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Structurally bound lipids in peat humic acids

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Abstract

Humic acids from highly decomposed peat were subjected to oxidation with alkaline cupric oxide (CuO) at 170°C (single oxidation). Oxidation products were isolated as three fractions, oxidized humic acids, fulvic acids and lipophilic compounds. Isolated oxidized humic acids were subsequently re-oxidized (sequential oxidation) under the same conditions, and released lipophilic compounds were isolated. Lipids released during single and sequential oxidations were determined using capillary gas chromatography and mass spectrometry. Identified compounds accounted for 3.3 and 0.12 wt.% of humic acid content, respectively. Lipid profiles of lipophilic fractions released during single and sequential oxidation were markedly different. Lipids released by single oxidation contained triterpenoids (83%), shorter-chain fatty acids (11%) and β-sitosterol (6%). In contrast, sequential oxidation released various diterpenoid acids (53%) of coniferous resin origin, various unsaturated C_{18} fatty acids (20%), and a series (C_{22}–C_{34}) of *n*-alkanes (20%). The consistent distribution of *n*-alkane fraction with a minor predominance of even-chain homologues (CPI 0.95), a high proportion (77%) of dehydroabietic acid and its degradation products, and a high preponderance of abietane over pimarane structures in the diterpenoid acid fraction all indicate substantial microbial activity within the humic acid matrix. Since polyfunctional compounds were predominant in monomeric lipids released from humic acids, the current data indicate that these compounds may potentially bind to humic acids at two or more points.

Keywords: Humic acids; Humified peat; Cupric oxide oxidation; Diterpenoid acids; *n*-alkanes; Unsaturated fatty acids; Triterpenoids

1. Introduction

Humic substances are comprised of three operationally defined fractions, water soluble fulvic acids, acid-precipitated humic acids and water insoluble humins (Aiken et al., 1985). Humic substances are ubiquitous organic constituents present in a wide range of soils, peats, sediments and aquatic ecosystems. In agriculture, humic substances are fundamental to good soil properties and the control of nutrient and trace element balances (Stevenson, 1985; Violante et al., 1999). However, humic substances can also be detrimental, particularly during municipal water treatment, due to the production of mutagens in the presence of molecular chlorine (Vartiainen, 1986; Kronberg, 1987; Becher et al., 1991).

Humic substances appear to exist as polyfunctional macromolecules (Steinberg and Münster, 1985; Peuravuori, 1992), and constantly change their structural conformations due to interactions involving the carboxylic acid, phenolic and alcoholic hydroxyl functional groups present. They are formed in situ during chemical, physical and microbial degradation of biogenic compounds (Steinberg and Münster, 1985; Stevenson, 1985; Ikan et al., 1986; Rasyid et al., 1992) due to the activities of biosynthetic enzymes (Batistic and Mayaudon, 1970; Morita, 1981; Witthauer and Klöcking, 1981; Fuchsman, 1983; Bollag and Liu, 1985; Katase and Bollag, 1991; Lassen et al., 1991) indigenous enzymes (Witthauer and

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Klocking, 1981; Fuchsman, 1983; de Haan, 1983; Steinberg and Münster, 1985; Bertino et al., 1987; Amador et al., 1989), UV light (Schnitzer, 1982; Katase, 1985; Steinberg and Münster, 1985; Kotzias, et al., 1987; Amador et al., 1989, Backlund, 1991; de Haan and de Boer, 1991) and adsorptive abiotic transformations in clay soils (Bollag and Liu, 1985; Stevenson, 1985; Okamura, 1991; Miodragovic et al., 1992).

2. Origin of the lipids in humic acids

Lipids in humic acids originate from aliphatic components of macromolecules and are important for conformational flexibility (Steinberg and Münster, 1985). Organic materials including pesticides (Stevenson, 1985), PAHs (polyaromatic hydrocarbons) (Morehead et al., 1986), PCB (polychlorinated biphenyl) (Chiou et al., 1987) and lipids (Ogner and Schnitzer, 1970; Khan and Schnitzer, 1972; Kemp, 1973; van Vleet and Quinn, 1979; Aiken et al., 1985; McKnight et al., 1985; Bertino et al., 1987; Martin and Gonzales-Vila, 1988; Almendros and Sanz, 1989; Becher et al., 1989; del Rio et al., 1989) are capable of attaching to humic acids by various mechanisms. Adsorptive associations tend to occur due to hydrogen bonding, ion-exchange or van der Waals forces, the extent of which is dependent on the source (Morehead et al., 1986) and chemical nature (Chiou et al., 1987) of humic substances and on the polarity of adsorbed compounds. Although relatively minor components of humic substances (Mathur and Farnham, 1985; Tegelaar et al., 1989b), biogenic lipids may have an important role in humic acid synthesis due to the presence of hydroxy and dicarboxylic fatty acids that potentially facilitate esterification of carboxylic acid and hydroxyl groups in the humic acid molecules, respectively. Various lipids, accounting for up to a few percent units of the starting material, have been identified following humic acid oxidation (Schnitzer and Ortiz de Serra, 1973; Martin et al., 1981; Anderson et al., 1985; Ishiwatari, 1985), reductive degradation (Michaelis et al., 1989) and transesterification (Almendros and Sanz, 1989).

Oxidation based on alkaline cupric oxide (CuO) is routinely used to determine lignin derived aromatic components of humic acids (Ertel and Hedges, 1984). The composition of phenolic monomers released from the milled peat sample subjected to CuO oxidation (Hänninen et al., 1986), $KMnO_4$ oxidation (Hänninen and Paajanen, 1989) and alkaline degradation (Hänninen and Niemelä, 1991, 1992) has been previously reported.

In addition to phenolic compounds, alkaline CuO oxidation also releases bound lipids from the humic acid matrix. Since oxidation is performed under mild conditions, the molecular structure of the lipids remains intact (Hänninen et al., 1986), and the oxidation method, therefore, is a valuable approach for the determination of humic acid lipid content.

In the current study the composition and content of lipids released in two sequential alkaline CuO oxidations of humic acids derived from a milled, humified peat were assessed.

3. Experimental

3.1. Peat sample

Milled peat from the Haukineva bog (Peräseinäjoki, Western Finland) was supplied by Vapo Ltd. The peat sample was characterized by botanical content, age, elemental analysis, and degree of humification (Hänninen, 1987).

3.2. Isolation of humic acids

The procedure used to isolate humic acids from peat has previously been described in detail (Hänninen et al., 1986). Peat was air-dried, ground, passed through a 2 mm sieve and sequentially solvent-extracted with water (24 h) and chloroform (24 h). A 10 g portion of washed, solvent-extracted peat was extracted 10 times with 100 ml portions of fresh 0.1 M NaOH. The extracts were combined and centrifuged for 2 h at 14,000 rpm. The supernatant was acidified with 6 M HCl to pH 2. Humic acids were isolated by centrifugation (4500 rpm, 5 min), redissolved in minimal amounts of 0.1 M NaOH and purified by dialysis against distilled water. Purified humic acids were subsequently freeze-dried, weighed, placed in minibags and stored in the dark. Elemental analysis was performed using a Carlo-Erba analyzer. Ash content determination and functional group analyses were carried out according to Hänninen (1987).

3.3. Humic acid oxidation (single oxidation)

Two 500 mg subsamples of humic acids were oxidized in a 150 ml stainless steel autoclave equipped with a Teflon cartridge, magnetic stirrer and heating mantle (Hänninen et al., 1986). Once dissolved in 2 M NaOH (60 ml), humic acids were oxidized using 2.5 g of CuO and 250 mg of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ at 170°C for 3 h (refer to Fig. 1). The reaction mixture was cooled to ambient temperature, centrifuged, and the clear supernatant was acidified to pH 1.5 with 6 M HCl. Oxidized humic acids were isolated by centrifugation and resuspended in 0.1 M NaOH, purified by dialysis, freeze-dried, placed in minibags and stored in the dark (fraction II). Following the removal of fraction II, the remaining supernatant was subjected to ethyl acetate (200 ml) extraction in a liquid-liquid extractor for 24 h, and the resultant water phase was concentrated, dialysed and

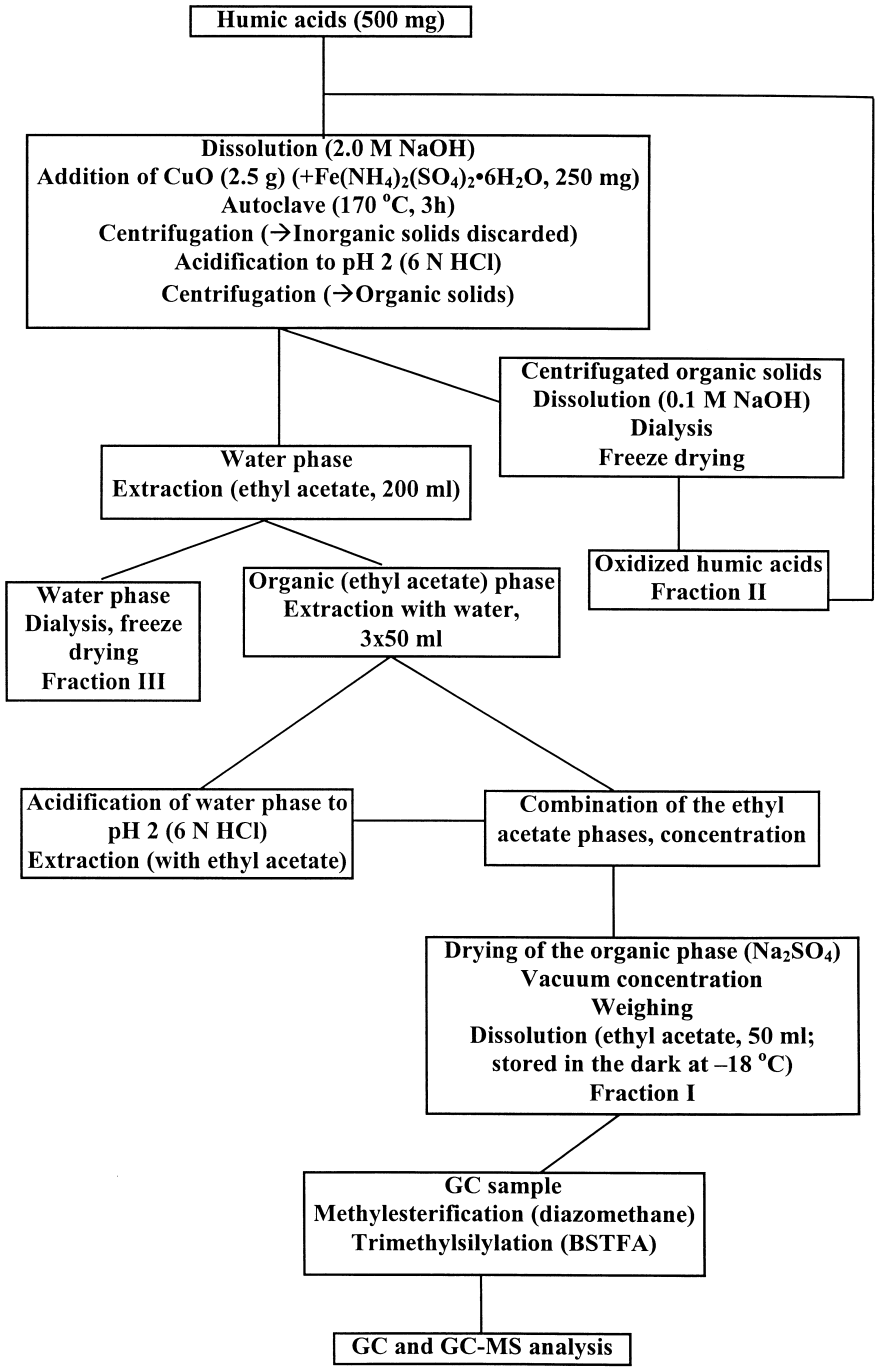

Fig. 1. Experimental procedures used for sequential oxidation of humic acids contained in highly decomposed peat.

freeze dried (fraction III). The ethyl acetate phase was rinsed with water (3×50 ml), and the resultant aqueous phases were combined, acidified with 6 M HCl to pH 1.5 and subjected to ethyl acetate (3×25 ml) extraction. Following extraction, organic phases were combined, dried with Na_2SO_4, concentrated, weighed, redissolved in 50 ml of ethyl acetate and stored in the dark at −18°C (fraction I).

The ^{13}C-NMR spectra of original (Fig. 2a) and oxidized humic acids (Fig. 2b) were obtained with a Bruker 250 MHz NMR spectrometer using 0.1 M NaOD dissolved in D_2O as a solvent and deuterated dimethylsulfoxide

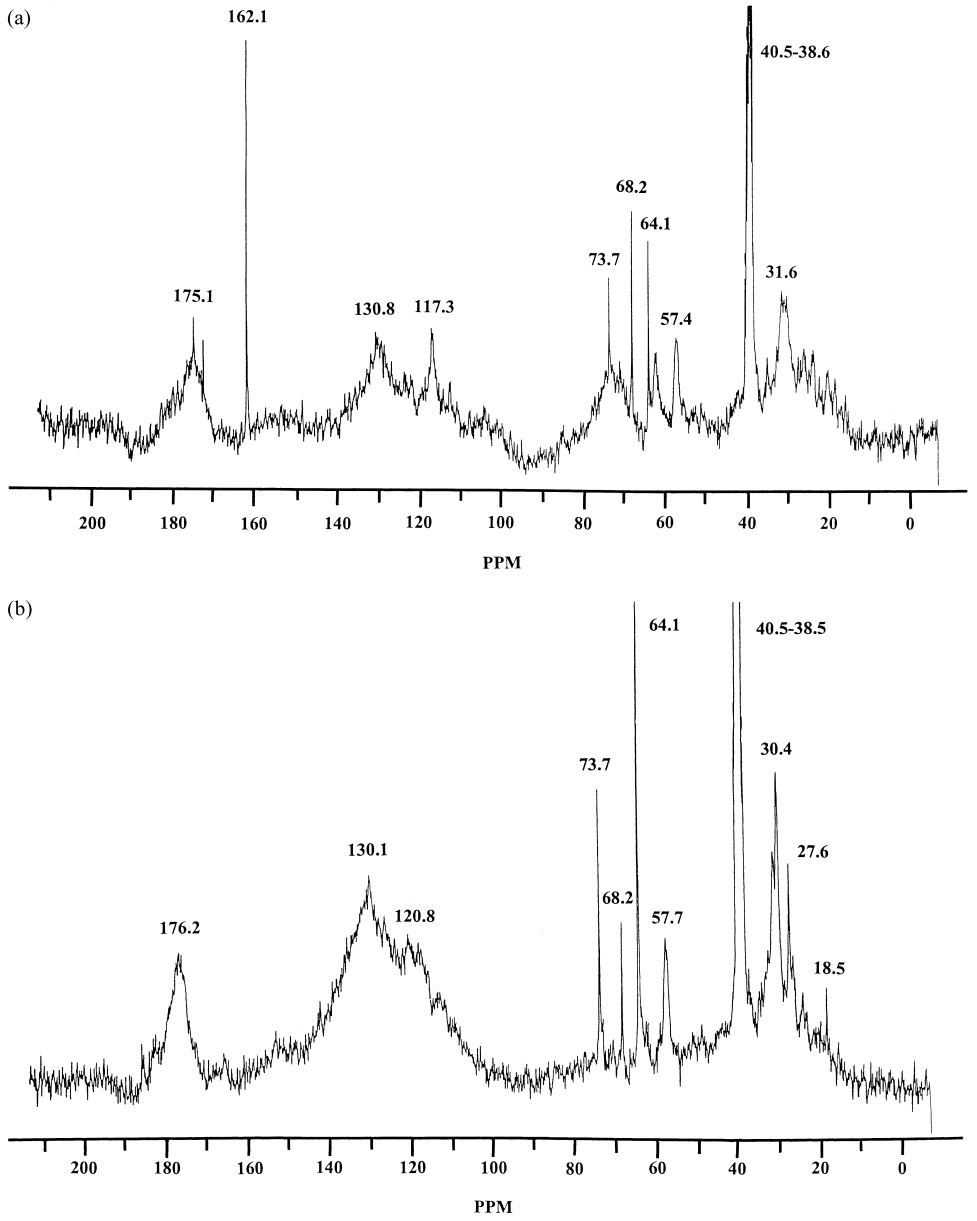

Fig. 2. ^{13}C NMR spectra of original (a) and oxidized humic acids (b) (dissolved in 0.1 M NaOD in D_2O).

(DMSO-d_6) as an internal standard (Hänninen, 1987; Hänninen and Paajanen, 1989).

3.4. Oxidation of oxidized humic acids (sequential oxidation)

Samples (200 mg) of fraction II, containing oxidized humic acids were subjected to further oxidization using the same procedures described for original humic acids. The organic fraction produced after ethyl acetate extraction was concentrated to approximately 1 ml, sealed under nitrogen and stored in the dark at −18°C before being submitted for chemical analysis.

3.5. Quantification of lipids

Lipids from the ethyl acetate extraction of the original humic acids were quantified with a Hewlett-Packard 5710A gas chromatograph fitted with a chemically bonded SE-54 fused silica capillary column (25 m, 0.32 mm i.d., 0.25 μm phase thickness; Nordion, Finland) using nitrogen (1.6 ml/min) as the carrier gas. Lipids from

ethyl acetate extraction of oxidized humic acids were quantified using a Micromat HRGC 412 (Nordion, Finland) gas chromatograph fitted with a SE-54 fused silica capillary column (25 m, 0.20 mm i. d., and 0.10 μm phase thickness; Nordion, Finland) using hydrogen (0.3 ml/min) as the carrier gas. Gas chromatograph oven temperatures were programmed to increase linearly from 130 to 300°C at 4°C/min with bold-times of 2 and 16 min at 130 and 300°C, respectively. Temperatures at the injection port and detector were maintained at 300 and 350°C, respectively. Separated lipids were monitored with flame ionization detectors on both gas chromatographs. Prior to analysis, carboxylic acids were converted to corresponding methyl esters with diazomethane and hydroxy compounds to their corresponding trimethylsilyl (TMS) ethers using BSTFA reagent [*N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS)] (Fluka, *purum*; Buchs, Switzerland) according to Lehtonen and Ketola (1993).

Mass spectra of lipids separated by gas chromatography were obtained using a VG Analytical 7070E mass spectrometer coupled to a Dani 3800 HRGC and VG 11-250 data processor (Ketola et al., 1986). The lipids were separated under the same chromatographic conditions used for lipids extracted from humic acids. Qualitative lipid analysis was based on comparison of unidentified compound retention times (relative to methyl *n*-heptadecanoate) with corresponding retention times obtained using authentic standards, comparison of unidentified compound mass spectral data with that of authentic standards or in some cases published data (Takeda et al., 1968, 1969; Zinkel et al., 1969; Holmbom et al., 1974; Simoneit, 1977; Ekman, 1979). Once identified, compounds were quantified using *n*-heptadecanoic acid (99%; Sigma, St. Louis, MO, USA) as an internal standard. Relative mass responses used for quantification were based on the most probable corresponding authentic straight-chain and cyclic lipid monomers.

4. Results

4.1. Peat and humic acid characteristics

Peat was found to be highly decomposed, as indicated by measurements of between H6 and H7 on the von Post (1922) scale, and was approximately 2000 years old based on ^{14}C dating (Hänninen, 1987). Only 30% of peat plant cell content could be botanically identified, and was found to contain material derived from coniferous trees (70%), deciduous trees (10%), *Eriophorum* (10%), *Sphagnum* (6%) and *Carex* (4%) species (Hänninen, 1987). Peat contained 48.0% C, 1.4% N, and 8.0% chloroform soluble lipids, on a dry weight basis.

Milled, humified peat contained 44 wt.% humic acids on a dry basis. Elemental analysis indicated that the original humic acids contained 56.5% C and 2.0% N. The ash content was 1.2% on a dry weight basis. Functional group analysis indicated a higher proportion of hydroxyl (total and phenolic 5.1 and 0.8 meq/g, respectively) than carboxyl groups (2.5 meq/g).

4.2. Extraction of free lipids

Solvent-extraction of peat with chloroform prior to the isolation of humic acids removed structurally unbound lipids. This lipid fraction, generally referred to as free lipids, is typically determined following saponification (Lehtonen and Ketola, 1993). The free lipid fraction contains both fatty acids (C_{13}–C_{34}, primarily even numbered chain lengths) and ω-hydroxy fatty acids (C_{12}–C_{28}, entirely even numbered chain lengths) which together form typically 70–90% of the total unbound lipid contents in humified peats. The free lipid fraction also contains relatively high amounts of fatty alcohols (C_{12}–C_{34}, mainly even carbon numbered chain lengths) and sterols (primarily as β-sitosterol). In addition, this fraction includes α,ω-alkanedioic acids (C_9–C_{28}), *n*-alkanes (C_{16}–C_{36}, predominantly as odd numbered chain lengths), *n*-alkan-2-ones (methyl ketones, C_{17}–C_{35}, with a predominance of odd numbered chain lengths) and several triterpenoids, mainly in the form of betulin and betulinic acid.

4.3. Humic acid oxidative fractionation

Humic acids were separated into three major fractions by the alkaline CuO oxidation procedure (Hänninen, 1987), chemically defined as a base soluble and acid insoluble fraction (oxidized humic acids), a base and acid soluble fraction and an ethyl acetate soluble fraction. Equivalent chemically distinct fractions were also released during the sequential oxidation of oxidized humic acids.

Following, oxidation, separation, purification and freeze drying procedures, 27.5 wt.% of original humic acids were recovered in oxidized form as a base soluble, acid insoluble fraction. Oxidized humic acids contained 46.3% C and 0.8% N. Recoveries of original and oxidized humic acids as ethyl acetate soluble fractions were 26.6% and 12.5%, respectively (Hänninen, 1987; Hänninen and Paajanen, 1989).

4.4. Release of lipids during humic acid oxidation

Oxidation of humic acids isolated from humified (H6-7) peat released lipids 3.15% on dry weight basis (refer to Table 1). This lipid fraction contained a high proportion of triterpenoids (83%), primarily as betulinic acid and other polyfunctional (bi-, tri- and tetra-) constituents. Shorter-chain saturated fatty acid homologues (C_{14},C_{16}, C_{18}) dominated the aliphatic component of released

Table 1
Lipids released from humic acids in highly decomposed humified peat during alkaline CuO oxidation

		μg/g	%
Fatty compounds		3300	10.5
Acids	%	3100	9.9
n-$C_{14:0}$	22.6	700	
n-$C_{16:0}$	22.6	700	
n-$C_{18:0}$	16.1	500	
n-$C_{19:0}$	3.2	100	
n-$C_{20:0}$	6.5	200	
n-$C_{22:0}$	12.9	400	
n-$C_{23:0}$	9.7	300	
n-$C_{24:0}$	6.5	200	
n-$C_{26:0}$	100.1	trace	
n-$C_{30:0}$		trace	
Alcohols		200	0.6
n-$C_{24:0}$		200	
Triterpenoids		26200	83.2
Acids			
3β-Hydroxylup-20(29)-en-28-oic (betulinic) acid		10300	
Ketones			
Friedelan-3-one		400	
x-Hydroxyfriedelan-3-one		6000	
x(y)-Acetoxy-3-oxotaraxer-14-ene-y(x)-ol and oxotriterpane[a]		9100	
Alcohols			
Lup-20(29)-en-3β-ol (lupeol)		400	
Sterols		2000	6.3
24-Ethylcholest-5-en-3β-0l (β-sitosterol)		2000	
Total amount		31500	100

[a] Values reported are for both compounds combined.

lipids, while n-tetracosanol and 24-ethylcholest-5-en-3β-ol accounted for 6% of total fatty monomers and total lipids, respectively.

The major triterpenones could only be partially identifed as a hydroxyfriedelan-3-one (TMS ether, M^+ 514), an acetoxy-3-oxotaraxer-14-enol (TMS ether, M^+ 570) and an oxotriterpane (TMS ether, M^+ 426). The precise origins of acetoxy-3-oxotaraxer-14-enol are unknown, since it may be formed either directly or as a result of acetylation of the corresponding oxotriterpenediol (or triterpenetriol) in the ethyl acetate solution (e.g. Ageta and Arai, 1983; Das and Mahato, 1983). An abundance of 3-oxotriterpenoid compounds tends to suggest that hydroxyl groups on the C-3 position are targeted during CuO oxidation.

Triterpenoid resins occur widely in higher plants (Chandler and Hooper, 1979; Pant and Rastogi, 1979; Das and Mahato, 1983), and in deciduous trees in particular (Ekman, 1983). These compounds appear to be relatively resistant to geochemical aging, since they have been identified in 140 million year old sediments. They are, however, finally altered to the corresponding hydrocarbons (Brassell et al., 1983). In the current study, CuO oxidation of humic acids released primarily bi-, tri- and tetrafunctional triterpenoids (i.e. lupeol, betulinic acid and x-hydroxyfriedelan-3-one, x(y)-acetoxy-3-oxotaraxer-14-ene-y(x)-ol precursors) and β-sitosterol thus suggesting their participation as structural components in humic acids forming reactions in humified peat. Triterpenoid compounds have not been identified during $KMnO_4$ oxidation of humic acids, because under such strong oxidative conditions the structure of triterpenoids would be partially or completely destroyed.

4.5. Release of lipids during oxidation of oxidized humic acids

Sequential (second) oxidation of humic acids resulted in the release of an additional 0.12% of lipids, equivalent to 3.6% of total analyzed lipid components, and 0.43% of oxidized humic acids. Products obtained from the oxidation of oxidized humic acids primarily contained diterpenoid resin acids (53.0%), fatty acids (24.7%) and n-alkanes (20.0%), as indicated in Table 2. Betulinic acid was the only triterpenoid present, accounting for 2.3% of released lipids. The diterpenoid acid fraction contained relatively high amounts of dehydroabietic acid (43.0%), a lower homologue of dehydroabietic acid (C_{18}, methyl instead of *iso*-propyl in the C-ring) (Simoneit, 1977) (13.2%), 8,15-isopimaradien-18-oic acid (Holmbom et al., 1974) (12.3%) and two des-B-dehydroabietic acids (Takeda et al., 1968, 1969; Zinkel et al., 1969; Holmbom et al., 1974; Ekman, 1979; del Rio et al., 1992), in the form of seco2 (2β-[2′-(*m*-isopropylphenyl)ethyl]-1β,3α-dimethylcyclohexanecarboxylic acid) and seco1 (2α-[2′-(*m*-isopropylphenyl)ethyl]-1β,3α-dimethylcyclohexanecarboxylic acid) in concentrations of 9.6 and 8.3%, respectively. The remaining diterpenoid fraction (13.6%) contained 6 minor diterpenoid acids, two of them being structural isomers of the seco1- and seco2-des-B-dehydroabietic acids. The fatty acid fraction had a high ratio of unsaturated to saturated fatty acids (3.8) and only contained acids with a chain length between C_{16} and C_{18}, with oleic (*cis*-9-octadecenoic) acid being quantitatively the most important (54.7%). The n-alkane fraction was evenly distributed across C_{22}–C_{34} chain lengths with a maximum at C_{24}, and had a slight predominance of even numbered chain lengths (carbon preference index, CPI 0.95; Kvenvolden, 1970).

Diterpenoid acids, typical constituents of coniferous resins (Holmbom, 1978; Ekman, 1980) are highly reactive, participating in the protection of wounded trees against microbial attacks. Under geochemical conditions, diterpenoid acids become less reactive by conversion to

Table 2
Lipids released from oxidized humic acids during alkaline CuO oxidation

	%	μg/g	%
Fatty acids		1060	24.7
n-$C_{16:0}$	10.4	110	
$C_{17:0}$	3.8	40	
n-$C_{18:1}$I	16.0	170	
n-$C_{18:1}$II (oleic acid)	54.7	580	
n-$C_{18:0}$	6.6	70	
n-$C_{18:2}$	8.5	90	
	100.0		
Diterpenoid acids		2280	53.0
$C_{17}H_{22}O_2$[a]	3.1	70	
Des-B-dehydroabietic acid (seco1)[b]	8.3	190	
8,15-Isopimaradien-18-oic acid	12.3	280	
Des-B-dehydroabietic acid (seo2)[b]	9.6	220	
$C_{18}H_{24}O_2$[a]	13.2	300	
8,15-pimaradien-18-oic acid	2.6	60	
8,13-Abietadien-18-oic acid + 13-Abieten-18-oic acid	2.2	50	
x-Abieten-18-oic acid I	1.8	40	
x-Abieten-18-oic acid II	3.9	90	
8,11,13-Abietatrien-18-oic (dehydroabietic) acic	43.0	980	
	100.0		
Triterpenoid acids		100	2.3
3β-Hydroxylup-20(29)-en-28-oic (betulinic) acid		100	
n-Alkanes		860	20.0
C_{22}	11.6	100	
C_{23}	10.5	90	
C_{24}	16.2	140	
C_{25}	14.0	120	
C_{26}	11.6	100	
C_{27}	9.3	80	
C_{28}	8.1	70	
C_{29}	7.0	60	
C_{30}	4.6	40	
C_{31}	3.5	30	
C_{32}	1.2	10	
C_{33}	1.2	10	
C_{34}	1.2	10	
	100.0		
Total amount		4300	100

[a] $C_{17}H_{22}O_2$ and $C_{18}H_{24}O_2$, lower dehydroabietic acid homologues.

[b] Seco1 and seco2, refer to 2α- and 2β-[2′-(m-isopropylphenyl) ethyl]-1β,3α-dimethylcyclohexane-carboxylic acids, respectively.

dehydroabietic acid, which is used as a distinct biogenic marker of coniferous residues in sediments (Brassell et al., 1983) and peat (del Rio et al., 1992). The current study suggests that diterpenoid acids are involved in the formation of humic acids in humified peats as primary structural components as indicated by their polyfunctional (bi-, tri- and tetra-) character and release under the more vigorous second step oxidative conditions.

Dehydroabietic (8,11,13-abietatrien-18-oic) acid (43.0%) was the major compound present in the diterpenoid fraction. Derivatives of microbial dehydroabietic acid degradation were also present in significant amounts (34.2%) in this fraction (refer to Table 2), and included two des-B-dehydroabietic acids (seco1 and seco2) and two lower (C_{18} and C_{17}) dehydroabietic acid analogues. Trace amounts of two additional des-B-dehydroabietic acids (seco0 and seco3) were probably formed by microbial isomerization of seco1 and seco2 acids. The rest of the diterpenoid fraction (22.8%) was comprised of 7 mono- or diunsaturated diterpenoid acids, with 8,15-isopimaradien-18-oic acid being quantitatively the most important. Degraded dehydroabietic acid $C_{18}H_{24}O_2$ (with *iso*-propyl being replaced by methyl in the C-ring) has also been identified in sediment from the Black sea (Simoneit, 1977).

4.6. ^{13}C-NMR spectra of starting materials

The ^{13}C-NMR spectrum of original humic acids (Fig. 2a) had signals indicative of aliphatic CH_3- and CH_2-carbons in the range of 15–35 ppm. The signal at 57.4 ppm was derived from methoxyl carbons. The broad signal between 65 and 80 ppm demonstrated the presence of CH_2–O-carbons in polysaccharide structures, while signals at the range of 117–131 ppm were due to carbon–carbon double bonds (aromatic or olefinic). For example, the aromatic carbons of *p*-hydroxybenzoic acid produced duplicate signals at 116.9, and singular signals at 129 and 132 ppm. A signal at approximately 175 ppm was associated with carbon atoms contained in carboxyl and carbonyl groups.

In contrast, spectra obtained from oxidized humic acids (Fig. 2b) had fewer distinguishing characteristics. Spectra associated with polysaccharide CH_2–O-carbons was decreased to only one distinct signal at 73.7 ppm, but the intensity and broadness of signals due to carbon–carbon double bonds were markedly increased. Signals of carbons due to carboxyl, carbonyl and aliphatic CH_3– and –CH_2– groups were similar to that obtained from original humic acids.

5. Discussion

5.1. Contribution of lipids to the structure of peat humic acids

Polyfunctional triterpenoids were the major components of lipids released by CuO oxidation, indicating that these compounds have a central role within the

humic acid structure of humified peat. High losses (75%) of humic acid during oxidation probably results from the breaking of intrastructural covalent bonds leading to the release of polyfunctional lipid and phenolic (Hänninen et al., 1986) moieties. Monofunctional lipids, i.e. saturated fatty acid compounds and triterpanone precursors seem to be released from the functional groups of the humic acid cluster.

Sequential humic acid oxidation resulted in the release of lipids with an exceptional fatty acid composition, with almost 80% being unsaturated and primarily in the form of $C_{18:1}$ acids (refer to Table 2). Polyfunctionality in general, and bi- and trifunctionality in particular, appears to be an important factor for maintaining the structural integrity of these fatty acids within the humic acid matrix. In peats, increases in humification result in a rapid decrease in oleic acid (Lehtonen and Ketola, 1993), such that a slow and continuous release from humic acids may potentially explain the low and relatively consistent oleic acid concentrations identified in extracts of humified peats.

Coniferous resin acids were the major component of the extracted lipid fraction released by sequential humic acid oxidation. The presence of dehydroabietic acid and associated degraded analogs, may possibly be explained by microbial transformation of polyfunctional diterpenoid acids previously bound to the humic acid matrix. Several observations tend to support the suggestion that coniferous resin acids are transformed during the formation of humic acids. Firstly, diterpenoid acids were only released during the oxidation of oxidized humic acids, tentatively indicating a role as primary lipid reactants for the formation of humic acids which are trapped within the humic acid matrix behind a slower forming outer surface and released during humic acid oxidation. Secondly, an almost entire predominance of the abietane rather than the pimarane structure and the high proportion (77%) of dehydroabietic acid and associated degradation derivatives in diterpenoid oxidation products, provides relatively strong experimental evidence to indicate microbial activity within the humic acid matrix (Simoneit, 1977; del Rio et al., 1992). Furthermore, distribution of dehydroabietic acid and its degraded (seco1 and seco2) acids is consistent with that identified in peat (del Rio et al., 1992) indicating release from humic acids following transformation of the matrix outer surface.

Previous studies have demonstrated that CuO oxidation of original humic acids produced phenolic (20.5 mg/g humic acids) compounds (Hänninen et al., 1986) and subsequent oxidation of oxidized humic acids produced additional phenolic (3.6 mg/g oxidized humic acids) compounds (Hänninen, 1987). This additional release of phenolic (aromatic) compounds only partly explains the increased NMR-signal intensity associated with carbon–carbon double bonds of sequentially oxidized humic acids. However, oxidation of oxidized humic acids did release unsaturated diterpenoid and fatty acids containing olefinic (aliphatic) double bonds, which account also for the increased intensity of the double bond signal.

5.2. *Potential sources of trapped* n-*alkanes*

The slight predominance of even compared to odd carbon chain lengths (CPI 0.95) of the *n*-alkane fraction released from sequential humic acid oxidation, excludes *n*-alkanes of peat as their source, since these have a markedly high predominance of odd carbon chain length homologues (Lehtonen and Ketola, 1993). Khan and Schnitzer (1972) evaluated hydrocarbons retained in humic acids, and reported that plant-derived *n*-alkanes were adsorbed on the outside of the matrix, while microbially synthesized *n*-alkanes occurred inside the humic acid structure. Thermal treatment of humic materials obtained from arctic marine sediments have also released *n*-alkanes (0.27% of parent humic substances) with an even distribution of C_{16}–C_{30} chain lengths and a slight predominance towards odd carbon numbered chain lengths (CPI 1.1) (Baker, 1973).

The type of matrix leading to microbial production of *n*-alkanes characterized by an even chain length distribution with slight predominance of even or odd numbered chain lengths remains unclear. A combination of interesterified fatty acids and fatty alcohols within the humic acid structure or trapped wax esters may potentially serve as microbial precursors which, through decarboxylation and reduction, result in the production of *n*-alkanes with such a profile. Tegelaar et al. (1989a) speculated that selectively preserved non-hydrolyzable aliphatic biopolymers of higher terrestrial plants and algae were the major precursors of aliphatic moieties in humic substances. The proposed structure of these biolipids consisted of a carbohydrate chain with 1,4-linkages between the sugar units, and secondary alkyl chains esterified to sugar hydroxyl groups, with an average alkyl chain/sugar unit ratio approaching unity. This proposed structure could also serve as a substrate for a specific, as yet unidentified micro-organisms, leading to a slow release of hydrocarbon side chains that may account for the production of *n*-alkanes with a profile consistent with that identified in the current study. Because of a high resistance to hydrolysis, this type of aliphatic biopolymer has been considered to be one source for kerogen formation (Tegelaar et al., 1989b). Amblés et al. (1991) demonstrated that cleavage of ester bonds of podzol and anmoor polar lipid fractions by using boron tribromide, resulted in the release of *n*-alkanes with even chain length distributions (C_{21}–C_{30}) and slight predominance of even carbon chain lengths (CPI's 0.95 and 1.0), that accounted for 2 and 1.8%, respectively of total released lipids. Recently, aliphatic biogenic macromolecules of plant origin (Almendros et al., 1996) and

resistant highly aliphatic microbial polymers (Lichtfouse et al., 1998) have been shown to occur in soil humins and soil organic matter, respectively. These findings strongly support the suggestion that the occurrence of highly aliphatic macromolecular biolipids represent potential substrates for intramolecular *n*-alkane production within the humic acid structure.

6. Conclusions

Sequential alkaline cupric oxide oxidations of humic acids of greatly decomposed (H6-7) peat released highly different amounts of lipids (equivalent to 3.2 and 0.4 wt.% of original and oxidized humic acids, respectively), and with totally different compositions. Variations in the composition of lipids released during oxidation of original and oxidized humic acids indicated that the participation of lipids in the formation of humic acids is markedly different. Diterpenoid and unsaturated fatty acids appear to be more readily incorporated into the humic acid structure than triterpenoids, saturated fatty acids or sterols. Neither α,ω-alkanedioic nor hydroxy fatty acids were identified, but one fatty alcohol (n-C_{24}-ol) existed as a minor contributor. The molecular distribution of *n*-alkanes tentatively suggests that they are synthesized within the macromolecular humic acid matrix, possibly due to microbial alteration of chemically bound or adsorbed aliphatic macromolecular biolipids. Distribution of acids derived from coniferous resin provided further evidence to support this suggestion. It is concluded that peat lipids, especially those possessing several functional groups in their molecular structure, are potential structural units of peat humic acids.

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