree of unsaturation and aromaticity, and lower aliphaticity than HA-38. An increase
251 in C/N is observed with depth, which in this hydromorphic environment may indicate a
252 preservation of peptide material. The higher C/N value for HA-38 suggests differences
253 in humification; organic N rather than organic C is lost preferentially during the
254 decomposition of OM during early sediment diagenesis (Meyers, 1994; Hedges and
255 Oades, 1997). This is a classical mechanism which could explain a C/N atomic ratio
256 increase during OM decomposition. Finally, the decreases in the value of the O/C ratio
257 suggest a lower degree of carboxylation with depth.

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259 3.2. Spectroscopic features

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261 No apparent differences were found between the conventional FT-IR spectra of the two
262 HAs. The spectra are typical for HSs, with broad bands and marked band overlapping.
263 This problem was overcome by using the resolution enhancement procedure described
264 above. In Fig. 1 the most informative part of the spectra is shown. According to
265 classical assignments (MacCarthy and Rice, 1985), the spectra show a contribution from

OH groups via a broad band maximizing at 3292 cm^{-1} (not shown) and a moderate contribution from C-H stretching bands of alkyl groups at $2925\text{--}2854\text{ cm}^{-1}$ (not shown), indicating the partly aliphatic nature of both HAs, carbonyl groups (absorption at 1750 and 1720 cm^{-1}) and aromatic and olefinic C=C bonds (bands centred at 1620 and 1510 cm^{-1}). Neat amide bands at 1650 and 1550 cm^{-1} (Farmer and Morrison, 1960) typical for algal-derived HAs (Huc et al., 1974) are also present. The occurrence of aromatic and carboxylic C=O absorptions could reveal the presence in the HAs of lignin (or related compounds) from decaying vascular plants. C–O stretching of polysaccharides appeared around 1030 cm^{-1} , indicating that the OM is in a very early stage of diagenesis (Calace et al., 1999). Assignment of this signal to Si–O stretching of silica compounds should be discarded due to the low silica content (ash content $< 5\%$) of the samples. A typical lignin pattern, consisting of bands centred mainly at 1510 , 1460 , 1420 , 1270 , 1230 and 1130 cm^{-1} (Farmer and Morrison, 1960) is evident. Some band ratios are depicted in Table 2; this points to a slightly higher aromatic character for HA-38, with higher values of the $2920/1510$ ratio (alkyl/aryl), whereas HA-14 shows greater functionality, with higher values of $1720/1510$ (C=O/aryl).

Fig. 2 shows the solid state ^{13}C NMR spectra for the two HA samples as well as one sample for which lipids were not extracted before HA extraction. As would be expected, the main difference in the HS without previous lipid extraction is in the signal corresponding to alkyl C ($0\text{--}45\text{ ppm}$). Table 3 shows the semi-quantitative distribution of the different C types in the lipid-free HAs. Resonances in the four major regions of the spectra are found: aliphatic C in the range 0 to 45 ppm , C of *O*-alkyl structures (mainly of carbohydrate origin) from 45 to 110 ppm , aromatic C between $110\text{--}160\text{ ppm}$, and carboxyl and carbonyl groups in the range 160 to 200 ppm . The spectral profiles suggest an appreciable amount of aromatic constituents. However, aromatic ethers and alcohols, i.e. from propanyl side chains of lignin units or tannins can also contribute to the intensity of spectral peaks at 156 ppm and 45 ppm (Preston et al., 1997). The spectra show typical signals centred at 172 ppm from carboxyl C, 56 ppm (methoxyl and α -amino Cs) and prominent signals at 32 ppm assigned to alkyl C in polymethylene structures from lipid polymers or condensed wax material. Also, a peak for C-2, C-3 and C-5 carbons in sugar-derived structures (73 ppm) is apparent. An indication of the presence of lignin is given by the signals corresponding to the region for *O*-substituted aromatic C between 160 and 140 ppm along with the methoxyl groups at 56 ppm (Lüdemann and Nimz, 1974; Wilson, 1987).

The integration values of the different C types (Table 3) only reflected small differences between the HAs from the two depths for most regions, except for aryl C which increased with sediment depth.

3.3. *Py-GC/MS*

Fig. 3 shows partial pyrograms of the two HAs in the absence (Fig. 3a) and presence of TMAH (Fig. 3b). The chromatographic conditions allow only the study of short chain compounds and therefore the distribution of series potentially extending further than C₁₆ are not discussed. Again, no noticeable qualitative fluctuations were observed in the pyrolytic behaviour of the two HAs and the pyrolyzate differences were mainly quantitative.

The compounds (Tables 4 and 5) were typical Curie–Point pyrolytic products of HAs from various origins (Martín et al., 1979, 2001; Almendros et al., 1993; Leinweber and Schulten, 1999), with phenolic lignin derived compounds as major pyrolysis products. Major compounds released were phenolic molecules that in most cases showed typical methoxyl substitutions, suggesting a clear origin from lignin, although a suberin contribution cannot be excluded (Nierop, 1998). In addition, phenolic compounds of nonspecific and/or multiple origin(s) such as phenol (**6**), vinylphenol (**20**) and C₁–C₅ alkyl phenols were detected.

Other minor pyrolysis compounds were present, although in lesser and varying amount: some N-bearing compounds with a probable protein origin (**15**, **26**) and furan derivatives arising from polysaccharides (**3**, **4**, **7**, **13**, **42**).

Products released from HAs via TMAH thermochemolysis confirm the occurrence of typical lignin derived methoxyphenols. As a result of its resistance to biodegradation, lignin has been frequently detected in geological materials using different procedures and is considered a source indicator for terrestrial plant input (González-Vila et al., 1994b). The result of a specific search for lignin derived products using single ion monitoring (sum of *m/z* 124+154, 138+168, 152+182, 150+180, 164+194, 160+196) is shown in Table 5 and corresponds to compounds dotted in Fig 3b. Compounds with guaicyl (G), syringyl (S) and *p*-coumaric nuclei were detected, indicating both the contribution of hardwood lignin and angiosperms to the sedimentary organic matter. The detection of methoxyphenol units with three to six carbon atom side chains suggests that the lignin is only partially degraded in this environment. It must also be

334 noted that suberin is also composed of some proportion of C₃-C₆ aromatic structures
335 (Kolattukudy, 1976).

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337 3.4. Wet chemical degradation

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339 No apparent differences were observed regarding the behaviour of the two HAs against
340 the wet chemical treatments. Table 6 shows the compounds identified after the two
341 oxidative treatments on the two HA samples, with an indication of their semi-
342 quantitative abundances. The study of the oxidation products obtained after sodium
343 Na₂S₂O₈ degradation, followed by oxidation with KMnO₄ was informative with respect
344 to elucidating bond strength and molecular structural organization in the HAs.
345 Persulfate oxidation, a mild degradation method, has been used for studying the most
346 accessible and easily released components of humic fractions (Martín et al., 1981,
347 1983). It is supposed to yield compounds associated with high molecular weight
348 structures via physical interactions (like molecular encapsulation) or through easily
349 cleaved covalent bonds, and resulted in ca. 60 % degradation of the initial HA, as
350 determined by weight loss. The oxidation yields mainly a series of *n*-alkanes (C₁₆-C₃₃
351 with a clear odd/even predominance and largely dominated by the longer chain
352 homologues), indicating a dominant plant input. The alkanes are probably trapped
353 within the macromolecular network (Amblès et al., 1996). Also, series of *n*-fatty acids,
354 both saturated (C₁₀-C₂₆ with strong even/odd predominance), unsaturated (maximum at
355 C₁₆ and C₁₈) were released, as well as branched (*iso* and *anteiso*) fatty acids which
356 indicate a microbial origin.

357 Oxidation with alkaline permanganate of the persulfate residues resulted in a much
358 more extensive degradation (no residue), affording predominantly aliphatic series of
359 α,ω -alkanedioic (C₆-C₁₂ with a maximum at C₉) and saturated alkanoic acids (C₁₀-C₃₁,
360 with maxima at C₁₆ and C₁₈ and strong even/odd predominance), as well as aromatic
361 compounds (phenols, hydroxydimethoxybenzene carboxylic acid and benzene di-, tri-,
362 tetra- and pentacarboxylic acids) in relatively much lesser amounts. The former
363 evidence the contribution of lipids to the macromolecular structure, whereas the
364 aromatic compounds were most probably derived from the aromatic backbone of the
365 HAs, including lignin moieties and other polyphenols (flavonoids and tannins). Benzene
366 polycarboxylic acids in humic acids remain a subject of controversy (Maximov et al.
367 1977; Almendros et al, 1989). In this study, benzene they were detected mainly after the

368 most drastic oxidative treatments (KMnO_4 after $\text{Na}_2\text{S}_2\text{O}_8$ and RuO_4), and are thought to
369 be formed upon oxidation of condensed polyaromatic structures or polysubstituted
370 aromatic rings (Quénée et al, 2005).

371 The high proportion of diacids may originate from the oxidation of ether bonds linking
372 the building blocks that form the core of the HA structure, as recently pointed out by
373 Quénée et al. (2005), when studying refractory OM from soils using wet chemical
374 degradation. In agreement with these authors, the main short chain diacids around C_9 ,
375 may reflect the occurrence in the HA of moieties originating from the condensation of
376 unsaturated lipids. Indeed, $\omega 8$, $\omega 9$ and $\omega 10$ double bonds commonly occur in biolipids
377 and the oxidative cross linking of such unsaturated lipids could contribute to the
378 formation of resistant geomacromolecules, as previously considered for the formation of
379 some humin moieties (Almendros et al, 1991; Almendros and Sanz, 1992; Quénée et al.,
380 2005).

381 Oxidation with RuO_4 preferentially oxidises aromatic carbons but also cleaves several
382 types of functional groups and releases the alkyl substituents of aromatic rings.
383 (González-Vila et al., 1994a, 2001; Blokker et al., 1998, 2000; Boucher et al., 1989;
384 Schouten et al., 1998; Bajc et al., 2001; Kribii et al., 2001; Quénée et al., 2005). Non-
385 substituted aromatic carbon atoms are oxidised to CO_2 , so that isolated aromatic rings
386 are entirely degraded. This means that in the present case the oxidation was not efficient
387 enough, since some benzene polycarboxylic acids were detected.

388 RuO_4 oxidation degraded ca. 90% of the original material, releasing series of *n*- α,ω -
389 alkanedioic acids, *n*-alkanes and *n*-fatty acids. The *n*-alkanes range from C_{16} to C_{33} .
390 The main series of *n*-alkanoic acids ranges from C_{10} to C_{28} , exhibiting a maximum at
391 C_{16} . In the series, long chain (C_{22} – C_{28}) homologues are not abundant and even carbon
392 number homologues predominate over the whole range. The distribution and the
393 abundance of the alkanoic acid series are similar to those observed for the permanganate
394 treatment that represent, both for the RuO_4 and permanganate reaction products, a minor
395 series of predominantly even numbered compounds with an extended range and a
396 maximum at C_{16} . The α,ω -alkanedioic acid series ranges from C_7 to C_{25} , with a low
397 contribution of long chain homologues and no clear cut odd or even carbon number
398 predominance. As discussed above, the short chain diacids may also originate from
399 alkyl bridges between aromatic rings. However, the differences in distribution vs. the
400 permanganate products might also indicate a direct contribution of suberin or any other

aliphatic biopolymer as the source of the long chain diacids, rather than alkyl bridges between aromatic units (Quénée et al., 2005).

4. Conclusions

The different approaches used show that the HAs isolated from the studied sedimentary sequence contains valuable information on the signature of aliphatic and aromatic biomacromolecules originally contributing to the deposited OM. Our data show the presence of lignin-derived residues that suggest a large input from terrestrial carbon throughout the core. The HA fraction released typical methoxyphenols with both guaiacyl and syringyl skeletons, pointing to the presence of a microbially reworked, not extensively demethoxylated lignin.

In general, a correlation between pyrolytic (Py-GC-MS) and spectroscopic (^{13}C NMR and FT-IR) data was found, so the reliability of the quantitative distribution of C atoms pertaining to alkyl and aromatic structures obtained from NMR was in acceptable agreement with the pyrolysis products released. Both spectroscopic techniques (FT-IR and ^{13}C -NMR) showed close similarities between the structural characteristics of the HA isolated at different depth along the core. Specific spectral features pointed to the presence of an important aliphatic domain in the structure of the HAs and to the presence of methoxyphenols from lignin. This observation was confirmed from Py-GC/MS analysis of the two HAs. Fragments arising from pyrolysis of protein and polysaccharide were detected in trace amounts, with the exception of some individual compounds. Both pyrolysis and thermoevaporation products can be considered biomarkers of the input from different biogenic sources, mainly terrestrial plants and plankton. In addition, the main TMAH thermochemolysis products detected were typical lignin derived methoxyphenols, with both guaiacyl and syringyl nuclei. The detection of $\text{C}_6\text{-C}_3$ units suggests that the lignin is only partially degraded in this environment. Different compounds arising from proteins and polysaccharides were also detected, although in lesser and varying amount. When the two pyrolytic techniques are compared, it appears that Py/TMAH emphasizes better the aliphatic and lignin contribution.

The major products from persulfate oxidation were series of *n*-alkanes ($\text{C}_{16}\text{-C}_{33}$ with clear odd/even predominance) and *n*-fatty acids, both saturated ($\text{C}_{10}\text{-C}_{26}$ with strong even/odd predominance) and unsaturated, which may arise from the above aliphatic

biomacromolecules. The major products from permanganate oxidation of the persulfate residue were α , ω -diacids (C_6 - C_{11}) originating from oxidation of the ether bonds linking the building blocks constituting the core of the structure. The aromatic compounds (phenols, methoxy- dimethoxybenzene carboxylic acid and benzene di-, tri-, tetra- and pentacarboxylic acids) in the permanganate degradation products most probably derived from the aromatic backbone of the HAs, that may also include lignin moieties as well as other polyphenols (flavonoids and tannins). The high yielding RuO_4 oxidation also released series of *n*-alkanes (C_{16} - C_{33}), linear saturated fatty acids (C_{10} - C_{28}) and α , ω -diacids (C_7 - C_{25}), as well as traces of benzene polycarboxylic acids. Regarding the usefulness of the various techniques used for the characterization of the sedimentary HA, they provide complementary information. Indeed spectroscopic techniques and analytical pyrolysis provide information on the backbone of the HAs and on their origin, whereas the wet oxidative degradations provide different information on the structural features of the HA, particularly on the nature of the linking between the building blocks. In general, the data support the idea that the HAs retain information about the signature of aliphatic and aromatic biomacromolecules contributing to the OM deposited. The presence of lignin-derived residues suggests a large input from terrestrial carbon throughout the core.

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TABLE CAPTIONS

Table 1

Elemental (ash free) composition (wt%) and atomic ratios for humic acids from two depths in core CM-5 of Guadiana sediments

Table 2

FT-IR ratios for humic acids from two different depths in core CM-5 of Guadiana sediments

Table 3

Intensity distribution (%) in solid-state ^{13}C NMR spectra of lipid-free humic acids from two different depths in core CM-5 of Guadiana sediments

Table 4

Compounds released after pyrolysis of humic acids from two different depths in core CM-5 of Guadiana sediments, with an indication of origin (Lg: lignins; Pp polyphenols; Pr proteins; Ps polysaccharides)

Table 5

Lignin derived compounds released after pyrolysis in presence of TMAH of humic acids from two different depths in core CM-5 of Guadiana sediments

Table 6

Distribution of products by oxidation with different reagents from humic acids from two different depths in core CM-5 of Guadiana sediments

Table 1

	C%	H%	N%	S%	O%	C/N ^a	H/C ^a	O/C ^a	S/C ^a
HA-14	42.74	4.88	3.31	7.20	41.87	15.5	1.4	0.7	0.06
HA-38	47.32	6.30	3.49	1.55	41.34	16.4	1.6	0.6	0.01
HA recent sediments ^b	41-61	4-7	0.6-4	0.1-7.6	40-45				

^a Atomic ratio.

^b Range found for HA from recent sediments (Povoledo et al., 1975; Ishiwatari, 1992).

Table 2

	3400/151	2920/151	1720/151	1460/151	1270/151	1030/151
	0	0	0	0	0	0
HA-14	1.1	1.2	1.3	1.1	1.1	0.6
HA-38	1.1	1.3	1.3	1.2	1.1	0.7

^a Relative to bands at 1510 cm⁻¹ (aromatic C=C stretch).

Table 3

	Chemical shift (ppm)						
	Carbonyl C 245-185	Carboxyl C 185-160	Aryl C 160-110	O-Alkyl-C 110-45	Alkyl C 45-0	O-Alk/Alk	Alk /Carboxyl
HA-14	3.1	12.0	37.9	21.7	25.3	0.6	2.1
HA-38	5.0	14.0	43.3	15.6	22.1	0.4	1.6

Table 4

Ref.	Compound	Origin
1	Benzene	Pp
2	Toluene	Pp/Pr
3	2- Acetylfuran	Ps
4	2,3-Dihydro-5-methylfuran-2-one	Ps
5	Styrene	Pp
6	Phenol	Pp/Lg/Pr
7	4-Hydroxy-5,6-dihydro-(2H)-pyran-2-one	Ps
8	2-Methoxytoluene	Pp/Pr
9	2,3-Dimethylcyclopenten-1-one	Ps
10	3-Methylphenol	Pp/Lg/Pr
11	4-Methylphenol	Pp/Lg/Pr
12	Guaiacol	Lg
13	Levogluconone	Ps
14	2,6-Dimethylphenol	Pp/Lg
15	Phenylacetonitrile	Pp
16	2,4-Dimethylphenol	Pp/Lg
17	Ethylphenol	Pp/Lg
18	4-Methylguaiacol	Lg
19	Catechol	Pp
20	4-Vinylphenol	Pp/Lg
21	<i>cis</i> -4-Propenylphenol	Pp/Lg
22	Ethylphenol	Pp/Lg
23	3-Methoxycatechol	Pp
24	4-Ethylguaiacol	Lg
25	Methylcatechol	Pp
26	Indole	Pr
27	1,4-Dideoxy- <i>D</i> -glycerohex-1-enopyranos-3-ulose	Ps
28	4-Vinylguaiacol	Lg
29	1-(4-Hydroxyphenyl)ethanol	Lg
30	4-Hydroxybenzyl alcohol	Pp
31	<i>trans</i> 4-Propenylphenol	Pp/Lg
32	Syringol	Lg
33	Eugenol	Lg
34	5-Ethylpyrogallol	Lg
35	4-Propylguaiacol	Lg
36	1,4-Dihydroxy-3-methoxybenzene	Pp
37	3-Methylindole	Lg
38	Vanillin	Lg
39	<i>cis</i> -Isoeugenol	Lg
40	4-Methylsyringol	Lg
41	<i>trans</i> -Isoeugenol	Lg
42	Levogluconane	Ps
43	1-(4-Hydroxy-3-methoxyphenyl)ethanol	Lg
44	Acetoguaiacone	Lg
45	Vanillic acid methyl ester	Lg
46	4-Ethylsyringol	Lg
47	Guaiacylacetone	Lg
48	4-Vinylsyringol	Lg
49	Propiovanillone	Lg
50	Guaiacyl vinyl ketone	Lg
51	4-Allylsyringol	Lg
52	<i>cis</i> 4-Propenylsyringol	Lg
53	Syringaldehyde	Lg
54	1-(3,5-Dimethoxy-4-hydroxyphenyl)propyne	Lg
55	<i>trans</i> 4-Propenylsyringol	Lg
56	3,5-Dimethoxy-4-hydroxybenzyl alcohol	Lg
57	Acetosyringone	Lg
58	<i>p</i> -Coumaric acid	Lg
59	Syringylacetone	Lg

Ref.	Compound	Origin ^a
1	4-Methoxybenzene	Lg (G)
2	<i>p</i> -Methoxytoluene	Pp/Lg/Pr
3	<i>o</i> -Methoxytoluene	Pp/Lg/Pr
4	Guaiacol	Lg (G)
5	4-Ethyl -1-methoxybenzene	Lg (G)
6	1,2 Dimethoxybenzene	Lg (S)
7	4-Methoxytoluene	Lg (G)
8	4 Methoxybenzenethylene	Lg (G)
9	Methylguaiacol	Lg (G)
10	3,4-Dimethoxytoluene	Lg (S)
11	4-Propenyl-1-methoxybenzene	Lg (G)
12	Ethylguaiacol	Lg (G)
13	4-Methoxybenzaldehyde	Lg (G)
14	1,2,3-Trimethoxybenzene	Lg (S)
15	4-Methoxyacetophenone	Lg (G)
16	4 Ethyl-1,2-dimethoxybenzene	Lg (G/S)
17	Syringol	Lg (S)
18	3,4-Dimethoxybenzenethylene	Lg (G/S)
19	1,3,5 Trimethoxybenzene	Lg (S)
20	4-Methoxybenzoic acid methyl ester	Lg (G)
21	3,4,5-Trimethoxytoluene	Lg (S)
22	Methylsyringol	Lg (S)
23	1-(4-Methoxyphenyl)-1-methoxyethane	Lg (G)
24	3,4-Dimethoxybenzaldehyde	Lg (G/S)
25	1-(3,4-Dimethoxyphenyl)-1-propene	Lg (G/S)
26	5-Vinyl-1,2,3-trimethoxybenzene	Lg (S)
27	Vanillic acid methyl ester	Lg (G)
28	Ethylsyringol	Lg (S)
29	3,4-Dimethoxyacetophenone	Lg (G/S)
30	1-(3,4-Dimethoxyphenyl)-2-propanone	Lg (G/S)
31	3,4 Dimethoxybenzoic acid methyl ester	Lg (G/S)
32	1,3,5-Trimethoxybenzaldehyde	Lg (S)
33	1-(3,4-Dimethoxyphenyl)-2-methoxypropane	Lg (G/S)
34	<i>cis</i> -1-(3,4 Dimethoxyphenyl)-2-methoxyethylene	Lg (G/S)
35	3,4 dimethoxyphenyl-2-acetone	Lg (G/S)
36	<i>cis</i> -1(3,4-Dimethoxyphenyl)-1-methoxyprop-1-ene	Lg (G/S)
37	<i>trans</i> -1(3,4-Dimethoxyphenyl)-1-methoxyprop-1-ene	Lg (G/S)
38	3,4,5-Trimethoxyacetophenone	Lg (S)
39	3,4,5-Trimethoxybenzoic acid methyl ester	Lg (S)
40	<i>trans</i> -1(3,4-Dimethoxyphenyl)-3 -methoxyprop-1-ene	Lg (G/S)
41	<i>cis</i> 1-(3,4,5-Trimethoxyphenyl)-2-methoxyethylene	Lg (S)
42	1,2,3,-Trimethoxyphenylpropan-3-one	Lg (S)
43	<i>cis</i> - 1-(3,4-Dimethoxyphenyl)-1,2,3-trimethoxypropane	Lg (G/S)
44	<i>trans</i> 1-(3,4,5-Trimethoxyphenyl)-2-methoxyethylene	Lg (S)
45	<i>trans</i> -1-(3,4-Dimethoxyphenyl)-1,2,3-trimethoxypropane	Lg (G/S)

46	Table 3	<i>cis</i> -1-(3,4,5-Trimethoxyphenyl)-1,2,3-trimethoxybenzene	Lg (S)
47		3-(3,4-Dimethoxyphenyl)-propenoic acid methyl ester	Lg (G/S)
48		<i>trans</i> -1-(3,4,5-Trimethoxyphenyl)-1,2,3-trimethoxybenzene	Lg (S)

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746 ^a G Guaiacyl, S Syringyl.

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750 **Table 6**

751

Family	Sequential oxidation				
	K ₂ S ₂ O ₈	KMnO ₄			
		HA-14	HA-38	HA-14	
<i>n</i> -Alkanes	C ₁₆ -C ₃₃ (C ₁₆) ^a	+++ ^c	+++	C ₂₃ -C ₃₃ (C ₂₉) ^a	+++
Branched fatty acids ^b	<i>Iso, anteiso</i>	+++	+++	<i>Iso, anteiso</i>	++
Unsaturated fatty acids ^b	C ₁₅ and C ₁₇			C ₁₅ and C ₁₇	
	C ₁₆ and C ₁₈	+++	+++	C ₁₆ and C ₁₈	++
Saturated fatty acids ^b	C ₁₀ –C ₂₆ (C ₁₆) ^a	+++	+++	C ₁₀ –C ₃₁ (C ₁₆₋₁₈) ^a	++
Branched α, ω-diacids ^b		n.d.	n.d.	C ₅	+
α, ω-Diacids ^b		n.d.	n.d.	C ₆ –C ₁₂ (C ₉) ^a	+++
Phenol compounds ^b		n.d.	n.d.	Methoxybenzene	+
Benzene polycarboxylic acids ^b				carboxylic acids	
		n.d.	n.d.	Di- to tetra-carboxylic acids	+

^a Maximum.

^b As methyl esters.

^c Semiquantitative assessment; + trace, +++maximum.

^d N.d.; not detected.

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FIGURE CAPTIONS

Fig. 1. FT-IR spectra and resolution enhanced spectra of HAs from two different depths in core CM-5 of Guadiana sediments.

Fig. 2. Solid state (CPMAS) ^{13}C NMR spectra of HAs from two different depths in core CM-5 of Guadiana sediments. (* = Spinning side bands).

Fig. 3. Partial chromatograms obtained after pyrolysis of HAs from different depth in core CM-5 in the absence (a) and presence (b) of TMAH. Numbers on peaks correspond to products listed in Table 4; methylated thermochemolysis products derived from lignin are listed in Table 5.

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Figure 1.

Figure 2.

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Figure 3.