Structural characteristics of marine sedimentary humic acids by CP/MAS ^{13}C NMR spectroscopy

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Abstract – Humic acids from sediments of different depositional environments have been studied by solid-state ^{13}C NMR and the results compared with the traditional wet chemical analysis. Results obtained are well in agreement with the previous literature reports that the carboxyl content measured by NMR correlated better with the total acidity, as well as with the carboxyl content obtained by wet chemical analysis after correction for amino acid carboxyl is made (following hydrolysis of peptide bonds). There is a large discrepancy between the NMR and wet chemical measurements of phenolics. NMR spectra was also indicative of branched paraffinic structures in the humic acids from the Arabian Sea; the humic acids of sediments from estuarine and coastal areas of the Bay of Bengal being dominated by carbohydrates and aromatic structures and to a lesser extent by paraffinic structures. These differences are attributed to their different biogeochemical origin. © Elsevier, Paris

humic acids / structural characteristics / CP/MAS ^{13}C NMR / Arabian Sea / Surficial sediment

Résumé – Caractéristiques structurales d'acides humiques de sédiments marins par spectroscopie CP/MAS ^{13}C RMN. Les acides humiques de sédiments ont été étudiés dans différents environnements de dépôts par RMN du ^{13}C (état solide) et les résultats ont été comparés avec les analyses chimiques traditionnelles en milieu liquide. Les données obtenues sont en accord avec la littérature, montrant une meilleure corrélation de la teneur en carboxyle mesurée par RMN avec l'acidité totale. Elle est également mieux corrélée avec la teneur en carboxyle obtenue par analyse chimique (milieu liquide), après correction des groupes carboxyle des amino-acides (par hydrolyse des liaisons peptidiques). Il y a une grande différence entre la RMN et les mesures chimiques en milieu liquide des phénols. Les spectres RMN sont également des indicateurs des structures ramifiées paraffiniques dans les acides humiques de la mer d'Arabie ; les acides humiques des sédiments estuariens et côtiers de la baie du Bengale sont dominés par les hydrates de carbone et les structures aromatiques, ainsi que, dans une moindre mesure, par les structures paraffiniques. Ces différences sont attribuées à une origine biogéochimique différente. © Elsevier, Paris

acide humique / caractéristique structurale / CP/MAS ^{13}C RMN / mer d'Arabie / sédiment superficiel

1. INTRODUCTION

Humic substances have been widely reported to contain proteins, carbohydrates, lipids and lignin-derived phenols that are undeniably of biochemical origin [1]. The presence of these compounds in humus is not surprising when we consider that they are believed to be formed through diagenetic transformation of organic matter derived from allochthonous sources. Marine humic and fulvic acids are predominantly autochthonous [22, 32]. Although the composition and structural components of marine humic substances have not been as extensively studied as those of soil humic substances, several major differences and similarities between them have been noted [25, 31]. Recent findings have further indicated that humic substances in each of soil, stream and marine environments are unique [18].

Different methods of instrumental analysis have been used for the characterisation of humic substances, includ-

ing: UV-Vis spectroscopy [4, 6]; infra-red spectroscopy [3, 6, 7, 12, 34]; pyrolysis followed by mass spectrometry, and gas chromatography-mass spectrometry [39]; ^{1}H and/or ^{13}C NMR spectroscopy [5, 40]; and CP/MAS ^{13}C NMR spectroscopy [12]. These papers show that even though ^{13}C NMR is the most valuable analytical technique, no single method is in itself sufficient for characterisation of humic substances. Rather, a combination of various methods is required. There have been only a few studies [24, 28] on the structural characteristics of humic acids from sediments of the Indian Ocean region; and much of this information is based on IR, ESR and wet chemical analysis, there being no reference to the use of NMR techniques. The main objective of the present study is to analyse the ^{13}C NMR spectra of sedimentary humic acids from different depths and depositional environments for their probable composition, and to relate the latter to origin. The second aim is to compare the wet and spectroscopic methods of analysis. Infra-red spectroscopy and GC- analysis of hydrolysed humic acids have also been used to substantiate the results obtained with the CP/MAS technique.

2. MATERIAL AND METHODS

2.1. Samples

Station locations and the characteristics of the sediment samples analysed for humic acids are given in *table I*. Surface sediment samples of about 5 cm thickness were collected. Samples from the Arabian Sea (HA$_3$ and HA$_4$) have a depositional environment which is influenced by arid land masses to the north and coastal highlands to the east, as well as by monsoonal rain, upwelling, depletion of oxygen and other factors. Bulk sediments in this region may be characterised as silty clay, clayey silt or sand, silt and clay [8, 21]. The sediment texture in the marginal areas reflects higher inputs of both coarse-grained terrigenous detritus and biogenic components (calcium carbon-

ate and opal). The terrigenous sediments are derived from the land via the rivers Indus, Narmada and Tapti, and aeolian transport. The southerly, deep areas far from these terrigenous sources have very fine-grained clays [16]. Hooghly estuary (HA$_2$) is a one-layer estuary, characterised by a semidiurnal tide with maximum amplitude of 5.5 m. Sediments from this station were clayey; sediment core from the coastal station of Bay of Bengal (HA$_1$) was fairly homogenous and composed of silt and clay with small amounts of calcareous shells and pebbles.

3. METHODS

The procedure for extracting humic acids from sediments has been detailed [20], but will be mentioned again briefly.

A known quantity of the sediment sample was extracted under inert atmosphere, first with a mixture of aqueous 0.05M NaOH and 0.05M Na$_4$P$_2$O$_7$, and then repeatedly with 0.05M NaOH until the extract was colourless. All the 0.05M NaOH extracts were combined. The residue was further extracted with 0.5M NaOH and finally refluxed with 0.5M NaOH for 20 h. All the 0.5M NaOH extracts were combined. The two extracts were separately pressure-filtered twice through GF/C glass fibre filters and then fractionated either directly or after dialysis into different molecular weight fractions, using Amicon ultrafiltration cell and Diaflo ultrafiltration membranes XM-300, XM-100, PM-30 and PM-10. Humic acids were converted into the protonated form by acidifying with 6N HCl to pH 2.0. The humic acid precipitated out was collected by centrifugation, dialysed against distilled water until neutral and freeze dried.

Phenolic, carboxylic and total acidities were measured using wet chemical methods as described by Schnitzer and Khan [29] and are listed in *table II*, which also lists elemental contents of the isolates. Elemental analyses

Table I. Location, depth and sediment texture, percentage of organic carbon and nitrogen, and their ratios in the sediments at different stations.

Area	station	location		depth m	sediment texture	C %	N %	C/N
		lat. N	long. E					
Bay of Bengal	BB-06	20 00.4	88 04.2	8	clay	0.20	0.013	15.4
Hooghly estuary	BB-02	20 23.2	87 04.2	43	clay	0.39	0.022	17.7
Arabian Sea	SK 37F8	12 35.24	74 04.89	890	clay	5.6	0.37	15.1
"	SK 44/11	15 52.5	74 24.00	1121	clay	4.8	0.27	17.7

Table II. Elemental and wet chemical functional group analyses of humic acids.

Analysis	HA$_1$	HA$_2$	HA$_3$	HA$_4$
Total acidity	5.2	17.8	3.2	4.2
Carboxyl	2.8	3.2	1.8	ND
Phenolic	2.4	14.6	1.4	ND
Carbon	50.7	49.7	50.3	53.3
Hydrogen	4.0	4.3	6.9	6.1
Nitrogen	3.3	3.4	2.5	4.1
C/H	12.7	11.5	7.3	8.7
C/N	15.3	14.6	20.1	13.0
C/O	1.2	1.1	1.2	1.4

Functional groups (meq. g^{-1}).
Elemental composition (%).
Ha -- Humic acids.

Table III. Relative distribution (mol%) of different groups of amino acids in the HA of the sediments.

Amino acids	HA$_3$	HA$_4$
Neutral	39.8	44.0
Acidic	23.1	20.9
Basic	17.9	16.9
Aromatics	10.7	12.5
Sulphur	4.6	5.6

Table IV. Relative mole % abundance of amino acids in HAs from sediment samples.

Amino acids	HA$_3$	HA$_4$
Acidic		
Aspartic acid	15.75	13.84
Glutamic acid	9.14	7.13
Basic		
Arginine	2.95	3.13
Histidine	1.72	1.87
Lysine	13.97	11.87
Neutral		
Alanine	14.98	12.79
Inoleucine	6.78	10.37
Leucine	7.54	8.72
Serine	2.94	3.7
Valine	traces	traces
Glycine	9.59	8.42
Threonine	traces	traces
Methionine	4.77	5.62
Aromatics		
Phenylalanine	6.66	7.82
Tyrosine	4.5	4.67

were carried out using a Perkin-Elmer 240B CHN analyser. Infra-red spectra were recorded on a Pye Unicam SP 1100 or Fourier Transform IR spectrophotometer using KBr pellets.

3.2 Amino acid and carbohydrate analysis of humic acids

The amino acid composition of the samples was determined after hydrolysis by refluxing with 6N HCl for 22 h at 110 °C, followed by removal of the humic residue by filtration through 0.4 µm Nucleopore filters. The hydrochloric acid was removed from the hydrolysate by rotavapor at 40 °C. The amino acids in the residue were dissolved in 500 µL of distilled water and again evaporated to dryness to remove traces of acid. The material dried over KOH under vacuum was derivatised using o-phthaldehyde and analysed using HPLC on a C-18 RP column maintained at 34 °C. The equipment (System Gold HPLC Beckman) consisted of two pumps, RP column, heater, fluorescence detector, a system controller and a computer-based (PS -30) data-handling system (System Gold software). A binary solvent system was used to resolve amino acids in 28 min. Solvent A consisted of 50 mM of sodium acetate solution and 30 % HPLC grade THF adjusted to pH 6.5 with acetic acid. Solvent B was glass-distilled MeOH. Peaks were integrated using System Gold software and peak areas from both calibration and sample runs were automatically transferred to spread sheets. Amino acid yields were calculated relative to amino butyric acid as internal standard (*tables III* and *IV*). The following amino acids were detected: threonine, serine, glycine, alanine, valine, iso-

leucine, leucine, aspartic acid, glutamic acid, lysine, histidine, arginine, thyrosine, phenylalanine and methionine.

Humic acids HA$_3$ and HA$_4$ (Arabian Sea samples) were analysed qualitatively for carbohydrate content after hydrolysis. The constituent monosaccharides were analysed by capillary GC equipped with a 25 m long Si capillary column OV 275 and flame ionization detector; 1 µL of the sample was injected at 160 °C initial temperature. After 8 min the oven temperature was raised at the rate of 4 °C min^{-1} to 200 °C. This temperature was maintained for 2 min and then raised again to 210 °C at the rate of 2 °C min^{-1} and held at 210 °C for 14 min. Nitrogen was used as a carrier gas at a pressure of 10.35×10^4 N m^{-2} Injector and detector were held at 300 °C. Glucose, xylose, galactose, mannose and rhamnose were the main components of the hydrolysate.

## 3.3 Nuclear magnetic resonance spectroscopy

The cross-polarization technique with magic angle spinning (CP/MAS) was used to obtain ^{13}C NMR spectra. Samples were thoroughly freeze-dried before examination by NMR. High-resolution solid-state spectra were obtained on a JEOL FX-200 instrument. Samples were spun at a rotor speed of up to 3.1 kHz at the magic angle of 54.7°. Radio frequency excitation fields were adjusted to 50 kHz. The magic angle and Hartman-Hahn conditions were adjusted using hexamethylbenzene as a standard. Field homogeneity was adjusted with adamantane (line width for adamantane less than 5 Hz). A 90° proton pulse of 5 μs was used at a contact time of 1s. Between 2,446 and 17,186 pulses were collected to obtain adequate signals viz:

HA$_1$-- 10,439; HA$_2$-- 17,186; HA$_3$-- 2,446; HA$_4$-- 3,220.

Chemical shifts were obtained by using aromatic carbons of hexamethylbenzene (132.3 ppm) as an external standard.

The ^{13}C NMR spectra (*figure 1*) were analysed by integrating areas of peaks from selected regions of the spectrum assigned to each functional group. The natural valleys between signals were used as integration cut-off
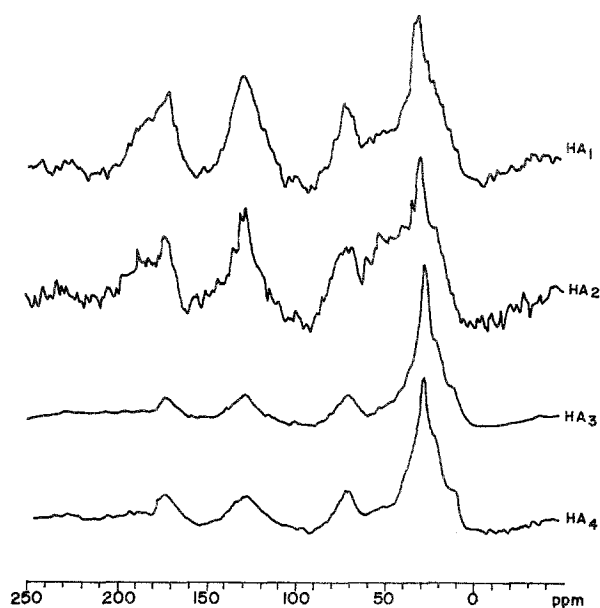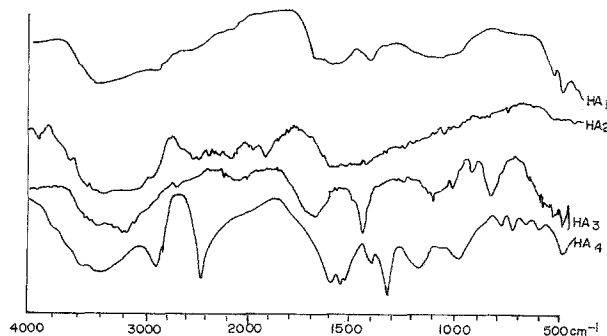
points [27, 30]. These measurements, which are listed in *table V,* have been used for comparative purposes only. In general, several structural types could be delineated: ketone and aldehyde carbons resonating at or around 202 ppm; carboxyl, ester and amide carbons at around 172 ppm; oxygenated aromatic carbons at or around 150 ppm; protonated and carbon-substituted aromatic carbon at around 130 ppm and anomeric dioxygenated carbon of carbohydrates and non-protonated tannins at 103 ppm or thereabouts. Alcoholic carbon of carbohydrates was also observed at or about 70–75 ppm. Sometimes, well-defined resonance from methoxyl carbon in syringyl and guaiacyl units of lignin and carbons α to nitrogen in proteins was observed at 55 ppm. Aliphatic resonances centred at around 30–35 ppm were also observed.

The presence of oxygenated structures such as carbohydrates and methoxyl carbon was confirmed by C-O absorption in the infra-red at 1140–1040 cm^{-1} (*figure 2*). The carbohydrate content of the hydrolysate of HA$_3$ and HA$_4$ was also determined, as mentioned by GC analysis.

4. RESULTS AND DISCUSSION

Since both NMR and wet chemical analytical data were available on most of the samples, the results of the two methods were compared wherever possible. The data for comparison are given in *tables II* and *V.* In general, -COOH values obtained by titration (*table II*) are lower than the amount of carboxyl carbon (172 ppm) measured by NMR (*table V*) which, except for HA$_3$ (Arabian Sea), more closely approximates the values obtained for total acidic groups after deducting the values obtained for
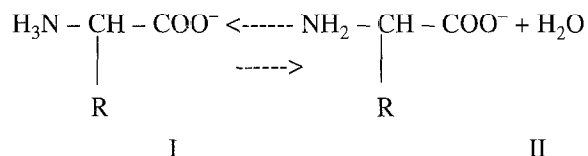

Figure 1. CP/MAS ^{13}C NMR spectra of humic acids. HA$_1$ BB - 06, HA$_2$ -- BB - 02, HA$_3$ --SK 37F8 (>300,000 m.wt.) and HA$_4$ -- SK 44/11 (>300,000 m.wt.) (70% reduction from original spectra).

Figure 2. IR spectra of HA$_1$ -- BB-06, HA$_2$ -- BB-02, HA$_3$ -- SK 37F8 (>300,000 m.wt.), HA$_4$-- SK 44/11 (>300,000 m.wt.)

Table V. Integrated areas (%) of ^{13}C NMR spectrum of humic acids

Sample	Approximate chemical shift (ppm)								COOH (Amino acid)
	195	172	155	129	106	73	55	32	
HA$_1$	1.8	21.3	1.2	20.2	0.85	14.2	6.2	34.25	2.9
HA$_2$	4.1	13.9	1.7	21.6	1.6	15.2	8.9	33.0	ND
HA$_3$	1.5	8.1	–	11.0	0.4	10.6	2.9	65.5	4.8
HA$_4$	2.0	9.2	–	12.4	0.6	10.4	3.4	62.0	3.5

amide carbonyl carbon of peptide (amino acid carboxyl). This is in agreement with the finding of Hatcher et al. [10] who concluded that the discrepancy between the two methods was due to an error in the wet chemical method of analysis as there was an apparent lack of phenolic carbon in the NMR spectra of their samples. Rasyid et al. [27] also made a similar observation in their study of the structural group composition of humic and fulvic acids with depth in sediment with similar geographical but different depositional environments; they attributed the discrepancy to the interference of groups like amide (-CONH-), ester (-COOR-) and to the amphoteric nature of the terminal amino acid residues of the peptide chain, which was not observed accurately by titration (proteins are one of the main constituents of humic acids).

In wet chemical analyses, carboxyl content is determined by titration with alkali. Under alkaline conditions the dipolar ion I (Zwitter ion) of the amino acid is converted into the anion II; (see the equation below); the base removes a proton from the ammonium ion, thus displacing the weaker base, the amine [19]. Therefore the amphoteric nature of the terminal amino acid should not affect the titration values and the discrepancy observed should be attributed to the amide carbonyl group of the peptide chain which resonates in the same region of the ^{13}C NMR as the carboxyl but is not observed by titration.

$$H_3N - \underset{\underset{R}{|}}{CH} - COO^- \xleftarrow{} \underset{\underset{R}{|}}{NH_2} - CH - COO^- + H_2O$$
$$\xrightarrow{}$$

$$I \qquad\qquad\qquad II$$

This also would partly explain the increase in the intensity of the resonances at 171 and 167 ppm reported by Wilson et al. [35] when the sample is left longer in NaOH.

The amino acid carboxyl content of the humic material has been determined in this study and hence the carboxyl content measured by NMR can be corrected for amide

carbonyl carbon carbon of peptides. These values are given in *table V*. In general, the values lie below the values for carboxyl carbon (172 ppm) by NMR such that the difference could be attributed to the carboxylic acid carbon. This value should be practically equivalent to the carboxyl content measured by wet chemical analysis provided the humic material does not contain much ester carboxyl. This has, in fact, been observed to some extent in the present investigation for HA$_3$.

Phenoxy content measured by NMR is quite small, amounting to 1.2 % in HA$_1$ and 1.7 % in HA$_2$; HA$_3$ and HA$_4$ are practically devoid of phenolics. Comparatively, wet chemical analysis shows a phenoxy content ranging from 1.4–14.6 meq g^{-1}. Thus, there is a poor agreement between the two analyses. ^{13}C NMR results obtained for the Hooghly estuary sample (HA$_2$) show that the carboxyl resonance (172 ppm) is significantly greater (13.9 %) than the phenoxy resonance (1.7 %), yet the wet chemical analysis shows much higher concentrations of phenolics (14.6) than the carboxyl (3.2). Moreover, significant resonance at 55 ppm (8.9) from aromatic methoxy carbon suggests that phenol is present mostly as methyl ether and not as free phenol. The signal at 55 ppm is also attributed to aliphatic carbon adjacent to the amino functional group as in amino acids. This means, as suggested by Rasyid et al. [27], that the determination of phenol by titration is in error. Alternatively, this could be partly explained by considering easy decomposition due to oxidation of free phenols to the corresponding quinones or semiquinones which exhibit ^{13}C resonance at around 195 ppm. Free radicals of semiquinone type have been reported from the aquatic environment [2]. Spectra of all the four samples show absorption at 195 ppm.

The total acidity of marine humic compounds has been reported to vary from 2.0 to 10.0 meq g^{-1}. Rashid and King [25] have also observed that well-developed soils possess considerably higher acidity than marine organic matter. They attributed the difference in acidic properties to the starting material rather than to the marine environ-

mental factors. The acidity obtained by wet chemical technique for the four humic samples of the present investigation is highest for HA_2 (17.8 meq g^{-1}) followed by HA_1 (5.2 meq g^{-1}), HA_4 (4.2 meq g^{-1}) and HA_3 (3.2 meq g^{-1}). This indicates that only the Hooghly estuary (HA_2) sample has a mainly terrestrial input, the remaining three being mainly of aquatic origin. The terrestrial origin of humus in HA_2 is further supported by NMR data where phenoxy/methoxy content of HA_2, as shown by the resonances at 155 ppm and 55 ppm is highest. This is to be expected, given the proximity of the site of collection to the terrestrial source.

Elemental analysis shows that estuarine (HA_2) and coastal samples (HA_1) have greater C/H ratios than the sea samples (HA_3 and HA_4), indicating a higher degree of aromaticity; the sea samples with lower C/H ratio are more aliphatic in nature. NMR data are well in agreement with these observations. The low aromatic content of Arabian Sea samples HA_3 and HA_4 reflects their origin in humus from a high rainfall region [36, 37].

In ^{13}C NMR, it is difficult to assign a specific region within the 0–50 ppm scale to any particular aliphatic structures, since the number of α, β and γ carbons adjacent to a particular carbon can shift its resonance over a wide range, and a great deal of overlap occurs [17]. This is the most intense region in all the spectra, indicating that paraffinic structures are the major contributors. In all four humic acids studied, the major peak in this region is centred at 32 ppm, which is the chemical shift of methylene carbons $-(CH_2)_{n-}$ in either long-chain paraffinic structure or cycloparaffin. The strong peak at 30 ppm has been observed by numerous other workers in marine humic substances [9, 10, 39] and in various soil humic acids [33]. Such a strong signal at 32 ppm indicates either that various carbons in different environments have the same or similar chemical shift or that a large number of similar structural units are present. The humps at 23 ppm and downfield at 34 ppm indicate paraffinic carbons in repeating configurations. The signal at 15 ppm represents the end methyl groups of aliphatic structures. Carbohydrates are clearly associated with HAs, though not always to the same extent, as evidenced by ^{13}C NMR resonance at 106 and 73 ppm. The carbohydrate concentration is higher in the coastal (HA_1) and estuarine (HA_2) samples as compared to the Arabian Sea samples, most probably because marine samples are more advanced diagenetically [13]. No wet chemical analysis data are available on

these samples for comparison, but qualitative GC data of HA_3 and HA_4 (Arabian Sea samples) on hydrolysis indicated that they are of similar composition, with glucose, xylose, galactose, mannose and rhamnose as the main components. HA_4 is richer in glucose with anomeric carbon resonance at 98.4 ppm (β isomer); and the concentration of galactose is highest in HA_4 with the anomeric carbon resonance at 102 ppm. The signal at 98.4 ppm could also be attributed to 3.6 anhydrogalactose units resulting from the elimination of the sulphate from L-galactose-6-sulphate residues under the alkaline conditions used for extraction. The presence of IR absorptions (figure 2) at 840 and 800 cm^{-1} in HA_3 further supports the presence of galactose-4- sulphate and 2-sulphate respectively. Infra-red absorption at 900 and 900–930 cm^{-1} is also suggestive of Arabian Sea samples containing sugars with 1.4 linkages. The absorption in the IR spectra of HA_4 at around 2400 cm^{-1} is characteristic of carbon dioxide.

The percentage distribution of different groups of amino acids relative to the total amino acids in the two humic acids studied is shown in table III. The humic acids show 40–45 % neutral, about 21–23 % acidic, 17–18 % basic, 11–13 % aromatic amino acids and from 4.6–5.6% sulphur containing amino acids. The percentage of neutral amino acids is well in agreement with the values reported by Rashid [26].

The relative concentrations of individual amino acids in different HAs are presented in table IV. Of all the basic amino acids analysed, lysine is the major basic amino acid in all the samples, and is relatively more abundant in the slope sample. Within the acidic fraction, aspartic acid concentration is higher than that of glutamic acid in all the samples. Aspartic acid is reported to be abundant in terrestrial humic hydrolysates and in sediments receiving large terrestrial organic input [15, 23], whereas glutamic acid is more abundant in plankton [14]. As far as the neutral fraction in concerned, the relative abundance of straight-chain amino acid, glycine and alanine is characteristic of all the samples; the total branched-chain amino acids valine, leucine and isoleucine form the second largest group of the neutral fraction. Within the total hydrolyacid fraction (threonine and serine) there is a predominance of serine, over threonine. These observations are in confirmity with the findings of the present study that the humic acids in the sediments of the Arabian Sea contain organic matter of aquatic as well as terrestrial origin.

5. CONCLUSIONS

1. Measurement of carboxyl content of HA samples by NMR equates better with total acidity measured by wet chemical methods. It also correlates better with the caboxyl content measured by wet chemical analysis after the correction for amino acid carboxyl is made (after hydrolysis of peptide bond).

2. The low level or virtual absence of phenoxy carbon in the NMR as compared to the wet chemical measurements seem to be due to easy oxidation of free phenols to the corresponding semiquinones or quinones.

3. Samples from the Arabian Sea with a low degree of aromaticity and high degree of aliphaticity in the form of branched alkyl chains indicated their origin mainly in aquatic humus, although some influence by organic matter of terrestrial origin cannot be precluded. Phenolics seem to be present in much higher concentrations in the estuarine sample than in the coastal sample, indicating that the former is mainly of terrestrial origin while the latter shows terrestrial origin with some marine input.

The NMR technique applied here indicates that non-destructive means whereby spectra are obtained, though qualitative in nature, provide a more realistic picture of the structural components of humic acids than any other method. NMR is thus an important tool in determining source of humics. Other methods, involving the stepwise degradation of humic acids, hydrolysis and oxidation followed by GC/MS, if combined, would give a better picture of the components of humic acids.

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