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Crown humus: Part I—The chemistry of the canopy organic matter of rain forests in Costa Rica

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In some tropical rain forests organic matter, derived from epiphytic plants, accumulates as heavy mats on tall tree tops (Crown), trunks and branches. Upon microbial and abiotic (chemical, geological) interactions, the canopy organic matter (COM) is transformed into Crown humus. Detailed chemical, chromatographic and spectroscopic analyses of seven COM samples (COM-1 to -7), collected from five different tree tops, and one soil sample collected from the corresponding forest floor (FF-1), from Monte Verde Reserve Forest, in Costa Rica, have been carried out in respect of their low M_w organic compounds and the contained humus. The nature and chemical characteristics of these arboreal humic substances are compared with those of their terrestrial and aquatic counterparts. Some striking similarities, particularly in the core structures of fulvic acids (FAs), occurring in three different natural habitats (arboreal, terrestrial and aquatic), are observed. Like those of terrestrial and aquatic -FAs, the molecular architecture of the Crown-FAs constitute metallo-organic complexes of mono-, di- and oligomeric oxygenated dibenzo-α-pyrones (**1-4, 9a, b, 11, Chart 1**). However, the ecological and geochemical conditions of the arboreal mini-ecosystem being different, are reflected in the simpler structures of a large variety of its secondary metabolites (**Tables I & II**) entrapped in the inner core and outer-surface of the Crown humus. The role of these compounds in the microbial (chemoheterotroph) metabolic sequence enroute to Crown humus is appraised.

Introduction

The increasing interest of ecologists and their frequent incursions into the canopy of tropical rain forests of the world have now validated[1,2] the forecast of the famous naturalist, William Beebe, who, about hundred years ago, said: "Yet another continent of life remains to be discovered, not upon the earth, but one to two hundred feet above it... there awaits a rich harvest for the naturalist who overcomes the obstacles—gravitation, insects and beetles, rotten trunks—and mounts to the summit of the jungle trees". Indeed, a familiar characteristic of many mid-to-high elevation tropical rain forests is their high biodiversity and rich biomass, such as of epiphytic plants (bryophites, lichens and club mosses), which survive and flourish because of moist conditions prevailing on the tree canopy. As these epiphytes die and decompose by microbial infestation, thick mats (up to 30 cm height) are formed on the host tree canopy, trunks and branches, high above the forest floor. The dead canopy organic matter (COM) is then transformed, by microbial and chemical and geological processes (abiotic), into Crown humus. The Crown humus serve as the rich nutrient source for the canopy dwellers. Also, they play important role in trapping and conserving mineral nutrients entering rain forests in mist, rain and as plant-leaf leachates. Crown humus inhibit nitrification of COM and thereby preserve the nitrogen resources which contribute to the growth and maintenance of the arboreal mini-ecosystem. Although COM, comprising Crown humus, dead mats of epiphytes and residual matters of canopy dwellers (insects and animals), has received considerable attention from ecological stand point[1-3], its chemistry has so far remained unexplored. As a first step in understanding this subject, we have

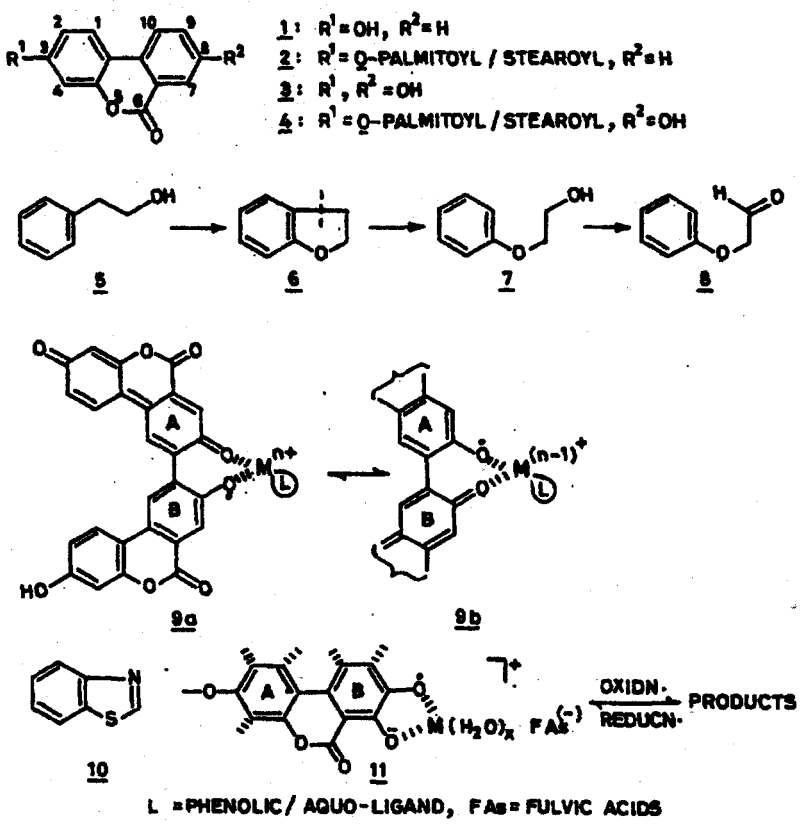
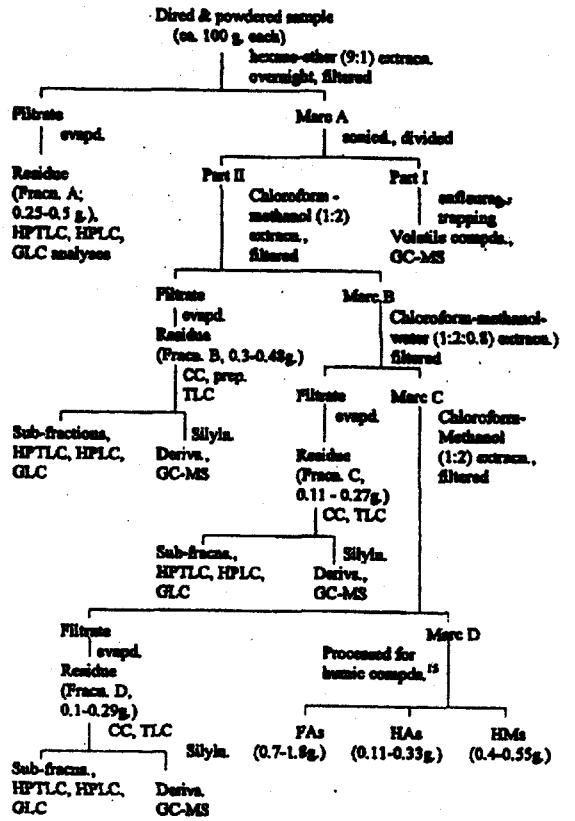


Chart 1

studied the nature and chemical characteristics of seven Crown humus samples, isolated from COM (COM -1 to -7), and of one corresponding forest floor soil sample (FF-1). The samples (COM and FF) were collected from two tropical rain forest (Monte Verde Reserve Forest) in Costa Rica. We have also compared these results with those of terrestrial and aquatic humus[4-7] to examine the link in humus formation in different natural habitats.

Results and Discussion

The canopy and forest floor samples (COM-1 to -7 and FF-1) were separately extracted with solvents of graded polarity (**Scheme I**) and the extracts were processed by column chromatography (CC), prep. TLC and ion-exchange technique for separation of the contained organic compounds. The constituents present in the outer-surface (Fraction A, **Scheme I**) and the inner-core (Marc A, **Scheme I**) of each sample were separately processed. The identities of the low M_w (mol. wt.) organic compounds were established by comprehensive chromatographic (HPTLC, HPLC, GLC) and spectroscopic (FT-IR, EI-MS, GC-MS) analyses of the isolated compounds and their silyl, acetyl and methyl ester derivatives, using authentic markers, where possible. The extractives from the different humus samples (see Experimental section) showed very similar finger prints and reflectance/UV spectral patterns in HPTLC/HPLC. The free elements present at the outer-surface and inner-core of these samples were analyzed by an electron probe micro analyzer (EPMA) and were also found to be qualitatively similar to those of terrestrial humus (**Table III**). The organic solvent-extracted samples (Marc D, **Scheme I**) were then processed for the three different types of humic substances[8], viz. fulvic acids (FAs), humic acids(HAs) and humins (HMs). Several volatile and labile entities were encountered in the inner core of the Crown humus, which were entrapped in the micropores/voids of FAs and HAs. These micro-molecules were isolated, as before[9], by enfluerage technique and characterized by GC-MS analysis. The general methods of isolation and modes of characterization of the organic constituents of COM and FF samples are given in Scheme I.



Scheme I — Modes of isolation and characterization of the organic constituents of COM-1 to 7 and FF-1

A large number and variety of low and medium M_w organic compounds were encountered (**Table. I**). Their close structural similarities, irrespective of the trees or forest floor from where these samples were collected, suggest their common metabolic origin. These organic compounds are essentially the outcome of microbial and abiotic transformations of the different COMs analyzed in this study.

The *n*- and branched-chain (*iso*-, ante-*iso*) hydrocarbons (C_{16}-C_{34}) (Table. I), obtained from the neutral lipid fractions of COM, showed appreciable relative abundance (*ca.* 66%) of the C_{20}-C_{34} compounds, with odd to even carbon ratio of nearly 1. This finding suggests major microbial contribution to their formation. Additionally, pristane (mean rel. abundance. 5.35%), phytane (2.54%), and squalene (0.33%) were obtained from the inner-core of the Crown humus-FAs. The inner-core of the humic substances (FAs and HAs), obtained from the COM, also contained lower carbon-containing hydrocarbons (C_9-C_{15}), alcohols (C_8-C_{15}) and aldehydes (C_8-C_{15}) (Table II).

Table I—Relative abundance of low M_w organic compounds of COM

Compound	Relative abundance[a], %	
	Outer surface[b]	Inner core[c]
Hydrocarbons	10.43	3.22
Free fatty acids	12.41	2.35
Wax esters	5.50	1.20
Monoalkyl glycerol	0.57	0.08
Monoalkyl acyl glycerol	5.87	1.14
Dialkyl glycerol	0.76	0.13
Dialkyl acyl glycerol	4.92	1.04
Triacylglycerol	3.61	0.82
Terpene/steroid alcohols	21.20	2.55
Terpene /steroid esters	2.84	0.83
Aromatic, phenolic acids	2.33	18.10
Hydroxyacetophenones	3.11	22.44
Heterocyclic compounds	0.28	1.03
Oxygenated dibenzo-α pyrones	7.74	28.92
Unidentified compounds	8.26	7.04
Polymeric (high M_w) compounds	10.13	9.10

[a]Determined by GLC and GC-MS analysis of silyl derivatives
[b]Mean of COM-1 to COM-7
[c]by sonication and solvent extraction (see Experimental Sec.)

In the major free fatty acids, the carbon chain lengths ranged from C_{12}-C_{24}; palmitic ($C_{16:0}$, relative abundance, 28%) and stearic ($C_{18:0}$, 14.5%) being the two most abundant members. The occurrence of appreciable number and amounts of the unstable polyunsaturated fatty acids (PUFA): 22:3, ω 9; 22:4, ω 6; 22:5, ω 3; 22:5, ω 6; 23:5, ω, 3; 24:5, ω 6, within the micropores of Crown humus-FAs, are a unique feature of COMs. These polyunsaturated acids are presumably produced in preparation for the 'winter fast' of the canopy dwellers. This postulate finds support from the higher abundances of PUFA in the COM samples collected in August-September (eve of winter) than those of the April-May (post winter) collections.

The mono-*O*-alkylglycerols (and their acetate derivatives) exhibited appreciable abundance of C_{10}-C_{29} (*n*-, *iso*-, ante-*iso*) chains, associated with polyunsaturated moieties (rel. abundance, 4-10%), in the range C_{10}-C_{27}. These compounds were encountered essentially in the inner core of the COMs and in the micropores/voids of the Crown humus-FAs. The 1, 2-di-*O*-alkyl glycerols (and equivalents) contained C_{20}-C_{34} carbon moieties, associated with C_{12}-C_{29} polyunsaturated components, occurring in the inner core of the COMs and in the micropores/voids of Crown humus-FAs.

Table II—Identities of low M_w organic compounds of COM

Compound type	Occurrence[a]	
	Outer surface	Inner core
Aliphatics		
Hydrocarbons	C_{16} C_{34} (*n*-& branched)	C_9-C_{15}(*n*-& branched squalene, pristane & phytane)
Alcohols	C_{18}-C_{28}	C_8-C_{15}
Aldehydes	–	*n*-heptanal- *n*-decanal[b]
Fatty acids	C_{12}-C_{24}	C_{22}-C_{24} (PUFA)
Alkyl chain of Glycerols		
Monoalkyl	C_{10}-C_{29} (*n*-& branched)	C_{10}-C_{27} (ployunsatd)
Dialkyl	C_{20}-C_{34} (*n*-& branched)	C_{12}-C_{29} (ployunsatd)
Aromatics		
Alcohols	-	*m*-cresol [b] phenylethyl (5) [b] 2- phenoxyethyl (7) [b]
Aldehydes	-	2- phenoxyethanal (*8*) [b]
Acids	benzoic, cinnamic	benzoic, cinnamic, phenylacetic
Phenolics	vanillic, ferulic acids; *o*-, *p*-hydroxyaceto phenones	vanillin[b], vanillic, *p*-OH-benzoic, ferulic acids; *o*-, *p*-OH-acetophenones
Heterocyclics		
Oxygen	- dibenzo-α-pyrones (1-4)	dihydrobenzofuran (6)[b] dibenzo-α-pyrones (1-4)
Sulphur	–	benzothiazole (10)[b]
Miscellaneous		
Acids , alcohols & amides	2-deoxytetronic, 3- hydroxybutyric, phosphoric; glycerol, ergosterol, sitosterols; urea, benzamide	2- deoxytetronic, 3- hydroxybutyric, phosphoric; sulphuric , glycerol, ergosterol, sitosterols; urea, benzamide

[a]Sturcture No. in parenthesis (Figure 1)
[b]Volatile compound, characterized by enfleurage technique and GC-MS analysis (see Experimental Section)
PUFA, polyunsaturated fatty acids, branched chain comprises *iso* and ante -*iso* moieties

FF-1 also exhibited very similar characteristics but of much lower abundance of the respective organic compounds. This is presumably because the organic compounds occurring in the forest floor soil (FF-1) owe their origin largely to the COM-washings and to the plant leachates.

The other important classes of organic compounds, isolated from the COMs and occurring in the micropores of the Crown humus-FAs, include aromatic, phenolic and heterocyclic metabolites (**5-8, 10**) and the metal ion complexes of oxygenated dibenzo-α-pyrones (**1-4, 9, 11, Chart 1.**) (**Tables I and II**). These compounds were earlier reported to be the marker constituents of terrestrial (*shilajit, makshika* and *abhraka*)[7-10] and aquatic (*makshika, munga*)[10,11] humus.

Because the arboreal soil is highly acidic[1] much of life (earthworms, beetles, frogs, toads) it supports is different from the terrestrial life. The higher acidity of the Crown humus, compared to those of terrestrial and aquatic humus, is also reflected in the much less complexity of its humic substances (FAs, **Table III**), compared to their terrestrial and aquatic counterparts[7-11].

The canopy-dwelling chemoheterotrophs (bacteria, extremophiles) utilize energy derived from the oxidation of transition metal ions, e.g. Fe^{2+}-e → Fe^{3+}, leading to the formation of pyrites. The oxidized metal ion(s) are, in turn, reduced by the co-occurring humic ligands (**9a⇌9b**) and maintain their red-ox status. The arboreal chemo-heterotrophs also derive energy from the oxidation of low M_w organic

Table III—Analytical profiles of arboreal and terrestrial humus

Parameter type	Humus source			
	COM-1 to -7	FF -1	Sh[6]	Mk[7]
1. *Organic compounds/ category*				
Low M_w compounds[b]	25.2	15.0	17.9	11.3
Fulvic acids (FAs)	48.1	45.5	21.4	12.8
Humic acids (HAs)	12.4	7.9	19.8	8.4
Humins (HMs)	14.3	31.6	40.9	67.5
2. *E_4/E_6 values*				
FAs	1.8±0.3	3.2±0.1	6.8±0.8	5.8±1.2
HAs	1.0±0.1	2.8±0.1	4.1±0.2	3.2±0.3
3. Metal ions (free & FAs complexes)/other elements	Fe, Ca, Mg, V, Mo, Al, Ti, Si, P, S	Fe, Ca, Mg, Al	Fe, Ca, Mg, Cu, Zn, Mn, Ti, Mo, V	Fe, Ca, Cu, As, Pb
4. *ESR data*				
g value	2.003±0.001	2.0028±0.002	2.0025±0.001	2.004±0.0005
spins/gm	3.1×10^{12}-1.35×10^{13}	$1\text{-}2 \times 10^{14}$	1×10^{14}-2.5×10^{16}	1×10^{11}-3×10^{18}
5. Number average mol. wt (M_n)[c] of FAs	655-811	735-2525	750-2250	680-975

[a] Mean of three replicates;
[b] Combined organic volatile constituents and solvent extractives (see Scheme I & Table I)
[c] By vapour pressure osmometry (VPO)[15]; viscosity measurement[16]; *COM*, canopy organic matter; Sh, *shilajit*; Mk, *makshika*; E_4/E_6, absorbance ratio at λ 465/665 nm

compounds and PUFA. The biochemical transformations that occur on canopy top soil can be grouped into four general categories. These reactions are: (i) oxidation-reduction; (ii) carbon-carbon (C-C) and carbon-heteroatom (C-X) bond formation (**6, 10**); (iii) C-C and C-X bond fission (**7, 8**); and metallo-organic molecular interactions.

The oxidation-reductions include hydrogenation of olefins and *O*-acyl groups of lipids (*O*-alkyl formation); with the concomitant loss of hydrogen from naphthenes to produce aromatic compounds (e.g. cresols, **Table II**); or loss of hydrogen from phenolic OH groups, thereby producing semiquinone radicals (ESR, **Table III**); oxidation of alcohols to aldehydes (**7-8**); and oxidative cleavage of lignins and phenyl propenoids into vanillin/vanillic acid and benzoic acid (**Table II**). The redox potential of some of the low M_w compounds of Crown humus has been estimated to be in the range of 0.5-0.7 V. They seem to be involved also in the reduction and kinetic stabilization of a variety of transition metal ions ($Fe^{3+/2+}$, $Mn^{3+/2+}$) and complex ions (MoO_4^-, VO_2^+).

The ability of the humic-ligands to complex formation (**9a⇌9b**) reduces the risk of systemic free radical generation (e.g. cytotoxic oxo-ferryl radical). These metallo-humate complexes are thermodynamically highly stable and are known to survive diagenic transformation over several millennia[12].

C-C and C-X bond formation in Crown humus is exemplified by the synthesis of low M_w compounds (**1-4, 6-10**) and polymerization and condensation reactions leading to medium and high M_w humic substances (FAs, HAs and HMs) (e.g. **11, Chart 1**).

The bond-fission reactions are represented by decarboxylation, decarbonylation (of aldehydes), deamination of aromatic-α-amino acids to give Ar-ethyl alcohols (and equivalents), hydrolysis of esters to produce free fatty acids and phosphoric acid, and cleavage of heterocyclic rings (**6→7**).

The metallo-organic interactions are represented by the formation of metallo-humate complexes (**9a, b**); organo-mineral phase reaction (FeS_2 formation from $Fe^{3+/2+}$ and C-S compounds)[10-13], and adsorption (intercalation/clathra-tion). The negative charges, which arise from the dissociation of carboxylate/phenate groups, are stabilized partly by the formation of electric double layer at the solute-solution interface and largely by delocalization of electrons with the extended Ar-conjugated moieties (**11, Chart 1**) of the Crown humus-FAs. Thus, like

those of terrestrial and aquatic humus, the resultant quinone-semiquinone-hydroquinone functions present in Crown humus contribute to the mobility and delocalization of electrons within the metal-humate complexes. The solvated metallo-humate complexes (**11, Chart 1**) ensures bioavailability of the metal ions for the growth of canopy soil biota.

The intermediates involved in the citric acid cycle (e.g. succinic, fumaric, oxalo-acetic, glyoxalic and oxaloglutaric acids) are all found present in the Crown humus. This is due to the low catabolic rate of the extant chemoheterotrophs. However, the low concentration of these compounds, at any point of time, suggests that they are utilized at a rate commensurate with their production. The balance in the low M_w organic constituents is finally shifted to permanent deposits by the slow chemical and geological transformations to the diagenically resistant Crown humus.

The glycerol backbone-containing *O*-alkyl ethers, which are replete in the Crown humus, act as thermal and mechanical insulators of the canopy soil biota. Among other compounds serving as the biological adjuvants of the chemoheterotrophs, the products of purine and pyrimidine metabolism are prominent. The high concentrations of urea and benzamide are the outcome of these transformations.

The low E_4/E_6 (light absorption at λ 465/665 nm) and the low $\overline{M}_n$ values of Crown humus-FAs ('proto-fulvic acids'), compared to those of terrestrial and aquatic humus (**Table III**)[6,7] suggest simpler heterogeneity and larger aromatic conjugation of the FAs core structures. The essentially similar molecular architecture of the Crown humus-FAs compared to those of terrestrial and aquatic humus, is exemplified by their ESR characteristics (g value, spins) (**Table III**).

Conclusion

The chemical and biological processes that underlie epiphytic and microbial interactions, for the growth and preservation of arboreal mini-ecosystem in rain forests and the contribution of Crown humus in these processes are highlighted. The strikingly common features of humic substances occurring in widely divergent natural habitats (terrestrial, aquatic and arboreal), point to the major role played by some ubiquitous bacteria (extremophiles, mesophilic microorganisms)[10-12,14] in their synthesis and metabolism. These constitute the pedological link in the humification process. The primitive forms of micro-organisms would have a remarkable ability to derive energy, necessary for their growth and maintenance, from external sources. One such external source constitutes the metallo-organic reaction leading to the formation of iron pyrite (FeS_2)—a key mineral constituent of the Ayurvedic *maharasas*[7,11]. The pyrite-driven energy flow has been postulated to be linked with the most pervasive features of extant metabolism[13]. The effects of the *maharasas* (rejuvenators) are manifested by the redox interplay in osmo-enzymes, catalyzed by the low and medium M_w humic substances[5-7]. When the functions of the osmo-enzymes (mitochondrial energy-generator) are impaired by the onslaughts of reactive oxygen species (ROS), the metallo-humate *maharasas*[7] rejuvenate them. The same/similar rejuvenating mechanism is conceivably involved in the biological functions of Crown humus.

The common pedology and characteristics of terrestrial, aquatic and arboreal humus manifest a universal scheme of bioscience towards the genesis and sustenence of life on earth.

Experimental Section

Test samples. The location of collection of the test samples was Monte Verde Reserve Forest (lower montane cloud forest, 10°18' N and 84°48' W), Costa Rica. The samples of canopy organic matter (COM -1 to -4) were collected from the inner crowns of four dominant host species, viz., *Dussia macrophyllata* (Fabaceae), *Ocotea tonduzii* (Lauraceae), *Meliosma vernicosa* (Sabiaceae) and *Pouteria viridie* (Sapotaceae), *ca.* 20-30 m above ground. The other three COM (-5 to -7) samples were collected from an adjacent patch of secondary forest that is occupied by a near-monospecific stand of 20 year-old (*ca.* 20 m above ground) trees—*Canostegia bernoulliana* (Melastomataceae); the forest floor (FF-1) sample (upper 10 cm of soil) was collected from just below the *Canostegia* trees.

Techniques

Column chromatography. Silicic acid (Mallinekdrodt, 100 mesh) was used as the adsorbent. Solvents of graded polarity, *n*-hexane, hexane-diethyl ether (95.5:0.5, 98:2, 95:5, 90:10) and diethyl ether, were used as eluents. The eluates were monitored by

HPTLC and similar fractions were combined for subsequent processing.

HPTLC-CAMAG TLC. (Plate material, silica gel 60 F_{254}) evaluation assembly (CATS 3.16/Scanner II V 3.14) was employed. The detection of spots was done by both quenching and fluorescence modes using authentic markers. Three solvent systems, viz. *n*-hexane-diethyl ether (99.5:0.5, Solvent-1), chloroform-methanol (90:10, Solvent-2) and *n*-butyl alcohol-acetic acid-water (4:1:2, Solvent-3) were routinely used as the developers.

HPLC. Waters Associates HPLC assembly into a RP-8 column, equipped with PDA and RI detectors, were employed. Methanol-water (80:20, Solvent-4) and acetonitrile (Solvent -5) were used as eluents.

GLC. The comparative analyses of the mixture of lipids and other low M_w (mol.wt.) organic compounds were carried out on a Hewlett-Packard (HP-5890) instrument, equipped with a flame-ionization detector, coupled to a microprocessor-controlled integrator (HP-3394A). Glass column (1.8 m × 0.2 cm i.d.), packed with 3% SE-30 non-polar liquid phase coated on chromosorb-W (HP) (80-100 mesh) was used for analysis of the hydrocarbons, acetates of alcohols, steroid-terpenoids, and acyl-alkyl glycerols. For the detection of dibenzo-α-pyrones, hydroxy-acetophenones, and other low M_w aromatic compounds the conditions employed were—oven 210°C (2 min-hold), 6°C/m increase upto 320°C (15 min-hold); detector, FID; 380°C; carrier gas , N_2, 30 mL/min. The fatty acids were analyzed as methyl esters on a diethyleneglycol succinate polyester (10% DEGS) column, coated on chromosorb-W (80-100 mesh) and packed into the glass column as above.

GC-MS. Gas chromatographic separation was performed on a OV-1 (30 m × 0.33mm) capillary column; oven temperature was programmed at 80°C (1min-hold) to 320°C (15 min-hold) at 4°C/min rise: injection temperature was 250°C. MS was obtained on a Hitachi M-4100 instrument at an ionization potential of 70 eV.

Electron probe micro-analyzer (EPMA). The analysis of metals and other elements (**Table III**) was performed on a Shimatzu EPMA-8705 instrument. The conditions were ACC .V (kV) 20.0; S.C. (micro-) 0.20, beam size, 30-150 μm.

Electron spin resonance (ESR). The ESR spectra of the humic substances (FAs, HAs and HMs) were recorded on a Varian-112 spectrometer, according to a previously described procedure[16]. Briefly, dried and finely powdered samples were taken, separately, in standard ESR tubes. The spectra were recorded at room temperature using 100 KHz modulation at a microwave frequency of 9.5 GHz.

Marker samples. The even number aliphatic acids (C_{12}-C_{28}) were obtained from Nacalai Tesque Inc., and 13-methyl pentacosanoic, 14-methyl pentacosanoic, 2-octanoic, palmitoelaidic, elaidic, azelaic, vanillic and ferulic acids, and the sterol samples were procured from Wako Pure Chemical Industries Ltd. (Japan). The oxygenated dibenzo-α-pyrones, hydroxyacetopenones and the heterocyclic compounds were earlier isolated and charaterized from terrestrial humus[4,6-7].

Isolation of organic constituents of COM and FF. The general method of isolation and mode of characterization of the organic compounds of COM -1 to -7 and of *FF*-1 are given in **Scheme I.**

The HPTLC finger- prints of the organic constitutents of COM -1 to -7 and of FF-1 were closely similar although of different intensities. Similar observations were made in respect of the organic constituents isolated from the microporic voids of the respective fulvic acids (FAs) (*vide infra*).

In a typical experiment, COM -1 (*ca.* 100 g., dried sample, sieved through a 2 mm-mesh) was extracted with hexane-diethyl ether (9:1) at room temperature, overnight. The extract was filtered and the filtrate was evaporated to give a gummy residue (Fraction A, 0.25 g). This fraction essentially contained lipids and was further analyzed by HPTLC and GLC, using markers.

The marc (Marc A) was then sonicated (Lab-Sonic, Sonicator; 50% of maximum speed; Duty cycle, 0.5 Sec; for 2 min × 3, at 10°C) and then divided into two parts (Parts I and II).

Enfleurage trapping. The volatile constituents, present in part I (inner core of COM-1, *ca.* 40g.), were adsorbed into a fresh fat mixture (triolein palmitate in 1:10 ratio). After about 24 h, the adsorbed constituents were extracted with acetonitrile and lyophilized. The residue was subjected to HPLC (using solvent-5) and GC-MS analyses. (**Table II**). The part II sample (*ca.* 58 g) was exhaustively extracted with solvents of graded polarity to give fractions B to D (**Scheme I**).

Fraction B. A portion (0.37 g) of this fraction was subjected to column chromatography over silicic acid

using chloroform (750 mL, residue from chloroform eluate was marked R-1, 0.086 g), acetone (2 L, R-2, 0.17 g.) and methanol (750 mL, R-3, 0.104 g) as eluents. R-1 to R-3 were resolved into further sub-fractions by prep. TLC using solvent-1 and -2 as developers. The TLC scrapings were eluted with methanol, the methanol extractives and their acetyl and methyl ester derivatives were subjected to GLC analysis. The results are reported in **Table II**.

The more polar fractions isolated from the outer surface and inner core (Fraction B) of COM -1 were silylated and then subjected to GC-MS analysis.

Silylation. A small portion (0.3 mg) of each sample was dissolved in chloroform- methanol (2:1, 50 µl). An aliquot (10µl) of this solution was treated with *N, O*-bis (trimethyl silyl)-Trifluoroacetamide (Wako) at 60°C for 1 hr. A portion (1µl) of the silyl derivatives was injected into the GC-MS assembly.

The GC-Scan nos and mass spectral (M^+ and fragment ions) patterns of the silyl ether, ester and ether-ester derivatives, as given in parentheses, corresponded with the following compounds:

3-hydroxy-butyric acid (Scan 814-816; *m/z*, 233 (M-15), 191, 147, 117, 73); urea (913-933; *m/z*, 204, 189, 147, 99, 73); benzoic acid (968-970; *m/z*, 194, 179, 135, 105, 77); phosphoric acid (1055-1057; *m/z*, 299, 147, 73); glycerol (1085-1088; *m/z*, 308, 293, 205, 147, 73); succinic acid (1122-1124; *m/z*, 247 (M-15), 147, 73); 2-phenoxy ethanol (1216-1218; *m/z*, 210, 195, 151, 117, 73); glutaric acid (1311-1313; *m/z*, 261 (M-15), 229, 204, 147, 97, 73); 2-deoxytetronic acid (1408-1410; *m/z*, 321 (M-15) 281, 233, 189, 147, 117, 73); vanillin (1544-1546; *m/z*, 224, 209, 194, 156, 73); vanillic acid (1990-1992; *m/z*, 312, 297, 267, 223, 193, 126, 73); ferulic acid (2508-2510; *m/z*, 338, 323, 249, 219, 187, 147, 73); ergosterol (4006-4008; *m/z*, 527 (M-15), 473, 383, 344, 281, 216, 191, 159, 129, 105, 73); sitosterol (4139-4141; *m/z* 496, 397, 357, 304, 255, 204, 159, 129, 73).

The GC-MS analysis also showed the presence of PUFA (C_8-C_{24}); the aromatic and phenolic acids, alcohols and ketones (**Table II**) as reported in case of *shilajit*[5,6] and Ayurvedic *makshika*[7].

Fraction C. A portion (0.05 g) of this fraction was subjected to column chromatography using solvent-1 and -2 as eluents. Eluates containing similar entities (HPTLC) were combined, concentrated and then subjected to prep. TLC (Solvents 1-3). HPTLC and HPLC analyses of the sub-fractions showed the presence of oxygenated dibenzo-α-pyrones (**1-4, Chart 1**) and *o*- and *p*-hydroxy-aceto-phenones. Their identities were established by UV, IR and MS spectral analyses, and GC-MS analyses of the respective *o*-silyl derivatives.

Fraction D. The major constituents of this fraction were in complex association with metal ions and minerals. One of these constituents, containing $Fe^{3+/2+}$ ions, was separated by prep. TLC. and paper electrophoresis as before[15]. Its identity (**9a, b**) was established by direct comparison (HPTLC, FT-IR) with an authentic sample[15].

Column chromatography of fractions C and D afforded further crops of the oxygenated dibenzo-α-pyrones (**1-4**) and metal ion complexes of their dimers (**9a, b**, M=Fe, Al, Ti).

Marc D. The powdered marc (**Scheme I**) was triturated with an aqueous solution of NaOH (0.1 *N*); N_2 was passed through the solution for 1 h. The solution was filtered. The filtrate was successively extracted with ethyl acetate and *n*-butyl alcohol. The organic solvent extractives showed (HPTLC, HPLC, GLC), the presence of low M_w organic compounds (**Table II**), now partially released from the micropores-voids of the humic substances. The aqueous alkaline mother liquor was cooled and then acidified with HCl to *p*H~3. The acidified solution was kept at room temperature overnight. The mixture of precipitated humic acids (HAs) and humins (HMs) was collected by centrifugation. The acidic supernatant was then extracted with ethyl acetate and *n*-butyl alcohol to isolate a further crop of the freshly released low M_w organic constituents from the micropores of fulvic acids (FAs). Comprehensive HPLC and GC-MS analyses of the mixture and their *O*-silyl derivatives exhibited the occurrence of the major constituents of fractions A to C in the micropores of the Crown humus -FAs.

Crown humus-FAs. The aqueous acidic mother liquor was adsorbed on activated charcoal (1:20). The adsorbed FAs was then eluted from the charcoal by washing with acetone (100 mL). The yellowish-brown acetone solution was evaporated to give Crown humus FAs as a light brown powder. The physical and analytical properties (**Table III**) of the FAs from COM and FF were closely similar to those of terrestrial humus-FAs[15].

Ion-exchange chromatography. The metal ions were separated from the metallo-humate complexes

following this technique. The Crown humus-FAs and FF-FAs samples (in 10-100 mg-portions) were dissolved in distilled water (10 mL). To each flask, 1 *N* KCl (0.5 mL) was added. Dowex resin (50 W × 8, 1 g) was separately saturated with K^+ ion. The solutions of FAs were transferred to K^+ -saturated Dowex resin in Erlenmeyer flasks. Each mixture was mechanically stirred at 25 ± 2°C for 1 hr. The exchange resin was then removed by filtration. The filtrate and washings were combined and evaporated. The free phenolic compounds thus generated were analyzed by HPTLC using markers. A portion was silylated and analyzed by GC-MS, when the presence of oxygenated dibenzo-α-pyrones (**1, 3**) and hydroxy-acetophenones (**Table II**) were detected. The metal ions were released from the ion exchange resin column by treatment with 2*N* HCl; the effluent was evaporated and the metal ions were analyzed by EPMA and atomic absorption spectroscopy (**Table III**).

Crown-HAs. The HAs were separated from the HMs-HAs mixture by dissolving in dilute aqueous solution of $NaHCO_3$ in which HMs were insoluble and were collected by filtration. The aqueous alkaline filtrate on acidification and evaporation gave HAs. Exhaustive solvent extraction (*n*-BuOH) of HAs afforded di- and oligomeric dibenzo-α-pyrones from their micropores.

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