VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ
BRNO UNIVERSITY OF TECHNOLOGY

FAKULTA CHEMICKÁ
ÚSTAV FYZIKÁLNÍ A SPOTŘEBNÍ CHEMIE

FACULTY OF CHEMISTRY
INSTITUTE OF PHYSICAL AND APPLIED CHEMISTRY

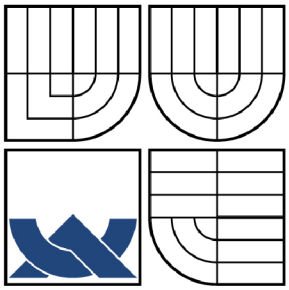
MOLECULAR STUDY OF LIPIDS IN HUMIC ACIDS BY SEQUENTIAL CHEMICAL DEGRADATION

DIPLOMOVÁ PRÁCE
MASTER'S THESIS

AUTOR PRÁCE
AUTHOR

Bc. RADKA BACHRATÁ

BRNO 2009



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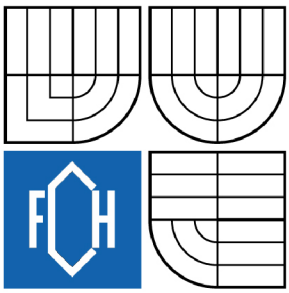

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Zadání diplomové práce

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Molecular study of lipids in humic acids by sequential chemical degradation

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4. Experimental data processing.
5. Results discussion and conclusion.

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Diplomová práce se odevzdává ve třech exemplářích na sekretariát ústavu a v elektronické formě vedoucímu diplomové práce. Toto zadání je přílohou diplomové práce.

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Bc. Radka Bachratá	doc. Ing. Martina Klučáková, Ph.D.	doc. Ing. Miloslav Pekař, CSc.
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- -
doc. Ing. Jaromír Havlica, DrSc.
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ABSTRACT

Soil lipids were shown to have relatively high resistance to biodegradation and therefore could provide information on organic matter sources and diagenetic processes. Soil organic matter is highly heterogeneous and different dynamic pools have been evidenced. However the links between these pools and molecular structure have not yet been established.
Lipid and bitumen analyses were performed for two samples (i.e. peat and lignite). Numerous compound classes (free hydrocarbons and free ketones, bound alkanols, bound fatty acids, bound ω-hydroxy acid, bound diacids and polycyclic compounds) were identified in both samples and their different distribution was determined. GC/MS analysis of the free and bound lipids has revealed different main sources depending on the differential degradation. Some similarities could be a strong sign of the preservation of a part of waxes and suberins from higher plants (long chained bound fatty acids.) Large differences in lipid molecular composition were observed between the samples, illustrating the importance of studying lipids from a young sediment (peat) and an older one (lignite).

ABSTRAKT

Lipidy v půdě vykazují poměrně vysokou resistenci k biodegradaci, mohou proto tak zajistit informace o zdrojích organické hmoty a diagenetických procesech. Půdní organická hmota je vysoce heterogenní a vyskytují se v ní různé dynamické systémy. Vztahy mezi těmito systémy a molekulární strukturou ještě nejsou úplně známy.
Analýza lipidů a bitumenu byla provedena u dvou vzroků (rašelina a lignit). Množství skupin sloučenin (volné uhlovodíky a volné ketony, vázané alkoholy, vázané mastné kyseliny, vázané ω-hydroxy kyseliny, volné dikyseliny a polycyklické sloučeniny) bylo idetifikováno pro oba vzorky a jejich distribuce byly určeny. GC/MS analýza volných a vázaných lipidů zjistila jejich různé zdroje během chemické degradace. Některé podobné znaky mohou být znakem uchovávání části vosků a suberinu z vyšších rostlin (dlouhé řetězce vázaných mastných kyselin). Velké rozdíly v molekulárním složení lipidů byly pozorovány mezi vzorky, dokládající význam studia lipidů z mladého sedimentu (rašelina) a starého (lignit).

KEYWORDS

lignite, peat, free and bound lipids, GC/MS

KLIČOVÁ SLOVA

lignit, rašelina, volné a vázané lipidy, GC/MS

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DECLARATION

I declare that this diploma thesis has been worked out by myself and I cited all used information sources correctly and completely.

PROHLÁŠENÍ

Prohlašuji, že jsem diplomovou práci vypracovala samostatně, a že všechny použité literární zdroje jsem správně a úplně citovala.

.....
signature of diploma thesis author

I would like to thank the laboratory UMR 6514 (Synthèse et Réactivité des Substances Naturelles) for their support, patient, motivation and friendly teaching environment. I would like to thank also my "home university" (Fakulty of chemistry, University of Technology, Brno). I recognize that this research would not have been possible without the financial assistance of the European Community programme for higher education (ERASMUS) and the Ministry of Education, Youth and Sports of the Czech Republic, and express my gratitude to those agencies.

CONTENTS

1. INTRODUCTION

Planet Earth and its biosphere have evolved together, and a chronicle of Earth's ecosystems and their geochemical cycles is recorded in sedimentary rocks spanning billions of years. A relatively new and very powerful approach to read these environmental signatures in ancient rocks is the study of molecular fossils, or biomarkers, within the context of the biochemistry and phylogeny of their origins.

Humic acids and humin often represent the major part of soil and sediment organic matter. Their chemical structure remains largely unknown, as a consequence of the complexity and heterogeneity of these "macromolecular" compounds. On the other hand, directly solvent-extractable lipids (named bitumen components in sediments) are almost always minor components of soil organic matter but they provide significant information regarding the sources of organic matter and its diagenetic processes occurring in soils and sediment yielding humic substances (i.e. humic acids). Moreover, the study of lipids is essential for understanding dynamic processes of the formation of humic substances.

2. STATE OF THE ART

2.1. Biomarkers and sedimentary organic matter

Biomarkers are organic compounds (such as lipids) that have particular biosynthetic origins and can be preserved in sediments and sedimentary rocks. The most valuable biomarkers are taxonomically specific, i.e., they can be assigned to a defined group of organisms, and are resistant to degradation. Reading the biomarker signatures in rocks can give information about the ancient record of deposit conditions. Biomarkers have also helped to reconstruct the first appearance of major groups of organisms, elucidate events of global climate change, record major perturbations and reorganization of geochemical cycles and document catastrophic losses in biodiversity. They are even used as tools to help in the discovery of major new petroleum reservoirs. The field of biomarker research is young and many new applications wait to be discovered [1].

2.1.1. Origin of biomarkers

The preservation of biomarkers in organic sediments was influenced by the conditions of deposit. The biological degradation of most proteins, nucleic acids and carbohydrates proceeds rapidly. However, a small fraction of organic matter escapes the remineralization process and accumulates. Molecules those are especially recalcitrant, such as pigments, lipids and many structural macromolecules, will become concentrated. With the onset of reductive conditions, the remaining sedimentary organic matter is degraded further by anaerobic heterotrophic organisms such as sulfate reducers, fermenters and methanogens; the chemical structure of the remains is consequently altered by biological and chemical processes. These alterations are referred to collectively as diagenesis [2].

Smaller molecular units and degradation-resistant macromolecules are cross-linked and form kerogen, an amorphous and exceedingly complex structural network of biochemical subunits. During the formation of kerogen, vulcanization reactions mediated by sulfur and polysulphides often play an important role in connecting smaller molecular units, such as lipids, to the macromolecular aggregate, thus protecting them against further structural alterations. Over millions of years, and with increasing burial depth and geothermal heat, most lipids will undergo structural rearrangement via cracking and isomerization reactions. These processes create a vast range of homologues and stereo- and structural isomers. Through reduction, elimination and aromatization the biomarkers typically lose all of their functional groups. The resultant products are geologically-stable hydrocarbon skeletons (Fig. 1) [1].

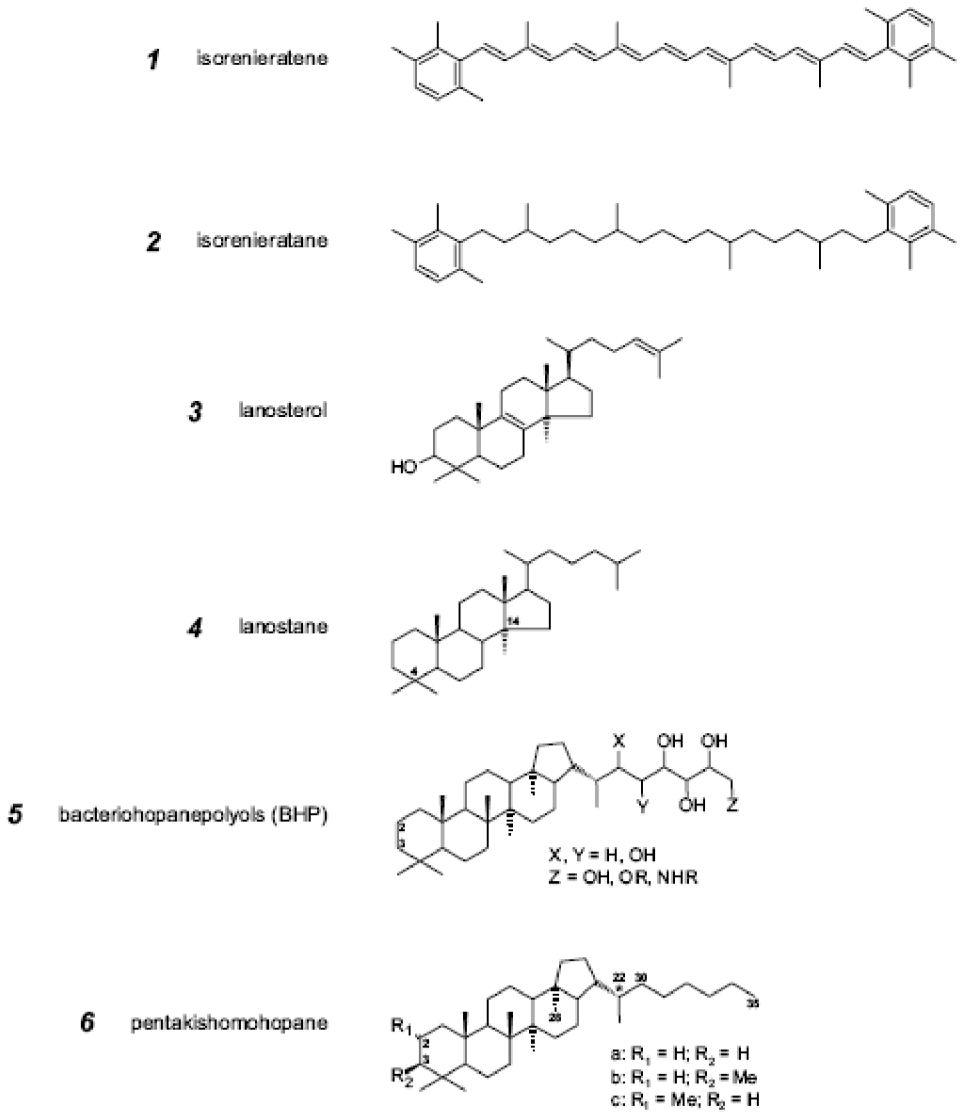

Fig. 1 *Examples of structure biolipids (1, 3 and 5) and their diagenetic hydrocarbon products (2, 4 and 6)* [1]

Bitumen is defined as the fraction of organic matter that can be extracted from sediments and sedimentary rocks using organic solvents, and it includes diagenetic components that have been thermally cracked, or released, from the kerogen. With increasing burial temperature and pressure, the thermal degradation of kerogen in organic-rich sedimentary rocks will generate enough liquid bitumen and natural gas for the expulsion of hydrocarbons in the form of petroleum. Petroleum reservoirs are, in fact, gigantic accumulations of biomarkers and other cracking products of sedimentary organic matter. The burial temperatures in the sedimentary unit exceed 150–250 °C. This is the upper survival temperature for biological molecules over geologic time [3].

The thermal destruction of biomarkers with deep burial is the primary complication in the search for biogenic molecular remains in very ancient, billion-year-old sedimentary rocks.

During the experimental analysis of biomarkers, sedimentary rocks are crushed to powder, and the powder is extracted with organic solvents such as chloroform, methanol or dichloromethane using conventional reflux extraction or automated solvent extractors (ASE). The bitumen extracts are usually yellow to dark brown, highly complex mixtures, containing many compounds. To simplify further analyses, the bitumen is fractionated into saturated hydrocarbons, aromatics, and polar compounds (usually those containing the heteroatoms O, N, and S) using normal-phase (SiO_2-gel) chromatography. The fractions are then analyzed by gas chromatography-mass spectrometry (GC/MS) (Fig. 2) [1].

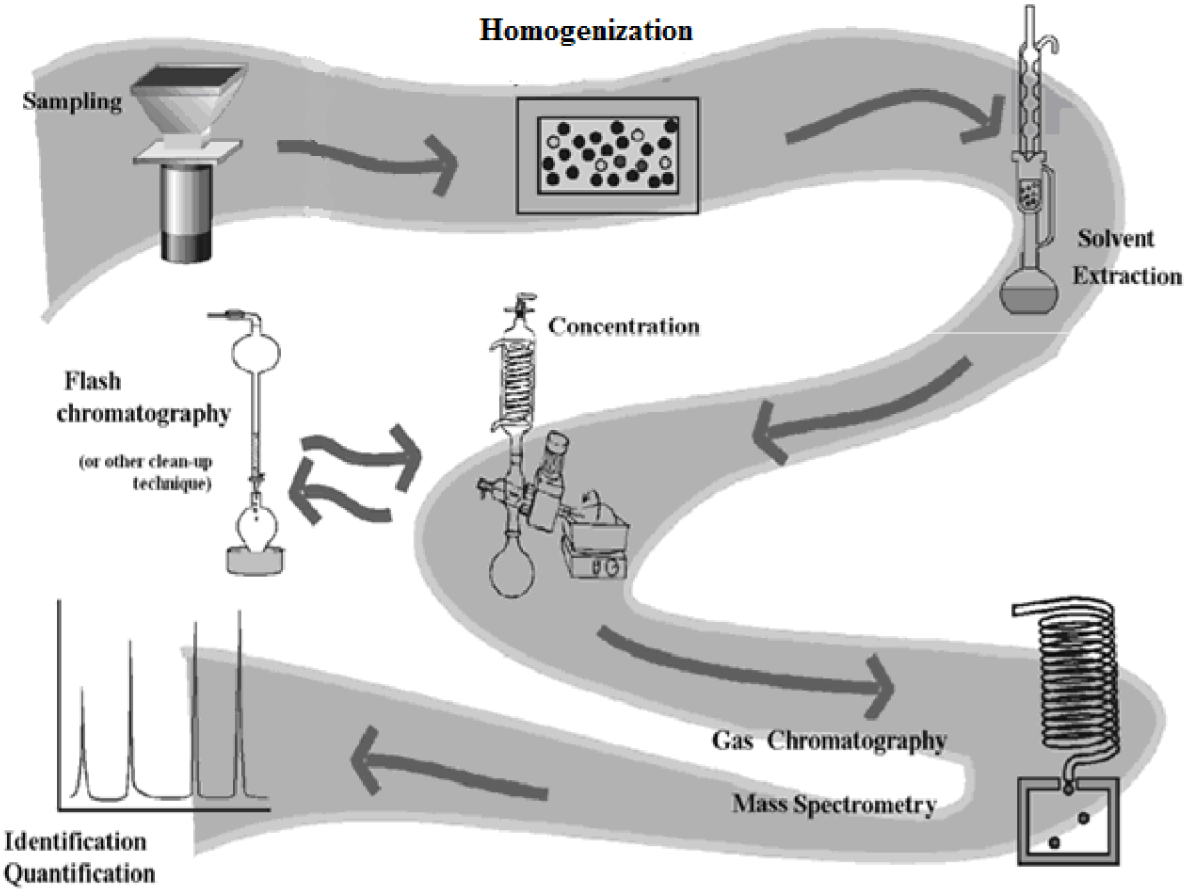

Fig. 2 *Scheme representing a common sampling, extraction and GC/MS procedure for extraction and characterization of bitumen and lipids* [4]

2.1.2. Molecular characteristics of biomarkers

Biomarkers are usually characterized by:
• A high degree of order in their molecular structures, resulting from the specificity of the biosynthetic processes.
• Small molecule building blocks.
• Precise sequence of assembly.
• Chirality of carbon centers and stereochemistry of the units.
• Distribution of isotopes in the molecule.
• Intra-molecular characteristics documented by structural identification and molecular isotope measurements.
• Inter-molecular variations assessed through compound distributions (e.g. abundance ratios) [5].

2.2. Lipids and bitumen

Lipids and bitumen are organic substances that are insoluble in water but extractable with non-polar solvents (e.g. chloroform, hexane, acetone, ether or benzene).
However, application of the term lipid can vary, sometimes being restricted to fats, waxes, steroids and phospholipids, and sometimes to fats alone. Simple organic compounds like aliphatic carboxylic acids and alcohols can be found among the lipids, but most lipids exist as combination of these simple molecules with one another (e.g. wax esters, triglycerides, steryl esters and phospholipids) or with other compound classes such as carbohydrates (glycolipids) and proteins (lipoproteins) [6].
The term bitumen is used for the fraction that can be extracted from sediments and sedimentary rocks [7]. For the rest of the thesis, the term lipids will refer to the term bitumen.

2.2.1. Role as biomarkers

It is possible to assess sedimentary contributions in contemporary environments from the microscopically identifiable remains of organisms, particularly inorganic skeletal material (e.g. calcareous and siliceous tests).
Unfortunately, these components do not always survive in older sediments, but molecular evidence may, particularly in form of biomarkers such as lipid-derived compounds that can be traced to particular biological precursor molecules.
The applications of molecular palaeontology (or chemotaxonomy) to ancient sediments requires an understanding of how individual compounds and groups of compounds can be used to identify contributions from extant organisms in recent (i.e. Holocene) sediments and of what changes these compounds undergo during diagenesis and subsequent catagenesis [7].

2.2.2. Free and bound lipids

Analysis of biomarkers in soils and sediments involves their extraction with the sediments with a suitable combination of organic solvents, usually followed by some separation procedure(s) in order and aid component identification by providing less complex mixtures.
Solvent extraction on its own removes the *free lipids*, but a proportion, the *bound lipids* fraction, remains bonded to insoluble polymeric material in the studied matter.
Bound lipids are subsequently extracted following hydrolysis of the sediment residue, which breaks the bonds between the remaining lipids and insoluble matrix. With increasing burial, the amount of free lipids usually decreases during the formation of insoluble kerogen, while changes in bound lipids reflect changes in kerogen composition.
The free lipids contain the simple lipids and the complex lipids. The complex lipids are substantial proportions of compounds in which smaller components are chemically bonded together, such as fatty acids and fatty alcohols in wax esters.
The amount of complex lipids in the free lipids fraction depends on the degree of hydrolysis that has occurred during diagenesis, a process that releases individual components (e.g. fatty or steryl esters respectively) [7].

2.3. Analysis of lipid biomarkers

In the section we present some studies and analysis of biomarkers in biogeochemistry. These examples are an attempt to illustrate the unique contributions that the analysis of lipid biomarkers can make to the field of biogeochemistry.

2.3.1. Extraction and isolation

Soil lipids, mostly originating from plants and microorganisms, have traditionally been extracted by non-automated extraction and separation methods, which produce several lipid fractions, operationally defined by polarity. Wiesenberg et al. [8] present a combination of fast, automated and reproducible techniques, adopted from organic geochemical studies, for preparative separation of individual soil lipid fractions with increasing polarity.
These techniques involve commercially available instruments, including accelerated solvent extraction (ASE) and a two-step automated medium-pressure liquid chromatography procedure. The method yields eight lipid fractions consisting of five fractions fully amenable to gas chromatography-mass spectrometry (GC/MS) (aliphatic hydrocarbons, aromatic hydrocarbons, ketones, alcohols, carboxylic acids), and three fractions of highly polar or high molecular weight compounds (bases, very long-chain wax esters ($C40_+$), high polarity compounds) that were not measurable with GC/MS under standard conditions.

Thus, automated separation can be a fast, effective and reproducible procedure for fractionation of complex mixtures of soil lipids into clean interference-free compound classes, directly suitable for a variety of molecular (e.g. GC/MS) and isotopic characterizations (e.g. gas chromatography coupled with isotope ratio monitoring mass spectrometry or accelerator mass spectrometry) [8].

The motive of Jansen and Nierop et al. [9] was to examine the efficiency of accelerated solvent extraction (ASE) to extract typical lipid biomarkers from a selection of soil horizons from a Dutch sandy soil under Corsican pine, using Soxhlet extraction as a reference. The biomarkers consisted of a selection from the following component classes:

- Straight-chain lipids
- Plant sterols
- Terpenoids

The comparison of ASE with Soxhlet extraction was done for this purpose.
Soxhlet extraction suffers from three main shortcomings:

- The necessity of using relatively large extractant volumes of usually 250 ml or more.
- Long analysis times of typically 16 h per analysis.
- A difficulty to automate.

A promising alternative is the relatively new technique of accelerated solvent extraction (ASE). In short, ASE extracts samples under elevated temperature, while elevated pressure ensures that volatile extractants remain liquid. ASE can be completely automated; it employs very small extractant volumes (normally 5–30 ml) and has typical extraction times of less than an hour. As such the technique has the potential to overcome the main shortcoming of Soxhlet extractions. However, while the use of ASE to extract organic contaminants from soils is now reasonably well-established, its application to the extraction of soil lipids has received very little attention so far.
The reason is that differences in extraction efficiencies for various types of lipids between ASE and other techniques would lead to a difference in the composition of the biomarker signal that is obtained.

The observed differences between ASE and Soxhlet extractions as well as the pressure effect can be explained by a decrease in polarity of the extractant due to the elevated pressure and temperature applied during ASE extractions as compared to Soxhlet extractions.
Future investigations comparing multiple extractants with different polarity ranges as well as other classes of lipids and other soil types with a different texture could be helpful in further examining the performance of ASE for this type of investigation [9].

Recent researches focus on careful study design and data analysis protocols. Authors offer detailed descriptions of study biomarker populations, analytical methods and data analysis, and highlight the use of practical data preprocessing.
The field is moving towards more methodical and structured approaches to biomarker identification.

2.3.2. Chromatography

Chromatography is concerned with separating a mixture of components during passage over the surface of an immobile material (a solid or liquid stationary phase) that has varying affinity for the different compounds in the mixture. The mixture is moved over the surface of the stationary phase (the process of *elution*) in a suitable fluid (a gas or liquid), which is termed the mobile phase, and separation of components results from their differing degrees of retention on the stationary phase.
The fractions of lipids can be achieved by liquid chromatography, using a simple column containing suitably activated aluminia or silica gel (the stationary phase) and various solvents as mobile phase, moving under gravity. As an example, using solvent with increasing polarity it is possible to elute sequentially from the column the saturated hydrocarbons (with hexane), aromatic hydrocarbons (with toluene) and resins (with methanol) [6].

2.3.2.1. Gas chromatography

In gas chromatography, the stationary phase is in the form of thin film lining the interior wall of a long, open tubular, capillary column. Coiled columns are typically made of vitreous silica.
The mobile phase is an inert gas (e.g. helium), passed through the column under pressure. This separation technique is strictly called gas-liquid chromatography because it involves a gaseous mobile phase (also termed the carrier gas) and a liquid stationary phase.
Ideally, the individual compounds present in a mixture emerge from the end of the column at varying intervals, with no two compounds eluting at the same time. Because a compound can move along the column only when in the gaseous phase, the time it takes to elute (its retention time) depends on its vapour pressure (i.e. boiling point) and its chemical affinity for the stationary phase (i.e. partition coefficient).
The higher a component´s vapour pressure (i.e. the higher the boiling point) and/or the higher its affinity for the stationary phase, the longer it will take to elute.
For a homologous series like the *n*-alkanes, each member has a similar affinity for the stationary phase, so the elution order is effectively governed by volatility, which decreases with increasing carbon number. The structure of a hydrocarbon can influence its interaction with the stationary phase and so diastereoisomers can potentially be resolved.
Increasing the column temperature uniformly throughout the analysis enables the compounds of lower volatility to elute within reasonable time, limiting the extent to which diffusion can spread them out on the column, causing peak broadening and low signal: noise ratios in the detection system [7].

2.3.2.2. Gas chromatography-mass spectroscopy

Flame ionization detector (FID) is suitable for the more abundant and readily identifiable components (which is sensitive to *c*.100 pg). Using this detector, components such as *n*-alkanes are identified from their recognizable elution patterns. But some compounds in sediments, such as terpenoidal and steroidal hydrocarbons, are generally present only in trace

quantities, at concentrations of around two orders of magnitude lower than the more abundant components, and require a more specific and sensitive detector, a mass spectrometer.
The technique is then referred to as gas chromatography-mass spectrometry (GC/MS). In the simplest form of GC/MS, compounds emerging from the column are bombarded by high-energy electrons, which expel an electron from each molecule, producing positively charged molecular ions, which tend to fragment into smaller, more stable ions, characteristic of the particular structural units present in the parent molecule. As an example, steranes yield an abundant fragment ion with a mass: charge (*m/z*) ratio of 217, monitoring of which provides the necessary sensitivity and specificity to examine sterane distribution. Other biomarker families have different characteristic fragment ions.
The plot of time versus intensity obtained from whatever detector is used is termed a *chromatogram* [7].

3. AIM OF THE WORK

The aim of this study was to characterize the free bitumen composition (the solvent extractable fraction) of the South-Moravian lignite (SML) from the locality Mikulčice (Czech Republic). The data are discussed in relation with the composition of the free lipids of a French Peat located in Frasne (Jura, France) to find out the link between a young sediment (peat) and an older one (lignite).

Apolar fractions isolated directly (simple lipids) or after chemical sequential degradations (complex lipids) from lignite and peat were analyzed by GC and GC/MS.

4. EXPERIMENTAL PART

4.1. Laboratory precautions

To avoid any source of contamination by mineral oils, greases, plasticisers from plastics and detergents. All operations should be carried out in glass equipment with, if required, ground-glass joints (do not grease). Separatory funnels or columns are best equipped with Teflon (PTFE) stopcocks.
All vials or tubes should be closed if necessary (for incubation, agitation, and centrifugation) with screw caps including a Teflon-covered liner. Never use cork, rubber, polyethylene or Para film.
Filtration of solutions should be made with an all-glass vacuum filtration apparatus equipped with filter membranes made of nylon or Teflon.
Except Teflon, all plastics must be avoided because they leach contaminants into the solution which can be detected as unknown spots on thin-layer plates or extra-peaks in GC chromatograms.
It should be emphasized that all the glassware must be cleaned using classical basic detergents in washing machine or sonicator bath but the washed vials or tubes should be first tested for the absence of contaminants with the current techniques.

4.2. Samples

4.2.1. Peat

Frasne peatland (46°49'N, 6°10'E), an undisturbed *Sphagnum*-dominated mire is situated in the Jura Mountains (France) (Fig. 3). The site is protected by the EU Habitat Directive of Natura 2000 and has been classified as a Regional Natural Reserve for more than 20 years.

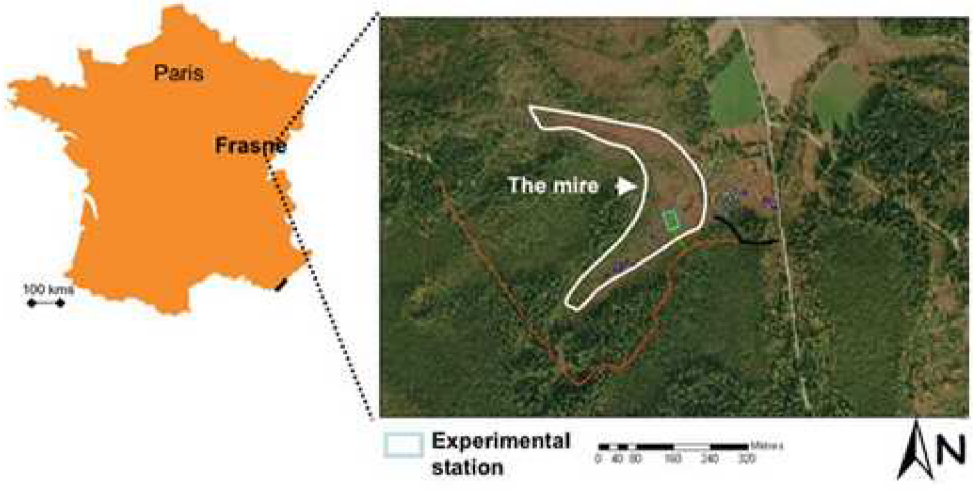

Fig. 3 *Location map of Frasne peatland* [10]

4.2.2. South-Moravian lignite (SML)

South-Moravian lignite (SML) from locality Mikulčice (Czech Republic) was used in our experiments (Fig. 4). It was received in the pre-dried and milled state from the power plant. First, it was dried at 105 °C for 24 h and then sieved. The lignite fraction captured between 0.1 and 0.2 mm sieves was used for subsequent experiments after moisture-equilibration at ambient conditions. Moisture content of lignite material used for the preparation of pastes was thus within the range 5–7%. Lignite particles are very sticky therefore particles smaller than 0.1 mm were present in the used lignite fraction [11].

Fig. 4 *Location map of South-Moravian Coalfield*

4.2.3. Elemental analysis

Elementary compositions of combustible matter of peat and SML are given in Table 1 (Lignit Hodonín, Ltd. unpublished data).
Comparing with the average composition of solid fuels, it is confirmed that SML belongs to the group of young brown coals and plant material forms Frasne peat.
Elemental analysis was kindly made in Pliva-Lachema Company, using standard methods of Carlo-Erba elementary analyzer (lignite) and in laboratory UMR 6514 using NC Analyzer – Flash FA 1112 Series (peat). Oxygen was calculated by difference [11].

Table 1 *Elemental composition of solid fuels, South-Moravian lignite (SML) and peat* [11]

	Carbon (weight %)	Hydrogen (weight %)	Oxygen (weight %)	Nitrogen (weight %)
wood	50	6	43	1
peat	60	6	33	1
brown coal	73	6	19	1
black coal	82	5	10	1
anthracite	94	3	2	1
SML	65,66	5,24	28,20	0,90
Frasne peat	52,85	6,79	38,27	2,09

4.3. Procedure of fractionation of total lipid extract

4.3.1. Lipid extraction

Before extraction, using a mortal and pestle, sediment sample was thoroughly homogenized.
The free lipids were obtained using traditional solvent extraction methods. The extraction was performed by Soxhlet with bidistilled chloroform (6×10 hours). The chloroform fractions were combined and evaporated to dryness under vacuum.

4.3.2. Lipid separation

The solvent fraction was dissolved again in chloroform to loading on pre-packed $SiO_2.2H_2O$ columns (SUPELCO Discovery® SPE DSC-Si Silica Tube).
Lipids were eluted with chloroform, 25 % acetone/chloroform, 100 % acetone, and methanol, respectively, and the latter fraction was collected as well as residue in silica gel.
The retention of lipids on the columns was a function of lipid polarity and of solvent strength and polarity.

Chloroform fraction was separated into neutral, basic and acid lipids. The neutral and basic parts (NB) were acetylated (see below) and separated on a SiO_2 column using diethyl ether/petroleum ether mixtures of increasing polarity for elution. The acid part (AS) was methylated (see below), acetylated and separated on a SiO_2 column. The apolar fractions (simple lipids) were directly analyzed using combined gas chromatography-mass spectrometry (GC/MS).

Fractions (25 % acetone/chloroform, 100 % acetone, methanol and residue in silica gel) were saponified (see below), methylated and acetylated. Separation was performed on SiO_2 column. Apolar fractions were directly analyzed by GC and GC/MS.

4.3.3. Lipid saponification (ester bond cleavage), methylation and acetylation

The remaining polar fractions (complex lipids) were submitted to saponification.
The products were again separated on SiO_2 column and then apolar fractions were directly analyzed.

Saponification – lipids were saponified by reflux for 6 h under nitrogen with 6 % KOH in methanol/distilled water 9/1 (v/v), then stirring overnight at room temperature to release bound lipids. The saponified lipids were extracted with chloroform after acidification with HCl (pH 1). The solvents were evaporated using a rotary evaporator. The fractions were transferred to preweight glass vials. They were dried under N_2 and weighted.

Methylation – fractions were methylated with trimethylsilyl-diazomethane ($TMS\text{-}CHN_2$). Briefly, to the dried sample of lipids (1 g) were added 4 ml of chloroform, 2 ml of methanol

and 2 ml of TMS-CHN_2. The sample was mixed on a vortex for 2 hours. The solvent were dried under N_2.

Acetylation – before analysis, alkanols and sterols were converted to acetates, to the dry lipids catalytic quantity of pyridine was added and an excess of acetic anhydride (1 ml). The mixture was heated for 20 min at 50 °C and cooled at room temperature overnight.

The reaction was stopped with ice-water (2 ml) to hydrolyse remaining acetic anhydride, and stirred for an additional two hours.

The products were extracted with chloroform. The organic extract (organic phase) was neutralized by saturated solution of $NaHCO_3$, and then by saturated solution of NaCl.

Anhydrous $MgSO_4$(s) was used as a drying agent for the organic phase which was finally concentrated with a rotary evaporator.

4.3.4. Ether bond cleavage with HI/cesium propionate

The remaining polar fractions after the previous step were submitted to hydrolysis with iodohydric acid.
The products were again separated on SiO_2 column and then apolar fractions were directly analyzed.

The lipids were suspended in 5 ml of a 57 % aqueous solution of HI. The mixture was stirred for 18 h at 100 °C under a N_2 atmosphere. The reaction mixture was neutralized with a saturated $NaHCO_3$ solution. I_2 formed during the reaction was eliminated with $Na_2S_2O_3$. After filtration, the reaction products were extracted with diethyl ether.
These products were allowed to react with 2 ml cesium propionate in dimethylformamide (DMF). The mixture was stirred for 24 h, at 40 °C, under an inert atmosphere. DMF was eliminated after adding 1 N HCl and evaporation. The propionate derivatives were methylated with TMS-CHN_2, and then were separated on a SiO_2 column.

Apolar fractions extracted from products were directly analyzed using gas chromatography (GC) and combined gas chromatography-mass spectrometry (GC/MS) (Fig. 5).

Separation was monitored using thin-layer chromatography (TLC) with commercial plates (ALUGRAM® SIL G/UV_{254} for TLC). The plates were developed in 8 % ethyl acetate in petroleum ether in lid-covered chamber. The spots were visualized by fluorescence (lamp U.V. 254 nm) or by using a phosphomolybdenic acid/EtOH solution.

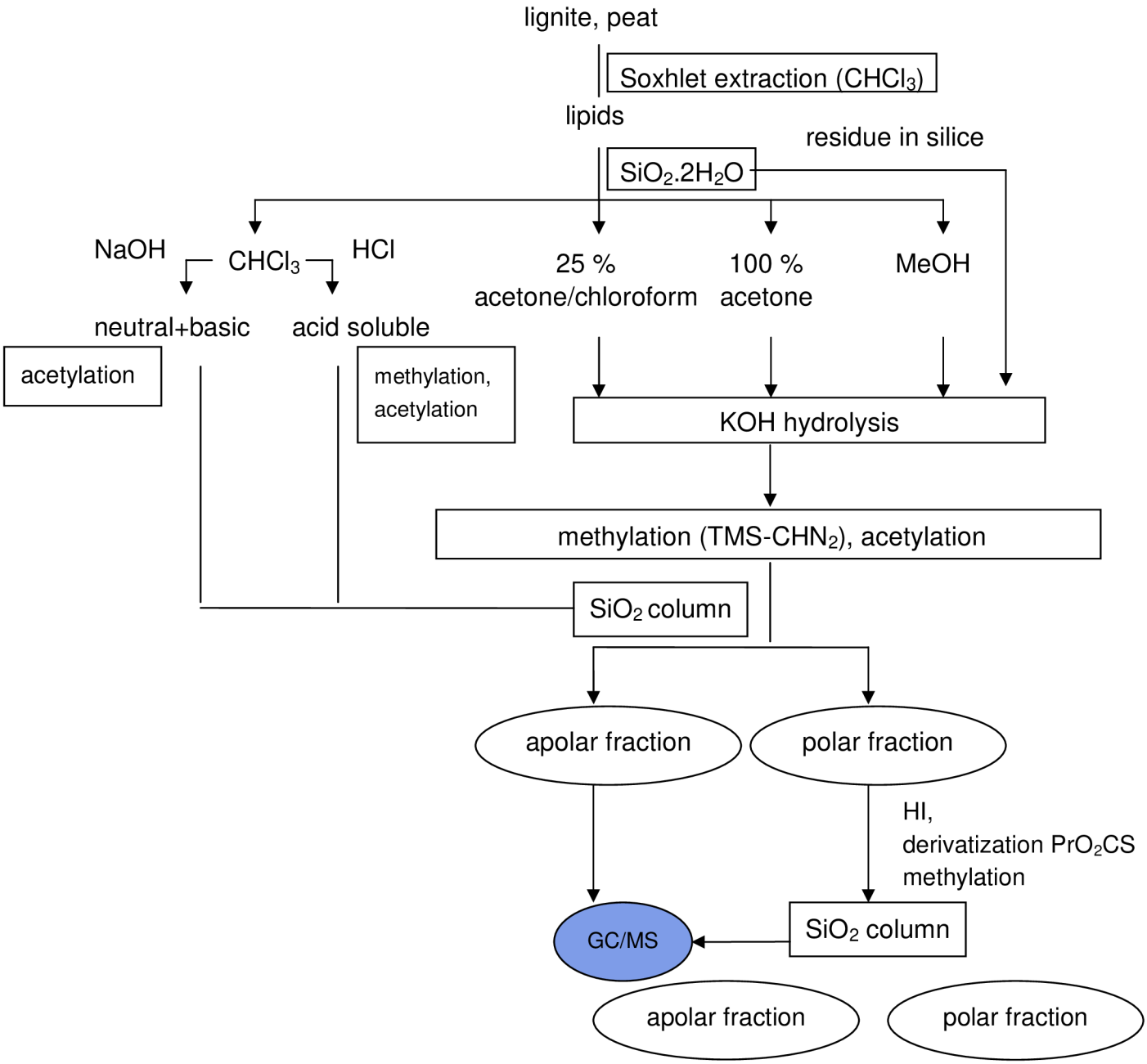

Fig. 5 *Separation of the various classes of lipids – sample preparation diagram*

4.4. Lipid identification (GC/MS)

The separated families were analyzed by capillary GC and GC-MS using a Hewlett-Packard 6890 GC (split injector, 250 °C; flame ionization Detector (FID), 300 °C) with a fused silica capillary column (SGE BPX 5 %, 30 m length, 0.25 mm id., 0.25 μm film thickness) and helium as carrier gas. The GC was temperature programmed from 60 to 300 °C at 5 °C min^{-1} (isothermal for 15 min final time). The GC/MS analyses were performed on a Trace GC Thermo Finnigan coupled to a Thermo Finnigan Automass (with the same GC conditions). The MS was operated in the electron impact mode with a 70 eV ion source energy and the ion separation was operated in a quadripolar filter. The various products were identified on the basis of their GC retention times, their mass spectra (comparison with standards) and literature data.

5. RESULTS AND DISSCUSION

5.1. Results of separation

The lipid fraction extract with Soxhlet amounts to 48,85 $mg.g^{-1}$ of lignite and lipid fraction extract amounts to 97,45 $mg.g^{-1}$ of peat.
Chloroform extract was separated into five fractions on silicic acid column (Table 2).

Table 2 *Quantitative results from separation of lipids (% of total used lipid extract)*

elutant	chloroform	25 % acetone/chloroform	100 % acetone	methanol	residue in silice	total Σ
lignite	43,6	45,6	4,0	1,0	2,1	96,3
peat	12,4	69,9	11,7	1,1	2,5	97,6

5.2. Molecular composition

GC/MS analysis of lipids from the peat and lignite reveals the occurrence of several series of components, including alkanes, ketones, alkanols, fatty acids, hydroxy acids and diacids. These main series are observed in the peat and lignite with different relative abundances and distributions. The information obtained from these different series is presented and discussed below in relation with their origin and differences between the peat and lignite.
The less polar fractions were separated from the chloroform fraction extract by SiO_2 column. Methylation and acetylation were also performed to improve the detection of the polar lipids. Finally, high molecular weight lipids which are not GC-amenable and thus cannot be directly analyzed were investigated through saponification and HI/cesium propionate treatment of the most polar lipid fraction and further GC/MS analysis.
The compounds were identified on the basis of retention time, mass spectra and by comparison with literature data.

5.2.1. Peat

5.2.1.1. Free hydrocarbons

The *n*-alkanes distribution in the lipid extracts, as reflected by ion chromatograms of *m/z* 99, is shown in Fig. 6, which is characterized by a strong dominance of nC_{31}. The hydrocarbons mainly correspond to the range C_{21}-C_{33}, with a major contribution of the odd carbon numbered long chain compounds. The odd-carbon-numbered long chain n-alkanes are typical constituents of epicuticular waxes of higher plant [12] [15]. Accordingly, they are classically used as biomarkers of higher plant input [8].

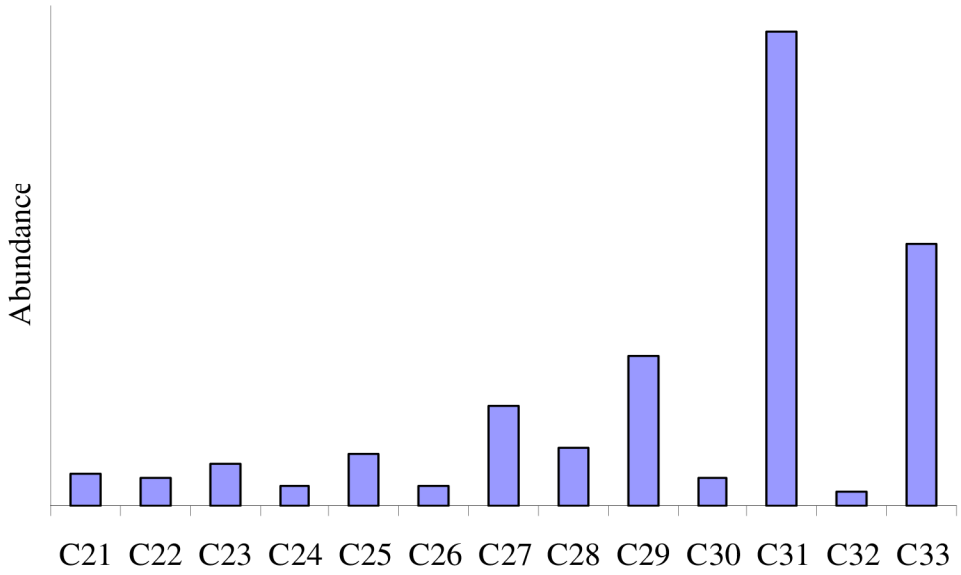

Fig. 6 *Distribution of n-alkanes in peat*

5.2.1.2. Free ketones

The presence of *n*-alkan-2-ones in the lipid extract was shown by selective detection of their specific ion (*m/z* 58). Straight chain *n*-alkan-2-ones ranging from C_{27} to C_{31} with an only odd carbon number predominance and a maximum at C_{29} and a submaximum at C_{31} were detected in the peat (Fig. 7). Straight chain *n*-alkan-2-ones are generally considered as not being directly formed as primary products by living organisms. The ketones exhibited the same distribution as the *n*-alkanes and were thus considered as having been formed by bacterial sub-terminal oxidation of the *n*-alkanes, as commonly assumed for sediments and soils. Another possible mechanism which was put forward to account for *n*-alkan-2-one formation is β-oxidation of fatty acids followed by decarboxylation. In the latter case, the distribution of the ketones should correspond to that of the fatty acids, minus one carbon [15]. In the present study, such relationships are not observed, either with the *n*-saturated fatty acid distributions

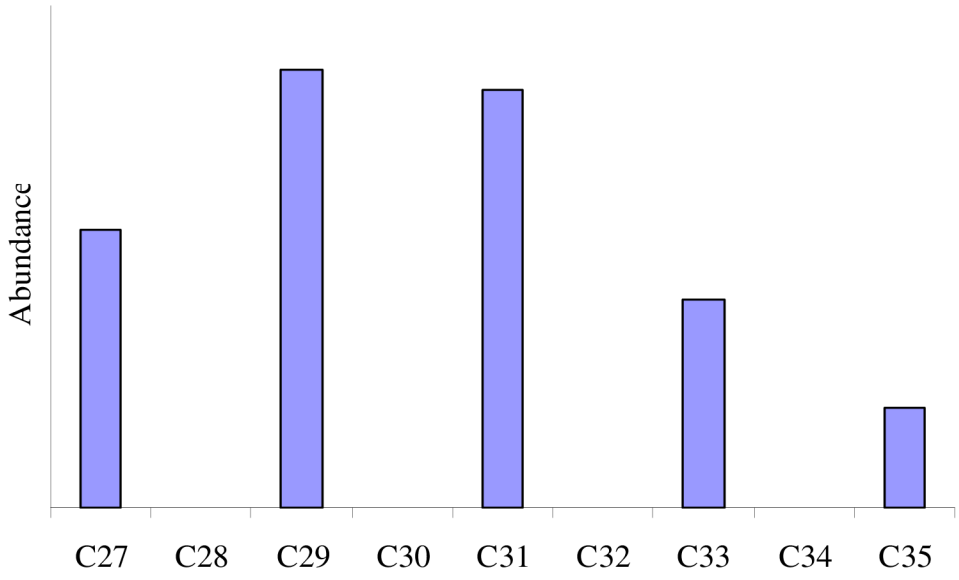

Fig. 7 *Distribution of n-alkan-2-ones in peat*

5.2.1.3. Bound alkanols

The distributions of bound *n*-alkan-1-ols, obtained after alkaline hydrolysis of the polar fractions, were illustrated using the *m/z* 61 ion fragmentograms (Fig. 8).

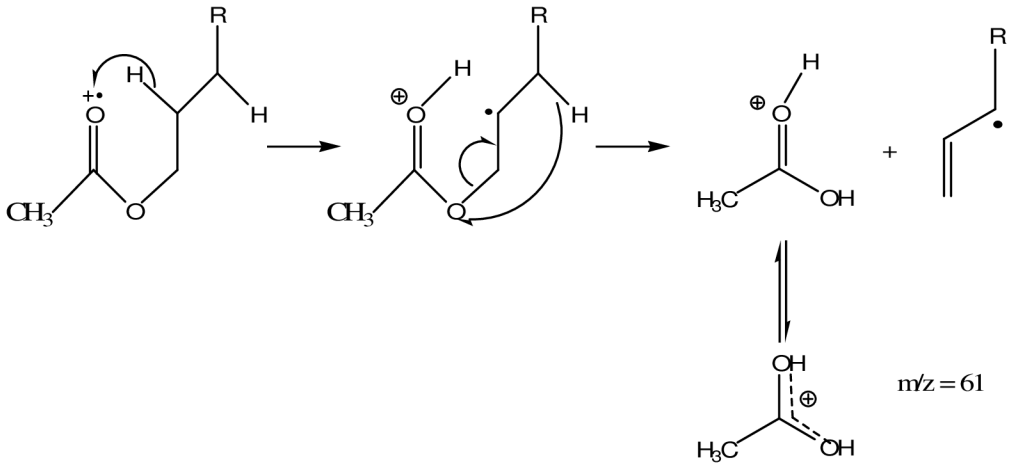

Fig. 8 *Significant mass fragment of alkanols*

The *n*-alkan-1-ols, ranging from C_{14} to C_{32} (C_{max} at C_{22}) in the fraction 25 % acetone/chloroform and dominated by even carbon numbered compounds, occur in the saponified lipids of the peat. Branched alkanols were also detected (Fig. 9). Long chain even numbered *n*-alkan-1-ols from C_{12} to C_{30} (C_{max} at C_{20}) were found in the fraction 100 % acetone (Fig. 10). Long chain n-alkan-1-ols (C_{20+}) with even karbon numbers have been reported in higher plant leaf waxes.

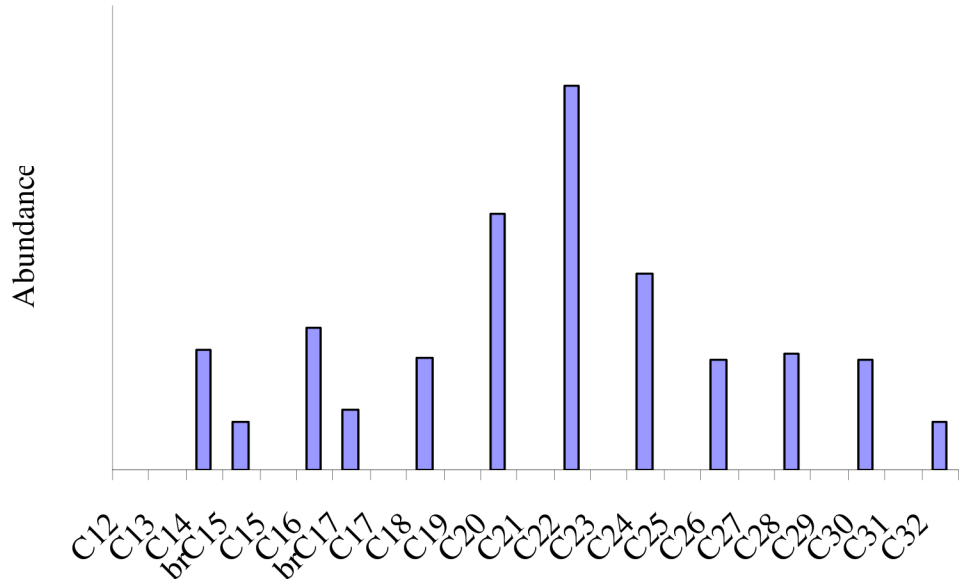

Fig. 9 *Distribution of n-alkan-2-ols in peat (25 % acetone/chloroform); br: branched*

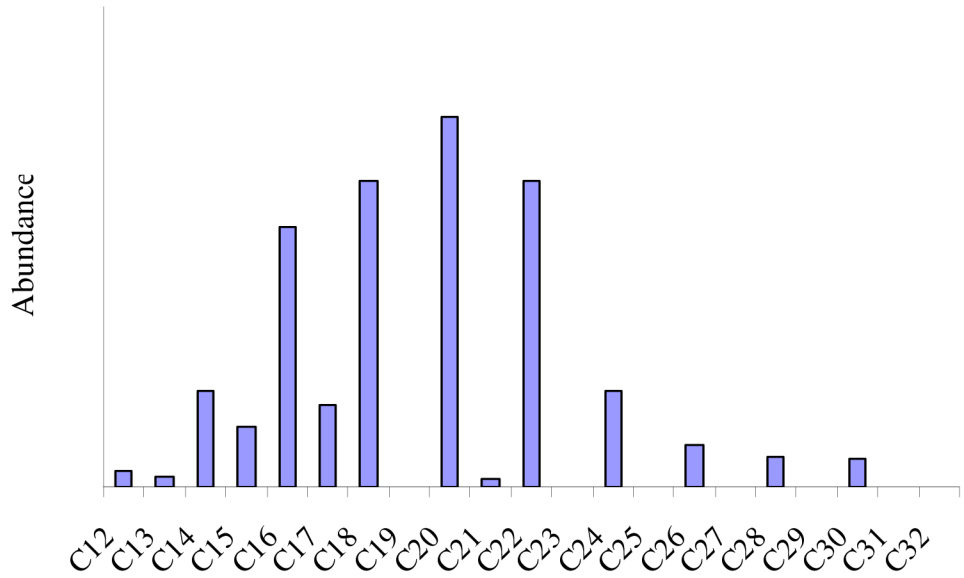

Fig. 10 *Distribution of n-alkan-2-ols in peat (100 % acetone)*

5.2.1.4. Bound fatty acids

The distributions of fatty acids, identified as methyl esters (FAMEs), were illustrated using the *m/z* 74 ion fragmentogram (Fig. 11). This ion arises from the characteristic McLafferty rearrangement.

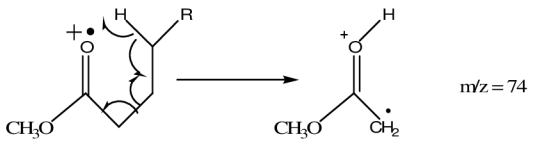

Fig. 11 *Significant mass fragments of FAMEs*

After saponification, carboxylic acids ranging from C_{12} to C_{34} (C_{max} at C_{22}) in the fraction 25 % acetone/chloroform and from C_{12} to C_{30} (C_{max} at C_{16}) in the fraction 100 % acetone with an even-over-odd carbon number predominance are obtained, as shown by the ion chromatogram at *m/z* 74 of the methylated acids (Fig. 12 and 13). In these two fractions is also noted the presence of branched acids and unsaturated $C_{16:1}$, $C_{18:1}$ components. The FAMEs profile showed a bimodal distribution with C_{max} at C_{16} and C_{22}. *Iso* and *anteiso* acids appear as minor components. Branched *iso*- and *anteiso*-C_{15} and C_{17} FAMEs are typical of microbial activity. Such even carbon numbered, long chain (>C_{18}) *n*-saturated fatty acids are considered as typical of higher plant epicuticular waxes and are commonly found in lipid extracts of whole soils [14].
These acids, observed after saponification, can originate either from the cleavage of ester linkages in high molecular weight and/or highly polar lipids or they can correspond to free acids.

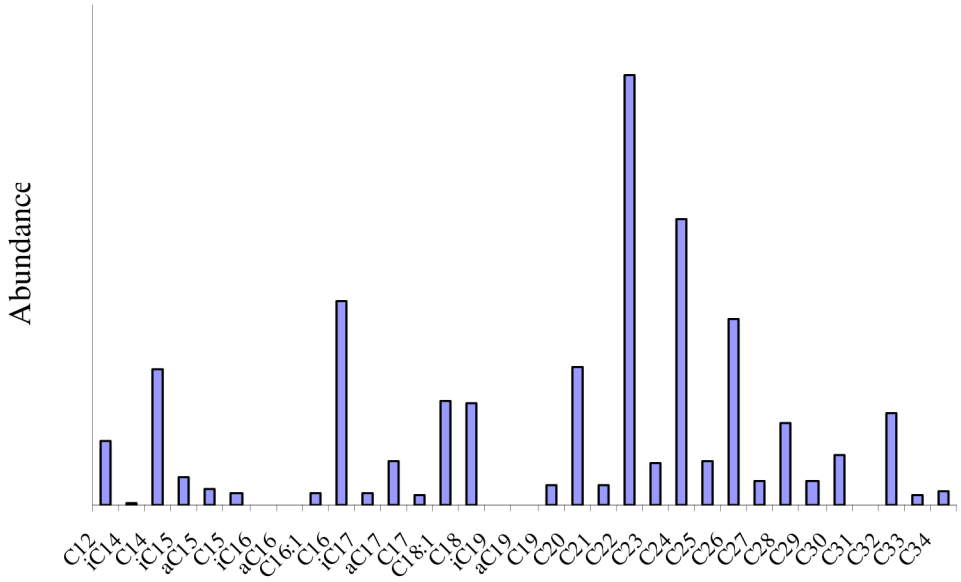

Fig. 12 *Distribution of FAMEs in peat (25 % acetone/chloroform); a: anteiso, i: iso; 16:1 and 18:1 are monounsaturated acids*

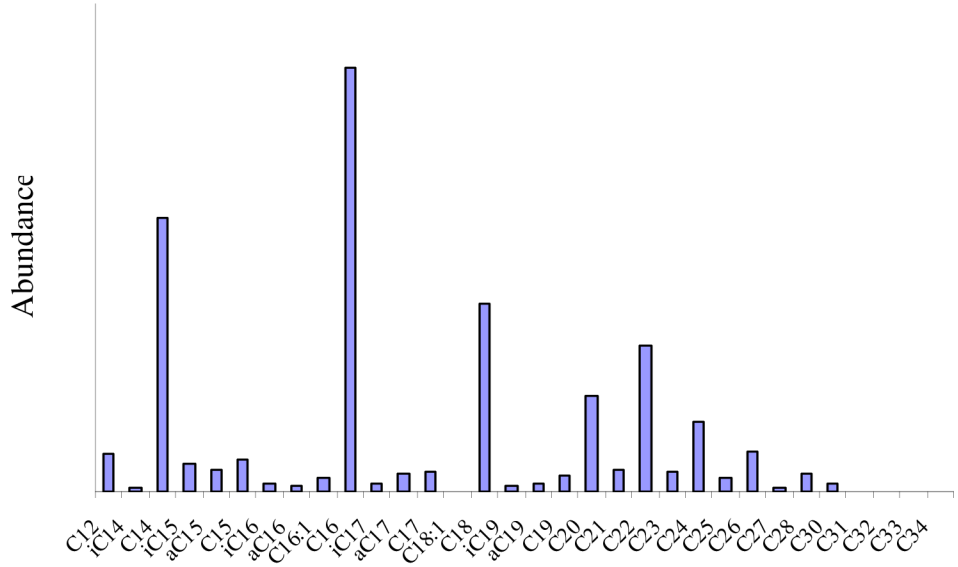

Fig. 13 *Distribution of FAMEs in peat (100 % acetone); a: anteiso, i: iso; 16:1 and 18:1 are monounsaturated acids*

In the fraction MeOH (Fig. 14) and residue in silice (Fig. 15), only the short chained FAMEs are present, revealing the microbial origin of the fatty acids isolated in these two fractions.

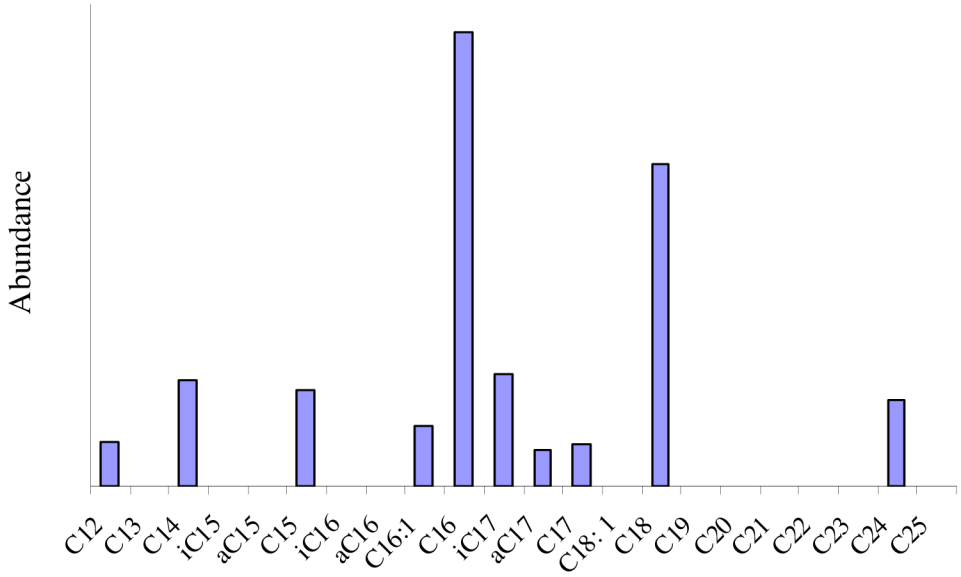

Fig. 14 *Distribution of FAMEs in peat (MeOH); a: anteiso, i: iso; 16:1 and 18:1 are monounsaturated acids*

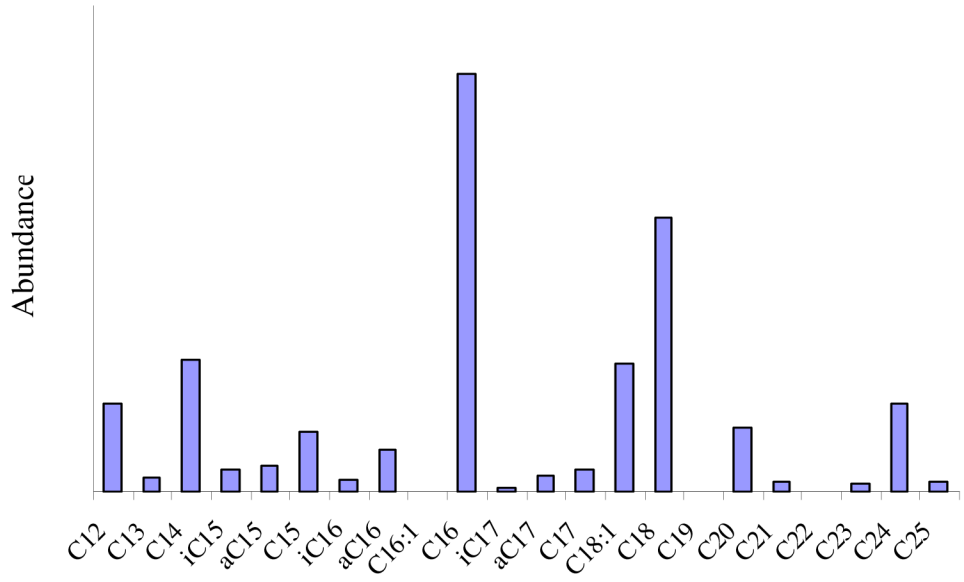

Fig. 15 *Distribution of FAMEs in peat (residue in silice); a: anteiso, i: iso; 16:1 and 18:1 are monounsaturated acids*

5.2.1.5. Bound hydroxy acids

The saponified lipids contain the series of ω-hydroxy acids. These hydroxy acids, exclusively occurring as ester-bound moieties in the lipids of the fractions, chiefly correspond to even carbon numbered compounds, ranging from C_{12} to C_{26}, showing a maximum at C_{16} (Fig. 16) and C_{22} (Fig. 17). The hydroxy acids profile ranges from C_{12} to C_{24} (C_{max} at C_{22}) in the fraction residue in silice (Fig. 18).
The ω-hydroxy acids, with similar distribution, were previously observed after saponification of the lipid extracts of acidic soils and trans-esterification of high molecular weight soil lipids. Several origins can be considered for these ω-hydroxy acids. Cutin and suberins are polyesters whose major monomers comprise even-carbon-numbered ω-hydroxy acids with chain length of C_{16} and C_{18} for cutins and from C_{16} to C_{26} for suberins. The occurrence of a series of ω-hydroxy acids in the saponified lipids thus probably reflects a contribution of the aforementioned polyesters and especially of suberins [15].
It must be noted that no ω-hydroxy acids are detected in the saponified lipids from the MeOH fraction.

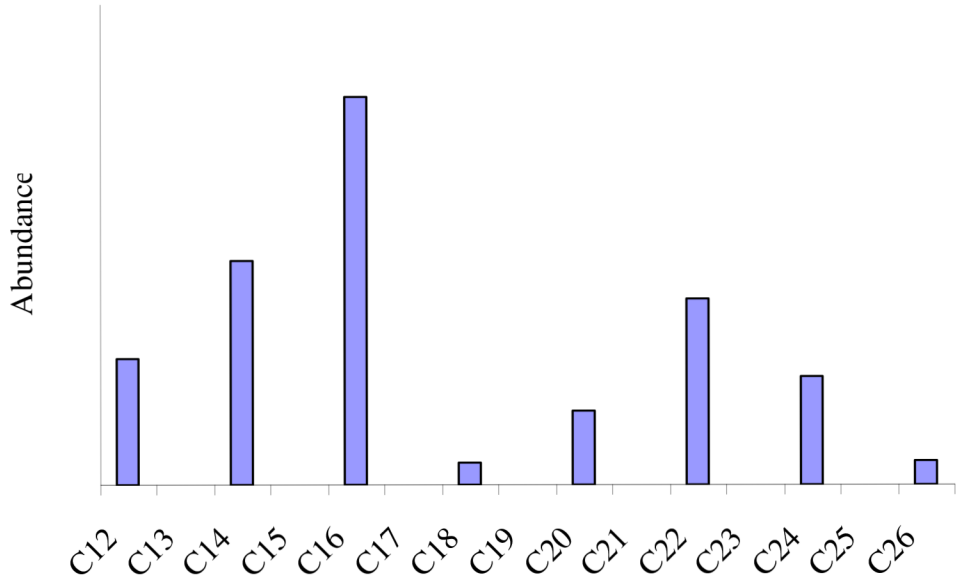

Fig. 16 *Distribution of ω-hydroxy acids in peat (25 % acetone/chloroform)*

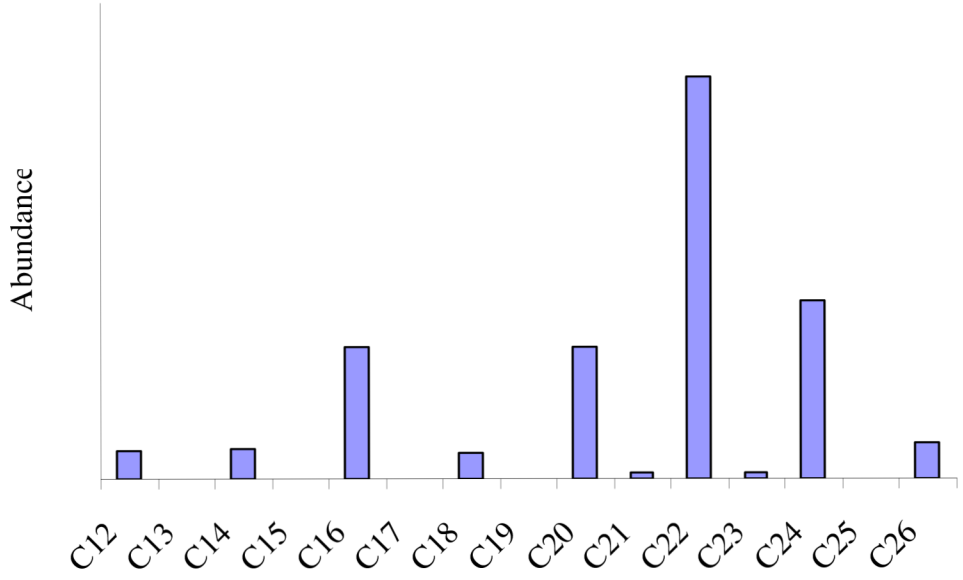

Fig. 17 *Distribution of ω-hydroxy acids in peat (100 % acetone)*

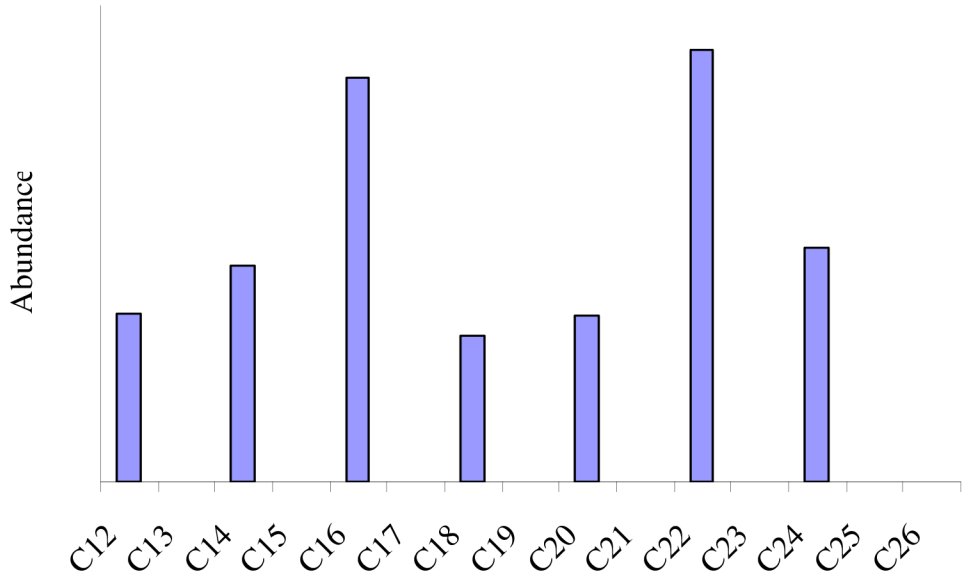

Fig. 18 *Distribution of ω-hydroxy acids in peat (residue in silice)*

5.2.1.6. Bound diacids

The distributions of diacids were illustrated using the *m/z* 98 (and *m/z* 74) ion fragmentogram (Fig. 19).

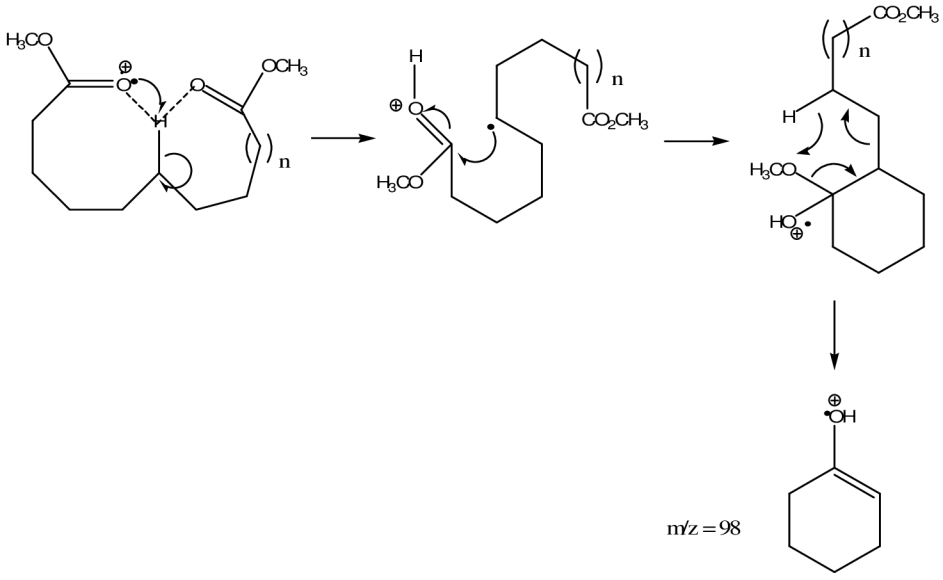

Fig. 19 *Significant mass fragment of diacids*

Whereas only a few diacids (C_{22} and C_{24}) were found in the fraction 25 % acetone/chloroform, a series of α,ω-diacids was observed in the saponified lipids of fraction 100 % acetone. This series ranges from C_{11} to C_{26} with even-over-odd carbon number predominance and a maximum at C_{20} (Fig. 20). Even carbon numbered α,ω-diacids in C_{16}-C_{24} range are major building blocks of suberin [14].

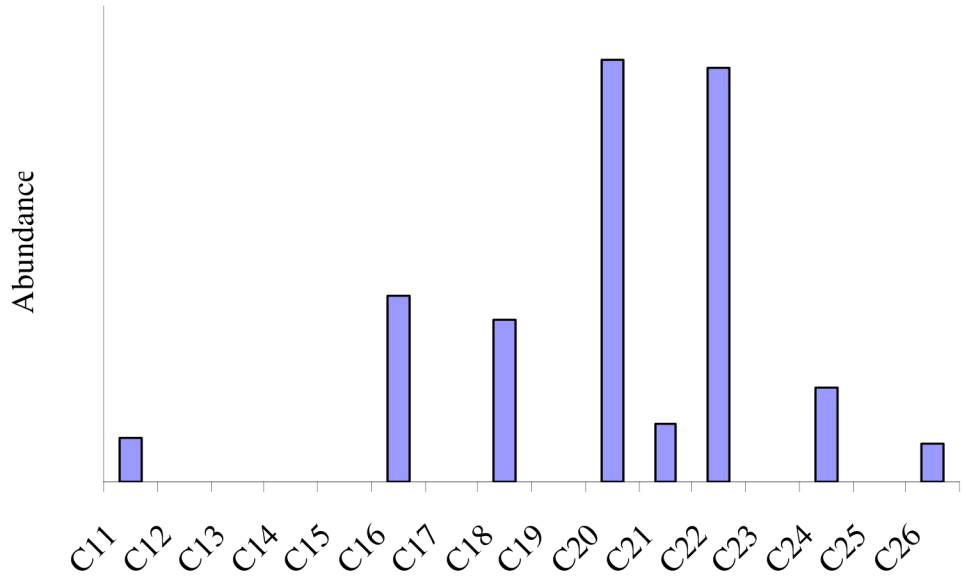

Fig. 20 *Distribution of diacids in peat (100 % acetone)*

5.2.1.7. Polycyclic compounds

Some polycyclic compounds were detected in the bound fractions. These structures are presented in Fig. 21. Campesterol, β-sitosterol and stigmasterol are sterols of higher plants (called phytosterol). Lupan-3-ol presents also plant origin [12] [13].

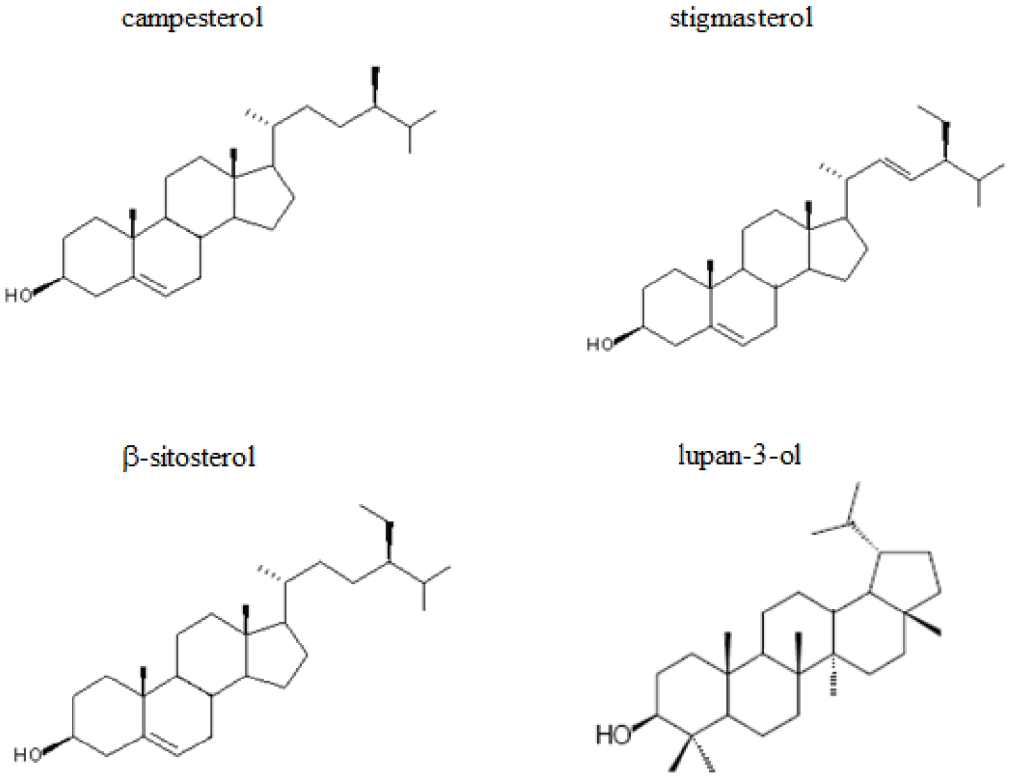

Fig. 21 *Polycyclic compounds found in peat (from GC/MS)*

5.2.2. Lignite

5.2.2.1. Free hydrocarbons

The n-alkanes distribution (Fig. 22) is characterized by a strong dominance of C_{29} and ranging from C_{22} to C_{33}. When the peat and lignite fractions are compared, the main difference is at maximum (for peat at C_{31}). The range is the same with peat and reflects a higher plant input [12].

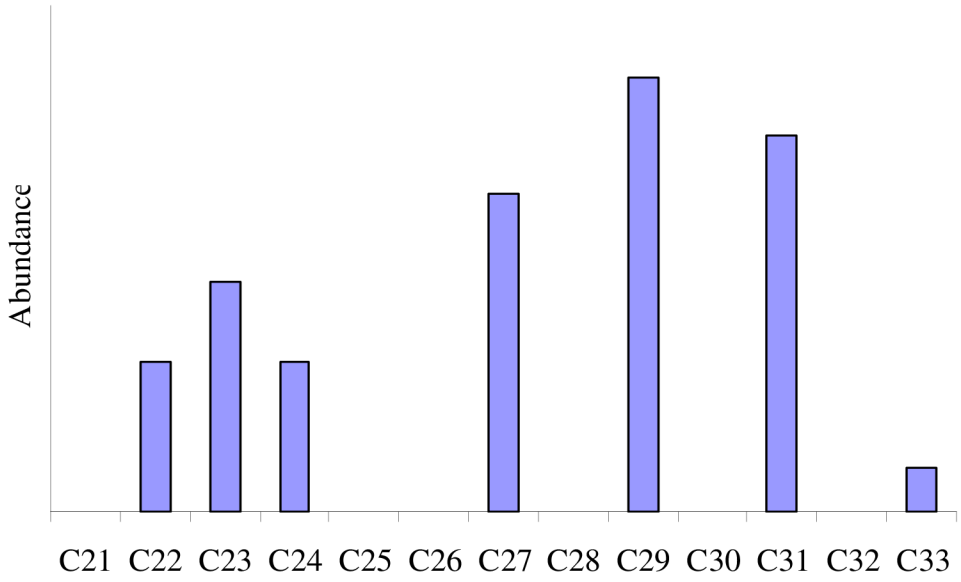

Fig. 22 *Distribution of n-alkanes in lignite*

5.2.2.2. Bound fatty acids

The fatty acids obtained after saponification are the esterified acids released by ester cleavage. The distributions of the FAMEs are reported in Fig. 23 and Fig. 24. FAMEs ranging from C_{12} to C_{28} (C_{max} at C_{16}) in the fraction 25 % acetone/chloroform and from C_{12} to C_{24} (C_{max} at C_{16}) in the fraction 100 % acetone with an even-over-odd carbon number predominance are obtained.
In the fraction MeOH (Fig. 25) and residue in silice (Fig. 26), only the short chained FAMEs are present, revealing the microbial origin of the fatty acids isolated in these two fractions.
The FAMEs profile shows a bimodal distribution with C_{max} at C_{16} and C_{26} (for the fraction 25 % acetone/chloroform).

Comparison with the distribution in the peat shows similar series. The above observation indicates the presence of branched acids and unsaturated acids.
These long ester-bound acids probably originate from higher plant components like wax esters and suberin-derived components. Short FAMEs are typical of microbial activity and are the most represented in the fraction 25 % acetone/chloroform for both samples.

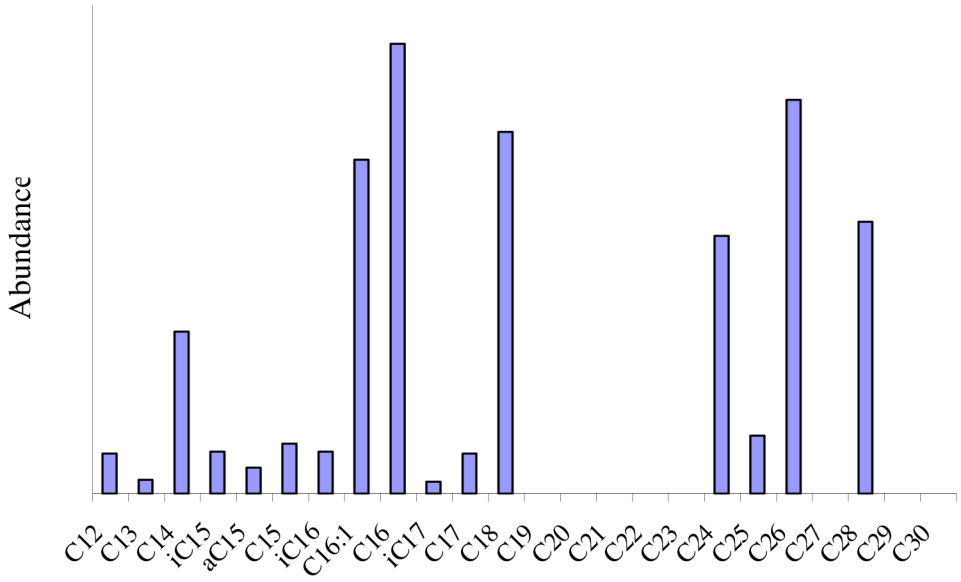

Fig. 23 *Distribution of FAMEs in lignite (25 % acetone/chloroform); a: anteiso, i: iso; 16:1 and 18:1 are monounsaturated acids*

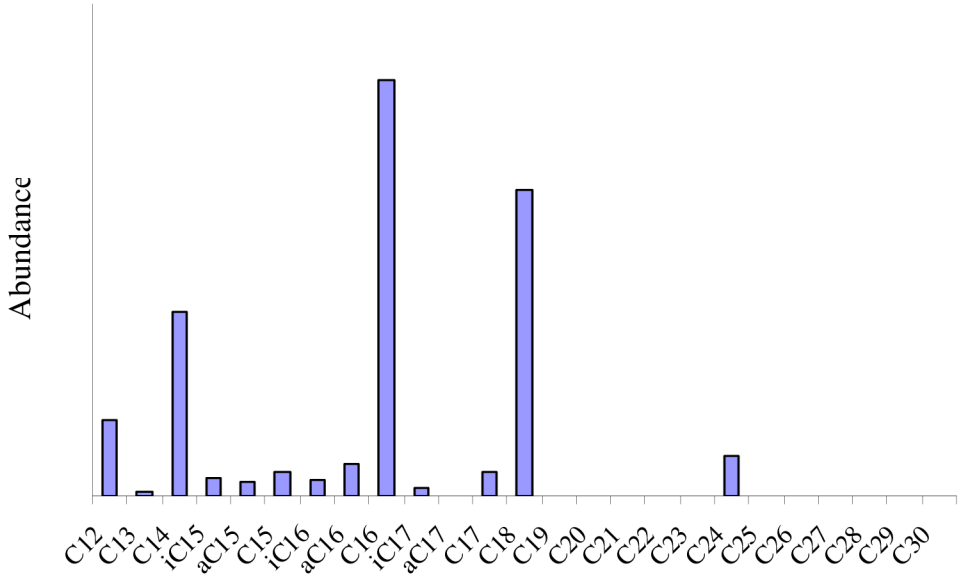

Fig. 24 *Distribution of FAMEs in lignite (100 % acetone); a: anteiso, i: iso; 16:1 and 18:1 are monounsaturated acids*

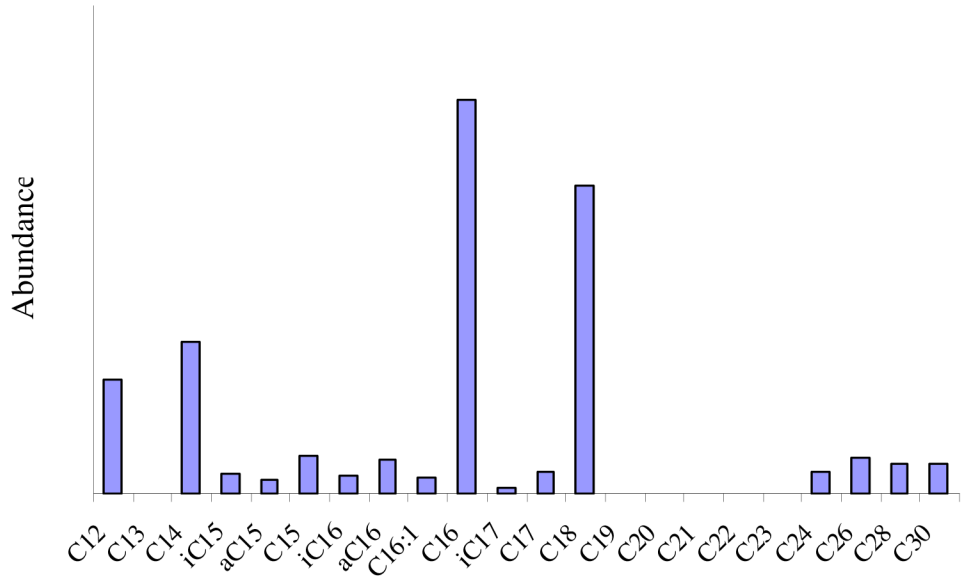

Fig. 25 *Distribution of FAMEs in lignite (MeOH); a: anteiso, i: iso; 16:1 and 18:1 are monounsaturated acids*

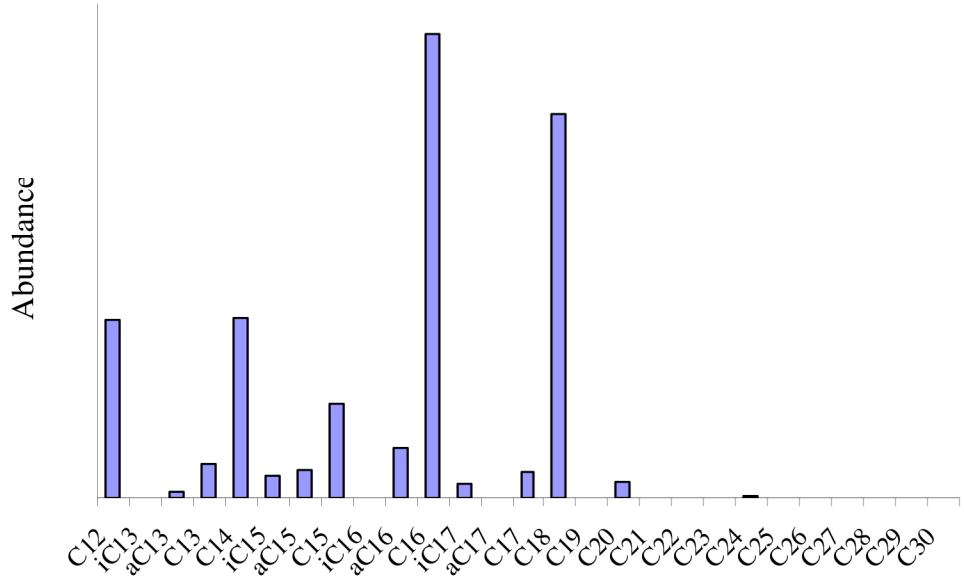

Fig. 26 *Distribution of FAMEs in lignite (residue in silice); a: anteiso, i: iso; 16:1 and 18:1 are monounsaturated acids*

5.2.2.3. Bound hydroxy acids

Several ω-hydroxy acids were detected in the saponified lipids from the fraction 25 % acetone/chloroform. These hydroxy acids chiefly correspond to even carbon numbered compounds (C_{15}, C_{16}, C_{24}, C_{26} and C_{28}), showing a maximum at C_{16}. The occurrence of a series of ω-hydroxy acids shows a contribution of suberins. No ω-hydroxy acids were found in the fraction 100 % acetone in comparison to peat.

5.2.2.4. Bound diacids

Whereas only a few α,ω-diacids ($C_{13,}$ C_{14}, C_{16}, C_{24} and C_{26}) were found in the fraction 25 % acetone/chloroform. This series ranges with even-over-odd carbon number predominance and a maximum at C_{16}. Similar trend was observed in the same fraction in peat. Even carbon numbered α,ω-diacids in C_{16}-C_{24} range are major building blocks of suberin [14].

5.2.2.5. Polycyclic compounds

Some polycyclic compounds were found in the lignite. These structures are presented in Fig. 27. Lupen-3-ol and lupan-3-one present a higher plant origin [12]. β-sitosterol was detected as well as in the peat.

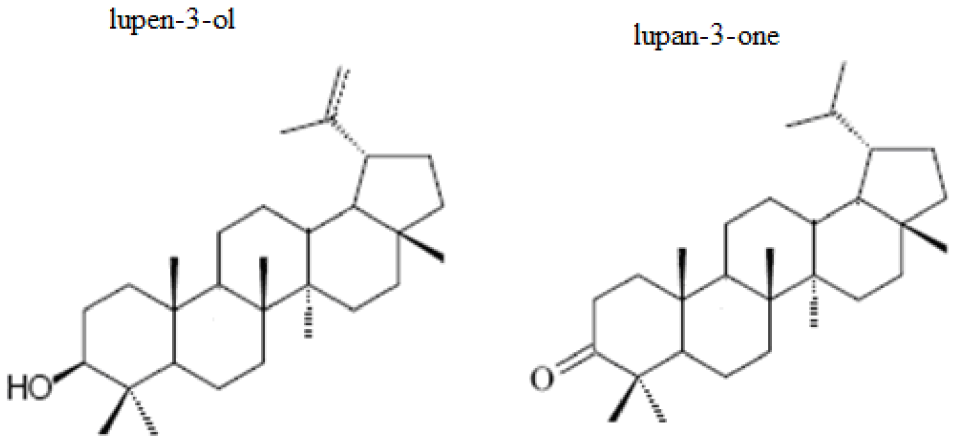

Fig. 27 *Polycyclic compounds found in lignite (from GC/MS)*

5.2.3. Ether bond cleavage with HI/cesium propionate

In order to confirm (or to invalidate) the presence of alkyl chains bound by ether groups, the remaining polar compounds after alkaline hydrolysis were treated with iodhydric acid. Alkyl iodides were then transformed into propionate derivatives with cesium propionate. In addition alkylpropionates show a highly characteristic MS fragmentation ion *m/z* 75 arising from a double hydrogen rearrangement of the molecular ion [14].

$$R - R' + HI \rightarrow RI + R'OH$$
$$R'I + ROH$$
$$RI + PrO_2Cs \rightarrow PrO_2R + CsI$$

GC/MS analyses are in progress.

6. CONCLUSION

Large differences in the distribution of the various lipid series (free hydrocarbons, free ketons, bound alkanols, bound fatty acids, bound hydroxy acids, bound diacids and polycyclic compounds) were observed between peat and lignite samples. The study provides information on the different lipid sources in these samples. Contribution from higher plant components (like waxes and wax esters), or microbially reworked plant components (like depolymerisation products of suberin and cutin) and direct microbial contributions (like fatty acids, alkanols and alkanes) were thus evidenced.

Comparison of lipid composition in the peat sample and lignite sample revealed marked differences for all the types of lipids. On the other hand, similarities could be a strong sign of the preservation of a part of waxes and suberins from higher plants (such as long chained fatty acids). The odd carbon numbered homologues observed in substantial amounts, probably originate form partial microbial reworking of the long chain even acids, through α-oxidation and subsequent chain shortening.

Future investigations comparing these results with results from HI treatment as well as quantification of different classes of lipids could be helpful in further insight on diagenetic processes.

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8. LIST OF SYMBOLS

c	concentration
g	gram
m	meter
m/z	mass-to-charge ratio
p	pico-

9. LIST OF ABBREVIANTIONS

a	anteiso
AS	acid soluble
ASE	accelerated solvent extraction
DMF	dimethylformamide
e.g.	example given
Et_2O	diethyl ether
FAMEs	fatty acid methyl ester
FID	flame ionization detector
GC/MS	gas chromatography-mass spectrometry
i	iso
i.e.	in effect
MeOH	methanol
NB	neutral basic
PrO_2Cs	cesium propionate
PTFE	polytetrafluoroethylene
SML	South Moravian lignite
SPE	solid phase extraction
TLC	thin-layer chromatography
TMS-CHN_2	trimethylsilyl-diazomethane
UV	ultraviolet