GEOLOGICA ULTRAIECTINA

Mededelingen van de
Faculteit Geowetenschappen
Universiteit Utrecht

No. 239

What are humic substances?

A molecular approach to the study of organic matter in acid soils

Dennis F. W. Naafs

2004

Illustration: Grayscale foto of south coast of Madeira Island from which the acid andic forest soil samples were obtained.

What are humic Substances?

A molecular approach to the study of organic matter in acid soils

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

Ter verkrijging van de graad van Doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W. H. Gispen,
ingevolge het besluit van het College voor Promoties in het openbaar te
verdedigen op donderdag 4 maart 2004 des middags te 12.45 uur.

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Geboren op 5 juni 1977
te Dordrecht

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The research in this thesis was carried out at the Faculty of Earth Sciences, Utrecht University, The Netherlands. The project was funded by the Institute for Paleoenvironment and Paleoclimate Utrecht (IPPU).

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ISBN: 90-5744-098-9

The wise man said just walk this way
To the dawn of the light
The wind will blow into your face
As the years pass you by
Hear this voice from deep inside
It's the call of your heart
Close your eyes and you will find
The passage out of the dark

The Scorpions, Crazy world – “Send me an angel”

“Training, talent, begeleiding, support en geluk zijn van onschatbare waarde voor het bereiken van de top van een col van de buitencategorie, maar zijn uiteindelijk slechts bepalend voor de snelheid waarmee men boven komt. OF men boven komt wordt volledig bepaald door het vermogen om af te zien buiten elk voorstellingsvermogen voor hen die nimmer naar de top gefietst zijn”.

Voor mijn ouders en grootouders

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Voorwoord

In dit proefschrift staan de belangrijkste bevindingen van mijn reizen door de organisch geochemische bodemwereld beschreven. Gedurende de afgelopen vier jaar heb ik onderzoek verricht naar organisch materiaal in zure bodems, met een nadruk op vulkanische bodems van Madeira. Wellicht een aardig detail is dat dit organisch materiaal, net als wijzelf, deel uit maakt van de koolstof kringloop op aarde…

Minstens zo belangrijk en enerverend als de reizen door de organisch geochemische bodemwereld zijn mijn wereldreizen die uiteindelijk over vier continenten voerden. Gedurende vier jaar werden onder andere steden als New-York, Washington, Miami, San Fransisco, Sydney, Hong-kong, Budapest, Londen, Venetië, Parijs, Lourdes, Aberdeen, Peking, Lhasa (Tibet), Kathmandu (Nepal), Maputu (Mozambique), Vicfalls (Zimbabwe) en Johannesburg aangedaan. Zo mogelijk nog indrukwekkender waren echter de koraalriffen voor de kust van Florida, Mozambique en het Grootbarriere Rif, de tropische regenwouden van Noord-Australië, de ruige kusten en smaragd groene bergen van Madeira, de Afrikaanse natuurparken, de Chinese muur en de machtige toppen van de Himalaya...

Aan een ieder die tijdens het lezen van dit proefschrift door de vele pagina’s vol data de indruk mocht krijgen dat promoveren een saaie bezigheid is raad ik dan ook aan de ogen een moment te sluiten en zijn gedachten over de aarde te laten zweven naar de vele plaatsen die tijdens de totstandkoming van dit proefschrift zijn bezocht…

Summary

Molecular studies on the composition of organic matter in soils are scarce. To date, most studies have analyzed the bulk of the organic matter and/or operationally defined soil organic matter (SOM) fractions such as the so called humic and fulvic fractions. In this thesis, a molecular approach to the study of organic matter in acid soils is presented, with a focus on andic, i.e. volcanic, soils. The fractionation of SOM in this study is based upon the chemical nature of the SOM fractions contributing to the total of SOM instead of solely on solubility in aqueous basic solutions. Analyses include both chemical extractions as well as pyrolysis-GC/MS and CPMAS ^{13}C NMR. Results are discussed in terms of their implications on the factors that determine the composition of organic matter in the acid soils studied (i.e. input, preservation/degradation and transport). In addition to obtaining molecular insights into SOM, the molecular approach to study SOM is standardized.

The outline of the thesis is arranged from low molecular weight SOM to high molecular weight SOM. In **Chapter 2**, variations with depth and season of solvent extractable lipids of an acid andic forest soil are studied. A decrease of the contribution of lipids per g TOC with depth is observed. The absence of specific bacterial markers may be an indication for reduced bacterial activity, most likely related to the low soil pH and the presence of Al and Fe (oxides). A strong decrease in both relative and absolute concentrations of leaf-derived compounds is observed when comparing the litter layer with the mineral soil. The presence of C_{22}-C_{32} *n*-alkanoic acids, C_{22}-C_{26} ω-hydroxy acids, C_{31} *n*-alkane and C_{22}-C_{32} *n*-alkanols observed in the sub-soil is suggested to be indicative of an important contribution by (grass) roots. In summer, a signal most likely reflecting the leaching of microbially derived products from the litter and/or aerial vegetation at the surface is observed.

In **Chapter 3**, the composition of total lipid extracts from different horizons in a podzol profile are compared. Organic matter is observed to accumulate mainly with aluminium in the Bh horizon. Independent of soil pH, lipids accumulate in organic rich horizons. A lipid signal related to the aerial parts, i.e. leaves and flowers, of *Calluna* is only observed in the O horizon. This "*n*-alkane, steroid and triterpenoid" signal is rapidly lost in the underlying Ah horizon due to (bacterial) oxidation. The other total lipid extracts observed are dominated by a root-derived signal. Overall, lipid data indicate that degradation (microbial oxidation) is an important process that should be taken into account in addition to leaching when describing the podzolization processes in soils.

The second section of the thesis (**Chapters 4, 5, 6** and **7**) deals with ester-linked compounds. **Chapter 4** describes changes in the molecular composition of ester-linked aliphatics with depth in an acid andic forest soil. These changes are compared with changes in the free lipid fraction, creating a link between the first and second section of the thesis. Thermally assisted hydrolysis and methylation in combination with gas chromatography/ mass spectrometry reveals a cutin over suberin dominated input in the top 5 cm. In contrast, sub-soil samples (20-50 cm) are dominated by a suberin-derived input. C_{22}, C_{24} (and C_{26}) ω-hydroxy acids (and C_{18}-C_{28} α, ω-dioic acids) are found to decrease relatively strongly with depth compared with other suberin building-blocks such as C_{16} ω-hydroxy acid. Their decrease with depth in the ester-bound fraction is 'reversely' linked with a strong increase in the free extractable lipid fraction (Chapter 2) which would justify their use as main indicators of a root–derived input to the free extractable lipid fraction.

In **Chapter 5**, the effects of pH adjustments after base hydrolysis and their implications for understanding organic matter in soils are studied. Results obtained after direct pH adjustment of the extract indicate that the relative amounts of identified compounds are, as expected, pH dependent, i.e. based on pK_a values and hydrophobicity. This phenomenon, which affects significantly the final results, may cause an under or over estimation of certain compound classes and a biased view on ester-related moieties in soils. Direct acidification of base extract in the presence of soil residue indicates that hydrophobic long-chain aliphatic moieties may become associated with the organic matrix left after base treatment. In addition, they may form new, insoluble, organic (macro) molecules or become associated with the inorganic matrix.

The results of this methodological study are used in **Chapter 6,** which describes a qualitative study on the chemical composition of ester-bound moieties in an acid andic forest soil. The solvent insoluble ester-bound moieties, released after base treatment from an acidic andosolic forest soil A-horizon from Madeira, have been sequentially extracted and analysed using gas chromatography and gas chromatography-mass spectrometry. The separation of all moieties released into several pH “subfractions” allowed the various products released to be studied in detail. The distributions observed for 2-alkanols, diols, *n*-alkanols, *n*-alkanoic acids, ω-hydroxy acids, trihydroxy acids, dihydroxy diacids and phenolic acids indicate a suberin dominated origin of ester related moieties. Besides plant biopolyesters, the detection of *iso* and *anteiso* alkanoic acids, together with short-chain di and ω-hydroxy acids suggests an additional bacterially-derived contribution.

The effects of pH on the preservation of ester-linked lipids are described in **Chapter 7**. The occurrence and distribution of ester-bound lipids in Dutch coastal dune soils along a pH gradient are reported. Comparison of thermally assisted hydrolysis and methylation (THM) with base hydrolysis data confirms that base hydrolysis is a very efficient way to analyse ester-linked compounds in soils. The compounds released from SOM upon base hydrolysis comprised largely suberin-derived lipids, which were clearly related to oak roots. Compared with fresh (fine) roots, which are largely responsible for the input of SOM in the subsoils, unsaturated and epoxy compounds decreased, probably due to oxidation. The amounts of the saponifiable fraction increased upon acidification, while hardly any changes were observed in compositions. This accumulation, without significant compositional alteration of ester-bound moieties in acidic soils, indicates that plant biopolyesters can be preserved in soils.

The final and third section of this thesis (**Chapters 8**, **9** and **10**) deals with the bulk and high molecular weight fraction of SOM. In **Chapter 8,** molecular information about the bulk of organic matter in andic soils is obtained using Curie-point pyrolysis and CPMAS ^{13}C NMR. A combination of both techniques reveals a significant contribution of fungal polysaccharide moieties (i.e. chitin). Pyrolysis data further show that these, and other, polysaccharide moieties are thermally altered. In addition, the presence of the biopolyesters cutin and suberin, the non-hydrolyzable biopolymers cutan and suberan, and polypeptides is confirmed by both analysis methods. Relatively high amounts of chitin pyrolysis products may be derived both from fungi and arthropods indicating that, in addition to fungi, arthropods are important for the reworking of organic matter. Low amounts of lignin detected are related to the low soil pH which is known to be favoured by (lignin-degrading) fungi. Condensed aromatics, possibly in the form of charcoal were found to be a significant contribution to the total of SOM, although underestimated by the methods used.

Chapters 9 and **10** deal with the characterization of chemically resistant soil organic matter fractions. In **Chapter 9**, the molecular composition of chemically resistant soil organic matter in andic soils is studied. Both NMR and pyrolysis data reveal an important contribution from condensed aromatic structures, possibly charcoal, in combination with an aliphatic signal most likely derived from non-hydrolysable aliphatic SOM. The chemically resistant SOM fraction isolated will most probably contribute significantly to the soil carbon millennium pool in andic soils.

Based on the results described in **Chapter 9**, **Chapter 10** reports on the molecular composition of chemically resistant aliphatic biopolymers. The aliphatic molecular building blocks consist of mono-, di-, tri- and tetra-functionalized aliphatic compounds reflected by the presence of series of C_9-C_{32} *n*-alkanoic acids, C_7-C_{30} α, ω-alkanoic diacids, C_{21}-C_{30} keto-α, ω-alkenoic diacids and C_{16}-C_{27} diketo-α, ω-alkanoic diacids in the RuO_4 extract. The formation of chemically resistant aliphatic material in soils through a process of crosslinking within biopolyesters, such as cutin and suberin, is considered unlikely.

Finally, **Chapter 11** synthesizes the previous chapters and describes the conclusions from the molecular approach to the study of organic matter in soils (SOM). In summary, the work described in this thesis which is based on chemical extractions, chemolysis and pyrolysis-GC/MS in combination with CPMAS ^{13}C NMR, provides new molecular insights into SOM. Analyses of total lipid extracts, in addition to analyses of detailed specific lipid fractions, are shown to provide a more complete insight into the lipid chemistry, and therefore on the total of processes that determine the composition of OM in soils. Research on both the free lipid and the ester-bound fraction, revealed that roots are the main source of OM in acid soils. An input from the surface vegetation is only important within the first few centimeters of the soil. In general, ester-bound SOM is shown to be preserved in acid soils. On a molecular level, however, ester-bound unsaturated and epoxy moieties are shown to be quickly degraded in soils. Ester bound ω-hydroxy acids were shown to be a source of free extractable ω-hydroxy acids revealing a direct link between the ester-bound end free lipid fractions. Analyses on the bulk of SOM reveal a relatively high contribution of fungal and/or thermally altered plant polysaccharides in addition to polypeptides, aliphatic SOM and a condensed aromatic fraction. The non-hydrolyzable material is shown to consist of a condensed aromatic fraction and an aliphatic fraction. The latter was shown to consist of mono-, di-, tri- and tetra-functionalized aliphatic building blocks.

Using the molecular data obtained, the main factors that determine the composition of organic matter in the acid soils studied are discussed, i.e. input, preservation/degradation and transport. It is suggested that humic substances are a group of operationally defined fractions comprised of both recognizable plant material and their degradation products, together with a fraction of SOM that has not been characterized at the molecular level. The preservation of SOM in andic soils is suggested to result from complexation with allophane/imogolite and ferrihydrite, complexation with free aluminum, aluminum toxicity to microorganisms, an acid soil pH and, for some SOM fractions, intrinsic structural resistance. Based on the molecular data presented in this thesis, a model consisting of four different cycles of SOM turnover in an acid andic soils is suggested.

Chapter 1

General introduction

1.1 Introduction

One of the major carbon pools playing an important role in the short-term global carbon cycle is soil organic matter (SOM) (Post et al., 1982). Globally, there is substantially more carbon in the top metre of soil than in the overlying vegetation or in the atmosphere (Oades, 1988; Jenkinson et al., 1992). The annual net primary terrestrial production is about 60 Gt and equals the amount of litterfall and the annual exchange of C between the earth's surface and the atmosphere (Hedges and Oades, 1997). Most SOM decomposes into CO_2 and recirculates within a few years into the atmosphere (Post et al., 1982). A minor part of the SOM is, however, assimilated into microbial tissues or converted into resistant SOM with a longer residence time (Paul and Clark, 1996; Coûteaux et al., 1998).

Several processes are known to influence the preservation and/or degradation of organic matter in soils, including microbial degradation, oxidation and hydrolysis. In addition to these processes, the structural recalcitrance of organic matter against biodegradation will also influence its preservation in soils (Largeau and de Leeuw, 1993). The rate of the degradation/preservation processes in soils is directly affected by pH, the concentration of free aluminium, the presence of oxyhydroxy minerals, soil texture, temperature, nutrient status, moisture and oxygen content, composition of the litter/SOM, etc. (Stevenson, 1994). Together with input and transport, the degradation/preservation processes determine the composition and residence time of organic matter in soils.

1.2 The sources and composition of soil organic matter

The bulk of SOM is insoluble in water and composed of a complex mixture of dead and living biological material derived from above and belowground plants, microorganisms, fungi and animals (Schnitzer and Kahn, 1972; Stevenson, 1994; Kögel-Knabner, 2002). Products resulting from a wide variety of degradation and transformation processes of the above mentioned materials contribute as well. From all these sources, vascular plants are the main source of organic carbon to soils through litterfall and roots (Oades, 1993; Kögel-Knabner, 2002). In general, it is assumed that up to half of the organic matter input to soils is through root systems of plants. In addition to being introduced by roots and leached from surface litter, fresh plant material is mixed into the soil by earthworms and other animals (Darwin, 1838, 1881; Cadée, 2003; Oades, 1993).

Characterization of SOM, or operationally defined SOM fractions (see section 1.4), is mainly performed by using pyrolysis, NMR and/or selective chemical treatments (Kögel-Knabner, 2000; Hatcher et al., 2001). The results led to the recognition that the main components of SOM are lipids, polysaccharides, proteins, (poly)phenols such as tannins, liginin, chitin, hydrolysable (cutin, suberin) biopolymers and non-hydrolysable (cutan, suberan) biopolymers and dense aromatic structures such as char (Saiz-Jiminez and de Leeuw, 1984, 1986a, b, 1987a; Kögel et al., 1988; Kögel-Knabner et al., 1992a, b; Knicker et al., 1996; van Bergen et al., 1997, 1998a; Augris et al., 1998; Nierop and Buurman, 1999; Nierop et al., 2001a, b; Derenne and Largeau, 2001; Kögel-Knabner, 2002). However, it is important to realize that more than two-thirds of the organic carbon in soils has not been characterized at the molecular level (Hedges and Oades, 1997).

1.3 Classical methods of fractionating SOM

Common approaches to study SOM include the classical humus fractionation (e.g. Davies et al., 1969; Grasset and Ambles, 1998; Augris et al., 1998) and selective chemical treatments (e.g. Schnitzer and Schuppli, 1989; Melakani et al., 1997). The so called humic substances extracted by the classical humus fractionation (Fig. 1.1) have further been analysed using techniques such as FTIR, pyrolysis-GC/MS and NMR. The fulvic acid fraction was shown to consist mainly of polysaccharide and/or carbohydrates and polyphenols, while the humic acid and humin fractions were found to be complex mixtures of several biopolymers such as polysaccharides, partially degraded lignins, peptides and lipids (Saiz-Jiminez, de Leeuw, 1986). In addition to compositional analyses of the operationally defined humic fractions (Fig. 1.1), size exclusion chromatographic studies led to the recognition that humic substances should be regarded as supramolecular associations of self assembling heterogeneous and relatively small molecules derived from the degradation and decomposition of dead biological material (Piccolo, 2001). These supramolecular structures were suggested to be stabilized predominantly by weak dispersive forces instead of covalent linkages (Piccolo, 2001).

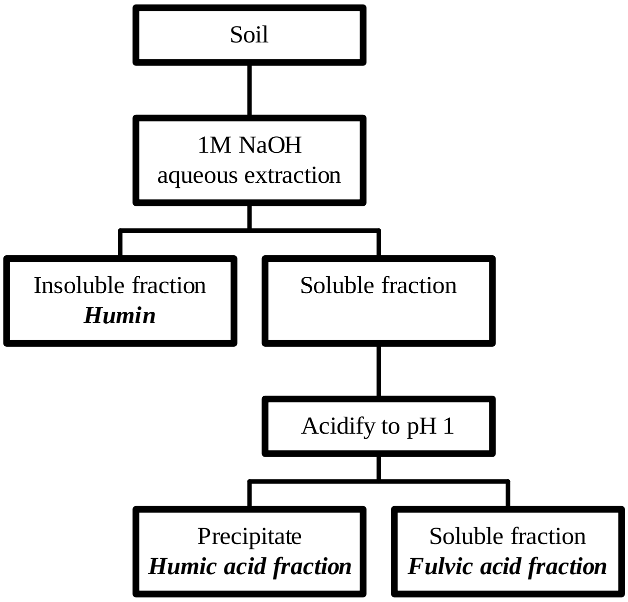

Figure 1.1 Schematic representation of classical humus extraction (Swift, 1996)

Due to the absence of specific isolation and characterization techniques for most SOM fractions, to date molecular research has focussed mainly on free extractable lipids and, to a lesser extent, the ester-bound (lipid) fraction. In addition, high molecular weight soil organic compounds such as lignin have been studied using a combination of pyrolysis and oxidation techniques (e.g. Saiz-Jiminez, de Leeuw, 1984b, 1986; Hatcher et al., 1995; Hatcher, 1987).

1.4 Free extractable and ester-bound lipids.

1.4.1 Free extractable lipids

Lipids are, by definition, organic compounds insoluble in water but soluble in common organic solvents (Dinel et al., 1990). Three main sources of plant-derived aliphatic compounds have been distinguished in soils: (a) free (extractable) lipids, (b) the biopolyesters cutin and suberin and (c) non-hydrolyzable biopolymers, such as cutan and suberan (Nip et al., 1985; Tegelaar et al., 1989, 1995; Augris et al., 1998; Nierop; 1998).

Free extractable lipids include hydrocarbons, ketones, *n*-alkanols, fatty acids, hydroxy acids, steroids, terpenoids, wax esters and acyl glycerols, as well as phospholipids and lipopolysaccharides (e.g. Dinel et al., 1990; Zelles et al., 1992; Stevenson, 1994). The total free extractable lipid fraction of soil organic matter (SOM) normally constitutes a small portion (2-50 g.kg^{-1}) of the total SOM

(Stevenson, 1982). However, this fraction can be very important, for example when retarding the decomposition of SOM (Amblès et al., 1993). Moreover, lipids exhibit an inherent diagnostic value with respect to SOM, both endogenous and exogenous, and the pedological processes to which it is subjected (Bull et al., 2000a).

Lipid research is quite well developed as compared with research on other SOM fractions because of the relative ease of extracting this fraction from soils. Both total lipid extracts (TLEs) as well as specific lipid fractions have been analysed. Vegetation, litter and soils have been extracted and the TLEs characterized (van Bergen et al., 1997, 1998a; Bull et al., 1998, 2000a, b). In addition, variations with depth (Jambu et al., 1993; Amblès et al., 1994a, 1998; Bull et al., 2000a, b), leaching (Amblès et al., 1998) oxidation/biodegradation (Jambu et al., 1991, Amblès et al., 1993, 1994b), diagenetic behaviour (Bull et al., 1998) and effect of pH (van Bergen et al., 1998a; Bull et al., 2000a), mineral fertilizers (Jambu et al., 1987) and clay minerals (Jambu et al., 1995; Amblès et al., 1989, 1997) on the composition of lipids as well as the influence of lipids on soil properties (Jambu et al., 1978; Amblès et al., 1997) have been described for several soil types. Despite the relatively detailed research and their high diagnostic value, total lipid studies of complete andic and podzol profiles have yet to be reported.

1.4.2 Ester-bound lipids

Both suberin and cutin are considered to be important sources of solvent-extractable lipids. Moreover, it has been shown that the greater part of the alkyl signal in the ^{13}C NMR spectra after thorough extraction with organic solvents, can be ascribed to cutin and suberin (Kögel-Knabner et al., 1992; Riederer et al., 1993). Cutin is present in cuticles covering all aerial parts of higher plants. The other biopolyester, suberin, is an abundant, complex, intractable, cell wall polymeric network that forms both protective and wound-healing layers in barks, woody stems and underground parts, i.e. roots (Kolattukudy, 1980, 1984; Walton, 1990). Molecular studies concerning both cutin and suberin have mainly focussed on fresh plant material such as leaves (Holloway, 1983a) and cork (Holloway, 1983b; Walton, 1990). These studies revealed that cutin and suberin are closely related chemically, since both consist mainly of hydroxy and epoxy-substituted alkanoic acids. However, there are a number of differences in their monomeric and tertiary structure (Matzke and Riederer, 1991; Tegelaar et al., 1995). Cutins are characterized by substantial amounts of dihydroxyhexadecanoic acids, whereas suberins contain considerable amounts of α, ω-dioic acids and long-chain ($>C_{18}$) monomers (Matzke and Riederer, 1991; Kolattukudy, 2001). In addition, suberin is suggested to consist of both a polyphenolic and poly aliphatic domain (Bernards, 2002).

Research into the molecular composition of biopolyesters in soils is usually performed using thermally assisted hydrolysis and methylation (THM) in the presence of TMAH (Nierop, 1998, 2001b), although alkaline hydrolysis under phase catalysis conditions (Grasset and Ambès, 1998) and BF_3/methanol-catalyzed transesterification (Almendros and Sanz, 1991; Riederer et al., 1993) have also been used occasionally. Apart from revealing the general molecular composition of biopolyesters in soils, these studies led to the recognition that the input of suberins from root litter, as compared with cutin, increases with soil depth (Riederer et al., 1993; Nierop, 1998). Apart from the latter studies, detailed molecular studies on the chemical composition of ester-bound lipids in soils and roots and the influence of soil conditions such as pH on this composition are still scarce.

1.5 Acid soils

A major part of the world soils are acidic (FAO, 1998). In general, the low pH is known to influence to a great extend the composition of the organic matter found in these soils. In this study, three acid soils have been analysed; a podzol, a coastal dune soil and, most importantly, an andic soil. For (sandy) soils it is generally known that with decreasing soil pH, SOM accumulates through reduced microbial activity (Wardle, 1992; Motavalli et al., 1995; Andersson and Nilsson, 2001). However, to what extent this increase in quantity accounts also for a change in SOM quality is only partly understood. For instance, fungi become more dominant compared with bacteria when soil pH decreases (Dinel et al., 1990; Bumpus, 1993; Bååth and Anderson, 2003). This may affect the composition of SOM considerably. For free (extractable) soil lipids it is well-documented that their relative amounts increase when soil pH is reduced (Jambu et al., 1985; Dinel et al., 1990; Stevenson, 1994). However, whether non-extractable lipids, in particular ester-bound moieties, are preserved at low soil pH as well, is as yet unclear. Furthermore, an acid soil pH is known to influence the degradation/preservation of high molecular weight SOM fractions such as lignin (van Bergen et al., 1997, 1998a). Futhermore, to what extend the composition of SOM in andic soils is influenced by the specific andic soil conditions is also still largely unknown.

1.5.1 Andic soils

Andisols cover between 0.76%-0.84% of the world’s land area (Leamy et al., 1980) and are typically associated with the weathering of recent volcanic ash deposits in humid, temperate environments (Parfitt and Kimble, 1990; Lowe, 1997). Despite their apparent small contribution to the total of soils in the world, they are extremely important for the global carbon cycle because of their capacity to stabilize large quantities of organic carbon for thousands of years (Torn et al., 1997).

The composition of SOM in andic soils is thought to be mainly influenced by the input and protective capacities strongly associated with the presence of both high levels of free aluminum together with sorption to allophane, imogolite and ferrihydrite (Boudot et al., 1986; Saggar et al., 1994; Shoji et al., 1993, Parfitt et al., 1997; Torn et al., 1997). Once introduced into the soils, the specific andic soil processes will influence the transformations and decomposition of the fresh litter and root material. In addition to soil processes, thermal alteration of organic matter during forest fires may influence the composition of SOM in these profiles. Most remarkable, in the andic soil profile used, enhanced preservation of SOM results in an accumulation of organic matter up to 7 wt%.

Andic soil samples were obtained from Madeira Island (Portugal). Madeira is located in a fully (Atlantic) oceanic domain between 32^{o}38’ and 32^{o}52’N and 16^{o}39’ and 17°16’ W, approximately 600 km from the African coast, at the same latitude as Casablanca. Soil samples were taken from andic profiles located on this island that have been classified as either being Umbric or Haplic Andisols (FAO, 1998) depending on their color (Madeira et al., 1994).

1.5.2 Sandy soils

Podzols cover approximately 15% of the world’s land area and typically appear in Europe’s temperate areas (Andreux, 1996). Many soils show evidence of podzolization, a process that involves a pronounced downwards translocation of iron, aluminum and organic matter to form characteristic soil horizons (van Breemen and Buurman, 2002). From top to bottom, a podzol profile consists of up to five major horizons, two of which may be absent (in italics): a litter layer (O), *a humose mineral topsoil (Ah), caused by biological mixing,* a leached eluvial layer (E), an accumulation layer of organic matter in combination with iron and aluminum (Bh, Bhs, Bs) and *thin bands with organic matter accumulation in the subsoil* (van Breemen and Buurman, 2002). Podzols form both in well-drained soils (xero-podzols) and in soils with a shallow, fluctuating water-table (hydro-podzols). Podzol samples were taken from a Haarpodzol (group: xero-podzols) (De Bakker and Schelling, 1989) at the Veluwe near Kootwijk/Assel, The Netherlands. This type of soil has formed in wind blown sands, a very poor parent material, which was deposited during the Pleistocene.

In addition to a podzol, a coastal dune soil was used. Although not significant as a major world soil type, the coastal dune soil profile was selected for its unique soil pH transition over time from basic calcareous to acid. The well documented vegetation history in combination with this pH transition created a unique opportunity to study the effects of soil pH on the composition of SOM. The coastal dune soil study sites are situated in the Amsterdamse Waterleiding Duinen, a sandy dune area at the southeast of Zandvoort, west of Amsterdam (The Netherlands). This area consists of olden and younger dunes that had initially calcareous sand with similar mineralogical composition and texture (Nierop and Verstraten, 2003). With time, the vegetation caused the profiles to become acidic. All coastal dune sites used have been classified as Cambic Arenosols (FAO, 1998).

1.6 Objectives

Molecular studies on the composition of organic matter in (andic) soils are scarce. To date, most studies have analysed the bulk of the organic matter and/or operationally defined SOM fractions such as the so called humic and fulvic fractions (see section 1.3). It should be noted that these operationally defined fractions are nothing more than a combination of the main SOM fractions, such as polysaccharides, lignin and aliphatic biopolymers, together with a yet uncharacterized SOM fraction, based upon their solubility in aqueous basic solutions. Studies that include molecular characterization of SOM have been mostly concentrated either on the free extractable or ester-bound lipid fraction (see section 1.4) or on the bulk of SOM using a combination of NMR and pyrolysis-GC/MS (e.g. van Bergen et al., 1997; Nierop, 1999) in sandy and clay soils.

In this study, a molecular approach to the study of organic matter in acid soils is presented (Fig. 1.2), with a focus on andic, i.e. volcanic, soils. The fractionation of SOM in this study is based upon the chemical nature of the SOM fractions contributing to the total of SOM instead of solely on solubility in aqueous basic solutions. Results are discussed in terms of their implications on the factors that determine the composition of organic matter in the acid soils studied (i.e. input, preservation/degradation and transport). In addition to obtaining molecular insights into SOM, the molecular approach to study SOM is standardized.

1.7 Outline of the thesis

The outline of the thesis is arranged from low molecular weight to the more complex high molecular weight SOM fractions. The first section (**Chapters 2** and **3**) deals with total lipid extracts. In **Chapter 2**, variations with depth and season in free lipids of an acid andic forest soil are studied. In **Chapter 3**, the composition of total lipid extracts from different horizons in a podzol profile are compared.

The second section of the thesis (**Chapters 4, 5, 6** and **7**) deals with ester-linked compounds. **Chapter 4** describes changes in the molecular composition of ester-linked aliphatics with depth in an acid andic forest soil. These changes are compared with changes in the free lipid fraction, creating a link between the first and second section of the thesis. In **Chapter 5**, the effects of pH adjustments after base hydrolysis and their implications for understanding organic matter in soils are studied. The results of this methodological study are used in **Chapter 6** which describes a qualitative study on the chemical composition of ester-bound moieties in an acid andic forest soil. The effects of pH on the preservation of ester-linked lipids are studied in **Chapter 7** in which the occurrence and distribution of ester-bound lipids in Dutch coastal dune soils along a pH gradient are reported.

In **Chapter 8,** molecular information about the bulk of organic matter in andic soils is obtained using Curie-point pyrolysis and CPMAS ^{13}C NMR. Data obtained are discussed in terms of their implications on the accumulation of organic matter in andic soils.

The last section of the thesis (**Chapters 9** and **10**) deals with the characterization of chemically resistant soil organic matter fractions. In **Chapter 9**, the molecular composition of chemically resistant soil organic matter in andic soils is studied. Based on the results obtained, **Chapter 10** describes the molecular composition of chemically resistant aliphatic biopolymers.

Finally, **Chapter 11** synthesizes all previous chapters and describes the conclusions from the molecular approach to the study of organic matter in soils on the factors that determine the composition of organic matter in the acid soils studied (i.e. input, preservation/degradation and transport).

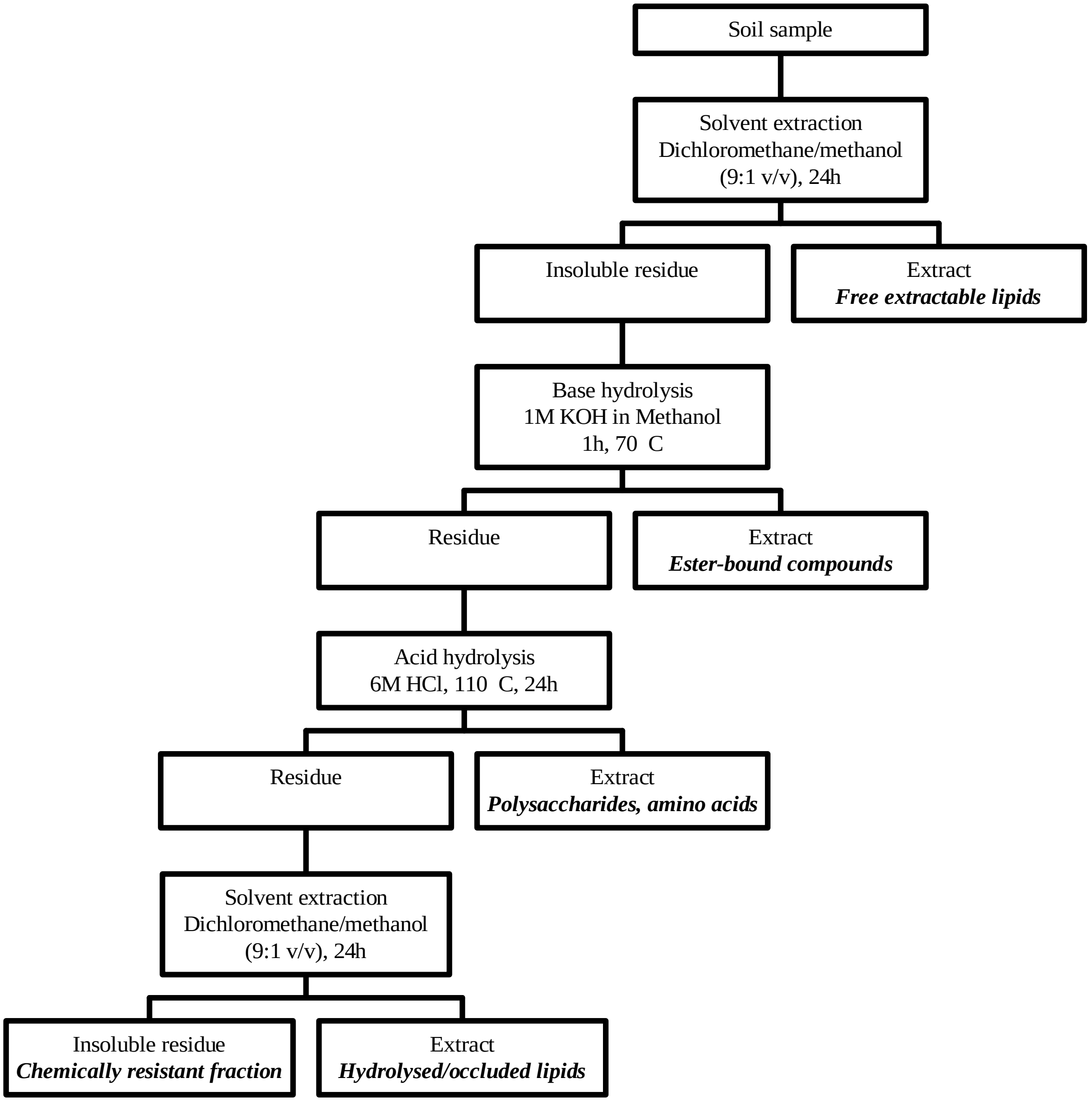

Figure 1.2 Schematic representation of sequential extraction used in this thesis

Chapter 2

Variations with depth and season; Solvent-extractable lipids in an acid andic forest soil

Total lipid extracts from an acid andic soil profile located on Madeira Island (Portugal) were analysed using gas chromatography (GC) and GC-mass spectrometry (GC/MS). The profile was covered mainly by grass. Bulk soil characteristics determined included soil pH (H_2O) ranging from 4.5 to 4.0 and TOC, ranging from 84 to 30 g.kg^{-1}. A decrease of the contribution of lipids per TOC with depth was observed. The absence of typical bacterial markers might be an indication for reduced bacterial activity, most likely related to the low soil pH and the presence of Al and Fe (oxides). The distribution observed in the top layer with a dominant C_{26} *n*-alkanol, steroids and triterpenoids, reflected mainly an input by grass leaves. A strong decrease in both relative and absolute concentration of these leaf derived compounds was observed when comparing the litter layer with the mineral soil. The presence of C_{22}-C_{32} *n*-alkanoic acids, C_{22}-C_{26} ω-hydroxy acids, C_{31} *n*-alkane and C_{22}-C_{32} *n*-alkanols observed in the sub-soil is indicative of an important contribution by (grass) roots. In summer, a signal most likely reflecting the leaching of microbially derived products from the litter and/or aerial vegetation at the surface was observed.

2.1 Introduction

Andisols cover between 0.76%-0.84% of the world’s land area (Leamy et al., 1980) and are typically associated with the weathering of recent volcanic ash deposits in humid, temperate environments (Parfitt and Kimble, 1990; Lowe, 1997). Despite their apparent small contribution to the total of soils in the world, they are extremely important for the global carbon cycle because of their capacity to stabilize large quantities of organic carbon for thousands of years (Torn et al., 1997).

Vascular plants are the main source of organic carbon to these and other soils through litterfall and roots (Oades, 1993). Relative inputs by these two pathways vary with plant type. For grasses, roots are more important than for trees (Oades, 1993). In general, it is assumed that up to half of the organic matter input to soils is through root systems of plants. In addition to being introduced by roots and leached from surface litter, fresh plant material is mixed into the soil by earthworms and other animals (Oades, 1993).

The characteristic vegetation of Mediterranean ecosystems is especially favourable for the accumulation of waxes, lipids and resins in soil (Almendros et al., 1996). Such accumulation is important in soils. For example, lipids on the surface of soil particles can cause such particles to become hydrophobic and thereby decrease the rate of organic matter degradation as a whole (Jambu et al., 1995).

Literature data concerning the molecular composition of the total free (extractable) lipid fraction are scarce (Almendros et al., 1996; Bull et al., 1998). The total lipid fraction of soil organic matter (SOM) can be isolated readily from soils with no substantial alteration by means of solvent extraction. This fraction normally constitutes a small portion (2-50 g.kg^{-1}) of the total SOM (Stevenson, 1982). However, as mentioned this fraction can be very important, for example when retarding the decomposition of SOM (Amblès et al., 1993). Moreover, lipids exhibit

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an inherent diagnostic value with respect to SOM, both endogenous and exogenous, and the pedological processes to which it is subjected (Bull et al., 2000a).

Lipids are, by classical definition, organic compounds insoluble in water but soluble in common organic solvents. In addition to being present as free extractable compounds in soils, lipids are part of complex organic structures such as biopolyesters. Extraction of these bound lipids requires more vigorous extraction procedures than solvent extraction and therefore bound lipids are not considered in this paper.

Lipids include fatty acids, *n*-alkanols, hydroxy acids, ketones, steroids, terpenoids, acyl glycerols and hydrocarbons, as well as phospholipids and lipopolysaccharides (e.g. Dinel et al., 1990; Zelles et al., 1992; Stevenson, 1994). These compounds originate from both plants and animals as products of deposition, decomposition and exudation, as well as from various pedogenic sources, including fungi, bacteria and mesofauna (Bull et al., 2000a). However, as aforementioned, the main source of lipids in soils is normally the vegetation (Oades, 1993; van Bergen et al., 1997).

In this paper, total lipid extracts obtained from an andic soil profile on Madeira Island, covered mainly by grass, were analyzed using gas chromatography (GC) and GC-mass spectrometry (GC/MS). The results are discussed in terms of the relationship between the chemical composition of total lipid extracts (TLEs) and specific inorganic andic soil properties, i.e. soil pH and aluminum concentrations. In addition, the origin of compounds identified together with variations in their chemical composition with depth and season will be evaluated.

2.2 Materials and methods

2.2.1 Soil profiles

Madeira Island (Portugal) is located in a fully (Atlantic) oceanic domain between $32^o38'$ and $32^o52'$N and $16^o39'$ and 17°16’ W, approximately 600 km from the African coast, at the same latitude as Casablanca. Soil samples were taken from andic profiles located on this island that have been classified as either being Umbric or Haplic Andisols (FAO, 1998) depending on their color (Madeira et al., 1994).

The profile studied is situated on a hill (slope 30^oW) near the village of Poiso (altitude 1175 m). The vegetation consisted mainly of grasses (55%), ferns (20%), deciduous trees, i.e., oak and birch (20%), and some mosses (5%). Tree- and some grass roots were found to a depth of about 80 cm, but most of the intensive “root-activity” was found in the top 0-40 cm consisting mainly of grass fibrils. Three horizons were distinguished; a thin O-horizon (0-2 cm), an A-horizon (2-73 cm) and an E/B horizon (73- 90 cm), the latter being formed by weathered basalt bedrock material. The deeper layer, i.e. C-horizon, was formed by a non-friable, compact layer of weathered basalt, the parent material for this Andisol (Madeira et al., 1994) and therefore not encountered. The O-horizon consisted mainly of litter and vegetation, i.e. grass, moss, twigs, leaves, etc. The reddish A horizon (5YR4/6) was characterized by a non-friable structure, clay aggregates with a diameter up to 1 cm, and highly weathered basalt fragments up to a diameter of 10 cm. Many roots were found in this horizon, ranging from mainly very fine grass roots in the top 30 cm to coarser roots, up to a diameter of 2 cm, at greater depth, i.e. 30-70 cm.

2.2.2 Sampling, sample pre-treatment, total organic carbon and solvent extraction

In February 2001, about 500 gram of sample was taken every 10 cm up to a depth of 70 cm (including the O-horizon). In June 2000, samples had already been taken from a depth of 15-50 cm. On Madeira, the samples were air dried in the dark and wrapped in aluminium foil. After 1 week, the samples were transported to the Netherlands and subsequently oven dried at 60 °C and sieved over a 2 mm and a 250 μm sieve to remove large roots and basalt fragments. Total organic carbon contents (TOC) of the dried and sieved (<250 μm) samples were measured using a Fisions Instruments NA 1500 NCS analyzer, with a cycle time of 180 s, a source temperature of 190 °C and an oxygen flow of ca. 30 $l.min^{-1}$.

Approximately ten gram of the sieved (<250 μm) samples was Soxhlet extracted using dichloromethane/methanol (DCM/MeOH) (9:1 v/v) for 24 h. The collected and combined DCM/MeOH phase was rotary evaporated to complete dryness. The dry extract was dissolved in DCM/isopropanol (2:1 v/v) and filtered using a Pasteur pipette packed with defatted wool, 0.5 cm Na_2SO_4 and 2 cm SiO_2 and dried using N_2. Free hydroxyl and carboxylic acid groups present in an aliquot were derivatized to their corresponding trimethylsilyl (TMS) ethers and esters respectively, using BSTFA (*N*,*O*-bis(trimethylsilyl) trifluoroacetamide, containing 1% trimethylchlorosilane and heated for 1 h at 70°C). The derivatized aliquots were dried using N_2 and dissolved in hexane. An aliquot of a standard solution containing 0.15 $\mu g.\mu l^{-1}$ 10-nonadecanone was added and the total extract analyzed by GC and GC/MS. The residue was air-dried.

2.2.3 Gas chromatography (GC)

GC analyses were performed using a Hewlett-Packard 6890 series gas chromatograph equipped with a CP-sil 5CB silica column (50m x 0.32mm, film thickness 0.12μm). Derivatized extracts (1.0 μl) in hexane were injected on-column. The oven temperature was programmed from 70 °C to 130 °C at 20 °C min^{-1} and from 130 °C to 320 °C (isothermal for 20 min) at 4 °C min^{-1}. Compounds were detected using a flame ionisation detector at 325 °C. Helium was used as carrier gas.

2.2.4 Gas chromatography-mass spectrometry (GC/MS)

Gas chromatography-mass spectrometry analyses were performed using a Hewlett-Packard 5890 series II gas chromatograph connected to a Fisons instruments VG platform II mass spectrometer operating at 70 eV, scanning the range *m/z* 50-650 with a cycling time of 0.65 s. The capillary column and temperature programme were as described for the GC analyses. Compound identification was based on published data (e.g. Holloway, 1982; Walton, 1990; Killops and Frewin, 1994; van Bergen et al., 1997).

2.3 Results

Total organic carbon, total nitrogen, C/N molar ratios and pH (H_2O) values are summarized in Table 2.1. TOC, expressed as $g.kg^{-1}$ of the total sieved (<250 μm) soil mass, ranged from 84 in the top-soil to 30 in the hard basaltic E/B-horizon. Total nitrogen, also expressed as weight percentage of the total sieved (<250 μm) soil mass, showed a decrease from 7 to 4 $g.kg^{-1}$. C/N molar ratios revealed a relatively minor variation from 13.4 to 11.8. In addition, for all samples an acid soil pH (H_2O) ranging from 4.5 to 4.0 was measured.

Table 2.1 Organic bulk soil characteristics and pH (H_2O)

Depth (cm)	TOC (g.kg^{-1})	N (g.kg^{-1})	C/N molar ratio	pH (H_2O)
0	84	7	13.4	4.5
0-10	79	7	13.6	4.1
10-20	71	7	12.4	4.0
20-30	67	6	13.0	4.2
30-40	61	6	11.8	4.1
40-50	56	5	12.2	4.1
50-60	44	4	12.9	4.2
60-70	30	5	7.5	4.1

Mass spectral characteristics of steroidal and triterpenoid constituents mentioned in the text are listed in Table 2.2. Steroids identified included camposterol ($C_{28:1}$), stigmasterol ($C_{29:2}$) and β-sitosterol ($C_{29:1}$) (Figs. 2.1 and 2.2), the latter component being the most abundant steroid identified. Triterpenoids include β-amyrin, lupeol, lupa-2,20(29)-diene and two triterpenyl acids (Table 2.2). Except for β-sitosterol, steroids and triterpenoids were only identified in the winter sample taken from the first 5 cm (Fig. 2.1a). β-Sitosterol together with C_{26} *n*-alkanol dominated the total lipid extract obtained from this layer. Other compounds identified in this sample included long-chain ($>C_{25}$) *n*-alkanes with a strong odd over even predominance, even *n*-alkanols ranging from C_{20} to C_{28}, and even *n*-alkanoic acids ranging from C_{16} to C_{26}. In addition, C_{22} and C_{24} ω-hydroxy acids were identified.

Table 2.2 Characteristic mass fragments of steroidal and known and unknown triterpenoid components (analyzed as their TMS derivatives)

Component (in order of elution)	Characteristic fragment ions (*m/z*)	$[M]^+$
24-Methylcholest-5-en-3β-ol[1] (Camposterol) *(S-$C_{28:1}$)*	75, 129, 213, 255, 261, 343, 367, 382, 457	472
24-Ethylcholest-5,22-dien-3β-ol[1, 2] (Stigmasterol) *(S-$C_{29:2}$)*	83, 129, 255, 351, 379, 394, 469	484
Olean-12-en-3β-ol[1, 2, 3] (β-Amyrin) *(T1)*	73, 189, 190, 203, 218, 279, 408	498
24-Ethylcholest-5-en-3β-ol[1,] 3 (β-sitosterol) *(S-$C_{29:1}$)*	129, 255, 275, 357, 381, 396, 471	486
Lupeol[1, 2] *(T2)*	73, 189, 190, 203, 218, 369, 393, 408, 483	498
Unknown titerpenoid *(T3)*	55, 124, 203, 218, 257, 271, 288, 355, 413, 483	520?
Lupa-2, 20(29)-diene[1] *(T4)*	121, 135, 147, 161, 189, 203, 241, 297, 339, 365, 393	408
4,4,14α-trimethyl-9β,19-cyclo-5α-ergost-24-en-3β-ol[2, 3] (24-Methylenecycloartenol)	95, 135, 175, 300, 353, 379, 407, 422	512
Unknown titerpenoid *(T5)*	73, 203, 320, 393, 410, 428, 513	528?
C_{30} Triterpenyl acid[2] *(Ta1)*	73, 133, 189, 203, 279, 320, 483, 585	600
C_{31} Triterpenyl acid[2] *(Ta2)*	73, 133, 189, 263, 320, 483, 585	?

Indications as used in Fig. 2.3 are given between brackets in *italics*, [1](Killops and Frewin, 1994), [2](van Bergen et al., 1997), [3](Grandmougin-Ferjani et al., 1999).

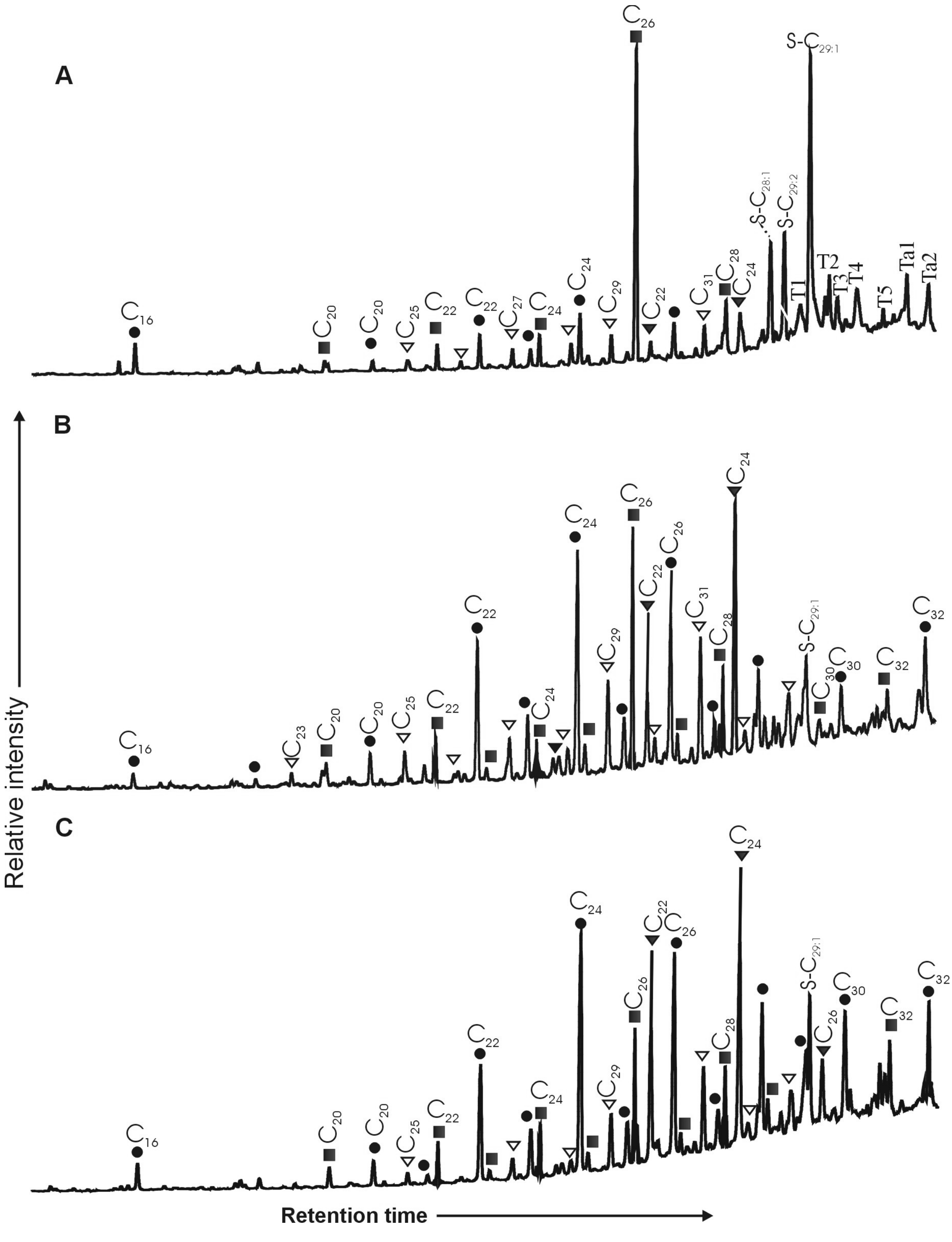


Figure 1. Gas chromatograms of total lipid extracts (TLEs) obtained in the winter from the grass location at depths of: (a) 0-5 cm (O-horizon), (b) 10-20 cm (top Ah horizon) and (c) 20-30 cm (Ah horizon). ■: *n*-alkanols, ●: *n*-alkanoic acids,▽: *n*-alkenes,▼: ω-hydroxy acids, ○: ketones, ▲: α,ω-alkanedioic acids. C_x above the peaks refers to the total number of carbon atoms. S-C_x refers to steroids with x referring to the total number of carbon atoms. Number after colon refers to the total number of double bonds. Tx and Tax refers to triterpenoids and triterpenoic acids respectively, with x referring to their number as assigned in the text and Table 2.

In the winter, from the litter layer to the top of the mineral horizon (10-20 cm), long-chain (>C_{20}) fatty acids, ranging from C_{16} to C_{32} maximizing at C_{22}, C_{24}, C_{26} and C_{28} with a strong even predominance, together with C_{22} and C_{24} ω-hydroxy acids became relatively more abundant (Fig. 2.1b). In addition, C_{23}-C_{33} *n*-alkanes, maximizing at C_{31} with a strong odd over even predominance together with even dominated C_{20}-C_{32} *n*-alkanols, still maximizing at C_{26}, were identified.

Even deeper in the profile, i.e. 20-30 cm, C_{22} and C_{24} ω-hydroxy acids became the most abundant compounds identified in this winter sample, together with C_{20}-C_{32} *n*-alkanoic acids (Fig. 2.1c). Other compounds identified in relatively minor concentrations included C_{20}-C_{32} *n*-alkanols with a strong even over odd predominance maximizing at C_{26} and C_{23}-C_{33} *n*-alkanes, maximizing at C_{31} with a strong odd over even predominance.

Monoacids identified in the summer extract (Fig. 2.2) were dominated by a bimodal distribution. Short-chain (<C_{20}) components were characterized by C_{16} acid, other dominant short-chain components identified included C_{14}, C_{18}, $C_{16:1}$ and $C_{18:1}$ fatty acids, the double bond located at position 9. Long-chain (>C_{20}) fatty acids were dominated by C_{22}-C_{28} members. In addition to short-chain monoacids, short-chain (<C_{20}) *n*-alkanols including C_{14}, C_{16}, $C_{18:1}$ and C_{18} were found in considerable relative amounts in the summer extract. Long-chain *n*-alkanols, like the *n*-alkanes, resembled those identified in the winter extract (Figs. 2.1b and 2.1c). Other compounds identified in relatively minor amounts in the summer extract included C_9 α,ω-alkanedioc acid and the C_{27} methylketone.

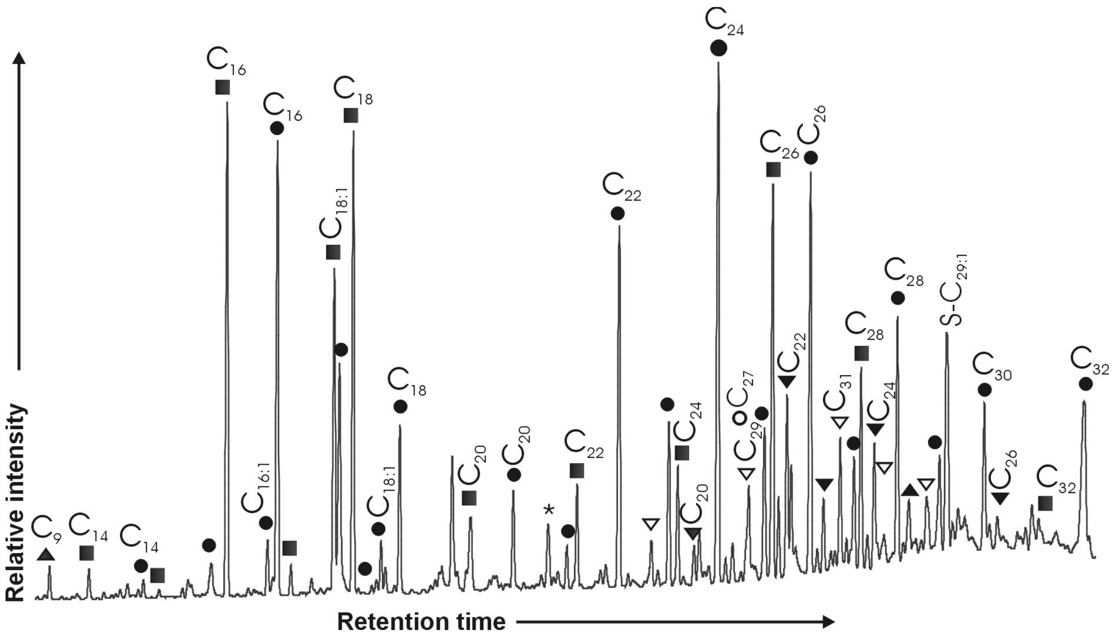


Figure 2. Gas chromatograms of total lipid extract (TLEs) obtained from the grass location sampled in summer at a depth of 15-50 cm. ■: *n*-alkanols,●: *n*-alkanoic acids, ▽: *n*-alkenes,▼ :ω-hydroxy acids,○ : ketones,▲: α,ω-alkanedioic acids. C_x above the peaks refers to the total number of carbon atoms. S-C_x refers to steroids with x referring to the total number of carbon atoms. Number after colon refers to the total number of double bonds.

2.4 Discussion

2.4.1 Total organic carbon vs depth and the contribution of lipids to TOC

Total organic carbon decreases with depth, (Fig. 2.3). From the litter layer to the top of the A-horizon around a depth of 10 cm, and from the A to the E/B-horizon around 45 cm a relatively stronger decrease in TOC with depth was observed (Table 2.1 and Fig. 2.3). The first transition is caused by the decomposition of litter (e.g. Cortez et al., 1996) and mixing with the inorganic matrix, whereas the latter is caused by a strong decrease in the number of roots observed in the hard basaltic E/B-horizon.

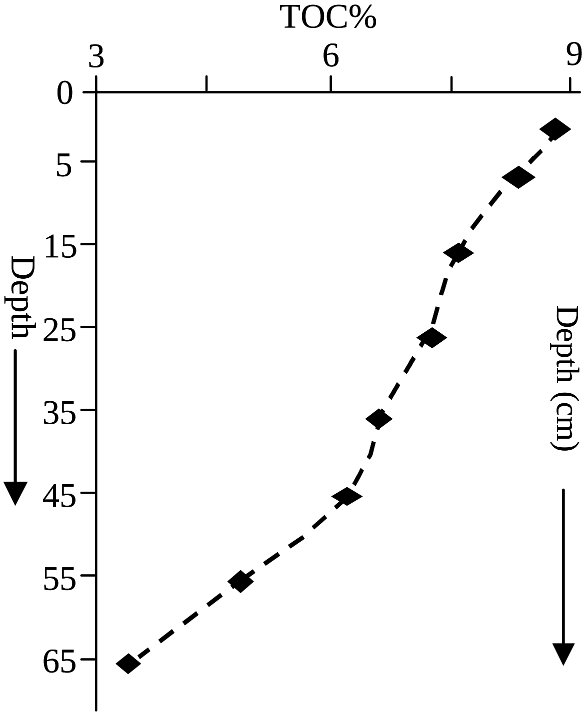


Figure 2.3 Total organic carbon (TOC%) plotted *vs* depth (cm).

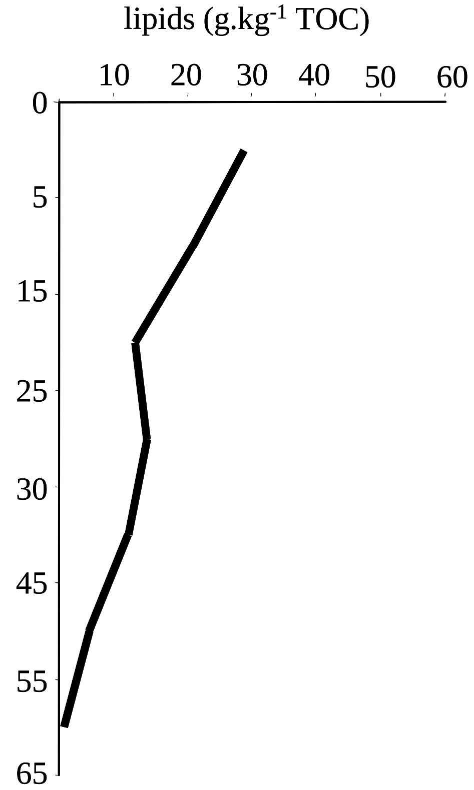


Figure 2.4 Contribution of free extractable lipids to the total organic carbon content (TOC) *vs* depth (cm).

The decrease in the contribution of free lipids to TOC with depth (Fig. 2.4), has been observed by others (e.g. Dinel et al., 1990; Jambu et al., 1991, 1993; Amblès et al., 1993, 1998). This phenomenon is suggested to be mainly caused by (I) a substantially lower input of lipids by roots deeper in the soil profile compared with the litter layer (most important in first 20 cm), together with (II) selective biodegradation of lipid molecules and possibly (III) a simultaneous (partial) incorporation of lipids and their microbial degradation products into macromolecular entities (Amblès et al., 1991). In addition, clays have been shown to bind lipids to their organo-mineral matrix (Amblès et al., 1989). Considering the very high complexing capacity known for all allophanic soils (e.g. Dahlgren et al., 1993), the transfer of some free lipids to organo-mineral complexes might also have contributed to the decrease in free lipids with depth observed.

2.4.2 C/N molar ratios and factors influencing the preservation of lipids in acid andic soils

Acid soils are known to have a low bacterially activity (Wardle, 1992; Motavalli et al., 1995; Andersson and Nilsson, 2001). In general, the amount of nitrogen relative to soil organic carbon increases with depth due to microbial activity and selective degradation of N-poor moieties (e.g. van Bergen et al., 1998b; Marseille et al., 1999). However, C/N ratios decreased only slightly with increasing depth (Table 2.1) suggesting, together with TOC data obtained (Table 2.1 and Fig. 2.3), that SOM was not strongly decomposed with increasing depth in the andic profile. This phenomenon is most probably related to the high protective properties against biodegradation of all types of organic matter in andic soils. These protective capacities are strongly associated with the presence of both high levels of free aluminum together with sorption to allophane, imogolite and ferrihydrite (Boudot et al., 1986; Shoji et al., 1993, Parfitt et al., 1997; Torn et al., 1997). In addition to aluminum toxicity and complexation, the preservation of lipids is favoured by a low soil pH (e.g. Jambu et al., 1985; Dinel et al., 1990).

2.4.3 Origin of compounds identified

Steroids and triterpenoids. Steroids and triterpenoids identified, dominated by β-sitosterol (Fig. 2.5) have frequently been identified in soil and leaf/litter extracts (e.g. Killops and Frewin, 1994; van Bergen et al., 1997; Bull et al., 1998, 2000a). In addition to an input from vascular plants, steroids are known to be derived from fungi (Weete, 1974, 1976; Grandmougin-Ferjani et al., 1999). β-sitosterol is by far the most abundant sterol identified in arbuscular mycorrhizal fungi (Grandmougin-Ferjani, 1999), although substantial amounts of C$_{28}$ sterols have also been found to be indicative of fungal activity

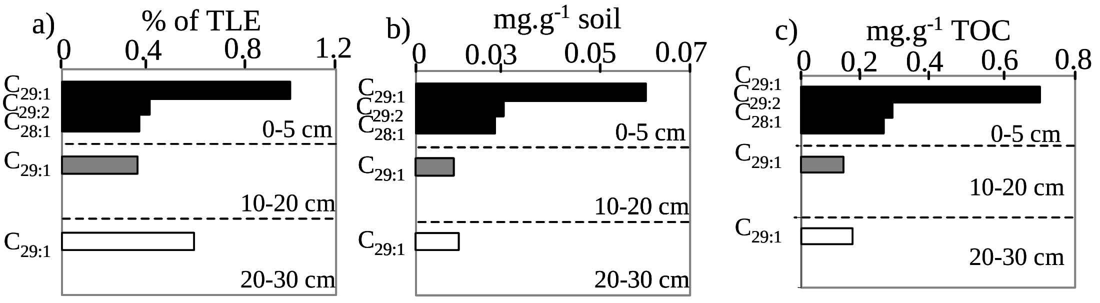


Figure 2.5 Diagrams reflecting changes in: (a) relative concentration (as % of TLE) (b) absolute concentrations (mg.g^{-1} soil) and (c) changes normalized to TOC (mg.g^{-1} TOC) with depth of b-sitosterol (C29:1), stigmasterol (C29:2) and camposterol (C28:1).

Weete, 1976). Other steroids identified in association with fungi in smaller or trace amounts include cholesterol, camposterol, stigmasterol, 24-methylene-cycloartenol, α-amyrin and β-amyrin, their amounts probably depending on production by the plant partner (Grandmougin-Ferjani, 1999). The steroid distribution (Fig. 2.1a) may therefore, in addition to a contribution from vascular plants, be derived from fungi in the litter layer.

n-Alkanes. The (>C$_{25}$) odd dominated alkane pattern observed (Figs. 2.1 and 2.2) is traditionally ascribed to epicuticular waxes and protective layers on vascular plants and commonly observed in lipid extracts from soils and/or plant leaves (e.g. Jambu et al., 1991; Kolattukudy, 1980b; Amblès et al., 1989; van Bergen et al., 1997, 1998a;

Marseille et al., 1999). These compounds can, however, also be produced by certain fungal populations (Weete, 1972, 1976; Marseille et al., 1999). Comparing lipid extracts from mineral soils covered by Quercus with extracts from Quercus leaves revealed enhanced contributions of odd long-chain C_{29}, C_{31} and C_{33} n-alkanes not directly related to the leave lipids (Nott, 1996 BSc thesis). Moreover these latter alkanes, maainly C31 and C33, were significantly 13C enriched (up to 4 per mill) in the soil compared with those from the leaves. A fungal contribution of 13C enriched odd long-chain alkanes could explain such data (van Bergen, Bull, Nott, Poulton and Evershed, unpublished results).

The dominance of C_{31}, most strongly seen in the samples taken from the mineral horizon (Figs. 2.1b, 2.1c and 2.6a), has been observed before in grass roots and rhizomes (Marseille et al., 1999). The relatively low abundance of alkanes in the the top-soil when compared with *n*-C_{26} alkanol and β-sitosterol (Fig. 2.1a) also typically reflects the input by grasses (van Bergen et al., 1997, 1998a).

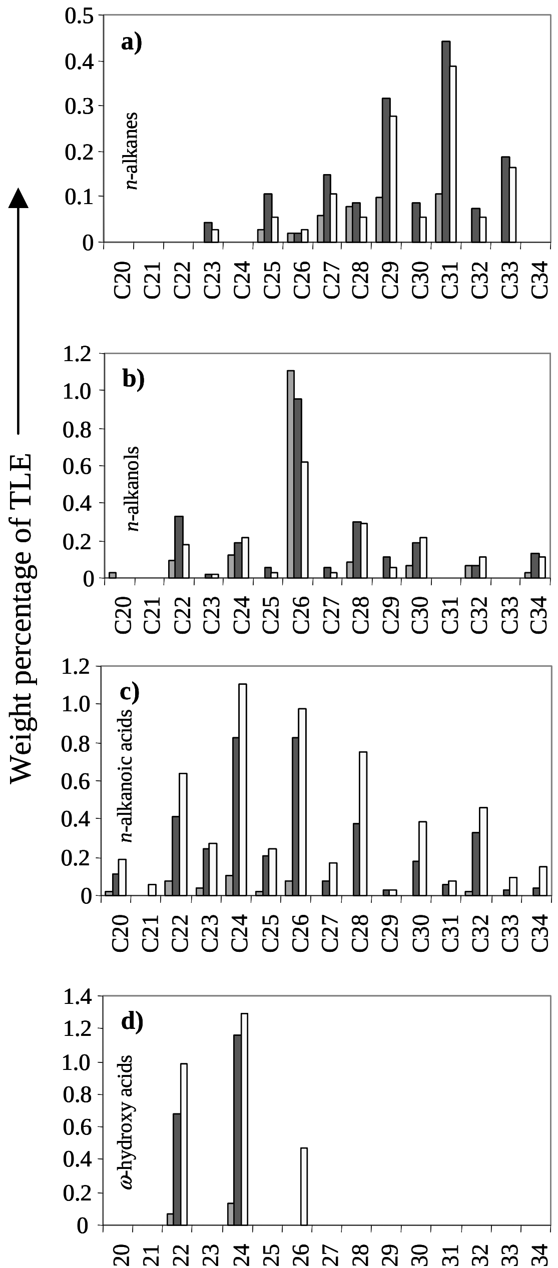
n-Alkanols. The distribution found in the top layer (Figs. 2.1a and 2.6b) with a very dominant contribution from C_{26} alcohol, typically reflects an input by grasses (van Bergen et al., 1997, 1998a; Bull et al., 2000a; Nierop et al., 2001a). From the litter layer to the mineral horizon, *n*-alkanols other than C_{26}, ranging from C_{20} to $C_{32,}$ become relatively more abundant (Fig. 2.6b). *n*-Alkanols identified in grass root thermochemolysis-GC traces (Nierop et al., 2001a) included C_{26}, C_{28} and C_{30} members in similar relative concentrations as found for the 20-30 cm sample (Figs. 2.1c and 2.6b), strongly suggesting a grass root dominated input in the mineral horizon. Moreover, *n*-alkanols ranging from C_{15} to C_{32} without a predominant C_{26} member have been identified in base-hydrolysates from this profile (Chapter 6).

C_{14} to C_{20} compounds, including a C_{18} *n*-alkenol, were only identified in the summer extract (Fig. 2.2). These compounds are most likely derived from lower plants, fungi, spore waxes or hydrolysis products of esters by microbial action (Weete, 1976; Colina-Tejada et al., 1996; Amblès et al, 1998).

Fatty acids (n-alkanoic acids). A distribution of long-chain ($>C_{20}$) fatty acids, ranging from C_{16} to C_{32} and maximizing at C_{22}, C_{24}, C_{26} and C_{28} with a strong even predominance (Figs. 2.1, 2.2, 2.6c), is commonly observed in extracts from acid soils covered by higher plant vegetations (e.g. Almendros et al., 1996; van Bergen et al., 1998a), such as grasses (Bull et al., 2000b).

Thermally assisted hydrolysis and methylation (THM) of grass roots (Nierop et al., 2001a) revealed a very similar distribution of fatty acids as observed in the A-horizon samples (Figs. 2.1b, 2.1c and 2.6c). Moreover, base hydrolysis of solvent insoluble soil residues of this profile revealed the presence of C_{12} to C_{30} fatty acids (Chapter 6). These ester-linked compounds, mainly derived from root biopolyesters, i.e. suberins (e.g. Riederer et al., 1993), could be released into the soil upon (microbial) hydrolysis. In addition, straight-chain components of fungal origin could be a source of *n*-alkanoic acids but they only range from C_{10} to C_{24} (Weete, 1974). Based on their distribution, a contribution from the oxidation of *n*-alkanes or *n*-alkanols (Amblès et al., 1994a), is unlikely.

Short-chain ($<C_{20}$) monoacids identified in the summer extract (Fig. 2.2) have been found to be mainly of microbial origin (Bridson, 1985; Parlanti et al., 1994; Marseille et al., 1999), although C_{16}, C_{18}, $C_{18:1}$ and $C_{16:1}$ fatty acids have also been identified in arbuscular fungi (Madan et al., 2002; Ruess et al., 2002). Hydrolysis of primary esters, including triglycerides, could also release short-chain ($<C_{20}$) acids into the soil (Bridson, 1985; Marseille et al., 1999).



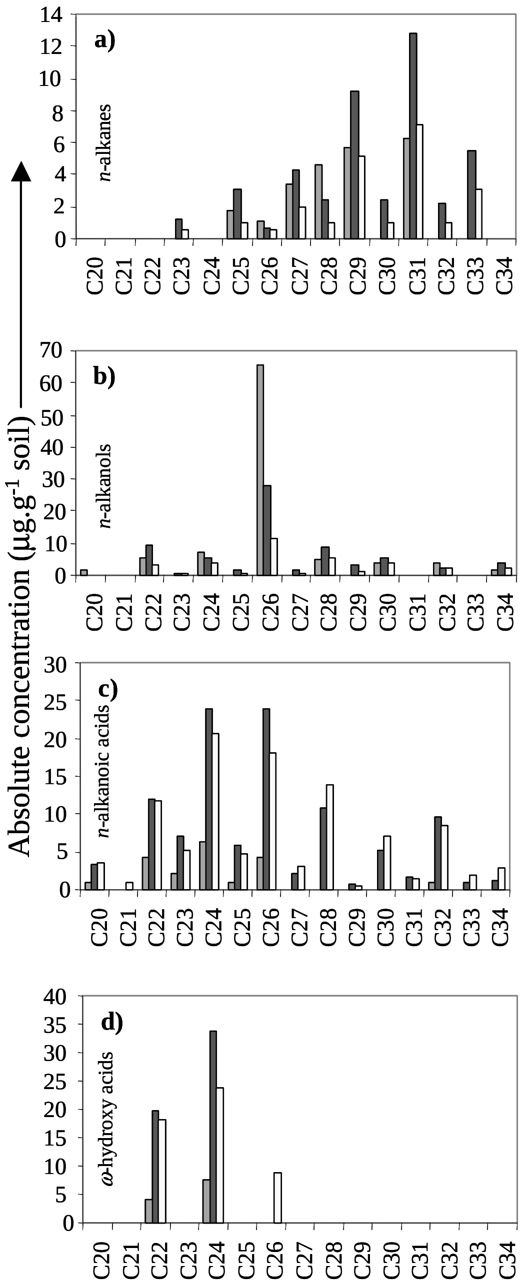


Figure 2.6 Diagrams reflecting changes in the relative (as % of TLE) with depth of: (a) *n*-alkanes, (*b*) *n*-alkanols, (*c*) *n*-alkanoic acids and (d) *w*-hydroxy acids. ▨ : litter layer, ■ : 10-20 cm, □ : 20-30 cm.

Figure 2.7 Diagrams reflecting changes normalized to absolute concentrations (as mg.g-1 soil) with depth of: (a) *n*-alkanes, (*b*) *n*-alkanols, (*c*) *n*-alkanoic acids and (d) *w*-hydroxy acids. ▨ : litter layer, ■ : 10-20 cm, □ : 20-30 cm.

ω-Hydroxy acids. One obvious precursor for the C_{22}, C_{24} and C_{26} ω-hydroxy acids identified mainly in the samples from the mineral soil A-horizon (Figs. 2.1b and 2.1c), is the biopolyester suberin (van Bergen et al., 1998a; Bull et al., 2000b). This root biopolyester has been reported to contain substantial amounts of either the C_{22} and C_{24} homologues (e.g. Kolattukudy, 1980a; Walton, 1990; Matzke and Riederer, 1991; Riederer et al., 1993; Nierop, 1998).

Base-hydrolysis of solvent insoluble residues from these soils as well as solvent extracted (grass) root residues (Bull et al., 2000b) released ω-hydroxy acids ranging from C_8 to C_{28}, including C_{22}, C_{24} and C_{26} ω-hydroxy acids (Chapters 5, 6). The presence of C_{22} and C_{24} ω-hydroxy acids in THM data of grass leaves (Nierop et al., 2001a), suggests a grass leaf derived input, in addition to roots, of these ω-hydroxy acids in the litter layer. Considering the resemblance between the distribution of free fatty acids and ω-hydroxy acids, i.e. dominant C_{22}, C_{24} and C_{26} components, oxidation of free fatty acids as a source for ω-hydroxy acids cannot be excluded.

α,ω-alkanedioic acids. The presence of a C_9 α,ω-alkanedioic acid in the summer extract (Fig. 2.2) is remarkable considering that these compounds are unusual constituents of extractable lipids. The short chain length suggests microbial β-oxidation (Watkinson and Morgan, 1990) of unsaturated alkanoic acids and/or mid-chain hydroxy acids (Regert et al., 1998; Nierop et al., 2003), which are common cutin and suberin monomers (e.g. Kolattukudy, 1980a; Holloway, 1982). Upon such oxidation, unsaturated alkanoic acids and/or dihydroxy fatty acids with mid-chain hydroxyl groups in either the C_7, C_8, C_9 or C_{10} position (del Río and Hatcher, 1998) can produce short-chain alkanedioic acids.

2.4.4 Variations with depth

Lipid signal in top- vs. sub-soil. The most striking change in the chemical composition of total lipid extracts with depth is the sharp decrease in both relative and absolute concentrations of steroids (Fig. 2.5) and triterpenoids going from the litter to the top of the A-horizon. Such a decrease in steroid and terpenoids was observed previously when lipids from forest soil litter layers or leaf extracts were compared with those from the underlying A horizon (Jambu et al., 1993; Amblès et al., 1994a, b; van Bergen et al., 1997; Bull et al., 2000a). It has been suggested that these compounds can be easily mineralized in the soil (Almendros et al., 1996; van Bergen et al., 1997). Assimilation by arthropods (Nes et al., 1997; Bull et al., 2000a) may also contribute considering their significant abundance in this soil profile (Chapter 8). In addition, their absence in soluble lipid fractions can be caused by their condensation into more stable, insoluble structures (Amblès et al., 1991, 1996; van Bergen et al, 1997) or may result from chemically alteration to form modified steroids and triterpenoids. Evidence for the latter two processes has, however, not yet been found (Chapter 8).

The total decrease of stigmasterol ($C_{29:2}$) compared with that of β-sitosterol ($C_{29:1}$) (Fig. 2.5) is probably related to the higher susceptibility towards oxidation of di-unsaturated steroids (Dragun, 1988). In addition, β-sitosterol has been found in base-hydrolysates obtained from soil samples taken from a depth of 15-50 cm (Chapter 6). Thus, hydrolysis of these ester-linked steroids could be an extra source of free β-sitosterol in the sub-soil.

Considering that epicuticular waxes present on and in the protective layers on vascular plants are the most likely source for *n*-alkanes in these soils (see section 4.3.2), we suggest that these layers are still relatively intact in the litter layer. Only after mixing with the acid mineral soil and with time, the *n*-alkanes are released from these layers into the top of the mineral soil (10-20 cm), thereby increasing their absolute (Fig. 2.7a and 2.8a) and relative concentrations (Fig. 2.6a). In addition, the fungal contribution could increase.

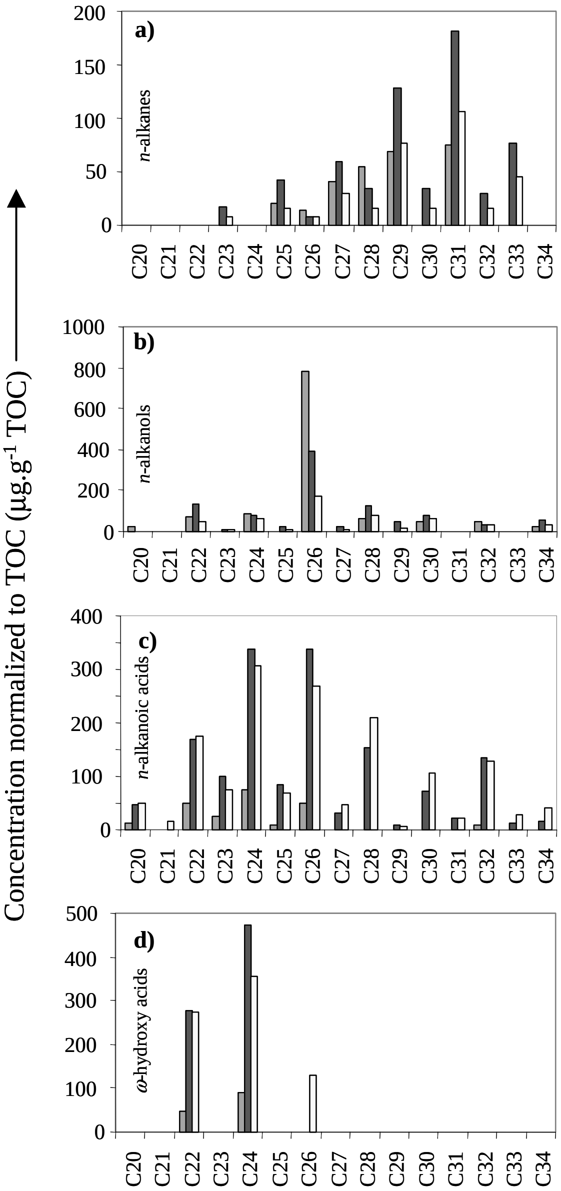


Figure 2.8 Diagrams reflecting changes normalized to TOC (mg.g-1 TOC) with depth of: (a) *n*-alkanes, (*b*) *n*-alkanols, (*c*) *n*-alkanoic acids and (d) *w*-hydroxy acids. ▨ : litter layer, ■ : 10-20 cm, ☐ : 20-30 cm.

The decrease in both relative and absolute concentration of C_{26} *n*-alkanol at greater depth (Figs. 2.6b, 2.7c and 2.8c) might be the result of (I) a reduced input at greater depth, (II) limited transport of this compound in the profile, (III) microbial degradation, and/or (III) a simple kinetic factor, the more abundant component decreasing faster than the other (Marseille et al., 1999). The other *n*-alkanols seem to have either a slower degradation, or an additional input at depth.

The *n*-alkanoic acids clearly show a significant increase in both relative (Fig. 2.6c) and absolute concentration (Figs. 2.7c and 2.8c) with depth, most likely indicating that these compounds, unlike for example C_{26} *n*-alkanol, have their major input in the mineral soil. The accumulation of C_{22}, C_{24} and C_{26} *ω*-hydroxy acids (Figs. 2.6d, 2.7d and 2.8d) has been observed to parallel the accumulation of *n*-alkanoic acids (Figs. 2.6c, 2.6d, 2.7c, 7d, 8c and 8d) in acid soils before (Bull et al., 2000a), suggesting that both *ω*-hydroxy and *n*-alkanoic acids have a similar source, i.e. roots.

Microbial activity. Indications for a limited bacterial activity in the andic profile could be the absence of specific lipid bacterial markers such as short- al., 1991), short-chain ($<C_{20}$) *n*-alkanols (Jambu et al., 1993), short-chain ($<C_{20}$) *n*-alkanoic acids and *iso* and *anteiso* fatty acids (Boon et al., 1977; Perry et al., 1979), C_{30+} hopanoids (Rohmer et al., 1980), and methylketones, i.e. microbial oxidation products of *n*-alkanes (Allen et al., 1971, Amblès et al., 1993). Moreover, the strong odd over even predominance observed for *n*-alkanes and the strong even over odd predominance for both *n*-alkanols and *n*-alkanoic acids are constant with depth (Figs. 2.6, 2.7 and 2.8). If microbial activity would be affecting these compounds, a decrease in these predominances is expected. The detection of steradienes, i.e. $C_{28:2}$ and $C_{29:2}$,

and a triterpadiene, i.e. lupa-2,20(29)-diene also seems to indicate a slow (microbial) transformation of these diagenetic intermediates (Killops and Frewin, 1994).

A low soil pH is known to increase the fungal contribution (Bumpus, 1993, Bååth and Anderson, 2003). Therefore, in the acid soil profile studied, the populations of filamentous fungi and actinomycetes are probably more abundant (as reflected in e.g. the *n*-alkane distribution), whereas in neutral and slightly alkaline soils, bacteria and other types of fungi are favoured (Dinel et al., 1990). Moreover, significant contributions from arbuscular mycorrhizal fungi to soil organic matter carbon pools in andic soils have been reported (Rillig et al., 2001).

Transport of compounds through the andic profile. During an experimental leaching study of soil lipids, Amblès *et al.* (1998) showed that hydrocarbons could be leached as soluble components from the litter layer into the soil profile. Despite their low solubility in water lipids can be present in true solution, colloidal dispersion and micelles (Nierop and Buurman, 1998; Piccolo et al., 1996a). The strong decrease in the steroid, triterpenoid and C_{26} alkanol signal from the top of the profile (0-5cm) to the deeper layers (Figs. 2.5 and 2.7b) may, in addition to a quick mineralization or assimilation, indicate a limited transport of these compounds through the profile. Considering the availability of enough precipitation to transport these compounds (Madeira et al., 1994), a very low mobility of organic compounds observed in andic profiles (Aran et al., 2001) is most likely caused by the strong sorption of organic compounds. Such sorption phenomena are well-known for (andic) soils containing large amounts of reactive mineral phases, such as Al and Fe hydrous oxides (Ulrich and Stumm, 1988; Ochs et al., 1994; Parfitt, 1999), especially near their pK_a, i.e. pH 4-5 (Hingston et al., 1967).

2.4.5 Seasonal influence

Differences observed between the chemical composition and distribution of both fatty acids and n-alkanols in the summer (Fig. 2.2) and winter extract (Fig. 2.1b) very closely resemble the differences found in the distribution of these compounds in summer and winter leachates (Colina-Tejada et al., 1996) and lipid extracts (Amblès et al., 1994b). A microbial origin for all short-chain ($<C_{20}$) compounds in these leachates and extracts has been suggested. Aa microbial origin becomes even more likely considering that $C_{16:1}$ and $C_{18:1}$ fatty acids identified together with C_9 diacid and a C_{27} methylketone (Fig. 2.2) are intermediate components in biodegradation processes (Parlanti et al., 1994; Amblès et al., 1993).

2.5 Conclusions

Total lipid extracts obtained from an andic soil profile on Madeira Island, covered mainly by grass, have been analyzed using gas chromatography (GC) and GC-mass spectrometry (GC/MS). The decrease in the contribution of free lipids to the TOC on going from the litter to the mineral A horizon is most likely indicative of a substantially lower input of lipids by roots compared with the litter layer.

The lipid distribution observed in the top layer, characterized by a very dominant contribution from C_{26} alcohol together with steroids and triterpenoids, typically reflects an input from grass leaves. The signal from long-chain *n*-alkanoic acids (C_{22}-C_{32}), ω-hydroxy acids (C_{22}-C_{26}), C_{31} *n*-alkane and *n*-alkanols (C_{22}-C_{32}), observed in the samples taken from the mineral A horizon, is indicative of an important contribution by (grass) roots. This contribution clearly increased with increasing depth, while a strong decrease was observed in the C_{26} alcohol, steroid and triterpenoids concentrations.

The absence of typical bacterial lipid markers could be indicative for limited bacterial activity. Possible molecular evidence of fungal activity, especially in the litter layer, has been found in the distributions of steroids, *n*-alkanes and *n*-alkanoic acids. In summer, a signal reflecting the leaching of microbially derived products from the litter and/or aerial vegetation at the surface is observed.

Overall, the lipid data obtained provide new detailed molecular insights into the origin and fate of SOM and further our understanding of the processes affecting andic soils.

Acknowledgements. This study was supported by the Institute for Paleoenvironment and Paleoclimate Utrecht (IPPU). Dr. K. G. J. Nierop is thanked for his constructive review of an earlier version of this paper. The reviewers are sincerely thanked for their careful reviews and useful comments.

Chapter 3

Total lipid extracts from characteristic soil horizons in a podzol profile

The podzolization process is studied through lipids in 9 characteristic podzol horizons. Organic matter accumulates particularly with aluminium in the Bh horizon, while the hard, cemented Bs horizon below this is mainly formed by iron oxides. The low soil pH seems to have no great influence on the preservation of lipids as reflected by the absolute amounts present ($mg \cdot g^{-1}$ soil) and the presence of bacterial lipid markers throughout the profile. Independent of soil pH, lipids accumulate in organically enriched horizons. Albeit, high molecular weight organic compounds accumulate to a relatively greater extend than lipids in these horizons. A lipid signal related to the aerial parts, i.e. leaves and flowers, of *Calluna* is observed only in the O horizon. This "*n*-alkane, steroid and triterpenoids" signal is quickly lost in the underlying Ah horizon due to (bacterial) oxidation. The other total lipid extracts obtained are dominated by root-derived compounds. In sub-soil horizons high in organic matter, i.e. the Ahb and Bh horizons, root-derived friedoolean and steroid compounds dominate the total lipid signal. Organic matter poor, degraded horizons, i.e. the E2, Bhs, Bs and B/C horizons, are dominated by C_{22} and C_{24} ω-hydroxy acids, long-chain ($>C_{20}$) *n*-alkanoic acids with a strong even over odd predominance and C_{22} and C_{24} *n*-alkanols. Steroid and root-derived triterpenoids with a friedooleanan structure have been removed from these horizons through degradation. Based on TOC content and lipid composition, the formation of an E1 horizon has started, but is not yet complete. In the Ahb horizon, a contribution from buried vegetation to the total lipid signal is still present, although degradation and an input from roots have significantly altered the original signal. Overall, lipid data indicate that degradation (microbial oxidation) is an important process that should be taken into account, in addition to leaching, when describing podzolization processes in soils.

3.1 Introduction

Many soils show evidence of podzolization, a process that involves a pronounced downwards translocation of iron, aluminum and organic matter to form characteristic soil horizons (van Breemen & Buurman, 2002). From top to bottom, a podzol profile consists of up to five major horizons, two of which may be absent (in italics): a litter layer (O), *a humic mineral topsoil (Ah), caused by biological mixing*, a leached eluvial layer (E), an accumulation layer of organic matter in combination with iron and aluminum (Bh, Bhs, Bs) and *thin bands with organic matter accumulation in the subsoil* (van Breemen & Buurman, 2002). Podzols form both in well-drained soils (xero-podzols) and in soils with a shallow, fluctuating water-table (hydro-podzols).

Breakdown of litter and exudation by plants and fungi result in a large variety of organic compounds introduced to the soil. Part of the decomposing products is soluble in water and can be transported with percolating rainwater to deeper parts of the soil. The remaining organic matter at the soil surface forms the humified part of the litter layer. It can be mixed with the mineral soil to form an Ah horizon where it is further

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broken down by microflora, also supplying soluble organic substances. Soluble percolating organic compounds include high molecular weight substances with various carboxylic and phenolic groups, as well as low molecular weight (LMW) organic acids, such as aliphatic acids and simple sugars (van Breemen & Buurman, 2002). In well-drained acid soils, iron and aluminum often move as soluble organo-metal chelating complexes (e.g. Schnitzer & Skinner, 1963; Jansen, 2003) or as a positively charged mixed Al_2O_3-Fe_2O_3-SiO_2-H_2O sol (Farmer, 1984). Several processes, a combination of which will play a role in all soils, may halt the movement of dissolved organic matter and the associated metals, Al and Fe. Complexed LMW organic acids can be decomposed by micro-organisms (Lundström et al., 2000), the high molecular weight (HMW) organic-metal complex may become saturated with metals and precipitate (Schnitzer & Skinner, 1963) and/or the water transport may stop (van Breemen & Buurman, 2002). Furthermore, LMW organic compounds, including a variety of lipids, may become associated with organic matter already present in relatively organic-rich soil horizons, i.e. Ah and/or Bh horizons. In the proto-imogolite theory of podzolization (Farmer, 1982), allophane and associated iron oxides are deposited from the moving sol. Negatively charged organic sols and solutions precipitate on the (so formed) positively charged sesquioxide-coated surfaces of the Bs horizon to create a Bh horizon (Farmer, 1982, 1984).

Soil lipids are, by definition, organic compounds insoluble in water but soluble in common organic solvents. They include *n*-alkanoic acids, *n*-alkanols, hydroxy acids, ketones, steroids, terpenoids, acyl glycerols and hydrocarbons, as well as phospholipids and lipopolysaccharides (Stevenson, 1982). These compounds originate from both plants and animals as products of deposition, decomposition and exudation, as well as from various other pedogenic sources, including fungi, bacteria and mesofauna (Bull et al.., 2000a). Lipids accumulate in acid soils such as podzols (Jambu et al.., 1985). In these, as well as in other soils, their composition is influenced by a wide range of processes, including bioturbation, oxidation, microbial degradation and hydrolysis. The rate of these processes is directly affected by soil pH, moisture, microbial biomass, etc. (van Bergen et al., 1997, 1998). So far, lipid research in podzols has mainly focussed on specific lipid fractions, e.g. *n*-alkanes, *n*-alkanols and *n*-alkanoic acids, mainly in litter and A horizons (e.g. Amblès et al., 1998; Jambu et al., 1993). To our best knowledge, no studies on the composition of total lipid extracts throughout the whole podzol profile have been undertaken.

In this paper, the composition of total lipid extracts from 9 characteristic podzol horizons from the Veluwe area (a natural park, located in the centre of The Netherlands) were determined using gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). In addition, soil pH (H_2O), ammonium oxalate- and sodium pyrophosphate extractable aluminium and iron were determined. Results are discussed in terms of the origin of the compounds identified and the processes that effect their distribution in the podzol profile.

3.2 Materials and methods

3.2.1 Site description

Samples were taken from a Haplic Podzol (FAO, 1998) located at the Veluwe near Kootwijk/Assel, The Netherlands, which has formed in wind blown sands, deposited during the Pleistocene. Vegetation is dominated by heather (*Calluna vulgaris*) and moss together with small birch trees. The profile is characterized by a litter layer (O) underlain by several horizons typical of podzols (Fig. 3.1). recognizeable roots and root-fragments were found only to a depth of about 52 cm (start of the Bs horizon).

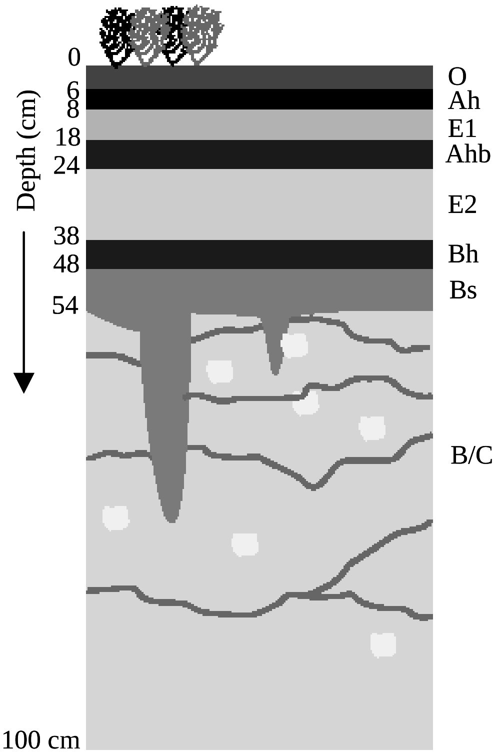

Figure 3.1 Schematic representation of the podzol profile sampled. Characteristic horizon symbols are indicated at the right.

3.2.2 Sampling, sample pre-treatment and inorganic analyses

Samples were taken in early September 1999 from each horizon and from in between the Bh and Bs horizons (referred to as the Bhs horizon in this paper). Samples were oven dried at 60 °C and sieved over a 2mm and a 250µm sieve to remove roots. Soil pH (H_2O) of the soil was measured in the supernatant of a suspension (1:2.5 sieved (<250 µm) sample: water) using a Scott Geräte pH meter CG 805. Sodium pyrophosphate extractable Fe and Al were determined by shaking 0.5 g of sieved (<250 µm) soil overnight (16 h) with 50 ml of 0.1 M Na pyrophosphate solution. Oxalate-extractable Fe, Al and Si were determined by shaking (4 h in the dark) 0.5 g of sieved (<250 µm) soil with 25 ml 0.2 M ammonium oxalate solution. Concentrations of Al, Fe and Si were measured using a Perkin Elmer Optima3000 ICP-OES.

3.2.3 Organic analyses

Total organic carbon contents (TOC%) of sieved (<250 µm) soil were measured using a Fisons Instruments NA 1500 NCS analyzer, with a cycle time of 180 s, a source temperature of 190 °C and an oxygen flow of ca. 30 lmin^{-1}. Approximately 15-25 g of the sieved (<250 µm) samples was extracted using a Soxhlet apparatus with dichloromethane/methanol (DCM/MeOH) (9:1v/v) for 24 h. The DCM/MeOH extract collected was taken to complete dryness in a rotary evaporator. The dry residue obtained was dissolved in approximately 2 ml DCM/isopropanol (2:1 v/v), filtered in a Pasteur pipette packed with defatted wool, 0.5 cm Na_2SO_4 and 2 cm SiO_2, and dried in a stream of N_2. Free hydroxyl and carboxylic acid groups present in an aliquot were derivatized to their corresponding trimethylsilyl (TMS) ethers and esters respectively, by heating for 1 h at 70 ^{o}C with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane. The derivatized aliquots were dried under N_2 and dissolved in hexane. An aliquot of a standard solution containing 0.18 µg µl^{-1} 10-nonadecanone was added. The remaining part of the extract was evaporated to dryness in air.

3.2.4 Gas chromatography (GC)

Derivitized total lipid extracts in hexane (1 µl) were analyzed using gas chromatography (GC). GC analyses were performed using a Hewlett-Packard 6890 equipped with a CP-sil 5CB silica column (50 m x 0.32 mm, film thickness 0.12 µm). Extracts were injected onto the column. The oven temperature was programmed from 70°C to 130°C at 20°C min^{-1} and from 130°C to 320°C (isothermal for 20 min at 320^{o}C) at 4°C min^{-1}. Compounds were detected using a flame ionisation detector (FID) at 325°C. Helium was used as carrier gas.

3.2.5 Gas chromatography-mass spectrometry (GC/MS)

GC/MS analyses were performed using a Hewlett-Packard 5890 series II gas chromatograph connected to a Fisons instruments VG platform II mass spectrometer operating at 70 eV, scanning the range *m/z* 50-650 in a cycle of 0.65 seconds. The capillary column and temperature programme were as described for the GC analyses. Identification of the compounds was carried out using their mass spectral data and a NIST library or by interpretation of the spectra and the GC retention times. In addition, compound identification was based on published data (e.g. Holloway, 1982; Killops & Frewin, 1994; van Bergen et al., 1997).

3.3 Results

3.3.1 Inorganic soil analyses

Values for pH (H_2O) ranged from 4.6 to 3.5 (Table 3.1). From the O horizon (4.6) to the Ah horizon (3.7), the pH (H_2O) decreased, only to rise again to 4.6 in the E2 horizon. Active iron (Fe_{ox}) was highest in the Bs horizon and lowest in the E2 horizon. The amount of organically complexed Al (Al_{py}) was highest in the Bh horizon and, like the organically-complexed Fe, was low in the E2 horizon.

Table 3.1 Inorganic soil analyses

Horizon	**O**	**Ah**	**E1**	**Ahb**	**E2**	**Bh**	**Bhs**	**Bs**	**B/C**
pH (H_2O)	4.6	3.7	3.9	3.8	4.6	3.5	3.5	4.0	4.1
Al_{ox}%	0.11	0.31	0.02	0.23	0.02	1.81	0.33	0.84	0.41
Al_{py}%	0.12	0.14	0.04	0.10	0.02	0.82	0.28	0.39	0.15
Fe_{ox}%	0.07	0.23	0.02	0.18	0.01	0.87	0.61	1.54	0.07
Fe_{py}%	0.07	0.17	0.03	0.07	0.02	0.42	0.52	0.75	0.03

3.3.2 Total organic carbon (TOC) and quantification of total lipid extracts

The largest TOC contents (>5%) were found in the O, Ah, Ahb and Bh horizons, while both E horizons together, with the Bs and B/C horizons, contained less than 2% TOC (Table 3.2). TOC contents of residues after solvent extraction showed a similar trend to those of the unextracted samples. The yields of total lipid extracts (mg g^{-1}) soil ranged from 0.02 to 2.8, the highest amounts again found in the humic horizons. The Ah horizon was making the largest contribution of lipids to the TOC on a mass basis.

Table 3.2 Organic soil analyses

Horizon	O	Ah	E1	Ahb	E2	Bh	Bhs	Bs	B/C
TOC (wt%)	10.1	7.8	2.0	5.3	0.6	6.6	1.7	1.2	0.4
TOC Sox. Res. (wt%)	9.6	7.3	1.6	4.9	0.5	5.7	1.2	1.1	0.4
TLE (mg.g^{-1} soil)	2.2	2.8	0.3	1.1	0.1	1.6	0.2	0.1	0.1
TLE (mg.g^{-1} TOC)	21.9	35.7	13.0	20.8	22.3	24.2	12.9	3.9	6.7

3.3.3 The composition of total lipid extracts from all 9 characteristic horizons

The extract from the O-horizon was dominated by a series of long-chain (>C_{25}) odd-over-even dominated *n*-alkanes (Fig. 3.2a), the most abundant being C_{31} and C_{33}. In addition, steroids and triterpenoids (Table 3.3) were detected including taraxerol, camposterol, stigmasterol, β-sitosterol, C_{29} ketosteroid, α-amyrin, lupeol, friedoolean-3-one, an unidentified triterpenoid and a C_{30} triterpenyl acid. Other compounds, present in relatively lower concentrations, were even-over-odd predominated C_{18}-C_{26} *n*-alkanols, *n*-alkanoic acids, ranging from C_{14}-C_{26}, C_{15} *iso* and *anteiso* acids, C_{18} isoprenoid methylketone and short-chain (<C_{25}) *n*-alkanes without an odd-over-even predominance.

C_{31} and C_{33} *n*-alkanes, together with their co-eluting C_{29} and C_{31} methylketones, were also relatively abundant in the extract of the Ah horizon (Fig. 3.2b). In addition, a C_{29} ketosteroid together with triterpenoids including taraxerone, friedoolean-3-one and an unknown steroid dominated the last part of the chromatogram. Even-over-odd dominated C_{12}-C_{32} *n*-alkanoic acids and C_{23}-C_{28} odd-over-even dominated methylketones were found in relatively large concentrations. Apart from saturated linear fatty acids, C_{15} *iso* and *anteiso* acids and one C_{18} *n*-alkenoic acid were found. *β*-sitosterol was present only in relatively low concentrations, together with even C_{20}-C_{26} *n*-alkanols, the C_{18} isoprenoid methylketone, C_{22} and C_{24} *ω*-hydroxy acids, and short-chain (<C_{25}) *n*-alkanes without an odd-over-even predominance.

Table 3.3 Characteristic mass fragments and IUPAC names of steroidal and known and unknown triterpenoid compounds (analyzed as their TMS derivatives)

Compound (in order of elution)	**Characteristic fragment ions (*m/z*)**	**$[M]^+$**
Friedoolean-2-ene[1] *(T1)*	69, 95, 205, 218, 231, 243, 257, 274, 287, 395	410
Unidentified triterpenoid *(T2)*	73, 190, 204, 218, 269, 359, 393, 483	498
24-Methylcholest-5-en-3β-ol[1, 2] (Camposterol) *(S-$C_{28:1}$)*	75, 129, 213, 255, 261, 343, 367, 382, 457	472
Friedoolean-14-en-3-one *(T3)* (Taraxerone)[1]	69, 81, 95, 109, 189, 191, 203, 205, 273, 409	424
Friedoolean-14-en-3β-ol *(T4)* (Taraxerol)[1]	121, 135, 189, 204, 269, 284, 359, 374, 393, 408, 483	498
24-Ethylcholest-5, 22-dien-3β-ol[1, 2, 3] (Stigmasterol) *(S-$C_{29:2}$)*	83, 129, 255, 351, 379, 394, 469	484
24-Ethylcholest-5-en-3β-ol[1, 2, 3] (β-sitosterol) *(S-$C_{29:1}$)*	129, 255, 275, 357, 381, 396, 471	486
24-Ethylcholestan-3β-ol *(S-C_{29})*[1]	107, 215, 257, 305, 359, 383, 398, 473	488
Ursan-12-en-3β-ol[2] (α-Amyrin) *(T5)*	73, 95, 189, 190, 203, 218, 279, 393, 408, 483	498
Lupeol[2, 3] *(T6)*	73, 190, 203, 218, 369, 393, 408, 483	498
Steroid[1] *(S1)*	95, 109,187, 205, 259, 274, 286, 393	408
Friedooleanan-3-one[1] *(T8)*	69, 81, 95, 109, 123, 273, 302, 341, 379, 411	426
Triterpenoid[1] *(T9)*	69, 81, 95, 109, 121, 137, 205, 218, 313, 327, 341, 409, 424, 481	496
24-Ethyl-5-cholestan-3-one *(KS-C_{29})*[1] (C_{29}-ketosteroid)	69, 81, 95, 109, 123, 147, 163, 177, 207, 231, 257, 275, 317, 395	414
C_{30} Triterpenyl acid[3] *(Ta1)*	73, 133, 189, 203, 279, 320, 483, 585	600

[1](van Smeerdijk and Boon, 1987), [2](Killops and Frewin, 1994), [3](van Bergen et al., 1997), letters (Tx, Sx) used in Fig. 3.2 shown in parenthesis.

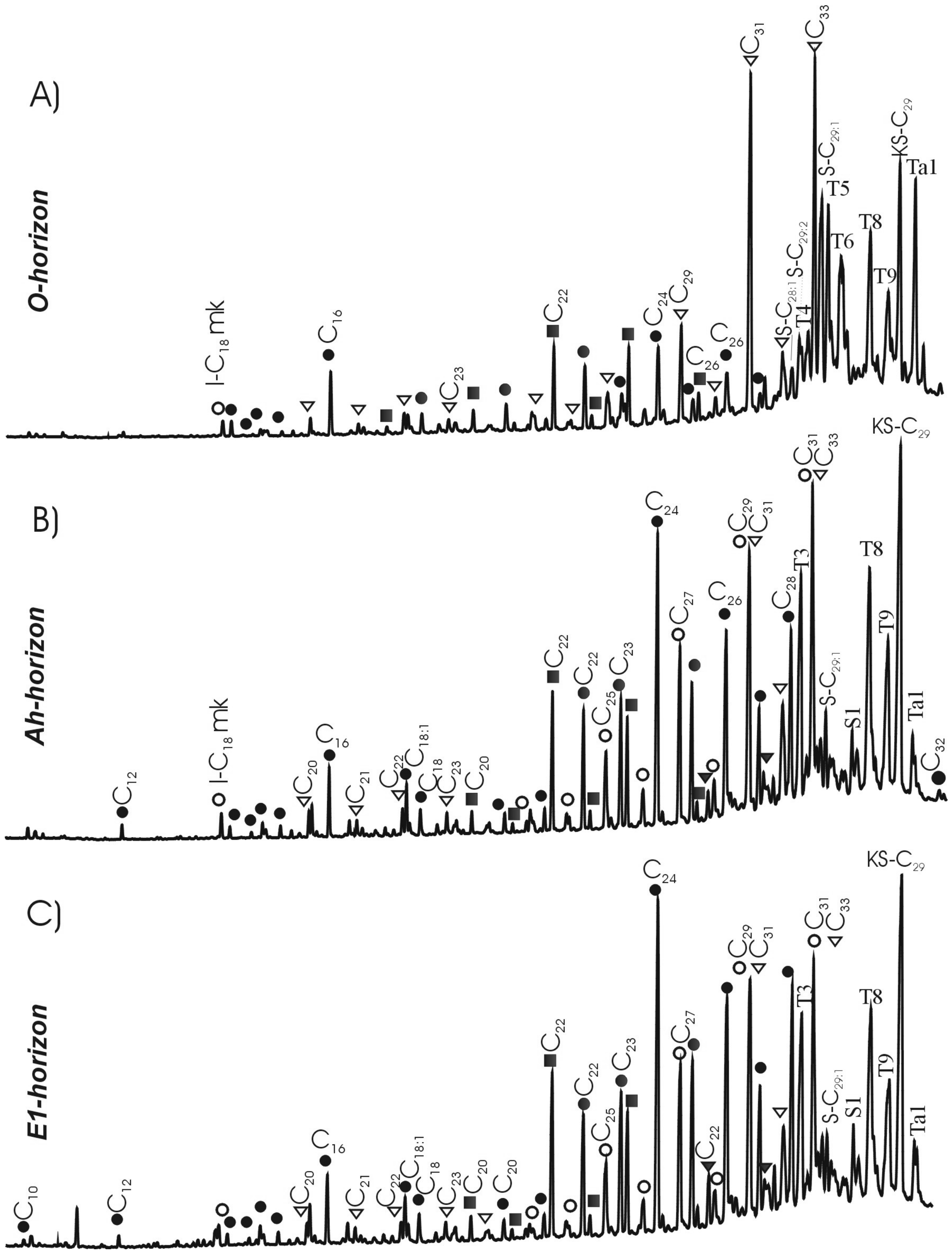

Fig. 3.2 Gas chromatograms of total lipid extracts (TLEs) obtained from: O-horizon (a), Ah horizon (b), E1 horizon (c), Ahb horizon (d), E2 horizon (e), Bh horizon (f), Bhs horizon (g), Bs horizon (h) and B/C horizon (i). Key: ■ : *n*-alkanols, ● : *n*-alkanoic acids, ▽ : *n*-alkanes, ▼ : ω-hydroxy acids, ○ : ketones, ***su*** : saccharide, I-C_{18} mk: isoprenoid methylketon, Ph: phthalate. C_x refers to the total number of carbon atoms. S-C_x refers to steroids and KS-C_x refers to keto-steroids with x referring to the total number of carbon atoms. Number after colon refers to the total number of double bonds. Tx and Tax refers to triterpenoids and triterpenoic acids respectively, with x referring to their number as assigned in the text and Table 3.

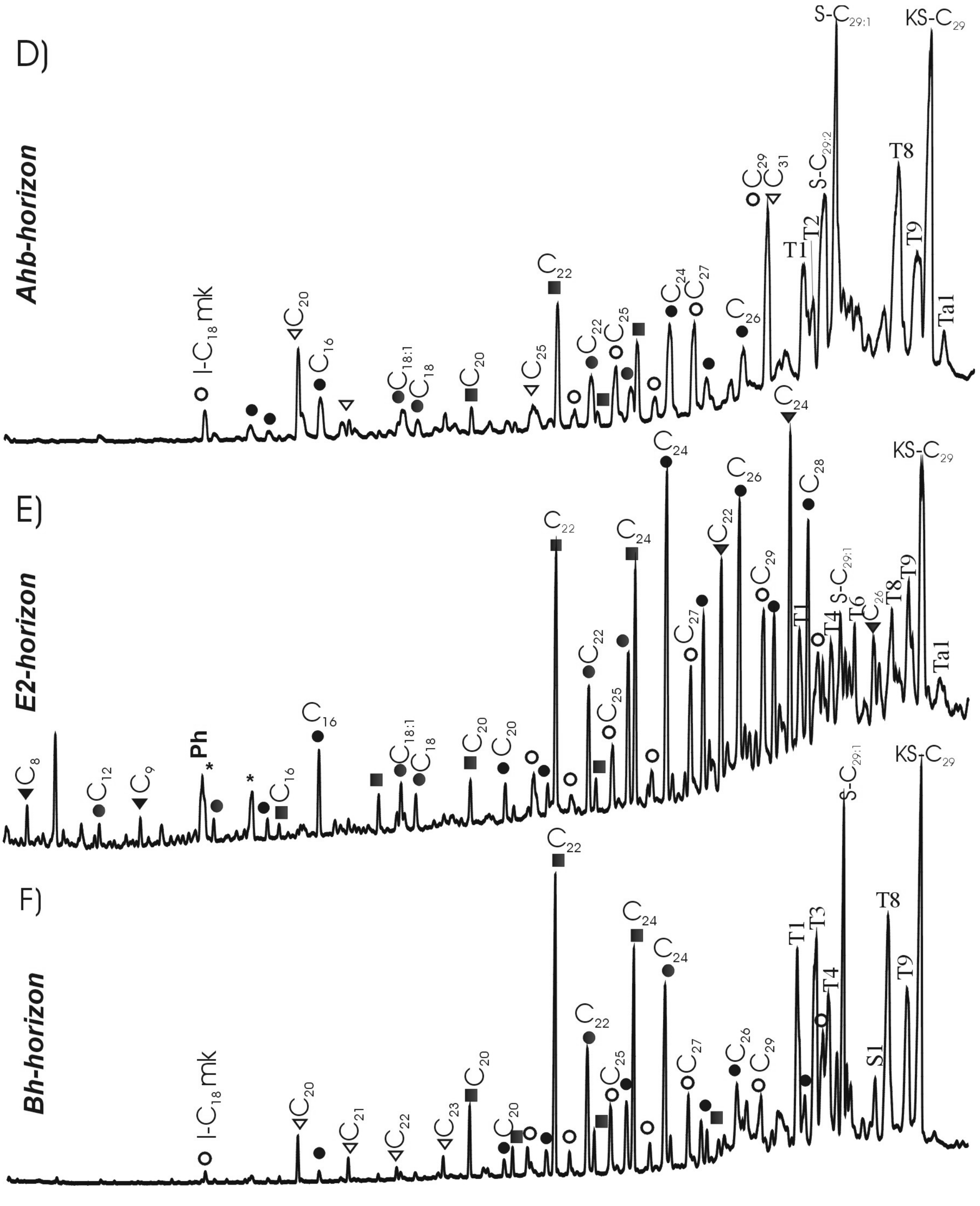

Figure 3.2 d, e, f.

The only difference between the extract from the E1 horizon (Fig. 3.2c) and that from the Ah horizon (Fig. 3.2b) was the increase in relative concentration of the *n*-alkanoic acids. In contrast, these acids were much less important in the Ahb extract (Fig. 3.2d), which extract was characterized both by β-sitosterol and the C_{29} ketosteroid. Other important compounds were friedoolean-2-ene, T2, friedoolean-3-one, T9, stigmasterol, C_{22} and C_{24} *n*-alkanols, and the co-eluting C_{31} *n*-alkane and C_{29} methylketone.

In the E2 horizon, the relative contribution of steroids and triterpenoids was smaller again, whereas the even-over-odd dominated *n*-alkanoic acids, *n*-alkanols and C_{22} and C_{24} ω-hydroxy acids were more abundant (Fig. 3.2e). Two short-chain, i.e. C_8 and C_9, ω-hydroxy acids were identified together with phytol, C_{23}-C_{31} methylketones and C_{12} and C_{14} *n*-alkanoic acids.

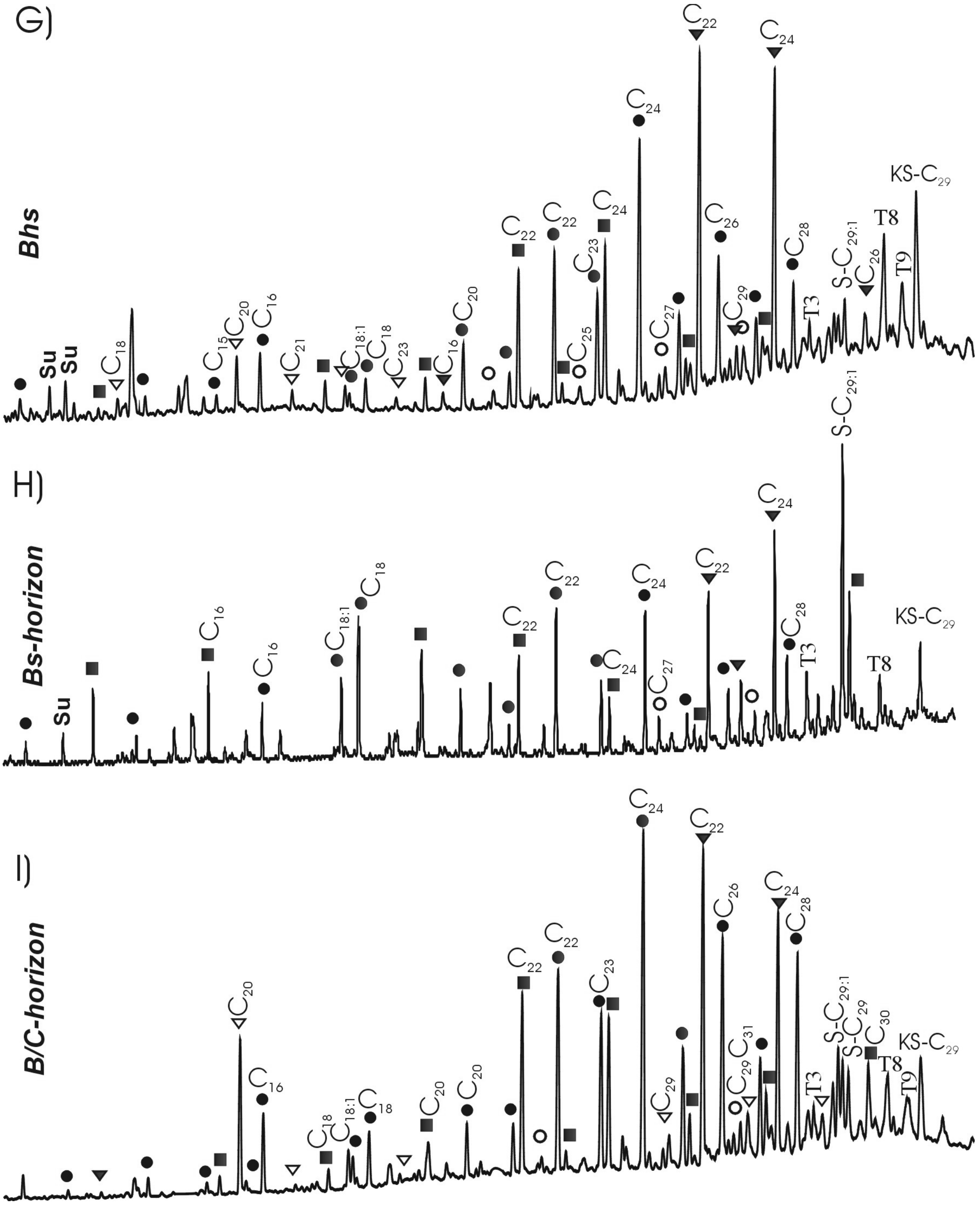

Fig. 3.2 g, h, i.

β-sitosterol and a C_{29} ketosteroid, together with a number of triterpenoids including friedoolean-2-ene, taraxerone, taraxerol and friedoolean-3-one, characterized the extract obtained from the Bh horizon (Fig. 3.2f), similar to the Ahb horizon extract (Fig. 3.2d). Other relatively abundant compounds included C_{22} and C_{24} *n*-alkanols and *n*-alkanoic acids. In addition to a series of odd-over-even dominated ketones (C_{23}-C_{31}), *n*-alkanes without a clear odd-over-even dominance (C_{20}-C_{23}) were identified.

C_{22} and C_{24} ω-hydroxy acids were found to dominate the extract obtained from the Bhs horizon in addition to an even-over-odd dominated series of *n*-alkanoic acids

(C_{10}-C_{28}) and *n*-alkanols (C_{14}-C_{28}; Fig. 3.2g). Compounds present in relatively smaller concentrations included *n*-alkanes (C_{18}-C_{23}), β-sitosterol, taraxerone, friedoolean-3-one, the C_{29} ketosteroid, as well as ketones (C_{23}-C_{29}) and two saccharides.

The Bs-horizon total lipid extract was characterized by a series of even-over-odd dominated C_{12}-C_{28} *n*-alkanoic acids maximizing at C_{18}, C_{22} and C_{24} (Fig. 3.2h). In addition, a series of even-over-odd dominated *n*-alkanols ranging from C_{14} to C_{28} and maximizing at C_{20}, C_{22} and C2,4 was found. Other compounds detected were β-sitosterol, C_{22} and C_{24} ω-hydroxy acids, C_{27} and C_{29} ketones, a saccharide, taraxerone and friedoolean-3-one.

Like the sample obtained from the Bhs horizon (Fig. 3.2g), long-chain (>C_{20}) *n*-alkanoic acids together with C_{22} and C_{24} ω-hydroxy acids characterized the B/C horizon extract (Fig. 3.2i). Although there was a slight increase in the relative concentration of C_{20} *n*-alkane and the long-chain *n*-alkanoic acids, the B/C extract resembled to a great extent the extract obtained from the overlying Bhs horizon (Fig. 3.2h).

3.4 Discussion

3.4.1 Inorganic and bulk organic soil analyses

The largest amounts of aluminium and iron complexed with organic matter, i.e. Al_{py} and Fe_{py} are found in the B horizons (Bh, Bs; Table 3.1). According to these data, soluble organic matter transported down the profile is immobilized in particular by aluminium in the Bh horizon (Lundström et al ., 1995; Wesselink et al., 1996). The Al_{ox} and Fe_{ox} data, in combination with Al_{py} and Fe_{py} data, show that the Bs horizon is mainly formed by Fe oxides (Table 3.1).

Acid soils are known to enhance the preservation of lipids (Jambu et al., 1985; van Bergen et al., 1998; Bull et al., 2000a). All concentrations of extractable lipids, with the exception of the Ah sample, where it is 35.7 mg g^{-1} TOC (Table 3.2), lie within the range of 2-25 mg g^{-1} TOC normally found for soils (Stevenson, 1982). The low soil pH therefore seems to have no great influence on the preservation of lipids in the soil profile studied.

The change of TOC% with depth (Fig. 3.3) clearly indicates the presence of organic matter poor layers (E1 and E2), an accumulation of organic matter (Ah, Bh), and a litter layer (O). The Bhs, Bs and B/C samples contain minor amounts of organic matter, indicating that its accumulation occurs mainly above the hard cemented Bs horizon (de Coninck, 1980). In addition to these characteristic horizons (van Breemen & Buurman 2002), accumulated organic matter was found in the buried A horizon (Ahb). The newly formed E1 horizon on top of this Ahb horizon (Fig. 3.1) still contains 2% TOC (Table 3.1 & Fig. 3.3) suggesting that the formation of this horizon has started, but is not yet complete.

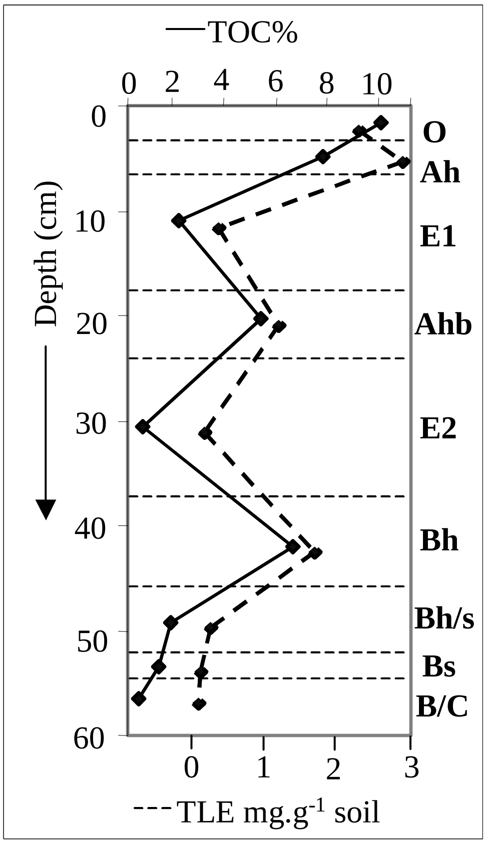

Figure 3.3 Total organic carbon content (TOC%) and TLE (mg.g^{-1} dry soil) *vs* depth. TOC% is represented by the solid line, TLE by the dashed line. Characteristic horizons are indicated between dashed lines, horizon codes are found at the right.

The absolute amounts of extracted lipids (mg g^{-1} soil; Fig. 3.3), show a good correlation with the TOC contents (Fig. 3.4). When the Ah horizon is excluded, the correlation is even better ($r^2 = 0.97$) showing that lipids accumulate in organic matter rich horizons. Comparison of the contribution of lipids to TOC with the TOC content reveals that lipids do not accumulate relative to other soil organic matter in these horizons. In other words, SOM compounds other than lipids have accumulated even more than lipids in podzol B horizons (Schmidt et al., 2000). The relatively great contribution of lipids to the total amount of organic carbon in the E2 horizon (Table 3.2) could be caused by the relatively low leachability of hydrophobic compounds, such as lipids, compared with more hydrophilic organic compounds. Alternatively, it could be due to the very low TOC content which would exaggerate any errors in this ratio. The difference between the theoretical yield calculated using the difference in TOC% between the unextracted and solvent extracted samples (Table 3.2), and the absolute weight of the total lipid extracts, is caused by losses during the work-up procedure of the extract. The large contribution of free lipids in the Ah horizon parallels the accumulation of aliphatic compounds in A horizons of acidic soils observed previously (Jambu et al., 1993) due to the hydrolysis of wax esters (Jambu et al., 1993).

3.4.2 Molecular composition of total lipid extracts (TLEs) from characteristic horizons

Composition of the TLE from the O horizon. Dominant long-chain (>C_{27}) alkanes with a strong odd-over-even predominance (Fig. 3.2a) are traditionally ascribed to epicuticular waxes and protective layers on vascular plants and commonly observed in lipid extracts from vegetation and soils (Jambu et al., 1991; Amblès et al., 1989).

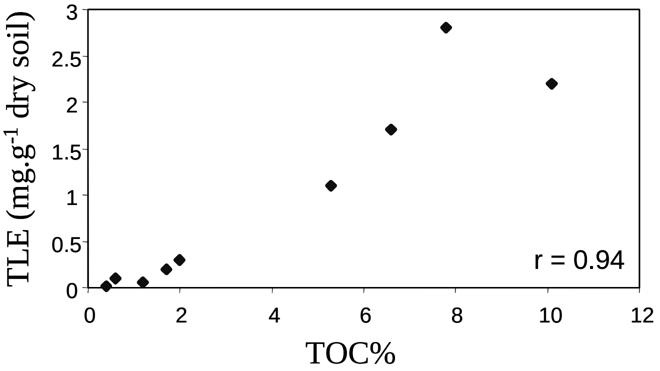

Fig. 3.4 TLE (mg.g^{-1} dry soil) *vs* TOC%

Moreover, the relatively high amounts of C_{31} and C_{33} alkanes (Figs 3.6c, 3.7c), with smaller contributions of C_{27} and C_{29}, are characteristic of heather (*Calluna)* flowers and leaves (Nierop et al., 2001). A heather-derived input is also reflected in the C_{22} and C_{24} *n*-alkanol distribution, characteristic for *Calluna* stem wood and roots (Nierop, 1998; Nierop et al., 2001) as well as *Ericaceae* rootlets (van Smeerdijk & Boon, 1987).

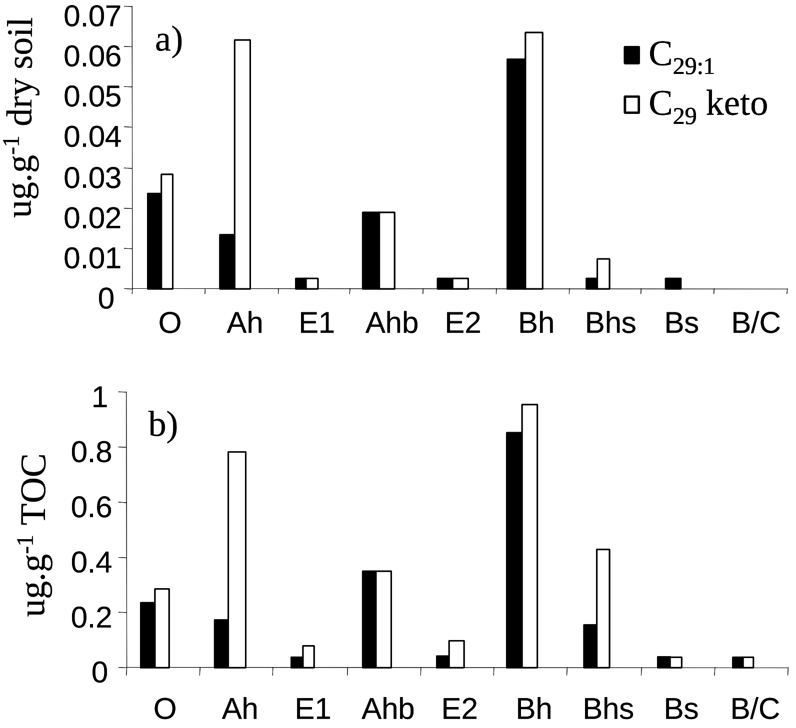

Figure 3.5 Absolute concentration (ug.g^{-1} dry soil) (a), and relative concentration (ug.g^{-1} TOC) of b-sitosterol and C_{29} ketosteroid (b), per horizon.

The *n*-alkanoic acids identified can have various natural origins such as plant, fungal or bacterial origins (Amblès et al., 1994). Straight-chain compounds of fungal origin are similar to those of plant origin, but they only range from C_{10} to C_{24}, often characterized by the presence of unsaturated C_{16} and C_{18} acids (Weete, 1974; Ruess et al., 2002). Furthermore, long-chain ($>C_{20}$) *n*-alkanoic acids in this top-soil layer may originate from the hydrolysis of wax esters (van Bergen et al., 1998) or the oxidation of a variety of other compounds such as *n*-alkanes and *n*-alkanols (Mouçawi et al., 1981; Amblès et al., 1994a, b), processes which are enhanced by a low soil pH (van Bergen et al., 1998a) and the presence of hydrous iron oxides (Mouçawi et al., 1981). The strong even-over-odd dominance and chain-length distribution observed (Fig. 3.2a) imply that odd-chain compounds such as *n*-alkanes are unlikely sources.Based on the same criteria, and the dominance of both C_{22} and C_{24} acids, a contribution from the oxidation of *n*-alkanols is more likely.

The *n*-alkanes ranging from C_{20} to C_{27}, with a carbon preference index close to unity, are more probably of microbial origin (Amblès et al., 1989), although they have also been identified in association with *Ericaeae* rootlets (van Smeerdijk & Boon, 1987). A microbial input is also obvious from the *iso*- and *anteiso*- C_{15} fatty acids (Boon et al., 1977), and possibly by the detection of C_{18} alkenoic acid (Parlanti et al., 1994).

The steroids and triterpenoids identified have frequently been detected in soil and leaf or litter extracts (Killops & Frewin, 1994; van Bergen et al., 1997; Bull et al., 1998). However, it is important to note that lupeol, taraxerol, α-amyrin and the triterpenoic acid are more associated with aerial vegetation (Killops & Frewin, 1994). On the other hand, all steroid compounds identified, together with friedoolean-3-one and T9, have been recognized as significant constituents of heather roots (van Smeerdijk & Boon, 1987; Nierop et al., 2001), although heather leaves and flowers do not contain these compounds in significant amounts (Nierop et al., 2001). In addition to their vascular plant origin, C_{29} steroids are known to be derived from fungi (Weete, 1976; Grandmougin-Ferjani et al., 1999). Keto-steroids are oxidation products of sterols (Goad, 1991) and have been identified in association with heather rootlets (van Smeerdijk & Boon, 1987). The C_{18} isoprenoid methylketone is a degradation product of phytol, which has been found before in total lipid extracts from aerial vegetation (van Bergen et al., 1997; Bull et al., 2000a).

Composition of the TLE from the Ah horizon. Both the relative (Figs 3.2b, 3.5b) and absolute (Fig. 3.5a) amounts of β-sitosterol decrease from the litter to the Ah horizon. A decrease in sitosterol, as well as other steroids and terpenoids associated with aerial parts of vegetation (Killops & Frewin, 1994), was seen when lipids from forest soil litter layers or leaf extracts were compared with those from the underlying A horizon (Jambu et al., 1993; Amblès et al., 1994a, b; van Bergen et al., 1997; Bull et al., 2000a). It is suggested that these compounds are easily mineralized in the soil (van Bergen et al., 1997) or that their decrease in soluble lipid fractions can be caused by their condensation into more stable, insoluble moieties (Gobé et al., 2000). In addition, they may be chemically altered to form modified steroids and triterpenoids (van Bergen et al., 1997).

The significant increase in concentration of the C_{29} ketosteroid (Fig. 3.5) and taraxerone from the O to the Ah horizon probably results from oxidation (van Bergen et al., 1997). In addition, taraxerone (T3), friedoolean-3-one (T8) and T9 triterpenoids have also been found in heather rootlets (van Smeerdijk & Boon, 1987; Nierop et al., 2001) reflecting a direct input. In general, more unsaturated compounds are more susceptible to (microbial) degradation. The total decrease of stigmasterol ($C_{29:2}$) compared with that of β-sitosterol ($C_{29:1}$) is therefore related to the degree of unsaturation. In addition, a source of β-sitosterol has been found in ester-linked lipids obtained from soil samples taken from a depth of 15-50 cm (Chapter 6).

Another group of lipids that decreases rapidly from the O to the Ah horizon are the *n*-alkanes (Figs 3.6c, 3.7c), as observed in acid soils (Marseille et al., 1999). This decrease in *n*-alkane concentration, is accompanied by an increase in the concentration of methylketones (Figs 3.2b, 3.6d & 3.7d), which are not known as primary plant products. Considering that *n*-alkanes are a likely substrate for *in situ* microbial β-oxidation based on similarities found in the distribution of ketones and *n*-alkanes (Amblès et al., 1993), and that such similarities are found in the podzol profile (Fig. 3.2a), it is assumed that methylketones in the Ah horizon are derived from such oxidation. The relatively minor contribution of methylketones to the total amount of lipids in the litter layer (Fig. 3.6d) is expected because methylketones are usually only detected in such layers in trace amounts (Amblès et al., 1993). Furthermore, because of their biodegradation, the amount of methylketones determined in soil is much lower than the amounts produced from *n*-alkanes (Amblès et al., 1993).

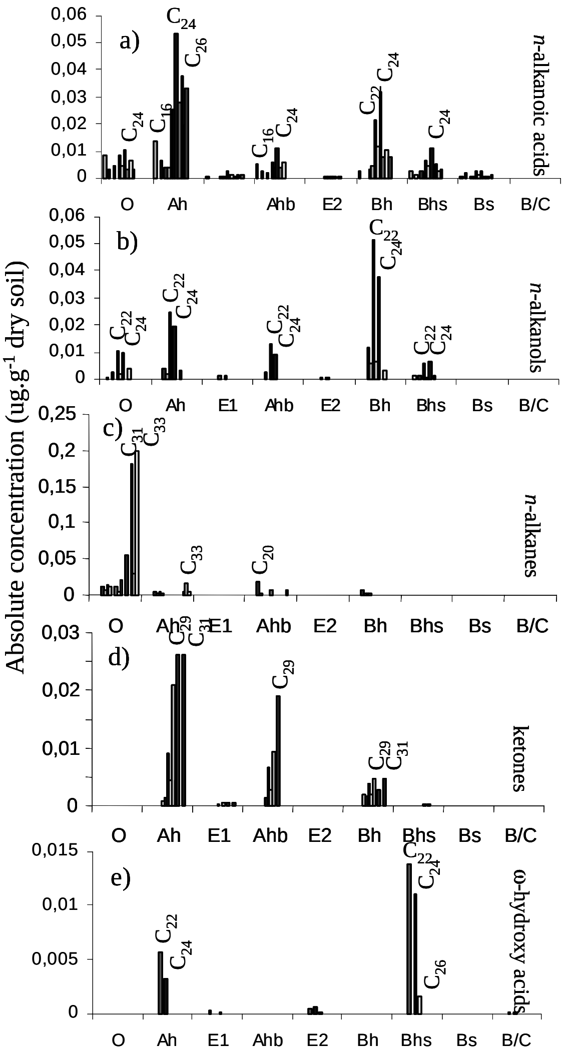

Figure 3.6 Absolute concentration (ug.g^{-1} dry soil) of C$_{16}$-C$_{33}$ lipid compounds per horizon: (a) *n*-alkanoic acids, (b) *n*-alkanols, (c) *n*-alkanes, (d) ketones and (e) w-hydroxy acids.

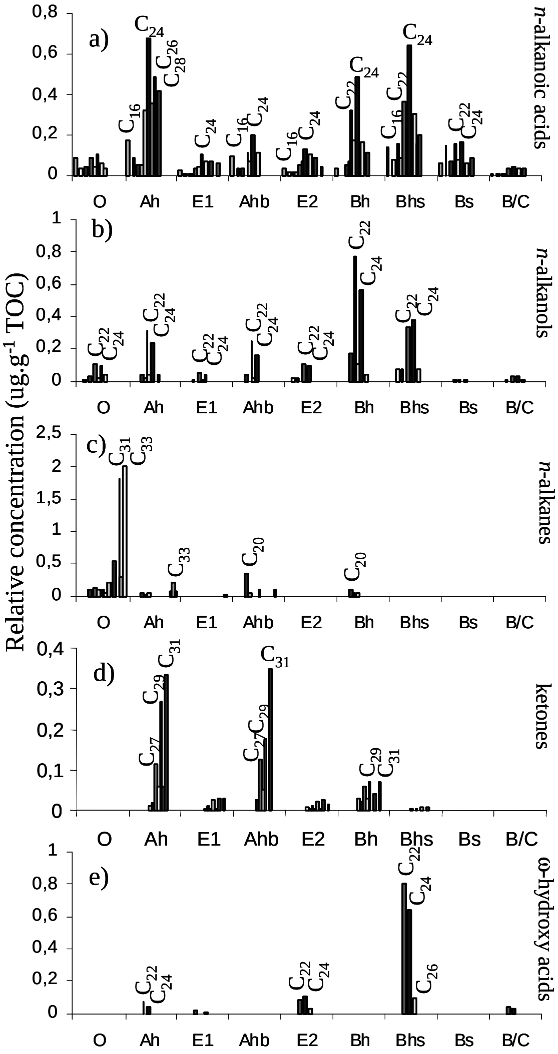

Figure 3.7 Relative concentration (ug.g^{-1} TOC) of C$_{16}$-C$_{33}$ lipid compounds per horizon: (a) *n*-alkanoic acids, (b) *n*-alkanols, (c) *n*-alkanes, (d) ketones and (e) w-hydroxy acids.

The increase in absolute (Fig. 3.6a) and relative (Fig. 3.7a) concentrations of long-chain (>C$_{20}$) *n*-alkanoic acids (cf. Amblès et al., 1994b), together with the increase in the total amount of lipids (Fig. 3.3), indicates that these compounds are produced in the Ah horizon. Hydrolysis of wax esters (van Bergen et al., 1998) and an input from roots (Bull et al., 2000b) are the most likely processes involved in this production. The presence of hexadecanoic acid and C$_{18}$ *n*-alkenoic acid could be related to an input from mosses (Nierop et al., 2001), but has also been observed in root- and rhizosphere-derived total lipid extracts (Bull et al., 2000a, b). Moreover, (<C$_{20}$) acids, including C$_{14}$, C$_{16}$, C$_{18:1}$ and C$_{18}$ fatty acids, were found to be the dominant acids leached from the litter and Ah horizon during an experimental study of podzol soil lipids (Amblès et al., 1998).

The absolute (Fig. 3.6b) and relative (Fig. 3.7b) increase in the concentration of *n*-alkanols is most likely to be the result of both an input from heather roots, considering their relatively large abundance in heather root pyrolysates (van Smeerdijk & Boon, 1987; Nierop, 1998; Nierop et al., 2001) and from the hydrolysis of wax esters (Jambu et al., 1993). The latter process is enhanced by the low soil pH (Wolfe et al., 1989). The detection of C_{22} and C_{24} ω-hydroxy acids (Figs 3.6e and 3.7e) in this horizon reveals an input derived possibly both from aerial vegetation as well as from roots (Bull et al., 2000b).

Composition of the TLE from the E1 horizon. As mentioned, the composition of the total lipid extract from the E1 horizon (Fig. 3.2c) resembles to a great extent that from the overlying Ah horizon (Fig. 3.2b). Taking into account the relatively high TOC content (2%) for a leached horizon (Fig. 3.3), this strongly suggests that the process of formation of this E1 horizon is not yet complete, although both absolute and relative amounts of lipids are very small (Figs 3.6, 3.7). Normalized to the TOC% there is, apart from the decrease in *n*-alkanoic acids and *n*-alkanols, a strong decrease in the concentration of ketones (Fig. 3.7d) caused by a relative rapid biodegradation as intermediates in the *n*-alkane degradation pathway (Amblès et al., 1993).

Composition of the TLE from the Ahb horizon. The Ahb extract again shows a signal dominated by steroids and triterpenoids (Fig. 3.2d), with abundant contributions from β-sitosterol (see also Fig. 3.5), C_{29} ketosteroid, and friedoolean-2-ene (T1), T2, friedoolean-3-one (T8) and T9 triterpenoids. All these compounds have been identified in heather rootlets (van Smeerdijk & Boon, 1987). Relative to the TOC%, β-sitosterol (Fig. 3.5b), *n*-alkanols (Fig. 3.7b), and ketones (Fig. 3.7d) show a significant increase. These compounds are enriched in the buried A-horizon either as remnants of the buried vegetation, or as the result of an input by roots (Nierop et al., 2001). A contribution from above-ground litter lipids through transport to Ah horizons in sandy soils, on the other hand, has been found to be negligible. Possible evidence for a contribution from previous vegetation could be the increase in both absolute (Fig. 3.6d) and relative (Fig. 3.7d) methylketone concentrations most probably derived from the oxidation of *n*-alkanes (Amblès et al., 1993; van Bergen et al., 1997).

Composition of the TLE from the E2 horizon. C_{22} and C_{24} ω-hydroxy acids, in combination with long-chain ($>C_{20}$) acids and C_{22} and C_{24} *n*-alkanols, are the lipids that remain in the E2 horizon. In addition to these root-derived compounds (Nierop, 1998; Chapter 2), C_8 and C_9 ω-hydroxy acids were detected, probably as a result of the oxidation (Regert et al., 1998) of unsaturated suberin building blocks (Nierop et al., 2003). It should be noted that the distributions of both *n*-alkanoic acids and ω-hydroxy acids resemble those observed in samples of rhizosphere and mineral horizons (Bull et al., 2000a, 2000b).

Methylketones also remain in this horizon as oxidation products of alkanes (Amblès et al., 1993). Due to this intensive leaching or degradation, absolute amounts of all compounds identified in this horizon are very small (Fig. 3.6). Normalized to TOC%, however, it is shown that the decrease in the contribution of lipids to the TOC% is mainly caused by a decrease in steroids (Fig. 3.5b), triterpenoids and ketones (Fig. 3.7d). Considering the labile nature of these compounds (van Bergen et al., 1997) and their hydrophobicity, (microbial) degradation of these compounds seems the most likely explanation for this decrease. The contributions of *n*-alkanoic acids (Fig. 3.7a), *n*-alkanols (Fig. 3.7b) and ω-hydroxy acids (Fig. 3.7e) on the contrary remain relatively similar, or even increase. These compounds therefore reflect a root-derived input that is hard to degrade (and/or leach). No evidence of accumulation of lipids derived from the soil surface, such as long-chain ($<C_{20}$) *n*-alkanes, has been observed in the E2 horizon. Thus, the E2 signal could be considered to be derived of an input by roots.

Composition of the TLE from the Bh horizon. Just as observed for the O and Ahb horizon, the organic matter rich Bh horizon is characterized by β-sitosterol and C_{29} ketosteroid, a series of triterpenoids, and C_{22} and C_{24} *n*-alkanols (Fig. 3.2f). Comparison with the large TOC content (Fig. 3.3) and both absolute and relative amounts of lipid compounds identified, clearly indicates that organic matter, including lipids, accumulate in this horizon. Podzol B-horizons may contain more root-derived than illuviated organic matter (van Breemen & Buurman, 2002).

The contribution of litter-derived organic matter to the sub-soil in sandy soils was found to be negligible (Nierop et al., 2001). Moreover, roots were found to develop preferentially in podzol Bh horizons (de Coninck, 1980). Thus, the significant increase in the contribution of steroids (Fig. 3.5), triterpenoids and C_{22} and C_{24} *n*-alkanols (Figs 3.6b, 3.7b), all known to be derived from heather roots (van Smeerdijk & Boon, 1987; Nierop & Buurman, 1999; Nierop et al., 2001), can be interpreted as resulting from the accumulation of root-derived lipids produced *in situ*. In addition to this root-derived signal, a bacterial contribution is again reflected in the presence of short-chain alkanes and methylketones. It should be noted that, similar to the observations by Nierop & Buurman, (1999) with respect to the insoluble organic matter fraction, no evidence was found for a contribution than lipid compounds transported from the top of the profile.

Composition of the TLE from the transition from the Bh to the Bs horizon. In the sample from the transition layer between the Bh and Bs horizons (Bhs; Fig. 3.2g), the total lipid signal again changes dramatically into a signal dominated by ω-hydroxy acids (Figs 3.6e, 3.7e) and *n*-alkanoic acids, as observed for the E2 horizon (Fig. 3.2e). The sharp decrease in TOC content (Fig. 3.3) indicates that no accumulation of organic matter took place in this horizon, again strongly suggesting that the signal observed is characteristic for a degraded root-derived input. The microbial signal in this horizon comprises short-chain alkanes and methylketones. It has been suggested that complexed OM is degraded in this horizon to yield Al and Fe which then precipitates in the underlying Bs horizon (Lundström et al., 1995). We suggest that this degradation may also effect the biopolyester suberin, resulting in the release of relatively large amounts of long-chain ($>C_{20}$) ω-hydroxy acids (Figs 3.5e, 3.6e) characteristic of an input by roots to the lipid signal in soils (Bull et al., 2000b; Chapter 2).

Composition of the TLEs from the Bs and B/C horizons. Both the TOC contents (Fig. 3.3) and absolute amounts of lipids (Figs 3.3, 3.6) are very small in the Bs horizon (Fig. 3.2h). The signal observed most probably reflects the very small remnant of lipids or a minor contribution from lipids that penetrate into this hard layer from the overlying Bhs horizon (Fig. 3.2g). Again, most root-derived steroids and triterpenoids have been degraded.

The total lipid signal from the B/C horizon (Fig. 3.2i) again resembles that of the other horizons with a small TOC content, i.e. the E2, Bhs and Bs horizons (Fig. 3.3). The lipid signal consists of compounds derived from degraded remnants of old roots that were present in this horizon before it was cut off from the rest of the profile by the formation of the hard, cemented Bs horizon (de Coninck, 1980).

Implications of lipid data for the podzolization process. The lipids in the Bh horizon are mainly derived from a fresh input by roots that accumulate in this horizon. The limited penetration of surface-vegetation derived lipids into the profile indicates that the influence of vertical transport of lipids as compared to (bacterial) degradation is negligible. The detection of lipid degradation products such as ketones, and bacterial markers such as short-chain alkanes and fatty acids throughout the profile, further indicates that (bacterial) degradation of lipids is an important process in this podzol profile. Considering that both steroids and triterpenoids have been shown to degrade rapidly within soils (van Bergen et al., 1997), this is a likely explanation for their small concentration in podzol E horizons. After degradation of most of these steroids and triterpenoids, a signal derived from bacteria and root-derived long-chain fatty acids and ω-hydroxy acids remains in the E horizon. These latter compounds reflect *in situ* lipid input produced by the degradation of remnants of root suberins and remain after degradation of the rest of the root-derived lipids.

3.5 Conclusions

The composition of total lipid extracts from 9 characteristic horizons in a podzol from the Veluwe area (The Netherlands) has been analysed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). In addition, soil pH (H_2O), ammonium oxalate- and sodium pyrophosphate extractable aluminium and iron have been determined. From these analyses it is concluded that:

1) Lipids accumulate in horizons with high organic matter contents, but there is no increase relative to other soil organic matter.
2) An *n*-alkane, steroid and triterpenoid signal related to the aerial parts, i.e. leaves and flowers, of *Calluna* is observed only in the O horizon. This signal is quickly lost in the underlying Ah horizon due to (bacterial) oxidation.
3) All total lipid extracts, except that from the O-horizon, are dominated by root-derived lipids.
4) In sub-soil horizons with high organic matter contents, i.e. the Ahb and Bh horizons, root-derived friedoolean and steroid compounds dominate the total lipid signal.
5) C_{22} and C_{24} ω-hydroxy acids, long-chain ($>C_{20}$) *n*-alkanoic acids with a strong even-over-odd dominance and C_{22} and C_{24} *n*-alkanols, characteristic of an input from roots, dominate the horizons with low contents of organic matter, i.e. the E2, Bhs, Bs and B/C horizons. Steroid- and root-derived triterpenoids with a friedoolean structure, once present in these horizons, have been degraded.
6) Based on TOC content and molecular lipid composition, the formation of an E1 horizon has started, but is not yet complete.
7) In the Ahb horizon, a contribution from buried vegetation to the total lipid signal is still present, although degradation and an input by roots have significantly altered the original signal.
8) Overall, lipid data indicate that degradation of lipids (mainly oxidation) is an important process that should be taken into account, in addition to leaching, when describing the so called “podzolization process” in soils.

Acknowledgements. This study was supported by the Institute for Paleoenvironment and Paleoclimate Utrecht (IPPU). M. van Alphen and T. Zalm are thanked for their help during GC and GC/MS analyses. Drs. J. Kool is thanked for supplying additional data and graphs. Two reviewers are thanked for their useful comments and suggestions. Both the associate editor Dr. L. Peterson and the editor-in-chief Dr. P. Loveland of the European Journal of Soil Science are sincerely thanked for their help with the final editing and formatting, as well as useful comments and suggestions on the contents of the paper.

Chapter 4

Changes in the molecular composition of ester-bound aliphatics with depth in an acid andic forest soil

Changes in the molecular composition of ester-bound aliphatics with depth in an acid andic forest soil are studied. Thermally assisted hydrolysis and methylation using tetramethylammonium hydroxide in combination with gas chromatography/mass spectrometry revealed a cutin over suberin dominated input in the top 5 cm. By contrast, sub-soil samples (20-50 cm) are dominated by a suberin-derived input. C_{22}, C_{24} (and C_{26}) ω-hydroxy acids (and C_{18}-C_{28} α, ω-dioic acids) are found to decrease relatively strongly with depth compared with other suberin building-blocks such as C_{16} ω-hydroxy acid. Their decrease with depth in the ester-bound fraction is 'reversely' linked with a strong increase in the free extractable lipid fraction which would justify their use as main indicators of a root–derived input to the free extractable lipid fraction. In addition, their relatively strong decrease in the ester-bound fraction suggests that these compounds are more easily released from the suberin structure in soils than other ester-bound aliphatic building-blocks.

4.1 Introduction

Three main sources of plant-derived aliphatic compounds have been distinguished in soils: (a) free (extractable) lipids, (b) the biopolyesters cutin and suberin and (c) non-hydrolyzable biopolymers, such as cutan and suberan (Nip et al., 1985; Tegelaar et al., 1989a, 1995; Augris et al., 1998; Nierop; 1998). In an earlier study, it was shown that ester-linked lipids account for approximately 2.5 wt% of the TOC in the acid andic forest soil studied (Chapter 6). Nierop et al. (2003) found that in acid sandy soils, ester-linked lipids may even account for up to 12.6 wt% of the TOC. Moreover, it appeared that the contribution of ester-linked aliphatics increases with decreasing soil pH (Nierop et al., 2003).

Through (microbial) hydrolysis, ester-linked aliphatics are released after which they are quickly degraded or become part of the free extractable aliphatic fraction in soils. To date, mainly "refractory" ω-hydroxy acids (and α, ω-dicarboxylic acids) observed in the free extractable lipid fraction have been ascribed to suberin as a predominant source (Bull et al., 2000b).

ω-Hydroxy acids have been suggested to be more easily released from the biopolyester structure than other ester-linked aliphatic building-blocks upon base hydrolysis as well as decomposition in soil (Lopes et al., 2000; Nierop et al., 2003). They may form a more accessible part of the biopolyester (Nierop et al., 2003). In addition, di- and tri-hydroxy functionalised acids have been suggested to be less susceptible to hydrolysis as being part of the core of the biopolyester structure (Nierop et al., 2003).

During an earlier study, free extractable C_{22}, C_{24} and C_{26} ω-hydroxy acids were observed to increase significantly with depth (Fig. 4.1; Chapter 2). In this paper, changes in the molecular composition of ester-linked aliphatics with depth in the same acid andic forest soil profile are studied. Samples were Soxhlet extracted to remove the earlier studied (Chapter 2) free extractable lipids and the residues were

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subsequently analysed using thermally assisted hydrolysis and methylation (THM) using tetramethylammonium hydroxide (TMAH) in combination with gas chromatography/mass spectrometry (GC/MS).

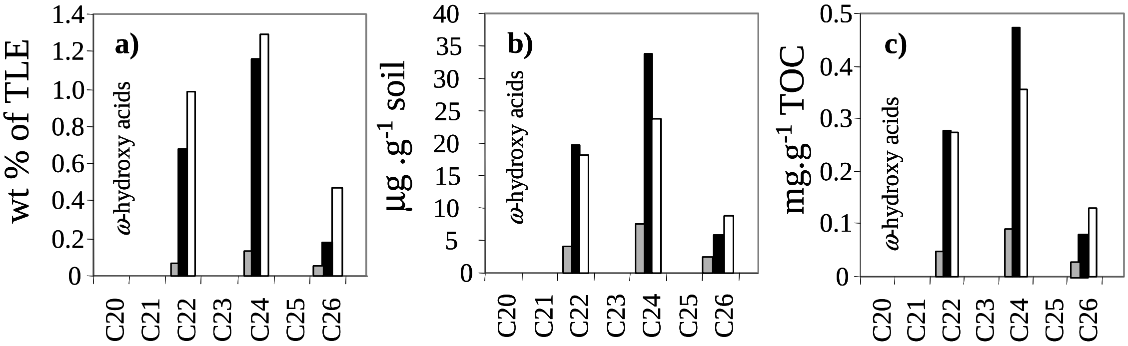

Figure 4.1. Diagrams reflecting changes with depth in the concentration of free ω-hydroxy acids; (a) relative concentration (as % of TLE), (b) absolute concentration (as mg.g^{-1} soil) and (c) normalized to TOC% (mg.g^{-1} TOC). Key: ▨: 0-5 cm, ■: 10-20 cm, □: 20-30 cm. (from Naafs et al., in press).

4.2. Materials and methods

4.2.1 Soil profile

Madeira Island (Portugal) is located in a fully (Atlantic) oceanic domain between 32°38’ and 32°52’N and 16°39’ and 17°16’ W, approximately 600 km from the African coast, at the same latitude as Casablanca (Morocco). Soil samples were taken from andic profiles located on this island that have been classified as either being Umbric or Haplic Andisols (FAO, 1998) depending on their color (Madeira et al., 1994).

The profile studied is situated on a hill (slope 30°W) near the village of Poiso (altitude 1175 m). The vegetation consisted mainly of grasses (55%), ferns (20%), deciduous trees, i.e., oak and birch (20%), and some mosses (5%). Tree- and some grass roots were found to a depth of about 80 cm, but most of the intensive “root-activity” was found in the top 0-40 cm consisting mainly of grass fibrils. Three horizons were distinguished; a thin O-horizon (0-2 cm), an A-horizon (2-73 cm) and an E/B horizon (73- 90 cm), the latter being formed by weathered basalt bedrock material. The deeper layer, i.e. C-horizon, was formed by a non-friable, compact layer of weathered basalt, the parent material for this Andisol (Madeira et al., 1994) and therefore not sampled. The O-horizon consisted mainly of litter and vegetation, i.e. grass, moss, twigs, leaves, etc. The A horizon (colour 4/6 5YR) was characterized by a non-friable structure, clay aggregates with a diameter up to 1 cm, and highly weathered basalt fragments up to a diameter of 10 cm. Many roots were found in this horizon, ranging from mainly very fine grass roots in the top 30 cm to coarser roots, up to a diameter of 2 cm, at greater depth, i.e. 30-70 cm.

4.2.2 Sample pre-treatment, total organic carbon measurement and solvent extraction

Soil samples were air dried, wrapped in aluminium foil and subsequently oven dried at 60 °C and sieved over a 2 mm and a 250 μm sieve to remove large roots and basalt fragments. The pH (H_2O) of the soil was measured in the supernatant suspension of a 1:2.5 sieved (<250 μm) sample to water ratio using a Scott Geräte pH meter CG 805. Total organic carbon contents (TOC%) of the dried samples were measured using a Fisons Instruments NA 1500 NCS analyzer, with a cycle time of 180 s, a source temperature of 190 °C and an oxygen flow of ca. 30 l/min. Approximately 15-25 gram of the sieved (<250 μm) samples was Soxhlet extracted to remove extractable lipids using dichloromethane/methanol (DCM/MeOH) (9:1v/v) for 24 h (for details and analysis of the free extractable lipid fraction see Chapter 2). The residue was air dried and used for further analyses.

4.2.3 Thermally assisted hydrolysis and methylation (THM)

Thermally assisted hydrolysis and methylation (THM) analyses were performed by adding a droplet of a 25% solution of tetramethylammonium hydroxide (TMAH) in water to a Soxhlet extracted residue that was pressed onto a ferromagnetic wire with a Curie temperature of 600 °C. The 'wet' sample was dried using a 100 W halogen lamp and subsequently inserted into a Horizon Instruments Curie-Point pyrolyser. Samples were heated for 5 s at 600 °C. The pyrolysis unit was connected to a ThermoQuest Trace GC 2000 gas chromatograph. Products were separated by a fused silica column (J & W 30 m x 0.32 mm, film thickness 0.25 μm, i.d.) coated with DB-5. Helium was used as carrier gas. The GC oven temperature was programmed from 40 °C (1 min) to 320 °C (isothermal for 15 min) at a rate of 7 °C min^{-1}. The GC column was coupled to a Finnigan Trace MS mass spectrometer operating at 70 eV, scanning the range *m/z* 60-600 with a cycling time of 1 s. Identification of the compounds was carried out using their mass spectral data (Table 4.1) and by interpretation of the spectra and the GC retention times.

Table 4.1 Characteristic mass fragments of methylated compounds identified in THM total ion current.

Compound	Characteristic mass fragments (*m/z*)
ω-Hydroxy alkanoic acids	74, 87, 98, 111, 143, $[M-64]^+$, $[M-47]^+$, $[M-32]^+$, $[M-15]^+$
ω-Hydroxy alkenoic acids	55, 67, 81, 95, 109, 121, 137, $[M-64]^+$, $[M-47]^+$, $[M-32]^+$
(9, ω)-Dihydroxy alkanoic acid	71, 109, 173, 201, $[M-32]^+$, $[M-15]^+$
(10, ω)-Dihydroxy alkanoic acid	71, 109, 173, 215, $[M-32]^+$, $[M-15]^+$
(9, 10, ω)-Trihydroxy alkanoic acid	71, 81, 187, 201
Unknown (sugar?)	71, 75, 101, 111, 187, 185, 275, 307

4.3. Results and discussion

Total organic contents decrease from 7.9 wt % in the top- soil sample (0-5 cm) to 5.6 wt % in the sample taken from a depth of 40-50 cm. Soil pH (H_2O) values are all acidic and vary only little between 4.2 and 4.0. All samples analysed contain a series of even numbered saturated ω-hydroxy acids ranging from C_{16} to C_{28}, accompanied by C_{18} ω-hydroxyalkenoic acid. In addition, C_{16} 10,16-dihydroxy acid, C_{18} 9,10,18-trihydroxy acid and series of C_{16} to C_{24} α, ω-alkanedioic acids and C_{14} to C_{28} *n*-alkanoic acids have been identified, both including unsaturated compounds (see Table 4.1 for characteristic mass fragments). The origin of ester-linked compounds identified in the sub-soil, i.e 15-50 cm, is discussed in detail in Chapter 6.

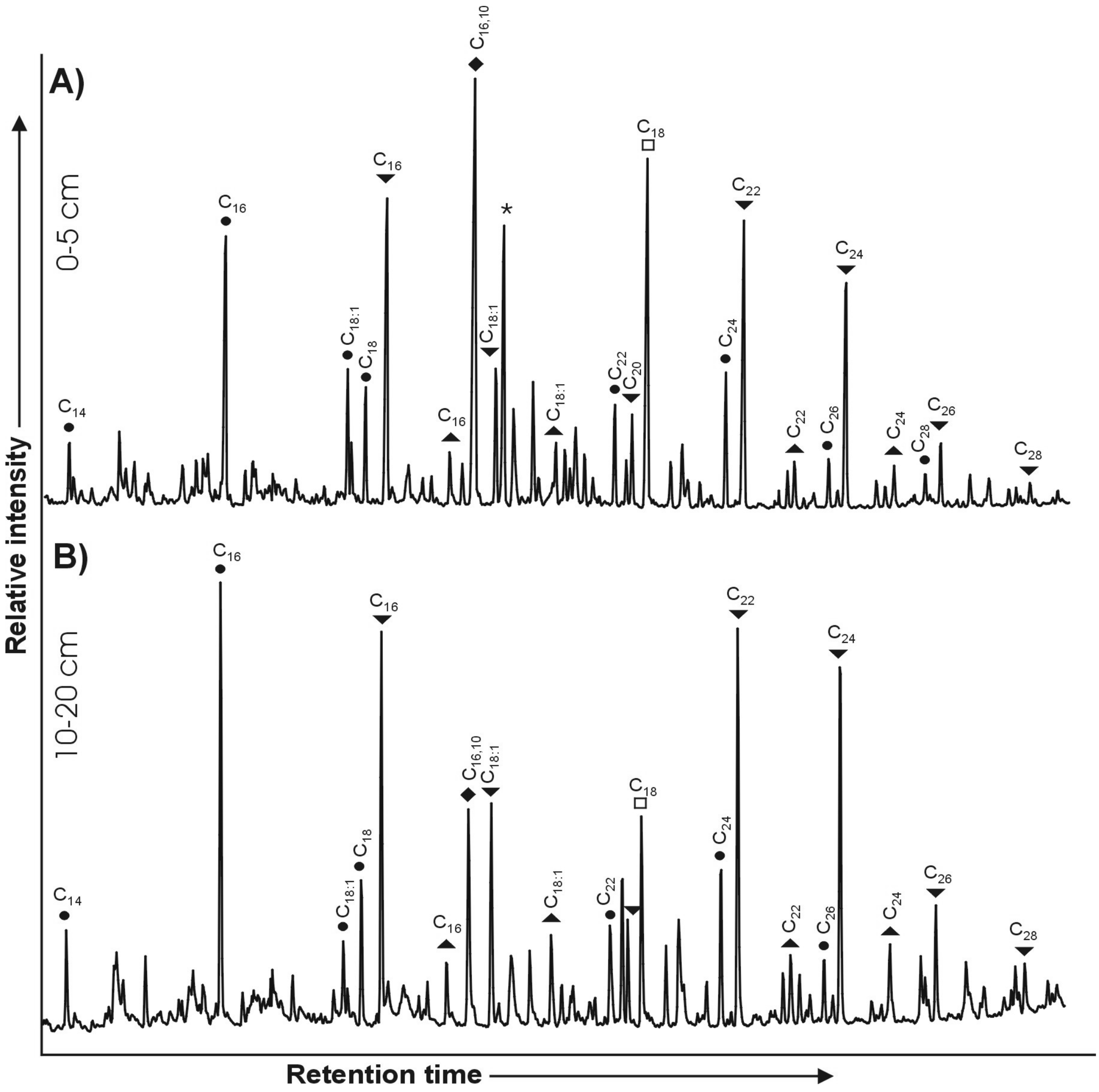

Figure 4.2 Partial total ion current of THM products of residues after Soxhlet extraction at depths of: (a) 0-5 cm, (b) 10-20 cm, c) 20-30 cm and (d) 40-50 cm. Key: ● : *n*-alkanoic acids, ▼ : ω-hydroxy acids, ▲ : Diacids, ◆: dihydroxy acids, □: trihydroxy acids, * : unknown. C_x above the peaks refers to the total number of carbon atoms. Number after the comma refers to the position of the hydroxy group. Number after colon refers to the total number of double bonds.

4.3.1 Cutin *vs*. suberin input

Cutin is present in cuticles covering all aerial parts of higher plants, while suberin forms both protective and wound-healing layers in barks, woody stems and underground parts, i.e. roots (Kolattukudy, 1980a, 1984; Walton, 1990). Comparative studies revealed that cutin and the aliphatic domain of suberin are closely related chemically, since both consist mainly of hydroxy and epoxy-substituted alkanoic acids (Kolattukudy, 1980a; Holloway, 1982, 1983; Walton, 1990; Matzke and Riederer, 1991; Riederer et al., 1993).

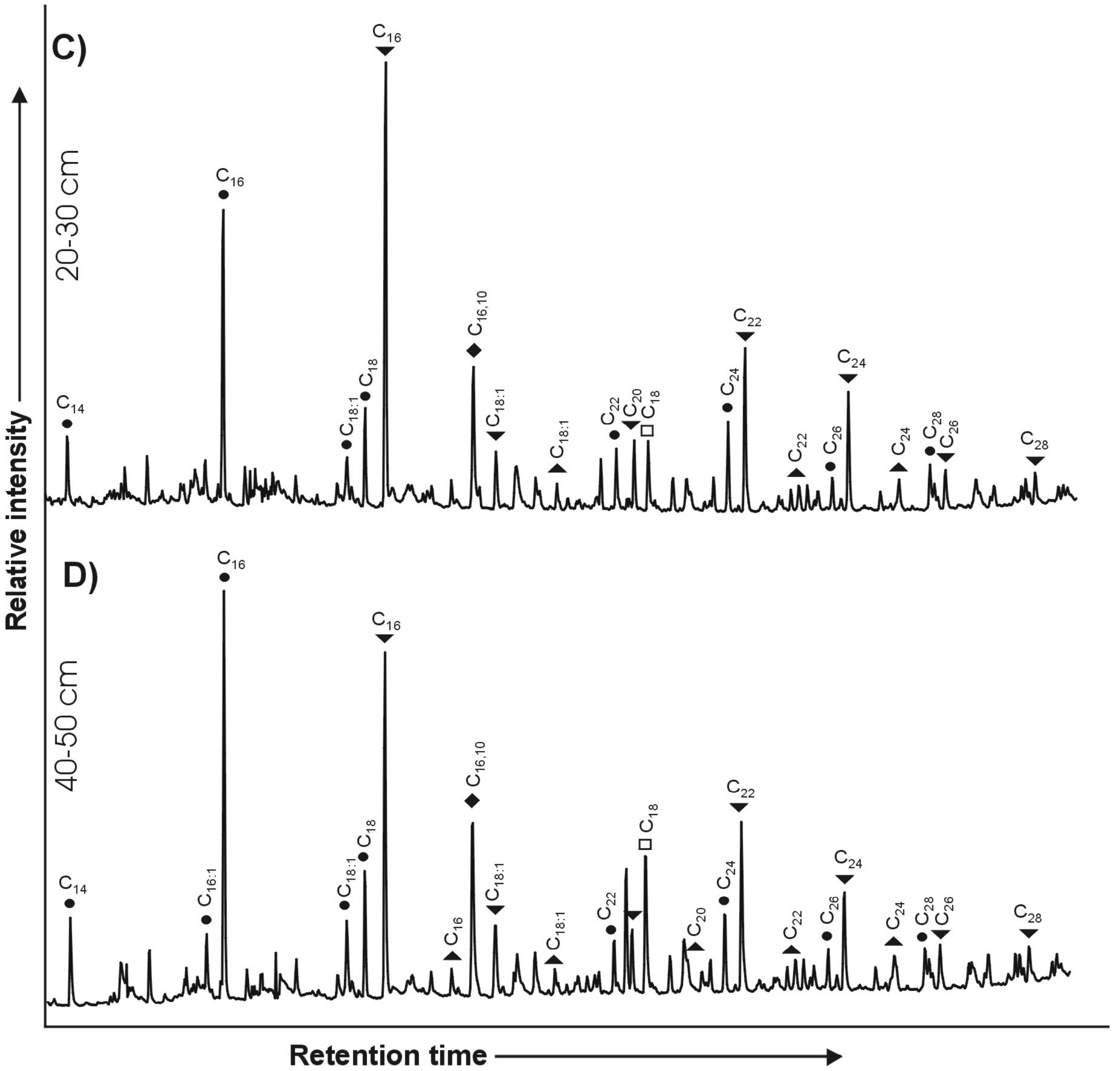

Figure 4.2 c,d.

However, there are a number of differences in their aliphatic monomeric and tertiary structure (Tegelaar et al., 1995). Dihydroxy-hexadecanoic acids (i.e. C_{16} family, Kolattukudy, 1980a) and 9,10-epoxy-18-hydroxy acids (i.e. C_{18} family, Kolattukudy, 1980a) have been identified as major components of plant cutins (Kolattukudy, 1980a; Holloway, 1982; Ryser and Holloway, 1985; Matzke and Riederer, 1991). The aliphatic domain (Bernards and Lewis, 1998) of most suberins on the contrary is dominated by α, ω-dioic acids and ω-hydroxy acids (Kolattukudy, 1980a; Matzke and Riederer, 1991; Riederer et al., 1993) with only small amounts of the mid-chain functionalised hydroxy-alkanoic acids. Taking these differences into account, a cutin over suberin dominated derived input is clearly reflected in the top-sample (0-5 cm; Fig. 4.2a) by the dominance of C_{16} 9,16 and C_{16} 10, 16-dihydroxy acid and C_{18} 9,10,18-trihydroxy acid. The significant cutin over suberin derived input decreases, however, with depth (Figs. 4.2b-d). The sub-soil samples are dominated by a suberin-related, i.e. root-derived, input reflected by the presence of long-chain ($>C_{16}$) ω-hydroxy acids and α, ω-alkanedioic acids.

In addition to a change in the cutin/suberin ratio with depth, a change in vegetation input with depth has been observed to cause similar changes in the composition of ester-linked aliphatics with depth (Nierop, 2001). An increase in the input by grass roots over that of tree roots could therefore also cause a relative dominance of C_{16} ω-hydroxy acid over C_{22} and C_{24} hydroxy acids (Nierop, 2001). However, considering the dominance of grasses over trees at the sample site, a diagenetic change in the cutin/suberin ratio is a more likely explanation for the changes observed.

4.3.2 The molecular composition of root-derived ester-linked lipids *vs* depth

Root material (i.e. suberin) has been considered as a predominant source of aliphatic ω-hydroxy acids in the free extractable lipid fraction in soils (Bull et al., 2000b). With depth, however, a strong relative decrease in concentration of ester-linked C_{22}, C_{24} (and C_{26}) ω-hydroxy acids is one of the most striking features (Figs. 2.2b-d). Directly associated with this decrease, the ω-hydroxy acids show an increase with depth in the free extractable lipid fraction (Fig. 4.1). Assuming an increase in the hydrolysis of biopolyesters with depth, these data indicate a 'reverse' link between the occurrence of free extractable- and ester-linked C_{22}, C_{24} (and C_{26}) ω-hydroxy acids. Moreover, such a direct link would justify their use as main indicators of a root–derived input to SOM in the free extractable lipid fraction (Bull et al., 2000b; Chapter 2).

Another rather significant feature is the decrease in relative concentration of the ω-hydroxyoctadecenoic acid (ω-$C_{18:1}$) . Nierop *et al.* (2003) showed that this compound is a major building block of oak root suberins. In soils, this compound is quickly degraded (Nierop et al., 2003) as revealed by its absence contrary to the C_{22}, C_{24} (and C_{26}) in the free lipid fraction. The only compound observed in the free lipid fraction that may reflect a contribution from hydrolysed $C_{18:1}$ ω-hydroxy acids is C_9 α, ω-alkanedioic acid (Chapter 2). Within the andic profile, the ester-linked ω-$C_{18:1}$ acid is more quickly hydrolysed than most other suberin building blocks as revealed by its relatively strong decrease with depth (Figs. 2.2b-d). Together with the relatively strong decrease in the ester-linked fraction of C_{22}, C_{24} ω-hydroxy acids and, to a lesser extent, C_{18}-C_{28} α, ω-dioic acids, this again suggests that these long-chain (>C_{16}) ω-hydroxy and α, ω-dioic acids are more easily released from the biopolyester structure than other ester-linked aliphatic building-blocks (Lopes et al., 2000; Nierop et al., 2003), including C_{16} ω-hydroxy acid (Fig. 4.2). They may form a more accessible part of the biopolyester, while di- and tri-hydroxy functionalised acids, as well as C_{16} ω-hydroxy acid (Ray and Stark, 1998), are less susceptible to hydrolysis as being part of the core of the biopolyester structure as suggested earlier (Nierop et al., 2003).

In general, the more depth-specific THM data obtained (Fig. 4.2) confirm the base-hydrolysis data obtained during an earlier study on a mixed sub-soil sample, i.e 15-50 cm (Chapter 6).

Acknowledgements. This study is N2 contribution no. 3 and was supported by the Institute for Paleoenvironment and Paleoclimate Utrecht (IPPU) and the Centre for Geo-ecological Research (ICG).

Chapter 5

Effects of pH Adjustments after Base Hydrolysis: Implications for Understanding Organic Matter in Soils

The composition of soil organic matter (SOM) released during a treatment with base, i.e. a 1M KOH in 96% methanol solution, from an acidic forest soil was studied using gas chromatography and gas chromatography-mass spectrometry. To provide detailed insight into how pH adjustments of the extract obtained can affect the organic matter released, our approach involved: (I) extraction with DCM of the methanol/water extract obtained at pH values ranging from 13 to 1 after direct pH adjustments, (II) sequential acidification of the methanol/water extract obtained and extraction with DCM at pH values ranging from 11 to 1, and (III) acidification after base treatment of the reaction mixture, i.e. soil and extract, to pH 3 followed by a second alkali hydrolysis of the residue and adjustment of the reaction mixture to pH 7. Results obtained after direct pH adjustment of the extract (experiment I) indicated that the relative amounts of identified compounds are, as expected, pH dependent, i.e. based on pK_a values and hydrophobicity. This phenomenon, which affected significantly the final results, may cause an under or over estimation of certain compound classes and a biased view on ester-related moieties in soils. Direct acidification of base extract in the presence of soil residue (experiment III) indicated that hydrophobic long-chain aliphatic moieties may become associated with the organic matrix left after base treatment, form new, insoluble, organic (macro) molecules or become associated with the inorganic matrix upon acidification of a base extract in the presence of a soil residue. An association with the inorganic soil matrix is discussed as another possible mode of occurrence of ester-related moieties in soil in addition to common known modes of occurrence of ester-related moieties in soils, e.g. the biopolyesters cutin and suberin. A possible contribution of ester-related acid and hydroxy substituted aliphatic monomers, in addition to aliphatic biopolymers, to the precipitate formed at the DCM-water/methanol interface is discussed as well.

5.1 Introduction

One of the major carbon pools playing an important role in the short-term global carbon cycle is soil organic matter (SOM) (Post et al., 1982). Globally, there is substantially more carbon in the top metre of soil than in the overlying vegetation or in the atmosphere (Oades, 1988; Jenkinson et al., 1992). The bulk of SOM is insoluble in water and composed of a complex mixture of dead and living biological material derived from above and belowground plants, microorganisms, fungi and animals (Schnitzer and Kahn, 1972; Stevenson, 1994). Products resulting from a wide variety of degradation and transformation processes of the above mentioned materials contribute as well. Most plant, animal and microbial organic material decomposes into CO_2 and recirculates within a few years into the atmosphere (Post et al., 1982). A minor part of the OM is assimilated into microbial tissues or converted into resistant SOM with a longer residence time (Paul and Clark, 1996; Coûteaux et al., 1998).

To determine which fraction of SOM comprises resistant material, several organic matter fractionation methods have been used. Common approaches include the classical humus fractionation (e.g. Davies et al., 1969; Grasset and Ambles, 1998;

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Augris et al., 1998), and selective chemical treatments (e.g. Schnitzer and Schuppli, 1989; Melakani et al., 1997). Selective chemical treatments usually start with a separation of low molecular weight (LMW) and high molecular weight (HMW) compounds using an organic solvent extraction. After drying, the HMW residue is treated with base to hydrolyse ester-bound moieties. To remove polysaccharides and amino acids, the residue obtained is subjected to acid hydrolysis and finally freeze-dried (e.g. Tegelaar et al., 1995). The fraction of SOM that is not removed during these extraction steps is widely considered to be the, chemically, resistant SOM fraction (e.g. Tegelaar et al., 1995; van Heemst et al., 1996; Kögel-Knabner et al., 1992; van Bergen et al., 1998b). Few investigators have looked at the influence of the methodology used on the results obtained (e.g. Piccolo, 1988; Kaiser and Zech, 1996; Poirier et al., 2000). However, the extent to which the methodology to fractionate SOM may influence our view of SOM composition, remains a crucial aspect.

In this paper we present the results of a qualitative study on the effect of pH adjustments during base treatment of an acidic forest soil, i.e. an Andosol, on the products released. The base released fraction is studied using gas chromatography (GC) and GC-mass spectrometry (GC-MS). The results obtained will also be discussed in terms of their implication for SOM studies.

5.2 Materials and methods

5.2.1 Soil profile

This study focussed on a forest soil from Madeira Island. Samples were taken from a hill near the village of Poiso (altitude 1175 m). The vegetation consisted mainly of grasses and ferns, minor contributions being dandelion, oak and beech. Tree- and some grass roots were found to a depth of about 80 cm, but most of the intensive root-activity was found in the top layer (0-40 cm), consisting mainly of grass fibrils. The dark red coloured A horizon used, i.e. 15-50 cm, was characterized by a non-friable structure, clay aggregates with a diameter up to 1 cm, and highly weathered basalt fragments up to a diameter of 10 cm. A non-friable, compact layer of weathered basalt formed the deeper layers. This weathered basalt is the parent material for this forest soil (see Madeira et al., 1994, for additional information).

5.2.2 Sample pre-treatment, total organic carbon, soil pH, oxalate and pyrophosphate extractions

Soil samples were taken, air dried and wrapped in aluminium foil to avoid organic contamination. Fresh soil samples were initially oven dried at 60°C, and sieved over a 2 mm and a 250 μm sieve to remove large roots and basalt fragments. Total organic carbon content (TOC%) of the dried soil sample was measured using a Fisions Instruments NA 1500 NCS analyser, with a cycle time of 180 s, a source temperature of 190°C and an oxygen flow of c.a. 30 l/min. The presence of active aluminium in this soil was assessed by measuring the pH increase, i.e. pH (NaF), of a 1M NaF solution upon a two-minute reaction in a 50:1 1 M NaF solution to sieved (<250 μm) sample ratio. The pH (H_2O) of the soil was measured in the supernatant suspension of a 1:2,5 sieved (<250 μm) sample to water ratio. To determine sodium pyrophosphate extractable Fe and Al, 0.5 g of sieved (<250 μm) sample was shaken overnight (16 h) with 50 ml 0.1 M Na pyrophosphate solution. Acid oxalate extractable Fe, Al and Si were determined through shaking (4 h in the dark) of 0.5 g of sieved (<250 μm) sample with 25 ml 0.2 M acid ammonium oxalate solution in oxalate. Concentrations in the extracts obtained were measured using a PerkinElmer Optima3000 ICP-OES.

5.2.3 Solvent extraction

Approximately 100 gram of sieved (<250 μm) sample was Soxhlet extracted using dichloromethane/methanol (DCM/MeOH) (9:1v/v) for 24 h. The extract was filtered using a Pasteur pipette packed with defatted wool, 0.5 cm Na_2SO_4 and 2 cm SiO_2 to remove water, soil particles and highly polar compounds. An aliquot of this filtered extract (total lipid extract; TLE) was derivatized using BSTFA (N, O-bis(trimethylsilyl)-trifluoro-acetamide, containing 1% trimethylchlorosilane; heated for 1 h at 70°C), dried using N_2, dissolved in hexane and analysed using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The residue was air-dried.

5.2.4 Base hydrolysis followed by HCl treatment of extract to different pH values

Dry Soxhlet residue (see above) was base treated for 1 h at 70°C using a 1M KOH in 96% methanol (MeOH) solution (Fig. 5.1). After cooling, extract and base hydrolysis residue were separated through centrifugation (3000 rpm, 3 min). The residue obtained was extracted with water (3x), MeOH/water (1:1 v/v; 1x), MeOH (2x) and dichloromethane (3x) at room temperature for 5 minutes using an ultrasonic probe. After each extraction step, the suspensions were centrifuged and the supernatants subsequently removed and collected in a separation funnel.

The pH of part of the collected extract (pH 13.7) was adjusted to pH 13 using a 12 M HCl solution (Fig. 5.1, part I). Dichloromethane (DCM) was added to this extract to obtain phase separation. The DCM layer was collected and phase separation with DCM was repeated. The collected and combined DCM phases were rotary evaporated to complete dryness. This extract was dissolved in DCM/isopropanol (2:1 v/v) and filtered using a Pasteur pipette packed with defatted wool, 0.5 cm Na_2SO_4 and 2 cm SiO_2, dried using N_2, derivatized using BSTFA and analysed using GC and GC-MS (Fig. 5.1, part I).

The same procedure was repeated for other parts of the extract, which were directly adjusted to pH values ranging from 11 to 1, with steps of 2 pH units (Fig. 5.1, part I).

5.2.5 Base hydrolysis followed by HCl treatment and step-wise extraction

Dry Soxhlet residue was treated with base and the extract and base hydrolysis residue were separated through centrifugation (see above). The pH of the collected extract (pH 13.7) was adjusted to pH 11 using a HCl solution (12 M) (Fig. 5.1, part II). DCM phase separation, filtration and preparation for GC and GC-MS were as described above. However, this time the pH of the MeOH/water phase in the separation funnel was subsequently adjusted to pH 9, 7, 5, 3 and 1. After each adjustment, the same procedure of phase separation, derivatisation and preparation for GC and GC-MS, was repeated as described above (Fig. 5.1, part II).

5.2.6 Base hydrolysis followed by HCl treatment in the presence of soil

Again, dry Soxhlet residue was base treated (see above). After cooling, the reaction mixture, i.e. soil residue and extract, was acidified to pH 3 using 2M hydrochloric acid in MeOH (Fig. 5.1, part III). After centrifugation (3 min 3000 rpm) the supernatant was removed. After extraction of the base hydrolysis residue with water and organic solvents (see above), DCM was added to the extract in a separation funnel to obtain phase separation. The DCM layer was collected and the phase separation with DCM was repeated. The combined DCM phase was rotary evaporated to complete dryness. This extract was filtered and prepared for GC and GC-MS as described above (Fig. 5.1, part III).

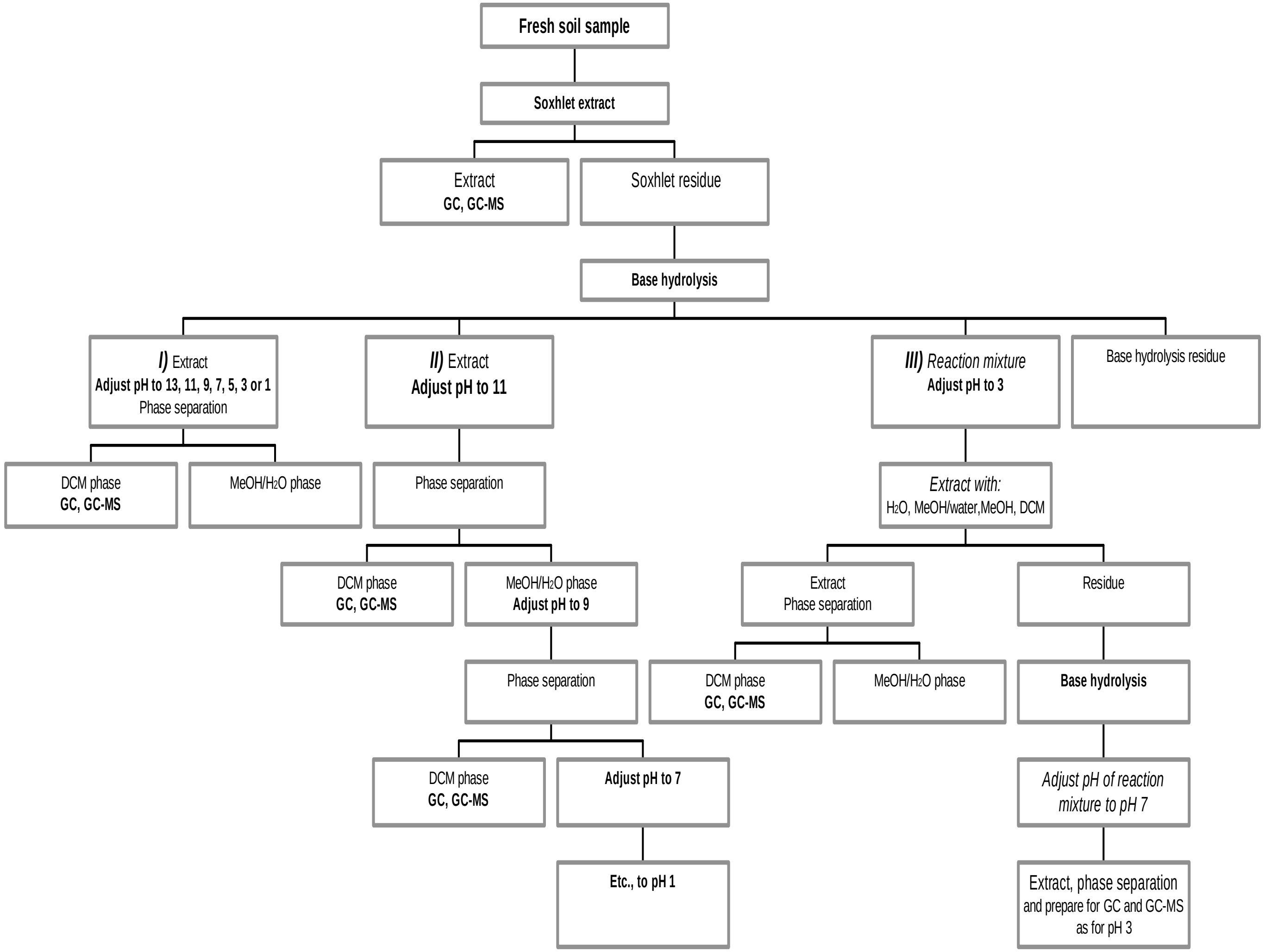

Figure 5.1 Schematic flow chart indicating the different base treatments: I direct acidification of the extract, II step wise acidification of the extract, III acidification of reaction mixture.

The soil residue obtained through this first treatment was again base-treated, after which the reaction mixture was acidified to pH 7 and subsequently extracted with water (3x), methanol/water (1:1 v/v; 1x), methanol (2x) and DCM (3x) (Fig. 5.1, part III). The extract obtained was prepared for GC and GC-MS as described above.

5.2.7 Gas chromatography (GC)

GC analyses were performed using a Hewlett-Packard 6890 series gas chromatograph equipped with a CP-sil 5CB silica column (50m x 0.32mm, film thickness 0.12µm). Derivatized extracts (1.0 µl) in hexane were injected on-column. The oven temperature was programmed from 70°C to 130°C at 20°C min^{-1} and from 130°C to 320°C (isothermal for 20 min) at 4°C min^{-1}. Compounds were detected using a flame ionisation detector (FID) at 325°C. Helium was used as carrier gas.

5.2.8 Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses were performed using a Hewlett-Packard 5890 series II gas chromatograph connected to a Fisions instruments VG platform II mass spectrometer operating at 70 eV, scanning the range *m/z* 50-650 in a cycle of 0.65 s. The capillary column and temperature programme were as described for the GC analyses. Compound identification was based on data published in the literature (e.g. Kolatukudy and Agrawal, 1974; Kolattukudy, 1980a; Holloway, 1982; Riederer et al., 1993; Walton, 1990).

5.3 Results

5.3.1 Total organic carbon (TOC), pH (H_2O), pH (NaF), extracts and DCM precipitate

The TOC% of the dried soil sample, the Soxhlet residue and the saponified residue, were respectively 7.1, 6.5 and 3.0. The saponified residue obtained after extraction and centrifugation consisted of two fractions: a dark, fine (approximately 20%) and a light, coarse fraction (approximately 80%). These two fractions were homogenised prior to TOC measurements. The pH (NaF) was 11.7 and the pH (H_2O) 4.9. Na pyrophosphate extractable Al and Fe were 0.46 and 0.13 (±0.01) $mmol.g^{-1}$ soil respectively. Acid oxalate extractable Al, Fe and Si were 0.61, 0.23, 0.04 (±0.01) $mmol.g^{-1}$ soil, respectively.

Colours of extracts obtained and supernatants are summarised in Tables 5.1 to 5.3. The MeOH/water layer contained suspended material, which, after standing overnight, accumulated at the interface between the DCM and MeOH/water layers. In the text, this dark brown/grey material will be referred to as “DCM precipitate”. This same phenomenon has also been observed by others (e.g. Tegelaar et al., 1995). The DCM precipitate was centrifuged and consisted of a fine (approximately 85%) and a coarse fraction (approximately15%). Some of the precipitate contained white needles (most probably salt) after centrifugation and air-drying.

Table 5.1 Colour of H_2O, H_2O/MeOH, MeOH and DCM extracts

H_2O	H_2O	MeOH/H_2O	MeOH	MeOH	DCM
Dark green/black	Light brown/green	Light brown/yellow	Light orange	Light yellow	Light orange/yellow

Table 5.2 Colour of MeOH/H_2O and DCM fractions obtained through direct pH adjustment

Layer	pH 13 & 11	pH 9	pH 7	pH 5	pH 3	pH 1
MeOH/H_2O	Brown/black	Brown/black	Dark yellow	Dark yellow	Dark yellow	Dark red/brown
DCM	Light yellow	Light Orange/yellow	Yellow	Yellow	Yellow	Dark red

Table 5.3 Colour of MeOH/H_2O and DCM fractions obtained through step-wise extraction

Layer	pH 11	pH 9	pH 7	pH 5	pH 3	pH 1
MeOH/H_2O	Brown/black	Brown/black	Brown/black	Brown/black	Brown/black	Brown/red
DCM	Light yellow	Light yellow	Light yellow	Light yellow	Light yellow	Red

5.3.2 Soxhlet extract (TLE)

The yellow coloured TLE obtained after Soxhlet extraction and filtration weighted 10.4 mg, approximately 0.01% of the dry weight of the dried soil extracted; 0.15% based on TOC%. The gas chromatogram of the TLE revealed a large variety of compound classes and was dominated by C_{12} to C_{34} n-alkanoic acids, maximising at C_{16} and C_{24}, and C_{14} to C_{32} n-alkanols, maximising at C_{16}, C_{18} and C_{26} (Fig. 5.2). Compounds identified in relatively low concentrations included ω-hydroxy alkanoic acids (C_9, C_{20}-C_{27}), n-alkanes (C_{27}-C_{33}, odd predominance) and 24-ethylcholesterol (β-sitosterol). Identified series of n-alkanols, α, ω-alkanediols, n-alkanoic acids and ω-hydroxy alkanoic acids showed a strong, even over odd, predominance for the $>C_{18}$ members. Series of $<C_{18}$ n-alkanoic acids were characterised by the presence of unsaturated C_{16} and C_{18} members together with C_{15} and C_{17} iso and anteiso branched compounds (not indicated in Fig. 5.2).

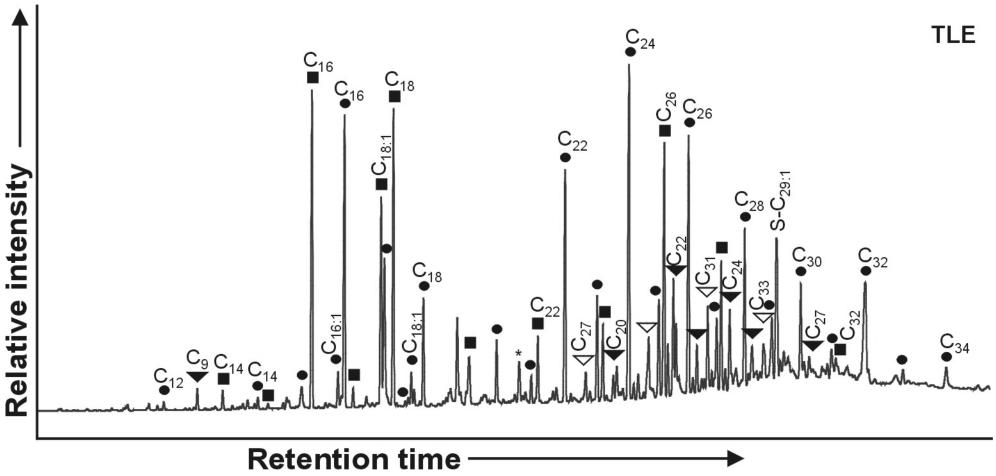

Figure 5.2 Gas chromatograms of Soxhlet extract. * : Phthalates,▽: *n*-alkanes,■: *n*-alkanols,●: *n*-alkanoic acids,▼: ω-hydroxy acids. C_x above the peaks refers to the total number of carbon atoms. Number after the comma refers to the position of the hydroxy group. Number after colon refers to the total number of double bonds. S-C_x refers to steroids with x referring to the total number of carbon atoms.

5.3.3 Base hydrolysis followed by direct pH adjustment of extract

Lipid content and weight of all DCM extracts are summarised in Table 5.4, gas chromatograms of pH 11, 7 and 3 fractions are presented in Fig. 5.3. The largest quantity of extract was obtained at pH 1. Significant amounts were also found at pH 5 and 3. The various chromatograms revealed large differences in overall composition. However, as found in the TLE, identified series of *n*-alkanols, diols, *n*-alkanoic acids and ω-hydroxy alkanoic acids showed a strong, even over odd, predominance for the >C_{18} members.

Table 5.4 Lipid composition of pH fractions obtained through direct pH adjustment, net weight, and weight fractions based on TOC% of Soxhlet residue.

Compound classes	pH 13	pH 11	pH 9	pH 7	pH 5	pH 3	pH 1
Weight (mg)	4.7	2.9	1.9	9.8	16.7	14.5	39.2
Weight (%)	0.87	0.54	0.35	1.8	3.1	2.7	7.2
n-Alkanols	C_{16}-C_{32} (C_{22})	C_{16}-C_{32} (C_{26})	C_{16}-C_{28}	C_{16}-C_{28}	C_{16}-C_{28}	C_{16}-C_{26}	C_{16}-C_{32}
2-Alkanols	C_{18}, C_{19}, C_{20}	C_{18}	C_{18}	---	---	---	C_{18}
α, ω-Diols	C_{12}-C_{26} ($C_{16,}$ C_{18})	C_{12}-C_{26} (C_{16}, C_{18})	C_{16}, C_{18}	C_{16}, C_{18}	C_{16}, C_{18}	C_{16}, C_{18}	---
n-Alkanoic acids	C_{12}-C_{30} (C_{16})	C_{12}-C_{30} (C_{16})	C_{14}-C_{28} (C_{16})	C_{14}-C_{26} (C_{16})	C_{14}-C_{26} (C_{16})	C_{14}-C_{24} (C_{16})	C_{14}-C_{28} (C_{16})
ω-Hydroxy acids	C_{16}-C_{28} (C_{16}, C_{22})	C_{16}-C_{28}	C_{16}-C_{28} (C_{16})	C_{14}-C_{28} (C_{16})	C_{8}-C_{28} (C_{16})	C_{8}-C_{24} (C_{16})	C_{7}-C_{30} (C_{16})
(ω-1)Hydroxy acids	C_{28}	---	---	---	---	---	---
α, ω-Diacids	---	--	---	C_{12}	C_{9}, C_{12}	C_{8}-C_{21} (C_{9})	C_{7}-C_{16} (C_{9})
Dihydroxy acids	C_{16}	C_{16}	---	C_{16},$C_{18:1}$ ($_{10}$ C_{16})	C_{16},$C_{18:1}$ ($C_{16,10}$)	C_{16},$C_{18:1}$ ($C_{16,10}$)	$C_{16,10}$
Trihydroxy acids	C_{18}	---	---	C_{18}	C_{18}	C_{18}	---
Hydroxy diacids	---	---	---	---	C_{16}	C_{16}	---
Dihydroxy diacids	---	---	---	C_{18}	C_{18}	C_{18}	---
Aromatic acids	---	---	---	---	*C, D, E*	*C, D, E*	*C, D, E*
Steroids	C_{29}	C_{29}	C_{29}, $C_{29:1}$	C_{29}, $C_{29:1}$	C_{29}, $C_{29:1}$	C_{29}	C_{29}
Phytol	C	---	---	---	---	---	---
Methyl Esters	C_{24}	---	---	---	---	---	$C_{9,14,16,20,24}$

C_x-C_y ranging from C_x to C_y, compounds between brackets are the most dominant homologues, number after colon refers to double bond, number after comma refers to position of hydroxy group.

The extract obtained after direct adjustment to pH 13 (Table 5.4) was characterized by homologous series of *n*-alkanols, α, ω-alkanediols, *n*-alkanoic acids and ω-hydroxy alkanoic acids. *n*-Alkanols ranged from C_{16} to C_{32}, maximising at C_{22} and C_{24}, α, ω-alkanediols from C_{12} to C_{26} maximising at C_{16} and C_{18}, and *n*-alkanoic acids from C_{12} to C_{30}, the C_{16} homologue being the most abundant. <C_{18} *n*-alkanoic acids were characterised by the presence of unsaturated C_{16} and C_{18} compounds together with C_{15} and C_{17} *iso* and *anteiso* branched compounds. Apart from saturated *n*-alkanoic acids, also $C_{16:1}$, $C_{18:1}$ and $C_{18:2}$ *n*-alkanoic acids were identified. ω-Hydroxy alkanoic acids ranged from C_{16} to C_{28}, maximising at C_{16} and C_{22}. Another dominant component was 24-ethylcholesterol. Other components, present in relatively lower concentrations included phytol, C_{18}-C_{20} 2-alkanols, C_{16} (9/10,16)-dihydroxy alkanoic acids and C_{24} acid methylated ω-hydroxy alkanoic acid. Fatty acid methyl esters (MEs) are known to be formed in a MeOH/water phase if left standing for more than one day in extreme pH conditions, i.e. extremely basic or acidic.

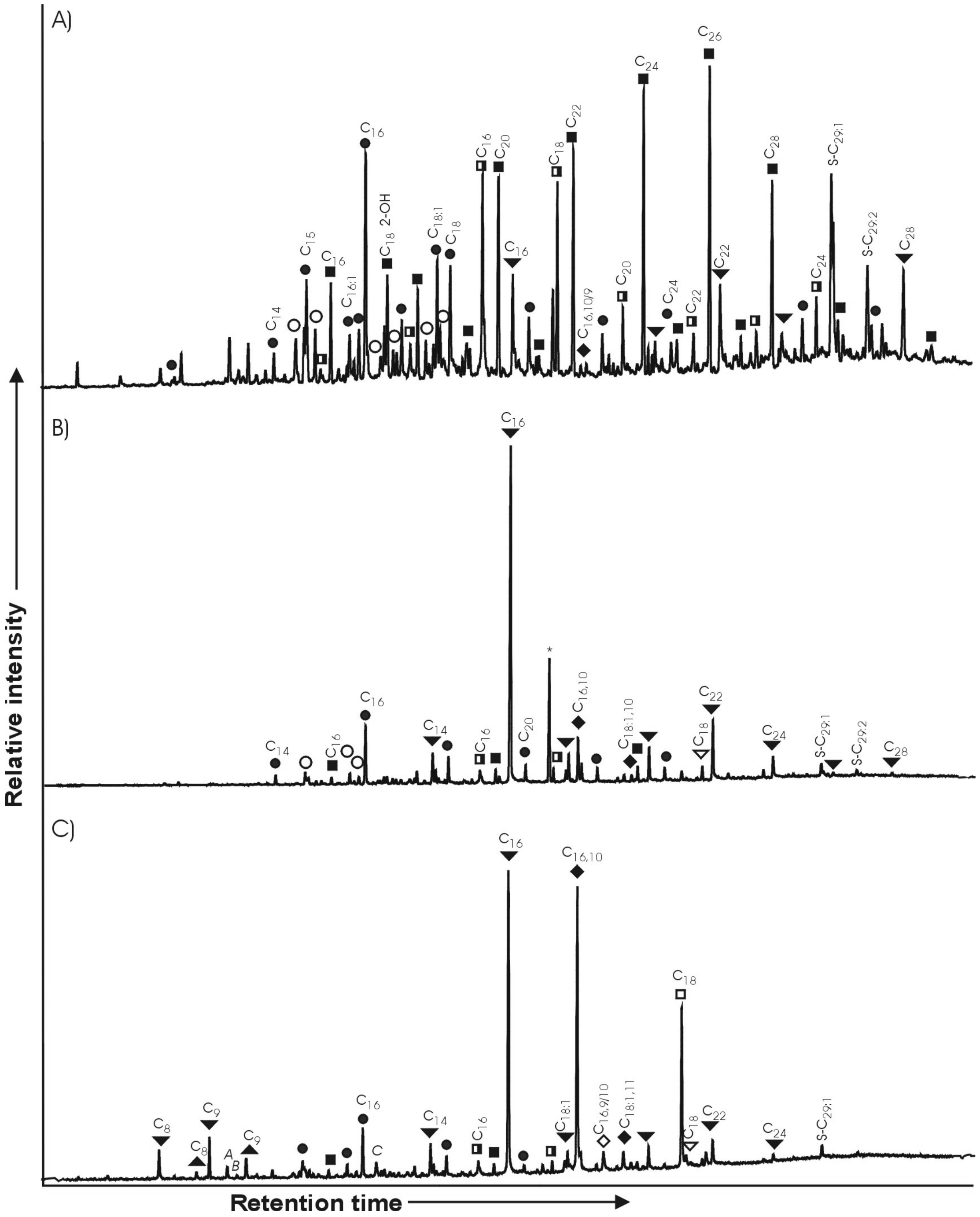

Figure 5.3 Gas chromatograms of direct acidification to (A) pH 11, (B) pH 7 and (C) pH 3. 2-OH: 2-alkanols,◨ : diols,◆: dihydroxy acids,□ : trihydroxy acids,◇: hydroxy diacids,▽ : dihydroxy diacids,○ : *iso* and *anteiso* branched compounds,▲ : Diacids, Ph : Phytol, *A*: 4-hydroxy-3-methoxy benzoic acid (diTMS), *B*: *p*-coumaric acid (diTMS; both *cis* en *trans* indicated), *C*: ferulic acid (diTMS; both *cis* en *trans* indicated); $C_{16,9/10}$ refers to a midchain hydroxy group either on position 9 or 10. For further information see text and symbol legend of Fig. 5.2.

The extracts obtained after direct adjustments to pH 11 (Fig. 5.3A, Table 5.4) and 9 (Table 5.4) were very similar to the extract described above (pH 13), the only striking differences being that long-chain *n*-alkanols (C_{20}-C_{28}) were dominant in the pH 11 extract, and that the C_{16} *n*-alkanoic acid, together with the C_{16} and C_{18} α, ω-alkanediols were more dominant in the extract obtained after adjustment to pH 9.

The pH 7 extract (Fig. 5.3B, Table 5.4) was dominated by the occurrence of C_{16} ω-hydroxy alkanoic acid. The rest of this extract was similar to those described above (pH 13-9), except for the occurrence of C_{12} α, ω-diacid, C_{18} (9,10,18)-trihydroxy alkanoic acid and C_{18} (9,10)-dihydroxy-α, ω-diacid.

Characteristic for the pH 5 extract (Table 5.4) were C_{16} ω-hydroxy alkanoic acid, C_{16} (10, 16)-dihydroxy alkanoic acid and, although relatively less dominant, C_{18} (9,10,18)-trihydroxy alkanoic acid. Other compounds present in relatively low concentrations included C_9 and C_{12} diacids, C_8 to C_{28} ω-hydroxy alkanoic acids, C_{16}, (9, 16) and $C_{18:1}$ (10/11, 18)-dihydroxy alkanoic acids, C_{16} (9/10)-hydroxy-α, ω-diacid, C_{18} (9, 10)-dihydroxy-α, ω-diacid, *n*-alkanols (C_{16} to C_{28}), *n*-alkanoic acids ranging from C_{14} to C_{26} maximising at C_{16}, aromatic acids (*A, B, C*), 24-ethylcholesterol, $C_{29:2}$ steroid and C_{16} and C_{18} α, ω-alkanediols.

The pH 3 extract (Fig. 5.3C, Table 5.4) was very similar to that of pH 5. The only differences were the absence of the long-chain ω-hydroxy alkanoic acids (C_{26}, C_{28}), C_{26} *n*-alkanol, C_{24} *n*-alkanoic acid, $C_{29:2}$ steroid, and the appearance of C_8 and C_9 diacids.

The pH 1 extract (Table 5.4) was dominated by C_{16}, C_{22} and C_{24} ω-hydroxy alkanoic acids together with C_{16} (10, 16)-dihydroxy alkanoic acid, although this dominance was not so strong as was found for the extract obtained after adjustment to pH 3. The rest of the extract had a similar composition compared with the extract obtained after adjustment to pH 3.

5.3.4 Base hydrolysis followed by step-wise extraction

Net weight and weight fractions, are presented in Table 5.5, together with the lipid contents of the pH fractions obtained. Yields obtained increased from pH 11 to pH 5, to decrease again to the lowest yield obtained after adjustment to pH 1. Gas chromatograms of fractions obtained after pH adjustment to pH 11, 7 and 3 are presented in Fig. 5.4. The DCM extract obtained after adjustment of the MeOH/water phase to pH 11 (Fig. 5.4A, Table 5.5) was characterised by $C_{29:1}$ and $C_{29:2}$ steroids and homologous series of n-alkanols ranging from C_{15} to C_{32} maximising at C_{20}, C_{14} to C_{30} n-alkanoic acids maximising at C_{16} (C_{15} and C_{17} iso and anteiso members also indicated), C_{12} to C_{26} diols (C_{16}), and C_{16} to C_{20} 2-alkanols, the C_{18} homologue being the most abundant. Relatively less abundant compounds included C_{16} and C_{28} ω-hydroxy alkanoic acids, C_{16} (9/10, 16)-dihydroxy alkanoic acid, phytol, a C_{27} steroid and acid methylated ω-hydroxy alkanoic acids (C_{16}, C_{24}) and n-alkanoic acids (C_{26}).

The extract obtained after adjustment of the DCM-extracted MeOH/water phase from pH 11 to pH 9 (Table 5.5) was mainly dominated by C_{16} ω-hydroxy alkanoic acid. Other characteristic compounds were C_{20} and C_{22} ω-hydroxy alkanoic acids and the C_{16} *n*-alkanoic acid. Other *n*-alkanoic acids identified were C_{12} to C_{26}, whereas ω-hydroxy alkanoic acids ranged from C_{14} to C_{28}. Compounds present in lower concentrations included *n*-alkanols (C_{24}, C_{26}, C_{28}), C_{18} (ω-1)-hydroxy alkanoic acid, C_{16} (10, 16) dihydroxy alkanoic acid, two methylated ω-hydroxy alkanoic acids (C_{16}, C_{22}) and diacids ranging from C_{19} to C_{28}.

Table 5.5 Lipid composition of pH fractions obtained through step-wise extraction.

Compound Classes	pH 11	pH 9	pH 7	pH 5	pH 3	pH 1
Weight (mg) 35.8*	2.1	5.5	8.5	9.9	8.4	1.4
Weight (%) 2.8	0.17	0.43	0.66	0.77	0.65	0.11
2-Alkanols	C_{16}-C_{20} (C_{18})	---	---	---	---	---
Diols	C_{12}-C_{26} (C_{16})	---	---	---	---	---
n-Alkanols	C_{15}-C_{32} (C_{20})	C_{24}-C_{28}	---	---	---	C_{16}
n-Alkanoic acids	C_{14}-C_{30} (C_{16})	C_{12}-C_{26} (C_{16})	C_{12}-C_{17}	C_{16}	---	C_{14}-C_{18}
ω-Hydroxy acids	C_{16}, C_{28}	C_{14}-C_{28} (C_{16})	C_8, C_9, C_{14}, C_{16} (C_{16})	C_7-C_{14} (C_9)	C_7-C_{14} (C_9)	C_7-C_{14} (C_9)
Dihydroxy acids	C_{16}	$C_{16,10}$	C_{16}, C_{18}, $C_{18:1}$ ($C_{16,10}$)	C_{14}-C_{18} ($C_{16,10}$)	C_{14}-C_{18} ($C_{16,10}$)	C_{14}-C_{18} ($C_{16,10}$)
(ω-1)-Hydroxy acids	---	C_{18}	C_{16}	C_8	---	---
α, ω-Diacids	---	C_{19}-C_{28}	C_{15}-C_{24}	C_6-C_{24} (C_9)	C_7-C_{24} (C_9)	C_7-C_{25} (C_9)
Trihydroxy acids	---	---	C_{18}	C_{18}	C_{18}	C_{18}
Hydroxy diacids	---	---	---	C_{14}, C_{16}, $C_{18:1}$,(C_{16})	$C_{14,\ 16}$, $C_{18:1}$	C_{14}-C_{18} ($C_{16,\ 9/10}$)
Dihydroxy diacids	---	---	---	C_{18}	C_{18}	C_{18}
Aromatic acids	---	---	---	*C, D, E*	*C, D, E*	*C, D, E*
Steroids	C_{27}, C_{29}, $C_{29:1}$	---	---	---	---	---
Phytol	C_{20}	---	---	---	---	---
Methyl Esters	C_{16},C_{24},C_{26}	C_{16}, C_{22}	---	---	---	---

C_x-C_y ranging from C_x to C_y, compounds in parentheses are the most dominant homologues, number after colon refers to double bond, number after comma refers to position of hydroxy group, slash forward to other positions.

The same dominance of C_{16} ω-hydroxy alkanoic acid was observed in the pH 7 extract (Fig. 5.4B, Table 5.5). Other compounds identified were C_{14} ω-hydroxy- and C_{16} (10, 16)-dihydroxy alkanoic acid. *n*-Alkanols or α,ω-alkanediols were no longer identified in this extract. Relatively less abundant compounds included *n*-alkanoic acids (C_{12} to C_{17}), ω-hydroxy alkanoic acids (C_8, C_9), C_{16} (9, 16)-, C_{18} (9/10, 18)- and $C_{18:1}$ (10/11, 18)-dihydroxy alkanoic acids, C_{16} (ω-1)-hydroxy alkanoic acid, C_{18} (9, 10, 18)-trihydroxy alkanoic acid and α,ω-diacids, ranging from C_{15} to C_{24}.

The pH 5 extract (Table 5.5) was dominated by both C_{16} (10, 16) dihydroxy and C_{18} (9, 10, 18) trihydroxy alkanoic acid. Identified series of compounds present in relatively low concentrations included a series of ω-hydroxy alkanoic acids ranging from C_7 to C_{14} maximising at C_9, a series of C_6 to C_{24} diacids, also maximising at C_9, and a series of (9/10, ω)-dihydroxy alkanoic acids ranging from C_{14} to C_{18}, (10, 16)-dihydroxyhexadecanoic acid being the most abundant homologue, and a small series of C_{14} to $C_{18:1}$ (9/10)-hydroxy-α, ω-diacids. Less abundant compounds included C_8 (ω-1)-hydroxy alkanoic acid, C_{18} (9, 10, 18)-trihydroxy alkanoic acid, C_{18} (9, 10)-dihydroxy-α, ω-diacid, C_{16} *n*-alkanoic acid and aromatic acids (*A*, *B*, *C*). The only striking difference between the pH 3 extract (Fig. 5.4C, Table 5.5) and that obtained after adjustment to pH 5 was the dominance of C_{18} (9, 10, 18) trihydroxy alkanoic acid and C_{18} (9, 10)-dihydroxy-α, ω-diacid. Besides this, no *n*-alkanoic acids were identified and the early eluting compounds had become relatively more abundant.

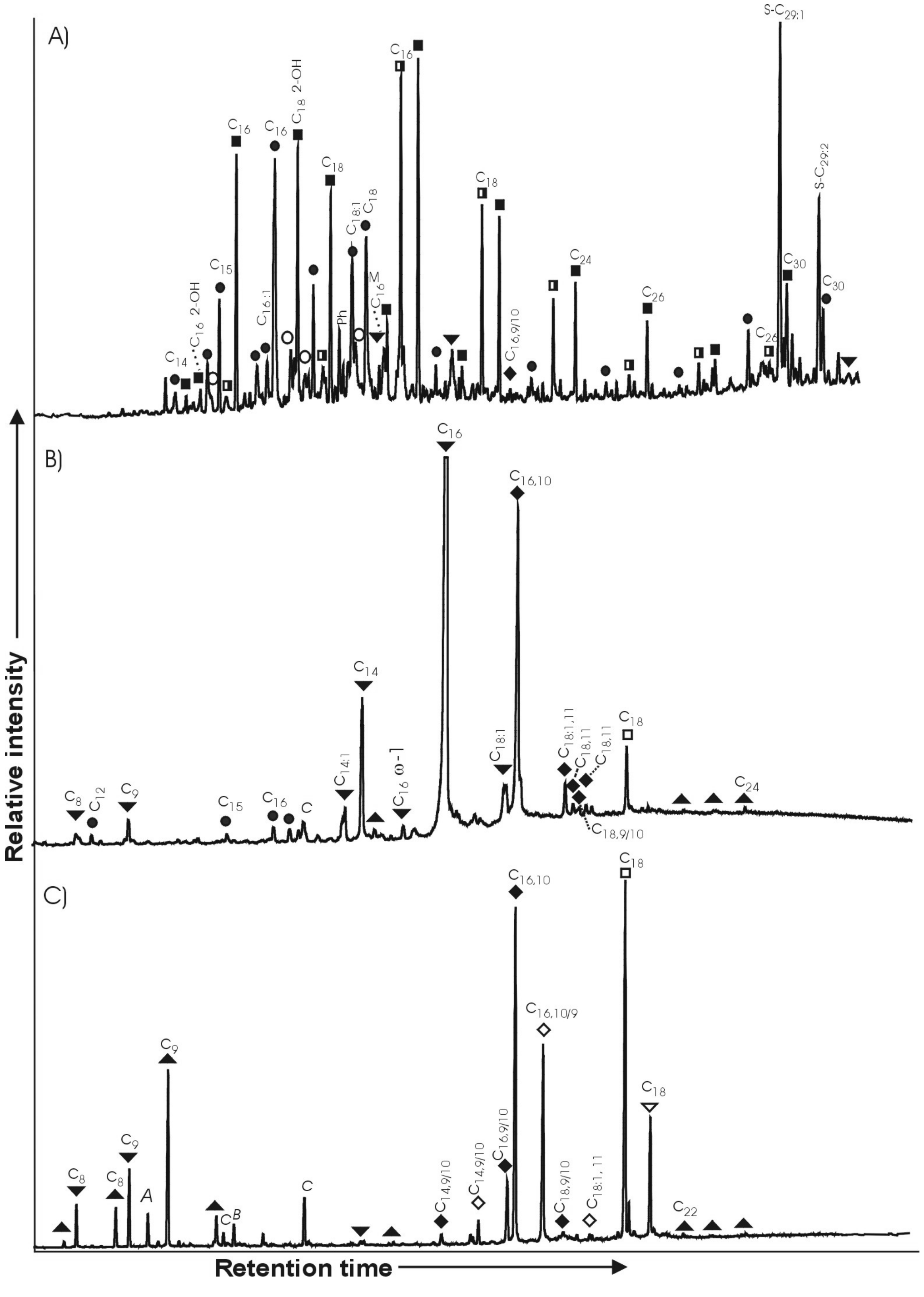

Figure 5.4 Gas chromatograms of step-wise acidification to (A) pH 11 (B), pH 7 and (C) pH 3. For further information see text and symbol legend of Figs. 2 and 3.

The last extract obtained through step-wise extraction, pH 1 (Table 5.5), was very similar to that obtained after adjustment to pH 3. The relative concentrations were somewhat different compared with the pH 3 fraction; the early eluting compounds had become relatively even more abundant. Besides this, small amounts of *n*-alkanoic acids (C_{14}-C_{18}) were identified.

5.3.5 Base hydrolysis followed by HCl treatment in the presence of soil

The base extract obtained after adjustment of the pH of the reaction mixture, i.e. soil residue and extract, to pH 3 was dominated by C_8, C_9 and C_{10} α, ω-diacids (Fig. 5.5A). n-Alkanoic acids ranged from C_{12} to C_{18}, maximising at $C_{18:2}$. Eluting early in the diagram were several aromatic acids (e.g. A, B). Other characteristic lipids included 24-ethylcholesterol and C_8 and C_9 ω-hydroxy alkanoic acids.

The residue obtained after the pH 3 treatment was again subjected to base hydrolysis but following, the pH of the reaction mixture was adjusted to pH 7 (Fig. 5.1, part III). The extract obtained was dominated by homologous series of *n*-alkanoic acids (C_{10}-C_{32}) maximising at C_{12}, and ω-hydroxy alkanoic acids (C_{14}-C_{22}) maximising at C_{14} and C_{16} (Fig. 5.5B). Relatively less abundant lipids identified included *n*-alkanols (C_{12}, C_{28}-C_{32}), and aromatic acids (i.e. *F*, *A*).

Comparable results were obtained for a podzolic Bh horizon that was obtained from the Veluwe near Kootwijk/Assel, the Netherlands. The fraction obtained after base hydrolysis and pH adjustment of the reaction mixture to pH 3 was also dominated by short chain ω-hydroxy alkanoic acids and aromatic acids, while the extract (pH 7) obtained after base hydrolysis of the pH 3 residue was again characterized by long-chain ($>C_{12}$) ω-hydroxy and *n*-alkanoic acids (data not shown).

5.4 Discussion

Based on the soil profile description, measured pH (NaF), pH (H_2O), Na pyrophosphate and acid oxalate data, the soil used was classified as a Fulvic or Melanic Andosol (to distinguish between these two, additional information would be necessary) (FAO, 1998). The Al_{ox}/Al_{py} ratio of 0.75 in combination with the soil pH (H_2O) of 4.9 further indicates that the sample analysed was taken from an alu-andic horizon (FAO, 1998).

5.4.1 Origin of identified compounds in the Soxhlet extract (TLE)

n-Alkanols ranging from C_{22} to C_{34} with a strong even predominance and n-alkanes ranging from C_{27} to C_{33} with a strong odd predominance are characteristic of higher plant waxes and some suberins (Amblès et al., 1989; Walton 1990; van Bergen et al., 1998a). The occurrence of short-chain ($<C_{22}$) n-alkanols (Fig. 5.2) is most probably the result of microbial activity (Jambu et al., 1993). n-Alkanoic acids, ranging from C_{12} to C_{36} with a strong even predominance, most likely originate from the oxidation of plant derived lipids and a contribution from hydrolysed wax biopolyesters (i.e. cutin) and biopolyesters from roots (i.e. suberin) (Holloway, 1982: Walton, 1990; van Bergen et al., 1998a; Bull et al., 2000b). The relatively small contribution of C_{15} and C_{17} iso and anteiso alkanoic acids, together with short-chain ($<C_{20}$) n-alkanoic acids without a clear even predominance, suggests a relatively small microbial contribution (Amblès et al., 1994a). The identified 24-ethylcholesterol is of plant origin (Amblès et al., 1989; Goad, 1991). C_9 ω-hydroxy alkanoic acid is suggested to be a product resulting from oxidation of the C=C
double bond of unsaturated fatty acids (Simic et al., 1992). The abundance and distribution of fatty acids is in concordance with TLE data from other acidic soils (Bull et al., 2000a) as opposed to alkaline soils (van Bergen et al., 1997).

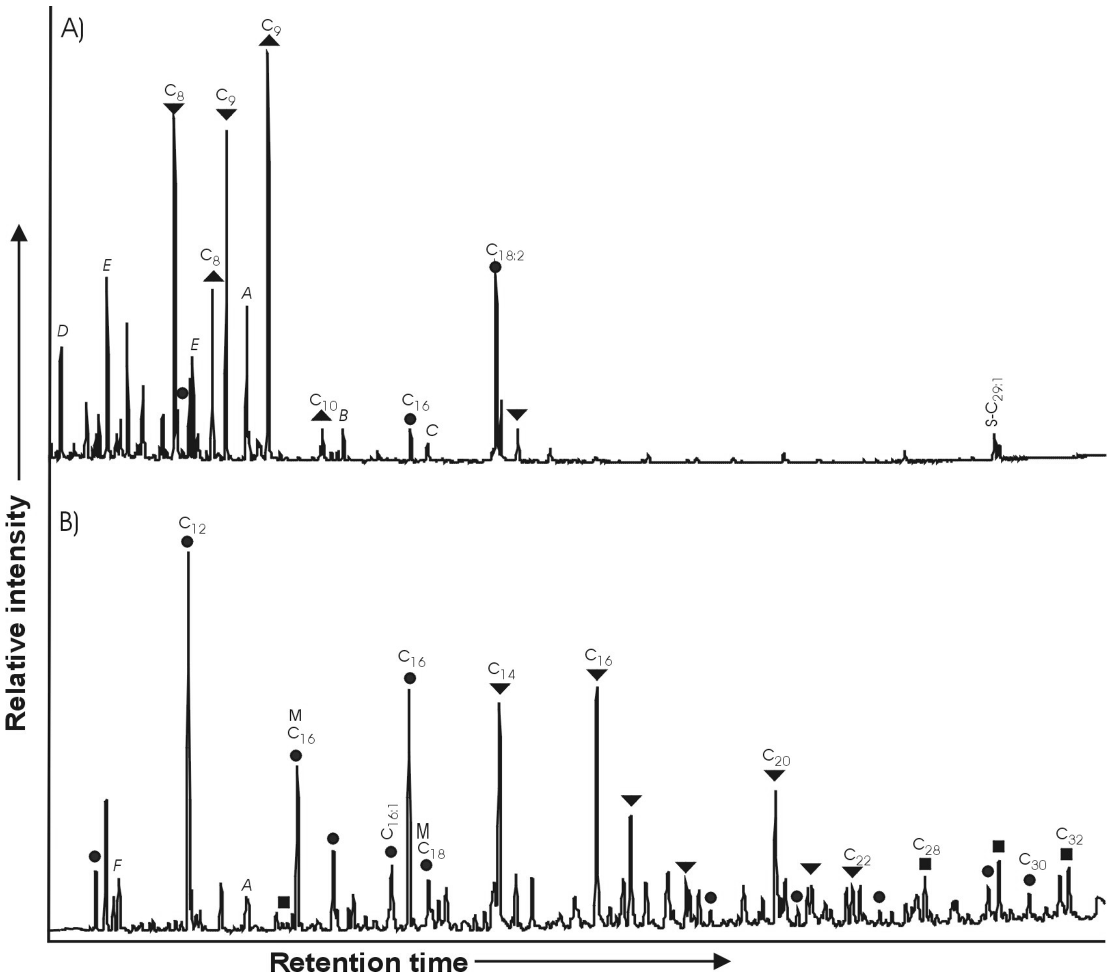

Figure 5.5 Gas chromatograms of A) the base extract after acidification of the reaction mixture to pH 3 and B) subsequent base extract of pH 3 residue after acidification of reaction mixture to pH 7. *D*: 4-hydroxy benzoic acid (diTMS), *E*: 3, 5-dimethoxy *p*-coumaric acid, *F*: 4-hydroxy benzoic acid methyl ester (TMS). For further information see text and symbol legend of Figs. 5.2, 5.3 and 5.4.

5.4.2 Relative concentrations of identified compounds after base treatment

The separation of organic compounds, released during a standard base treatment, into different pH fractions (experiments I and II) is based on their pK_a values and hydrophobicity. Organic acids will most probably enter the hydrophobic DCM phase as the pH of the MeOH/water layer equals, or becomes even lower than their respective pK_a values because they become more hydrophobic once protonated. Long chain acids are indeed obtained in higher pH fractions than the shorter chain or more functionalised acid compounds (Figs 5.3 and 5.4). In general, for both extraction experiments (I and II), the total yield is the largest at moderate and low pH values (7, 5, 3 and 1) (Fig. 5.6). This is not surprising, considering the fact that most organic acids have pK_a values in this region.

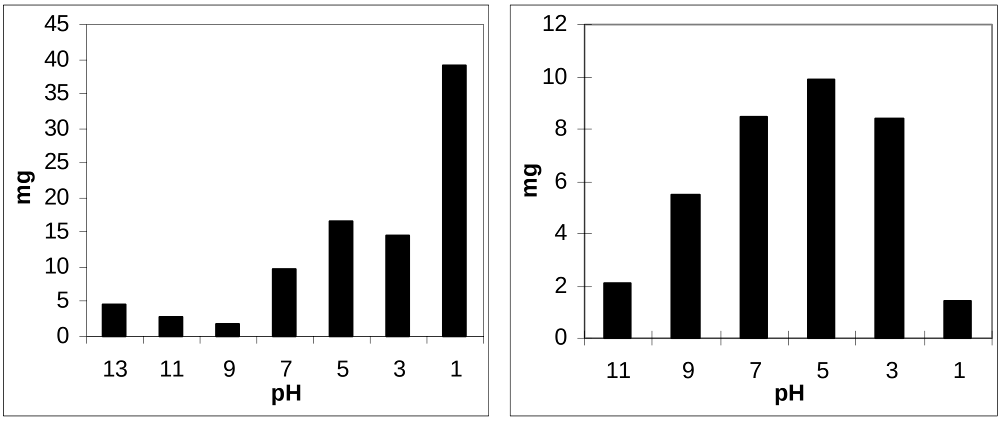

Figure 5.6. Total yield as function of pH obtained through (a) direct pH adjustment and (b) step wise extraction.

The direct pH adjustment results (experiment I, Fig. 5.1) clearly indicate that the relative amounts of identified compounds are pH dependent (Fig. 5.3). At high pH (i.e. 13, 11 and 9), extracts are dominated by *n*-alkanols, α, ω-alkanediols, *n*-alkanoic and ω-hydroxy alkanoic acids. At moderate and low pH (i.e. 7, 5, 3 and 1), extracts are dominated by ω-hydroxy alkanoic acids, (10,16)-dihydroxyhexadecanoic acid and (9,10,18)-trihydroxyoctadecanoic acid. In addition, the early eluting fraction at low pH (i.e. 5, 3 and 1) is dominated by short-chain (<C_{12}) ω-hydroxy alkanoic acids, phenolic acids and α, ω-diacids.

Direct adjustment to a low pH (Fig. 5.3C) leads to an extract dominated by phenolic, ω-dihydroxy and trihydroxy alkanoic acids, thereby neglecting the presence of *n*-alkanols and *n*-alkanoic acids (e.g. Tegelaar et al., 1995). However, after minor direct adjustment, i.e. pH 13, 11 and 9, these compound classes comprise an important part of the total amount of lipids identified (Fig. 5.3A). In Figures 6A and 6B, the total weight of the extracts obtained is shown as a function of the pH to which the MeOH/H_2O phase was adjusted. These two figures indicate that although the total yield at these high pH values is not as important as the yield obtained at moderate and low pH values (i.e. 7, 5, 3 and 1), the total weight of high pH fractions comprises up to 21% of the total yield obtained through step-wise extraction. These results strongly suggest that both *n*-alkanols and *n*-alkanoic acids are more important compounds in the ester bound aliphatic fraction in this soil than would have been estimated only from the more common used low, i.e. pH 4 to 1, fractions (e.g. Tegelaar et al., 1995; Nierop et al., 2001b). The very high yield obtained after direct adjustment to pH 1 can be expected but remains to be treated with caution. Besides the expected large contribution of all lipid compounds protonated at this extremely low pH, it might also be caused by the presence of water in the extract or by compounds not amenable by GC and GC-MS, including moderate and high molecular weight compounds, e.g. humic substances also extracted during the base treatment.

The low abundance of the *n*-alkanols and *n*-alkanoic acids at moderate and low pH is explained by the fact that their signal is ”buried” by the signal of phenolic acids, ω-dihydroxy and trihydroxy alkanoic acids in the fractions obtained through direct adjustment. To avoid this problem, step-wise extraction of the extract is suggested. In a step-wise extraction, less abundant compounds (e.g. C_{18} dihydroxy-α, ω-diacid, identified dihydroxy acids) can also be studied (Chapter 6), due to the separation of all compounds released upon base treatment into several pH sub fractions (Fig. 5.4). The subsequent removal of compounds protonated (pK_a dependent) after each pH adjustment avoids all compounds to end up in the same fraction, thereby causing only the most abundant components to be detected. However, it should be noted that direct acidification of the extract to pH 1 (experiment I) remains a very practical method to obtain a rapid insight into the bulk composition of LMW ester-related moieties released after a standard base treatment.

5.4.3 Mode of occurrence in soils (I): Biopolyesters

n-Alkanoic acids, n-alkanols and aromatic acids, together with C_{24} and C_{26} diols, C_{12}-C_{26} ω-hydroxy alkanoic acids, C_{16} and C_{18} dihydroxy alkanoic acids, C_{18} trihydroxy alkanoic acid, C_{18} dihydroxy α, ω-diacids, C_{16} hydroxy α, ω-diacids and C_{16}-C_{24} α, ω-diacids are known from hydrolysed natural biopolyesters, i.e. suberins and/or cutins (Kollattukudy, 1980a; Walton, 1980; Holloway, 1982; Matzke and Riederer 1991; Amblès et al., 1991; Riederer et al., 1993; Bernards et al. 1995; Bernards and Lewis, 1998). Their relative contribution to the total amount of lipids hydrolysed depends very much on the type and amount of root input (Kollattukudy, 1980a; Walton, 1990; Holloway, 1982; Matzke and Riederer, 1991; Riederer et al., 1993; Nierop and Buurman, 1999).

Input by roots is probably the most important source of insoluble organic matter in acidic mineral soils with virtually no biological mixing of above ground plant material (Bull et al., 2000b; Nierop, 1998). Suberized tissue is probably also the most important source for ester-bound aliphatics in our soil sample because it was taken from a depth of about 30 cm, at which intensive root activity was one of the major visible characteristics. It is therefore suggested that most products released upon base treatment are related to suberins, i.e. C_{16}-C_{20} 2-alkanols, C_{14} and C_{18} α, ω-diacids, C_{14} dihydroxy acid and C_{26} and C_{28} α, ω-diacids.

24-Ethylcholesterol ($C_{29:1}$) has been identified in depolymerisates from soils (e.g. Riederer et al., 1993; Amblès et al., 1991; 1996; Gobé et al., 2000) and suberins (e.g.Walton, 1990). Therefore, the other steroids identified, i.e. C_{27} and $C_{29:2}$, are most probably also derived from (ester bound) moieties (Mahato et al., 1982; Gobé et al., 2000), the exact nature of which is unclear.

C_9 α, ω-diacid, and most probably all the short-chain ω-hydroxy alkanoic acids and α, ω-diacids, probably originate from the enzymatic oxidation of the C=C double bond of unsaturated acids (Simic et al., 1992; Chan, 1987; Gillan and Johns, 1982; Grasset and Amblès, 1998). These hydroxy alkanoic and α, ω-diacids are suggested to be preserved through chemical bonding, most likely via ester linkages, into insoluble polymeric matrices (Regert et al., 1998). Hydroxycinnamic acid derivates are known to be presumably linked to the suberin matrix either through ester or amide linkages (Bernards et al., 1995), although other sources, e.g. ligno celluloses (Smith and Harris, 2001) can not be excluded.

5.4.4 Mode of occurrence in soils (II): Additional sources

Comparison of the, to pH 7 adjusted soil/MeOH reaction mixture (Fig. 5.5B) with the extract obtained through direct adjustment to pH 3 (Fig. 5.5A), indicates that hydrophobic moieties such as long-chain ω-hydroxy, dihydroxy and trihydroxy alkanoic acids precipitate as soon as they become protonated (i.e. after acidification to a low pH) in the presence of a soil matrix, i.e. the soil residue. This phenomenon has also been observed by others (Grasset and Amblès, 1998). Once they have precipitated, they are no longer extractable using organic solvents (i.e. MeOH, DCM) and water. Comparable results were found during a parallel study on acidic podzolic samples from the Veluwe, the Netherlands.

This may indicate that these compounds somehow become associated with the organic matrix left after base treatment, form new, insoluble, organic (macro) molecules or become associated with the inorganic matrix. Once more, it should be emphasized that without the presence of a soil residue this phenomenon does not occur. The question arises as to the impact of exposing potentially reactive mineral surfaces to a suspension in which small particles and molecules of OM are in solution (Baldock and Skjemstad, 2000). It should be emphasized that the OM in solution not only comprises small lipid compounds such as individual alkanoic acids and hydroxy alkanoic acids. The treatment with base, i.e. 1M KOH in MeOH, will also cause other organic substances to go into solution (e.g. phenolics, saccharides and flavanoids) (Saiz-Jiminez, 1996; Miyajima et al., 2001). Humic substances are known to be strongly sorbed on allophane (e.g. Yuan et al., 2000; Farmer and Lumsdon, 2001). Acidification of the reaction mixture will therefore most probably cause a co-precipitation of mainly humic substances, i.e. large micellar aggregates composed of smaller molecules ranging from high to low molecular weight (Piccolo et al., 1996a, b), and LMW hydrolysis products. Such a co-precipitation may lead to the formation of the above mentioned new, insoluble, organic (macro) molecules in which the LMW hydrolysis products might be directly attached to the organic matrix or become trapped inside new formed structures (Grasset and Amblès, 1998).

Moreover, under the humid weathering environments of Madeira Island, the colloidal fraction is typically dominated by Al-organic complexes or allophane/imogolite, depending on the pH and the organic matter characteristics of the weathering environment (Madeira et al., 1994). Al-organic complexes occur preferentially in pedogenic environments that are rich in organic matter and have a pH value of 5 or lower (Shoji et al., 1985; FAO, 1998; Dahlgren, 1994; Wesselink et al., 1996). Non-crystalline hydrous oxides such as allophanes, especially found in slightly acidic and acidic soils such as Andosols, are likely to provide more sites for organic matter adsorption in soils than the surfaces of micaceous clay minerals (Dahlgren, 1994). Allophanes have a very high adsorption capacity for organic molecules and the exceptionally high levels of organic matter common to all soils of high allophane content appear to indicate that there is a very important interaction between the organic matter and inorganic materials, i.e. amorphous Al and Fe structures, in (such) soils (Shoji et al., 1985; Boudot et al., 1988; Kaiser and Zech, 1999; Oades, 1988; Greenland, 1971; Martin et al., 1966, 1972). Based on this latter aspect in combination with the strong metal complexing ability known for aromatic acids, hydroxy alkanoic acids and α, ω-diacids (Davies et al., 1969; Hue et al., 1986; Ochs et al., 1994; Knauss et al., 1997; Dynes and Huang, 1997; Ainsworth et al., 1998; Wang et al., 2000; Kaiser and Guggenberger, 2000 and references therein), we suggest that the these acidic moieties may also interact with the inorganic soil matrix, e.g. a kind of Al/Fe interaction (Ulrich et al., 1988; Ochs et al., 1994), as soon as the pH of the reaction mixture, or soil, becomes equal to 5 or lower (e.g. pH 3, experiment III). Further studies to test these hypotheses are currently being undertaken.

Despite the suggested behaviour of the acidic moieties mentioned above, only long- chain hydroxy alkanoic acid moieties seem to become associated with the residual soil matrix upon acidification of the reaction mixture (Fig. 5.5, experiment III), thereby causing only hydrophilic short-chain diacids and aromatic acids to be eventually identified using GC and GC-MS (Fig. 5.5A). This phenomenon may be caused by a competitive sorption of hydrophilic and hydrophobic fractions, leading to a preferentially strong binding of hydrophobic moieties under conditions of limited available sorption sites (Kaiser and Zech, 1998a, b).

Moreover, their behaviour causes these long-chain aliphatics to remain in the soil residue after base-treatment followed by direct acidification of the reaction mixture (experiment III) instead of being removed. Since a standard base-treatment is part of the extraction method that eventually leads to a chemically resistant SOM fraction, precipitation of long-chain aliphatics after this base-treatment may cause a bias of the view on chemically resistant SOM. The precipitation of long chain aliphatics in the soil residue after base treatment followed by acidification of the reaction mixture to a low pH (this paper) will cause the aliphatic signal of residual chemically resistant SOM fractions to be erroneously construed as being too aliphatic due to a contribution of ester-related compounds that became incorporated during base treatment. Therefore, these results emphasize the importance of a separation of base extract and soil residue prior to pH adjustments to obtain a more complete insight into SOM.

5.4.5 DCM precipitate

The precipitate at the DCM/Methanol-water interface has been suggested to consist almost exclusively of aliphatic biopolymers (Tegelaar et al., 1993, 1995). The formation of this aliphatic material is suggested to be explained by the accumulation of the biopolymers suberan and cutan; i.e. suberan would exhibit a “humic acid-like behaviour” where it, like humic acids, precipitates after acidifying the 1M KOH in MeOH solution in the separation funnel (Tegelaar et al., 1995). However, this explanation is unlikely, considering the presence of the DCM precipitate at both low and high pH values. If the material would exhibit “humic acid-like behaviour”, it should only precipitate at low pH.

Piccolo *et al*. (1996b, 1999) suggested that humic substances are formed because micelle-like aggregates, having few dispersed negative groups, can get closer to each other in solution than fulvic substances, which may be regarded as stable micelle-like materials in which there are enough acidic functional groups to keep the fulvic micelles in solution. When the humic moieties come close to each other, favourable stereochemical configurations lead to the formation of hydrophobic interactions that are strong enough to bind these humic molecules together and to cause their flocculation into aggregates of apparent high molecular weight (Piccolo et al., 1996b, 1999). Because both humic and fulvic substances are extracted during a base treatment in addition to LMW hydrolysis products, this flocculation, based on hydrophobic interactions, may explain the formation of the DCM precipitate, which is formed directly as water extractable organic matter is added to the MeOH phase in the separation funnel. Piccolo *et al*. (1996b, 1999) further indicated that humic micelle-like structures may be thought of as having the hydrophilic components (i.e. carboxyl groups) mainly at the solution interface, whereas the hydrophobic constituents (i.e. alkyl groups) form a hydrophobic phase at the micelle’s interior (Tantord, 1980). This behaviour may explain the accumulation of the DCM precipitate at the hydrophobic/hydrophilic DCM-water/MeOH interface, where hydrophobic constituents can enter the DCM phase, while hydrophilic components remain in the water/MeOH layer. We suggest that acid and hydroxy-substituted aliphatic monomers, derived from the hydrolysed ester-fraction in the analysed soil sample, together with micelle-like structures of humic material, may gather at the DCM-water/MeOH interface until they are hydrophobic enough (i.e. protonated) to enter the DCM phase. At the interface they may be present as fatty acid salts. These salts are known to produce alkenes and alkanes upon pyrolysis (Hartgers et al., 1995), which may be an additional explanation for the alkenes and alkanes found in the pyrolysates of these fractions (Tegelaar et al., 1993, 1995).

5.5 Conclusions

The influence of pH adjustments on the composition of soil organic matter (SOM) released during base treatments from an acidic andic forest soil, was studied using GC and GC/MS. The abundance and distribution of fatty acids in the TLE is in concordance with TLE data from other acidic soils as opposed to alkaline soils. Results obtained after direct pH adjustment of the extract (experiment I) indicated that the relative amounts of identified compounds are, as expected, pH dependent, i.e. based on pK_a values and hydrophobicity. Therefore, *n*-alkanols and *n*-alkanoic acids are the most dominant compound classes identified at high pH. ω-Hydroxy, ω-dihydroxy and trihydroxy alkanoic acids become dominant at lower pH values while phenolic and short-chain ($<C_{12}$) ω-hydroxy and α, ω-diacids are found only after acidification to the lowest pH values (i.e. pH 3 and 1). This phenomenon may cause an under or over estimation of certain compound classes and a biased view on ester-related moieties in soils. Compounds obtained after acidification of the extract to moderate and low pH make up the most important part of the total amount of lipids hydrolysed during base-treatment. However, the total weight of high pH fractions comprised up to 21% of the total yield.

Besides common known modes of occurrence of ester-related moieties in soils, e.g. the biopolyesters cutin and suberin, we believe that aromatic acids, ω-hydroxy alkanoic acids and α, ω-diacids, are in part also derived from complexes between organic and inorganic constituents, e.g. Al/Fe organic complexes. This because these moieties are known for their strong metal complexing ability and considering that we did not discriminate between possible sources of compounds released during the standard base treatment, i.e. biopolyesters or other sources.

Direct acidification after base treatment of the reaction mixture (experiment III), i.e. soil and extract, to pH 3, released only part of the hydrolysed compounds due to precipitation of hydrophobic long-chain aliphatic moieties. This may indicate that these compounds somehow become associated with the organic matrix left after base treatment, form new, insoluble, organic (macro) molecules or become associated with the inorganic matrix upon acidification of a base extract in the presence of a soil residue.

The DCM precipitate, formed at the DCM-water/MeOH interface, is suggested to contain acid and hydroxy substituted aliphatic monomers, derived from the hydrolysed ester-fraction in the analysed soil sample, in addition to micelle-like structures of humic material, rather than consist almost exclusively of aliphatic biopolymers (i.e. suberan/cutan).

Based on all the findings presented in this paper, step-wise extraction (experiment II) after separation of base extract and soil residue is recommended because all hydrolysed moieties are removed (i.e. can not contribute erroneously to chemically resistant SOM) and the various products released can be studied in detail providing greater insight into the chemical composition of ester-related moieties in soils. However, it should be noted that direct acidification of the extract to pH 1 (experiment I) remains a vary practical method to obtain a rapid basic insight into the bulk composition of LMW ester-related moieties released after a standard base treatment.

Acknowledgements. This study was supported by the Institute for Paleoenvironment and Paleoclimate Utrecht (IPPU). We also thank A. Oonincx for her help during analysis of the podzol sample. M. van Alphen is thanked for his help during GC and GC/MS analyses.

Chapter 6

A qualitative study on the chemical composition of ester-bound moieties in an acid andic forest soil

The solvent insoluble ester-bound moieties, released after base treatment from an acidic andosolic forest soil A-horizon from Madeira, were sequentially extracted and analysed using gas chromatography and gas chromatography-mass spectrometry. The separation of all moieties released into several pH “subfractions” allowed the various products released to be studied in detail. The distributions obtained for 2-alkanols, diols, *n*-alkanols, *n*-alkanoic acids, ω-hydroxy acids, trihydroxy acids, dihydroxy diacids and phenolic acids indicate a suberin dominated origin of ester related moieties in this andosolic soil A-horizon. However, a small contribution from plant cutins can not be ruled out completely. Besides plant biopolyesters, the detection of *iso* and *anteiso* alkanoic acids, together with short-chain di and ω-hydroxy acids suggests an additional bacterial-derived contribution. In addition to being attached to intact biopolyesters or molecules trapped in the insoluble organic macromolecular network present in soils, we suggest that hydroxy, *n*-alkanoic, aromatic and diacid moieties released upon base treatment of andosolic soils may occur as Al/Fe organic complexes.

6.1 Introduction

Organic carbon in soils is widely considered to be a complex mixture of a variety of plant, animal and microbial derived compounds (e.g. Schnitzer and Kahn, 1972; Stevenson, 1994). CPMAS ^{13}C NMR studies of soils indicate that considerable portions of the organic carbon in forest soil organic matter are of an aliphatic nature (e.g. Kögel-Knabner et al., 1992), although recent data suggest that such interpretations may have to be treated with caution (Poirier et al., 2000). Three main sources of plant-derived aliphatic compounds have been distinguished in soils: (a) free (extractable) lipids, (b) the biopolyesters cutin and suberin and (c) non-hydrolyzable biopolymers, such as cutan and suberan (Nip et al., 1985; Tegelaar et al., 1989a, 1995; Augris et al., 1998; Nierop; 1998).

Soil lipids are mainly of plant origin, with a minor contribution resulting from bacterial activity (Amblès et al., 1983, 1989; van Bergen et al., 1997; Bull et al., 1998) or from the transformation of plant residues by soil microflora (Amblès et al., 1985). Both suberin and cutin are considered to be important sources of solvent-extractable lipids, which may account for as much as 30% of the alkyl carbon in forest soils. Moreover, it has been shown that the greater part of the alkyl signal in the ^{13}C NMR spectra after thorough extraction with organic solvents, can be ascribed to cutin and suberin (Kögel-Knabner et al., 1992; Riederer et al., 1993).

The chemical composition of these biopolyesters has been studied using chemical degradation methods such as alkaline hydrolysis (e.g. Holloway, 1982;Tegelaar et al., 1995; Bull et al., 2000b), treatment with BF_3/CH_3OH (e.g. Kolattukudy, 1980a; Matzke and Riederer, 1991; Riederer et al., 1993; Kögel-Knabner et al., 1992), pyrolysis in the presence of tetramethylammonium hydroxide (TMAH) (e.g. Nierop, 1998; Santos Bento et al., 2001) and phase transfer catalysed hydrolysis (Grasset and Amblès, 1998).

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Cutin is present in cuticles covering all aerial parts of higher plants. The above mentioned studies led to the recognition that cutin is a biopolyester mainly based of hydroxy fatty acids. Long-chain *n*-alkanoic, ω-hydroxy, dihydroxy, trihydroxy and epoxy-hydroxy acids constitute the major aliphatic monomers. Of these compounds, dihydroxyhexadecanoic acids (i.e. C_{16} family, Kolattukudy, 1980a) or 9,10-epoxy-18-hydroxy acids (i.e. C_{18} family, Kolattukudy, 1980a) have been identified as major components (Kolattukudy, 1980a; Holloway, 1982; Ryser and Holloway, 1985; Matzke and Riederer, 1991). In addition to these aliphatics, small amounts of phenolic acids such as *p*-coumaric and ferulic acid might also be esterified to cutins (Kolattukudy, 1980a; Holloway, 1982).

The other biopolyester, suberin, is an abundant, complex, intractable, cell wall polymeric network that forms both protective and wound-healing layers in barks, woody stems and underground parts, i.e. roots (Kolattukudy, 1980a, 1984; Walton, 1990). Among the various cell wall polymeric assemblies, suberization stands out as something of an anomaly because it involves the deposition of both polyaromatic and polyaliphatic moieties (Kolattukudy, 1980a). These two moieties of suberized tissue are spatially separate (Bernards and Lewis, 1998). The polyaromatic domain of suberized tissues comprises covalently cross-linked hydroxy-cinnamic acids and their derivatives, principally amides (Bernards et al., 1995; Bernards and Lewis, 1998). The aliphatic domain is dominated by α, ω-dioic acids and ω-hydroxy acids. Other aliphatic monomers identified in suberins include *n*-alkanoic acids, dihydroxy diacids, trihydroxy acids, epoxy hydroxy acids and *n*-alkanols (Kolattukudy, 1980a; Ryser and Holloway, 1985; Matzke and Riederer, 1991; Riederer et al., 1993; Cordeiro et al., 1998).

Comparative studies have been undertaken into the composition of cutins and suberins to develop criteria for differentiating between the aliphatic portions of the two types of biopolyesters by means of the chemical composition of the depolymerisates (e.g. Matzke and Riederer, 1991; Kolattukudy, 1980a; Holloway, 1982, 1983; Walton, 1990; Bull et al., 2000b). Such criteria are necessary, considering the importance of clarifying the contribution of roots and leaves, with respect to the question whether soils act as a sink or a source for atmospheric CO_2 (Gorissen and Cotrufo, 2000) and the importance of distinguishing cutin from suberin as a source of lipids and aliphatics in soils (e.g. Nierop, 1998; Bull et al., 2000b). The comparative studies revealed that cutin and suberin are closely related chemically, since both consist mainly of hydroxy and epoxy-substituted alkanoic acids. However, there are a number of differences in their monomeric and tertiary structure (Tegelaar et al., 1995). Cutins are characterized by substantial amounts of dihydroxyhexadecanoic acids, whereas suberins contain considerable amounts of α, ω-dioic acids and long-chain ($>C_{18}$) monomers (Matzke and Riederer, 1991). Based on these differences, a set of eight variables (i.e. mass percentages of several compound classes such as *n*-alkanoic, α, ω-alkanedioic and ω-hydroxy acids) was used to distinguish the relative contributions of suberin and cutin to (soil) organic matter (Matzke and Riederer, 1991).

These criteria have been used to study the basic processes determining the fate of these biopolyesters in soils (e.g. Riederer et al., 1993) and to assess the origin and fate of aliphatic organic compounds in terrestrial soils (e.g. Nierop, 1998; Bull et al., 2000b). For these kinds of studies, which intend to provide greater insight into the chemical composition of soil organic matter (SOM), it is of crucial importance that the various products released after base treatment, i.e. ester related moieties, can be studied in detail.

In this chapter we present the results of a qualitative study on the chemical composition of ester-related compounds in an acidic andosolic forest soil A-horizon. The ester related compounds, released after a standard base treatment, have been separated into six pH subfractions through a sequential pH extraction. In such a sequential extraction, all hydrolysed moieties are removed and the various products released can be studied in detail providing greater insight into the chemical composition of SOM (Chapter 5). The six pH subfractions obtained have been studied using gas chromatography (GC) and GC-mass spectrometry (GC-MS). The results will be discussed in terms of the origin of compounds identified and their mode of occurrence in this andosolic forest soil.

6.2 Materials and methods

6.2.1 Soil profile

The soil sample used was taken from an andosolic A-horizon from Madeira island. The sample was obtained from a hill near the village of Poiso (altitude 1175 m). The vegetation consisted mainly of grasses and ferns, minor contributions being dandelion, oak and beech. Tree- and some grass roots were found to a depth of about 80 cm, but most of the intensive “root-activity” was found in the top 0-40 cm consisting mainly of grass fibrils. The A horizon used, i.e. 15-50 cm, was characterized by a non-friable structure, clay aggregates with a diameter up to 1 cm, and highly weathered basalt fragments up to a diameter of 10 cm. The deeper layers were formed by a non friable, compact layer of weathered basalt, the parent material for this andosol (Madeira et al., 1994).

6.2.2 Sample pre-treatment, total organic carbon measurement and solvent extraction

The soil sample was air dried and wrapped in aluminium foil. The soil sample was subsequently oven dried at 60°C and sieved over a 2 mm and a 250 μm sieve to remove large roots and basalt fragments. Total organic carbon content (TOC%) of the dried soil sample was measured using a Fisions Instruments NA 1500 NCS analyser, with a cycle time of 180 s, a source temperature of 190°C and an oxygen flow of e.a. 30 l/min. Approximately hundred gram of sieved (<250 μm) sample was Soxhlet extracted using dichloromethane/methanol (DCM/MeOH) (9:1v/v) for 24 h. The residue was air dried.

6.2.3 Base hydrolysis followed by sequential extraction

Twenty gram of the dried residue was base treated for 1 h at 70°C using a 1M KOH in 96% MeOH solution (Fig. 6.1). After cooling, extract and residue were separated through centrifugation (3 min 3000 rpm) and the supernatant was removed and collected in a separation funnel. The residue was further extracted with water (3x), methanol/water (1:1 v/v; 1x), methanol (2x) and dichloromethane (3x). After each extraction step, the suspensions were centrifuged and the supernatants added to the previously collected supernatant. The pH (13.7) of the collected extract was adjusted to pH 11 using HCl (37%). DCM (80 ml) was added to the extract to obtain phase separation. The DCM layer was collected and the phase separation with DCM (200-300 ml) was repeated. The collected and combined DCM phase was rotary evaporated to complete dryness. The dry extract obtained was dissolved in DCM/isopropanol (2:1 v/v) and filtered using a Pasteur pipette packed with defatted wool, 0.5 cm Na_2SO_4 and 2 cm SiO_2 and dried using N_2. An aliquot of these filtered extracts was derivatized using BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide, containing 1% trimethylchlorosilane; heated for 1 h at 70°C), dried using N_2, dissolved in hexane and analysed using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

The pH of the MeOH/H_2O phase in the separation funnel was subsequently adjusted to pH 9, 7, 5, 3 and 1. After each adjustment, the same procedure of phase separation, derivatisation and preparation for GC and GC-MS, was repeated as described above (Fig. 6.1).

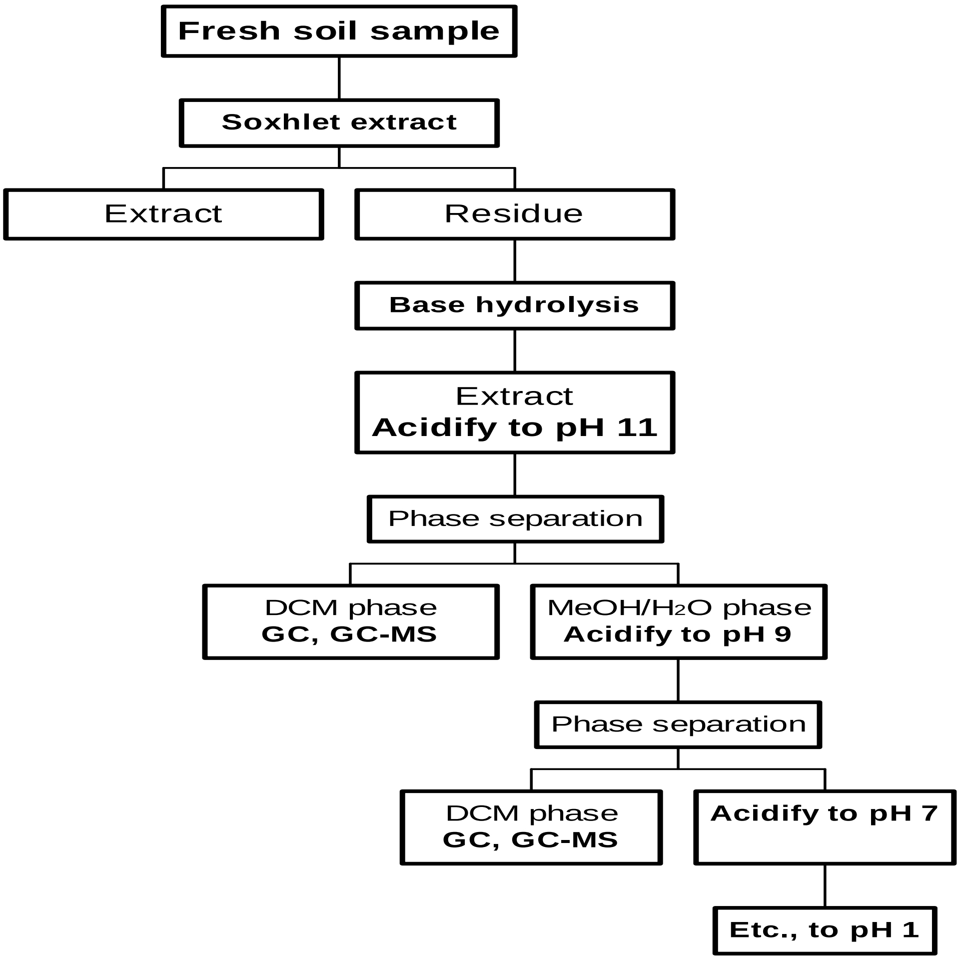

Figure 6.1 Schematic flow chart of chemical treatments indicating Soxhlet extraction subsequently followed by base-treatment of the residue and step wise acidification of so obtained base extract.

6.2.4 Gas chromatography (GC)

GC analyses were performed using a Hewlett-Packard 6890 series gas chromatograph equipped with a CP-sil 5CB silica column (50m x 0.32mm, film thickness 0.12µm). Derivatized extracts (1.0 µl) in hexane were injected on-column. The oven temperature was programmed from 70°C to 130°C at 20°C min^{-1} and from 130°C to 320°C (isothermal for 20 min) at 4°C min^{-1}. Compounds were detected using a flame ionisation detector (FID) at 325°C. Helium was used as carrier gas.

6.2.5 Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses were performed using a Hewlett-Packard 5890 series II gas chromatograph connected to a Fisions instruments VG platform II mass spectrometer operating at 70 eV, scanning the range *m/z* 50-650 in a cycle of 0.65 s. The capillary column and temperature programme were as described for the GC analyses. Compound identification was based on data published in the literature (e.g. Kolatukudy and Agrawal, 1974; Kolattukudy, 1980a; Holloway, 1982; Riederer et al., 1993; Walton, 1990).

6.3 Results and discussion

The separation of organic compounds, released during a standard base treatment, into different pH fractions is based on both their pK_a values and hydrophobicity. Separation based on pK_a is probably more important for short-chain moieties than for long-chain moieties, which are mainly separated based on their hydrophobicity. Organic acids will most probably enter the hydrophobic DCM phase as the pH of the MeOH/H_2O layer equals, or becomes even lower than their respective pK_a values. The TOC% of the dried soil sample was 7.1%. Net weight and weight fractions of this TOC% are presented in Table 6.1, together with the lipid contents of the pH fractions obtained. The total lipid yield obtained through sequential extraction after base treatment constituted 2.52% of the TOC in the soil sample. Yields obtained for each pH fraction are plotted in Fig. 6.2, gas chromatograms of pH fractions obtained are presented in Figure 6.3. Yields obtained increase from pH 11 to pH 5, to decrease again to the lowest yield obtained after adjustment to pH 1. Compounds obtained in the pH 11 fraction constitute no more than 6% by weight of the total yield obtained, against 28% for the pH 5 fraction. Altogether, 75% of the total yield is obtained in the pH 7, 5 and 3, fractions which are dominated by ω-hydroxy, dihydroxy, trihydroxy and hydroxydiacids (Fig. 6.3). However, in a step-wise extraction, also less abundant compounds (e.g. *n*-alkanols, C_{18} dihydroxy-α, ω-diacid, identified dihydroxy acids) can be studied, because of the separation into several pH “subfractions”. Note that the pH 7 fraction (Fig. 6.3c) is somewhat overconcentrated with respect to C_{16} ω-hydroxy acid to allow other compounds to be visualised.

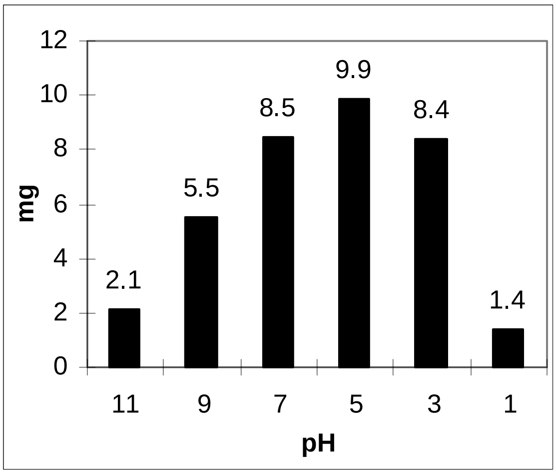

Figure 6.2 Yields obtained versus pH of subfractions obtained through step wise extraction of the base extract.

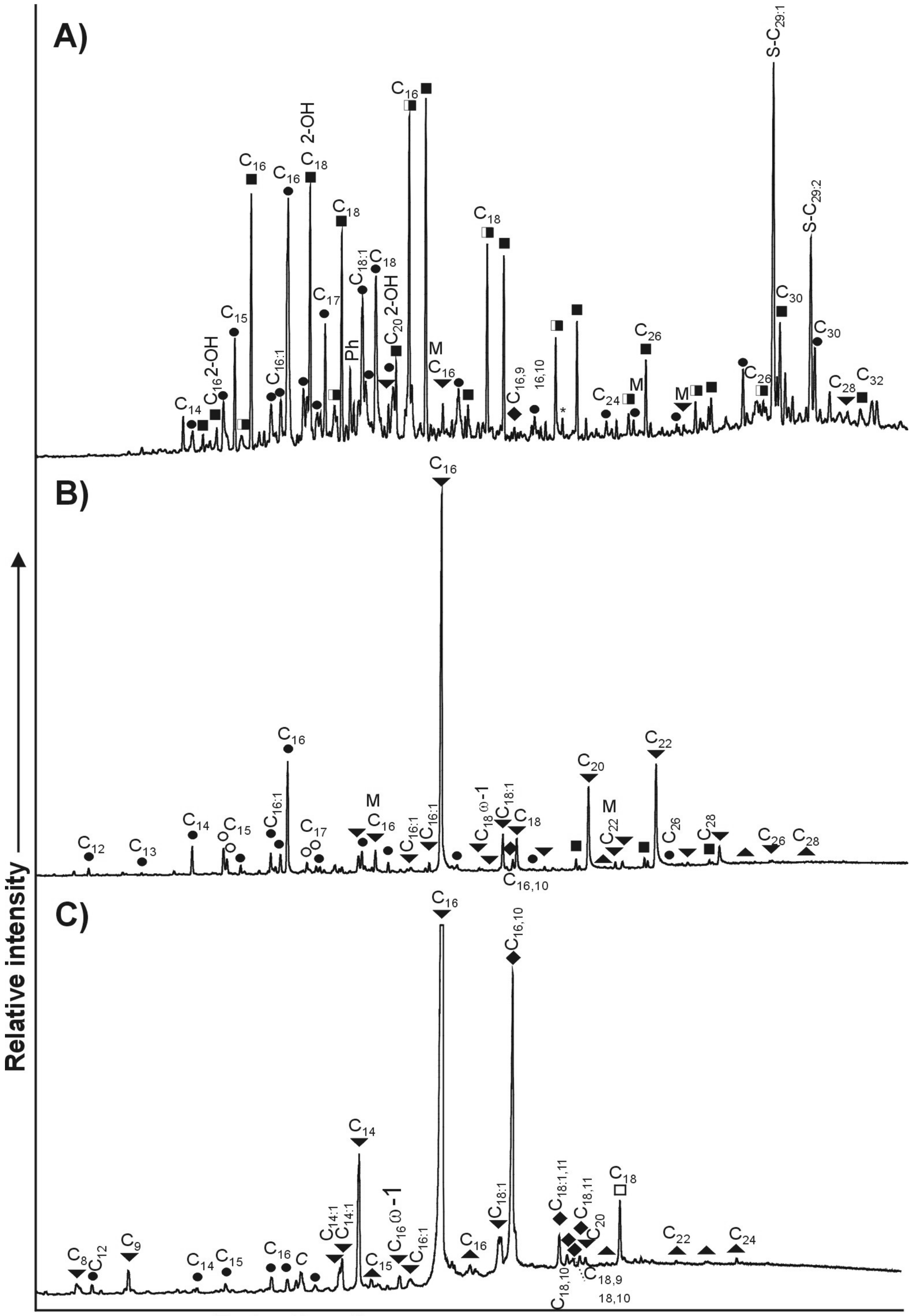

Figure 6.3. Gas chromatograms of base extracts after pH adjustment to (A) pH 11, (B) pH 9, (C) pH 7, (D) pH 5, (E) pH 3, (F) pH 1. * : Phthalates, ■: *n*-alkanols, ●: *n*-alkanoic acids,▼: ω-hydroxy acids,▲: Diacids, 2-OH: 2-alkanols,◨: diols,◆ : dihydroxy acids,□ : trihydroxy acids,▽: hydroxy diacids,◇: dihydroxy diacids,○ : *iso* and *anteiso* branched compounds, Ph : Phytol. *A*: 4-hydroxy-3-methoxy benzoic acid, *B*: *cis* en *trans* *p*-coumaric acid (diTMS), *C*: *cis* en *trans* ferulic acid (diTMS). C_x above the peaks refers to the total number of carbon atoms. Number after the comma refers to the position of the hydroxy group. Number after colon refers to the total number of double bonds. $C_{16,9/10}$ refers to a midchain hydroxy group either on position 9 or 10. S-C_x refers to steroids with x referring to the total number of carbon atoms.

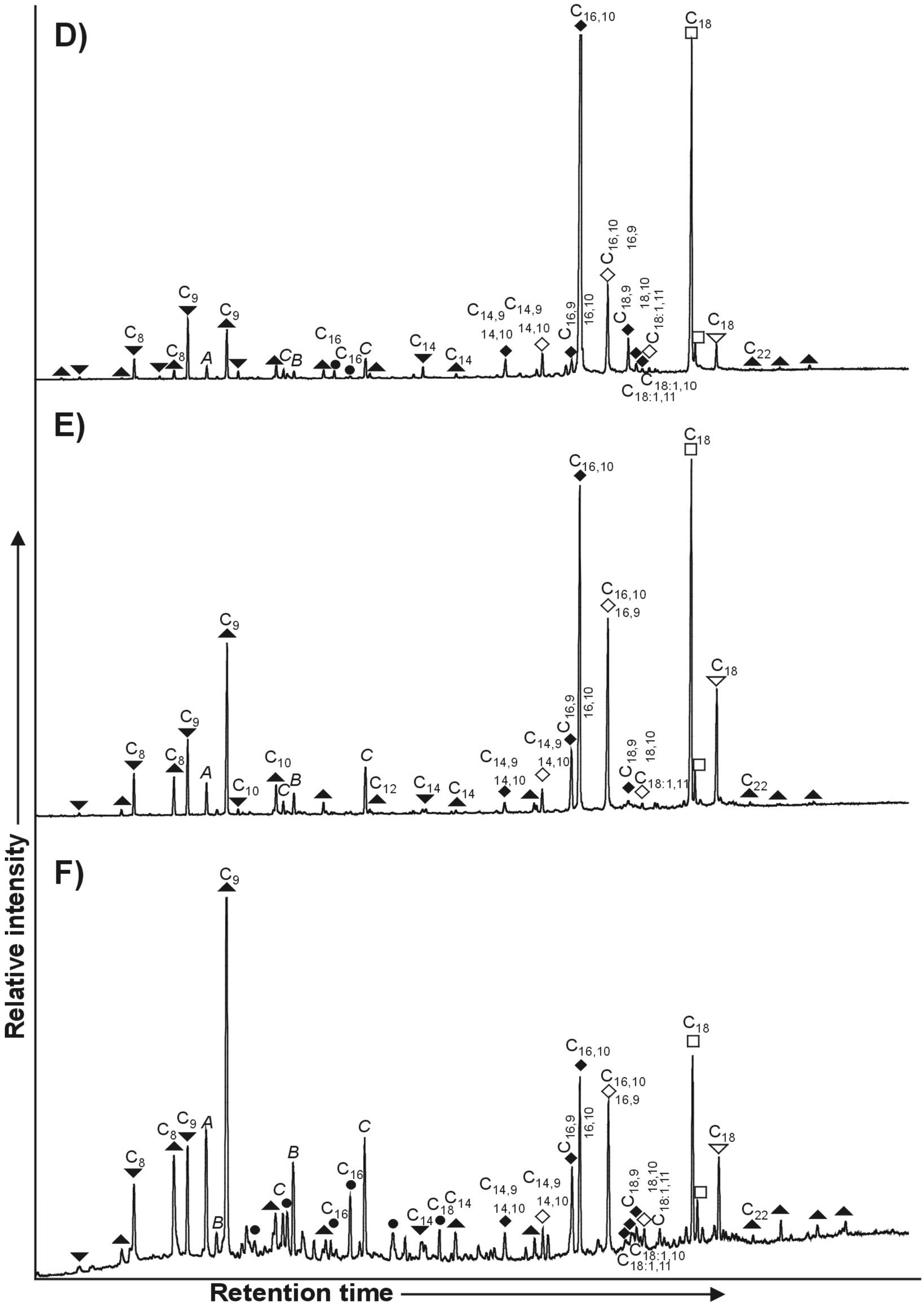
D)
E)
F)
Relative intensity
Retention time
C8
C9
A
B
C
C10
C12
C14
C16
C18
C22
C14,9
14,10
C16,9
16,10
C16,10
16,9
C18,9
18,10
C18:1,11
C18:1,10
C18:1,11

Fig. 6.3 d, e, f.

6.3.1 Alcohols

n-Alkanols are found in both the pH 11 and pH 9 fraction, although their relative contributions to the total of lipids identified was found to be far more important in the pH 11 fraction (Table 6.1, Fig. 6.3A). Homologues in the C_{15} to C_{32} range are observed and show a strong, even over odd predominance, the C_{20} homologue being most abundant. This wide range and predominance of C_{20}, together with the relative importance of shorter-chain, i.e. C_{15}-C_{20}, members has been reported in hydrolysis products of suberins (e.g. Ryser and Holloway, 1985; Walton, 1990) and of humin (Grasset and Amblès, 1998). The distribution is, however, somewhat unusual considering the more commonly found dominance of, suberin derived, C_{22} and C_{24} *n*-alkanols (e.g. Walton, 1990; Nierop, 1998; Riederer et al., 1993). According to Grasset and Amblès (1998), identification of *n*-alkanols, mainly in the C_{12}-C_{30} range, with dominant C_{16}, C_{18}, C_{20}, C_{22}, C_{24} and C_{26} members together with a prominent *iso* C_{15} alkanol component may direct to an important bacterial input. However, no *iso* or *anteiso* members that would confirm such a contribution were identified in the hydrolysate of the andosolic Soxhlet residue. In general, *n*-alkanols are known biopolyester-derived compounds, and the presence of homologues containing more than 18 carbon atoms is considered to distinguish suberin from cutin (Kolattukudy, 1980a; Matzke and Riederer, 1991), although C_{16} *n*-alkanols have also been identified in plant suberins (Walton, 1990).

Table 6.1 Lipid contents of pH fractions obtained through step-wise extraction, net weight, weight fractions based on TOC% of dried soil sample, and totals.

Compound Classes	pH 11	pH 9	pH 7	pH 5	pH 3	pH 1
Weight (mg) 35.8*	2.1	5.5	8.5	9.9	8.4	1.4
Weight (%) 2.5*	0.15	0.39	0.60	0.70	0.60	0.10
n-Alkanols	C_{15}-C_{32} (C_{20})	C_{24}-C_{28}	---	---	---	---
2-Alkanols	C_{16}-C_{20} (C_{18})	---	---	---	---	---
Diols	C_{12}-C_{26} (C_{16})	---	---	---	---	---
Steroids	$C_{29:1}$, $C_{29:2}$	---	---	---	---	---
n-Alkanoic acids	C_{14}-C_{30} (C_{16})	C_{12}-C_{26} (C_{16})	C_{12}-C_{17}	C_{16}	---	C_{14}-C_{18}
Di-acids	---	C_{19}-C_{28}	C_{15}-C_{24}	C_6-C_{24} (C_9)	C_7-C_{24} (C_9)	C_7-C_{25} (C_9)
Aromatic acids	---	---	---	*A, B, C*	*A, B, C*	*A, B, C*
ω-Hydroxy acids	C_{16}, C_{28}	C_{14}-C_{28} (C_{16})	C_8, C_9, C_{14}, C_{16} (C_{16})	C_7-C_{14} (C_9)	C_7-C_{14} (C_9)	C_7-C_{14} (C_9)
(ω-1)-Hydroxy acids	---	C_{18}	C_{16}	C_8	---	---
Dihydroxy acids	C_{16}	$C_{16,10}$	C_{16}, C_{18}, $C_{18:1}$ ($C_{16,10}$)	C_{14}-C_{18} ($C_{16,10}$)	C_{14}-C_{18} ($C_{16,10}$)	C_{14}-C_{18} ($C_{16,10}$)
Trihydroxy acids	---	---	C_{18}	C_{18}	C_{18}	C_{18}
Hydroxy diacids	---	---	---	C_{14}, C_{16}, $C_{18:1}$,(C_{16})	$C_{14,\ 16}$, $C_{18:1}$	C_{14}-C_{18} ($C_{16,\ 9/10}$)
Dihydroxy diacids	---	---	---	C_{18}	C_{18}	C_{18}
Phytol	C_{20}	---	---	---	---	---
Methyl Esters	C_{16},C_{24},C_{26}	C_{16}, C_{22}	---	---	---	---

*Totals, C_x-C_y ranging from C_x to C_y, compounds in parentheses are the most dominant homologues, number after colon refers to double bond, number after comma refers to position of hydroxy group, slash forward to other positions. *A*: 4-hydroxy-3-methoxy benzoic acid, *B*: *p*-coumaric acid, *C*: ferulic acid. Methyl esters (MEs) are known to be formed in a MeOH/H_2O phase if left standing for more than one day.

2-Alkanols identified range from C_{16} to C_{20} (Table 6.1, Fig. 6.3A). Only even numbered compounds have been identified, C_{18} being the major homologue. So far, only odd numbered 2-alkanols, i.e. C_{15} to C_{21}, have been reported in polymeric materials obtained from several plants and appear to be more common in suberins than in cutins (Kolattukudy, 1980a; Walton, 1990).

C_{12} to C_{26} α, ω-diols, maximizing at C_{16}, contribute to the first pH fraction obtained (Table 6.1, Fig. 6.3A). C_{12} and C_{14} (Matzke and Riederer, 1991; Riederer et al., 1993) together with C_{16} to C_{22} α, ω-diols, maximizing at C_{16} or $C_{18:1}$ (Walton, 1990) have been identified in suberin depolymerisates. We therefore suggest that other long-chain diols identified in the andosolic A-horizon depolymerate, i.e. C_{24} and C_{26}, are also derived from plant suberins.

β-sitosterol has been identified in depolymerisates from soils (e.g. Riederer et al., 1993; Amblès et al., 1991; 1996; Gobé et al., 2000) and suberins (e.g.Walton, 1990). Therefore, the other steroid identified, i.e. $C_{29:2}$, is most probably also derived from (ester bound) moieties (Gobé et al., 2000), the exact nature of which is unclear.

6.3.2 Acids

The distribution of *n*-alkanoic acids, ranging from C_{12} to C_{30}, is characterized by a strong, even over odd predominance for the long-chain (>C_{18}) members (Table 6.1, Fig. 6.3). *Iso* and *anteiso* branched C_{15} and C_{17} alkanoic acids are identified in the pH 9 fraction (Fig. 6.3B) and reflect, together with the distribution of C_{14}-C_{18} fatty acids maximizing at C_{16}, a bacterial input (Amblès et al., 1994a; Grasset and Amblès, 1998 and references therein). The distribution of long-chain, i.e. C_{20}-C_{32}, fatty acids with an even predominance is associated with higher plant input (e.g. Matsuda, 1978; Amblès et al., 1994a; Bull et al., 1998). *n*-Alkanoic acids occur as minor components in the cutin depolymerization products of most plants. These cutin-derived fatty acids are predominantly even carbon numbered, ranging from C_{12} to C_{18}, maximizing at C_{16} or $C_{18:1}$ (Holloway, 1982). Longer homologues (>C_{20}) are suggested to be mainly derived from suberins (Kolattukudy, 1980a; Walton, 1990; Ryser and Holloway, 1985; Matzke and Rieder, 1991; Bull et al., 2000b). The overall distribution of fatty acids identified in this acidic andosolic A-horizon has been observed previously in fractions obtained after alkaline hydrolysis of plant (Bull et al., 2000b) and soil residues (Riederer et al., 1993; Grasset and Amblès, 1998), although the long-chain (>C_{18}) compounds usually show a more pronounced dominance of the C_{22} and/or C_{24} member. Despite the lack of such a dominance, long-chain (>C_{18}) *n*-alkanoic acids identified in the andosolic A-horizon analysed are most probably derived from a suberin input because it was taken from a depth of about 30 cm, at which intensive root activity was one of the major visible characteristics.

Short chain diacids range from C_6 to about C_{14}, the most abundant member being C_9. Such a distribution has previously been observed in humin depolymerisates (e.g. Grasset and Amblès, 1998; Almendros et al., 1991). C_9 diacids have been suggested to originate from the enzymatic oxidation of Δ^9-unsaturated acids such as oleic acid (e.g. Regert et al., 1998; Grasset and Amblès, 1998). Thus, we believe that the short-chain diacids, together with short-chain ω-hydroxy acids (see later), are derived from bacterial degradation. Long-chain ($\geq C_{16}$) diacids are found only in relatively low concentrations, without a clear maximum (Fig. 6.3). The occurrence of some long-chain diacids, i.e. C_{16}, C_{18}, has been reported in plant cutins (Holloway, 1982), but they are commonly associated with a contribution from suberins (e.g. Kolattukudy, 1980a; Walton, 1990; Matzke and Riederer, 1991; Santos Bento et al., 2001).

In the lower pH fractions, i.e. pH 5, 3 and 1 (Figs 6.3D, E, F) aromatic acids are found. These acids include *p*-coumaric and ferulic acids. As mentioned in the introduction, these simple phenolic acids are known to be covalently bound to cutins (Kolattukudy, 1980a; Holloway, 1982) and, together with the 4-hydroxy-3-methoxy benzoic also identified, believed to be part of the aromatic domain of suberins (Kolattukudy, 1980a; Bernards et al., 1995; Bernards and Lewis, 1998).

6.3.3 Hydroxy acids

ω-Hydroxy acids contribute to all the pH fractions obtained (Table 6.1, Fig. 6.3). The long-chain (>C_{14}) members showing a strong, even over odd predominance, range from C_{14} to C_{30} and are indentified in the higher pH fractions, i.e. pH 11, 9 and 7 (Figs 6.3A, B, C). Only few, i.e. C_{16}, C_{18}, and C_8 (ω-1)-hydroxy acids have been identified as minor components in the andosolic depolymerisates (Table 6.1). ω-Hydroxy acids are very common constituents of cutins and often comprise 5-10% of the total monomers. The principal compounds are usually C_{16}, C_{18} $C_{18:1}$ and $C_{18:2}$ (Holloway, 1982). However, the presence of a high proportion of long-chain ($\geq C_{16}$) ω-hydroxy acids, maximizing at C_{16}, C_{18}, C_{22} and C_{24}, is mainly associated with suberins (Kolattukudy, 1980a; Walton, 1990). The same feature has also been shown by Matzke and Riederer (1991), who identified C_{16}-C_{26} ω-hydroxy acids in depolymerisates from periderms and roots and C_{12}-C_{16} ω-hydroxy acids in needle depolymerisates. In the hydrolysed andosolic Soxhlet residue, this dominance of $C_{18:1}$, C_{22} and C_{24} homologues in addition to C_{16} is, however, less pronounced. Bull *et al.* (2000b) suggested that the preferential loss of the C_{22} component is most likely due to biotic degradation, perhaps by micro-organisms which preferentially degrade the C_{22} component. Another explanation could be the variation in chemical composition amongst suberins, which range from mainly C_{22} to mainly C_{16} and C_{18} (Ryser and Holloway, 1985).). The relatively low abundance in the andosolic hydrolysate of 18-hydroxyoctadec-9-enoic acid is most likely the result of microbial β-oxidation or auto-oxidation of the double bond (Mlaker and Spiteller, 1996). This would most probably lead to short-chain ω-hydroxy acids (Simic et al., 1992; Chan, 1987; Gillan and Johns, 1982; Mlaker and Spiteller, 1996; Regert et al., 1998). In parallel with the observations made about short-chain diacids, these hydroxy acids range from C_7-C_{10}, maximising at C_9. They are suggested to be preserved through chemical bonding, most likely via ester linkages, into insoluble polymeric matrices (Regert et al., 1998). We believe that these compounds are indirectly derived from plant suberins considering that suberins are more important contributors to deeper soil horizons (Nierop, 1998; Bull et al., 2000b). However, an indirect cutin source can not be entirely ruled out.

Dihydroxy acids obtained by saponification of the solvent extracted andosolic sample range from C_{14} to C_{18} with 10,16-dihydroxyhexadecanoic acid being the most abundant component. All C_{14} and C_{16} dihydroxy acids identified have one of the hydroxyl groups in the ω-position and the other located at positions near the middle of the aliphatic chain at position 9 or 10, while C_{18} dihydroxy acids were found with the secondary hydroxyl located at position 10 or 11, the latter also containing a double bond. The principal component identified in cutins and suberins is usually 10,16- or 9,16-dihydroxy hexadecanoic acid (Kolattukudy, 1980a; Holloway, 1982, 1983; Ryser and Holloway, 1985). Smaller amounts of 8,16- and 7,16 isomers have also been identified. The C_{18} acids, which are less common dihydroxy acids, show a wide range of positional isomers from 7,18- through to 12,18-dihydroxy octadecanoic acid (Holloway, 1982). C_{14} acids occur only as minor components in the lower, i.e. pH 5, 3 and 1, pH fractions (Figs 6.3D, E, F). These acids are most probably also suberin derived acids, based on the importance of roots in our sample, although more highly oxygenated polyhydroxy acids are usually only minor components of plants suberins and more commonly associated with plant cutins (Matzke and Riederer, 1991).

The presence of substantial amounts of C_{18} 9,10,18-trihydroxy acid is the result of a contribution of both C_{18} trihydroxy acids originally present in suberized tissue (e.g. Walton, 1990; Nierop, 1998; Santos Bento, 2001) and those generated by hydrolysis of the corresponding C_{18} epoxide (Holloway, 1982). Both *threo*- and *erythro*- forms of the acid are detected (Figs 6.3D, E, F).

Hydroxy hexadecanoic diacids with a hydroxyl at position 9 or 10 have frequently been reported as minor components in cutin depolymerization products (Holloway, 1982). Besides the C_{16} acid, C_{14} and $C_{18:1}$, hydroxy diacids are also identified, although in relatively lower concentrations. These acids could be derived from cutins, but it should be noted that vertical chemical transport is extremely limited in this type of soils (Ugolini et al., 1988; Aran et al., 2001). We therefore suggest that these compounds are also suberin derived monomers considering the more likely suberin derived input of compounds in this soil sample. However, a contribution from cutins can not be ruled out completely considering that physical transport may not be excluded.

Like C_{18} trihydroxy acids, 9,10-dihydroxyoctadecane-1,18-dioic acids are derived both from original dihydroxy and epoxy substituted compounds. They are known to occur more commonly and in much larger amounts in suberins (e.g. Holloway, 1982; Santos Bento et al., 2001).

6.3.4 Mode of occurrence of the ester-related compounds

Compounds present in the hydrolysates of the solvent extracted sample may have been part of the biopolyester domains of suberins (and/or partly cutins). Lipids, i.e. fatty acids and sterols, can, however, also become ester bound to the insoluble organic matrix (Amblès et al., 1991; Regert et al., 1998) or be present as trapped molecules in the insoluble macromolecular network present in soils (Grasset and Amblès, 1998; Gobé et al., 2000), although the precise chemical/physical mechanism for this trapping is still unclear. These trapped lipids are suggested to be predominantly of bacterial origin (Grasset and Amblès, 1998), e.g. *iso* and *anteiso* C_{15} and C_{17} fatty acids.

Short-chain ω-hydroxy and diacids can be attached to the insoluble polymeric organic network but may also link to the inorganic matrix. Non-crystalline hydrous oxides such as allophanes, especially found in slightly acidic and acidic soils such as andosols, have a very high adsorption capacity for organic molecules (Dahlgren, 1994). The exceptionally high levels of organic matter common to all soils of high allophane content appear to indicate that there is a very important interaction between the organic matter and amorphous Al and Fe entities in (such) soils (e.g. Shoji et al., 1985; Boudot et al., 1988; Boudot, 1992; Kaiser and Zech, 1999; Saggar et al., 1996; Oades, 1988; Aran et al., 2001). In addition, aromatic, ω-hydroxy and diacids are known to have a strong metal complexing ability (e.g. Davies et al., 1969; Ochs et al., 1994; Schulten et al., 1996; Ainsworth et al., 1998; Kaiser and Guggenberger, 2000 and references therein). With this in mind and considering that we did not discriminate between sources, i.e. biopolyesters or other sources, we believe that short-chain ω-hydroxy, aromatic and diacids identified may also be derived from metal, i.e. Fe and Al, organic complexes (Chapter 5). Further studies to test this hypothesis are currently being undertaken.

6.4 Conclusions

In this study, a solvent extracted acidic andosolic forest soil A-horizon has been treated with base and subsequently sequentially extracted. The pH subfractions obtained have been studied in detail using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The subsequent removal of protonated compounds (pK_a dependent) after each pH adjustment avoids all products released to end up in the same fraction.

One would expect the hydrolysate of the solvent extracted sample to be dominated by suberin derived compounds, since the andosolic sample was taken from a depth of about 30 cm, at which intensive root activity was one of the major characteristics. The distributions obtained for 2-alkanols, diols, *n*-alkanols, *n*-alkanoic acids, ω-hydroxy acids, trihydroxy acids, dihydroxy diacids and phenolic acids confirm such a strong suberin-derived origin, although a, probably relatively small, cutin related input can not be ruled out completely. Apart from these plant biopolyesters, the presence of *iso* and *anteiso* alkanoic acids, together with short-chain di and ω-hydroxy acids suggests an additional bacterial-derived contribution. Finally, we suggest that hydroxy, *n*-alkanoic, aromatic and diacid moieties released upon base treatment of andosolic soils probably occur as Al/Fe organic complexes in addition to being attached to intact biopolyesters or molecules trapped in the insoluble organic macromolecular network present in soils.

Acknowledgements. This study was supported by the Institute for Paleoenvironment and Paleoclimate Utrecht (IPPU). M. van Alphen is thanked for his help during GC and GC/MS analyses. Prof. Dr. J. W. de Leeuw, Dr. A. Amblès and Dr. I. D. Bull are sincerely thanked for their constructive reviews of this chapter.

Chapter 7

Occurrence and distribution of ester-bound lipids in Dutch coastal dune soils along a pH gradient

The base hydrolysable fraction of roots and soil organic matter (SOM) present in mineral subsoils from oak forests (coastal dunes, the Netherlands) with a soil pH varying from pH ($CaCl_2$) 6.9 to 3.5 were analysed both quantitatively and qualitatively. Comparison of thermally assisted hydrolysis and methylation (THM) using tetramethylammonium hydroxide (TMAH) with base hydrolysis data confirmed that base hydrolysis is a very efficient way to analyse ester-linked compounds in soils. The compounds released from SOM upon base hydrolysis comprised largely suberin-derived lipids, which were clearly related to the oak vegetation and, in particular, to oak roots. Compared with fresh (fine) roots, which are largely responsible for the input of soil organic matter (SOM) in the subsoils, unsaturated and epoxy compounds decreased in soil, probably due to oxidation. The amounts of the saponifiable fraction increased upon acidification, while hardly any changes were observed in their compositions. This accumulation, without significant compositional alteration of ester-bound moieties in acidic soils, indicates that these chemically labile compounds can be preserved in soils.

7.1 Introduction

Soil organic matter (SOM) plays an essential role within the global carbon cycle. Although 80-90% of carbon is recycled as CO_2 into the atmosphere one year after litterfall, more carbon is present as SOM than as CO_2 and land biota together (Hedges and Oades, 1997). Hence, the soil compartment possesses a great potential to slow down decomposition rates. Both decomposition and humification are influenced by numerous factors that affect the activity of the decomposer community including soil texture, temperature, nutrient status, moisture and oxygen content, composition of the litter/SOM and soil pH (Stevenson, 1994).

For sandy soils it is generally known that with decreasing soil pH, SOM accumulates through reduced microbial activity. However, to what extent this increase in quantity accounts also for a change in SOM quality is only partly understood. For instance, fungi become relatively dominant compared with bacteria when soil pH decreases (Dinel et al., 1990; Bumpus, 1993), which may imply a considerable SOM differentiation as affected by soil acidification. Indeed, for free (extractable) soil lipids it is well-documented that their relative amounts increase when soil pH is reduced (Jambu et al., 1985; Dinel et al., 1990; Stevenson, 1994). However, whether non-extractable lipids, in particular macromolecular ester-bound moieties, are preserved at low soil pH as well, is as yet unclear.

Although ester-bound moieties are considered as (bio)chemically labile compounds (Derenne and Largeau, 2001), they are frequently found in soils (including sandy soils) and their importance often increases with humification (e.g. Kögel-Knabner et al., 1989; Almendros and Sanz, 1991; Riederer et al., 1993; Nierop, 1998; 2001; Grasset and Amblès, 1998; Nierop and Buurman, 1999; Bull et al., 2000b; Chefetz et al., 2002; Grasset et al., 2002; Rumpel et al., 2002). Notwithstanding other possible sources, the majority of ester-bound moieties in soil

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has been related, in those studies, to the biopolymers cutin and suberin. Together with waxes and sometimes cutan, cutin constitutes the cuticle that covers all aerial parts of plants (leaves, needles, flowers, fruits). Cutin constitutes mainly of C_{16} and C_{18} ω-hydroxyalkanoic acids with and without mid-chain hydroxy or epoxy groups in addition to *n*-alkanoic acids (Walton, 1990; Kolattukudy, 2001). All protective and wound-healing layers in barks, woody stems and underground parts (roots, tubers, stolons) that are not protected by cutin contain suberin. Originally, suberin was suggested to be an aliphatic biopolymer similar to cutin with a substantial contribution of phenolic acids or lignin (Kolattukudy, 1980a). Currently, it is believed that suberin is comprised of an aliphatic polyester and a polyphenolic domain, which are spatially segregated (Bernards and Lewis, 1998; Bernards and Razem, 2001). The nature of the polyphenolic domain is, however, still a matter of debate (Bernards, 2002). In addition to the building blocks present in cutin, the aliphatic part of suberin consists of ω-hydroxyalkanoic acids with chain lengths $\geq C_{20}$. Moreover, *n*-alkanols, α,ω-alkanediols and α,ω-alkanedioic acids with predominant chain lengths of C_{16}-C_{24} distinguish suberized tissues from those that are cutinized (Kolattukudy, 2001).

In a previous study it was found that increasing soil acidification and age led to an increase of the ‘suberin/lignin ratio’ in mineral subsurface horizons in Dutch coastal dune soils (Nierop and Verstraten, 2002). As an explanation, it has been hypothesized that cutin and suberin, i.e. labile ester-bound aliphatics, may form stable cross-links resistant to extensive decomposition (cf. Kögel-Knabner, 1993). The question arises whether the apparent relative increase of suberin in aforementioned soils is due to such stable bonds, or that esters are resistant enough to be preserved intact in soil.

The objective of the present paper is to study variations in (a) the quantitative contribution of ester-bound, non-extractable lipids to SOM and (b) the composition of ester-bound lipids, in relation to soil pH. Root and soil samples were taken from sandy subsurface soils where below-ground biomass almost entirely sources the SOM input (primarily fine roots). Soxhlet extracted residues of root and soil samples were subjected to base hydrolysis according to the methods presented in Chapters 5 and 6. The obtained extracts were analysed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). In addition, thermally assisted hydrolysis and methylation (THM) using tetramethylammonium hydroxide (TMAH) was applied to both Soxhlet extracted samples before and after base hydrolysis to verify the efficiency of the saponification.

7.2 Materials and methods

7.2.1 Sites description, sample collection and preparation

The study sites are situated in the Amsterdamse Waterleiding Duinen, a sandy dune area at the southeast of Zandvoort, west of Amsterdam (The Netherlands). At the end of the 19th century only a small part of the area was covered by vegetation. To prevent soil erosion and sand drifting, trees were planted. With time the soil at the Amsterdamse Waterleiding Duinen became acidified. At present some parts of the area have a completely decalcified soil (top 1 meter), whereas other sites are only partly decalcified and have a low pH (3-4) in the topsoil and a pH of around 7 in the subsoils where lime is still present.

The two study sites are dominated by common oak (*Quercus robur* L.) and their soils are characterized as Cambic Arenosols (FAO, 1990). Originally, oaks were naturally present, but plantation has also taken place. Duivendrift (DUI) is a forest where trees were planted in the early 1900s and has a current transition of decalcified soil material to carbonate-containing sand at a depth of 25 cm. Hoek van Klaas (HvK) has a present average decalcifying depth of 95-115 cm and was used previously for wood production. Although the exact plantation dates of the first trees are not precisely known (sometime in the beginning of the 19th century), the current trees are of a similar age as those of DUI.

Because both sites are located within close proximity (2 km) climatologic aspects were equal, as was the mineral composition of the parent material. As a consequence, apart from age of the forests and possible variations in plant growth and litter production, soil pH will be the only important factor determining SOM formation. Soil samples were dried at 30 °C for 24 h. and subsequently sieved over 1 mm to remove root fragments. Soil pH was measured in 0.01 M $CaCl_2$ (1:2.5 w/v). Due to the very low carbon contents (0.25-0.35%), which were too low to analyse SOM properly, we separated the sand from the rest of these soil samples by means of a density separation as described by Nierop and Buurman (1998) resulting into organic rich (> 95%) residues. Roots were collected during sampling of the soil samples and after sieving (see above). Therefore, only roots that were in the soil profiles, and thus representative parent materials of SOM, were taken from the field. Oak roots of 2-5 mm next to fine roots were present. After drying and milling, pyrolysis and THM of both types of oak roots gave similar distributions of all pyrolysis/THM products, and only minor differences in the ratio of aliphatic/aromatic compounds (fine roots having relatively more aliphatics). Therefore, a mixture of both root samples was used for further analysis as presented in this study. The soil samples analysed were derived from the subsurface horizons, i.e. the horizons below the A(E)h horizons, and are referred to as DUI-C1 [BC horizon (sampled at 8-25 cm depth) from DUI]; DUI-Ck [Ck horizon (sampled at 25-35 cm depth) from DUI]; HvK-C1 [(A)C horizon (sampled at 10-18 cm depth) from HvK].

7.2.2 Base hydrolysis

Root and soil samples were subjected to a saponification procedure according to Chapter 6. Prior to hydrolysis, about 2 g of each sample was extracted by a mixture of dichloromethane (DCM) and methanol (MeOH) (9:1 v/v) for 24 h. using a Soxhlet apparatus. The residues were air-dried. Approximately 1 g of the residue obtained was suspended in 1 M KOH in MeOH and refluxed for 1 h at 70°C. After cooling, the reaction mixture was centrifuged and the supernatant was transferred into a separation funnel. The residue was extracted with water (3x), MeOH/water (1:1) (1x), MeOH (2x) and DCM (3x). Each extraction was followed by centrifugation and all were collected into the separation funnel. The final extract was acidified with HCl to pH 2. Subsequently, the DCM layer was separated from the water/MeOH layer, which was again extracted with DCM (2x).

The DCM fractions were combined and after evaporation of the solvent, the extracts were re-dissolved in DCM/*iso*-propanol (2:1 v/v), eluted over (anhydrous) Na_2SO_4 and silica to remove water and very polar compounds and the solvent removed under a gentle stream of nitrogen. The so obtained dry extracts were weighed to calculate the total yield of ester-bound lipids. Aliquots were derivatised with *N*,*O*-bis(trimethylsilyl) trifluoro acetamide (BSTFA) containing 1% trimethylchlorosilane, dried using nitrogen, re-dissolved in hexane, and analysed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS).

7.2.3 Gas chromatography (GC)

GC analyses were performed on a Hewlett Packard 6890 series gas chromatograph. Derivatised extracts (1.0 μl) were separated by a fused silica column (Chrompack, 50 m, 0.32 mm i.d.) coated with CP-Sil 5CB (film thickness 0.12 μm). After on-column injection at 70°C the oven was heated at 20°C/min to 130°C followed by a rate of 4°C/min to 320°C and held there for 20 min. Compounds were detected by a flame ionisation detector (FID) at 325°C. Helium was used as carrier gas.

7.2.4 Gas chromatography/mass spectrometry (GC/MS)

GC/MS analyses were carried out on a Hewlett Packard 6890 series gas chromatograph coupled to a VG mass spectrometer (mass range *m/z* 50-650, ionisation energy 70 eV, cycle time 0.65 s). The capillary column and GC program were the same as for the GC analyses. Compound identification was based on data published in the literature (e.g. Kolattukudy and Agrawal, 1974; Kolattukudy, 1980a; Holloway, 1982; Walton, 1990; Riederer et al., 1993).

7.2.5. Thermally assisted hydrolysis and methylation (THM)

Thermally assisted hydrolysis and methylation (THM) was performed by adding a droplet of a 25% solution of tetramethylammonium hydroxide (TMAH) in water to a sample that was pressed onto a Curie-point wire, after which the sample was dried using a 100 W halogen lamp, and subsequently inserted into a Horizon Instruments Curie-Point pyrolyser. Samples were heated for 5 s at 600°C. The pyrolysis unit was connected to a ThermoQuest Trace GC 2000 gas chromatograph and the products were separated by a fused silica column (J & W, 30 m, 0.32 mm i.d.) coated with DB-5 (film thickness 0.25 µm). Helium was used as carrier gas. The oven was initially kept at 40°C for 1 min, then heated at a rate of 7°C/min to 320°C and maintained at that temperature for 15 min. The column was coupled to a Finnigan Trace MS mass spectrometer (mass range *m/z* 60-600, ionization energy 70 eV, cycle time 1 s). Identification of the compounds was carried out by their mass spectra using a NIST library or by interpretation of the spectra and by their GC retention times.

7.3 Results and discussion

Yields of extractable compounds released after base hydrolysis and pH values of soil samples are presented in Table 7.1. With decreasing soil pH in the sequence calcareous DUI-Ck (pH 6.9) - short-term acidified DUI-C1 (pH 4.3) - long-term acidified HvK-C1 (pH 3.8), an increase of base hydrolysable compounds is obvious, which coincides with the increase observed for the ‘suberin/lignin ratio’ in these same soils (Nierop and Verstraten, 2002). Moreover, a similar trend was noticed for the Soxhlet extractable (free) lipids in these (Nierop, Naafs and van Bergen, unpublished results) and other soils studied (e.g. Jambu et al., 1985; Dinel et al., 1990; Stevenson, 1994) implying that similar mechanisms may be responsible for the accumulation of non-extractable ester-bound moieties with decreasing soil pH.

It should be noted that the conditions under which saponification takes place resemble to some extent the classical extraction of humic acids and fulvic acids (Swift, 1996). Therefore, in addition to low molecular weight ester-derived products that can be identified using GC and GC-MS, large quantities of other compounds are released from SOM during base hydrolysis (Chapter 5).

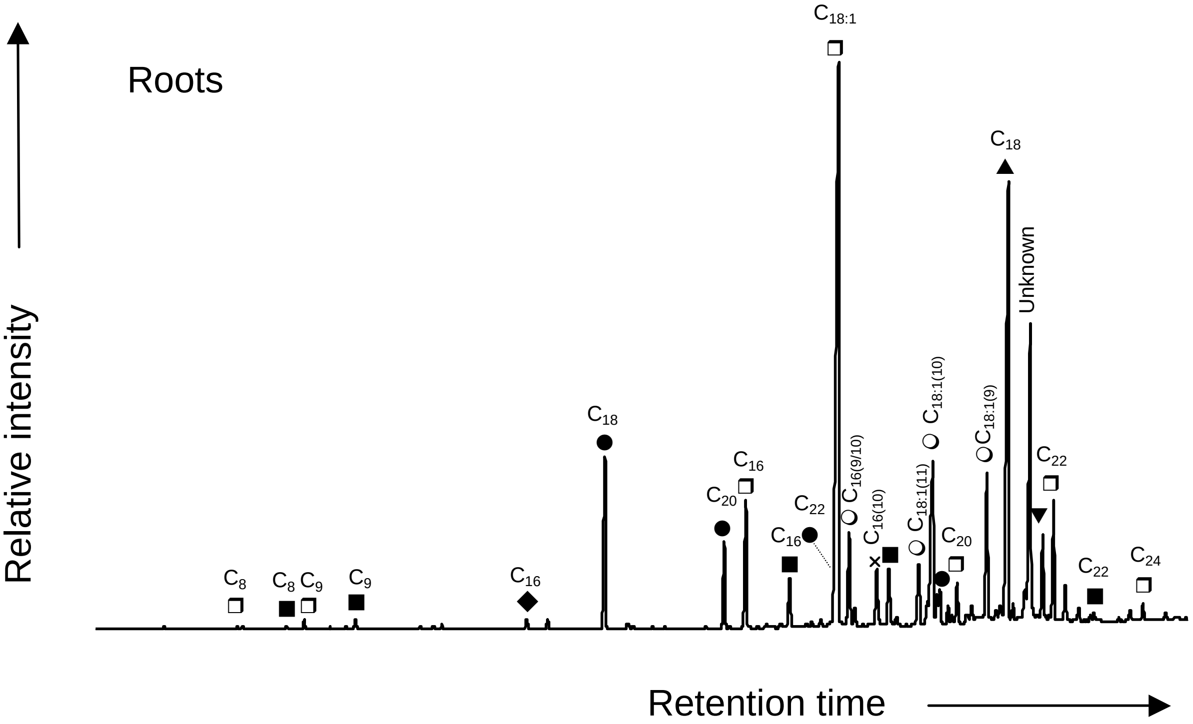

Figure 7.1 Partial gas chromatogram of root depolymerisate. Legend: ◆: alkanoic acid; ●: 1-alkanol; □: *ω*-hydroxyalkanoic acid; ■: *α,ω*-alkanedioic acid; ×: hydroxy- *α,ω*-alkanedioic acid; ○: dihydroxyalkanoic acid; ▼: 9,10-dihydroxy-1,18-octadecanedioic acid; ▲: 9,10,18-trihydroxyoctadecanoic acid. $C_{n:1}$: indicates chain length, when appropriate to the number of double bonds. $C_{n(9/10/11)}$: indicates chain length, and the position of the mid-chain hydroxy group. All compounds measured as trimethylsilyl esters and/or ethers.

7.3.1 Roots

The main compounds observed in the hydrolysates of the roots comprised *ω*-hydroxyalkanoic acids (C_{16}-C_{24}), alkanols (C_{18}-C_{24}), di- and trihydroxyalkanoic acids (C_{16}, C_{18}) and minor amounts of *α,ω*-alkanedioic acids (C_{16}-C_{22}) and an alkanoic acid (C_{16}), all of which were even in chain length (Fig. 7.1). All these compounds are typical constituents of suberin present in the cork layers of bark and twigs of *Quercus robur* (Holloway, 1983; Matzke and Riederer, 1991). The low contribution of *α,ω*-alkanedioic acids, dominated by the C_{16} member, is in line with reported data for cork (Holloway, 1983; Matzke and Riederer, 1991). However, the overall distribution of the ester-bound compounds in roots is very different from that in barks and twigs. For example, in roots the abundance of alkanols decreased in the sequence C_{18}-C_{20}-C_{22}-C_{24}, whereas in the cork of oak branches C_{20} dominated, followed by C_{22}, C_{24}, C_{18} and C_{26}, respectively (Holloway, 1983). The latter (C_{26}) was not even detected in the root depolymerisate. Furthermore, the roots had a relatively high contribution of alkanols, while virtually no alkanoic acids were observed, which is different from the reported suberin composition for bark and branches where alkanols and alkanoic acids had similar (low) concentrations (Holloway, 1983; Matzke and Riederer, 1991).

The most prominent compound released after base hydrolysis of roots was the $C_{18:1}$ (Δ^9) *ω*-hydroxyalkanoic acid, with additional contributions of C_{16}-C_{24} *ω*-hydroxyalkanoic acids. The relative concentration of the $C_{18:1}$ member is much higher than for bark or branches (Holloway, 1983; Matzke and Riederer, 1991). Another prominent and very characteristic constituent of the aliphatic part of suberin is

9,10,18-trihydroxyoctadecanoic acid, which is abundant in all plant parts (Matzke and Riederer, 1991). Other prominent building blocks were 9,16- and 10,16-dihydroxyhexadecanoic acid, 9,18-, 10,18- and 11,18-dihydroxyoctadecen-9-oic acids, 10-hydroxyhexadecane-1,16-dioic acid and 9,10-dihydroxyocadecane-1,18-dioic acid. The dihydroxyhexadecanoic acids are minor building blocks of oak suberins, while it is one of the main constituents of oak cutin (Holloway, 1983; Matzke and Riederer, 1991). The dihydroxyoctaden-9-oic acids were reported by Santos Bento et al. (2001) as artefacts of (or partially hydrolysed?) 18-hydroxy-9,10-epoxyoctadecanoic acid. In addition, 9,10,18-trihydroxyoctadecanoic acid could be partially derived from this epoxy derivative, which is hydrolysed under the reaction conditions used (Holloway, 1982). Furthermore, we have reason to believe that the ‘unknown’ compound is a derivative from the epoxy compound as well. It exhibits C_{18} hydroxyalkanoic mass fragments in its spectrum (*m/z* 103 (terminal hydroxyl), 117 (terminal acid), 204 and 217 (hydroxyalkanoic acid), 303 (C_9 chain bearing two hydroxyl groups) and 317 (C_9 chain bearing a hydroxyl and an acid group), but at present we are unable to reconstruct its precise molecular structure (Fig. 7.2).

In addition to all long-chain aliphatic components, small but distinct peaks were noticed for C_8 and C_9 ω-hydroxyalkanoic acids and α,ω-alkanedioic acids. Their presence is somewhat remarkable because these compounds were supposed to be markers of oxidative degradation of unsaturated compounds (e.g. Grasset and Amblès, 1998; Chapter 6) and therefore not very likely to occur in the (fresh) root biopolyesters. Possible explanations are that parts of the living roots were already subjected to microbial attack, or that little oxidation has taken place during the base hydrolysis and subsequent work-up procedure. No phenolic compounds were detected, implying that the polyphenolic part of suberin, if present, is not hydrolysable.

7.3.2 SOM composition in relation to soil pH

Table 7.1 summarizes all identified moieties, and the order of intensity within a given compound class. All these compounds have been found by others after base hydrolysis of SOM (Bull et al., 2000b; Chapters 5, 6), transesterification through BF_3/MeOH (Almendros and Sanz, 1991, Riederer et al., 1993) or thermally assisted hydrolysis and methylation using TMAH (e.g. Nierop, 2001; Santos Bento et al., 2001). However, although some other studies revealed the presence of alkanols, alkanoic acids and alkanedioic acids in SOM, hydroxyalkanoic acids were not always identified (Grasset and Amblès, 1998; Chefetz et al., 2002; Grasset et al., 2002). A possible explanation may be that these hydroxyalkanoic acids were further oxidized into alkanedioic acids in these latter studies.

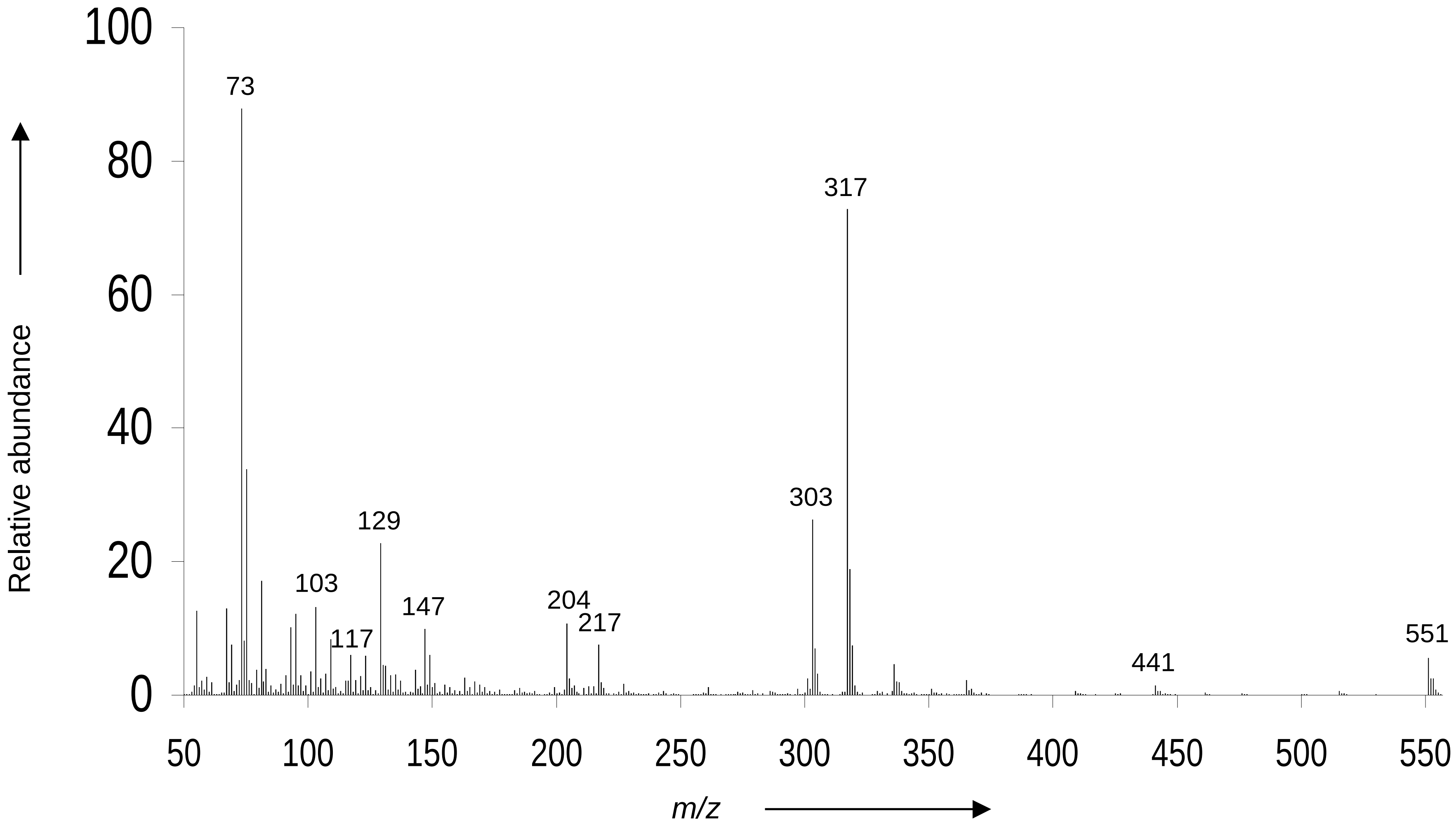

Figure 7.2 Mass spectrum of unknown compound.

Table 7.1 Yield and composition of extracts after base hydrolysis.

Compound[1]	Roots	DUI-Ck	DUI-C1	HvK
Soil pH ($CaCl_2$)	-	6.9	4.3	3.8
Weight% ester-bound fraction[2]	10.42	2.90	4.09	12.61
n-Alkanoic acids	C_{16}	C_{16}, C_{15}, $C_{18:1}$, C_{18}, C_{14}, C_{17}, $C_{19:1}$	C_{16}, C_{15}, C_{18}, $C_{18:1}$, C_{14}, C_{17}, $C_{19:1}$	C_{16}, $C_{18:1}$, C_{18}
1-Alkanols	C_{18}, C_{20}, C_{22}, C_{24}	C_{18}, C_{20}, C_{22}, C_{24}	C_{18}, C_{20}, C_{22}, C_{24}	C_{18}, C_{20}, C_{22}, C_{24}
ω-Hydroxyalkanoic acids	$C_{18:1}$, C_{16}, C_{22}, C_{20}, C_{24}, C_{18}, C_{9}, C_{8}	$C_{18:1}$, C_{16}, C_{22}, C_{20}, C_{9}, C_{24}, C_{8}	$C_{18:1}$, C_{16}, C_{22}, C_{20}, C_{9}, C_{24}, C_{8}	$C_{18:1}$, C_{16}, C_{22}, C_{9}, C_{20}, C_{24}, C_{8}
α,ω-Alkanedioic acids	$C_{18:1}$, C_{16}, C_{22}, C_{9}, C_{8}	C_{16}, C_{9}, $C_{18:1}$, C_{8}, C_{22}	C_{16}, C_{9}, $C_{18:1}$, C_{8}, C_{22}	C_{16}, C_{9}, C_{8}, $C_{18:1}$, C_{22}
Hydroxy-α,ω-alkanedioic acids	$C_{16(10)}$	$C_{16(10)}$	$C_{16(10)}$	$C_{16(10)}$
Dihydroxyalkanoic acids	$C_{18:1(10)}$, $C_{18:1(9)}$, $C_{16(9/10)}$, $C_{18:1(11)}$	$C_{16(9/10)}$, $C_{18:1(9)}$, $C_{18:1(11)}$, $C_{18:1(10)}$	$C_{16(9/10)}$, $C_{18:1(9)}$, $C_{18:1(11)}$, $C_{18:1(10)}$	$C_{16(9/10)}$, $C_{18:1(11)}$, $C_{18:1(10)}$, $C_{18:1(9)}$
Trihydroxyalkanoic acids	C_{18}	C_{18}	C_{18}	C_{18}
Dihydroxy-α,ω-alkanedioic acids	C_{18}	C_{18}	C_{18}	C_{18}
Aromatic acids		*p*-Coumaric, Vanillic, Ferulic	Vanillic, *p*-Coumaric, Ferulic	

[1] Chain lengths in order of decreasing abundance for each compound class. $C_{n:1(x)}$ indicates the chain length (n), the number of double bonds (:1) and the position of the mid chain hydroxy group (x).

[2] Weight fraction (%) of bound lipids per gram root/SOM.

All soil depolymerisates resembled each other closely, and only small differences could be noticed (Fig. 7.3). The calcareous soil (DUI-Ck) had clear contributions of alkanoic acids in the ester-bound fraction, which decreased from the short-term acidified soil DUI-C1 to the long-term acidified soil (HvK-C1), where hardly any alkanoic acids could be detected. The chain lengths of the alkanoic acids varied from C_{14} to C_{19} with prominent contributions of unsaturated and branched members, which suggest that these acids are not derived from suberin, but from microbes, particularly bacteria. These acids are present, among others, in phospholipids either esterified or as ethers (Zelles, 1999). When these moieties are not extracted, they appear in the Soxhlet residues and, finally, as a consequence of the saponification in the ester-bound fractions. Amblès et al. (1991) and Grasset and Amblès (1998) also found these bacterial acids, but accompanied with significant, if not dominant, amounts of even numbered long-chain ($> C_{20}$) alkanoic acids. The same trend as for the alkanoic acids, i.e. decrease in relative concentration with decreasing soil pH, was found for the aromatic acids, e.g. vanillic acid and other hydroxybenzoic acids. They may be derived from *p*-coumaric and ferulic acid, but also degradation products from lignin cannot be excluded. Part of them could also originate from the polyphenolic domain of the suberized tissue (Bernards, 2002). Nevertheless, the ester-bound aromatics were less well preserved under prolonged acidic conditions.

7.3.3 Roots vs. SOM

Compared to the roots, the C_{18} compound in the soil depolymerisates, unsaturated at Δ^9, decreased with respect to the saturated building blocks, which has been observed for beech forests as well (Nierop, 2001). Such a decrease is probably due to oxidation of the double bond (e.g. Grasset and Amblès, 1998; Regert et al., 1998; Chapter 6). Evidence for such a process is provided by the C_8 and C_9 ω-hydroxyalkanoic acids and α,ω-alkanedioic acids, which are more abundant in depolymerisates of soil samples (Fig. 7.3) than that of the oak roots (Fig. 7.1). Moreover, the chain-length distribution of these C_9 acids typically reflects the degradation of Δ^9 compounds. Next to these $C_{18:1}$ units, the compounds identified as 9,18- and 10,18-dihydroxyoctadecen-9-oic acids and the unknown compound were lower in relative concentration in the SOM hydrolysates compared with those released from the roots. Considering that epoxy compounds are thought to be the first intermediates in the oxidation of double bonds (Watkinson and Morgan, 1990) and that their presence decreased upon humification relative to its trihydroxy counterparts (Riederer et al., 1993), we suggest that all these compounds are derived from epoxy compound(s). In contrast to 9,18- and 10,18-dihydroxyoctadecen-9-oic acids, the building block from which the 11,18- analogue originates seems to be unaffected in soil, which suggests that this compound may have a different origin and/or is less susceptible to oxidative degradation.

7.3.4 Residues before and after saponification

The question arises whether the base hydrolysis released all or at least a representative part of ester-bound units from the roots and SOM. Therefore, thermally assisted hydrolysis and methylation (THM) – sometimes referred to as thermochemolysis – using tetramethylammonium hydroxide (TMAH) was applied before and after saponification. THM has been proven to be a very useful and efficient tool to analyse ester-bound polymers including cutin and suberin as ‘pure’ compounds as well as in SOM (de Leeuw and Baas, 1993; del Rio and Hatcher, 1998; Nierop, 1998; 2001; Santos Bento et al., 2001).

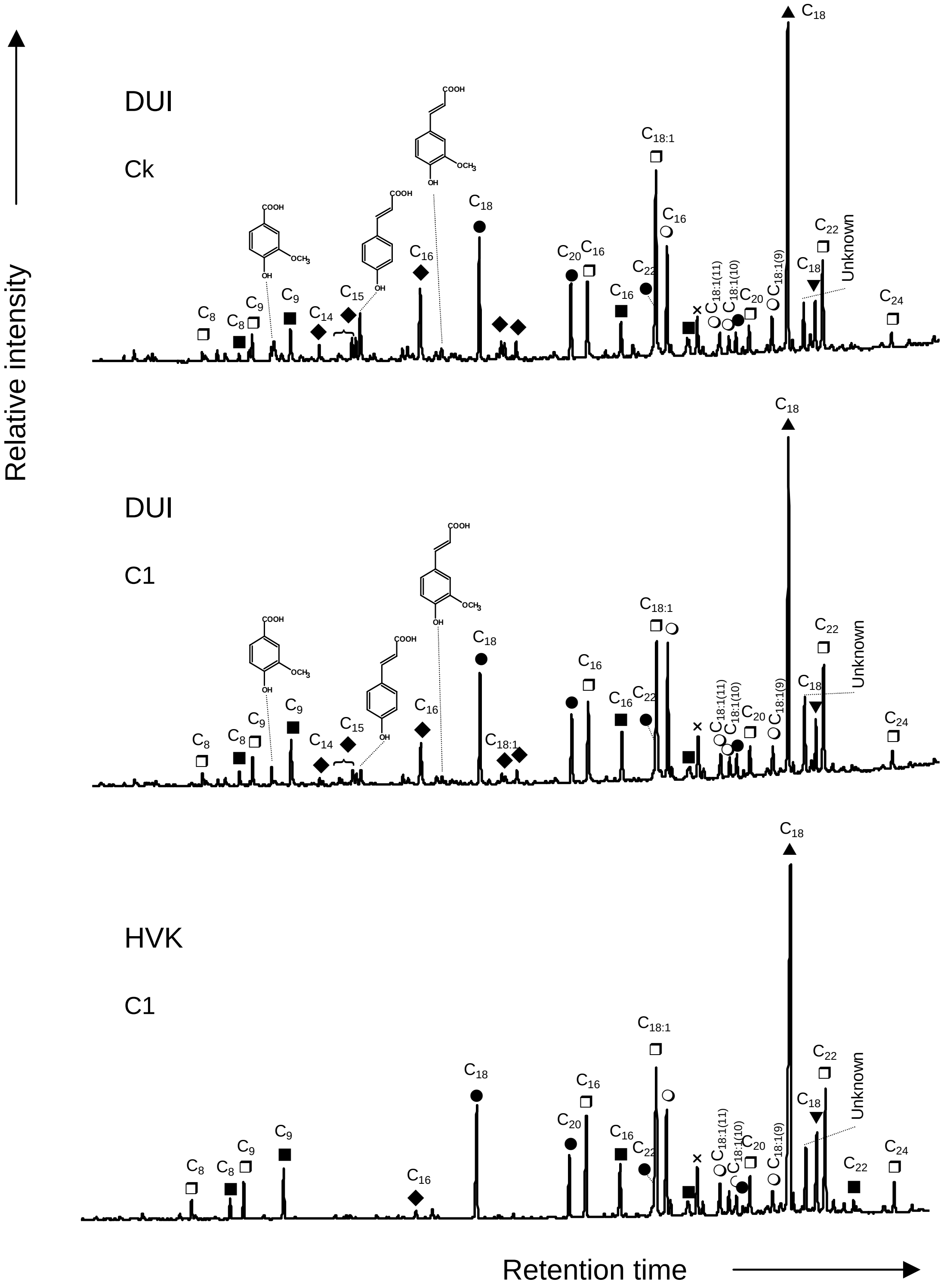

Figure 7.3 Partial gas chromatogram of soil depolymerisates. Legend: ◆: alkanoic acid; ●: 1-alkanol; ❐: *ω*-hydroxyalkanoic acid; ■: *α*,*ω*-alkanedioic acid; ✕: hydroxy- *α*,*ω*-alkanedioic acid; ❍: dihydroxyalkanoic acid; ▼: 9,10-dihydroxy-1,18-octadecanedioic acid; ▲: 9,10,18- trihydroxyoctadecanoic acid. $C_{n:1}$: indicates chain length, when appropriate to the number of double bonds. $C_{n(9/10/11)}$: indicates chain length, and the position of the mid-chain hydroxy group. All

In the residues after Soxhlet extraction, significant amounts of aliphatics were noticed and dominated the chromatograms upon THM as exemplified for DUI-Ck in Fig. 7.4. These aliphatics comprised methyl ethers and esters of all alkanols, alkanoic acids, and mono-, di- and trihydroxyalkanoic acids as identified in the soil depolymerisates, albeit in a slightly different distribution. Part of these differences was likely due to differences in instrumentation and variations in response factors (e.g. alkanol as methyl ether with TMAH vs. trimethylsilyl ether after BSTFA). In addition, hydrolysis may have induced the formation of compounds (e.g. the dihydroxyoctadecen-9-oic acids as outlined earlier) that do not appear upon THM, and vice versa.

Rather remarkable is the relatively high contribution of 10,16-dihydroxyhexadecanoic acid in the gas chromatograms after base hydrolysis, because this is a relatively unimportant suberin moiety. Moreover, upon THM the dihydroxyhexadecanoic acids display a less intense peak, ca. 50% of the intensity compared with what is detected after hydrolysis and subsequent GC analysis (Naafs and Nierop, unpublished results). It is even more remarkable that other compounds such as 9,10,18-trihydroxyoctadecanoic acid and all other hydroxyalkanoic acids exhibit very similar responses to both methods. At this point we can only speculate why the dihydroxyhexadecanoic acids react differently. It could be that they form a less accessible part as suggested by Lopes et al. (2000), sterically hindered, or the real core of the macromolecule so that the more drastic KOH hydrolysis, with its much longer reaction time, is more capable to release the acids than the THM technique. The fact that they increase relatively in the soil samples with respect to the roots suggests that they form recalcitrant entities in soil.

For all samples, a large majority of the ester-linked aliphatic moieties disappeared after base hydrolysis. Nevertheless, the release of so-called occluded or trapped lipids, which are not bound as esters, cannot be excluded (Grasset and Amblès, 2002), although all compounds released are typical (ester-bound) suberin-derived moieties. Only small amounts of aliphatics could still be identified in residues of roots and HvK-C1, while even smaller quantities were detected in those of DUI-Ck and DUI-C1 (Fig. 7.4). After base hydrolysis, lignin-derived aromatics prevailed in the residues. These (methylated) methoxyphenolics displayed similar distributions before and after saponification, indicating that they were hardly affected by the treatment, which is consistent with the relative small amounts found for the phenolic units in the GC traces after saponification.

7.3.5 Implications for SOM formation

The unequivocal difference between roots (Fig. 7.1) and SOM (Fig. 7.3) provides evidence for a more or less non-uniform degradation of the aliphatic esters. However, once in the soil compartment, there is little differentiation in the ester-bound fraction. Together with the increasing amount of this fraction upon soil acidification (and time) this suggests that these (bio)chemically labile compounds survive humification without much alteration. As a consequence, aliphatic ester-bound moieties, in this particular study

closely related with the aliphatic section of suberin, constitute a greater part of SOM in acidic sandy soils as compare to calcareous soils, and seem to become more important upon SOM formation as it increased from 2.90% (DUI-Ck) to 12.61% (HvK-C1).

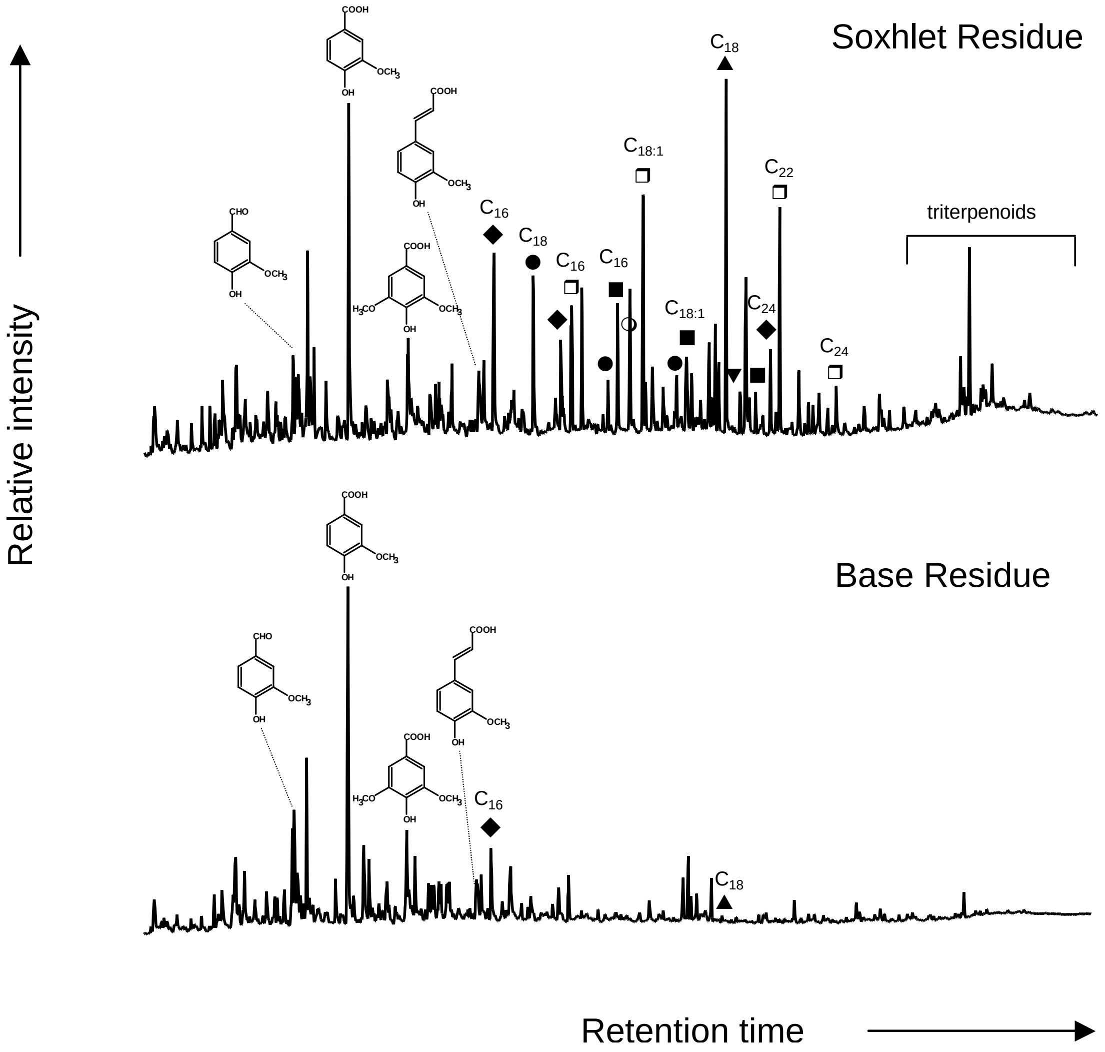

Figure 7.4 Partial gas chromatogram of DUI-Ck residues after Soxhlet extraction (above) and subsequent base hydrolysis (beneath). Legend: ◆: alkanoic acid; ●: 1-alkanol; ☐: *ω*-hydroxyalkanoic acid; ■: *α,ω*-alkanedioic acid; ○: 9,16- and 10,16-dihydroxyhexadecanoic acid; ▼: 9,10-dihydroxy-1,18-octadecanedioic acid; ▲: 9,10,18- trihydroxyoctadecanoic acid. $C_{n:1}$: indicates chain length, and when appropriate the number of double bonds. All compounds measured as methyl esters and/or ethers.

Very little is known about the degradation of suberin in soil. From studies that examined the cutin degradation by fungi it has become clear that the optimal cutinase activity to decompose cutin lies at neutral to slightly alkaline pH (Kolattukudy, 2001). An analogous pH dependence of polymeric break down can be anticipated for suberin that may explain the increasing resistance of the esters with decreasing soil pH because exactly the same enzymes have been found to degrade (the aliphatic part of) suberin, which has similar types of binding as cutin. Generalizations considering the SOM formation are, however, not realistic because decomposition and humification processes in different soils, especially those with a relatively high amount of clay and silt, will obviously have a large contribution of physicochemical SOM protection mechanisms in addition to a pH effect (e.g. Six et al., 2002). Still, the apparent ability of ester-bound moieties to survive biodegradation as such may also be important in the sequestration of C in soils other than sand dominated ones. Therefore, we suggest that ester-bound fractions can be of great importance on a relatively short-term scale in the global carbon cycle, especially when acidic soils are concerned.

As aforementioned, Nierop and Verstraten (2002) found that the ‘suberin/lignin ratio’ increased in the course of acidification for the soils studied. In addition, Poirier et al. (2002) observed that the non-hydrolysable part of SOM lacked lignin remnants that dominated the resistant fraction of fresh plant material. Moreover, in relatively old soils, the contribution of cutin/suberin-derived aliphatic moieties was highest in the subsoil, which had the greatest ^{14}C age implying a high degree of stabilisation (Rumpel et al., 2002). In the past, it has been proposed that cutin and suberin could survive biodegradation through increased cross-linking (e.g. Kögel-Knabner, 1993). However, the large increase of suberin-derived compounds when going from calcareous soil (DUI-Ck) to the older acidic soil (HvK-C1), in combination with the lack of such compounds in the residues after saponification, does not suggest that ester-bound aliphatics accumulate with a need of more stable (non-hydrolysable) bonds.

7. 4 Conclusions

Ester-bound aliphatic moieties mainly derived from root suberin accumulate without very significant compositional changes upon long-term soil acidification in sandy soils under oak in coastal dune ecosystems in the Netherlands. Compared with fresh (fine) roots, largely responsible for the input of SOM in the subsoils, only the unsaturated and epoxy compounds present in suberin are subjected to biodegradation in soil, probably via oxidation. The fact that ester-bound moieties are well preserved in soil indicates that these chemically labile compounds are able to accumulate in the soil compartment. THM confirmed that base hydrolysis is a very efficient approach to analyse ester-bound components.

Acknowledgements. This study was supported by the Institute for Paleoenvironment and Paleoclimate Utrecht (IPPU). Pim F. van Bergen and Jan W. de Leeuw are thanked for their constructive remarks on an earlier draft. Furthermore, we express our gratitude to A. Amblès, an anonymous referee and the Associate Editor (P. Farrimond) for their careful reviews.

Chapter 8

Characterization of organic matter in an acid volcanic sub-soil using Curie-point pyrolysis and solid state CPMAS ^{13}C NMR spectroscopy

Curie-point pyrolysis-GC/MS in combination with solid-state CPMAS ^{13}C NMR is used to analyze the organic matter present in an acid andic sub-soil. The sample is characterized by an acid pH of 4.2 and a high TOC content of 6.7. Curie-point pyrolysis data are combined with NMR data and reveal a significant contribution of fungal polysaccharide moieties. Pyrolysis data further show that these, and other, polysaccharide moieties are thermally altered. In addition, the presence of the biopolyesters cutin and suberin, the non-hydrolyzable biopolymers cutan and suberan, proteins and polypeptides is confirmed by both analysis methods. Relatively high amounts of chitin pyrolysis products may be derived both from fungi and arthropods indicating that, in addition to fungi, arthropods are important for the reworking of organic matter. Low amounts of lignin detected are related to the low soil pH which is known to be favoured by (lignin-degrading) fungi. Charcoal was found to be a significant contribution to the total of SOM, although underestimated by the methods used. Preservation of organic matter in this acid andic sub-soil is suggested to result from an increased resistance against microbial degradation by thermal alteration and complexation of SOM with aluminium and iron.

8.1 Introduction

Andisols cover between 0.76%-0.84% of the world's land area (Leamy et al., 1980) and are typically associated with the weathering of recent volcanic ash deposits in humid, temperate environments (Parfitt and Kimble, 1990; Shoji et al., 1993, 1996; Lowe, 1997). Despite their apparent small contribution to the total of soils in the world, they are significant for the global carbon cycle because of their capacity to stabilize large quantities of organic carbon for thousands of years (Torn et al., 1997).

Vascular plants are the main source of organic carbon to these and other soils through litterfall and roots (Oades, 1993). Once introduced into the soils, specific andic soil processes will influence the transformations and decomposition of the fresh litter and root material (Chapter 2). In addition to soil processes, thermal alteration of organic matter during forest fires influences the composition of SOM in these profiles.

In this chapter, Curie-point pyrolysis-GC/MS in combination with solid-state CPMAS ^{13}C NMR is used to analyze the organic matter present in an acid andic sub-soil. Results are discussed in terms of the influence of both andic soil properties and forest fires on the composition of the organic matter.

D. F. W. Naafs, P. F. van Bergen, H. Knicker, J. W. de Leeuw. *Soil Biology & Biochemistry, submitted.*

8.2 Experimental

8.2.1 Materials

Madeira Island (Portugal) is located in a fully (Atlantic) oceanic domain between 32°38’ and 32°52’N and 16°39’ and 17°16’ W, approximately 600 km from the African coast, at the same latitude as Casablanca (Morocco). The sample used was taken from an andic profile located on this island that has been classified as either being Umbric or Haplic Andisols (FAO, 1998) depending on their colour (Madeira et al., 1994).

The profile studied is situated on a hill (slope 30°W) near the village of Poiso (altitude 1175 m). The vegetation consisted of mosses (5%), grasses (55%), ferns (20%) and deciduous trees, i.e. oak and birch (20%). Tree- and some grass roots were found to a depth of about 80 cm, but most of the intensive “root-activity” was found in the top 0-40 cm consisting mainly of grass fibrils. Three horizons were distinguished; a thin O-horizon (0-2 cm), an A-horizon (2-73 cm) and an E/B horizon (73- 90 cm), the latter being formed by weathered basalt bedrock material. The deeper layer, i.e. C-horizon, was formed by a non-friable, compact layer of weathered basalt, the parent material for this Andisol (Madeira et al., 1994) and therefore not sampled. The O-horizon consisted mainly of litter and vegetation, i.e. grass, moss, twigs, leaves, etc. The A horizon (color 4/6 5YR) was characterized by a non-friable structure, clay aggregates with a diameter up to 1 cm, and highly weathered basalt fragments up to a diameter of 10 cm. Many roots were found in this horizon, ranging from mainly very fine grass roots in the top 30 cm to coarser roots, up to a diameter of 2 cm, at greater depth, i.e. 30-70 cm.

8.2.2 Sampling, sample pre-treatment, total organic carbon measurement

In September 2001, about 500 gram of sample was taken every 10 cm up to a depth of 70 cm (including the O-horizon). Based on earlier studies (Chapters 2, 6), the sample taken from the depth interval of 20-30 cm was selected for this study. On Madeira, the sample was air dried in the dark and wrapped in aluminium foil. After 1 week, the sample was transported to the Netherlands and subsequently oven dried at 60°C, sieved over a 2 mm and a 250 μm sieve to remove large roots and basalt fragments. Total organic carbon content (TOC%) of the dried and sieved (<250 μm) sample was measured using a Fisions Instruments NA 1500 NCS analyzer, with a cycle time of 180 s, a source temperature of 190°C and an oxygen flow of ca. 30 l/min. The pH (H_2O) of the soil was measured in the supernatant suspension of a 1:2.5 sieved (<250 μm) sample to water ratio.

Approximately ten gram of the sieved (<250 μm) sample was Soxhlet extracted using 100 ml dichloromethane/methanol (DCM/MeOH) (9:1v/v) for 24 h to remove extractable lipids. The residue was air-dried and used for further study.

8.2.4 Solid state CPMAS ^{13}C NMR spectroscopy

Prior to solid state ^{13}C CPMAS NMR measurements, the Soxhlet residue was treated with cold HCl/HF to remove the mineral matrix. 50 ml 38% HF was added to the sample after which the reaction mixture was shaken for 2h at 250 rpm. 250 ml water was added and the suspension obtained centrifuged. 30 ml 30% HCl was added to the residue after which another 250 ml of water was added. The suspension obtained was centrifuged and subsequently washed with water (3x) till neutral pH.

The solid state ^{13}C CPMAS NMR spectra were obtained on a Bruker DSX 200 operating at a frequency of 50.3 MHz using zirconium rotors of 7 mm OD with KEL-F-caps. The cross polarization magic angle spinning (CPMAS) technique was applied during magic-angle spinning of the rotor at 6.8 kHz. A ramped ^{1}H-pulse was used during contact time in order to circumvent spin modulation of Hartmann-Hahn conditions (Peersen et al., 1993). A contact time of 1 ms and a 90° ^{1}H pulse with of 6.5 μs were used for all spectra. The ^{13}C-chemical shifts were calibrated to tetramethylsilane (= 0 ppm) and were calibrated with glycine (176.04 ppm). Assignments of characteristic peaks was based on published data (e.g. Hatcher, 1987; Wilson and Hatcher, 1988; Nierop et al., 1999; Kögel-Knabner, 2002).

8.2.5 Curie-point pyrolysis- gas chromatography-mass spectrometry (Py-GC/MS) Prior to analyses, the sample was pressed onto a ferromagnetic wire with a Curie temperature of 610°C. The interface temperature of the pyrolysis unit was set at 200°C and the pyrolysis time was 10 s. Py-GC/MS analyses were performed using a FOM-5LX Curie-Point pyrolyser connected to a Hewlett-Packard 5890 series gas chromatograph, equipped with a CP-sil 5CB-MS silica column (50 m x 0.32 mm, film thickness 0.4 μm), and a Fisons instruments VG platform II mass spectrometer operating at 70 eV, scanning the range *m/z* 50-650 with a cycling time of 0.65 s. The GC oven temperature was programmed from 40°C (5 min) to 310°C (isothermal for 10 min) at a rate of 5°C min^{-1}. Helium was used as carrier gas. Compound identification was based on mass spectral data (Pouwels et al., 1987, 1989; Pouwels and Boon, 1990; van Smeerdijk and Boon, 1987; Ralph and Hatfield, 1991; Stankiewicz et al., 1996; van Bergen et al., 1996, 1997, 1998a, b; Nierop et al., 2001b) and retention time comparisons with reference data.

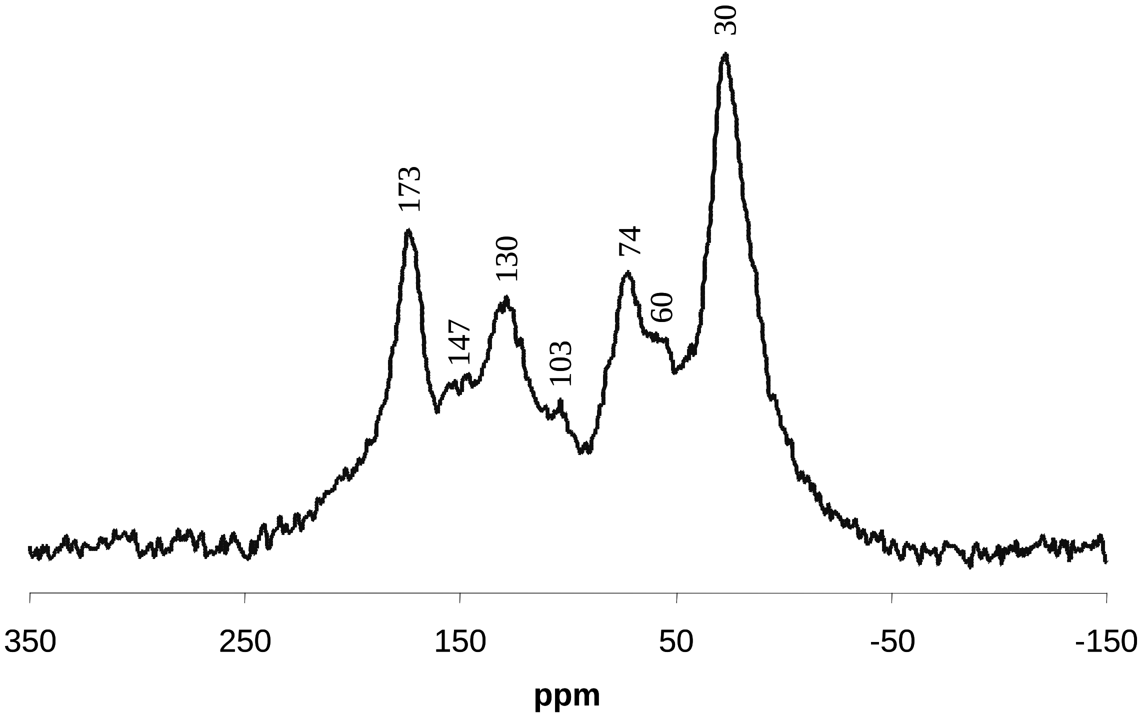

Figure 8.1 Solid sate CPMAS ^{13}C NMR spectrum of solvent extracted acid andic soil.

8.3 Results

Total organic carbon (TOC) expressed as weight percentage of the total sieved (<250 μm) soil mass, and total organic nitrogen, also expressed as weight percentage of the total sieved (<250 μm) soil mass, were 6.7 % and 0.6 % respectively. In addition, an acid soil pH (H_2O) of 4.2 was determined. TOC, total organic nitrogen, C/N molar ratios and pH (H_2O) values of the complete profile have been described elsewhere (Chapter 2).

The 30 ppm peak in the alkyl C region (0-45 ppm) dominated the solid state CPMAS ^{13}C NMR spectrum (Fig. 8.1) and is assigned to methylene C. Peaks at 60, 74 and 103 ppm indicating the O-alkyl C (45-110 ppm region), at 130 ppm indicating aromatic C (110-160 ppm region) and at 173 ppm indicating carbonyl/carbonyl/amide C (160-220 ppm region) were also significant.

The andic sub-soil pyrolysate is dominated by furans (Fig. 8.2 peaks 1, 16, 26, 47; Table 8.1). Other relatively abundant pyrolysis products include phenol (29), the anhydrohexoses levoglucosenone (41) and levoglucosan (81), polyaromatic products (50, 76, 86, 88, 93, 94, 99, 105) and nitrogen containing products such as pyrrole (10), benzeneacetonitrile (44) and indole (64). In addition, C_{10}-C_{26} alkene/alkane pairs, a few fatty acids maximizing at C_{16}, methoxyphenols (51, 63, 67, 79, 82) and acetamido functionalised products (65, 69, 83) were identified in relatively lower amounts (Fig. 8.2).

Table 8.1 Mass spectral data pyrolysis products

No.	Compound	Source	M^+	Characteristic (*m/z*)
1	2-Methylfuran	Ps	*82**	81, 54
2	Acetic acid	Ps	*60*	45
3	2-Butanal	Ps	*70*	69
4	Pentan-3-one	Ps	86	58, 57
5	Benzene	Ar	*78*	77
6	C_7 *n*-alkane	Al	100	*57*
7	2,3-Pentanedione	Ps	100	*69*
8	N-methylpyrrole	Pp	*81*	80, 69, 53
9	Pyridine	Pp	*79*	52
10	Pyrrole	Pp	*67*	
11	Toluene	Ar	92	*91*
12	(2H)-Furan-3-one	Ps	*84*	55, 54
13	3-Furaldehyde	Ps	*96*	95
14	Acetamide	Chi	*59*	
15	2,4-Pentadienal	Ps	*82*	54, 53
16	2-Furaldehyde	Ps	*96*	95, 67
17	Methylpyrroles		81	*80*, 53
18	2-Hydroxymethylfuran	Ps	98	81, *55*
19	Cyclopent-1-ene-3,4-dione		*96*	68, 54
20	C_2 alkyl benzenes	Ar	106	*91*, 77
21	(5H)-Furan-2-one	Ps	84	69, *55*
22	Vinylbenzene (styrene)	Ar	*104*	103, 78, 51
23	2-Methyl-2-cyclopenten-1-one	Ps	96	*67*, 53
24	2-Acetylfuran	Ps	110	*95*
25	2,3-Dihydro-5-methylfuran-2-one	Ps	*98*	70, 69, 55
26	5-Methyl-2-furaldehyde	Ps	*110*	109, 81, 53
27	Benzonitrile	Aa	*103*	76

28	unknown	Ps	*114*	58
29	Phenol	Pp/Lg	*94*	66, 65
30	2-Carboxyaldehyde-(1H)-pyrrole	Chi	*95*	94, 66
31	2,3-Dimethylcyclopent-2-en-1-one	Ps	110	109, *105*
32	2-Hydroxy-3-methyl-2-cyclopenten-1-one	Ps	*112*	84, 71
33	C_{10} *n*-alkane	Al	142	71, 57
34	2,3-Dimethylcyclopent-2-en-1-one	Ps	110	95, 81, *67*
35	Indene		*116*	115
36	Acetophenone		120	*105*, 77
37	2-Methylphenol	Pp/Lg	*108*	107, 90, 79, 77
38	2-Propan-2-one-tetrahydrofuran	Ps	*128*	85, 72
39	3/4-Methylphenol	Pp/Lg	*108*	107, 90, 79, 77
40	2-Methoxyphenol (Guaiacol)	Lg	124	*109*, 81
41	Levoglucosenone	Ps	126	*98*, 96, 68
42	Unknown	Ps	*99*	*56*
43	Unknown		*132*	131, 103, 82, 81
44	Benzeneacetonitrile	Aa/Pp	*117*	116, 90
45	Unknown		138	*92*, 65
46	Methylindene		*130*	*129*, *115*
47	5-Hydroxymethyl-2-tetrahydrofuraldehyde-3-one	Ps	144	128, 85, *69*, 57
48	Unknown	Ps	142	127, *100*, 69
49	C_2-phenol	Ar/Lg	122	*107*, 105, 77
50	Naphthalene	Pa	*128*	
51	4-Methyl-2-methoxyphenol	Lg	*138*	*123*, 95, 77
52	Unknown	Ps	130	114, 101, 85, *69*
53	Acetylpyridone	Chi	137	*109*, 95, 81, 68
54	1,2-benzenediol (catechol)	Ar/Lg/Ta	*110*	92, 64
55	Acetomidofuran	Chi	125	125, *83*, 54, 53
56	Unknown		134	105, *91*, 78
57	4-Vinylphenol	Ar/Lg	*120*	119, 91
58	C_2-alkylbenzene		106	*105*, 78
59	3-(2 Methylpropyl)-5-(2 methylpropyl)-3,4-dihydro-2H-pyrrole-2,4-dione	Aa	181	166, 84, *69*, 56
60	Anhydropentose	Ps	*132*	*104*, 103, 78, 77
61	Isoquinoline		*129*	128, 102
62	C_6 Pyrrole?		151	*136*, 109, 80
63	4-Ethyl-2-methoxyphenol	Lg	152	*137*, 122
64	Indole	Pp	*117*	90
65	3-Acetamido-5-methylfuran	Chi	139	*97*, 69
66	C_1 naphthalene		*142*	*141*, 115
67	4-Vinyl-2-methoxyphenol	Lg	*150*	*135*, 107, 77
68	Unknown		166	134, *105*, 91, 77
69	3-Acetamido-2/4-pyrone	Chi	153	*111*, 83, 82
70	Unknown		160	159, 145, 103, *74*
71	Unknown	Chi	152	*127*, 110, 55
72	n-Hydroxyphenylacetamide	Chi	151	*109*, 80, 81

73	Unknown		163	120, *91*
74	Unknown	Ps	158	141, 87, *74*
75	Methylindole	Pp	131	*130*, 105, 77
76	Biphenyl	Pa	154	*152*, *151*
77	Unknown		138	98, 69, *55*
78	C_2 naphthalene		156	141, *101*, 74, 71
79	*trans* 4-(2-Propenyl)guaiacol	Lg	164	149, 131, 104, *55*
80	Unknown		158	*133*, 104, 78
81	Anhydroglucosan (levoglucosan)	Ps	162	98, 73, *60*
82	4-Acetyl-2-methoxyphenol	Lg	166	*151*, 123, 108
83	3-Acetamido-6-methyl-n-pyrone	Chi	167	*125*, 83, 54
84	Anhydrohexose	Ps	*162*	147, 144, 130, 91
85	C_{13} methylketon		198	138, 71, *58*, 59
86	Naphthole	Pa	*144*	*115*, 116
87	Phthalate		177	*149*
88	Fluorene	Pa	*166*	*165*, 83, 82
89	2,5-Diketopiperazine derivatives	Pp	168	125, 97, *70*
90	Diketodipyrrole	Pp	*186*	93, 65
91	1-Pristene		266	126, 69, *57*, *56*
92	C_{14} *n*-Alkanoic acid	Lp	228	129, *73*
93	Phenanthrene	Pa	*178*	
94	Anthracene	Pa	*178*	
95	4-Alkyl-2,6-dimethoxyphenol/Caffein		*194*	109, 82, 67, 55
96	C_{15} n-Alkanoic acid	Lp	242	129, *73*
97	Diketopiperazine derivate	Pp	*154*	125, 97, 70
98	Diketopiperazine derivate	Pp	194	*70*
99	C_1 anthracene/C_1 phenanthrene	Pa	*192*	191, 105
100	C_{16} *n*-Alkenoic acid methyl ester	Lp	270	87, *74*
101	C_{16} *n*-Alkenoic acid	Lp	254	236, *73*
102	C_{16} *n*-Alkanoic acid	Lp	256	129, *73*
103	Polyaromatic hydrocarbon	Pa	*204*	202, 101
104	Fluorantene	Pa	*202*	101
105	Pyrene	Pa	*202*	101

* Numbers in *italics* refer to the base peak in the mass spectrum. Key: Ps: Polysaccharide, Pp: polypeptide, Al: aliphatic biopolymer, Chi: chitin, Lg: lignin, Ta: tannin, Aa: amino acid, Ar: aromate/thermally altered polysaccharide, Pa: polyaromatic, Lp: lipid.

8.4 Discussion

8.4.1 Solid state ^{13}C CPMAS NMR spectroscopy

The peak around 30 ppm is assigned to alkyl C in hydrolysable polyesters, i.e. cutins and suberins, and non-hydrolysable alkyl-based biopolymers such as cutan and suberan (Nip et al., 1986b; Tegelaar et al., 1989b, 1995) but could also be related to the presence of C in amino acids. The signal around 74 ppm is attributed to the presence of C_3 sugar units, including signals from carbon atoms 3 and 5 in chitins (Hemsley et al., 1994; Kramer et al., 1995). The shoulder at 63 ppm and the peak at 103 ppm are derived from C_5 (pentose) or C_6 (hexose) and hemicellulose carbons that are structurally analogous to cellulose C_1 carbon, respectively (Kögel et al., 1988). As hexose units are part of the chitin structure, the latter shoulder and peak may also be derived from carbon 6 and carbon 1 in chitins respectively (Schaefer et al., 1987; Hemsley et al., 1994; Kramer et al., 1995). The relatively minor peaks around 147 and 152 ppm are assigned to O-substituted (OH and OCH_3 groups) aromatic carbons of guaiacol and syringol units, whereby the small shoulder at 56 ppm is most probably derived from the C in methoxy groups (Hatcher, 1987). Signals at 104 ppm (chitin carbon 1) and 82 ppm (chitin carbon 4) are representative of chitin content (Hemsley et al., 1994; Kramer et al., 1995, 1998). Carboxylic acid and amide C make up the second most important peak at 173 ppm. It should be noted that insect cuticles give a strong resonance around this value (Kramer et al., 1995; Schaefer et al., 1987). The main peak in the aromatic C part of the spectrum, around 130 ppm, is assigned to C in condensed aromatic structures, possibly indicating the presence of charcoals (Hatcher et al., 1989; Haumaier and Zech, 1995; Freitas et al., 1999). In addition, organic compounds in fungal spores may also have contributed to the aromatic signal (Hemsley et al., 1994).

8.4.2 Curie-point pyrolysis; polysaccharides

In the andic sub-soil pyrolysate many furans (1, 12, 13, 16, 18, 21, 24, 25, 26, 38, 47, 55, 65), a pyran (28), an anhydropentose (60), three anhydrohexoses (41, 81, 84), and other compounds such as acetic acid (2), 2-butanal (3), and 2,4-pentadienal (15) have been identified (Table 8.1, Fig. 8.2). All these products are considered to be derived from polysaccharides (Saiz-Jiminez & de Leeuw, 1984a; Pouwels et al., 1987, 1989; Pouwels and Boon, 1990), which is confirmed by the CPMAS ^{13}C NMR data (section 3.1).

The dominance of polysaccharide derived pyrolysis products is remarkable considering that polysaccharide products are dominant only in pyrolysates from fresh plant material (e.g. Murayama, 1984; van Bergen et al., 1997, 1998a). In soil sample pyrolysates, polysaccharide products are usually less dominant (e.g. van Bergen et al., 1997, 1998a; Nierop, 1998; Nierop and Buurman, 1999; Nierop et al., 2001b). Significant contributions of 2-furaldehyde and 5-methyl-2-furaldehyde (Fig. 8.2) have been observed before in soil polysaccharide pyrolysates (Saiz-Jiminez & de Leeuw, 1984a). However, comparison with pyrolysis-GC traces of SOM from an Ah horizon underneath a grass vegetation in Dutch sandy soils (Nierop and Buurman, 1998; Nierop et al., 2001b) reveals the absence of relatively large amounts of hexose derived pyrolysis products (e.g. pyrans, anhydrohexose, levoglucosan) in the andic sub-soil pyrolysate (Table 8.1, Fig. 8.2). The same absence has been noted before in acid soil pyrolysates from underneath grass vegetations (e.g. van Bergen et al., 1998a). In stead of these hexose (C_6) derived products, the extract is dominated by furans. The absence of levoglucosan may result from the presence of alkali-metals in the sample that inhibit its formation (Evans and Milne, 1987).

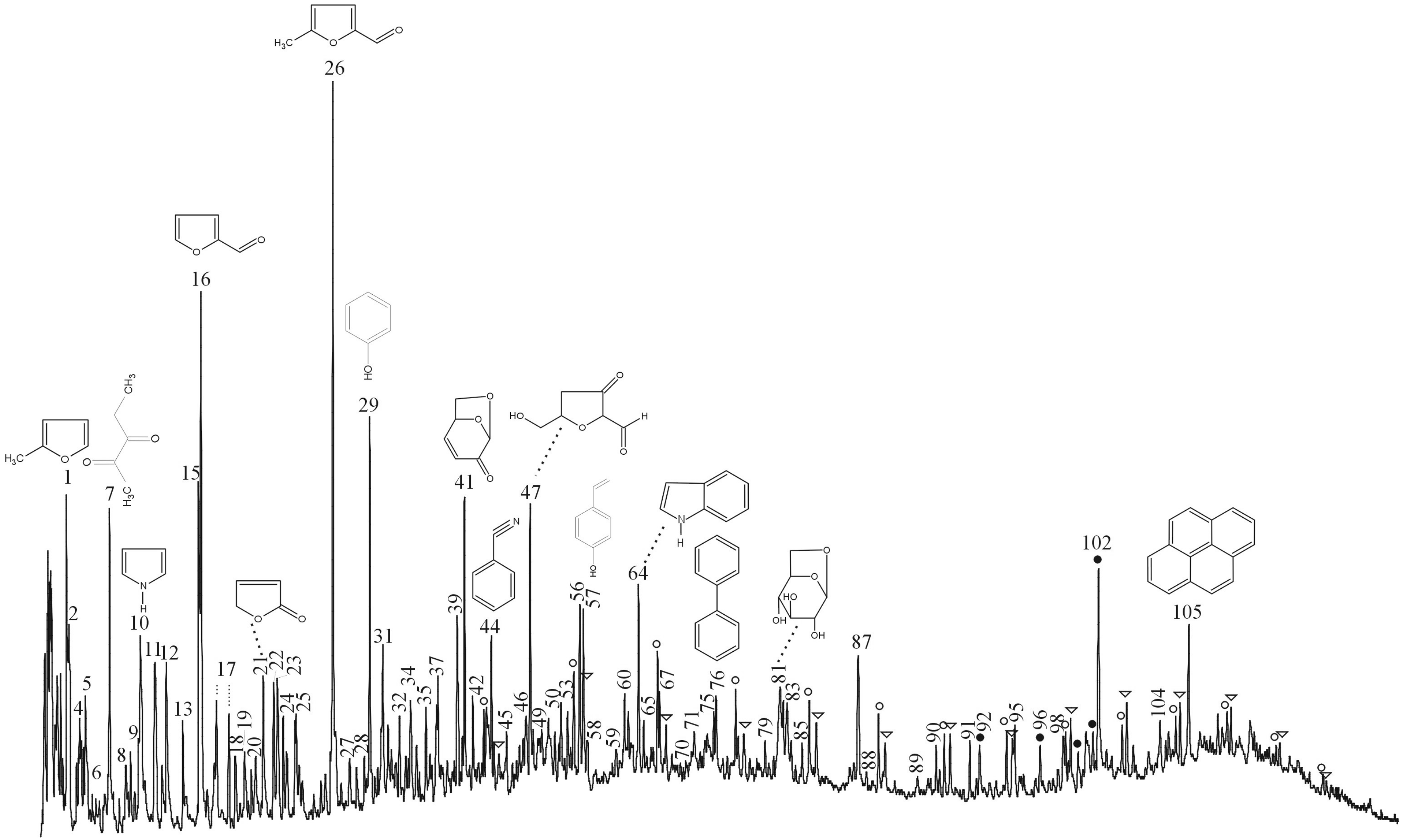

Figure 8.2 Total ion current trace of the 610 °C Curie-point pyrolysate. Numbers above peaks refer to Table 1. Key: ▽ : *n*-alkene, ○ : *n*-alkane, ● : *n*-alkanoic acid.

8.4.2.1 Microbial contribution to the polysaccharide signal

Although specific monosaccharides cannot be attributed as markers for either plant or microbial species (Cheshire, 1979), pyrolysis of *Agraricus bisporus* fruit bodies and mycelia generated lots of relatively small pyrolysis products, including furans (C. H. Vane, personal comment).

Pentose sugars have been encountered in particular in acid soils (Oades, 1984). Lipid, as well as other data, indicate that acid (andic) soils are characterised by reduced bacterial activities (Boudot et al., 1989; Boudot, 1992; Cerri and Jenkinson, 1981; Wardle, 1992; Motavalli et al., 1995; Andersson and Nilsson, 2001; Chapter 2). Moreover, an acid soil pH (4.2) is known to increase the fungal contribution (Bumpus, 1993) and pyrolysis-GC/MS of fungal biomass produces high amounts of furans, including 2-furaldehyde (16) and 5-methyl-furaldehyde (26) (Gutierrez et al., 1995). Both white- and brown-rot fungi are known to degrade polysaccharides (C_6) in lignocellulose (Blanchette et al., 1994; Eriksson et al., 1990). Such degradation may explain the low contribution of C_6 based polysaccharide pyrolysis products in the andic sub-soil (Fig. 8.2) as compared with e.g. a sandy soil (Nierop and Buurman, 1999). Based on these findings, the dominance of furans (Fig. 8.2), together with the low contribution of C_6 based polysaccharide pyrolysis products, seems to reflect an important *in situ* fungal influence on composition of the organic matter.

8.4.2.2 Thermal alteration of polysaccharides (cellulose)

Another explanation for the furan dominance in the pyrolysate may be thermal alteration of polysaccharides under the influence of forest fires. On Madeira island, as well as in numerous other places, forest fires are known to influence the composition of SOM (e.g. Almendros et al., 1998, 1992). After sufficient heating of polysaccharides, the composition of the pyrolysate changes from levoglucosan (C_6) dominated to one characterized by alkyl furans, benzoid aromatics and condensed aromatics (Pastorova et al., 1994). These changes are caused by the formation of a new intermediate polymer from the original glycan cellulose structure that is built of phenol and furan elements formed by rearrangement and condensation reactions (Pastorova et al., 1994). In addition to benzene and phenol derivatives, a series of condensed aromatics have been identified in the andic soil pyrolysate (Fig. 8.2; section 3.5) indicating that thermally alteration of SOM is a likely process in this andic soils sample. In addition to a contribution from thermally altered polysaccharide structures, forest fire combustion products, including furans, are incorporated into soils and can be released upon evaporation during pyrolysis (Saiz-Jiminez, 1994).

8.4.2.3 Preservation of polysaccharides in acid andic subsoils

Although microbial polysaccharides synthesized *in situ* have been suggested responsible for the presence of persistent polysaccharides in mineral soils (Huang et al., 1998), the question arises as to why polysaccharides, being relatively “labile” compounds, can be so dominant in this andic sub-soil. In general, the preservation of SOM in andic soils is strongly associated with (i) complexation of OM with both iron and aluminium (Shoji et al., 1993; Aran et al., 2001), (ii) a reduced bacterial activity resulting from the presence of free aluminium and iron (Boudot et al., 1989, 1992; Torn et al., 1997), (iii) a low soil pH (Stevenson, 1994) and (iv) a high phosphorus deficiency (Boudot et al., 1986, 1988). Reduced bacterial degradation of polysaccharides could therefore result in a relative accumulation of these “labile” compounds. In addition, thermally altered polysaccharide structures in soils are protected from degradation by their resistance against microbial degradation as a result of changes in the polysaccharide molecular structure (Almendros et al., 1988, 1992).

8.4.3 Curie-point pyrolysis: aliphatic compounds

The presence of C_9-C_{26} alkene/alkane pairs (Fig. 8.2) reflects a contribution from hydrolysable polyesters, i.e. cutins and suberins, and non hydrolysable biopolymers such as cutan (Nip et al., 1986b, 1987), or suberan (Tegelaar et al., 1993, 1995; Nierop, 1998). In addition, salts of fatty acids (Hartgers et al., 1995) and clay-organic complexes (Nierop and van Bergen, 2002) are known to produce short-chain alkenes and alkanes upon pyrolysis. These pyrolysis results (Fig. 8.2) indicating the presence of alkyl chains are in line with the CPMAS ^{13}C NMR data (Fig. 8.1). During an earlier study, the biopolyesters cutin and suberin were found to contribute up to 2.5% of the TOC in this andic sub-soil (Chapter 6). The rest of the aliphatic peak in the NMR spectrum may therefore be attributed to non-hydrolysable aliphatic biopolymers.

Lipids, normally found as evaporates in SOM pyrolysates (e.g. van Bergen et al., 1997; Nierop, 1998) have been almost completely removed during the solvent extraction prior to pyrolysis. The few fatty acids found, maximizing at C_{16} (Fig. 8.2), have been identified in Soxhlet extracted soil samples underneath a grass vegetation (van Bergen et al., 1998a). Considering their distribution, they are either occluded or sorbed acids released upon pyrolysis (Saiz-Jiminez & de Leeuw, 1984a) or contaminants. In addition, a short-chain ($<C_{20}$) dominated alkanoic acid distribution maximising at C_{16} has been found to be characteristic for post-fire samples (Almendros et al., 1988).

8.4.4 Curie-point pyrolysis: nitrogen containing compounds

Chitin is a highly ordered copolymer of 2-acetamido-2-deoxy-β-D-glucose and 2-amino-2-deoxy-β-D-glucose that differs from other polysaccharides by the presence of nitrogen in its structure (Muzzarelli and Muzzarelli, 1998). Chitin derived nitrogen containing products (14, 30, 53, 55, 65, 69, 71, 72, 83; Table 8.1, Fig. 8.2), may be derived both from chitin-glucans present in the cell wall of fungi (Ramos-Sanchez et al., 1988; Windig et al., 1982) and/or chitin in arthropods (Stankiewicz et al., 1996). The relatively large amount of chitin derived products when compared with other soil pyrolysates (e.g. van Bergen et al., 1997, 1998a), indicates once again that fungi are important in this andic sub-soil. In addition, a contribution from arthropods (e.g. collembolas, ants) may be significant. These latter organisms are important in soils (Bull et al., 2000a) and may be responsible for biological mixing of fresh materials from the top-soil into the profile (i.e. bioturbation).

Toluene, pyrrole, (alkyl)phenols, probably phenol and N-containing-compounds such as indole and methylindole may result from a contribution of amino acid moieties, probably present as polypeptides and proteins (Bracewell and Robertson, 1984; Tsuge et al., 1985; Boon and de Leeuw, 1987; van Heemst et al., 1999). These nitrogen compounds may also be present in chitin-protein complexes of arthropods in addition to a plant source. As described for the carbohydrate fraction of SOM (section 8.4.2.3), the stability towards microbial degradation of N-containing SOM compounds may have been increased by heating (Knicker et al., 1996) resulting in the transformation of N-containing compounds.

8.4.5 Curie-point pyrolysis: aromatic compounds

The relatively low contribution of lignin derived (Saiz-Jiminez & de Leeuw, 1984a) methoxy phenols (51, 63, 67, 79, 82; Table 8.1, Fig. 8.2), might be related to the low soil pH, which has been suggested to enhance the degradation of lignin in mineral soils (Nierop and Verstraten, 2003). A low lignin contribution is also witnessed by the low abundance of the 56 ppm peak in the NMR data (Fig. 8.1) (Hatcher, 1987). This low pH and the degradation of lignin may be strongly related to fungi the presence of which is reflected in both the polysaccharide pyrolysis products (section 4.2) and specific nitrogen containing chitin pyrolysis products (section 4.4). Although brown-rot fungi are selectively metabolising polysaccharide moieties over those of lignin (Blancette et al., 1994), white-rot fungi can degrade lignin and cellulose simultaneously or selectively degrade lignin present in lignocellulose (Eriksson et al., 1990; Vane et al., 2001).

Condensed tannins might be expected to contribute to the organic matter in this sub-soil sample as well. The absence of specific tannin pyrolysis products (e.g. 1,2-benzenediol and 4-methyl-1,2-benzenediol; Galletti and Reeves, 1992) and specific resonances in the NMR spectrum (105, 130, 145 and 153 ppm; Wilson and Hatcher, 1988; Preston et al., 1994) may indicate that strong sorption of tannins to Al/Fe-oxyhydroxides prevents the transport of these polyaromatic compounds to the sub-soil.

8.4.6 Dense aromatic structures; charcoal

In addition to possibly thermally altered cellulose pyrolysis products (see section 3.2.2), polyaromatic products (50, 76, 86, 88, 93, 94, 99, 105) derived from dense aromatic structures present in charcoal (add ref, Pastorova et al., 1994) have been identified. As mentioned, such products are expected in these soils considering that forest fires occur frequently on the island. Apart from an input from fresh charcoal, the polyaromatic signal (Table 8.1, Fig. 8.2) might be related to charcoal material formed during the deposition of hot volcanic ash, resulting in the burial and burning of the old vegetation. ^{14}C carbon dating of this charcoal material might help to distinguish these two sources in the future. In the NMR spectrum, the presence of condensed aromatic moieties is reflected by the peak at 130 ppm (Hatcher et al., 1989; Haumaier and Zech, 1995; Freitas et al., 1999). However, both pyrolysis and NMR techniques used in this study are discussed to underestimate the contribution of charcoal like materials (Skjemstadt et al., 1996, 1996; Poirier et al., 2000), implying that the contribution of these types of materials is most probably more important than reflected by our data.

Research to estimate the contribution of charcoal to these soils is currently undertaken because this carbon fraction may represent a significant sink of atmospheric CO_2 (Kuhlbusch and Crutzen, 1995). Apart from being intrinsically refractory (Baldock and Smernik, 2002), charcoal may be stabilized by organo-mineral complexation (Glaser et al., 2000). Such complexation may cause SOM, in the form of charcoal, to enter the soil carbon millennium pool identified in andic soils (Jenkinson et al., 1992; Torn et al., 1997).

8.5 Conclusions

Curie-point pyrolysis data are in line with the NMR data and reveal relatively high amounts of polysaccharide derived furans in this acid (pH 4.2) andic soil sample (TOC content 6.7%). These pyrolysis products result from a contribution of fungal and/or thermally altered plant polysaccharide structures. A significant fungal contribution, enhanced by the low soil pH, is further reflected by the strong degradation of lignin and specific nitrogen containing chitin pyrolysis products. The aliphatic signal reflects the presence of the biopolyesters cutin and suberin as well as the non-hydrolyzable biopolymers cutan and suberan. Relatively high amounts of chitin pyrolysis products may, in addition to fungi, be derived from arthropods indicating that these latter organisms are important for the reworking of organic matter in these soils. Other nitrogen containing products are derived from both proteins and polypeptides. Although probably underestimated by the techniques used, charcoal represents a significant contribution to the SOM. Preservation of organic matter in this acid andic sub-soil is suggested to be caused by an increased resistance against microbial degradation by thermal alteration of SOM and complexation of SOM with aluminium and iron.

Acknowledgements. This study was supported by the Institute for Paleoenvironment and Paleoclimate Utrecht (IPPU). T. Zalm is thanked for his help with Py-GC/MS analyses.

Chapter 9

Chemically resistant organic matter in an acid volcanic sub-soil

The chemically resistant soil organic matter fraction, isolated from an acid andic sub-soil, has been analysed using Curie-point pyrolysis in combination with CPMAS ^{13}C NMR. Both NMR and pyrolysis data reveal an important charcoal contribution in combination with an aliphatic signal most likely derived from non-hydrolysable aliphatic biopolymers. The chemically resistant soil organic matter fraction isolated will most probably contribute significantly to the soil carbon millennium pool in andic soils.

9.1 Introduction

Andisols cover between 0.76%-0.84% of the world's land area (Leamy et al., 1980) and are typically associated with the weathering of recent volcanic ash deposits in humid, temperate environments (Parfitt and Kimble, 1990; Shoji et al., 1993, 1996; Lowe, 1997). Despite their apparent small contribution to the total of soils in the world, they are significant for the global carbon cycle because of their capacity to store large quantities of organic carbon for thousands of years (Torn et al., 1997).

Stabilization of organic carbon in andic soils is suggested to arise mainly from the protective effects of relatively large quantities of both free aluminium and iron as well as their amorphous mineral phases in these soils (Torn et al., 1997). In addition to these protective effects, the structural recalcitrance of organic matter against biodegradation as such will also influence its preservation in soils (Largeau and de Leeuw, 1995). Although chemical resistance does not automatically imply resistance against biodegradation in soils (Nierop and Verstraten, 2003), the sequential removal of relatively labile SOM such as polysaccharides will yield the fraction of SOM that is more likely to be resistant in soils. In this study, the chemically resistant soil organic matter fraction is isolated from an acid andic sub-soil sample and analysed using Curie-point pyrolysis in combination with CPMAS ^{13}C NMR.

9.2 Experimental

9.2.1 Materials

Madeira Island (Portugal) is located in a fully (Atlantic) oceanic domain between 32^{o}38' and 32^{o}52'N and 16^{o}39' and 17°16' W, approximately 600 km from the African coast, at the same latitude as Casablanca (Morocco). Soil samples were taken from andic profiles located on this island that have been classified as either being Umbric or Haplic Andisols (FAO, 1998) depending on their colour (Madeira et al., 1994).

The profile studied is situated on a hill (slope 30^{o}W) near the village of Poiso (altitude 1175 m). The vegetation consisted of mosses (5%), grasses (55%), ferns (20%) and deciduous trees, i.e. oak and birch (20%). Tree- and some grass roots were found to a depth of about 80 cm, but most of the intensive "root-activity" was found in the top 0-40 cm consisting mainly of grass fibrils. Three horizons were distinguished; a thin O horizon (0-2 cm), an A horizon (2-73 cm) and an E/B horizon (73- 90 cm), the latter being formed by weathered basalt bedrock material. The deeper

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layer, i.e. C horizon, was formed by a non-friable, compact layer of weathered basalt, the parent material for this Andisol (Madeira et al., 1994). The O horizon consisted mainly of litter and vegetation, i.e. grass, moss, twigs, leaves, etc. The A horizon (color 4/6 5YR) was characterized by a non-friable structure, clay aggregates with a diameter up to 1 cm, and highly weathered basalt fragments up to a diameter of 10 cm. Many roots were found in this horizon, ranging from mainly very fine grass roots in the top 30 cm to coarser roots, up to a diameter of 2 cm, at greater depth, i.e. 30-70 cm.

9.2.2 Sampling, sample pre-treatment, total organic carbon measurement

In September 2001, about 500 g of sample was taken every 10 cm up to a depth of 70 cm (including the O-horizon). Based on earlier studies (Chapters 2, 6), the sample taken from the depth interval of 20-30 cm was selected for this study. On Madeira, the sample was air dried in the dark and wrapped in aluminium foil. After 1 week, the sample was transported to the Netherlands and subsequently oven dried at 60°C, sieved over a 2 mm and a 250 μm sieve to remove large roots and basalt fragments. Total organic carbon content (TOC) and total nitrogen (TN) of the dried and sieved (<250 μm) sample were measured using a Fisons Instruments NA 1500 NCS analyzer, with a cycle time of 180 s, a source temperature of 190°C and an oxygen flow of ca. 30 l min^{-1}.

9.2.3 Isolation of the chemically resistant residue

Approximately 10 g of sieved (<250 μm) soil was Soxhlet extracted (Chapter 2) using dichloromethane/methanol (DCM/MeOH) (9:1v/v) for 24 h to remove extractable lipids (Fig. 9.1).

The residue was base treated for 1 h at 70°C using a 1M KOH in 96% MeOH solution (Chapters 5, 6) to remove base hydrolysable compounds such as biopolyesters (Fig. 9.1). After cooling, extract and residue were separated through centrifugation (3 min 3000 rpm). The residue was further extracted with water (3x), methanol/water (1:1 v/v; 1x), methanol (2x) and dichloromethane (3x).

The residue thus obtained was Soxhlet extracted using dichloromethane/ methanol (DCM/MeOH) (9:1v/v) for 24 h to remove any remaining extractable lipids and subsequently subjected to an acid hydrolysis using 6M HCl for 24h at 110^{o}C to remove mainly polysaccharides and polypeptides (Fig. 9.1). After cooling, extract and residue were separated through centrifugation (3 min 3000 rpm). The residue was further extracted with water (3x) untill the pH of the supernatant was approximately 7.

To remove any remaining ester-linked moieties, the residue was again base treated for 1 h at 70°C using a 1M KOH in 96% MeOH solution (Fig. 9.1). After cooling, extract and residue were separated through centrifugation (3 min 3000 rpm). The residue was further extracted with water (3x), methanol/water (1:1 v/v; 1x), methanol (2x) and dichloromethane (3x).

Prior to analyses, the residue was again Soxhlet extracted using dichloromethane/ methanol (DCM/MeOH) (9:1v/v) for 24 h to remove any remaining extractable lipids (Fig. 9.1). The chemically resistant residue thus obtained was air-dried and used for further study.

9.2.4 Solid state CPMAS ^{13}C NMR spectroscopy

Prior to solid state ^{13}C CPMAS NMR measurements, the chemically resistant residue was treated with cold HCl/HF to remove the mineral matrix. 100 ml 40% HF was added to approximately 10 g of the residue. The suspension was shaken for 2h at ± 250 rpm. 300 ml water was then added and the sample centrifuged. The residue was washed with 30 ml 30% HCl and centrifuges to remove fluoride gels. The residue was washed with 60 ml water (3x) and centrifuged until neutral pH.

The solid state ^{13}C CPMAS NMR spectra were obtained on a Bruker DSX 200 operating at a frequency of 50.3 MHz using zirconium rotors of 7 mm OD with KEL-F-caps. The cross polarization magic angle spinning (CPMAS) technique was applied during magic-angle spinning of the rotor at 6.8 kHz. A ramped ^{1}H-pulse was used during contact time in order to circumvent spin modulation of Hartmann-Hahn conditions (Peersen et al., 1993). A contact time of 1 ms, a 90^{o} ^{1}H pulse of 6.5 μs and a pulse delay of 400 ms were used for all spectra. The ^{13}C-chemical shifts are given relative to tetramethylsilane (= 0 ppm) and were calibrated with glycine (176.04 ppm). Assignments of characteristic peaks was based on published data (e.g. Hatcher, 1987; Kögel et al., 1988; Wilson and Hatcher, 1988; Nierop et al., 1999; Baldock and Smernik, 2002).

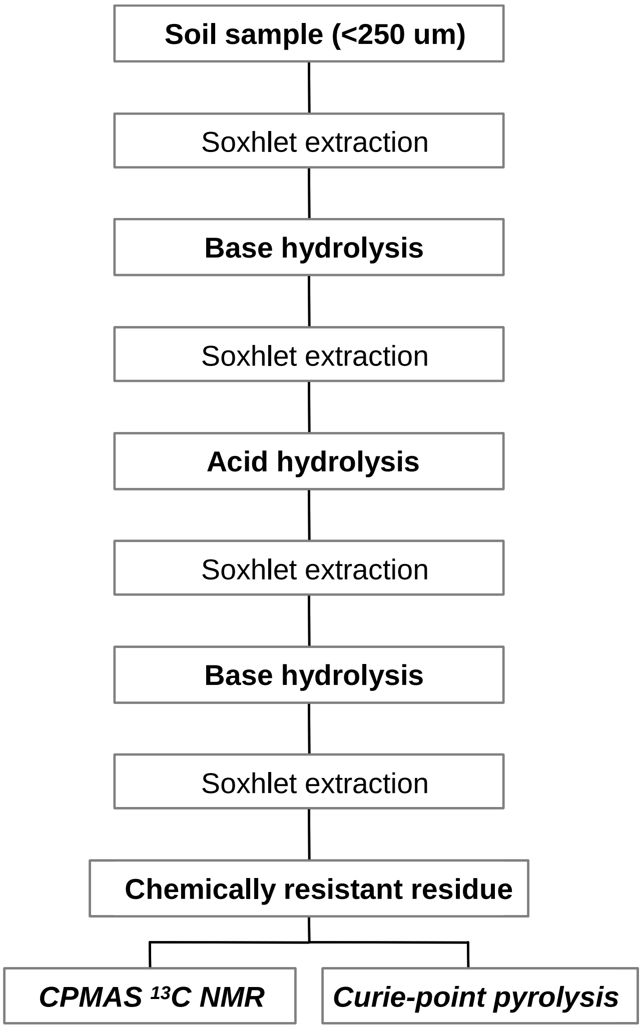

Figure 9.1 Schematic representation of the isolation of the chemically resistant residue.

9.2.5 Curie-point pyrolysis- gas chromatography-mass spectrometry (Py-GC/MS) Prior to analyses, the sample was pressed onto a ferromagnetic wire with a Curie temperature of 610°C. The interface temperature of the pyrolysis unit was set at 200°C and the pyrolysis time was 10 s. Py-GC/MS analyses were performed using a FOM-5LX Curie-Point pyrolyser connected to a Hewlett-Packard 5890 series gas chromatograph, equipped with a CP-sil 5CB-MS silica column (50 m x 0.32 mm, film thickness 0.4 μm), and a Fisons instruments VG platform II mass spectrometer operating at 70 eV, scanning the range *m/z* 50-650 with a cycling time of 0.65 s. The GC oven temperature was programmed from 40°C (5 min) to 310°C (isothermal for 10 min) at a rate of 5°C min^{-1}. Helium was used as carrier gas. Compound identification was based on mass spectral data and retention time comparisons with reference samples.

9.3. Results

TOC and TN, both expressed as weight percentage of the total sieved (<250 μm) soil mass, were 6.7 % and 0.6 % respectively for the untreated sample. In addition, an acid soil pH (H_2O) of 4.2 was determined. TOC, TN, C/N molar ratios and pH (H_2O) values of the complete profile have been described elsewhere (Chapter 2).

TOC and TN were 3.6 % and 0,1 wt % respectively for the chemically resistant fraction. After treatment with HF/HCl to remove the mineral matrix, the TOC increased to 16.0 %, the total nitrogen to 0.7 %.

Table 9.1 Mass spectral data of main pyrolysis products

No.	**Compound**	**Source**	**M$^+$**
1	Acetic acid	Ps	60
2	Benzene	Pp/Pr	78
3	Pyridine		79
4	Toluene	Pp/Pr	92
5	Unkown		82
6	Vinylbenzene (styrene)	Pp/Pr	104
7	Benzaldehyde		106
8	Benzonitrile	Pp	103
9	Phenol	Pp/Lg/Pr	94
10	Indene		116
11	2-Methyl phenol	Pp/Lg/Pr	108
12	3/4- Methylphenol	Pp/Lg/Pr	108
13	2-Methoxyphenol	Lg	124
14	Naphthalene	Pa	128
15	Unknown		132
16	Biphenyl	Pa	154
17	Fluorene	Pa	166
18	Unknown		264
●	Fatty acid	Lp	
○	*n*-Alkene	Abp	
▽	*n*-Alkane	Abp	

Ps: polysaccharides, Pp: polyphenols, Pr: proteins, Lg: Lignin, Pa: polyaromatic structures, Lp: lipids, Abp: aliphatic biopolymer

9.3.1 solid state CPMAS ^{13}C NMR and Curie-point pyrolysis-GC/MS

The solid sate CPMAS ^{13}C NMR spectrum was dominated by a main signal ain the aromatic C region (160-110 ppm) peaking at 130 ppm (Fig. 9.2). The second most abundant peak was identified in the alkyl region (30 ppm). In addition, peaks were identified around 55 most tentatively assigned to methoxyl C and N-alkyl C. The signal at 170 ppm derives from carboxyl C bound to aromatic rings. Its high intensity indicates a high oxidation state of the sample. Besides these signals, two spinning side bands arise due to imcomplete removal of chemical shift anischopy of aromatic C.

The pyrolysis-GC/MS total ion current (TIC) was dominated by series of alkene/alkane pairs ranging from C_8 to C_{32}, together with benzene (2), pyridine (3), toluene (4), styrene (6) and phenol (9), the latter being the most abundant compound identified (Fig. 9.3; Table 9.1). In addition, some monoaromatic compounds (peaks 10, 11, 12, 13), a nitrogen containing product (8) and polyaromatic compounds were found including naphthalene (14), biphenyl (16) and fluorene (17).

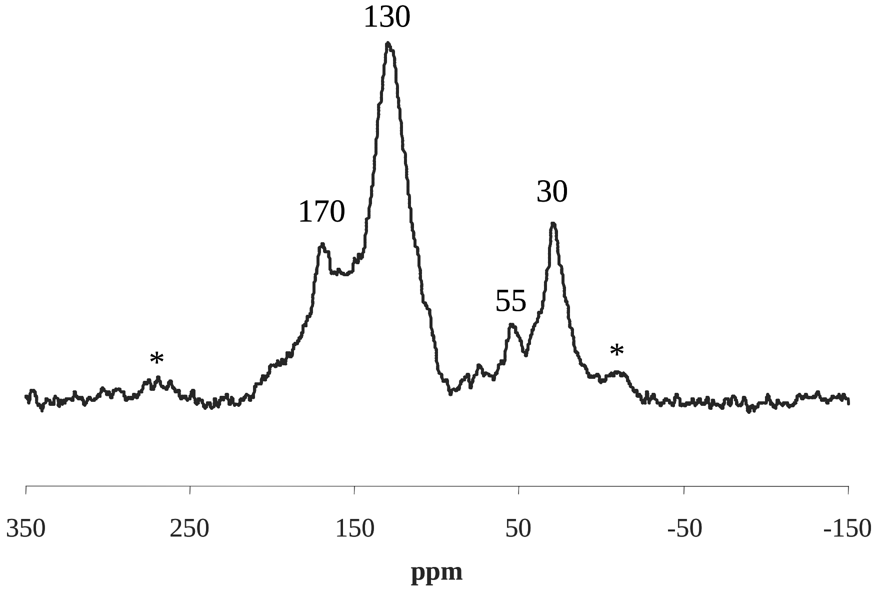

Figure 9.2 CPMAS 13C NMR spectrum chemically resistant residue. * refers to spinning side band.

9.4. Discussion

The sequential chemical treatments used result in a decrease in TOC content of 47 % and a TN decrease of 86%. The relatively larger decrease in TN results from the removal of relatively labile N-containing compounds such as peptides as well as inorganic nitrogen (e.g. nitrate), whereas part of the organic C containing compounds seems to be more chemically resistant. It should be noted that both the TOC and TN values of the chemically resistant residue have been influenced by the dissolution of part of the mineral matrix resulting in relative increase of both TOC and TN values. Allophane is known to dissolve in acid solutions (Parfitt and Kimble, 1990). Treatment of the sample with 6M HCl for 24 h at 110 °C will therefore result in the partial dissolution of the mineral matrix. Calculations used to estimate the contribution of the chemically resistant residue should therefore take into account the decrease in weight (i.e. 87%) of the sample during the chemical treatments. Based both on the TOC and the weight loss, the contribution of

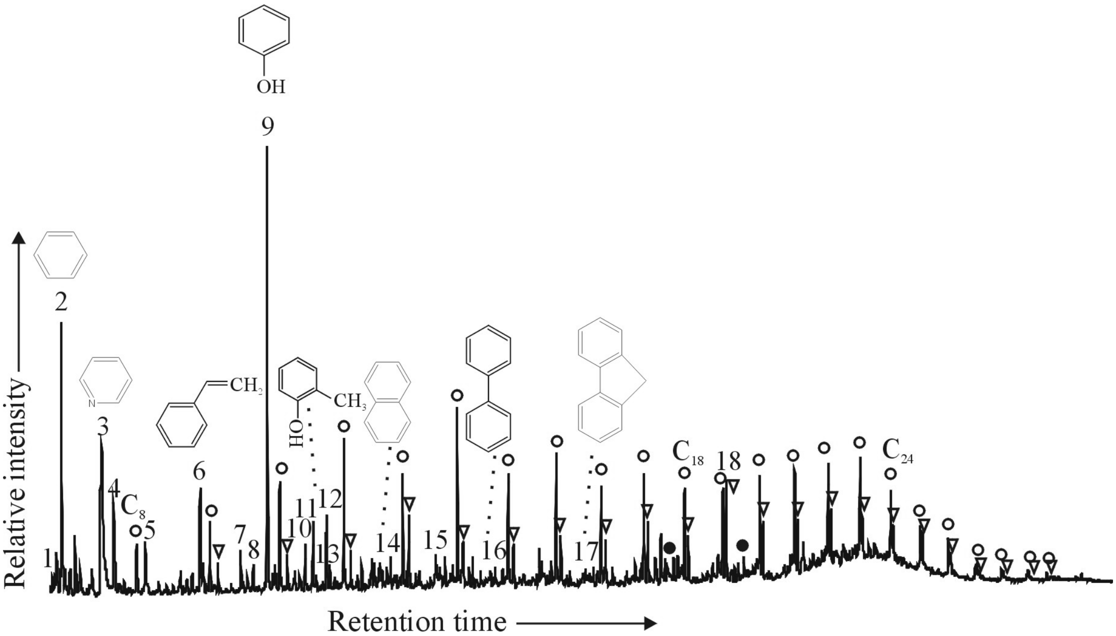

Figure 9.3 Curie-point pyrolysis-GC/MS total ion current (Curie temperature of 610 oC) of the chemically resistant residue. Numbers above peaks refer to Table 1.

chemically resistant fraction obtained to the total of soil organic matter is approximately 3 wt%. These results are in line with previous research by Poirier et al. (2002), who found the non-hydrolysable organic carbon fraction in an ancient tropical soil to account for ca. 5% of the total carbon. Due to losses during the work-up procedure, the real contribution of chemically resistant SOM is probably more than the 3% calculated.

Previous research (Chapter 8) including pyrolysis-GC/MS and CPMAS ^{13}C NMR revealed that the majority of organic matter in this acid andic sub-soil is comprised of fungal and/or thermally altered plant polysaccharide moieties. A significant fungal contribution, enhanced by the low soil pH, was further reflected by the strong degradation of lignin and specific nitrogen-containing chitin pyrolysis products. In addition to these latter polysaccharide and chitin derived signal, signals reflecting the presence of non-hydrolyzable biopolymers, proteins, polypeptides and condensed aromatic compounds were found to contribute significantly (Chapter 8).

9.4.1 Solid state ^{13}C CPMAS NMR spectroscopy

The CPMAS ^{13}C NMR the spectrum (Fig. 9.2), reveals a pattern typically assigned to C in condensed aromatic structures probably representing charcoals (Hatcher et al., 1989; Haumaier and Zech, 1995; Knicker et al., 1996; Freitas et al., 1999). The occurrence of the two spinning side bands is due to an anisotropy associated with the presence of magnetic species in the mineral phase of the material and has been observed before (Knicker et al., 1996; Freitas et al., 1999).

The peak around 30 ppm is assigned to alkyl C in non-hydrolysable alkyl-based biopolymers such as cutan and suberan (Nip et al., 1986b; Tegelaar et al., 1989b, 1995). In addition, cycloalkane structures may be formed by thermal alteration of aliphatic materials in soils (Knicker et al., 1996). Peaks around 55 ppm and 170 ppm are most probably derived from C in methoxyl groups (Hatcher, 1987) and carboxyl groups respectively (Kögel et al., 1988; Kögel-Knabner, 1997). Very minor signals between 60 and 90 ppm are assigned to a minor contribution of polysaccharides (Kögel et al., 1988).

9.4.2 Curie-point pyrolysis-GC/MS

All ester-linked moieties have been removed during the two base hydrolyses. Therefore, the presence of C_8-C_{32} alkene/alkane pairs (Fig. 9.3) most likely reflects a contribution non-hydrolysable biopolymers such as cutan (Nip et al., 1986b, 1987), and suberan (Tegelaar et al., 1989b, 1995; Nierop, 1998). These non-hydrolysable biopolymers are considered to be responsible for the accumulation of resistant aliphatics in soils (Tegelaar et al., 1989b; Nierop et al., 2001b) and have been identified in residues after severe chemical extractions before (Almendros et al., 1999; van Bergen et al., 1998). The detection of nitrogen containing pyrolysis products reflects the presence of resistant nitrogen containing heterocyclic structures that result after substantial thermal alteration of polypeptides (Knicker et al., 1996). The 2-methoxyphenol (13) is derived from a left-over of lignin (e.g. Saiz-Jiminez and de Leeuw, 1986a) or tannin (van Bergen et al., 2003).

Polyaromatic products identified are probably derived from condensed aromatic moieties of charcoal (Pastorova et al., 1994; Behar and Hatcher, 1995), which are also reflected in the NMR spectrum by the peak at 130 ppm. Charcoal is expected in these soils considering that forest fires occur frequently on the island. Moreover, it has been identified in the non-hydrolyzable fraction of SOM before (Poirier et al., 2002). Biomass burning leads to a significant production of charred materials (Seiler and Crutzen, 1980). During burning, or better thermal alteration process, well-known plant and soil organic matter compounds, such as cellulose, are transformed into (poly) aromatic structures (e.g. Pastorova et al., 1994). Apart from an input from fresh charcoal, the polyaromatic signal might be related to charcoal material formed during the deposition of hot volcanic ash, resulting in the burial and burning of the old vegetation. ^{14}C carbon dating of this charcoal material (Poirier et al., 2002) might help to distinguish these two sources in future.

Both pyrolysis and NMR techniques used in this study are known to underestimate the contribution of charcoal like material (Skjemstadt et al., 1996, 1996; Poirier et al., 2000), implying that the contribution of this material is most probably even more important than reflected by our data.

9.4.3 Isolation of chemically resistant residue

The methods used in this study to isolate a chemically resistant residue differ significantly from the so called classical humus fractionation (e.g. Swift, 1996) that has often been used to fractionate SOM in (volcanic) soils to date. In stead of separating the SOM into operationally defined humic, fulvic and humin fractions, the SOM in this study is separated into specific chemical fractions that can be analysed on the molecular level. For example, free lipids selectively removed by the first Soxhlet extraction as well as ester-linked compounds removed by the base-treatment (Fig. 9.1) have been studied in detail on the molecular level (Chapters 2, 5, 6). Moreover, the base-hydrolysis used was shown to be a very efficient method to remove and analyse ester-bound compounds in soils (Nierop et al., 2003). Amino acids released during the acid hydrolysis have also been studied (Naafs et al., unpublished results).

A comparison of pyrolysis data of the total SOM (Chapter 8) and the pyrolysis data presented in this study indicates that the specific sequential chemical SOM extraction used is a very effective way to remove chemically labile SOM fractions such as polysaccharides, peptides and biopolyesters.

9.4.4 Stabilization of OM in andic soils

Selective enrichment and molecular characterization of the chemically resistant SOM fraction in soils presented in this study provides a powerful tool to the study of long-term storage of carbon in soils. Radiocarbon dating of charcoals and the non-hydrolysable fraction of SOM revealed ages of 1000-8300 years (Glaser, 1999; Poirier et al., 2002). This chemically resistant carbon fraction, isolated through sequential removal of chemically labile SOM fractions in this study, may represent a significant sink of atmospheric CO_2 (Kuhlbusch and Crutzen, 1995). Charred material has been found to contribute significantly to SOM in volcanic soils before (Kumada, 1983; Shindo, 1986; Kuhlbusch and Crutzen, 1995). Apart from being intrinsically refractory (Shindo, 1991; Baldock and Smernik, 2002), charcoal may be stabilized by organo-mineral complexation in these soils (Glaser et al., 2000). In general, organic matter in andic soils is thought to be stabilized through the formation of Al-organic complexes and sorption to allophane, imogolite and ferrihydrite (e.g. Shoji et al., 1993; Saggar et al., 1994, 1996; Parfitt et al., 1997, 1999; Baldock and Skjemstad, 2000). Such complexation may explain the strong relationship between ^{14}C age of SOM and mineral surface as found by Torn *et al*. (1997). In addition to being intrinsically refractory, complexation may therefore cause SOM, in the form of charcoal, to enter the soil carbon millennium pool identified in andic soils (Jenkinson et al., 1992; Torn et al., 1997). Overall, fire-induced sequestration of carbon from the short-term biological to the long-term geological cycle may represent an important sink for atmospheric CO_2 (Kuhlbusch and Crutzen, 1995). Further isolation and molecular characterization of chemically resistant SOM together with for example ^{14}C dating will provide essential knowledge about the long-term storage of carbon in soils.

Acknowledgements. This study was supported by the Institute for Paleoenvironment and Paleoclimate Utrecht (IPPU). T. Zalm is thanked for his help with Py-GC/MS analyses.

Chapter 10

The molecular composition of non-hydrolyzable aliphatic soil organic matter

The non-hydrolyzable fraction of soil organic matter isolated during an earlier study (Chapter 9) was subjected to RuO_4 oxidation providing detailed insights into the molecular structure of the aliphatic soil organic matter in this fraction. The aliphatic molecular building blocks consisted of mono-, di-, tri- and tetra- functionalized aliphatic compounds reflected by the presence of series of C_9-C_{32} *n*-alkanoic acids, C_7-C_{30} α, ω-alkanedioic acids, C_{21}-C_{30} keto-α, ω-alkanoic diacids and C_{16}-C_{27} diketo-α, ω-alkanoic diacids in the RuO_4 extract. A comparison was made between the molecular building blocks of non-hydrolyzable aliphatic SOM and the molecular buiding blocks of biopolyesters observed during an earlier study within the same sample (Chapter 6). As a result, the formation of chemically resistant aliphatic material in soils through a process of cross-linking within biopolyesters is considered unlikely.

10.1 Introduction

The use of both acid and base hydrolysis in combination with analyses such as pyrolysis- gas chromatography- mass spectrometry (Py-GC-MS) and ^{13}C NMR, have shown clearly that soil organic matter contains a non-hydrolyzable aliphatic fraction (Saiz-Jiminez and de Leeuw, 1987b; Kögel-Knabner et al., 1992; Augris et al., 1998). This fraction is suggested to be derived from insoluble, non-hydrolyzable highly aliphatic biopolymers present in the cuticle and periderm of higher plants (de Leeuw & Largeau, 1993) i.e. cutans and suberans, respectively (Nip et al., 1986b; Tegelaar et al., 1989a, 1995). These biopolymers are usually present in low amounts in modern plants (Kögel-Knabner, 2002) but, since they appear to be resistant against degradation, can be enriched upon degradation of soil organic matter (de Leeuw & Largeau, 1993; Tegelaar et al., 1989d).

Studies of the aliphatic, non-hydrolyzable fraction in soils have, until now, mostly been performed using flash pyrolysis and ^{13}C NMR (e.g. Augris et al., 1998; Poirier et al., 2000). However, specific chemical degradation studies are scarce. Analyses on a molecular level have been hampered because of two main issues. First, the material needs to be isolated from the complex mixture of soil organic matter and the inorganic soil matrix. Second, the material needs to be subjected to some treatment which depolymerizes the macromolecular network to yield its molecular building blocks (Schouten et al., 1998).

In this study, the non-hydrolyzable fraction of soil organic matter isolated from an andic sub-soil during an earlier study (Chapter 9) was subjected to RuO_4 oxidation. This chemical degradation technique is known to completely oxidize aromatic compounds and to cleave ether bonds to their corresponding acids (Schouten et al., 1998). To the best of our knowledge, this procedure for the first time reveals detailed information about the molecular structure of the non-hydrolyzable aliphatic fraction in soils. The molecular building blocks obtained were analyzed using both GC and GC/MS.

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10.2 Materials and methods

10.2.1 Materials

The sample used for this study was taken from an acid (pH ≈ 4) andic profile located on Madeira Island (Portugal) and described in detail earlier (Chapter 2). Briefly, the sample used was taken from a depth of 20-30 cm, at which grass root fibrils were dominant.

10.2.2 Sample pre-treatment and total organic carbon measurement

The sample was oven dried at 60°C, sieved over a 2 mm and a 250 μm sieve to remove large roots and basalt fragments. Total organic carbon content (TOC) and total nitrogen (TN) of the dried and sieved (<250 μm) sample were measured using a Fisons Instruments NA 1500 NCS analyzer, with a cycle time of 180 s, a source temperature of 190°C and an oxygen flow of ca. 30 $l.min^{-1}$.

10.2.3 Isolation of the non-hydrolyzable residue

The isolation of the chemically resistant, i.e. non-hydrolyzable, residue has been described in detail earlier (Chapter 9). Briefly, approximately 10 grams of sieved (<250 μm) soil was Soxhlet extracted using dichloromethane/methanol (DCM/MeOH) (9:1v/v) for 24 h to remove extractable lipids (Chapter 2). The extracted residue was subsequently base treated for 1 h at 70°C using a 1M KOH in 96% MeOH solution (Chapters 5, 6) to saponify and remove base hydrolyzable compounds such as biopolyesters. The residue thus obtained was Soxhlet extracted to remove any remaining extractable lipids and subsequently subjected to an acid hydrolysis using 6M HCl for 24h at 110°C to remove mainly polysaccharides and polypeptides. To remove any remaining ester-linked moieties, this residue was again base treated. The residue thus obtained was once again Soxhlet extracted to remove any remaining extractable lipids.

Prior to the solid state CPMAS ^{13}C NMR measurements and the ruthenium tetroxide oxidation (RuO_4), the chemically resistant residue was treated with cold HF/HCl to remove the mineral matrix. The non-hydrolyzable residue thus obtained was air-dried and used for further study.

10.2.4 Solid state CPMAS ^{13}C NMR spectroscopy

The solid state CPMAS ^{13}C NMR spectra were obtained with a Bruker DSX 200 operating at a frequency of 50.3 MHz using zirconium rotors of 7 mm OD with KEL-F-caps (cf. Chapter 9). The cross polarization magic angle spinning (CPMAS) technique was applied during magic-angle spinning of the rotor at 6.8 kHz. A ramped ^{1}H-pulse was used during contact time in order to circumvent spin modulation of Hartmann-Hahn conditions (Peersen et al., 1993). A contact time of 1 ms and a 90° ^{1}H pulse with of 6.5 μs were used for all spectra. The ^{13}C-chemical shifts were calibrated to tetramethylsilane (= 0 ppm) and were calibrated with glycine (176.04 ppm).

10.2.5 Ruthenium tetroxide (RuO_4) oxidation

The ruthenium tetroxide oxidation used in this study was modified from procedures reported earlier (Boucher et al., 1991; Blokker et al., 2000). The HF/HCl treated chemically resistant residue (100 mg, TOC = 16.0%) was ultrasonically suspended in a mixture of 4 ml chloroform, 4 ml acetonitrile and 6 ml H_2O (pH 3-4) respectively. After addition of 2 g $NaIO_4$ (to establish a co-oxidant : sample ratio of 20:1) and 25 mg $RuCl_3.3H_2O$, the two phase system was allowed to react in an ultrasonic bath for 1 h and then stirred for 24 h at room temperature. After these 24 h, 2 ml H_2O (pH ≈ 1) and 6 ml hexane were added. The organic layer was removed after centrifugation (3000 rpm, 3 min). Ruthenium salts were precipitated from this organic extract by step-wise addition of 0.5 ml methanol. The aqueous layer and the residual material were further extracted with 8 ml hexane/dichloromethane (1:1 v/v) (2x). The organic layers after centrifugation were added to the first organic extract obtained. The combined organic extract was mixed using a Pasteur pipette to precipitate all ruthenium salts and subsequently centrifuged. The supernatant was washed with 0.5 ml $Na_2S_2O_3$ (5% in H_2O). After addition of 1 ml H_2O (pH 3-4), the two-phase system was centrifuged. The supernatant was evaporated to dryness using a rotary evaporater, dissolved in dichloromethane/isopropanol (2:1 v/v) and filtered using a Pasteur pipette packed with defatted wool, 1.5 cm Na_2SO_4 and 0.5 cm SiO_2 and dried using N_2. Carboxylic acid groups present were methylated using diazomethane. The methylated sample was dried using N_2 and dissolved in dichloromethane. The total extract was analyzed using GC and GC/MS.

10.2.6 Curie-point pyrolysis- gas chromatography-mass spectrometry (Py-GC/MS)

Prior to analyses, the sample was pressed onto a ferromagnetic wire with a Curie temperature of 610°C. The interface temperature of the pyrolysis unit was set at 200°C and the pyrolysis time was 10 s. Py-GC/MS analyses were performed using a FOM-5LX Curie-Point pyrolyser connected to a Hewlett-Packard 5890 series gas chromatograph, equipped with a CP-sil 5CB-MS silica column (50 m x 0.32 mm, film thickness 0.4 μm), and a Fisons instruments VG platform II mass spectrometer operating at 70 eV, scanning the range *m/z* 50-650 with a cycling time of 0.65 s. The GC oven temperature was programmed from 40°C (5 min) to 310°C (isothermal for 10 min) at a rate of 5°C min^{-1}. Helium was used as carrier gas. Compound identification was based on mass spectral data and retention time comparisons with reference samples.

10.2.7 Gas chromatography (GC)

GC analyses were performed using a Hewlett-Packard 6890 series gas chromatograph equipped with a CP-sil 5CB silica column (50m x 0.32mm, film thickness 0.12μm). Derivatized extracts (1.0 μl) in hexane were injected on-column. The oven temperature was programmed from 70 °C to 130 °C at 20 °C min^{-1} and from 130 °C to 320 °C (isothermal for 20 min) at 4 °C min^{-1}. Compounds were detected using a flame ionisation detector at 325 °C. Helium was used as carrier gas.

10.2.8 Gas chromatography-mass spectrometry (GC/MS)

Gas chromatography-mass spectrometry analyses were performed using a Hewlett-Packard 5890 series II gas chromatograph connected to a Fisons instruments VG platform II mass spectrometer operating at 70 eV, scanning the range *m/z* 50-650 with a cycling time of 0.65 s. The capillary column and temperature programme were as described for the GC analyses. Compound identification was based on published data (e.g. Blokker, 2000).

10.3 Results

TOC and TN were 3.6 % and 0,1 wt % respectively for the non-hydrolyzable fraction (Chapter 9). After treatment with HF/HCl to remove the mineral matrix, the TOC increased to 16.0 %, the TN to 0.7 % (Chapter 9). Based on both the TOC and the weight loss, the contribution of the chemically resistant fraction obtained to the total of soil organic matter is approximately 3 wt% (Chapter 9). Based upon the weight of the total extract obtained after RuO_4 oxidation, about 3% of the carbon in this chemically resistant residue is released using the RuO_4 oxidation.

Curie-point pyrolysis (Curie-temperature of 610 ^{o}C) and CPMAS ^{13}C NMR data indicate that the chemically resistant residue is comprised of aliphatic moieties in addition to a dominant contribution of dense aromatic structures, most probably in the form of charcoal (Chapter 9). The mass chromatogram of *m/z* 83+ 85 obtained from the Curie-point pyrolysis-GC/MS total ion current of the chemically resistant residue reveals a series of alkene/alkane doublets ranging from C_6 to C_{31} (Fig. 10.1). Interesting features in the alkene/alkane distribution are the sharp decrease in intensity after C_{23} and the maxima around C_{11}, C_{13} and C_{15}.

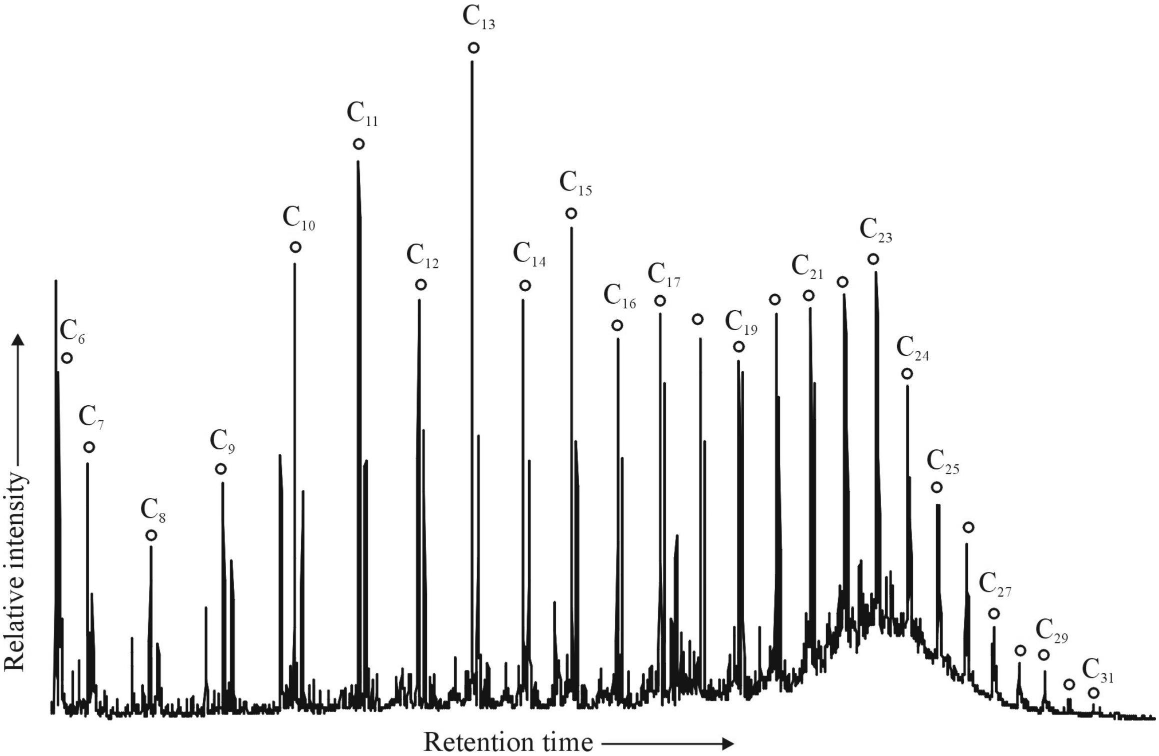


Figure 10.1 Mass chromatogram of m/z 83+85 obtained from the Curie-point pyrolysis-GC/MS total ion current (Curie temperature of 610 oC) of the chemically resistant residue (cf. Chapter 9). Numbers above peaks refer to chain length of the alkene/alkane doublets, denotes the position of an alkene/alkane doublet.

The total ion current of the RuO_4 extract is dominated by series of *n*-alkanoic acids and α, ω-alkanoic diacids (Fig. 10.2). *n*-Alkanoic acids range from C_9 to C_{32}, maximizing at C_{14} and C_{16} and show a decrease in relative intensity after C_{30}. α, ω-Alkanoic diacids range from C_7 to C_{30}, maximize at C_9 and show a sharp decrease in abundance after C_{24}. In addition to these compounds, series of coeluting isomers of diketo-α, ω-alkanoic diacids with different keto group positions ranging from C_{16} to C_{27} and coeluting keto-α, ω-alkanoic diacids with different keto group positions ranging from C_{21} to C_{30} are observed (Fig. 10.2). It is noted that the position of the first keto group in these compounds is located at position 8, 9, 10 or 11. The second keto group, if present, is located at position ω8, ω9, ω10 or ω11. Besides the series of compounds mentioned, a mid-chain C_{30} diketone was identified together with two phthalates (Fig. 10.2). Based on mass spectral data only, the presence of C_{14} and C_{15} diketo-α, ω-diacids with the keto groups located at positions 4 and ω4 as well as some α-methyl, ω-ethyl esters and possibly mid-chain acids (Blokker et al., 2001) is suggested. These latter compounds are present in relatively minor amounts.

10.4 Discussion

Curie-point pyrolysis data in combination with ^{13}C CPMAS NMR analyses indicated that the sequential chemical SOM treatments of SOM used are very effective to remove chemically labile SOM fractions such as polysaccharides, peptides and biopolyesters (Chapter 9) that may interfere with the results obtained for the RuO_4 oxidation of the chemically resistant residue. The contribution of the chemically resistant, i.e. non-hydrolyzable, residue to the total of organic matter in the andic sub-soil is comparable with results obtained by others (Augris et al., 1998). The presence of *n*-alkene/alkane doublets in the C_6-C_{31} range (Fig. 10.1) has been observed earlier in Curie-point flash pyrolysates of lipid-free soils (van Bergen et al., 1997) and the non-hydrolyzable fraction of a forest soil (Augris et al., 1998). Both these studies considered these compounds to be derived from a contribution of cutans and/or suberans.

The contribution of RuO_4 degradation products as compared with the fraction not characterized after this degradation may seem relatively small. However, one should realize that CPMAS ^{13}C NMR data in combination with pyrolysis data indicate that the major part of the chemically resistant residue, i.e. the starting material, is comprised of condensed aromatic moieties (Chapter 9) which are completely oxidized to CO_2 during the RuO_4 treatment (Schouten et al., 1998). Moreover, RuO_4 oxidation yields of complex organic matter are often relatively low and lie in the same order of magnitude as the yield obtained here (cf. Schouten et al., 1998; Blokker, 2000). Most important, despite this relatively low recovery the experiment still provides valuable representative information about the building blocks of aliphatic biopolymers analyzed (Schouten et al., 1998; Blokker et al., 1998).

10.4.1 Molecular building blocks

The compounds identified in the RuO_4 extract contain two types of functional groups: carboxylic and keto groups (Fig. 10.2). RuO_4 is capable of cleaving ether-bonds by oxidation of the ether-linked carbon atoms (Schouten et al., 1998; Sharpless et al., 1981; Blokker, 2000). Isolated mid-chain ether linkages are oxidized by RuO_4 to ketones (Table 10.1), whereas vicinal ether linkages and double bonds are completely oxidized to two carboxylic acid moieties (Table 10.1; Schouten et al., 1998; Blokker et al., 2000). However, the presence of double bonds in the chemically resistant aliphatic SOM would have resulted in the detection of alkadienes in the pyrolysate. In addition, the presence of double bonds within the chemically resistant fraction is considered unlikely because of their high reactivity in soils (Nierop et al., 2003). Ether linkages are therefore considered the most likely bond types. RuO_4 is also known to convert primary alcohols to carboxylic acids (Schouten et al., 2000).

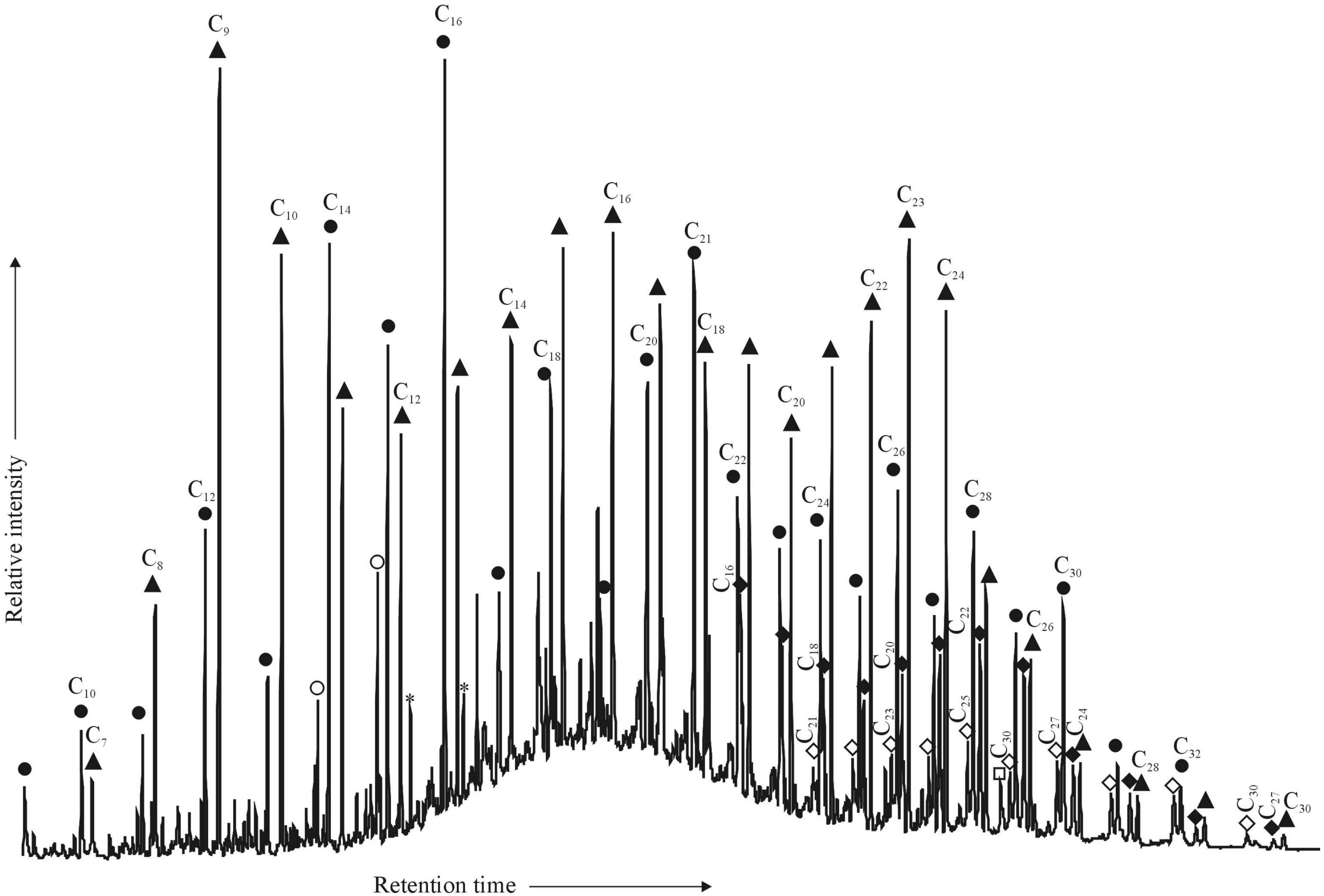
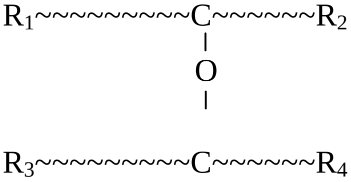
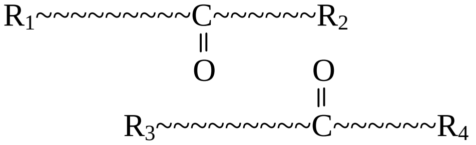


Figure 10.2 Total ion current of Ruthenium tetroxide extract after derivatization. Key:● : *n*-alkanoic acids,▲ : alkanoic diacids, ◆ : diketo-alkanoic diacids, ◇: diketo acids, □ : diketone,○ : ketoacid, * : phthlate. C_x above the peaks refers to the total number of carbon atoms.

Table 10.1 RuO_4 products and their origin (Schouten et al., 1998; Blokker, 2000)

Original compound	RuO_4 product(s)
R_1~~~~~~~~~C=C~~~~~~R_2	R_1~~~~~~~~~COOH + HOOC~~~~~~R_2
R_1~~~~~~~~~C-O-C~~~~~~R_2	R_1~~~~~~~~~COOH + HOOC~~~~~~R_2
R_1~~~~~~~~~COH	R_1~~~~~~~~~COOH
 $\begin{array}{c} R_1\sim\sim\sim\sim\sim\sim\sim\sim\sim C\sim\sim\sim\sim\sim\sim R_2 \\ \vert \\ O \\ \vert \\ R_3\sim\sim\sim\sim\sim\sim\sim\sim\sim C\sim\sim\sim\sim\sim\sim R_4 \end{array}$	 $R_1\sim\sim\sim\sim\sim\sim\sim\sim\sim \underset{O}{\underset{\Vert}{C}}\sim\sim\sim\sim\sim\sim R_2 \quad R_3\sim\sim\sim\sim\sim\sim\sim\sim\sim \overset{O}{\overset{\Vert}{C}}\sim\sim\sim\sim\sim\sim R_4$

Pyrolysis data can be used to provide structural information, although aspects such as the effect of cross-linking within the OM studied and rearrangements occurring during pyrolysis should be taken into account (van Bergen, 1999; van Bergen et al., 1999). Monocarboxylic acids identified in the RuO_4 extract are related to moieties originally present in the aliphatic matrix linked through one ether linkage. When comparing their distribution (Fig. 10.3) with the alkene/alkane pairs produced upon pyrolysis (Fig. 10.1), it becomes clear that the maximum chain length of the alkene/alkane pairs is related to the presence of C_{32} alkyl compounds attached to the aliphatic biopolymer structure. A maximum chain length around C_{32} was also observed in pyrolysis data of the aliphatic biopolymer cutan (Tegelaar et al., 1989c). In addition, maxima at C_{14} and C_{16} for the *n*-alkanoic acids (Fig. 10.3) are reflected in the pyrolysis data by maxima at C_{13} and C_{15} (Fig. 10.1), although to a minor extent.

The presence of α, ω-dicarboxylic acids is related to alkyl compounds originally linked within the aliphatic biopolymer structure on both ends through ether linkages. In addition, these compounds can be derived from free primary alcohols linked by an ether linkage to the non-hydrolyzable aliphatic SOM. Their specific distribution (Fig. 10.4) clearly reveals a decrease after C_{24}, which is also reflected in the pyrolysis data (Fig. 10.1). The relation between pyrolysis and RuO_4 data again indicates that the RuO_4 oxidation provides valuable representative information about the aliphatic building blocks of the material analyzed. The maximum at C_9 is most probably related to the former position of a double bond or hydroxy group, often observed in plant derived soil organic matter e.g. in root suberins (Chapter 7).

The last group of compounds identified (Fig. 10.1), those containing keto groups, represent compounds linked through mid-chain ether linkages in the original biopolymer structure. The location of the keto-group, i.e. the ether linkage in the original structure, is highly specific at position 8, 9, 10 or 11 or at position ω8, ω9, ω10 or ω11. Double bonds observed within biopolyesters have been suggested to be the starting point of the ether-cross linking of aliphatic biopolymers observed in marine environments (Blokker et al., 1998). The specific position of the ether linkages is, however, the only structural resemblance between the chemically resistant aliphatic fraction and plant cutins and suberins. Most important, the chain-length distribution of cutin and/or suberin building blocks (e.g. Holloway, 1982, 1983; Kolattukudy, 1980a; Chapter 6) differs significantly from that of the non-hydrolyzable aliphatic SOM analyzed (Fig. 10.2). The formation of chemically resistant aliphatic material through a process of crosslinking within the biopolyester matrix as suggested earlier (Kögel-Knabner et al., 1992) seems therefore unlikely. An important mid-chain coupling between the condensed aromatic and the aliphatic fraction in the residue would result in the formation of more mid-chain acids (Blokker et al., 2001) and is therefore also considered of limited importance, if present.

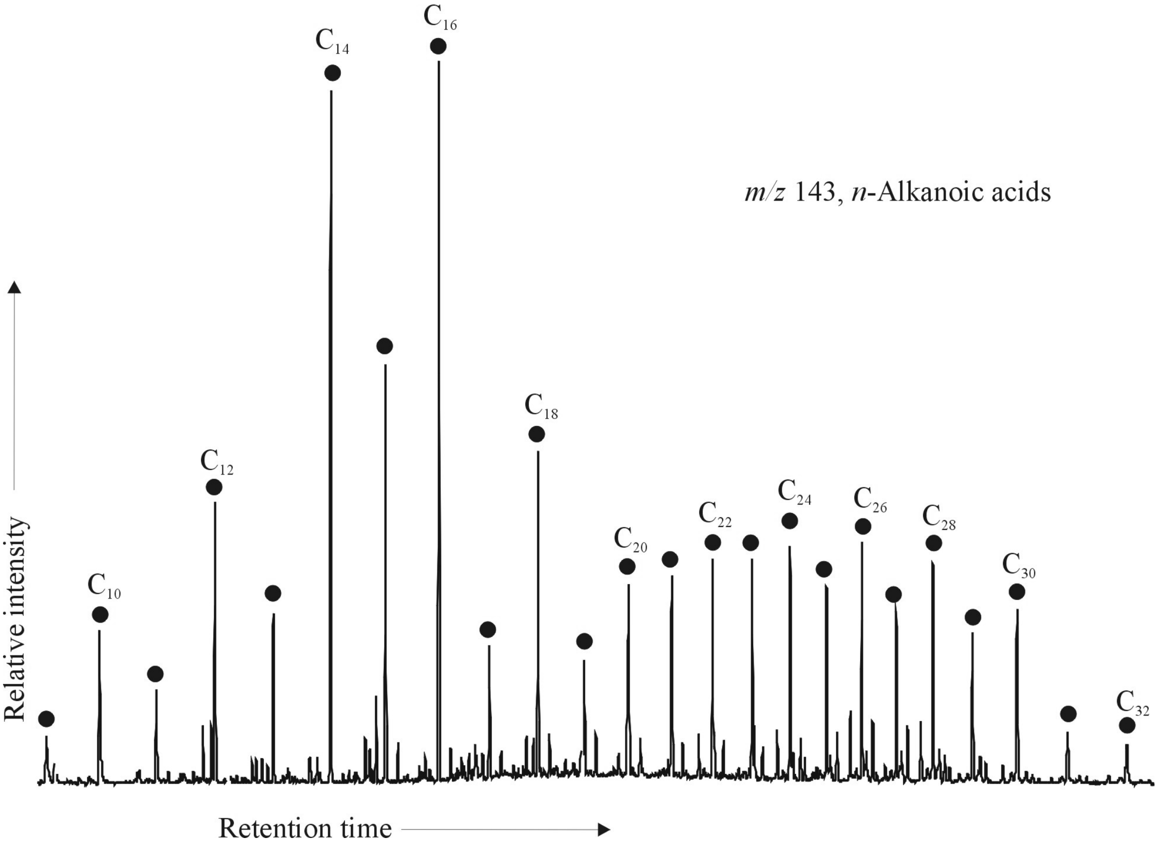


Figure 10.3 Mass chromatogram of *m/z* 143 obtained from the GC/MS total ion current of the Ruthenium tetroxide extract after derivatization. Numbers above peaks refer to chain length of the n-alkanoic acids.

10.4.2 Structure of non-hydrolyzable SOM and implications for further studies

The molecular building blocks obtained through the RuO_4 oxidation can be used to construct a model of the original macromolecular aliphatic structure. In general, the non-hydrolyzable aliphatic SOM present in the andic soil studied is formed by aliphatic moieties, linked by vicinal ether linkages creating a two dimensional structure and by mid-chain ether linkages creating a three dimensional structure. The mono carboxylic acids are most probably present as aliphatic compounds linked to the peripheral part of the aliphatic structure. Both α, ω-diacids and keto functionalized acids are more related to the core of the aliphatic material considering their ability of cross-linking.

A comparison between the pyrolysis data (Fig. 10.1) with published pyrolysis data from cutan (Tegelaar et al., 1989c) and suberan (Tegelaar et al., 1995) reveals both similarities and differences. Both pyrolysis of cutan and suberan as well as the non-hydrolyzable aliphatic SOM produce alkene/alkane doublets ranging from C_6 to C_{30} (and further). The chain length distribution of cutan pyrolysis products, however shows no relatively sharp decrease after C_{23}. In stead, a relatively smooth decrease from C_{27} to C_{35}

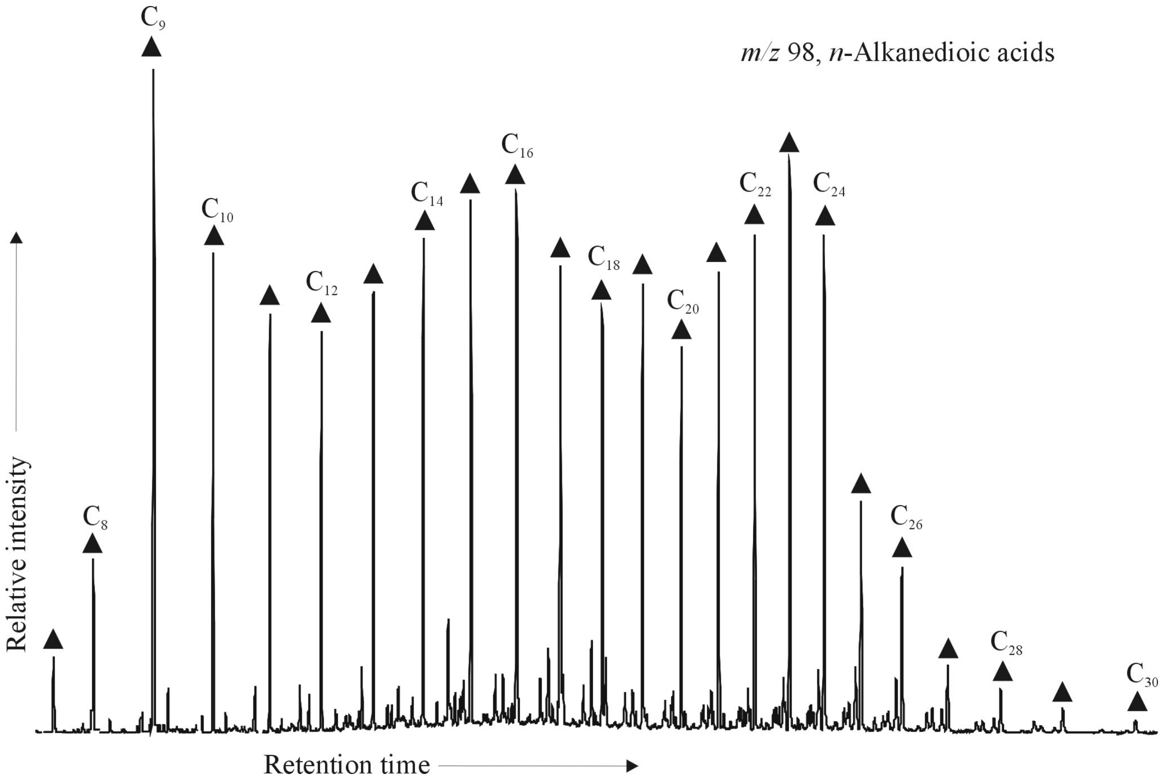


Figure 10.4 Mass chromatogram of *m/z* 98 obtained from the GC/MS total ion current of the Ruthenium tetroxide extract. Numbers above peaks refer to chain length of the alkanoic diacids. For compounds with chain length $<C_{10}$, the mass chromatogram has been adjusted according to relative intensity in Figure 10.2.

was observed (Tegelaar et al., 1989c). The chain length distribution of suberan pyrolysis products on the other hand revealed a sharp decrease after C_{20} (Tegelaar et al., 1995), comparable to the decrease observed in the pyrolysis data presented in this paper after C_{23} (Fig. 10.1). In addition, a maximum around C_{10}/C_{11} was observed in the suberan derived alkene/alkane doublets (Tegelaar et al., 1995), whereas no short-chain ($<C_{20}$) maximum was observed in the cutan derived alkene/alkane doublets (Tegelaar et al., 1989c). Overall, the pyrolysis data from suberan isolated from *Betula japonica* (Tegelaar et al., 1995) resemble more closely the data obtained for the non-hydrolyzable aliphatic SOM (Fig. 10.1). Suberan is therefore suggested to be the most likely source of the non-hydrolyzable aliphatic SOM fraction. The differences observed in the location of the maxima within the alkene/alkane chain-length distributions are most likely related to differences between suberan isolated from *Betula japonica* and the precursor (suberan) of the non-hydrolyzable aliphatic OM in the andic profile.

Finally, the molecular characterization of non-hydrolyzable aliphatic SOM as shown in this paper provides new opportunities for the study of organic matter in soils. Further research could focus on possible differences in the molecular composition of aliphatic non-hydrolyzable SOM between different vegetation species in soils. Molecular characterization of selectively preserved aliphatic biopolymers within sediments could distinguish this probably well preserved terrestrial input from a marine (algeanan) input and could be used as a more robust tracer for a terrestrial input than the more common used long-chain *n*-alkanes. Other interesting projects could focus on possible changes in the molecular structure upon degradation/preservation of these compounds in soils over time. Isotopic studies, involving compound specific ^{14}C and ^{13}C analysesare most interesting to obtain further insights into the turnover and role of non-hydrolyzable aliphatic SOM in soils and may help to distinguish between cutan and suberan derived inputs.

Acknowledgements. This study was supported by the Institute for Paleoenvironment and Paleoclimate Utrecht (IPPU). T. Zalm is thanked for assistance with Py-GC/MS analyses. Dr. Peter Blokker is sincerely thanked for his explanation and help with RuO_4 oxidations. Hendrik-Jan Bosch is thanked for his help with developing the RuO_4 oxidation procedure used in this paper.

Chapter 11

Conclusions

In the preceding chapters, a molecular approach to the study of organic matter in acid soils, with a focus on andic, i.e. volcanic, soils has been presented. Results have been discussed with respect to the factors that determine the composition of organic matter present in these soils (i.e. input, preservation/degradation and transport). In addition to obtaining molecular insights into SOM, a molecular approach to study SOM is suggested. In this concluding chapter, all data obtained from the various studies will be used to provide a more complete view on both the composition of SOM and the factors that determine its composition in acid soils. In the first two sections, focus will be on the free extractable lipid fraction and on ester-bound moieties in acid soils. The bulk and high molecular weight SOM fractions are discussed in the third section. In the final section of the conclusions, the answer to the title question in combination with mineralogical properties, factors influencing the preservation/ degradation of SOM in acid andic soils and recommendations for future research are discussed.

11.1 Free extractable lipids

Lipids exhibit an inherent diagnostic value with respect to SOM, both endogenous and exogenous, and the pedological processes to which SOM subjected. In this thesis, total lipid extracts have been analysed (according to procedures used by van Bergen et al., 1997, 1998a; Bull et al., 1998, 2000a, 2000b), instead of specific lipid fractions, e.g. *n*-alkanols or *n*-alkanoic acids (e.g. Amblès et al., 1989, 1993; Jambu et al., 1993, 1995). Analysing and comparing total lipid extracts, in addition to detailed analyses of specific fractions, has the advantage of obtaining a more complete view on lipid chemistry in soils, and therefore on the total of processes that determine the composition of SOM.

11.1.1 Input of free extractable lipids in an acid andic forest soil

A substantially lower input of lipids by roots deeper in the soil profile compared with the litter layer is observed. The distribution observed in the top layer with a very dominant contribution of C_{26} alcohol typically reflects an input by grass leaves. In addition, fungal and bacterial derived compounds have been observed in the litter (Chapter 2).

In addition to the surface grass-leaf signal, the signal from long-chain *n*-alkanoic acids (C_{22}-C_{32}), ω-hydroxy acids (C_{22}-C_{26}), C_{31} *n*-alkane and *n*-alkanols (C_{22}-C_{32}), observed in the samples taken from the mineral A horizon, is suggested to be indicative of an important input by (grass) roots. This contribution clearly increased with increasing depth (Chapter 2). Thermally assisted hydrolysis and methylation (THM) analyses of Soxhlet extracted samples revealed a ‘reverse’ link between the occurrence of free extractable- and ester-linked root derived C_{22}, C_{24} (and C_{26}) ω-hydroxy acids (Chapter 4) which justifies their use as main indicators of a root–derived input to SOM in the free extractable lipid fraction. Furthermore, this link indicates that hydrolysis of ester-linked lipids in soils is another source for free extractable lipids in addition to the earlier mentioned grass leafs, roots, fungi and bacteria. Most important, the research presented in this thesis revealed that roots are the main lipid contributors in andic sub-soils and that an input from the surface vegetation is only important within the first few top centimetres of the profile (Fig. 11.1).

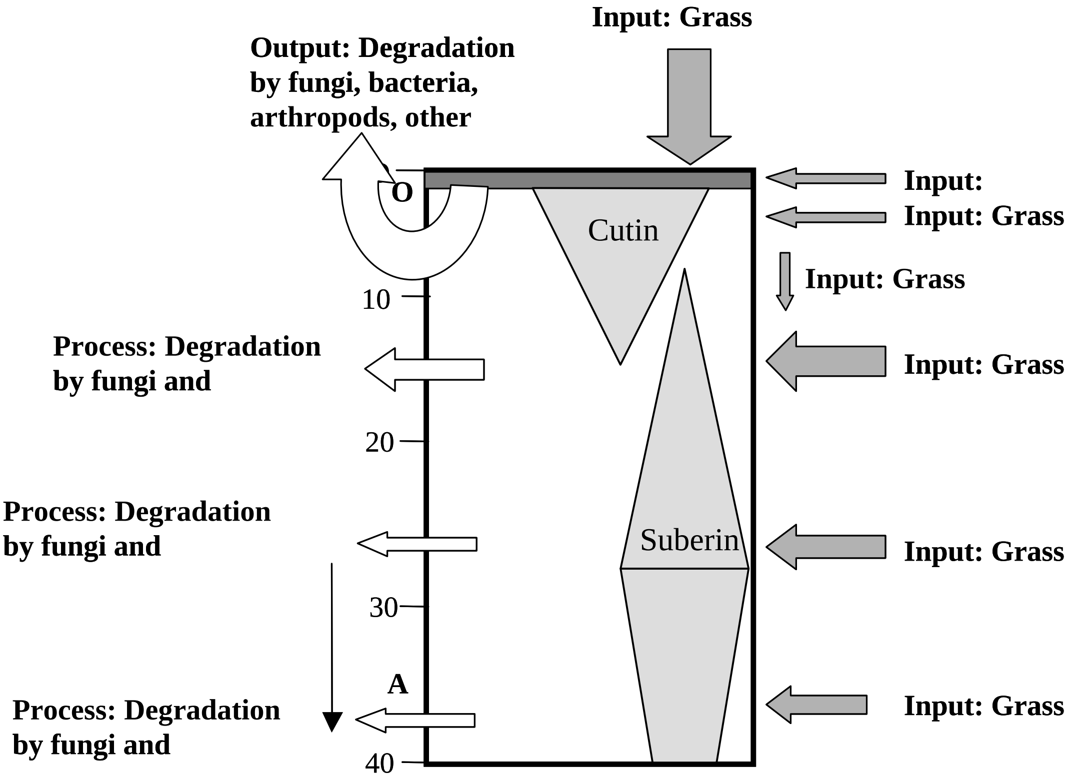


Figure 1.1 Schematic representation of soil processes in the andic profile based upon the toal lipid data obtained.

11.1.2 Degradation/stabilization of free extractable lipids in an acid andic forest soil In addition to well-known lipid degradation pathways such as microbial oxidation, the loss of steroids (and triterpenoids) on going from the litter to the A-horizon, may to a great extent be related to direct assimilation by arthropods (Nes et al., 1997; Bull et al., 2000a), a route of loss independent of low pH and the presence of aluminium (Chapter 2). This decrease, together with the C_{26} alkanol signal from the top of the profile (0-5cm) to the deeper layers, further indicates limited transport of lipids through the andic profile (Fig. 11.1). The absence of typical bacterial lipid markers within the profile suggests limited bacterial activity (Chapter 2) in the soil. Fungi on the other hand are known to be the predominant type of microorganisms in acid soils (Stevenson, 1994; Bumpus et al., 2003) and therefore play most probably an important role in the degradation of lipids within the acid andic soil.

In general, a reduced lipid degradation has been suggested within acid soils (e.g. Jamby et al., 1985; Dinel et al., 1990) because of a lower bacterial activity (Stevenson, 1994). Within the andic soil, lipids may further be stabilized through interactions with the allophane matrix present (Chapters 2 and 5). It should be noted that, due to the overall accumulation of SOM within andic soils, lipids have not been found to accumulate relative to other SOM compounds (this thesis).

In summer, a strong signal reflecting the leaching of microbially derived products from the litter and/or aerial vegetation at the surface is seen in addition to the original plant signal which determines the winter data (Chapter 2).

11.1.3 Total lipid extracts from characteristic horizons in a podzol profile

An *n*-alkane, steroid and triterpenoid signal related to aerial parts, i.e. leaves and flowers, of *Calluna* is only observed in the O horizon. This signal is rapidly lost in the underlying Ah horizon because of (bacterial) oxidation. All other total lipid extracts are dominated by root-derived lipids. These results are in line with previous reports on the source of high molecular weight organic compounds in podzols revealing a root dominated input in the sub-soil instead of compounds transported from the litter layer (Nierop and Buurman, 1999).

In the organic-rich sub-soil horizons, i.e. the Ahb and Bh horizons, root-derived friedolean and steroid compounds dominate the total lipid signal. In the Ahb horizon, a contribution from buried vegetation to the total lipid signal is still present, although degradation and an input by roots have significantly altered the original signal. C_{22} and C_{24} ω-hydroxy acids, long-chain ($>C_{20}$) *n*-alkanoic acids with a strong even over odd predominance and C_{22} and C_{24} *n*-alkanols, characteristic of an input from roots, dominate the organic-poor horizons, i.e. the E2, Bhs, Bs and B/C horizons. Steroid- and root-derived triterpenoids with a friedolean structure, once present in these horizons, have been degraded.

Within the podzol profile studied, organic matter mainly accumulates in the Bh horizon in combination with aluminium. In general, lipids in the podzol profile accumulate in organic-rich horizons, but not relative to other soil organic matter. Overall, lipid data indicate that degradation (mainly oxidation), is an important process that should be taken into account in addition to leaching when describing the so called “podzolization process” in soils.

Using the total lipid data obtained, a schematic representation of soil processes within the podzol profile can be reconstructed (Fig. 11.2). From this model it becomes clear that vertical transport of lipids is most probably of minor importance within this profile. Input by roots and preservation of organic matter due to the accumulation of organic matter (including lipids) in combination with aluminium appears to be far more important than leaching of organic compounds through the profile. These results are in line with earlier observations made for the insoluble fraction of soil organic matter (Nierop and Buurman, 1999) indicating that vertical transport of organic matter from the top of the profile is of minor importance compared with an *in situ* input by roots.

11.1.4 Recommendations for future soil lipid research

Lipids are probably the most well defined group of compounds amongst soil organic matter. However, specific data on the molecular composition of lipids of specific plants, microbes and fungi is, scarce. Complete lipid signatures for each species would greatly aid to our knowledge of soil processes and the origin of lipids identified in soil TLEs. The presence of specific organisms could then relatively easily be detected through the lipid record, assuming species specific signatures. In addition, processes such as transport and degradation could be studied in detail by using isotopically ^{13}C and ^{14}C labelled lipids revealing more information about for example the accumulation of organic mater in specific soil horizons. Most important for the acid soils studied, specific turnover rates for different lipid compounds can be obtained by monitoring the degradation of specific lipid compounds such as fatty acids, hydroxy acids, alkanols and steroids.

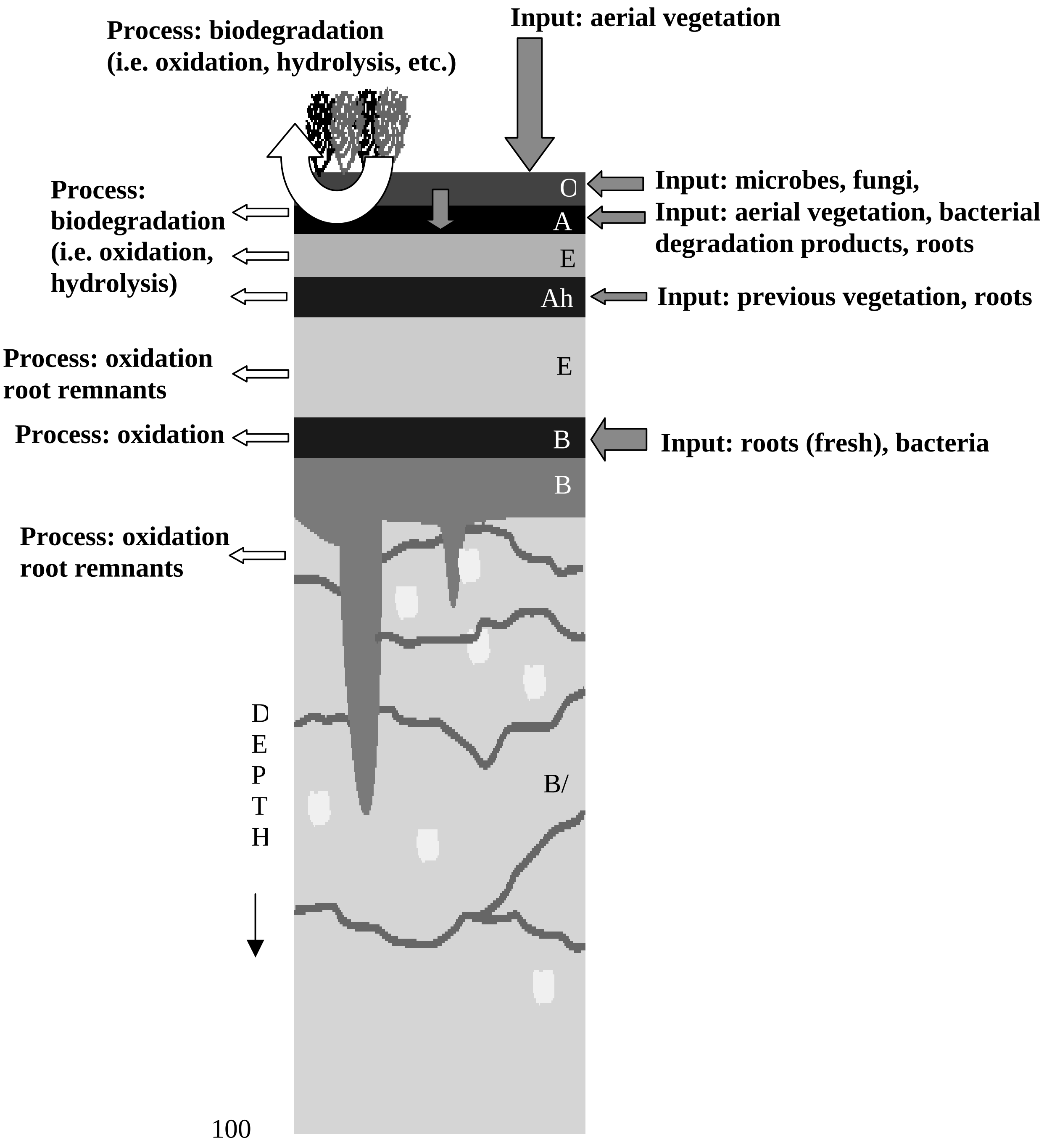


Figure 11.2 Schematic representation of soil processes in the podzol profile based upon the total lipid data obtained.

11.2 Ester-bound moieties in acid soils

Previous studies revealed that the biopolyester fraction can contribute significantly to OM in forest soils (e.g. Kögel-Knabner et al., 1992; Nierop, 1998). All free extractable lipids have been removed prior to THM and/or base hydrolysis methods applied in this thesis. Such a sequential approach is highly recommended to avoid interference of free extractable compounds with ester-bound compounds.

11.2.1 Standard approach to isolate and analyse solvent-insoluble ester-bound moieties in soils

Comparison of thermally assisted hydrolysis and methylation (THM) using tetramethylammonium hydroxide (TMAH) with base hydrolysis data confirmed that base hydrolysis (1M KOH in methanol, 70 ^{0}C, 1 h) is an efficient way of removing ester-linked compounds in soils (Chapter 7). Moreover, this method also allows detailed analyses of the moieties released.

Once the base treatment has been completed, step-wise pH extraction of the base extract (pH 14) after separation of base extract and soil residue is recommended (Chapter 5). The step-wise pH extraction removes all hydrolysed moieties from the base extract according to their pK_a values. The various products released can subsequently be studied in detail providing greater insight into the chemical composition of ester-related moieties in soils. For example *n*-alkanoic acids, *n*-alkanols and α, ω-alkanediols, that would otherwise remain undetected due to their low relative concentrations, can be studied in detail (Chapters 5 and 6). However, it should be noted that direct acidification of the extract to pH 1 after standard base treatment remains a vary practical method to obtain rapid general insights into the bulk composition of low molecular weight ester-related moieties released.

11.2.2 Origin and mode of occurrence of ester-bound compounds

The biopolyester cutin is present in cuticles covering all aerial parts of higher plants, while suberin forms both protective and wound-healing layers in barks, woody stems and underground parts, i.e. roots (Kollattukudy, 1980, 1984; Walton, 1990). Although comparative studies revealed that cutin and suberin are closely related chemically, there are a number of differences in their building blocks and tertiary structure (Kollattukudy, 1980). Taking these differences into account, a cutin over suberin derived input is clearly reflected in the andic top-sample (0-5 cm; Fig. 11.1). This significant cutin-derived input is, however, quickly lost in the sub-soil samples (10-40 cm; Fig. 11.1) indicating that vertical transport of biopolyesters within the acid andic profile is of limited importance (Chapter 4). These results are in line with previous research indicating that in contrast to podzolization, transport of Al, Fe and dissolved organic matter is not significant in andic soils (Ugolini and Dahlgren, 1991). The detailed molecular distributions obtained for 2-alkanols, diols, *n*-alkanols, *n*-alkanoic acids, ω-hydroxy acids, trihydroxy acids, dihydroxy diacids and phenolic acids reveal a strong suberin-derived origin for the andic sub-soil samples, although a, relatively small, cutin related input can not be ruled out completely (Chapters 4 and 6). Ester-bound compounds in Dutch coastal sandy dune sub-soils are also mainly derived from root suberins (Chapter 7).

11.2.3 Changes in the chemical composition of ester-bound compounds in soils

Ester-bound aliphatic compounds mainly derived from root suberin accumulate in acid sandy soils without significant compositional changes (Chapter 7). Within the acid soils studied, however, the ester-linked ω-$C_{18:1}$ acid is more quickly hydrolysed than most other suberin building blocks (Chapters 4 and 7). Moreover, unsaturated and epoxy compounds present in relatively large concentrations in fresh plant root suberins are degraded quickly once introduced into the soil, most probably through oxidation (Chapter 7). Together with the relatively strong decrease in the ester-linked fraction of C_{22}, C_{24} ω-hydroxy acids and, to a lesser extent, C_{18}-C_{28} α, ω-dioic acids, this suggests that unsaturated and epoxy compounds, as well as long-chain ($>C_{16}$) ω-hydroxy and α, ω-dioic acids are more easily released, i.e. hydrolysed, from the biopolyester structure than other ester-linked aliphatic building-blocks, including C_{16} ω-hydroxy acid. They may form a more accessible part of the biopolyester, while di- and tri-hydroxy functionalised acids, as well as C_{16} ω-hydroxy acid, comprise a part of the biopolyester less susceptible to biodegradation.

11.2.4 Effect of soil acidification on the preservation of ester-bound compounds in soils

As mentioned, ester-bound aliphatic moieties, mainly derived from root suberin, are shown to accumulate upon long-term soil acidification in sandy soils (Chapter 7). The fact that ester-bound moieties are well preserved in acid soils further indicated that these chemically labile compounds can accumulate in soils and contribute significantly to SOM. In the most acid coastal dune soil, ester-bound compounds are found to contribute as much as 12% to the TOC. In the acid andic profile, ester-bound compounds constitute approximately 3% of the TOC (Chapter 6). This lower contribution may not necessary imply less preservation of ester-bound compounds in the andic profile but may well result from an increased preservation of other SOM fractions such as polysaccharides (Chapter 8).

11.2.5 Recommendations for further research on ester-linked lipids in soils

With the availability of new approaches to perform detailed molecular analyses of ester-linked lipids in soils (Chapters 4, 5, 6 and 7), it is important to further our understanding on the molecular characterization of different plants creating an overview of molecular characteristics. Such a data set provides more insights into the origin and fate of ester-linked lipids in soils. Both cutins and suberins should be analysed. Changes in the molecular composition, in addition to total degradation, should be monitored in soils to obtain clearer insights into on the degradation/preservation of ester-linked compounds in relation to soil conditions such as pH (Chapter 7). Moreover, model studies should be performed using ^{13}C and ^{14}C isotopically labelled suberins and cutins isolated from plants. Both biopolyesters could be labelled using either enriched atmospheric growth conditions or by the addition of isotopically enriched LMW organic acids or polysaccharides to the growth environment. These labelled compounds should be introduced into several well defined soil systems after which changes on the molecular level with time in relation to specific soil conditions can be monitored. In this way, turnover times could be determined for specific ester-linked compounds. In addition, the labelled ester building blocks can be monitored upon degradation to see whether they are incorporated in microbes or other SOM compounds, become part of the free extractable lipid fraction, or are completely oxidized to CO_2. The same materials could also be used to run experiments on the transport of ester-linked compounds within soils.

11.3 High molecular weight soil organic matter in acid andic soils

11.3.1 Composition of bulk soil organic matter

Curie-point pyrolysis data in combination with the NMR data of the solvent insoluble fraction of SOM reveal a relatively high contribution of fungal and/or thermally altered plant polysaccharide structures in addition to proteins and polypeptides (Chapter 8). A significant fungal contribution, enhanced by the low soil pH (Bumpus, 1993; Bååth and Anderson, 2003), is further reflected by the strong degradation of lignin and specific nitrogen containing chitin pyrolysis products. Aliphatic compounds identified were related to the biopolyesters cutin and suberin as well as the non-hydrolyzable biopolymers cutan and suberan (Chapters 8 and 9). Finally, although probably underestimated by the techniques used, condensed aromatics represent a significant contribution to SOM (Chapters 8 and 9) from this site.

11.3.2 Isolation and composition of non-hydrolyzable fraction

In stead of separating the SOM into operationally defined humic, fulvic and humin fractions (see Chapter 1), SOM in this study is separated into specific chemical fractions that can be analysed on the molecular level. For example, free lipids selectively removed by the first Soxhlet extraction (Chapter 2), as well as ester-linked compounds removed by the base-treatment (Chapter 6) have been studied in detail on the molecular level. A comparison between pyrolysis data of the total SOM (Chapter 8) and data of the non-hydrolyzable fraction (Chapter 9) reveals that the specific sequential chemical SOM extraction procedure used is a very effective way to remove chemically labile SOM fractions such as polysaccharides, peptides and biopolyesters. The fraction so isolated consists of chemically stable entities and will therefore provide essential insights into the long-term storage of carbon in soils (Chapter 9).

A combination of both NMR and pyrolysis data reveals that the non-hydrolyzable fraction comprises an important contribution of condensed aromatic structures in combination with an aliphatic signal most likely derived from non-hydrolysable aliphatic biopolymers (Chapter 9). Molecular analyses using RuO_4 oxidation reveal that the molecular building blocks of the non-hydrolyzable aliphatic SOM are comprised of mono, di, tri and tetra functionalized aliphatic moieties (Chapter 10). Using these molecular building blocks a model of the original aliphatic biopolymer structure consisting of aliphatic compounds linked through both vicinal and mid-chain ethers has been reconstructed. In addition, based on the molecular information, the formation of chemically resistant aliphatic material in soils through a process of cross-linking within biopolyesters is considered unlikely (Chapter 10).

11.4 Organic matter in acid andic soils

11.4.1 What are humic substances?

Soil organic matter is thought to consist of two major types of compounds by many researchers in the field of organic soil chemistry. The first type are the non humic substances, consisting of relatively well-defined compounds such as lipids and carbohydrates (Stevenson, 1994). The second group is suggested to consist of humic substances, which are described as a series of high-molecular weight, yellow to black substances formed by secondary synthesis reactions such as the Maillard reaction (Stevenson, 1994). The separation of soil organic matter in three operationally defined fractions, i.e. humic acids, fulvic acids and humin, using the classical humus extraction (see Introduction) has further contributed to the structural mystery on the molecular level, for example through the formation of artefacts (Burdon, 2001).

It has been suggested that SOM is completely comprised of recognizable plant, microbial or fungal derived compounds in various states of decomposition (Burdon, 2001). In soils, these compounds may become associated to form complex mixtures. These complex mixtures would be held together by relatively weak molecular interactions such as van der Waals bonds (Piccolo et al., 1996a; Piccolo, 2001). In line with this latter model, a combination of NMR and pyrolysis studies (e.g. Saiz-Jiminez and de Leeuw, 1986b; Saiz-Jiminez, 1996; Kögel-Knabner et al., 1988; Hatcher et al., 1981, 2001) on the chemical composition of humic substances, lead to the recognition that the operationally defined fractions contain recognizeable biological compounds biosynthesized by living organisms.

Further research on the composition of SOM (e.g. van Bergen et al., 1997; 1998a, b; Nierop 1998, 2001), together with the molecular approach presented in this thesis, suggests that humic substances are more like a group of operationally defined fractions comprised of both recognizeable fresh plant materials and their degradation products, together with a fraction of SOM that has not been characterized at the molecular level. Whether this latter fraction is comprised of not yet molecularly characterized fresh plant materials and their degradation products, or of newly formed macromolecular structures is still a matter of debate. However, as shown in this thesis, progress is made when it comes to characterizing SOM on the molecular level. With each step, the fraction of SOM belonging to the so called “black box” with possibly newly formed macromolecular structures becomes smaller, while the fraction directly related to fresh plant materials and their degradation products increases. In addition, intermolecular reactions leading to the formation of new macromolecular structures, i.e. humic and fulvic acids, are unlikely to occur for many reasons (Burdon, 2001).

A way to test whether new macromolecules are formed through reactions within the soil is the introduction of isotopically labelled specific soil organic matter compounds such as polysaccharides, lipids, biopolyesters and biopolymers to several well-defined soil systems and monitor changes at the molecular level using a combination of techniques including off-line pyrolysis. Using the molecular approach presented in this thesis, recognizeable molecular building blocks of soil organic matter can be monitored in the soil. Molecular characterization techniques are already used to observe molecular changes upon decomposition of specific soil organic matter fractions such as lignin (e.g. van Bergen et al., 1998a) and suberins (Chapters 4 and 7). Extending and improving these studies, as well as the approach presented in this thesis will hopefully lead to a complete molecular characterization of SOM and will replace the operationally defined fractions.

11.4.2 Mineralogical properties

The mineral fraction of soils has a profound effect on the quantity and quality of organic matter in (volcanic) soils (Parfitt et al., 1997). The accumulation of active forms of Al and Fe is one of the most important properties involved in the central concept of Andisols (Shoji et al., 1993). Active Al and Fe in andic soils occur primarily as allophane/ imogolite, ferrihydrite or as Al/Fe organic complexes (Shoji et al., 1993).

Under the humid weathering conditions of Madeira (Madeira et al., 1994), the composition of the colloidal fraction in the andic profile forms a continuum between pure Al/Fe organic complexes and pure allophane/ imogolite and ferrihydrite (Shoji and Fujiwara, 1984; Dahlgren et al., 1994), depending on the pH and organic matter content. Organic matter under these conditions effectively competes for Al (more than Fe, which is more stable in oxidezed form; Childs et al, 1991) with silica to form Al-organic complexes instead of allophane/ imogolite (Dahlgren et al., 1994). Allophane/ imogolite forms preferentially in weathering environments of less leaching, with a pH in the 5-7 range (Dahlgren et al., 1994). Both allophane/ imogolite as well as ferrihydrite are known for their very high specific surface areas and complexing capacities (Childs and Parfitt, 1987; Childs, 1992). In the acid pH range (pH<5), as observed in the Madeira profile (Chapters 2 and 5), Al organic complexes are preferentially formed (Shoji and Fujiwara, 1984; Dahlgren et al., 1994). As a result, organic matter in the Madeira soil can be complexed with free aluminium, or be complexed with allophane/ imogolite and ferrihydrite, depending on the soil pH and organic matter content.

Although the main focus of this thesis has been on the organic soil constituents, both soil pH data (Chapters 2 and 5) as well as pyrophosphate and oxalate data (Chapter 5) in combination with published (Madeira et al., 1994) and unpublished data on Al and Fe (Naafs et al., unpublished results) indicate that in the andic soil used, organic matter is complexed with both free Al and with allophane, most likely in the form of imogolite (Naafs et al., unpublished results) and ferrihydrite.

11.4.2 Factors influencing the preservation/degradation of SOM in acid andic soils Andic soils are widely known to contain relatively high amounts of organic carbon. As a result of the increased preservation of SOM, the mean residence time of SOM in andic soils is much greater than of SOM in other soils (Shoji et al., 1993). In this section the most important factors that determine the preservation/degradation of SOM in acid andic soils are presented. Together with the input, these factors determine the composition of SOM in volcanic soils, taken into account the limited role of transport within these soils (this Thesis; Ugolini and Dahlgren, 1991; Aran et al., 2001).

Complexation with allophane/imogolite and ferrihydrite. In general, the high amounts of SOM in volcanic soils are thought to be related to stabilization of SOM through complexation (Yuan et al., 2000) with allophane/imogolite and ferrihydrite (Theng, 1980; Shoji et al., 1993; Torn et al., 1997; Parfitt et al., 1997). The stabilization effect is due partly to adsorption on mineral surfaces by which a large part of the SOM is protected from microbial decomposition (Oades, 1988) and partly to physical inaccessibility of SOM within pores of microaggragates (Tisdall and Oades, 1982; Parfitt et al., 1997). In general, SOM is protected from exposure to the soil enzymatic environment by complexation with the mineral phase (Boudot et al., 1992). Besides a direct complexation of SOM with the mineral phase, sorption of degradation enzymes to active Al and Fe phases (Tate and Theng, 1980) and phosphorous deficiency of micro-organisms caused by high P retention (Boudot et al., 1986, 1988; Brahim, 1987; Shoji et al., 1996) will also slow down SOM degradation. Complexation/sorption may further cause steric hindrance of functional groups to decomposer microorganisms (Wada, 1977; Shoji et al., 1996).

It should be noted that complexation with the mineral phase is not only important for macromolecular SOM, but has also been shown to be important for low molecular weight compounds, e.g. small-chain acids such as citric acid (Boudot et al., 1992) and lipids (Kummert and Stumm, 1980; Ulrich and Stumm, 1987). In addition, complexation of SOM with the mineral phase is most probably also causing the limited vertical transport of organic matter compounds observed within andic soils (Chapters 2 and 4; Ugolini and Dahlgren, 1991).

Complexation with free aluminium (and iron). Complexation with free Fe is relatively unimportant because Fe has a greater stability as Fe oxyhydroxides compared to Fe organic complexes (Childs et al, 1991). Direct complexation of Al with SOM plays an important role within this soil but is hard to distinguish from complexation of SOM with Al and Fe minerals. The flocculation of Al organic complexes has been shown to provoke a protective effect against microbial breakdown (Boudot et al., 1986, 1989). Both mechanisms of complexation, i.e. with free Al and with Al/Fe minerals, occur in soils containing both free Al and allophane/imogolite (Parfitt et al., 1999). Complexation with free Al renders the SOM highly resistant to microbial attack (Wada, 1980; Shoji et al., 1993), most likely through Al toxicity to micro-organisms (Tokashiki and Wada, 1975).

Aluminium toxicity to microorganisms. Organic matter has also been found to accumulate in soils without significant amounts of allophane (Tokashiki and Wada, 1975). Aluminium is widely known to decrease degradation by micro-organisms through inhibiting enzyme activity (Sollins et al., 1996). Plant roots react to high concentrations of Al in the soil by the production of low molecular weight organic acids that complex with Al to prevent Al from entering the plant system (Lundström et al., 2000; and van Hees et al., 2000). Micro-organisms, however are not known to respond in such a way to high Al concentrations and will therefore be of less importance within the andic profile. Within the litter layer, however, Al concentrations are lower allowing microbial degradation of OM to occur in this layer on a larger scale than in the soil. Indications within the soil for a lower microbial activity are relatively low concentrations or absence of bacterial markers within the free lipid fraction (Chapter 2).

Acid soil pH. At typical soil pH CO_2 partial pressures, the minimum soil solution pH obtainable by carbonic acid is within the approximate range of 5.1-5.5 (Shoji et al., 1993). To get pH values lower than 5, as observed in the soils studied, requires the presence of a stronger proton donor, which in many soils are organic acids (Ugolini and Sletten, 1991). Organic acids have acidic functional groups with pK_a's ranging from approximately 2.6 to 6.6 (Gregor and Powell, 1988; Shoji et al., 1993). These acids could be produced in response to the high Al concentrations (see 11.4.2.3).

Bacterial enzyme activities are known to decrease upon acidification, possibly in relation to an increased concentration of Al^{3+} at low pH (Lähdesmäki and Piispanen, 1992; Sollins et al., 1996). Fungi on the other hand are known to become more dominant compared with bacteria when soil pH decreases (Dinel et al., 1990; Bumpus, 1993; Bååth and Anderson, 2003). So, at low pH, fungal productivity is enhanced while bacterial activity is decreased. Within the acid andic profile, chitin derived pyrolysis products, known to be part of fungal polysaccharides have been found to be a relatively large important contribution (Chapter 8). In addition, the lignin derived signal was low, again suggesting a relative important fungal activity within this soil (Chapter 8) as compared with bacteria. Moreover, the shift from a dominant bacterial to a dominant fungal activity results in the degradation of specific SOM fractions preferred by fungi, e.g. lignin, and the production of fungal polysaccharides. On the other hand, compounds preferred by bacteria will be protected within the profile. For example, lipids are known to accumulate within acid soils (Mouçawi et al., 1981b; Jambu et al., 1985; Dinel et al., 1990).

Intrisic structural resistance. In addition to stabilization by external factors such as complexation and an acid soil pH, SOM contains a fraction of compounds that exhibit a relatively high resistance to degradation (de Leeuw & Largeau, 1993; Derenne and Largeau, 2001). In soils, this fraction is mainly formed by aliphatic insoluble biopolymers (van Bergen et al., 1997, 1998a; Nierop, 1998), referred to as cutan and suberan (Nip et al., 1986a, 1986b; Tegelaar et al., 1989c, 1995). Due to their structural resistance, these compounds have been suggested to be selectively enriched upon SOM degradation (de Leeuw & Largeau, 1993; Derenne and Largeau, 2001).

Research presented in this Thesis (Chapter 8) led to the recognition of thermally altered SOM compounds, e.g. polysaccharides in andic sub-soils. These compounds have also been suggested to inhibit an increased resistance against biodegradation (Almendros et al., 1988, 1992; Knicker et al., 1996).

The final SOM fraction with a high resistance to degradation originates from the incomplete combustion of organic materials. Research revealing a link between ^{14}C age (Fig. 11.3) and the molecular composition of SOM in Hawaii soils (Fig. 11.4; Naafs et al., unpublished results) indicates that this condensed aromatic fraction is preserved within soils on the millennium scale. First, relatively labile SOM compounds such as polysaccharides and proteins are degraded. On the long-term, however, even chemically more resistant compounds such as aliphatic biopolymers are degraded (Fig. 11.4; Naafs et al., unpublished data) leaving only condensed aromatic structures. Overall, fire-induced sequestration of carbon from the short-term biological to the long-term geological cycle may represent an important sink for atmospheric CO_2 (Kuhlbusch and Crutzen, 1995).

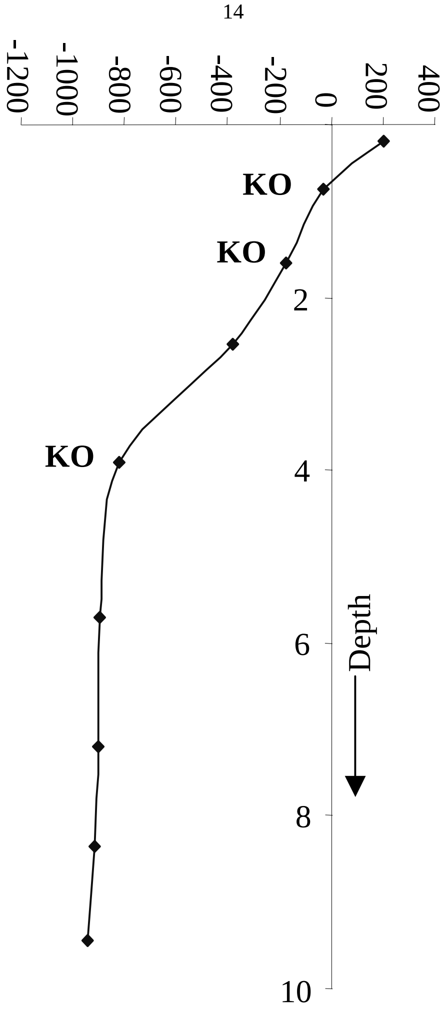


Figure 11.3 ^{14}C vs depth in a volcanic soil from Hawaii showing a clear decrease in ^{14}C with depth. From 0 to 40 cm a transition from relatively young to relatively old, depleted carbon is observed.

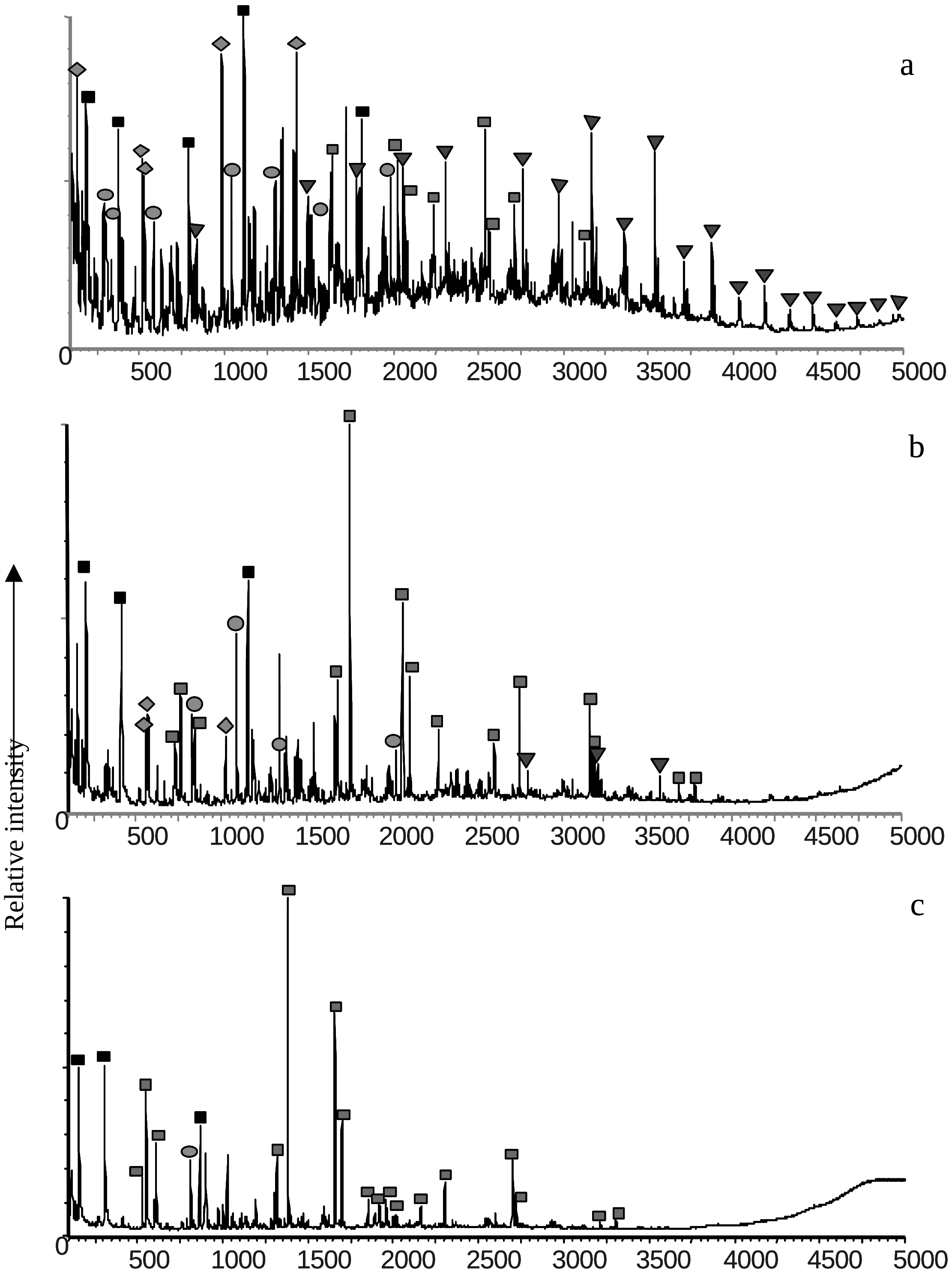


Figure 11.4 Pyrolysis GC/MS data of Hawaii depth profile (Fig. 11.3), (a) KO1, (b) KO2, (c), KO3. Key: ▼aliphatic derived; ◇polysaccharide derived, ■ condensed aromatics derived, ●polypeptide derived, ■polyphenolic derived pyrolysis product. A decrease of polysaccharide, polypeptide and aliphatic derived products with depth and ^{14}C is observed, while condensed aromatic products reveal a relative increase.

11.4.3 Preservation of SOM in acid andic soils from Madeira

An experimental study using ^{14}C labelled ryegrass which was introduced to an andic soil indicated that a relatively large portion of the OM is stabilized (Saggar et al., 1996). However, still up to half of the OM introduced was decomposed within a few weeks (Saggar et al., 1996), indicting that not all OM is stabilized. It should also be noted that allophane is known to stabilize not only plant derived SOM, but also protects microbial metabolites produced during the decomposition of SOM together with organic residues and microbial biomass (Saggar et al., 1994).

As a result of the factors mentioned above (section 11.4.2), the formation of micro-organisms is decreased in andic soils. Together with a stabilization and protection of the SOM through complexation/sorption and (thermally increased) structural recalcitrance, this decrease results in the accumulation of SOM within andic soils (Fig. 11.5). In this Thesis, for the first time molecular data are presented revealing that SOM is not accumulated as a whole in andic soils (Chapter 8). Instead, the accumulation of polysaccharides is shown to be the main factor responsible for the relatively high TOC values (Chapter 8). These polysaccharides may be both plant as well as fungal/microbial derived (Chapter 8).

In general, it is suggested that four different cycles of SOM turnover can be distinguished within acid volcanic sub-soils (Fig. 11.5). The first cycle accounts for the fast turnover of readily degradable SOM compounds. Lignin is also included in this cycle in the acid andic sub-soil studied due to the high fungal activity within this soil. The second cycle represents the slow turnover of compounds stabilized and protected by free Al and allophane/imogolite and ferrihydrite as well as those compounds assimilated into microbial biomass prior to stabilization (Fig. 11.5). The third cycle represents those compounds stabilized on the relatively long-term including thermally stabilized compounds and chemically resistant SOM (Fig. 11.5). The fourth and final cycle represent the extremely slow turnover of the most refractory material in volcanic soils. This material can be stored within soils for thousands of years (Fig. 11.5).

11.4.4 Future research

To date, complexation studies have been performed mostly by using ill- defined SOM fractions extracted from soils i.e. so called humic and fulvic acids, and synthetic allophane/imogolite (Parfitt et al., 1999; Farmer and Lumsdon, 2001) or free aluminium (Boudot et al., 1989). Future research projects could focus on the complexation of synthetic allophane/imogolite (Farmer and Lumsdon, 2001) with well-defined entities such as polysaccharides, proteins, biopolyesters and lipids (Naafs et al., unpublished results). Once produced and defined, complexes can be introduced into well-defined soil systems to study their turnover in relation to specific soil properties such as pH. Specific attention could also be paid to the kind of interaction between the organic compounds and the mineral phase (Filius et al., 1997; 2000) using for example spectroscopy techniques (Ainsworth et al., 1998).

The effect of vegetation and SOM on soils could be studied using plots located on the same location that consist of the same soil but are covered with different vegetation. In addition, different soil types/conditions covered with the same vegetation could be selected to study the influence of the soil type/conditions on the vegetation and the composition of SOM.

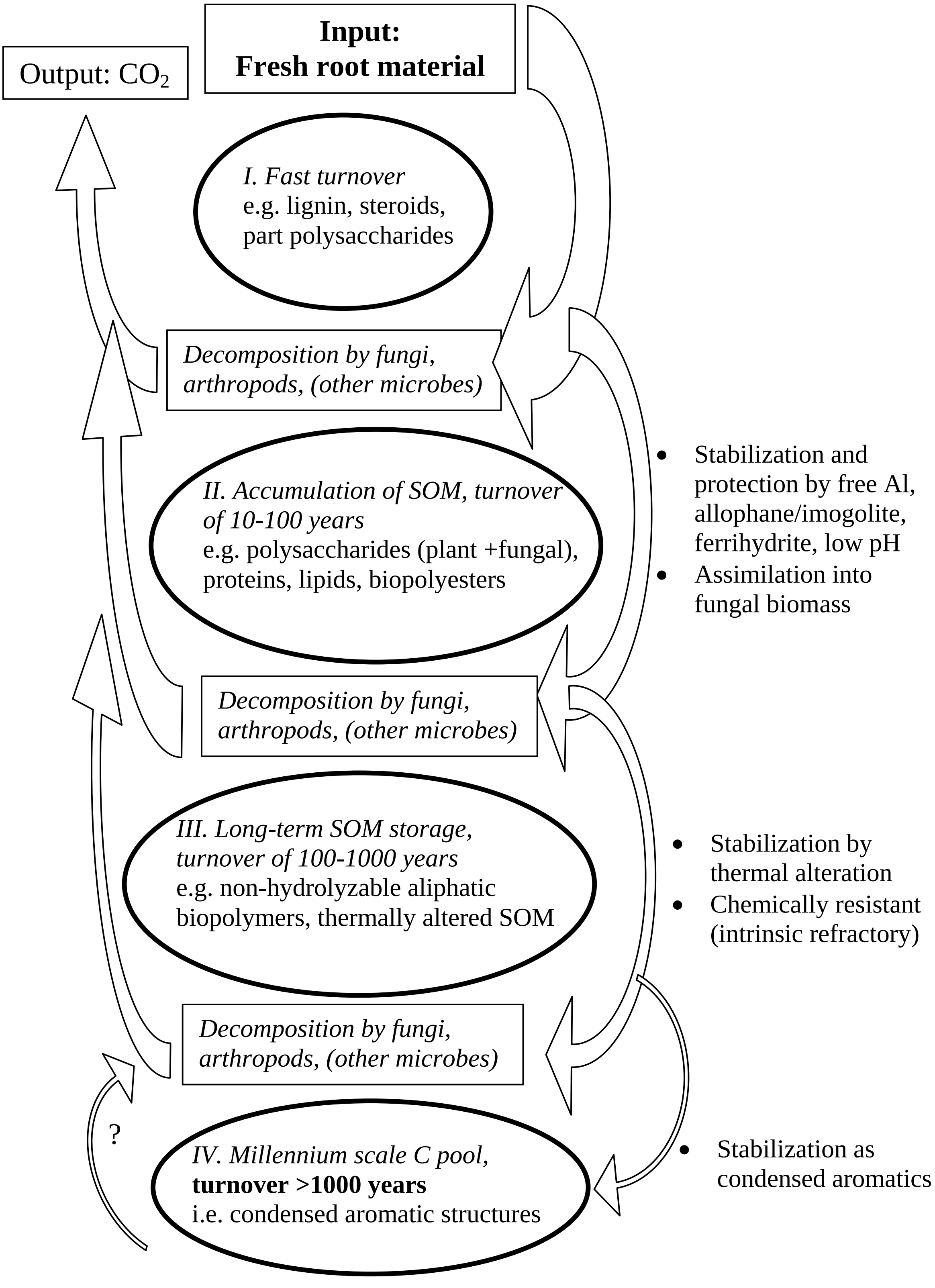


Figure 11.5 Schematic representation of proposed SOM cycles within an acid andic soil.

Studies on the degradation of non-complexed SOM compounds can be extended in two ways. First, the degradation of SOM compounds could be further studied per compound class, e.g. (poly)saccharides (Saggar etal., 1994), lignin (van Bergen et al., 1998a), polyesters (Chapter 7), lipids, low molecular weight acids (Boudot, 1992) and proteins in addition to monitoring changes in the total of organic carbon. Second, degradation studies should be extended to the molecular level to further reveal changes at the molecular level upon degradation as revealed for suberins described in this Thesis (Chapters 4 and 7).

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Samenvatting in het Nederlands (Summary in Dutch)

De indeling van dit proefschrift is gestructureerd van verbindingen met een laag moleculair gewicht naar de meer complexe hoog moleculaire verbindingen. Het eerste deel van dit proefschrift (**Hoofdstuk 2** en **3**) behandelt totaal extracten van lipiden.

In **Hoofdstuk 2** is gekeken naar variaties met diepte en seizoen in de extraheerbare lipiden fractie van een zure andische bosbodem. Hierbij is een afname van de bijdrage van lipiden per totaal organisch koolstof geconstateerd. De afwezigheid van typische bacteriële markers kan een indicatie zijn voor een verlaagde bacteriële activiteit, waarschijnlijk gerelateerd aan de lage bodem pH en de aanwezigheid van Al en Fe oxides. Een sterke afname in zowel de relatieve als absolute bijdrage van verbindingen afkomstig van bladeren is waar te nemen wanneer de strooisellaag vergeleken wordt met de minerale bodem. De aanwezigheid van C_{22}-C_{32} zuren, C_{22}-C_{26} ω-hydroxy zuren, C_{31} *n*-alkaan en C_{22}-C_{32} *n*-alkanolen, aanwezig in de ondiepe bodem, is waarschijnlijk gerelateerd aan een belangrijke bijdrage van gras wortels. In de zomer wordt een lipiden signaal gezien dat waarschijnlijk afkomstig is van de uitspoeling van microbieel gerelateerde producten vanuit de strooisellaag en/of de bovengrondse vegetatie.

In **Hoofdstuk 3** wordt de samenstelling van totaal extracten van lipiden van verschillende bodem horizonten in een podzol profiel vergeleken. Organisch materiaal accumuleert met name in combinatie met aluminium in the Bh horizont. Lipiden accumuleren onafhankelijk van de bodem pH in organisch rijke horizonten. Een lipiden signaal gerelateerd aan de bovengrondse delen van *Calluna*, zoals bladeren en bloemen, wordt alleen waargenomen in de O horizont. Dit “*n*-alkaan, steroide and triterpenoide” signaal gaat echter al snel verloren in de onderliggende A horizont door (bacteriële) oxidatie. De overige totaal lipiden extracten worden gedomineerd door componenten afkomstig van wortels. In het algemeen geven de lipiden data aan dat, naast uitspoeling, degradatie (microbiële oxidatie) een belangrijk proces is waarmee rekening gehouden dient te worden in studies naar podzolizatie processen in bodems.

Het tweede deel van dit proefschrift behandelt ester-gebonden verbindingen.

Hoofdstuk 4 beschrijft de veranderingen in de moleculaire samenstelling van ester-gebonden aliphaten met veranderende diepte in een zure andische bosbodem. Deze veranderingen worden vergeleken met veranderingen in de vrije lipiden fractie waardoor een connectie tussen het eerste en tweede deel van het proefschrift gerealiseerd wordt. Hydrolyse met behulp van warmte gevolgd door methylering in combinatie met gas-chromatografie/ massa spectrometrie laat zien dat in de bovenste 5 cm van de bodem cutine een belangrijker bron van ester-gebonden componenten is dan suberine. De diepere lagen (20-50 cm) echter worden gedomineerd door van suberine afkomstige verbindingen. Een relatief sterke afname van C_{22}, C_{24} (and C_{26}) ω-hydroxy zuren (en C_{18}-C_{28} α, ω-dizuren) wordt gemeten vergeleken met andere suberine bouwstenen zoals C_{16} ω-hydroxy zuur. Deze afname met diepte is omgekeerd evenredig met een sterke toename van deze verbindingen in de vrije lipiden fractie met diepte. Deze relatie tussen de vrije en ester-gebonden lipiden fractie lijkt het gebruik van bovengenoemde ω-hydroxy zuren als indicatoren voor een wortel gerelateerde bron van vrije lipiden (Hoofdstuk 2) te rechtvaardigen.

In **Hoofdstuk 5** worden de effecten van pH veranderingen na basische hydrolyse en hun implicaties op het begrip van bodem organisch materiaal in bodems bestudeerd. De verkeregen resultaten na direct aanpassen van de pH van het extract geven aan dat de relatieve hoeveelheden van geïdentificeerde verbindingen, zoals verwacht, pH afhankelijk zijn. Meer specifiek zijn deze relatieve hoeveelheden afhankelijk van de pK_a waarde van de verbindingen en hun hydrophobiciteit. Dit verschijnsel, dat de uiteindelijke resultaten aanzienlijk beïnvloedt, kan tot een over- of onderschatting van bepaalde verbindingen leiden en daarmee de kijk op ester-gerelateerde verbindingen in bodems foutief beïnvloeden. Direct aanzuren van het base extract in de aanwezigheid van het bodem residu geeft aan dat hydrophobe aliphatische verbindingen met lange koolstofketens een interactie aan kunnen gaan met de overgebleven organische matrix na de hydrolyse, deel kunnen gaan uitmaken van nieuwe onoplosbare organische (macro) moleculen, of een interactie kunnen aangaan met de anorganische matrix. Deze laatste optie wordt naar voren gebracht als een andere mogelijke wijze van voorkomen van ester-gerelateerde verbindingen in bodems ter aanvulling op reeds bekende wijzen van voorkomen zoals de biopolyesters cutine en suberine.

De resultaten van **Hoofdstuk 5** worden gebruikt in **Hoofdstuk 6** waarin de resultaten van een kwalitatieve studie naar de chemische compositie van ester-gebonden verbindingen in een zure andische bosbodem worden beschreven. De niet in organische oplosmiddelen oplosbare ester-gebonden fractie die vrij komt na basische hydrolyse van een andische A horizont van Madeira, is sequentieel geëxtraheerd and geanalyseerd met behulp van gas chromatografie en gas chromatografie/massa spectrometrie. De scheiding van alle vrijgekomen verbindingen in een aantal "pH subftacties" maakt het mogelijk de verschillende verbindingen in detail te bestuderen. De distributies van 2-alkanolen, diolen, *n*-alkanolen, liniaire zuren, ω-hydroxy zuren, trihydroxy zuren, dihydroxy dizuren en phenolische zuren wijzen op een door suberin gedomineerde input van ester-gerelateerde verbindingen. Naast deze bijdrage vanuit van planten afkomstige biopolyesters wijst de detectie van *iso* en *anteiso* vertakte zuren en relatief korte dizuren en *ω*-hydroxy op een bacteriële bijdrage.

De effecten van de pH op de preservatie van ester-gebonden lipiden worden bestudeerd in **Hoofdstuk 7** waarin het voorkomen en de distributie van ester-gebonden lipiden in Nederlandse duin bodems langs een pH gradiënt beschreven worden. Vergelijking van hydrolyse met behulp van warmte gevolgd door methylering met basische hydrolyse bevestigt dat basische hydrolyse een zeer efficiënte manier is om ester-gebonden verbindingen in bodems te bestuderen. De verbindingen die vrij komen tijdens basische hydrolyse bestaan voornamelijk uit van suberine afkomstige lipiden, die duidelijk gerelateerd zijn aan eikenwortels. Vergeleken met verse wortels die grotendeels verantwoordelijk zijn voor de input van bodem organisch materiaal in de diepere bodem, is er een afname te zien van onverzadigde en epoxy verbindingen in de bodem, waarschijnlijk door oxidatie. De bijdrage van de verzeepbare fractie aan het totaal van bodem organisch materiaal neemt duidelijk toe met toenemende verzuring, terwijl er nauwelijks veranderingen in de moleculaire samenstelling van deze fractie zijn waargenomen. Deze accumulatie zonder grote veranderingen in de moleculaire samenstelling van de ester-gebonden fractie geeft aan dat van planten afkomstige biopolyesters gepreserveerd kunnen worden in bodems.

Het laatste deel van dit proefschrift behandeld zoals gezegd de hoog moleculaire fractie van het bodem organisch materiaal. In **Hoofdstuk 8** wordt moleculaire informatie over de bulk van het organisch materiaal in andische bodems verkregen met behulp van Curie-point pyrolyse en CPMAS ^{13}C NMR. Beide technieken onthullen een significante bijdrage van polysaccharide verbindingen afkomstig van schimmels. De pyrolyse data laten verder zien dat deze en andere polysaccharide verbindingen thermische veranderingen hebben ondergaan. Naast polysaccharides wordt door een combinatie van beide technieken de aanwezigheid bevestigd van de biopolyesters cutine en suberine, de niet-hydrolyseerbare biopolymeren cutan en suberan, proteïnen en polypeptiden. Relatief grote hoeveelheden van pyrolyse producten afkomstig van chitine kunnen afkomstig zijn van zowel schimmels als insecten hetgeen aangeeft dat insecten, naast schimmels, van invloed kunnen zijn op de verwerking van het organisch materiaal. De relatief kleine hoeveelheid lignine die in deze bodems gevonden wordt is waarschijnlijk gerelateerd aan de lage bodem pH waarvan bekend is dat deze de groei van lignine afbrekende schimmels bevordert. Gecondenseerde aromatische structuren tot slot maken ook een belangrijk deel uit van het bodem organisch materiaal, al wordt deze bijdrage onderschat door de gebruikte methoden.

De laatste twee hoofdstukken van dit proefschrift (**Hoofdstuk 9** en **10**) beschrijven de moleculaire karakterisering van een deel van het chemisch resistent bodem organisch materiaal. In **Hoofdstuk 9** wordt de samenstelling van de bulk van het chemisch resistente organisch materiaal bestudeerd. Zowel NMR als pyrolyse data laten een belangrijke bijdrage van gecondenseerde aromatische verbindingen zien, in combinatie met een aliphatische bijdrage die waarschijnlijk afkomstig is van niet-hydrolyseerbare aliphatische biopolymeren. Het geïsoleerde chemisch resistente bodem organisch materiaal zal naar alle waarschijnlijkheid een belangrijke bijdrage leveren aan het millennium bodem koolstof reservoir in andische bodems.

Gebaseerd op de resultaten van **Hoofdstuk 9**, wordt in **Hoofdstuk 10** de moleculaire samenstelling van resistente aliphatische biopolymeren beschreven. De bouwstenen van deze biopolymeren bestaan uit mono, di, tri en tetra gesubstitueerde aliphatische verbindingen. Dit laatste kan afgeleid worden uit de aanwezigheid van C_9-C_{32} liniaire zuren, C_7-C_{30} liniaire α, ω-dizuren, C_{21}-C_{30} keto-α, ω-dizuren and C_{16}-C_{27} diketo-α, ω-zuren in het RuO_4 extract. Gebruikmakend van deze bouwstenen kan een model van de originele aliphatische biopolymeer structuur gereconstrueerd worden. Bovendien kan op basis van de moleculaire bouwstenen de vorming van chemisch resistente aliphatische biopolymeren in bodems door middel van cross-linking van biopolyesters vrijwel uitgesloten worden.

Tot slot worden in **Hoofdstuk 11** de belangrijkste bevindingen van alle voorgaande hoofdstukken samengevoegd. In dit hoofdstuk worden bovendien de belangrijkste conclusies beschreven van de in dit proefschrift gepresenteerde moleculaire benadering van de studie van organisch materiaal in bodems op de factoren die de samenstelling van het organisch materiaal in zure bodems bepalen (input, preservatie/degradatie en transport).

List of abbreviations

SOM	Soil organic matter
TOC	Total organic carbon
TN	Total nitrogen
GC	Gas chromatography
GC/MS	Gas chromatography/ mass spectrometry
NMR	Nuclear magnetic resonance
LMW	Low molecular weight
HMW	High molecular weight
THM	Thermally assisted hydrolysis and methylation
TMAH	Tetramethyl ammonium hydroxide
TMS	Trimethyl-silyl

Publications

This thesis is based on the following publications:

Chapter 2: Naafs, D. F. W., van Bergen, P. F., Boogert, S. J., de Leeuw, J. W., 2004. Variations with depth and season; Solvent extractable lipids in an acid andic forest soil. *Soil Biology & Biochemistry* 36, 297-308.

Chapter 3: Naafs, D. F. W., van Bergen, P. F., de Jong, M. A., Oonincx, A., de Leeuw, J. W., in press. Total lipid extracts from characteristic soil horizons in a podzol profile. *European Journal of Soil Science*.

Chapter 4: Naafs, D. F. W., Nierop, K. G. J., van Bergen, P. F., de Leeuw, J. W. Changes in the molecular composition of ester-linked aliphatics with depth in an acid andic forest soil. *Submitted to Organic Geochemistry*.

Chapter 5: Naafs, D. F. W., van Bergen, P. F., 2002. Effects of pH adjustments after base hydrolysis: implications for understanding organic matter in soils. *Geoderma* 106, 191-217.

Chapter 6: Naafs, D. F. W., van Bergen, P. F., 2002. A qualitative study on the chemical composition of ester-bound moieties in an acidic andosolic forest soil. *Organic Geochemistry* 33, 189-199.

Chapter 7: Nierop, K. G. J., Naafs, D. F. W., Verstraten, J. M., 2003. Occurrence and distribution of ester-bound lipids in Dutch coastal dune soils along a pH gradient. *Organic Geochemistry* 34, 719-729.

Chapter 8: Naafs, D. F. W., van Bergen, P. F., Knicker, H., de Leeuw, J. W. Characterization of organic matter in an acid volcanic sub-soil using Curie-point pyrolysis and solid state CPMAS ^{13}C NMR spectroscopy. *Submitted to Soil Biology & Biochemistry*.

Chapter 9: Naafs, D. F. W., van Bergen, P. F., Knicker, H., de Leeuw, J. W. Chemically resistant SOM in an acid andic sub-soil. *Submitted to Organic Geochemistry*.

Chapter 10: Naafs, D. F. W., van Bergen, P. F., de Leeuw, J. W. The molecular composition of non-hydrolyzable aliphatic soil organic matter. *To be submitted to Geochimica et Cosmochimica Acta*.

Other publications:

Nierop, K. G. J., Naafs, D. F. W., van Bergen, P. F. Occurrence and distribution of extractable lipids in Dutch coastal dune soils along a pH gradient. *Submitted to Soil Biology and Biochemistry.*

Naafs, D. F. W., Torn, M. S., van Bergen, P. F., de Leeuw, J. W. Changes in the molecular composition of SOM with increasing ^{14}C age in soils from the Hawaii island chain. *To be submitted to Nature*.

Dankwoord/ Acknowledgements

Voor hen die zich het voorwoord herinneren werd dit dankwoord niet toevalligerwijs geschreven in de Franse Pyreneeën. Tussen het trainen op de fiets door heb ik in dit schitterende gebied gedurende een week in juli eens rustig mijn gedachten over de afgelopen vier jaar laten gaan. Het resultaat daarvan ligt nu voor u en zal ongetwijfeld als meest gelezen deel van dit proefschrift zijn weg vinden naar al diegenen waar het met name voor bestemd is...

Allereerst het team dat de begeleiding voor zijn rekening heeft genomen. Pim, je hebt tijdens deze promotie veruit het grootste deel van de dagelijkse begeleiding op werkelijk perfecte wijze ter hand genomen. Naast het feit dat je een excellent docent bent, is het met name je persoonlijke benadering, doorspekt met Brabantse humor, die ik ook nu nog altijd zeer waardeer. Voeg daarbij dat ik, ondanks je razend drukke leven, nooit langer dan 10 dagen heb hoeven wachten op je altijd opbouwende commentaren op soms wel 40 pagina’s tellende manuscripten en het zal een ieder duidelijk zijn waarom je in de ogen van mijzelf en vele andere promovendi een welhaast beangstigende benadering van de perfecte begeleider bent.

Naast de dagelijkse begeleiding van Pim van Bergen is er de supervisie van mijn promotor Jan de Leeuw. Ondanks uw vele bestuurstaken en andere verplichtingen was u altijd exact op de hoogte van de ontwikkelingen en vorderingen van mijn promotie, uiteraard bijsturend waar nodig. Ook de steun, het advies en het vertrouwen tijdens de soms zeer gevoelige bestuurstechnische kwesties waar ik mee heb leren omgaan zijn zaken die ik zeer waardeer.

Het begeleidingsteam wordt gecomplementeerd door Klaas Nierop. Met name na het vertrek van Pim naar Shell ben je een steeds belangrijker rol gaan spelen. Naast de serie “N2” publicaties (waarvan er 2 in dit proefschrift zijn opgenomen), heeft dit geresulteerd in ontelbare e-mails (waarvan een paar toch nog wetenschappelijke inhoud), en vele waardevolle tips.

Na het begeleidingsteam kom ik bij de studenten, collega’s en ondersteunend personeel die hun bijdrage geleverd hebben aan het onderzoek. Allereerst de studenten; Steven Boogerd, Mieke de Jong, Johan Kool, Debby Los en Annemiek Oonincx. Zonder jullie inspanningen en persoonlijke inbreng was dit proefschrift niet alleen minder omvangrijk geweest, maar zou ik zeker ook een hoop lol in het lab misgelopen zijn! Kathrin Reimer, jij hebt met je aanwezigheid aan de zgn TNO zijde van het lab een grote bijdrage geleverd aan de buitengewoon prettige werksfeer op de afdeling en er mijn inziens voor een groot deel aan bijgedragen dat organische geochemie over een echt geintegreerd lab beschikt. Ook de technische ondersteuning van met name Mark van Alphen, Ton Zalm en Bart Zegers is uiteraard onmisbaar geweest.

Met Diana Menzel heb ik tijdens mijn gehele promotie de kamer en het lab gedeeld. Met name het feit dat je te allen tijde eerst aan de wens van anderen denkt alvorens aan je eigen belang toe te komen, alsmede je eerlijke commentaar (lees: charmante lachbui) op mijn pogingen over te schakelen op jouw eigen Duitse taal hebben gemaakt dat ik met veel plezier terugkijk op de tijd in W218. Met Hendrik-Jan heb ik met name veel gelachen om Amerikaanse stand-up comedians, veel geleerd over werken in het lab, maar wellicht nog wel het meest over internet, wereldpolitiek en niet te vergeten de aandelenhandel. Het hoofdstuk over de Ruthenium tetroxide extractie was er zonder jouw inbreng zeker niet geweest! De overstap van student naar kamergenoot van Johan Kool had met name een exponentiele toename op het gebied van linkse politiek en niet te vergeten computerkennis tot gevolg. Dank met name voor je hulp bij het maken van de uiteindelijke versie van dit proefschrift. Met de komst van Imogen Poole bij onze groep werd er niet alleen een zeer goed wetenschapster aan de groep toegevoegd. Met name de ervaring en rust alsmede de tijd voor een goed gesprek over bv de gang van zaken binnen het instituut of de voortgang van de promotie hebben je tot een waardevol persoon in deze promotie gemaakt.

Het woord humor is al diverse malen gevallen. Daarmee kom ik als vanzelf op mijn collega’s van de west gang Edith en Menno, aangevuld met Kike. Als trotse houder van de Gouden-Stoelpoot trofee (voor de absoluut veruit domste actie van de week/maand) kan ik melden dat de zeer regelmatige inbreng van jullie humor (over het niveau waarvan ik het uiteraard niet zal hebben) en de verdere leut en ongein tijdens koffie/thee breaks het leven op de faculteit zeker een stuk aangenamer hebben gemaakt!

Tijdens mijn promotie heb ik deel uitgemaakt van het promovendi platform. Met name dank aan de kern van het platform bestaande uit Edith, Sjoukje, Menno, Iwan en Gesa.

Op wetenschappelijk en ondersteunend gebied ben ik verder nog dank verschuldigd aan tal van andere personen waaronder; Peter Buurman voor de uitwisseling van ervaring en data en mijn opname in de Europese vulkaanbodemgroep waaraan hij leiding geeft; Heike Knicker for her help with NMR analyses contributing to two publications in this thesis; Margaret Torn for her enthousiasm and collaboration on the Hawaii project; Mijn collega promovendi, postdocs, docenten en professoren van geochemie waaronder Mariëtte (speciaal voor advies bij het afronden van het proefschrift), Niels, Hellen, Anniet en Ralf voor gezellige discussies tijdens de lunches en Guus Loch voor zijn introductie van vulkaanbodems; Pien van Minnen voor secretariële ondersteuning bij o.a. reisaanvragen en je verwoedde pogingen structuur aan te brengen in de chaos; Peter van der Linde voor het inzicht in het geven van goed onderwijs alsmede de prettige samenwerking tijdens het geven van ditzelfde onderwijs; de analisten van andere groepen waaronder Helen de Waard voor je accurate data en goede uitleg en Gijs Nobbe voor betrouwbare TOC data; de AV-dienst; Harry Veld; Roy, Ronald en Mimi voor jullie vrolijke aanpak van de dagelijkse zaken en het starten van mijn auto als ie weer eens met een lege accu stond (Mimi); het personeel van de schoonmaak, voor de vriendelijke groet en rustige werkwijze; Rodriguez Domingos from Madeira Island for his help during field research.

Naast de wetenschappelijke begeleiding en ondersteuning is er natuurlijk het minstens zo belangrijke thuisfront. De interesse voor chemie werd er als het ware met de paplepel ingegoten door mijn vader die er met name de laatste drie laatste jaren van de middelbare school als mijn docent scheikunde voor zorgde dat er een solide basis gelegd werd voor de latere studie en promotie. Het is dan ook geen toeval dat de monsters van Madeira waarop deze promotie gebaseerd is tijdens een gezamenlijke trip naar dit schitterende tropische eiland werden genomen. Verder bijzonder veel dank voor de vele bestuurlijke adviezen die meer dan eens zeer goed van pas kwamen. Tegenwicht aan de β-invloeden werd gelukkig dagelijks gegeven door mijn moeder die niet alleen van groot belang is geweest voor het logistiek mogelijk maken van een druk bestaan vol werk, sport en sociale activiteiten, maar ook de drijvende kracht is achter een multiculturele opvoeding die tijdens een internationaal georiënteerde promotie van onschatbaar belang is gebleken. Naast mijn ouders zijn er natuurlijk ook mijn broer en zussen die in de afgelopen vier jaar hun steentje absoluut hebben bijgedragen! Mobiliteit werd volledig verzorgd door Marinka en Samir die garant stonden voor de luxe als promovendus een auto te kunnen blijven rijden en deze ieder jaar op soms miraculeuze wijze weer door de keuring te slepen. Met mijn broer David heb niet alleen een vrolijk en waardig collega Aardwetenschapper op de faculteit, maar een zeker zo reislustig type in de familie die een bezoek aan het continent Australië mogelijk maakte. Mijn jongste zus Martine tenslotte is ook altijd te vinden voor een onvoorstelbare berg leut en ongein hetgeen bijzonder prettig is tussen al de wetenschap door! Uiteraard heb ik de belangstelling vanuit de rest van de familie ook altijd zeer gewaardeerd. Dank aan mijn oma's voor hum immer warme belangstelling. Een speciaal woord van dank is er voor mijn beide opa's Andries Verhoek en Frederik Naafs. Ondanks dat zij mijn promotie niet mee mochten maken zijn met name hun karakteristieke opvattingen, humor en levenslessen terug te horen te zien in mijn opvattingen en aanpak van vele zaken.

Sommige dingen zijn niet in woorden uit te drukken. Daarom zal ik hier volstaan met het noemen van je naam. Monique.

In de categorie welhaast familie valt uiteraard mijn goede vriend en paranimf Robbert Trouwborst. Meer dan 750 e-mails doorgaans doorspekt met humor ver beneden de maat vonden in vier jaar moeiteloos hun weg naar de VS en terug. Daarbij werd Philadelphia een logische stop onderweg naar bestemmingen elders in de VS om eens relaxed te ouwehoeren over de serieuze zaken van het leven, de laatste Youp van 't Hek humor of boeken van Mart Smeets uit te wisselen.

Mieke en Monique droegen tijdens deze promotie niet alleen data bij die, met name tot hun eigen verbazing, gepresenteerd zijn op een congres. Onze etentjes zo iedere twee maanden in Utrecht zijn een buitengewoon leuke besteding van de avond.

Een speciale plaats wordt ingenomen door Lucia Schram met wie ik veel beleefd heb en met wie vooral de mooie momenten tijdens de reizen naar Griekenland, Italië en Afrika unieke momenten waren.

Last maar zeer zeker not least zijn er dan nog al die personen die in de loop van de jaren garant stonden voor die momenten vol humor, afleiding en belangstelling waaronder: Ariën en Michelle; Ton en Antoinette; Familie Schram; Familie van Monique; Mijn voetbalteamgenoten; Verzorgster Ien; Mijn vrienden van mijn middelbare school; Leraren en collega's van mijn middelbare school.

Curriculum Vitae

Derk Ferdinand Werner Naafs (roepnaam Dennis) werd geboren op 5 juni 1977 te Dordrecht. In 1989 begon hij met het volgen van een VWO opleiding aan het Willem de Zwijger College te Papendrecht. In 1995 werd deze opleiding succesvol afgesloten met het bijbehorende VWO diploma. In dat zelfde jaar begon hij aan de studie Scheikunde aan de Universiteit Utrecht. Na binnen een jaar de propedeuse voor Scheikunde behaald te hebben werd de overstap gemaakt naar de doctoraal studie Geochemie, eveneens aan de Universiteit Utrecht. Vanaf september 1996 werd deze laatste studie doorlopen en in januari 2000 afgesloten met het judicium *cum laude*. Tijdens zijn afstuderen verrichtte hij onderzoek naar de karakterisering van ester-gebonden organisch materiaal in vulkanische bodems. De laatste maanden van zijn afstuderen was hij tevens werkzaam als docent wiskunde, natuurkunde en scheikunde aan het Willem de Zwijger College te Papendrecht. In februari 2000 werd hij voor een periode van 4 jaar aangesteld als promovendus bij de vakgroep Organische Geochemie aan de Universiteit Utrecht. Tijdens deze periode werd het in dit proefschrift beschreven onderzoek verricht op het gebied van moleculaire karakterisering van organisch materiaal in zure bodems. Naast dit onderzoek was hij actief in de begeleiding van het afstuderen van diverse studenten, assisteerde hij gedurende de eerste drie jaar van zijn promotie bij de werkcolleges aquatische chemie en chemie van oppervlakken, had hij de dagelijkse supervisie over het organisch geochemisch laboratorium en maakte hij deel uit van het promovendi platform. Vanaf maart 2004 is hij werkzaam bij Shell Global Solutions in Amsterdam.

I close my eyes
Only for a moment and the moment's gone
All my dreams
Pass before my eyes a curiosity
Dust in the wind
All we are is dust in the wind

Kansas, “Dust In The Wind”