An evaluation of the differences in the composition of humic acids in soils under oak and pine forest by GC-MS after mild degradation

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Abstract

Significant differences were found in the products released after eight samples of humic acids (HA's) isolated from soils under mediterranean pine and oak forests underwent mild degradation. A number of compounds, including typical degradation products of lignins, were found exclusively in the HA's from the soils under pine forests. The aliphatic constituents were more firmly retained in the HA's under oak, which presented mean values for aromatic acids lower than the humic samples from pine forests. Different types of diterpene resin acids were also found amongst the mild degradation products of HA's from the studied areas.

Introduction

The evolution of humus and the composition of humic colloids may vary greatly in relation to a series of parameters, such as vegetation, microbiological activity and edaphoclimatic or geological factors. Therefore, studies of the particular influence of vegetation on the composition of humic acids (HA's) require a comparison between areas that are homogeneous with regard to climatic and geomorphological factors as well as soils with a reduced biological activity where the biogenic compounds or the organic substances formed by the chemical alteration of plant biopolymers are preserved.

These conditions are present in the brown soils on triassic sandstones located in Guadalajara (Central Spain), under mediterranean pine or oak forests. Previous studies on the physico-chemical characteristics of HA's from these areas (Almendros and Velasco, 1984), suggested a different contribution of specific humification pathways under the dominant arboreal species.

In order to confirm these assumptions, HA's from the above soils were studied after ultrasonic

treatment in organic solvent followed by mild degradations with potassium persulphate. These methods are more suitable for the recognition of differences between the humic samples than other drastic degradative methods commonly used for structural studies (Schnitzer and Khan, 1972; Martín and González-Vila, 1981). The present paper describes the qualitative and quantitative differences between bound lipids and mild degradation products from both HA's, which will be discussed in terms of the influence of the original biomass on the chemical composition of the HA's.

Materials and methods

Material

Analytical characteristics and ecological factors of the soils studied were described earlier (Velasco and Almendros, 1984; Almendros and Velasco, 1984). A total of eight soil samples, four under conifer species (*Pinus pinaster* Sol. ssp. *mediterranea*) and four under oak species (*Quercus rotundifolia* Lam., *Q. lusitanica* Webb ssp. *faginea* DC. and *Q. pyre-*

naica Wild), were selected for the extraction of humic colloids.

All samples studied presented a tendency towards sandy textures and pH values between 4 and 7, C/N ratios between 20 and 40, and a total exchange capacity (pH:8) of between 12 and 18 meq.100 g⁻¹. Humus types ranged from *sandmull* (= 'sandy moder') (Duchaufour and Souchier, 1977) to *mor*.

For the isolation of HA's, air-dry soil samples were first extracted with light petroleum (to remove free lipids), treated with bromoform-ethanol 1:1 to remove the floating organic particles (free organic matter), and then extracted with 0.1N NaOH under N₂. The HA's were precipitated by acidifying the alkaline extract and were purified by repeated treatments with HCl-HF(50%)1% and centrifugation in alkaline pH.

Mild degradation of humic acids

Physical pretreatments. 400 mg of freeze-dried HA were first suspended in 50 cc of toluene and dispersed with a Branson ultrasonic disruptor Sonifier B-12 for 5 minutes. Extraction was continued in a Blount's micro-extractor with the same liquid for 12 h, and these operations were repeated for a week, using fresh solvent every day. The extracts were put together, the solvent was removed in a rotary evaporator, and the lipid fractions were methylated with ethereal diazomethane (Schnitzer, 1974). Since the composition of these bonded lipid fractions were too complex for an adequate gas-chromatographic separation, they were fractionated by preparative column chromatography with silicagel, first using hexane and then methylene chloride-hexane 3:1 as eluents.

Oxidative degradation. After physical pretreatments, persulphate degradation of HA's was carried out (Martín *et al.*, 1981). Degradation products were extracted in a liquid-liquid extractor with ethyl acetate (8 h). The organic phase was dehydrated with anhydrous Na₂SO₄, the solvent was partially distilled, and the degradation products were finally dried under N₂, then methylated with diazomethane and stored at -20°C under N₂. Because of the brownish colour of the methylated degradation products, they were puri-

fied by column chromatography using silicagel and methylene chloride as eluents.

Gas chromatography-mass spectrometry analyses

The separation and identification of the individual components in the methylated extracts from HA's and the methylated mixtures of degradation products were performed in a Hewlett-Packard 5992B gas chromatographic-mass spectrometric system with a 25-m cross-linked capillary column coated with OV-101. The oven temperature was programmed for between 10 and 270°C, carrier flow was 1 ml.min⁻¹, and ionizing voltage was 70 eV.

The relative abundance of peaks (as regards the total amount of volatile products of each sample) was computed for statistical comparison using the peak size detected by a FID in a Hewlett-Packard 5730A GC in which the samples had previously been injected under the same conditions.

Results and discussion

Organic compounds removed by physical pretreatments

After repeated ultrasonic shaking and toluene extractions, the HA's yielded an important fraction of loosely-joined lipid compounds. The proportion of removed lipids were significantly different depending on the type of vegetation: in pine samples it presented a mean value of 11.6%, while in oak samples only 2.7%.

The organic compounds identified (90–99%) were different types of hydrocarbons, fatty acids and resin acids (Figs. 1, 2 and Table 1). In samples under pine forests, the alkane series ranged from between C₁₄ and C₂₇, and showed a maximum of between C₁₆ and C₂₀ and no odd-chain predominance. The last two parameters are frequently considered as indicative of hydrocarbons of microbial origin associated with HA's (Schnitzer and Khan, 1972). In the samples under oak forests, the alkanes were not abundant and the CPI values were erratic, but presented a maximum near C₂₇, suggesting a higher presence of epicuticular waxes (Simoneit and Mazurek, 1982). These tendencies were also

Fig. 1. Gas chromatographic separation of lipid fraction eluted with methylene chloride from total amount of compounds removed by ultrasonic pretreatment in toluene of humic acids. *Top*: Humic acids from pine forest (injection ratio 1:400). *Bottom*: Humic acids from oak forest (injection ratio 1:100)

confirmed by the analysis of the fatty acid series: in the samples under pine the *n*-homologues had a carbon range of C_{12} – C_{24} , with the maximum at C_{14} or C_{16} and the more abundantly branched fatty acids were two isomers of the C_{17} acid (compounds No. 33 and 34), probably with configurations *iso* and *anteiso*, which also suggested a microbial influence in these aliphatic components of HA's.

The fatty acid composition of the samples under oak presented a bimodal distribution with maxima at C_{24} and C_{16} , the ratio of fatty acids ≥ 20 -C/fatty acids < 20 -C atoms being higher than in samples under pine. In this case, the more prominently branched fatty acids were two C_{15} isomers (No. 22 and 23). Unsaturated fatty acids (mainly $C_{16:1}$, $C_{18:1}$ and $C_{18:2}$) were also found in highly variable proportions (7–30% under pine and 4–8% under oak). Important compounds identified in these lipid fractions were the diterpene resin acids (10–33% of the polar subfraction in the lipids of HA from pine soils), the most common being dehydroabietic acid (No. 45). Other resin acids that were identified included an abietadienoic acid (No. 44), pimaric acid (No. 43), and an unusual resin acid assigned to a secodehydroabietic acid (No. 40)

Fig. 2. Gas chromatographic separation of products released by persulphate degradation after treatments with hot toluene. *Top*: Humic acids from pine forest (injection ratio 1:100). *Bottom*: Humic acids from oak forest (injection ratio 1:200)

(Zinkel *et al.*, 1971). The occurrence in small quantities of these compounds in samples under oak forests (No. 40, 43 and 45) may be explained by probable wind transportation from the neighbouring pine forests. In fact, these compounds are not useful biological markers for vegetation types in neighbouring areas because of their presence in aerosols (Simoneit and Mazurek, 1982).

Persulphate degradation of pretreated HA's

The compounds released by persulphate oxidation of both types of HA were different in nature as were the relative amounts of the different compounds, although the degradation percentages were very similar; mean values were 61.7% for *Quercus* HA's (6.9% soluble in ethyl acetate) and 58.9% for *Pinus* HA's (5.3% soluble in ethyl acetate).

In samples from pine forests (Table 1 and Fig. 2), 1,3 benzenedicarboxylic acid (Comp. No. 6) and methyl vanillate (Comp. No. 7) were the most common degradation products, while dimethoxybenzaldehyde (Comp. No. 5) was also very common in

Table 1. Organic compounds^a obtained after mild degradation of humic acids from forest soils under *Pinus pinaster* or *Quercus* sp.

No.	Compounds identified both in samples under Pine and Quercus	No.	Compounds found exclusively in samples under Pinus forest
3	n-Tetradecane (2)	1	Methyl 3 phenil propionate (P)
8	n-Pentadecane (S ^c)	2	Propane tricarboxylic acid trimethyl ester (P)
9	Methyl dodecanoate (laurate) (S)	4	1,2 Benzene dicarboxylic acid dimethyl ester (P)
10	Dimethoxy benzenecarboxylic acid methyl ester (I ^b) (P ^d)	5	Dimethoxybenzaldehyde (P)
11	Dimethoxy benzenecarboxylic acid methyl ester (II) (P)	6	1,3 Benzene dicarboxylic acid dimethyl ester (P)
12	n-Hexadecane (S)	7	Methyl vanillate (P)
13	Methyl tridecanoate (S)		
14	n-Heptadecane (S)	14	14-C branched fatty acid methyl ester (S)
18	14-C branched fatty acid methyl ester (I) (S)	16	Benzophenone (P)
19	Methyl tetradecanoate (myristate) (S)	17	Dimethoxy benzenecarboxylic acid methyl ester (III) (P)
21	n-Octadecane (SP)	20	1,2,3 Benzene tricarboxylic acid trimethyl ester (P)
22	15-C branched fatty acid methyl ester (I) (S)		
23	15-C branched fatty acid methyl ester (II) (S)	26	2-Carbomethoxy benzophenone (I) (P)
24	Methyl pentadecanoate (SP)		
25	1,3,5 Benzene tricarboxylic acid trimethyl ester (P)	30	Carbomethoxy benzophenone (II) (P)
27	Methyl hexadecenoate (palmitoleate) (SP)	31	Carbomethoxy benzophenone (III) (P)
28	n-Nonadecane (S)		
29	Methyl hexadecanoate (palmitate) (SP)		
32	n-Eicosane (SP)	36	Methyl octadecadienoate (linoleate) (S)
33	17-C branched fatty acid methyl ester (I) (SP)		
34	17-C branched fatty acid methyl ester (II) (SP)		
35	Methyl heptadecanoate (margarate) (P)		
37	Methyl octadecenoate (oleate) (SP)		
38	n-Heneicosane (SP)		
39	Methyl octadecanoate (stearate) (SP)		
40	Methyl 2S-(2'(m-isopropylphenyl)ethyl)-1R,3S-dimethyl cyclohexanecarboxylate (secodehydroabietate) (S)		
41	n-Docosane (S)		
42	Methyl nonadecanoate (S)		
43	Methyl 8(14),15-pimaradien-18-oate (SP)	44	Methyl 8,12-abietadien-18-oate (SP)
45	Methyl 8,11,13-abietatrien-18-oate (dehydroabietate) (SP)		
46	n-Tricosane (S)		
47	Methyl eicosanoate (arachidate) (SP)		
48	Diethyl adipate (SP)		
49	n-Tetracosane (SP)		
50	Methyl heneicosanoate (S)		
51	n-Pentacosane (SP)		
52	Methyl docosanoate (behenate) (SP)		
53	n-Hexacosane (SP)		
54	Methyl tricosanoate (S)		
55	n-Heptacosane (SP)		
56	Methyl tetracosanoate (lignocerate) (SP)		
57	n-Octacosane (P)		
58	Methyl pentacosanoate (SP)		
59	n-Nonacosane (P)		
60	Methyl hexacosanoate (cerotate) (SP)		
61	Methyl heptacosanoate (SP)		
62	n-Hentriacontane (P)		
63	Methyl octacosanoate (SP)		
64	Methyl nonacosanoate (SP)		
65	Methyl triacosanoate (S)		

^a As methyl esters.

^b Roman numbers indicate different isomers.

^c (S) Obtained after repeated sonication and extraction with hot toluene.

^d (P) Obtained by persulphate degradation after toluene treatment.

Chromatographic peaks representing less than 0.1% of total volatile products were not taken into consideration.

certain samples. Other compounds identified were different alkanes and fatty acids, methyl phenyl propionate (No. 1), resin acids (Nos. 40, 43, 44, 45), propane tricarboxylic acid (Comp. No. 2), and some benzophenones (Comps. 16, 26, 30 and 31). These latter compounds may be constituents of various higher plants, (Ribereau-Gayon, 1968), but as they were not detected among the compounds released after physical pretreatments, the origin of these molecules could be persulphate-induced reactions in alicyclic and aromatic structures.

HA from oak forest showed different oxidation products, the most common generally being C₁₆ and C₁₈ n-fatty acids (Nos. 29 and 39). Small amounts of phenolic and benzenecarboxylic acids were also detected. If compared with the results of other oxidation methods, such as the permanganate or alkaline cupric oxide ones, the different HA's showed not only differences in the aliphatic: phenolic:benzenecarboxylic acid ratios, but also in the types of compounds released by oxidation. Thus, Table 1 shows that several of the identified degradation products were present exclusively under pine forest (no specific compounds for oak forest were found). Most of these compounds characterizing HA's from soils under pine are typical degradation products of lignin, confirming previous suggestions that altered lignin polymers are present in the HA fractions of soils under pine forest in this area (Almendros and Velasco, 1984).

Because of the intrinsic limitation of the quantitative studies on HA's when using the present degradative techniques, absolute yields were not computed, but the GC's were used as 'fingerprints', considering the normalized size of the different peaks. Taking peak heights into account, the analyses of variance of the degradation products simultaneously present in samples from each type of forest (Table 2), showed that a higher proportion of a benzenetricarboxylic acid, some fatty acids (C₁₆, C_{16:1}, C₁₇, C_{18:1}) and a C₁₇ branched fatty acid were characteristic of the samples under oak forest while dehydroabiatic acid and two long-chain alkanes (C₂₅ and C₂₈) were predominant in pine forest.

On the other hand, differences were also found when comparing the proportion of the main groups of degradation products of HA's from each type of vegetation (Table 2): it was observed that aliphatic compounds (mainly fatty acids) tend to be more abundant in samples under *Quercus*, while the

Table 2. Persulphate degradation products simultaneously present in digest from humic acids of soils under *Pinus pinaster* and *Quercus sp.*, showing significant ($P < 0.05\%$) differences in their relative peak areas

No.	Individual compound ^a	
25	1,3,5 Benzene tricarboxylic acid trimethyl ester	(Q)
27	Methyl palmitoleate	(Q)
29	Methyl palmitate	(Q)
34	17-C branched fatty acid methyl ester	(Q)
35	Methyl margarate	(Q)
39	Methyl stearate	(Q)
45	Methyl dehydroabiatic acid	(P)
51	n-Pentacosane	(P)
57	n-Octacosane	(P)
Groups of compounds		
	Total aromatic	(P)
	Total fatty acids	(Q)
	Total resin acids	(P)
	Total benzenecarboxylic acids	(P)
	Total phenolic acids	(P)

^a methyl esters

(P, Q): more abundant in HA's from soils under *P. pinaster* or *Quercus sp.*, respectively.

small amounts of alkanes were erratic. As expected, resin acids were significantly more abundant in samples under conifers, while phenolic and benzenecarboxylic acids (the former considered to be degradation products more typical of lignins) predominated in samples under pine.

A classic problem in studies on the aliphatic structures of HA's is that an undefined proportion of lipid compounds retained in soil may be incorporated in the HA fraction during alkaline extraction. The influence of such circumstance may vary, but in the present study their effects are probably reduced after the previous extraction of the free soil lipids and the desimetric removal of plant particles using a mixture of organic solvents. In any case, the possible association with non-humic fatty compounds would probably affect only the composition of lipids removed by ultrasonication in toluene. Their composition, on the other hand, is comparable with that reported for HA extractives obtained upon supercritical gas extraction (Schnitzler *et al.*, 1986).

In conclusion, it was observed that several of the characteristics recognized after HA's from soils under different forests underwent mild degradation may be attributed to the direct and indirect effects of the composition of the original biomass. In

general, humification processes were simpler in soils under pine than under oak forest. In the first case, the preservation of compounds directly derived from plants was more evident, and the oxidative alteration of lignins may be a predominant pathway leading to humus accumulation.

The results of the degradation studies were also in agreement with the fact that the geographic distribution of *P. pinaster* in the areas under study became wider in recent centuries, invading disafforested areas of *Quercus*. This would produce the overlapping of biosynthetic or diagenetic compounds derived from pine, on a constant biogenic background representative of the original *Quercus* vegetation.

The association of different kinds of aliphatic compounds in the humic structures was remarkable. In samples under pine the lipid compounds tend to associate loosely (directly removable by physical treatments) but, in samples under oak, a higher chemical stability was demonstrated since most were obtained after persulphate degradation.

The tendency towards an unstable association of lipids (natural or pollutants) together with the humic polymer (Schnitzer and Khan, 1972) may explain the presence of diterpene resin acids in all the samples studied. As in the case of linear lipids, the physically-removable resin acids were probably 'entrapped' in the internal surfaces of three-dimensional humic polymers, but their presence in the persulphate digest from pretreated HA's suggested they might also be linked to HA, to some extent, by their carboxyl group. In accordance with the above criteria, the humification processes seem to be more efficient under oak forest, where biodegradation of

plant polymers was found to be more rapid and complete, and most of the aliphatic structures were not physically entrapped but chemically associated to HA's.

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