Analysis of fatty acids in soil and sediments

Deepika P Panawennage

University of Nevada, Las Vegas

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ANALYSIS OF FATTY ACIDS IN SOIL AND SEDIMENTS

by

Deepika P. Panawenage

Bachelor of Science
University of Colombo, Sri Lanka
2000

A thesis submitted in partial fulfillment
of the requirements for the

Master of Science Degree in Chemistry
Department of Chemistry
College of Sciences

Graduate College
University of Nevada, Las Vegas
December 2005
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Thesis Approval
The Graduate College
University of Nevada, Las Vegas

November 9, 2005

The Thesis prepared by

Deepika Pushpakanthi Panawennage

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Analysis of Fatty Acids in Soil and Sediments

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Examination Committee Chair

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Vernon Hodge

Graduate College Faculty Representative
ABSTRACT

Analysis of Fatty Acids in Soil and Sediments

by

Deepika Pushpakanthi Panawennage

Dr. Spencer M. Steinberg, Examination Committee Chair
Professor of Chemistry
University of Nevada, Las Vegas.

Fatty acids are one of the most important classes of naturally occurring organic compounds. Various organisms have distinctive fatty acid profiles. Therefore analyzing fatty acids present in soil and sediments can provide important information on sources of origin of the organic matter.

Analysis of fatty acids by gas chromatography requires derivatization to produce a derivative volatile enough for gas chromatography followed by mass spectrometric (GC/MS) analysis. In this work conventional acid catalyzed derivatization methods and tetramethylammonium hydroxide (TMAH) chemopyrolysis methods were examined and compared. Results obtained for Lake Mead sediments show the variation of fatty acids profile with depth, which leads to an important information on sources of organic matter present in the lake.
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CHAPTER 1

INTRODUCTION

Fatty acids are naturally found in soil. The lack of efficient methods to completely characterize these soil fatty acids in various soil components, limits the application of fatty acid data to soil chemistry and environmental problems. Because of the high resolution available in gas chromatography, fatty acids are generally analyzed by this technique. However to apply gas chromatography, compounds have to be volatile enough to get a good chromatographic resolution. Derivatizing fatty acids to the corresponding methyl esters can increase the volatility of the fatty acids. In this thesis, several derivatization methods including conventional acid catalyzed methylation techniques and chemopyrolysis techniques were compared.

1.1. Fatty Acids

Fatty acids are one of the most important classes of naturally occurring organic compounds, and can be found in every living organism. These compounds persist in sediments and soil after the death of the organism. Also many metabolic products of living organisms are composed, in part, of fatty acids (Brondz, 2002). Scarce amounts of fatty acids are available in “free form” and generally fatty acids exist in nature in combination with complex molecules through ester or amide bonds (Brondz, 2002; http://www.cyberlipid.org). These bound fatty acids are ionically or covalently linked to...
alcohol, glycol, glycerine, phosphatidyl glycerol, diphosphatidyl glycerol, phosphatidyl inositol, monogalactosyl glycerol, digalactosyl glycerol, galactosylglucosyl glycerol, aminosaccharides, proteins, or other molecules (Brondz, 2002). Only a small fraction of the total lipid fraction, obtained from extracting with a non-polar solvent, consists of long chain free carboxylic acids and most fatty acids exist as esters of glycerol or triacylglycerols (Solomons, 1996), Figure 1.1.

![Triacylglycerol](image)

Figure 1.1. Triacylglycerol. R1, R2 and R3 are long chain carbon chain alkyl groups and may contain one or more carbon-carbon double bonds (Solomons, 1996).

Triacylglycerols are found as oils of plants or animal fat. Hydrolysis of these compounds may produce mixtures of fatty acids (Figure 1.2).
Most natural fatty acids consist of an even number of carbon atoms, due to the fact that they are biologically synthesized from two carbon units. However, some fatty acids may have an odd number of carbon atoms or contain varieties of other functional groups such as alkyne, epoxy, hydroxy or keto groups and even ring structures (cyclopropane, cyclopropene, furan, and cyclohexyl) [http://www.cyberlipid.org]. In addition, most fatty acids are straight chain molecules (Solomons, 1996). Branched chain fatty acids do occur and have unsubstituted carbon chains and can have one or several methyl groups (Brondz, 2002). Branched methyl substituted fatty acids are commonly associated with bacteria and are often found in sediments. Their abundance decreases rapidly with depth (Matsuda, 1977). Branched fatty acids are not exclusive to bacteria. 14-Methylhexadecanoic acid was found to be characteristic for pine seed oil (Wolff, 1997). In animals, 2- and 4- methyl fatty acids are found in the uropygial gland of ducks (Kolattukudy, 1991) as well as in the guinea pig hardener gland (Yasugi, 1991). Large quantities of C14 to C30 fatty acids with a branch as well as odd-chains were found in
sponges (Carballeria, 1989). For example, 20-methyl-hexacosanoic acid was elucidated in sponges from Canary Islands (Nechev, 2002) while some monomethyl polyunsaturated fatty acids were found in marine sponges (Caballeira, 2001). Unusually branched fatty acids have also been isolated from the glycolipids fraction of fresh water sponges as minor components (Dembitsky, 2003). An example of unusual fatty acid of cyanobacterial origin, 12,12-Dimethyl-tridecanoic acid is shown in Figure 1.3 (http://www.cyberlipid.org).

![Figure 1.3. An Unusual Branches Fatty Acid.](http://www.cyberlipid.org)

Naturally occurring fatty acids may consist of saturated or unsaturated carbon chains. Cis-unsaturated fatty acids, have considerably lower melting points than those of saturated fatty acids because the cis configuration of the double bond puts a rigid bend in the carbon chain which interferes with crystal packing (Solomons, 1996). The Table 1.1 shows the list of most common saturated fatty acids. A few sources including sponges contain branched polyunsaturated fatty acids (Rezanka, 1989). For example, freshwater sponges were shown to contain di-, tri-, and tetramethyl substituted dienoic, tetraenoic, and hexaenoic fatty acids (Rezanka, 2002).
Table 1.1. Most Common Saturated Fatty Acids (http://www.cyberlipid.org).

<table>
<thead>
<tr>
<th>IUPAC Name</th>
<th>Trivial Name</th>
<th>Shorthand Designation</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butanoic</td>
<td>Butyric</td>
<td>4:0</td>
<td>88.1</td>
</tr>
<tr>
<td>Pentanoic</td>
<td>Valeric</td>
<td>5:0</td>
<td>116.1</td>
</tr>
<tr>
<td>Hexanoic</td>
<td>Caproic</td>
<td>6:0</td>
<td>144.2</td>
</tr>
<tr>
<td>Octanoic</td>
<td>Caprylic</td>
<td>8:0</td>
<td>172.3</td>
</tr>
<tr>
<td>Nonanoic</td>
<td>Pelargonic</td>
<td>9:0</td>
<td>200.3</td>
</tr>
<tr>
<td>Decanoic</td>
<td>Capric</td>
<td>10:0</td>
<td>228.4</td>
</tr>
<tr>
<td>Dodecanoic</td>
<td>Lauric</td>
<td>12:0</td>
<td>256.4</td>
</tr>
<tr>
<td>Tetradecanoic</td>
<td>Myristic</td>
<td>14:0</td>
<td>270.4</td>
</tr>
<tr>
<td>Hexadecanoic</td>
<td>Palmitic</td>
<td>16:0</td>
<td>284.4</td>
</tr>
<tr>
<td>Heptadecanoic</td>
<td>Margaric(daturic)</td>
<td>17:0</td>
<td>308.5</td>
</tr>
<tr>
<td>Octadecanoic</td>
<td>Stearic</td>
<td>18:0</td>
<td>340.5</td>
</tr>
<tr>
<td>Eicosanoic</td>
<td>Arachidic</td>
<td>20:0</td>
<td>368.6</td>
</tr>
<tr>
<td>Docosanoic</td>
<td>Behenic</td>
<td>22:0</td>
<td>396.7</td>
</tr>
<tr>
<td>Tetracosanoic</td>
<td>Lignoceric</td>
<td>24:0</td>
<td>410.7</td>
</tr>
<tr>
<td>Hexacosanoic</td>
<td>Cerotic</td>
<td>26:0</td>
<td>424.8</td>
</tr>
<tr>
<td>Heptacosanoic</td>
<td>Carboceric</td>
<td>27:0</td>
<td>432.9</td>
</tr>
<tr>
<td>Octacosanoic</td>
<td>Montanic</td>
<td>28:0</td>
<td>452.9</td>
</tr>
<tr>
<td>Triacontanoic</td>
<td>Melissic</td>
<td>30:0</td>
<td>481</td>
</tr>
<tr>
<td>Dotriacontanoic</td>
<td>Lacceroic</td>
<td>32:0</td>
<td>495</td>
</tr>
<tr>
<td>Tritiacontanoic</td>
<td>Ceramicelissic(psyllic)</td>
<td>33:0</td>
<td>509.1</td>
</tr>
<tr>
<td>Tetratriacontanoic</td>
<td>Geddic</td>
<td>34:0</td>
<td>523.1</td>
</tr>
<tr>
<td>Pentatriacontanoic</td>
<td>Ceroplastic</td>
<td>35:0</td>
<td></td>
</tr>
</tbody>
</table>

Fatty Acids Nomenclature: <number of carbon atoms>: <number of double bonds>

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1.2 History of Fatty Acids Analysis

In spite of the absence of sophisticated analytical instrumentation, investigators prior to 1935 made considerable contributions to our knowledge of fatty acid compositions of natural lipids (http:www.cyberlipid.org). Since the first works of Chevreul, M.E., and for about a century, chemists isolated lipids using only their solubility properties in different solvents. Chevreul, M.E. investigated the chemical nature of a sample of soap while he was working in the Vauquelin's laboratory as a preparator. During his academic career he published a long series of papers, on the saponification of fatty acid esters. By diluting soap from pig fat in water, he found that a portion of the fat dissolved and that a fraction of these materials deposited as small pearly crystals. Then this deposited substance was decomposed using acid, which resulted in a solid fatty substance with acidic properties in contrast to original neutral fat. Then “new” compound was named as “margarin” and later “margaric acid” (Chevreul, 1813). In his second paper he reported the presence of “glycerine” and concluded that fat is a combination of a glycerol and a carboxylic acid (Chevreul, 1815). His later work was mainly devoted to the saponification process. Chevreul, M.E. summarized all his research on fats in a monograph published in 1823. This monograph can be considered as the first book on lipochemistry. At this time chemical investigation techniques were poorly developed, therefore, Chevreul, M.E. may be considered not only as the father of lipochemistry, and also of the whole field organic chemistry.

Several techniques including distillation, crystallization and chromatographic that have been marked as more efficient methods for analysis of fatty acids. Once laboratory vacuum distillation was introduced, this technique was reported to be used to distill fatty
acids, esters etc. Separation of pure palmitic acid was carried out by high vacuum distillation in 1903 (Krafft, 1903). In 1906 (Haller et al.) reported the fractional distillation of methyl esters derived from coconut oil, without any quantification. In the same year 9-icosaenoic acid was isolated by fractional distillation of methyl esters from cod liver oil (Bull, 1906). From analytical data, obtained by fractional distillation components, the composition of coconut oil (Elsdon, 1913) and the composition of palm kernel oil (Elsdon, 1914) were calculated. Jamieson G.S. and Baughman, W.F. reported analytical fractionation of methyl esters to determine the composition of fatty acids in peanuts (Jamieson, G.S.; Baughman, W.F., 1921) and sunflowers (Jamieson, 1922). The fatty acid composition of soybean oil was also reported by this method (Baughman, 1922). Brown applied the same technique for beef brain lipids (Brown, 1930). Later, spinning-band columns were used to fractionate the same range of fatty acids chain lengths. This method was applied to isolate and to identify 32 compounds from wool wax (Weitkamp, 1945).

The first chromatographic separations of fatty acids were concerned with short chain acids (Smith, 1942; Elsden, 1946). Boldingh first used reversed polarity partition chromatography to separate fatty acids (Boldingh, 1948). Thereafter, even numbered C6-C12 acids were separated using this same technique (Wittenberg, 1957). Then the invention of gas liquid chromatography revolutionized the analysis of fatty acids. Since the development of gas chromatography this technique has become the most widely used technique for analysis of fatty acids (Christie, 1989). In 1952 James and co workers published a description of the separation of C1-C12 carbon chain length free fatty acids (James, 1952). Four years later, they discovered that methylating fatty acids before gas
chromatography, could enhance their volatility, and allow separation below 250 °C (James, 1956). At this time it was not possible to analyze complex mixtures, since it was not possible to apply higher temperatures to the detector of the instrument. When a liquid polyester phase was introduced as stationary phase, saturated and unsaturated fatty acids were conveniently separated (Orr, 1958). In 1963, the use of capillary columns with very thin films, of various polar phases, significantly improved the analysis of fatty acids (Horning, 1963). Free fatty acids with medium or long carbon chains, can be extracted from aqueous media using a small C_{18} bonded phase column. This solid phase adsorption method was used to isolate fatty acids ethyl esters from alcoholic beverages (Battistuta, et al, 1994). An alternative method of saponification using microwaves was introduced in 1998. In this method a closed reactor containing the lipid sample and ethanolic KOH solution was irradiated for 2-3 minutes time in a microwave oven (Pinerio, et al, 1998). When a small amount of sample is available, saponification with ethanolic KOH was unsuitable, so a basic solution of 1M tetramethylammonium hydroxide (TMAH) was found as an excellent reagent for hydrolysis (Woo, 1999). Gas chromatography coupled to mass spectrometry following headspace solid-phase micro extraction was used to determine C_{2}-C_{7} free fatty acids in raw sewage, and this approach was found to be a highly reproducible and a sensitive method (Abalos, 2000).

1.3 Fatty Acids as Biomarkers

Molecular biomarkers can provide insight into the history of a sample; they can provide information on past events and also about the future conditions, as the appearance of certain compounds can be interpreted as early warning signals of
environmental change. Usually biomarkers are easily recognized compounds, and that can be signatures of the environmental condition of a sample (Parrish, 2000). Biomarkers have been extensively studied in geochemistry, but now there is increasing interest for their application to ecology. Biomarker studies in environment samples greatly help with source identification of organic carbon in sediments (Volkman et al., 1998). Microbial biomarkers are indicators of microbial populations (Salomonova, 2003). Lipids and nucleic acids are very important constituents in investigating and characterizing organisms, because of their cellular abundance and chemical diversity in the microbial community (Drenovsky, 2004). Lipids occur in plants, animals and microorganisms, but in soil lipids mainly originate from plants and microorganisms. Anthropogenic sources of fatty acids, such as petrochemicals and incomplete combustion products of fossil fuel or coal dust, are also possible sources (Wiesenberg, 2004). Lipids are major cellular components and constitute of wide variety of structurally and functionally modified compounds (Drenovsky, 2004). In marine ecosystems most important lipid classes are triacylglycerols and phospholipids. These two components are biochemically related to each other, as they both possess a glycerol backbone to which 2 or 3 fatty acids are esterfied, and they also share a common precursor (Parrish, 2000). And triacylglycerols are very important biological energy storage substances (Fraser, 1989).

Bacteria, sponges and certain plants have the ability to synthesize a variety of structurally different fatty acids (Volkman, 1998; Wolff, 2001). Since many organisms have distinctive fatty acid profiles, fatty acids are of interest for characterizing the organisms in various environments (Moldovan, 2002). Understanding the diversity and abundance of fatty acids among living organisms is very useful to classify and determine
abundance and biological interactions between living organisms (Zhukova, 1999). For example, fatty acids with odd carbon chain lengths, such as 15 or 17 carbon atoms, and branched fatty acids are synthesized primarily from aerobic and anaerobic bacteria (Parkes and Taylor, 1983; Caudales and Wells, 1991; Harvey and Macko, 1997). C$_{24}$ and longer chain fatty acids are usually from higher plants (Pulchan, 2003). Some other examples of lipid biomarkers are shown in Table 1.2.

Variability in the distribution of lipid biomarkers, and their molecular isotopic compositions, in Altamaha estuarine sediments were analyzed by W. Shi et al in 2001. Results suggest that particulate organic matter from terrestrial plant and marine phytoplankton are differentially deposited in the sediments. Organic matter from terrestrial plant material is dominant at initial, upper estuary mixing zone while organic matter from marine sources was mainly deposited in more intensive mixing zone. Long chain fatty acids, which are mainly originated from vascular plants, were buried in sediments mainly in chemically bounded (saponification-released) form while compounds from marine algae remain in the sediments in free (so solvent-extractable) form. Further bacterial biomarkers are highly abundant, with high input of fresh organic material, and this implies close relationship between bacterial activity and fresh active organic matter (Shi et al, 2001).

By analyzing, abundance and distribution of fatty acids in hydrothermal vent sediments of the western Pacific Ocean, Yamanaka and Sakata (2004) suggest that concentration of fatty acids in vent-surrounding sediments were higher than those of non-vent sediments. This indicates the intense primary production and a large biomass at the active vents. Furthermore they observed that the composition of total fatty acids is rich in
bacterial biomarkers, especially monounsaturated fatty acids (Yamanaka and Sakata, 2004).

Table 1.2. Proposed lipid markers to represent taxonomic groups of microorganisms (Green, 2000; Vestal, 1989; Salomonova, 2003)

<table>
<thead>
<tr>
<th>Lipid marker</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>i15:0, a15:0, 15:0, 16:1(\omega)5, i17:0, 17:0, 18:1(\omega)7</td>
<td>Most of bacteria</td>
</tr>
<tr>
<td>Branched PLFAs</td>
<td>Gram-positive bacteria</td>
</tr>
<tr>
<td>Monounsaturated PLFAs</td>
<td>Gram-negative bacteria</td>
</tr>
<tr>
<td>10Me 18:0</td>
<td>Actinomycetes</td>
</tr>
<tr>
<td>18:2(\omega)6c, 18:3(\omega)6c, 18:3(\omega)3c</td>
<td>Fungi</td>
</tr>
<tr>
<td>16:2(\omega)8c, 16:1(\omega)6c</td>
<td>Methanotrophs I</td>
</tr>
<tr>
<td>18:1(\omega)8c, 18:1(\omega)8t, 18:1(\omega)6c</td>
<td>Methanotrophs II</td>
</tr>
<tr>
<td>10Me 16:0, cy18:0, ((\omega)7,8)</td>
<td>Desulfobacter</td>
</tr>
<tr>
<td>i17:1(\omega)7c, i15:1(\omega)7c, i19:1(\omega)7c</td>
<td>Desulfovibrio</td>
</tr>
<tr>
<td>17:1(\omega)6, 15:1</td>
<td>Desulfobulbus</td>
</tr>
<tr>
<td>i17:1(\omega)5, 10Me 18:1(\omega)6, 11Me 18:1(\omega)6</td>
<td>Thiobacillus</td>
</tr>
<tr>
<td>cy15:1</td>
<td>Clostridia</td>
</tr>
<tr>
<td>18:2(\omega)6</td>
<td>Cyanophytes</td>
</tr>
<tr>
<td>16:1(\omega)3t, 20:5(\omega)5, 20:5(\omega)3</td>
<td>Diatoms</td>
</tr>
<tr>
<td>16:1(\omega)13t, 18:3(\omega)3, 18:1(\omega)9</td>
<td>Green algae</td>
</tr>
<tr>
<td>18:1(\omega)9, 18:1(\omega)11, 18:3(\omega)3, 20:5(\omega)3, 26:0</td>
<td>Higher plants</td>
</tr>
<tr>
<td>20:2(\omega)6, 20:3(\omega)6, 20:4(\omega)6</td>
<td>Protozoa</td>
</tr>
</tbody>
</table>

Fatty Acids Nomenclature: <number of carbon atoms>: <number of double bonds>\(\omega\)<position of the double bond from the methyl end of the molecule. For example, 16:1\(\omega\)7 denotes 9-hexadecanoic acid, which has 16 carbon atoms with one double bond at 7\(^{th}\) position from the methyl group.

* c-cis, t-trans
* 10 Me- Methyl group at C10
* Prefixes i – iso, a-anteiso, cy –cyclopropyl fatty acids
* PLFA-phospholipid fatty acids
1.4 Analysis of Fatty Acids in Soil and Sediments

The difficulty of extracting representative lipid materials from soils, and in availability of adequate techniques to completely characterize lipid components in soils, still limits the information available from the chemical composition of soil lipids (Wiesenberg, 2004). A significant amount of research has been devoted to the development of methods for analysis of fatty acids, and recently these methods have advanced considerably. As new methods are introduced, conventional methods such as gravimetric, volumetric, spectrophotometric and colorimetric methods are becoming obsolete (Silva, 2004). Because of the high resolution available in the capillary gas chromatography column, determination of free fatty acids is most often carried out using gas chromatography (Christie, 1989). However for gas chromatographic analysis, compounds must have relatively high volatility, and this requirement can be achieved by analytical derivatization (Christie, 1993). Esterification or conversion of carboxylic acid group into an ester is the most common derivatization reaction used in analysis of fatty acids by gas chromatography, and Knapp summarized basic techniques of derivatization in 1979. Esterification enhances gas chromatographic properties, which improves sensitivity. In order to achieve an optimum conversion of fatty acids to esters, proper selection of a derivatization technique is very important (Rosenfeld, 2002). The derivatization of fatty acids remains a subject of ongoing interest in spite of its extensive application (Park, 2001; Ostrowska, 2000). Esterification is the most common technique to derivatize fatty acids. The resulting esters have higher volatility, good peak morphology and good chromatographic separation (Rosenfeld, 2002). The most commonly used derivatives are alkyl ester derivatives, such as methyl, ethyl, propyl etc.
(Brondz, 2002). However, methyl esters are most common. The wide ranges of available methylation and transesterification procedures, reagents, suitable equipment, and well-developed techniques are some of the reasons (Brondz, 2002) for the popularity of these derivatives. Methyl esters can be prepared by alkaline, acid or alkaline and acid catalysis (Silva, 2004).

1.4.1 Conventional Acid Catalyzed Derivatization

Esterification of bound fatty acids in the presence of alkaline catalysts, such as sodium methoxide, requires only short reaction time and often can be completed at room temperature (Silva, 2004; Drenovsky, 2004). However, the presence of acid catalysts can convert both free and bound fatty acids to esters (Silva, 2004; Drenovsky, 2004). HCl/MeOH, HCl/EtOH or H₂SO₄/isoPrOH can be used to derivatize saturated free fatty acids (Brondz, 2002). About 50 years ago, transesterification of fatty acids with HCl/MeOH was described by Stoffel et al (Stoffel et al., 1959). Boron derivatives such as BF₃ or BCl₃ are Lewis acid catalysts that can be used with MeOH, for converting fatty acids to methyl esters (Metcalfe, 1961; Minikin, 1967). BF₃/MeOH was reported to be more successful than that of milder BCl₃/MeOH (Morrison, 1964).

1.4.2 Pyrolysis Gas Chromatography Mass Spectrometry (Py-GC/MS)

Without any sample pretreatment, pyrolytic techniques followed by GC/MS analysis can provide detailed molecular level structural information on complex organic materials, and require only small amount of samples (Del Rio, 1998). However, it was found that carboxylic acids present in pyrolyzed samples, were often decarboxylated during the process. In addition, other significant structural modifications occurred as a result of thermal reactions, and which may lead to misinterpretation of the structure (Del Rio,
1998: Lehtonen, 2000). Pyrolysis in the presence of tetramethyl ammonium hydroxide (TMAH) can minimize some of these complications (Challinor, 1989). When a sample is pyrolyzed in the presence of TMAH, carboxylic and hydroxyl groups are methylated and form corresponding methyl esters or ethers. Volatile esters can be rapidly removed from the sample, thereby minimizing further structural changes. This will prevent decarboxylation and also increase volatility of the fatty acids that are liberated making them amendable to GC analysis (Del Rio, et al, 1998). Rapid formation of volatile derivatives also reduces pyrolysis time. Pyrolysis methods have been successfully used for structural characterization of lignin (Challinor, 1995; Clifford; et al, 1995). With TMAH, the overall degradation mechanism is termed chemopyrolysis rather than pyrolysis, as strongly basic TMAH not only acts as a methylating agent but also cleaves ester and ether bonds. Chemopyrolysis occurs at temperatures lower than traditional pyrolysis thereby reducing structural changes (Lehtonen, et al, 2000). The technique thermochemopyrolysis in the presence of TMAH has been introduced as an efficient method to analyze variety of natural macromolecules (Chefetz, et al, 2000). This method requires minimal amount of sample preparation, and is overall a very straightforward method.

Robb and Westbrook (1963) first showed that TMAH salt of carboxylic acids could be converted to corresponding methyl esters at a heated (330°-365°C) injection port of a GC. TMAH salts were prepared by titration or by ion exchange method and results obtained were only qualitative (Robb and Westbrook, 1963). The relationship between fatty acid compositions of zooplankton species was analyzed by thermochemolysis with TMAH (Ishida et al., 1998). McKinney et al. outlined a procedure for characterization of
lignin at a sub-pyrolysis temperature of 300°C, with TMAH, in a sealed glass ampoules (McKinney et al., 1995). A set of biogeomacromolecules (such as fresh and degraded gymnosperm and angiosperm woods, proteins, polyesters, humic substances and dissolved organic matter in natural water) was examined by off-line TMAH thermochemolysis. This methylates carboxylic acids and hydroxyl groups, and also induces cleavage of β–o-4 ether bonds in lignin (del Rio; et al., 1998). Their analysis focused mostly on qualitative aspects, but for lignin some quantitative results were reported. To discriminate free and bound fatty acids in wood pulps, TMAH and tetramethyl ammonium acetate (TMAAC) were used in chemopyrolysis (Hardell and Nilvebrant, 1999). The chemical nature and structural composition of some lake aquatic humic matter samples were elucidated using off-line TMAH chemopyrolysis by Lehtonen, et al., 2000. The major degradative products they obtained were methyl derivatives of phenols, alkylphenols, phenolic acids and aliphatic acids (Lehtonen, et al., 2000). Chefetz et al. studied structural changes of soil organic matter with soil depth also using off-line chemopyrolysis. And they concluded this technique is very useful to analyze non-extracted soil organic matter. Monomethoxyphenyl and dimethoxyphenyl were identified as the main lignin derived products in soil. In addition a large fraction of long-chain fatty acids were identified by this method (Chefetz et al., 2000). The TMAH chemopyrolysis method was successfully used to analyze marine sedimentary organic matter in a Newfoundland fjord, and they found out this method is a straightforward method to identify acidic or phenolic functional groups. The most abundant phenol in the near-shore sediments was identified as vanillic acid (Pulchan; et al, 2003). This technique
was also used for the characterization of residual lignin directly from softwood pulps (Ohra-aho et al., 2005).

1.5 Fatty Acids in Lake Mead Sediments

Hoover Dam, about the height of a 60-story skyscraper is a marvel of engineering, located in 60 miles southeast of Las Vegas (http://www.pbs.org; http://www.frommers.com). In the 1930s, when Hoover Dam was constructed on the Colorado River, it created America’s largest man-made reservoir, Lake Mead which is 110 miles long (http://www.pbs.org). The lake covers approximately 593 square kilometers (Nemr, 2004). This is one of the most important water resources in the west, and it provides water to Arizona, Nevada, California and northern Mexico (http://www.earthobservstory.nasa.gov). When full, the lake contains approximately 36 trillion liters or 9.3 trillion gallons (http://www.earthobservstory.nasa.gov). The Boulder, Virgin, Gregg Basins and Overton Arm are the four major basins in Lake Mead (http://www.earthobservstory.nasa.gov). Lake Mead provides recreational watercraft activities and domestic drinking and irrigation water for over 32 million people (http://www.waterontheweb.org/data/mead/). Water that flows to Lake Mead is primarily from the Colorado River (http://www.waterontheweb.org/data/mead/). The remaining three percent of the water is delivered by tributaries on the northern side of the lake and from Las Vegas Wash on the northwest side of the lake (http://www.waterontheweb.org/data/mead/). Las Vegas Wash carries the valley’s excess water through artificial wetlands on its way to Lake Mead (http://www.lvwash.org). Urban runoff contributes a significant amount of volume to the Wash, and may contain
various pollutants such as bacteria, oil, grease, pesticides and nutrients from fertilizers, since water in the wash has collected from streets and flood channels (http://www.lvwash.org/wash/). Urban runoff is generally attributed to excess water used in the urban landscape, draining of swimming pools to streets, the washing of vehicles and driveways (http://www.lvwash.org/wash). Sediments are picked up by Las Vegas Wash along its way to Lake Mead and deposited in Las Vegas Bay of Lake Mead (http://www.lvwash.org/important/). Suspended particles are generally a combination of inorganic and organic matter (http://www.lvwash.org/important/). In earlier studies, Lake Mead sediments have been analyzed for lignin and organic matter (Nemr, 2004). In this study, sediments samples with at several sediment subsurface depths and from several locations were analyzed for fatty acids. Since fatty acids are an important set of biomarkers, analyzing fatty acids present in Lake Mead sediments may provide important information on source and origin of organic matter (Steinberg; et al., 2003). Several cores were obtained from Las Vegas Bay and Boulder Basin of Lake Mead by vibracore (VC) or gravitycore (GC) methods (Nemr, 2004). Core locations are shown on Figure 1.4.
Chemopyrolysis followed by gas chromatography, mass spectrometry was mainly used to analyze fatty acids in sediments. This method involves heating organic material in the presence of TMAH. This procedure results in decomposition of organic matter into simpler products and methylation of these components so that these compounds are amendable to GC. The presence of fatty acids in sediments cores was indicated by the occurrence of ion fragment m/z 74 in a mass spectrum. Therefore in this study ion chromatograms corresponding to m/z 74 were used to identify fatty acids qualitatively as
well as quantitatively in various samples. For unsaturated fatty acids, the \((m/z)\) 74 ion is weak, so \(m/z\) 74 was not used exclusively. Retention time and comparison to standard library spectra were employed.

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CHAPTER 2

EXPERIMENTAL

2.1 Sampling

Sediment cores were collected from Las Vegas Bay and Boulder Basin, of Lake Mead, using a vibrating corer system with aluminum core liner, and gravity corer system with polycarbonate core liner as described in Nemr, 2004. Pahokee peat was obtained from International Humic Substances Society (IHSS). All samples were stored in the frozen state until use.

2.2 Reagents and Analytical Standards

All fatty acid methyl esters standards were purchased from Nu-Chek-Prep, Inc., MN, USA. Triglyceride mix #2 was purchased from Alltech-Applied Science Labs, which consists of trioctanoin, tridecanoin, tridodecanoin, tritetradecanoin and trihexadecanoin. Percentages by weight of each triglyceride were 20.08%, 19.66%, 20.84%, 19.67% and 19.73% respectively. Tetramethylammoniumhydroxide (TMAH) (25% in H$_2$O) was obtained from J.T.Baker Inc., and was used for offline analysis. TMAH (10% in MeOH) was obtained from Aldrich Chemicals Company and was used for online analysis. Individual fatty acid and triacylglycerol standard solutions were prepared in dichloromethane. Dilutions were also carried out using dichloromethane. All solutions were stored in refrigerator until use.
2.3 Analytical Derivatization

Determination of fatty acids requires analytical derivatization to form more volatile ester derivatives of the molecules, rendering them more amendable to gas chromatographic analysis. In this thesis, conventional acid catalyzed derivatization methods as well as both online and offline tetramethyl ammonium hydroxide (TMAH) chemopyrolysis techniques were compared. TMAH chemopyrolysis involved heating organic material in the presence of TMAH.

Conventional acid catalyzed derivatization involves refluxing soil/sediments sample in an acidic or basic medium. Refluxing in the presence of an acidic or basic catalyst, releases free fatty acids. In addition fatty acids bound to the insoluble macromolecular fraction via ester bonds may be released (Silva; et al, 2004). In our work aimed at enhancing the extraction and detection of these extracted fatty acids for gas chromatographic analysis and to compare conventional methods to chemopyrolysis. Esterification was done using both Lewis acid (BF₃) and Bronsted acid (HCl) catalysts with MeOH.

2.3.1 Online TMAH Chemopyrolysis

The soil/sediments sample (5-10 mg) was inserted between quartz wool plugs, into an open quartz tube (2mmx20mm), which was previously cleaned by heating to 1000°C. The sample was saturated with 10ul of 10% w/w TMAH (or TMAAC), and placed into a heated pyrolysis interface. The current was passed through the platinum wire surrounding the sample, which was then rapidly heated to 500°C for 20 seconds. This temperature was found as the optimum temperature, by comparing ester yields obtained from two other temperatures (400°C and 600°C). The resulting methylated fatty acids are swept by
a carrier gas (helium) into the GC column. Analyses were performed using the Pyroprobe 2000 with a model 1500 interface connected to a Varian 3400 GC and Saturn III Ion trap Mass Spectrometer.

Figure 2.1. Online chemopyrolysis (A) The Pyroprobe with the platinum wire (Steinberg, 2003). (B) The schematic diagram showing chemopyrolysis method
2.3.2 Offline TMAH Chemopyrolysis

Approximately 100 mg of sediment/soil sample was placed into a glass ampoule (obtained from Wheaton Company) and 25ul of 25% w/w TMAH in H₂O was added to ensure excess reagent for reaction. Then 100ul of distilled water was added to the ampoule. The sample was homogenized, dried in a vacuum dessicator, and then sealed under vacuum. Individual ampoules, wrapped with aluminum foil, and heated at 250°C for 30 minutes (30-minute time was found as the optimum time by comparing data obtained from different heating times). The resulting components were extracted using dichloromethane and filtered through a plug of silinized glass wool. The residue was dissolved in 50ul of dichloromethane, after evaporating the extraction solvent under nitrogen gas. Two micro liters of this sample was analyzed by GC/MS.

Figure 2.2. Flow chart showing the processes involve in offline TMAH chemopyrolysis
2.3.3 Methylation with BF$_3$/MeOH

Approximately 100 mg of dried sediment/soil sample was placed in a glass screw cap test tube with Teflon lined cap (obtained from VWR International). Samples were subjected to saponification with 2 ml of 6% KOH in MeOH (MeOH: H$_2$O ratio 9:1 v/v) heating at 70 °C for 6 hours in an aluminum heating block. The supernatant was separated by centrifugation and acidified to pH 2 using HCl. The resulting free fatty acids were extracted with 2 ml of ethyl ether. The ether was evaporated under nitrogen gas. The residue was heated at 70°C for 1 hour with 2 ml of 14% w/v BF$_3$/MeOH, to convert acids to their corresponding methylated derivatives. The solution was diluted with 5 ml of 5N NaCl solution, and extracted using dichloromethane. The solvent was evaporated to near dryness under nitrogen gas. The residue was dissolved in 200ul of dichloromethane, and 2ul of this sample was injected into the GC/MS.

Figure 2.3 BF$_3$.MeOH acid catalyzed derivatization procedure

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2.3.4. Methylation with HCl/MeOH

Soil/sediment samples were treated using the method described by Silva; et al., 2004, which was used to analyze fatty acids in potato crisps. Dried soil/sediment sample (100 mg) was weighed into a glass test tube with Teflon lined screw cap. Methanolic HCl 3 ml of 5% (This solution was prepared by adding required amount of acetyl chloride dropwise into a cooled methanol solution in an ice-water bath) was added and test tube was closed with a nitrogen atmosphere. The samples were heated in a heating block for 2 hours at 70°C, afterwards 4 ml of 6% K$_2$CO$_3$ was added, and the sample was extracted three times with ethyl ether. The combined organic layers were dried using anhydrous Na$_2$SO$_4$ and were then evaporated to dryness under nitrogen gas. The resulting residue was dissolved in 400ul dichloromethane, and 2ul of this sample was used for GC/MS analysis.

Figure 2.4 HCl/MeOH acid catalyzed derivatization method
2.4 Gas Chromatography

GC/MS analysis was performed using a Varian Saturn III GC/MS system. The gas chromatograph was a Varian 3400, and it was fitted with a split/splitless injector and fused silica column (Supelco EC-5, 0.25mm diameter, 25micron film). The GC was connected to Varian Saturn 3 Ion-Trap MS, through a heated transfer oven. Helium was used as carrier gas, and was maintained at 10 PSI throughout the analysis.

Temperature programs for both online and offline GC/MS analysis were as follows; initial temperature held for 6 minutes at 40°C and then increased to 250°C with 10°C/minute. After increasing to 280°C with 20°C/minute held for 15 minutes at 280°C. For online chemopyrolysis, a CDS Pyroprobe 2000 was used to pyrolyzed the samples. The sample was packed into 2 mm diameter quartz tube, and placed into a heated CDS model 5000 programmable GC interface. A current was passed through the platinum wire surrounding the tube and which was then rapidly heated to 500°C for 20 seconds. Helium carrier gas swept decomposition products into the GC column. The products were analyzed with a MS detector (Saturn Varian III Ion trap MS).

Isothermal products obtained by both online and offline techniques were identified by comparing collected mass spectra with the National Institute Standard Technology 2002 (NIST 2002) mass spectral library.

2.5. References


CHAPTER 3

RESULTS AND DISCUSSION

3.1 Analysis of Fatty Acids by GC/MS

Gas chromatography (GC) followed by mass spectrometry (MS) is a widely used analytical technique to analyze fatty acids. For GC analysis compounds must have relatively high volatility in order to have a reasonable elution time in the chromatogram. Derivatizing fatty acids into corresponding esters can increase volatility of fatty acids. Methyl esters are the most commonly used derivatives of carboxylic acids for analysis because of the wide range of available reagents for both esterification and transesterification of fatty acids.

\[
\begin{align*}
\text{CH}_3\text{O} & \text{C} \equiv \text{CH} \equiv \text{R} \\
\text{OH} & \text{CH}_3\text{O} \text{C} \equiv \text{CH} \equiv \text{R} \\
\text{m/z 74 ion} & \text{ CH}_2\equiv\text{CH} \equiv \text{R}
\end{align*}
\]

Figure 3.1. McLafferty rearrangement for formation of m/z 74 ion (Solomons, 1996).

Mass spectrometry provides structural information about components present in a sample. Mass fragment m/z 74, which results from a McLafferty rearrangement, is
characteristic for fatty acid methyl esters (Figure 3.1). Therefore the occurrence of m/z 74 in the ion chromatogram indicates not only the presence of fatty acids, but its intensity also is proportional to the quantity of fatty acids present in the sample.

Figure 3.2. Total ion current and m/z 74-ion chromatogram for off-line Py/GC/MS analysis of Pahokee peat sample.

Figure 3.2 shows total ion current and ion chromatogram corresponding to mass fragment 74, for chemopyrolysis GC/MS analysis of a Pahokee peat sample. The lengths of the carbon chain of fatty acids were identified by the masses of the parent ion corresponding to the particular peak, and by retention time. Retention times were determined by standard compound injections. Table 3.1 indicates the masses corresponding to fatty acids with various carbon chain lengths. Quantification of these
fatty acids were carried out using the peak areas obtained by integrating corresponding peaks, using the Varian Saturn software mass spectra interpretation routines.

Table 3.1. Masses corresponding to fatty acid methyl esters with various carbon chain lengths.

<table>
<thead>
<tr>
<th>Carbon Chain Length</th>
<th>Molecular weight of parent ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>214</td>
</tr>
<tr>
<td>14</td>
<td>242</td>
</tr>
<tr>
<td>15</td>
<td>256</td>
</tr>
<tr>
<td>16</td>
<td>270</td>
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<td>17</td>
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<td>27</td>
<td>424</td>
</tr>
<tr>
<td>28</td>
<td>438</td>
</tr>
</tbody>
</table>
3.2 Conventional acid catalyzed derivatization

Esterification in the presence of an acid catalyst was used to derivatize fatty acids. Even though esterification can be carried out under basic catalysis conditions, derivatization of total fatty acids (both free and bound fatty acids) is not possible in basic medium (Silva, 2004). In this work HCl as well as BF$_3$ were used with methanol as derivatizing agents. The resulting fatty acid methyl esters are amendable to gas chromatographic analysis, as they have higher volatility compared to corresponding fatty acids. The mechanisms of these fatty acids derivatizations are indicated in Figure 3.3.
Figure 3.3 Mechanisms of acid catalyzed derivatization of carboxylic acids (a) with BF₃/MeOH (b) with HCl/MeOH (Solomons, 1996)
The Pahokee peat sample was analyzed by GC/MS after derivatizing with these two acid catalyzed methods. Peak area per gram of sample versus length of the carbon chain of the fatty acids obtained by both methods are shown in Figure 3.4.

![Acid Catalyzed Derivatization](image)

**Figure 3.4** Comparison of BF₃/MeOH and HCl/MeOH results.

Even though both methods have given high peak areas per gram of sample, the HCl/MeOH derivatization method gave higher yields than that of BF₃/MeOH derivatization method, for long-chain fatty acids. Slight differences were observed at lower molecular weights, and more pronounced differences at higher molecular weights. The reason for this difference can be attributed to the differences in acidities of HCl and BF₃. Strong acid HCl may have higher chance of protonating the carboxyl acid group in fatty acids, which ultimately leads to formation of the corresponding methyl esters.
Further HCl may be strong enough to break down fatty acids associations, such as ester bonds present in soil/sediment samples.

3.3. Offline and Online Chemopyrolysis

Chemopyrolysis involves heating organic material in the presence of quaternary ammonium salts. This process results in decomposition of organic matter into the simpler components, and methylation of these simpler components. In online chemopyrolysis thermal decomposition products and methylated components are swept directly by a helium carrier gas into the GC column, where they are subsequently detected by MS.

Online chemopyrolysis was performed at three different temperatures (400°C, 500°C and 600°C) in the presence of TMAH, in order to find the optimum temperature for analysis of our samples. 500°C was found as the optimum temperature for analysis, because of higher yield of high molecular weight acids. However there is no significant difference between the various results (Figure 3.5).
To discriminate between free and bound fatty acids ratios TMAH and tetramethyl ammonium acetate (TMAAC) have been used. The theory behind this discrimination is differences in nucleophilicities of hydroxide and acetate groups (Hardell and Nilvebrant, 1999). The hypothesis is that acetate and a much weaker nucleophile will not liberate fatty acids from triglycerides and other esters. Proposed mechanisms of chemopyrolysis of carboxylic acids with TMAH and TMAAC are shown in Figure 3.8. However, before applying this technique to our samples; experiments were carried out both with TMAH and TMAAC with the standard triglyceride mix where only bound fatty acids are available. Unexpected results, which show significant methyl ester formation, with these standards, were obtained as shown in Figure 3.6. We were able to observe methyl ester production with TMAAC, which we did not expect as standard triglyceride sample does not contain free fatty acids and this leads to a different conclusion from previous
investigators. Although TMAAC is a weaker nucleophile than the hydroxide it can cleave some of the ester bonds present in the sample and methylate resulting carboxylic acids at higher temperature prevailing in the pyrolysis filament. However detection limits are significantly different.

![Graph](image)

**Figure 3.6.** Variation of Peak area with number of carbon in the alkyl chain of triacylglycerol in the presence of TMAH and TMAAC for On-Line Chemopyrolysis at 500°C. 

Offline chemopyrolysis was performed in a furnace at 250°C in the presence of TMAH and resulting methylated components were analyzed by GC/MS after extraction of the sample with dichloromethane. Results obtained by analyzing a peat sample for fatty acids with these two methods (online and offline TMAH) are shown in Figure 3.7.
Figure 3.7 Comparison of Online and Offline TMAH Chemopyrolysis by normalizing results to C16 results of Pahokee Peat.

Our results indicate that the offline method provides lower yield than the online method. Furthermore, higher carbon chain length fatty acids were often not detectable with the on-line method. The lower detection limit may be due to insufficient amount of TMAH, or not enough time in the furnace under pyrolysis conditions. However when we increased the amount of TMAH for the reaction, sealed ampoules exploded in the furnace, as TMAH can deteriorate the glass causing, the ampoule to burst. When we increased the reaction time, in the furnace, we did not observe better yields. Further, unlike in the online method, the loss of components during the extraction and the concentration procedures are unavoidable. When we compare the time required per analysis, as well as the amount of sample required per analysis, the online chemopyrolysis technique provides rapid and more reliable method for analysis of fatty acids when compared to offline chemopyrolysis.
Figure 3.8 Proposed mechanisms of chemopyrolysis of carboxylic acids (a) with TMAH (b) with TMAAC

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3.4 Comparison of chemopyrolysis with conventional acid catalyzed derivatization

Since analysis of fatty acids by GC/MS is dependent on the successful derivatization, in this work chemopyrolysis and conventional acid catalyzed derivatization methods were compared (Figure 3.9).

![Comparison of Derivatization Methods](image)

**Figure 3.9** Comparison of chemopyrolysis with conventional acid catalyzed derivation results.

Overall results show significant differences in detection limits of fatty acids and also distribution is defined by method. The detection limit of conventional acid catalyzed methods is higher than that of chemopyrolysis. However when we compare the analysis time and quantity of the sample required for analysis, online chemopyrolysis requires only 5-10 mg of sample and requires only 45 minutes per entire analysis. Therefore when we have to analyze large number of samples, such as environmental samples, the online chemopyrolysis may be a viable screening method.

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3.5 Fatty acids in Lake Mead sediments

The above methods can be used to investigate the fatty acids present in Lake Mead sediments and examine the variation of fatty acids distribution with depth in order to understand sediments dynamics in the lake. Variation of the amount of fatty acids with carbon chain length 14, 16 and 18 for core VC1 which was taken from near to the Las Vegas Wash (Figure 3.10) is shown in Figure 3.11.

Figure 3.10 Locations of Cores in Las Vegas Bay and Boulder Basin of Lake Mead

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Figure 3.11 Peak Area per gram of sediments sample with depth for fatty acids in core VC 01 with alkyl chain length (A) Carbon-14 (B) Carbon-16, and (C) Carbon-18 for off-line chemopyrolysis.

Obtained results (Figure 3.12) for same analysis for core VC-9 shows irregular variation of fatty acids with depth.
Figure 3.12 Peak Area per gram of sediments sample with depth for fatty acids in core VC 09 with alkyl chain length (A) Carbon-14 (B) Carbon-16, and (C) Carbon-18 for off-line chemopyrolysis.

3.6 References


CHAPTER 4

CONCLUSIONS

Analysis of fatty acids by Gas Chromatography Mass Spectrometry techniques depends on the successful derivatization of fatty acids, high-resolution chromatography and sensitive detection. When conventional acid catalyzed derivatization techniques and chemopyrolysis technique were compared, acid catalyzed methods have given higher yields than chemopyrolysis. However when all methods are compared, on-line chemopyrolysis is a rapid screening method to analyze fatty acids in environmental samples such as sediments. Off-line chemopyrolysis gives lower detection limits for analysis of fatty acids.

Our results obtained for core VC 01 in Lake Mead sediments show the highest abundance of fatty acids in depth 20-30 cm. However same analysis for core VC 09 shows irregular variation of fatty acids with depth and overall it increases with depth.
Figure A-1: The chromatogram obtained for Pahokee peat by online TMAH chemopyrolysis at 400°C (a): scan from 1350-1600 and (b): scan from 1600 to 2000
Figure A-2: The chromatogram obtained for Pahokee peat by online TMAH chemopyrolysis at 500°C (a): scan from 1350-1550 and (b) scan from 1600 to 2000.
Figure A-3: The chromatogram obtained for Pahokee peat by online TMAH chemopyrolysis at 600°C (a): scan from 1350-1550 and (b) scan from 1600 to 2000.
Figure A-4: The chromatogram obtained for Pahokee peat by online TMAH chemopyrolysis at 500°C (a): scan from 1350-1550 and (b) scan from 1600 to 2000

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Figure A-5: The chromatogram obtained for Pahokee peat by off-line TMAH chemopyrolysis

Figure A-6: The chromatogram obtained for Pahokee peat by BF$_3$/MeOH scan from 1100-1700

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Figure A-7: The chromatogram obtained for Pahokee peat by BF$_3$/MeOH scan from 1700-2200

Figure A-8: The chromatogram obtained for Pahokee peat by HCl/MeOH scan from 1250-1700

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Figure A-9: The chromatogram obtained for Pahokee peat by HCl/MeOH scan from 1700-2200

Figure A-10: The chromatogram obtained for triacylglycerol mix by on-line TMAH chemopyrolysis

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Figure A-11: The chromatogram obtained for triacylglycerol mix by on-line TMAAC chemopyrolysis

Figure A-12: The chromatogram obtained for core VC 1 (depth 24-26cm) by off-line TMAH chemopyrolysis

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Figure A-13: The chromatogram obtained for core VC 1 by off-line TMAH chemopyrolysis (a): depth 34-36cm and (b): depth 76-78cm
Figure A-14: The chromatogram obtained for core VC 1 (depth 138-140cm) by off-line TMAH chemopyrolysis

Figure A-15: The chromatogram obtained for core VC 9 (depth 15-16cm) by off-line TMAH chemopyrolysis
Figure A-16: The chromatogram obtained for core VC 9 by off-line TMAH chemopyrolysis (a): depth 73-74cm and (b): depth 101-102cm
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