The Characterisation of Antarctic Dissolved Organic Matter: Study of chemical structure of fulvic acid

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

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Sydney, Australia

March 2016

Dedication

"Science is not only a disciple of reason but, also, one of romance and passion."

- Stephen Hawking

Dedicated to my husband, Vahid, for his company and support during this difficult time in our lives."

Declaration of Originality

I declare that the work described in this thesis has been carried out by me and has not been submitted, in part or in full, to any other university or institution for any other degree.

Sahar Farzadnia

March 2016

Acknowledgements

This thesis has been written under the supervision of Dr. Christopher McRae to whom goes my most sincere gratitude for his patience, constant guidance and the science he taught me throughout the whole period of my PhD.

My warmest thanks go to all my colleagues and friends at Macquarie University and in particular to Zahra Khamseh Safa from Dr. McRae's research group for her warm friendship and continuous support. I am also very grateful to Mohammad Hashemi for his encouragement and many valuable scientific discussions and inputs through this project. I would like to thank my friends Raha Moossavi, Shabnam Tarahi and Dr. Nima Sayyadi for their support and friendship.

Also, I would like to acknowledge Dr.Wendy Loa who helped me to run my NMR samples. Her valuable advice and technical support are highly appreciated.

A special thank you goes to my parents and family without whom none of this work would have been possible. Most importantly, I would like to thank my husband and my son for their love, support and understanding.

Finally, I am greateful to dedicated faculty members, technicians and personel in CBMS and Macquarie University for making me always feel home and part of their group.

Abstract

Study of the chemical structure of dissolved organic matter (DOM) has always been of great interest due to its contribution to global carbon cycle and its recognition as a source marker. Fulvic acids are a complicated mixture of organic compounds accounting for about half of Natural Organic Matter (NOM) in fresh water and 15-20 % of the NOM in marine waters. Fulvic acid composition and structure varies depending on the geographic locations and sources of inputs, but it is generally believed that it consists of weak aliphatic and aromatic organic acids soluble in water at all pH conditions. Chemical characterisation of fulvic material from the Antarctic with its cold climate and absence of higher order plants has the advantage of simpler mixture contribution and pure microbial input to the DOM. This relative simplicity helps facilitate the data interpretation and speculating origin and formation pathways of Antarctic fulvic acids. In this study, we have comprehensively examined fulvic acids from two different areas in Antarctica. Pony Lake fulvic acid (PLFA) from McMurdo Sound area, Western Antarctica as IHSS Standard Reference fulvic acid and three other fulvic acids from Vestfold Hills, Eastern Antarctica were chosen as samples. For the first time these fulvic acids were analysed using a range of derivatisation methods coupled to gas chromatography/mass spectrometry. These methods complemented each other to demonstrate the broad range of components present in these complex fulvic acids. Our findings indicated the presence of various chemical components (apart from carbohydrates and sugars); many of which have not been previously reported in fulvic acids; they mainly include aliphatic linear and branched carboxylic acids, fatty acids (saturated and unsaturated), heterocyclic nitrogen-containing compounds, aromatics and terpenoids. Our results are the first report of the presence of unbroken tricyclic terpenoids among the products of three out of four fulvic acids tabulated. It also highlighted the feasibility of characterisation of DOM using gas chromatography without the need for depolymerisation using hydrolysis or other harsh treatments. Our results also showed that using a fractionation step prior to gas

chromatography can assist with providing additional information regarding molecular-level analysis of Antarctic fulvic acid as an important aquatic microbial end-member DOM. Furthermore, fractionation clearly revealed the presence of micelle-like structure in Antarctic fulvic materials supporting the theory of supramolecular structures for Humic substances.

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Acronyms

FA	Fulvic Acid
HA	Humic Acid
HSs	Humic Substances
DOM	Dissolved Organic Matter
NOM	Natural Organic Matter
SOM	Soil Organic Matter
HMWDOM	High Molecular weight Dissolved Organic Matter
DOC	Dissolved Organic Carbon
SPE	Solid Phase Extraction
UCM	Unresolved Complex Mixture
GC	Gas Chromatography
MS	Mass Spectrometry
HPLC	High Performance Liquid Chromatography
HILIC	Hydrophilic Interaction Liquid Chromatography
LC/MS	Liquid Chromatography/Mass spectrometry
ESI	Electro-spray Ionisation
IR	Infrared
NMR	Nuclear Magnetic Resonance
XPS	X-ray Photoelectron Spectroscopy
SEC	Size Exclusion Chromatography
QqTOF-MS	Quadrupole Time-Of-Flight Mass Spectrometry
FT–ICR	Fourier Transform Ion Cyclotron Resonance

COSY	Correlation Spectroscopy
TOCSY	Total Correlation Spectroscopy
NOESY	Nuclear Overhauser Effect Spectroscopy
HSQC	Heteronuclear Single Quantum Coherence
HMBC	Heteronuclear Multiple Bond Connectivity
DOSY	Diffusion Ordered Spectroscopy
HETCOR	Heteronuclear Correlation Spectroscopy
MALS	Multi Angle Light Scattering
ELSD	Evaporative Light Scattering Detector
VPO	Vapour Pressure Osmometry
XPO	X ray photoelectron spectroscopy
SRFA	Suwannee River Fulvic Acid
PLFA	Pony Lake Fulvic Acid
PNFA	Pendant Lake Fulvic Acid
MLFA	Mossel Lake Fulvic Acid
OLFA	Organic Lake Fulvic Acid
DHAA	Dehydroabietic acid
AA	Amino acid
n-BS	n-Butylsilane
BSTFA	(N,O-bis(trimethylsilyl) trifluoroacetamide
TMSD	Trimethylsilyldiazomethane
$B(C_6F_5)_3$	Tris(pentafluorophenyl)borane
TMAH	Tetramethyl ammonium hydroxide
ECF	Ethyl chloroformate

DCM	Dichloromethane
THF	Tetrahydrofuran
DMF	Dimethyl formamide

Chapter 1
Introduction

1.1 Introduction to Dissolved Organic Matter (DOM)

DOM is generally defined as organic substances in aqueous solutions passing through a membrane filter with nominal pore size of 0.45 μm (Zsolnay, 2003;Akkanen et al., 2005;Provenzano et al., 2010)**;** the fraction of DOM which is retained by this filter is called Ultra filtered dissolved organic matter (UDOM) or high molecular weight dissolved organic matter (HMW DOM) (Zsolnay, 2003;Frimmel, 2009). Although this definition is widely accepted among geochemists, it does not necessarily reflect the dissolved state of organic matter in natural waters as some times small plankton may not be retained by the filters; for example, 22–38% of the bacterial biomass, if unaccompanied by other material, pass through the filter (Lee et al., 1995). The source of DOM can be from either anthropogenic activities, or produced naturally. Naturally produced DOM is known as Natural Organic Matter (NOM)

Natural Organic Matter (NOM) is composed of a heterogeneous mixture of organic material derived from allochthonous (such as plant and soil material) and autochthonous (such as algae and phytoplankton) sources (Hedges et al., 1997;Mostofa et al., 2013). Both low molecular weight compounds such as organic acids as well as supramolecular structure including fulvic and humic acids (known jointly as humic substances) are found in natural waters DOM. DOM composition derived from oceanic and fresh water ecosystems differ significantly due to different inputs as well as intrinsic differences in the nature of these environments in terms of biota and physiochemical properties (Hedges, 1997).

Allochthonous DOM formed in freshwater and coastal seawater are dominated by humic substances (fulvic and humic acids) of terrestrial origin (Hedges, 1997;Mostofa, 2009 a), that are produced from the decomposition of vascular plants, root exudates and animal debris in soil. On the other hand, phytoplankton, including photosynthetic algae and bacteria are the primary source of dissolved organic matter fraction in lakes and oceans (autochthonous DOM) (Parlanti, 2000;Amado, 2007). Major biochemicals including carbohydrates, amino acids, amino sugars, proteins, lipids, organic acids and sterols also greatly contribute to NOM composition (Zhang, 2009)

1.1.1 Marine Dissolved Organic Matter

Marine dissolved organic matter refers to the complex mixture of molecules of varied origins that is found in seawater. Although the concentrations of the marine DOM in the oceans is quite low measured around 2 μM- 46 μM in terms of DOC (Hansell and Carlson, 2001), it comprises the main pool of non-living organic matter present in seawater. Unfortunately, this valuable source of organic matter is still one of the least understood organic reservoirs on the earth because their source and formation pathway is very ambiguous.

It is generally believed that the main source of marine DOM is photosynthesis (Ogawa, 2003;Perdue, 2009;Mostofa et al., 2013). Algae, cyanobacteria and phytoplankton as photosynthetic organisms are able to produce autochthonous DOM via photo-induced respiration (or photo-induced assimilation) and microbial respiration or assimilation in natural waters (Harvey, 1995;Thomas and Lara, 1995). DOM produced under this process contains bioavailable fraction that is consumable by heterotrophic bacteria, and therefore unable to accumulate (Mopper et al., 2007). On the other hand, a considerable part of marine DOM is composed of refractory (long-time stable) materials preserved in the ocean for thousands of

years. However, the mechanism by which they are formed is uncertain (Hertkorn et al., 2006;Jiao and Zheng, 2011).

It is interesting that the nature of refractory organic matter in marine environment is strictly bound to the concept of humic substances (HS). HS are known as complicated assemblage of organic components produced from the decomposition and transformation of biomaterials (MacCarthy, 2001). Recent characterisation studies of DOM isolated from seawater shows that humic substances are an important constituent of these material (Quan and Repeta, 2007). Apart from polysaccharides (hetero polysaccharides) which greatly contribute to the oceanic HMWDOM (Aluwihare et al., 1997), ^{1}H NMR data derived from this material indicates the presence of humic-like structures (Aluwiharea et al., 2002). Therefore, it seems that unraveling the mystery of humic substances is intimately linked with the successful characterisation of dissolved organic matter in the ocean or other aquatic environments.

1.2 Humic substances and their formation in the environment

As mentioned earlier, humic substances account for a major part of organic material in the environment as they comprise 50-80% of organic carbon in soil, natural waters and sediment (Perminova et al., 2005). They are produced by degradation and transformation of biomaterial (dead plant or microbial biomass) during a process named humification (Ogawa, 2003;Peña-Méndez et al., 2005). This naturally-occurring substance cannot be categorised within any common classes of organic compounds such as proteins, polysaccharides and polynucleotides since no definable building block has been proposed for them (Hedges, 2000;MacCarthy, 2001). Humic substances are yellow to black in colour, poly disperse, polyelectrolytic and refractory (Hayes and Clapp, 2001;MacCarthy, 2001). It was traditionally believed that humic material were geopolymers with large molecular weights (1 to 100 kDa). However, the introduction of supramolecular view of the chemical structure of humic

substances in recent years has challenged the polymeric view. In fact, humic substances consist of a large number of small molecules stabilised by weak dispersive forces instead of covalent linkages (Piccolo, 2001; Piccolo et al. 2001; Piccolo, 2002). Carbon, hydrogen, oxygen and nitrogen are elements always present in the humic substances regardless of their origin, although sulfur and phosphorus are sometimes observed in their structures. In fact, humic substances contain abundant oxygen-containing functional groups which heavily affect their chemical properties.

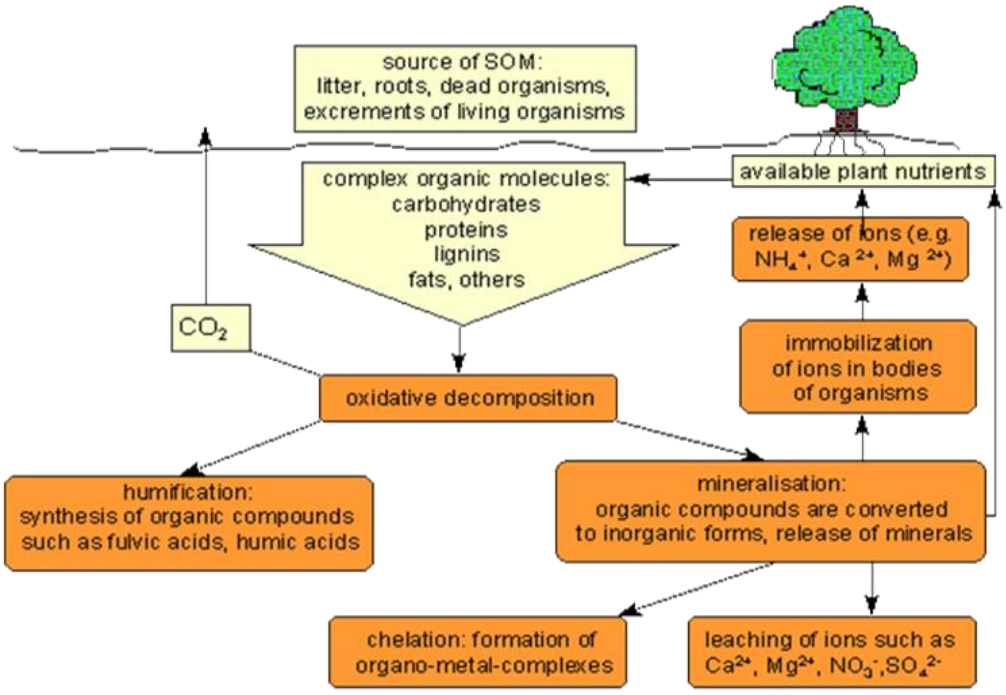

Figure 1.1 **Soil organic matter production pathways (Grunwald, 2014)**

1.2.1 Traditional theories on formation of humic substances

Humic substances are naturally produced by complicated and incompletely understood reactions. Several theories have been proposed in order to explain their formation in the environment mainly considering the contribution of lignin as a component found in plant biopolymers. Based on the classical theory of Waksman (Huang, 2009), reactive biomolecules such as proteins and carbohydrates are decomposed by microbial degradation, these residues in combination with refractory biopolymers including lignin, paraffinic macromolecules and

cutin produce high molecular weight material as humic substances. Subsequent oxidation of these materials leads to the formation of highly oxygenated components containing carboxylic acids, phenols and alcohols. As this trend continues, molecules become smaller as well as more hydrophilic so that they dissolve in basic or even acidic media. Accordingly, humin will be the primary product in this theory which in turn converts to humic and fulvic acid.

Another hypothesis based on a synthetic approach, suggests that a series of condensation and polymerisation reactions occurs on the previously-degraded components of plant biopolymers, resulting in the formation of higher molecular weight humic substances. For instance, polyphenols synthesised by fungi and other microorganisms combine with degradation products of lignin via oxidative polymerisation. This leads to formation of fulvic acid which can be a precursor of humic acid and humin (the reverse of the degradative theory mentioned above).

The third theory which appears as more compatible with the concept of microbially-derived humic materials indicates that a sugar-amine condensation known as Milliard reaction (Burdon, 2001;Huang, 2009) might be responsible for the formation of humic substances. According to this theory, reducing sugars and amino acids formed by microbial metabolism undergo condensation reactions to form glycosylamine, diacetyl and acetal derivatives, all highly labile and quite likely to be subjected to polymerisation and formation of brown-coloured products.

1.2.2 Supramolecualr theory of humic substances

Recently, a new theory, which considers humic substances to be a supramolecular association of molecules held together by hydrophobic interactions and hydrogen bonds, has been introduced (Piccolo 2001; Sutton and Sposito 2005). Consequently, the complex molecular mixture of humic substances, rather than high molecular weight polymers, was described as heterogeneous molecules self-assembling in apparent large molecular size materials. The

main feature of supramolecular theory is that dispersive forces such as van der Waals, p-p and ion-dipole interactions at neutral pH, and classical hydrogen bonds at lower pH are responsible for such assemblages (Piccolo and Splitter, 2003). This theory was strongly supported by previous works; for example it has been indicated that when acetic acid is added to HS that have been already dialyzed, further small components are released during subsequent dialysis (Nardi et al., 1988). The detached fractions showed specific biological properties in plants and tended to be more biologically active than the whole humic materials (Piccolo et al., 1992). These low molecular size fractions are of different chemical compositions from the bulk HS caused by conformational rearrangements.

Humic substances are traditionally divided into three main fractions based on their solubility in water. Humin is considered as the fraction of humic substances not soluble in water at any pH; humic acid, is not soluble under acid conditions (below pH 2), but becomes soluble at higher pH and finally fulvic acid which remains soluble under all pH conditions. Fulvic acid is the smaller molecular weight fraction of humic substances found in natural waters, soil and sediment. Fulvic acids' molecular weight varies from 600 to 900 Da. These fulvic acids largely affect the physical and biogeochemical processes in terrestrial and aquatic environments. This is because fulvic acid contains abundant oxygen containing functional groups especially carboxylic acids, phenols and alcohols in its structure; these polar functional groups are mainly responsible for the high solubility of fulvic acid in aqueous media as well as its metal complexation and chelating ability which facilitates geochemical transport of metals in the environment (Dobbs et al., 1989;Violante et al., 2010). Fulvic acid can also transfer and remove toxic metals and organic contaminants from water and soil (Marley et al., 1992).

It is believed that dissolved fulvic acids accounts for about half of the natural organic matter (NOM) in fresh water and 15-20 % of the NOM in marine waters (Aiken et al., 1996).

This high contribution of fulvic acid to one of the most heterogeneous natural materials known, justifies its importance and significance, so that unravelling the structure of fulvic acid hopefully assists with understanding a main portion of natural DOM content. Also, study of DOM from regions with the minimum external inputs enables more accurate assumption about its formation pathway and most importantly its origin.

1.3 Importance and distinction of Antarctic fulvic acid (microbial versus terrestrial origin)

Antarctica is an area with very unique characteristics, apart from its extreme temperature and permanent ice-covered lakes; it is well-known as a desert (Matsumoto, 1989;Mcknight et al., 1991;Aiken et al., 1996). This is because the very low precipitation in the majority of regions in Antarctica does not allow plants to grow; unlike other desserts the low temperature means evaporation is quite low, resulting in a permanent and thick ice cover on the land. Due to the absence of higher order plants and the minimal occurrence of terrestrial flora (mainly lichen), DOM form Antarctica is believed to be purely autochthonous organic matter. From a structural study perspective, this is an important characteristic because any material identified in the Antarctic DOM can be attributed to the limited non-land-based sources such as phytoplankton, algal material or bacterial utilization.

As mentioned earlier, DOM in the aquatic environments comes from different sources commonly categorised as two forms of allochthonous and autochthonous materials. Allochthonous materials normally occur in freshwater ecosystems where humic substances are formed from decomposition of plants and soil in the adjacent environment. In contrast, the source of DOM in seawater or lakes that usually lack higher order plants, is photosynthesis as well as algae or bacteria excretion in water and sediment; these types of DOM form autochthonous humic substances (Thurman 1985 b ;Aiken et al., 1992).

Being produced from distinct sources, there are obvious compositional differences in these two categories of naturally-occurring organic material that have attracted many

researchers. Various analytical and spectroscopic techniques such as fluorescence spectroscopy (Coble, 1996;Mcknight et al., 2001;Cory and McKnight, 2005), NMR (Kaiser et al., 2003;Thorn and Cox, 2009) and high-resolution mass spectrometry (Reemtsma and These, 2005;Reemtsma et al., 2008) have compared and highlighted the difference in the structure of terrestrially and microbial derived fulvic acids. For example, it was reported that oceanic fulvic acid has more aliphatic nature compared to fresh water fulvic acid (Stuermer and Payne, 1976;Chin et al., 1994). Also, terrestrial DOM has shown a higher molecular weight distribution than microbially derived DOM (Chin et al., 1997;Koch et al., 2005).

On the other hand some similarities were reported for these two types of DOM, for instance, Fimmen et al. (2007) compared the products of thermochemolysis of Antarctic Pony Lake fulvic acid with the fulvic acid isolated from freshwater lakes. Using comparative study of ^{13}C NMR and XPS, they concluded that quinone/hydroquinone compounds are found in both types of DOM. Furthermore, Koch et al. (2005) examined the DOM derived from algae and terrigenous origin and observed similarities in the mass spectra of both samples, reflecting their structural similarities, or in a broader sense the presence of a general chemical structures as refractory DOM, resistant to microbial decomposition and formed regardless of source and environment in which the examined organic matter occurred.

Allochthonous DOM is generally believed to have a more recalcitrant character than autochthonous DOM (Catalan et al., 2013) due to the presence of aromatics and lignin monomers which are more stable than material derived from phyto and bacterioplankton activities. However, production of refractory DOM from labile microbial DOM is also well-documented because there is evidence indicating that photodegradation of microbial DOM in surface waters contributes to the formation of the refractory fulvic acid (microbially-derived fulvic acid) in marine environment (Miller et al., 2009).

Differences in the origin and formation pathway of allochthonous versus autochthonous DOM is also evident in the distribution of non-living organic material in water ecosystems;

Purdue et al. (2009) indicated that a major part of organic carbon in the marine environment are distributed in non-living organic matter such as sediments and particulate organic matter while in freshwater systems around 25 % of organic carbon is found in biomass. This shows the clear relation between the production of non-living organic matter and processes such as photosynthesis rather than biota decomposition.

1.4 Methods for fulvic acid characterisation

In this section a literature review on various techniques used in previous fulvic acid structural study will be presented. To be more focused, mostly the studies regarding Antarctic fulvic acid will be discussed.

1.4.1 Spectroscopy- based methods

1.4.1.1 Infrared (IR) spectrometry

IR spectroscopy is a well-established technique, traditionally used to investigate the contribution of various functional groups in polymers and macromolecules (Boulet et al., 2007;Zhang et al., 2011;Shang et al., 2013). The development of fourier transfer infrared spectroscopy (FT-IR) as a reproducible and accurate technique has been a facilitator for the analysis of fulvic acids in recent years. The main advantages of using this technique, apart from the valuable information it provides about functional groups, are its easy operation and small quantities of samples for measurements. The latter can be of especial importance for precious samples such as natural organic matter. Looking at limitations, firstly the complex mixture of functional groups found in various chemical environments, results in broadening of IR bands. Moreover, the IR spectra of humic substances even of various origins show an overall similarity that sometimes is more prominent than the differences (Belzile et al., 1997). For example, in all humic substances spectra there are typically three main bands for OH group, C=O group and C-O stretches; other peaks are minor and appear as shoulders or very weak bands.

Despite the overall similarities in the IR spectra of various humic materials some significant differences based on the composition and origin of humic or fulvic acid can be observed. Further, the contribution of carbohydrates, proteins, aliphatic and aromatic constituents in their structures are also observable in the IR spectra. For instance, the 1620 cm^{-1} peak is attributed to aromatic ring C=C vibrations only observable in freshwater humic

substances IR spectra as sharp peaks and their intensity is 20 to 50 times lower than the C=O stretch of conjugated ketones (Wexler, 1967). Other indicator peaks are 1660 cm^{-1} (amide I) and 1550 cm^{-1} (amide II band) (Schmitt and Flemming, 1998;Muruganantham et al., 2009) as characteristics of amide linkage of proteins; if it is accompanied by 1370 cm^{-1} methyl-bending, it could be N-acetyl amino sugars not protein (Dereppe et al., 1980). Overall, although IR spectroscopy can provides useful information about functionalities and possible structures of humic substances, it is often the case that information obtained needs to be confirmed by other techniques due to the complexity of humic materials matrix and uncertainties mentioned earlier. Table 1.1 summarises the Infrared frequency peaks for various compounds in NOM (Leenheer, 2009).

Table 1.1 Infrared frequency assigned to different compounds in NOM

Compound Class	Peak Frequencies (cm^{-1}) and Structure (Φ = aromatic ring)
Carbohydrates	3400-3300 (O-H), 1100-1000 (C-O)
Humic substances	3600-3300 (O-H), 2700-2200 (COOH), 1760 (COOR), 1720 (COOH), 1660-1600 (C=C-C=O), 1280-1150 (Φ -O, COOH)
Hydrocarbons	2960 (CH_3), 2940(CH_2), 1460 (CH_2), 1380 (CH_3)
Lignin	1600 (Φ -C=O), 1510 (Φ), 1460 (CH_2), 1420 (Φ), 1267 (Φ -O), 1127 (O-CH_3), 1035 (Φ)
Lipids	2960 (CH_3), 2940 (CH_2), 1740 (COOR), 1720 (COOH), 1460 (CH_2), 1380 (CH_3)
Proteins	3100 (N-H), 1660 (Amide 1 peak, N-C=O), 1540 (amide 2 peak, N=C-O)
N-Acetyl amino sugars	1660 (amide 1 peak, N-C=O), 1550 (amide 2 peak, N=C-O), 1380(CH_3)
Aromatic sulfonic acids	1180, 1125, 1040, (Φ -SO_3H), 1010 (Φ -H)

1.4.1.2 NMR spectroscopy

Traditionally, NMR spectroscopy has been one of the commonly-used techniques for the characterization of fulvic acid. NMR is a non-destructive technique enabling the study of complex matrix of natural organic matter for the presence of specific functional groups and their surrounding environment. However, due to the heterogeneity of humic substances overlap of resonances in the NMR spectra and consequently broad signals are observed that decreases the sensitivity of analysis. The NMR parameters such as chemical shift, coupling constant J, amplitude A, and resonance width at half height are also affected due to the polydispersity and heterogeneity of this material (Hertkorn et al., 2002). Despite the difficulties associated, NMR results especially ^{13}C NMR and ^{15}N NMR techniques have provided valuable information to simplify the complexity associated with the characterisation of fulvic acids. Most importantly, the recent advances in 2D NMR techniques have resulted in considerable improvements towards determining the interactions between different functional groups and resolving the overlapped peaks and signals.

1.4.1.1.1 One dimensional NMR spectroscopy

The first NMR study of Antarctic fulvic acid goes back to the last century when fulvic acid isolated from two lakes Lake Fryxell and Lake Hoare located in the McMurdo Dry Valleys, Antarctica was examined using ^{13}C NMR and ^{1}H NMR by McKnight et al (1991). The quantitative spectra was obtained using inverse-gated decoupling; for both fulvic acids NMR indicated the presence of an aliphatic-dominated material and with conspicuous difference from fresh water fulvic acids. Moreover, the ^{13}C NMR spectra revealed that some aromatic moieties were present in these fulvic acids, that their origin and occurrence was not recognised. Later the authors studied the fulvic acid form Feather Pond at Cape Bird as well as Pony Lake, a eutrophic saline coastal pond both located on Ross Island (the sample isolated from the latter was supplied from then on by IHSS (International Humic Substances Society)

as a pure microbially-derived fulvic acid reference material for public use). The quantitative ^{13}C NMR data obtained for these fulvic acids generally appeared similar to fulvic acids from the dry valley lakes, but with more carbohydrate carbon. Pony Lake Fulvic Acid (PLFA) did not appear different from other fulvic acids based according to NMR data; however it differed from other Antarctic fulvic acids due to its higher nitrogen content. The high nitrogen content besides its pure microbially-derived nature inspired the further studies of PLFA using advanced ^{15}N NMR (liquid and solid states) and $^{13}C\{14N\}$ SPIDER NMR (for identification of carbons bonded to nitrogen), these recent studies has been able to reveal important information about the structure and building blocks of PLFA, one major finding was the presence of a well-resolved heterocyclic nitrogen peaks at 144 ppm and 172 ppm in CP/MAS ^{15}N NMR spectra (Thorn and Cox, 2009) which had not been observed in the previous solid state ^{15}N NMR of this fulvic acid (Mao et al., 2007) supposedly due to the shorter contact time used during recording of NMR experiment. Thorn and Cox (2009) also indicated the feasibility of study of PLFA by liquid state NMR using DEPT (Distortionless Enhancement of Polarisation Transfer) technique which is a spectral editing sequence for generating separate ^{13}C sub-spectra for methyl (CH_3), methylene (CH_2), and methine (CH) signals, that results enabled the detection of amide peak at 112.4 ppm and a heterocyclic nitrogen peak at 153.4 ppm. In a study by Fang et al. (2011), which focused on the identification of carbons bonded to nitrogen, the ^{13}C $\{^{14}N\}$ SPIDER NMR technique was applied on PLFA as well as several model compounds containing N in various functional groups; the results of these experiments in combination with the data obtained by ^{15}N NMR CP/MAS interestingly revealed the presence of –CO–NH–CO–NH- structures matching with hydantoin, or allantoin, derivatives. This work seems to be a decisive practice of 1D NMR for structural analysis of Antarctic fulvic acid, it not only added important information regarding nitrogen-containing compounds in this nitrogen-rich natural material, but promises further improvements in the

characterisation of fulvic acid using sophisticated NMR procedures including one and two dimensional techniques that could complement each other.

1.4.1.1.2 Two dimensional NMR spectroscopy

2D NMR spectroscopy is a technique that introduces a second frequency domain in order to provide information about bonding or spatial interactions between pairs of nuclei. For example, resonance between bond electrons can use to elucidate an atom's connectivity, whereas dipole-dipole coupling between neighbouring atoms provides information about spatial interactions (Hertkorn et al., 2001). Multi-dimensional NMR may be undertaken in liquid or solid states and are divided into various categories based on the applied homonuclear or heteronuclear shift correlations named total correlation spectroscopy (TOCSY), correlation spectroscopy (COSY), nuclear overhauser effect spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond connectivity (HMBC), diffusion ordered spectroscopy (DOSY), and heteronuclear correlation spectroscopy (HETCOR) (Simpson, 2001).

The use of two dimensional NMR including heteronuclear and homonuclear correlation for characterization of Antarctic fulvic acid is relatively new, although it has been widely employed for other humic and fulvic acids (Morris et al., 1999;Kingery et al., 2000;Haiber et al., 2001;Simpson et al., 2001;Deshmukh et al., 2007). The results of HMBC and TOCSY experiments on IHSS soil and freshwater humic and fulvic acid has identified four different types of carboxylic groups in the samples in mostly unsubstituted and branched aliphatic and alicyclic structures (Deshmukh et al., 2007), according to HMBC data some of carboxyl groups in soil material bear electron-withdrawing groups on neighbouring carbons. In a separate study ^{1}H-^{13}C HMQC of aquatic humic acids demonstrated major cross peaks indicative of Hα-Cα, Hβ-Cβ and Hγ-Cγ correlations of amino acids that refer to the fact that peptides are present as relatively large structures. Since no additional cross peaks were

observed in the HMQC spectrum, the authors concluded that peptides are not covalently connected to other structures (Haiber et al., 1999). The two-dimensional ^{13}C, ^{1}H-correlated NMR spectroscopy of Suwannee River Fulvic and Humic Acid Reference has provided evidence for a lignin degradation pathway by demonstrating its demethylated products (Haiber et al., 2001). Simpson (2002) carried out DOSY NMR experiments on Mississippi fulvic acid isolated from forest soil and standard peat humic acid and monitored the aggregation and associations in solution that was not possible to study by other methods. Another application involves using advanced 2D NMR such as double quantum filtered (DQF)-COSY and echo/anti-echohetero-nuclear single quantum coherence (HSQC) for studying pollutant sorption and bioremediation on oak forest fulvic acid (Simpson et al., 2002).

Hektron et al (2002) extensively examined soil humic and fulvic acids and compared their structural features using a combination of 1D and various 2D NMR techniques, his results revealed important findings regarding sub-structures of humic and fulvic acids, for instance, TOCSY and HSQC data revealed that the chemical environment of nitrogen is different in fulvic and humic acids even with their similar C to N ratios. Since no peptides and amino sugar peaks were detected, the nitrogenous material was assumed to be of heterocyclic structures. This study has also highlighted the similarity of carbohydrates structure in both fulvic and humic acids occurring in the soil.

Regarding Pony Lake FA, one recent work, known as an indirect detection technique applied on PLFA is $^{1}H–^{15}N$ HSQC; this technique correlates the interactions between nitrogens directly bonded to protons, for example it was able to show the interaction between heterocyclic nitrogens bonded to protons such as pyrroles and imides in PLFA structure in agreement with the results obtained by DEPT spectra (Thorn and Cox, 2009). However,

difficulties in the detection of such interactions due to the less sensitivity in the heterogeneous fulvic acid matrix and intrinsic low natural abundance of nitrogen are expected.

1.4.1.3 Fluorescence Spectroscopy

Fluorescence spectroscopy is a sensitive, simple and non-destructive method for study of humic substances content, requiring only a small amount of sample and very low concentrations (Mobed et al., 1996). Fluorescence spectroscopy can be used both qualitatively and quantitatively; it has been used for the quantification of humic substances in natural waters (Senesi and Testini, 1984; Senesi et al.,1991; Miano and Senesi, 1992; Michałowski et al., 2001;Sheng et al., 2007) or for determination of fulvic acid oxidation state (Klapper et al., 2002). The basic fluorescence of humic substances provides information about conformation, functional groups, aromaticity, heterogeneity and intramolecular and intermolecular interactions (Lochmuller and Saavedra, 1986). Total luminescence spectroscopy that also known as excitation-emission matrix (EEM) is another technique recently employed for humic and fulvic acids analysis; in this method fluorescence intensity is presented as a function of excitation wavelength on one axis and emission wavelength on the other. The presence of two fluorophores in EEM spectrum of Pony Lake fulvic acid was reported using this technique (Mcknight et al., 2001;D'Andrilli et al., 2013) reflecting the humic structure and protein-like structures. The comparison of these fluorophores with those obtained from EEM of other fulvic acids revealed that this technique can be used to as a powerful tool to discriminate between microbial-derived and terrestrial-derived materials and provided information about the nature and origin of humic substances (Mobed et al., 1996). The introduction of advanced fluorescence techniques such as synchronous fluorescence spectroscopy (SFS) or emission scan fluorescence (ESF) in combination with other techniques (Rodriguez et al., 2014) has also shown promise in revealing information not attainable using EEM Fluorescence spectroscopy.

1.4.1.4 Mass Spectrometry

The direct analysis (without derivatisation) of humic substances by mass spectrometry for acquiring molecular information was initiated with the work of McIntyre et al. (1997) who demonstrated the application of ESI-MS for the analysis of hydrophobic acids in drinking water. Subsequent studies indicated that experimental conditions (e.g., pH) and instrumental parameters had considerable impact on the molecular distribution of the mass spectrum (Brown and Rice, 2000). Further, the extreme complexity of the spectra obtained and difficulties in data interpretation meant that direct mass spectrometric analysis of humic substances demanded high resolution mass analysers or the combination of mass spectrometry with high-quality separation systems. Interestingly, high resolution alone was still found to be inadequate; when the combination of ESI with the ultrahigh resolution fourier transform ion cyclotron resonance (FT–ICR) mass spectrometer was undertaken on the humic and fulvic acids isolated from the Suwannee River (Fievre et al., 1997), resolution was still inadequate, necessitating pre-fractionation by HPLC .

Application of FT-ICR-MS has recently received much attention as a favorable detector for chromatographic systems used for the humic substances characterisation. Its main advantage involves accurate mass measurements and resolving individual humic ions with the ability to calculate their empirical formulas (Stenson et al., 2003). Reemtsma et al. (2006) coupled size exclusion chromatography with FT-ICR-MS and demonstrated that by plotting the number of carbon or hydrogen atoms of each IHSS fulvic acid reference material versus its molecular mass, valuable information about molecular composition can be extracted. Further study of deep Atlantic fractionated DOM by this group (Reemtsma et al., 2008) indicated important information in terms of the chemical nature of the nitrogenous molecules, such as the presence of reduced nitrogen species as alicyclic structures. More relevantly, PLFA and SRFA (Suwannee River Fulvic Acid) were analysed using ESI-FT-ICR MS

(D'Andrilli et al., 2013) and their empirical formula and building blocks compared, the results suggested that PLFA has species distributed over a broad range of O/C and H/C ratios, protein-and amino sugar-like components were also identified in the van Krevelen diagram, which were not observed for SRFA. Data obtained by ESI coupled to quadrupole time-of-flight mass spectrometry (ESI-QqTOF-MS) by Kujawinski et al. (2002) also appeared comparable to those obtained by ESI-FTICR-MS and this technique was shown capable of providing useful information especially in combination with NMR or other techniques.

Despite its popularity in recent years, direct mass spectrometric methods using ionization methods such as ESI are not without its limitations, for instance, non-ionizable compounds cannot be analysed using MS and ionization of terrestrial HS or DOM in the presence of complex non-homogeneous, supramolecular associations and molecular interferences can produce changeable and sometimes unreliable results (Nebbioso and Piccolo, 2013). So far, powerful detection systems such as FT-ICR-MS has been able to provide practical information about fulvic acid and similar NOM, however it is noteworthy that they always need to be hyphenated to efficient separations system in order to produce reproducible results and reliable mass spectrum interpretations. Furthermore, although elemental formulas are offered by FT-ICR-MS, the structures containing these elements and their origin remain mostly unknown (Reemtsma et al., 2006).

1.4.1.5 Other spectral techniques

There have been other spectroscopic methods utilised for structural analysis of humic substances, but being less popular than methods discussed earlier. Ultraviolet-Visible (UV-VIS) spectroscopy as a general method for qualitative and quantitative functional groups determination has found applications for humic substances analysis. For instance the ratio of absorbance at 254 nm (UV_{254}) and DOC concentration can be an estimation of the aromatic content of aquatic humic substances (Traina et al., 1990); moreover, the ratio of absorbance at

465 nm and 665 nm known as the E4/E6 ratio which is the slope of the absorbance tail of humic substances in the visible area is believed to be related to intra-molecular charge-transfer complexes (Del Vecchio and Blough, 2004).

Another technique worth mentioning is X-ray Photoelectron Spectroscopy (XPS) also known as ESCA (Electron Spectroscopy for Chemical Analysis). XPS capability in detection of different species of an element based on chemical shift of the binding energy of electrons present in the atoms can be an advantage to study of unknown materials. Therefore, XPS provides information about the relative abundance of elements in a solid sample as well as the different chemical form of the bound elements. It is also a non-destructive technique using very small amounts of sample and analysis can be completed within a short run time

Fortunately, the recognition of different elemental species by XPS has assisted emerging valuable information about structure of DOM and humic substances not attainable by other spectroscopic techniques such as NMR or IR. For instance, Monteil-Rivera and her colleagues using as a series of XPS analyses, have been able to reveal an overestimation in detection of carboxylic acids in humic acid appearing in ^{13}C NMR spectrum, apparently attributed to amides group (Monteil-Rivera et al., 2000). A few XPS studies were recently performed to elucidate the structural features of humic acids from aquatic and soil environments (Abe and Watanabe, 2004;Bubert et al., 2000). These studies identified features with respect to the nitrogen functionalities such as amides, amines, pyrroles and aromatic nitrogen in humic substances that highlighted the authority of XPS analyses in both qualitative and quantitative aspects.

1.5.2 Chromatographic- based methods

1.5.2.1 High performance liquid chromatography

Separation of humic substances using liquid chromatography (LC) is potentially a powerful method as samples can be studied without derivatization and with access to a range of separation mechanisms thanks to the various columns commercially available for different functional groups analysis. The most widespread method in this category has been reversed phase HPLC (RP-HPLC) commonly used in preparative or analytical modes. In a pioneering work on fulvic acids, Suwannee River FA from the International Humic Substances Society (IHSS) was separated by semi-preparative RP-HPLC into hydrophilic and hydrophobic fraction and subsequent analytical RP-HPLC-UV was performed to detect up to ten peaks in each fractions using acetonitrile/water gradient elution (Saleh and Ong, 1989). RP-HPLC using stepwise gradients of dimethyl formamide (DMF) in buffered aqueous mobile phase was demonstrated to be efficient for analysis of soil, peat and air particulate humic acids (Hutta and Gora, 2003).

High-performance immobilized metal ion affinity chromatography (HP-IMAC) was used as an additional fractionation for fulvic acids from different origins prior to RP-HPLC analysis. This fractionation was based on the differential affinity of fulvic acid constituents for the immobilized ion (in this case copper (II)) and subsequent analysis using RP-HPLC. The method yielded five general peaks detected by UV–VIS photodiode array and fluorescence detector (Wu et al., 2002 a;Wu et al., 2002 b).

HPLC systems interfaced with different sophisticated detectors have recently found widespread recognition for structural studies of humic substances. LC-NMR (^{1}H) and LC-SPE-NMR (^{1}H) was employed for the first time to study the DOM from freshwater environments (Simpson et al., 2004), the authors concluded that due to the relatively low sensitivity of NMR and complexity of DOM matrix, data obtained are required to be

combined with the molecular formulas obtained from MS studies. Numerous LC-MS studies for the characterisation of DOM and humic substances have been completed; as mentioned earlier FT-ICR mass spectrometers were widely employed in combination to HPLC and enabled constructing possible structures whose formula weights could be calculated and compared to detected peaks in high resolution mass spectra. Despite the advantages that FT-ICR-MS offers, identification of compounds can be challenging. Some of these challenges were demonstrated in a study characterising DOM from western Weddell Sea, Antarctica using HPLC coupled to FT-ICR-MS (Koch et al., 2005); the authors found out that even very narrow chromatographic areas can contain over one thousand different DOM components so that their identification was not fully achievable.

Other LC/MS techniques were also undertaken for NOM analysis; in a comparative study, fulvic acid standards from Suwannee River, Pony Lake, Elliot Soil, Waskish Peat, and Nordic Reservoir were studied by HPLC coupled to quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) (Mawhinney et al., 2009); the approaches used were not sensitive enough because TOF mass analyzer was not able to resolve discrete species, yielding mass peaks that were an average of all components and therefore provided little qualitative information regarding structures of studied fulvic acids.

Apart from detection system, a tendency toward utilisation of separation mechanism other than RP-HPLC was observed in the literature to induce more effective and meaningful interaction between natural organic matter and stationary phase. The work of Mawhinney et al. (2009) is an example of use of Allure organic acids column bearing polar embedded groups on stationary phase that allows the alkyl groups to remain protracted in 100% aqueous mobile phases (conventional C18 columns tend to collapse under such conditions) providing stable and reproducible retention. Hydrophilic interaction liquid chromatography (HILIC) is another choice that has recently applied on NOM (Woods et al., 2011). The mechanism of

separation in HILIC is similar to that of normal-phase HPLC with a polar stationary phase but the mechanism involves partitioning between the hydrophobic mobile phase and a layer of mobile phase enriched with water and dynamically immobilised on the stationary phase. Polar analytes enter this water layer and interact with the surface functional groups (McCalley, 2007;Hao et al., 2008). Relying on hydrophilic or polar interactions, HILIC is a promising method for the separation of polar analytes which are abundant in the aquatic environment and are difficult to separate using conventional chromatographic methods.

Another approach to resolve the chemical heterogeneity and accomplish a better resolution involves using two-dimensional liquid chromatography. The first attempt was made to analyse Suwannee River Fulvic Acids (SRFA) and Pony Lake Fulvic Acids (PLFA). In two separate 2D setups using either a conventional reversed-phase (RP), or a mixed-mode HILIC column in the first dimension, and a size-exclusion column in the second dimension, three detectors included UV, FLD (Fluorescence Detector) and ELSD (Evaporative Light Scattering Detector) (Duarte et al., 2012). The paper provided information regarding molecular weight distribution of fractions obtained under two different chromatographic condition including RP-HPLC and a technique named ‘’PALC’’ (PALC stands for *per* aqueous liquid chromatography and refers to the state in HILIC chromatography when the percentage of water in the mobile phase exceeds about 75%, under such condition retention of analytes resembles RP-LC and is described as per aqueous liquid chromatography). According to the result, the smaller molecular weight fractions belong to more hydrophobic material. Obtaining up to two times different molecular weights values for the same fractions under different retention circumstances (RP-C18 × SEC and PALC × SEC), the results have highlighted the impact of retention mechanism on MW properties and uncertainty associated with the condition of such experiments. The study also showed that PALC is a promising approach for fractionation and better separation of PLFA.

Other detectors that were used in combination to LC systems were FTIR (Landry and Tremblay, 2012) and in a few cases Multi Angle Light Scattering (MALS) (Wagoner and Christman, 1998), the information that these methods provided can be used as complementary to other techniques such as NMR and mass spectrometry in combination to GC and HPLC.

1.5.2.2 Gas chromatography in combination with thermal and chemical degradation

The high resolving power of gas chromatography has made it a commonly used technique for DOM analysis. When coupled to mass spectrometry (electron-ionization MS), it can be used to show up the volatile or easily derivatised components in complex mixtures such as humic substances. Humic substances are categorised as heterogeneous geopolymers and basically non-volatile, however the low molecular weight constituents incorporated into their structures could become volatile with the aid of chemical derivatisation, making them detectable by GC. The detection and identification of these components indirectly provide us with valuable structural information about the whole complicated matrix.

1.5.2.2.1 Thermal degradation - Pyrolysis and Thermochemolysis

Generally, pyrolysis is the most widespread thermal-degradation technique used in combination with GC/MS. It normally involves the breakdown of material to subunits that can be separated by GC. Py-GC-MS has also been extensively employed for fulvic acids characterisation especially for those of soil origin (Fezzey and Armitage, 2006), there are also a few studies regarding marine or microbial- based fulvic acids (Zsolnay and Harvey, 1985). Flash pyrolysis (Py-GC/MS) and pyrolysis–field ionization–mass spectrometry; have also found application for humic substances analysis (Leinweber and H.R., 1999;González-Vila et al., 2001). However, most of the recent applications of fulvic acid analysis using pyrolysis has been in the presence of methylation agents such as TMAH (Challinor, 1989;Martin et al., 1994;Martin et al., 1995) mainly because in TMAH assisted py-GC/MS, polar compounds containing phenolic hydroxyl and carboxylic acids groups are converted to methyl ester and

ether derivatives which are easily detected by GC/MS. However, when pyrolysis is solely used, it is often the case that polar compounds cannot be observed in the pyrolysates (Saiz-Jiménez, 1994). TMAH thermochemolysis as a single-step degradation coupled to GC/MS has also been employed for fulvic acid analysis and as stated above acted as an alternative to straight pyrolysis and is able to convert polar groups to their methylated derivative, rendering them more amenable to GC separation (de Leeuw and Baas, 1993;Hatcher and Cliford, 1994). Amide-containing structures present in nitrogen-rich fulvic and humic acids have been one of the main targets of TMAH-thermochemolysis analysis. This is because many humic substances contain peptides and as such need the cleavage of amide linkages using TMAH, these linkages shown to be susceptible to basic hydrolysis (Knicker and Hatcher, 1997;Knicker et al., 2001). Antarctic PLFA in its original and unfractionated form was also exposed to TMAH-GC/MS analysis (Fimmen et al., 2007); the compounds obtained from that experiment ranged from C7-C17 saturated and unsaturated fatty acids and dicarboxylic acids, benzoic acid derivatives as well as heterocyclic nitrogenous compounds such as aniline, pyrrole, pyrrolidone and pyrimidine derivatives, highlighting the capability and potential of this degradation method for fulvic materials.

1.5.2.2.2 Chemical Hydrolytic degradation

Application of hydrolysis methods to degrade humic substances are usually performed in order to analyse the contribution of carbohydrate or peptides in their structures or to remove these compounds prior to the additional characterisation. Acidic hydrolysis has not been extensively applied on fulvic acids as they are the smallest fraction of humic substances instead most studies were focused on NOM and specifically HMWDOM or soil organic matter (SOM). Common hydrolysis methods have been hydrochloric acid, sulfuric (Leenheer, 2009) or trifluoroacetic acid (Aluwihare et al., 2002). Recently, methanesulfonic acid has shown promise for soil organic matter hydrolysis, especially to release amino acids and amino sugars with high yield (85.6% of total nitrogen) higher than that obtained by traditional HCl

hydrolysis (30-40%) (Martens and Loeffelmann, 2003); this method, however, needs the application of relatively high temperature (136 °C). Yet, the selection of appropriate hydrolysis agent and condition is totally substrate-dependent and it could be hard to decide the best approach beforehand (Warren, 2013).

1.5.2.2.3 Chemical Oxidative and Reductive degradation

One of the most common issues in humic substance analysis is overcoming the intermolecular interactions that prevent the complete unfolding and separation of individual components in the complex assemblage. Many approaches, therefore aim to eliminate or decrease such interactions by elimination of active functional groups using reduction, oxidation and derivatisation methods. The main oxidative degradations examined on humic substance analysis include alkaline $KMnO_4$ (Davies and Lawson, 1966;Chesire et al., 1967;Chesire et al., 1968), chlorination (Frimmel and Schmiedel, 1993) and more commonly alkaline CuO oxidation (Ertel et al., 1984;Norwood and Chrlstman, 1987;Hautala et al., 1997). The latter was mostly employed for the identification of lignin structure and was shown successful for detection of aliphatic and aromatic moieties in fulvic acid (Norwood and Chrlstman, 1987); Alkaline CuO oxidation in combination with silylation was applied on Nordic aquatic reference fulvic acid characterisation and compared it with off-line TMAH pyrolysis, the results revealed that CuO oxidation should be categorised as a mild degradation because the products obtained by CuO oxidation were loosely bound moieties compared to those acquired using TMAH pyrolysis.

As an alternative approach, reductive degradations focuses on elimination of hydrogen bonding between abundant carboxylic, phenolic and alcoholic functional groups by reducing them to alcohols and alkanes. The reduction of soil humic sucstances was first performed using Zn-dust distillation (Schnitzer and Khan, 1972) and recently employing Zn dust fusion (Eglite et al., 2003). The other method used was sodium amalgam reduction (Mendez and

Stevenson, 1966) that appeared useful in the preservation of aliphatic structures. High and low pressure hydrogenation in combination with bromination was also attempted (Stuermer and Harvey, 1978). However, mostly constituents bearing several functional groups gave a low yield due to the incomplete reduction of each functional group. Moreover, the high temperatures and pressures usually used in the catalytic hydrogenation methods may induce structural rearrangements (Hayes et al., 1989).

A novel reduction technique using a one-step hydrogenation by an alkylsilane reagent has recently been applied on IHSS standard fulvic acids (Nimmagadda and McRae, 2007) and is the only method capable of the reduction of material bearing alcohols, aldehyde, ketone, carboxylic acids and esters to alkanes. This promising approach was shown to be specifically effective for poly-functional materials; however for sterically-hindered substrates high yields were not fully achieved.

1.6 Conclusion

Fulvic acids influence a range of physical and biogeochemical processes in aquatic environments. In order to better understand their roles and functions, there is a need to study their structures in detail and with respect to the input and sources contribute to their formation. A detailed structural study of Antarctic fulvic acid can assist with having a general picture of microbial DOM structures because Antarctica is an environment with very limited land-based anthropogenic activities, therefore information obtained regarding their structures could be simply attributed to the materials found in the surrounding environment.

Thanks to the recent advances in analytical and instrumental techniques such as high resolution mass spectrometry and sophisticated multi-dimensional NMR spectroscopy, now there are many more options available to the humic substances studies compared to a decade ago, however, despite the tremendous attempts made to unfold the structure of Antarctic fulvic acid using those techniques, there is still a gap in terms of molecular-level analysis. The complexity of these materials necessitates the selection of approaches that have the potential to produce meaningful results and are appropriate to the aims of the study. For instance, the molecular-level characterisation requires methods that enable better separation of components to assist with the identification of trace components present in the sample, such approaches generally place in the area of chromatographic techniques and most specifically gas chromatography.

To secure a successful separation by chromatography, some preliminary measures should be taken to enhance resolution and to facilitate the identification of compounds. In summary, to become successful in this task, coordination of consecutive degradation approaches in combination with physical and instrumental methods is required. It is also important to conduct other approaches such as fractionation and isolation to be able to

increase the efficiency of degradation methods and enhance the identification of the released products.

Based on the above discussion and with respect to the objective and scope of this thesis, the main analysis technique considered in this work involves substantial analysis using gas chromatography/mass spectrometry on the pre-fractionated fulvic acid material. Other chemical and spectral methods are also performed as complementary analyses.

1.7 Aims and scope of the thesis

The primary goal of the thesis was to chemically characterise nitrogen-rich fulvic acids from Antarctic lakes using substantial gas chromatography/mass spectrometry analyses and use the chemical information to gain a better understanding of the structure of this pure microbial-based dissolved organic matter. This was achieved through the following specific aims:

I. Develop and optimise chemical degradation methods using various reagent or combination of reagents to achieve an efficient GC amenable rendition of Pony Lake Fulvic Acid as a pure microbial DOM
II. Accurately identify the individual components obtained in the course or as a result of different degradation methods.
III. Perform fractionation of fulvic acid being examined and comprehensively analyse the fractionated material to investigate the impact of fractionation
IV. Apply the established and optimised procedure undertaken on PLFA for other Antarctic fulvic material (Pendant Lake Fulvic Acid) and compare the result associated with composition and structure.
V. Perform other spectral qualitative methods as additional experimental studies to gas chromatographic results to enable a conclusion to be drawn regarding structures

1.8 Thesis organisation

The thesis consists of four chapters: (1) Introduction; (2) The characterisation of bulk Pony Lake Fulvic Acid; (3) The characterisation of fractionated Pony Lake Fulvic Acid; (4) The characterisation of Antarctic Fulvic Acids from Vestfold Hills.

The second chapter of the thesis is dedicated to the structural study of the nitrogen-rich Antarctic Pony Lake Fulvic Acid using various chemical analysis methods in its original form (the spectral analysis was not performed, but was presented based on previous studies in the literature). Pony Lake (PLFA) is considered for this study because it is currently known as the commercial and generally-accepted Antarctic fulvic acid reference material, however despite its popularity its structure is still ill-defined especially in terms of the nature and contribution of nitrogen-containing compounds.

The goal of Chapter 3 is to demonstrate the result of the characterisation of "Fractionated PLFA" and to test the efficiency of fractionation to gain better resolution and enhanced separation and identification of peaks and components. RP fractionation was performed prior to the chemical and spectral analyses; also the chemical analyses in this section were selected considering the fact that PLFA has high nitrogen content, therefore our derivatisation methods were mostly aimed to target nitrogenous compounds.

Lastly, we attempted to characterise hydrophobic acids (correspondent to fulvic acids) isolated from water samples of Antarctic Lakes (Pendant, Mossel and Organic Lakes) located in Vestfold Hills, Antarctica. The results of this investigation are presented in Chapter 4. The experiments in this chapter followed the same approach as PLFA (fractionation prior to analysis) with respect to the advantages of fractionation. These fulvic-like materials are similarly nitrogen-rich and experiments were conducted to answer the question of whether fulvic acids with similar elemental composition (specifically Nitrogen content) with the similar origin share the same structure and building blocks.

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Chapter 2
The Characterisation of Bulk Pony Lake Fulvic Acid

2.1 Introduction

Pony Lake Fulvic Acid (PLFA) refers to a microbially-derived reference fulvic acid isolated from Pony Lake water. Pony Lake is located in Cape Royds, McMurdo Sound, Antarctica. The lake system formation goes back to 10000-20000 years ago (Doran et al., 1994) and was named after the ponies brought to Antarctica during Ernest Shackleton's Nimrod expedition in 1907 (IHSS, 2013).

As previously discussed in Chapter 1, the chemical properties of fulvic acid within a lake or any other aquatic environment are influenced by input from their surroundings including the soils and plants found in the area or algae and bacteria in the water or sediments. The ice free areas surrounding Pony Lake is a desert with no higher plants (Mcknight et al., 1994) and therefore the degradation of algal material in the lake sediments can be considered the main source of dissolved organic carbon (DOC). The very unique characteristics of this region combined with its extreme environment can assist with better understanding of the fulvic acid formation process.

It may be argued that the microbial origin of PLFA would promise a simpler structure and easier interpretation compared to fulvic acids originating from temperate climates with the high contribution of various compounds from various sources inputs. Moreover, the absence of anthropogenic activities including chemicals discharged from agricultural and industrial processes or medicinal and cosmetic products would decrease the complexity of DOM in regions such as Antarctica. However, whether these factors guarantee a simpler structure or an easier interpretation is doubtful. This is because the mechanism by which the

building blocks combine together is still unclear. Further, it is assumed that transformation or alteration of primary precursors may occur during the formation of humic substances, resulting in more complexity and more challenging identification. While PLFA has been extensively studies by spectroscopic techniques, very little work has been done at the individual molecular level and this work is the first attempt to comprehensively examine PLFA at the molecular-level.

2.1.1 General chemical properties of PLFA

2.1.1.1 Elemental Composition

The elemental composition of PLFA, as a well-known reference material, has been reported by IHSS and presented in Table 2.1. For comparison purposes, values for other standard soil and water reference fulvic acids are also shown.

Table 2.1 Elemental Compositions of IHSS reference fulvic acids (IHSS, 2013)

Fulvic acid Reference	**Ash%**	**C%**	**H%**	**O%**	**N%**	**S%**	**P%**
Pony Lake (1R109F)	1.25	52.47	5.39	31.38	6.51	3.03	0.55
Suwannee River (1R101F)	0.98	53.04	4.36	43.91	0.75	0.46	<0.01
Waskish Peat (1R107F)	0.16	53.63	4.24	41.81	1.07	0.29	0.12
Nordic Lake (1R105F)	0.45	52.31	3.98	45.12	0.68	0.46	<0.01
Pahokee Peat (1R103F)	1.58	52.12	3.23	43.93	2.43	0.53	0.01

A notable feature of the elemental composition of PLFA, compared with the other fulvic acids tabulated, is its relatively high nitrogen content. This suggests that PLFA contains a relatively high percentage of nitrogen-containing species. A major part of this study will be to investigate the nature and origin of these nitrogen-rich materials.

2.1.1.2 Carbon and Nitrogen Distribution based on NMR spectroscopy

One of the most commonly-used methods to estimate the distribution of different types of carbon in fulvic acid is NMR spectroscopy. The distribution of carbon, as estimated by ^{13}C

NMR spectroscopy, amongst the different functional group classes present in PLFA, is summarised in Table 2.2. Similarly, the distribution of nitrogen in PLFA, as estimated by CP/MAS ^{15}N NMR, is summarised in Table 2.3.

Table 2.2 Estimates of carbon distribution in various fulvic acid references based on the peak area as percent of total carbon in quantitative liquid state ^{13}C NMR[1]

Type of Carbon	Pony Lake	Suwannee River (Std. I) [2]	Nordic Lake
Ketone/quinone 230–190 ppm	6.0	7.0	10
Carboxyl/amide/ quinone 190–165 ppm	19.0	20.0	24.0
Aromatic / Olefinic 165–110 ppm	13.0	24.0	31.0
Acetal / Ketal 110–90 ppm	0.3	5.0	7.0
O-Alkyl 90–60 ppm	8.0	11.0	12.0
Aliphatic 60–0 ppm	53.0	33.0	18.0

[1] (Thorn and Cox, 2009), [2] 1S101F

Table 2.3 Estimates of nitrogen distribution in various fulvic acid reference based on Peak areas as percent of total nitrogen in semi quantitative solid state CP/MAS ^{15}N NMR spectra [1]

Type of Nitrogen	Pony Lake	Suwannee River (Std. I)[2]	Nordic Lake	Elliot soil
Heterocyclic 250–160 ppm	29.0	36.6	41.3	21.6
Amide/amino/ quinone 160–60 ppm	67.1	62.2	48.2	73.0
Amino sugar/ terminal AA 60–0 ppm	3.9	2.7	10.5	5.5

[1] (Thorn and Cox, 2009), [2]1S101F

Based on these data, PLFA appears to be more aliphatic in nature (lower aromaticity) when compared with the other fulvic acids tabulated. The general trend for nitrogen containing functions is, however, similar for the three aquatic and one soil fulvic acids; they all contain a high level of amides (possibly peptides) and amino quinones compared to heterocylics and amino sugars or terminal amino acids. This similarity is interesting because PLFA (with a higher nitrogen content compared to other fulvic acids) would be expected to show atypical contributions of nitrogen-containing compounds. It can be concluded,

therefore, that either the nature of the nitrogen compounds is alike in all fulvic acids, or, that ^{15}N NMR does not provide an accurate representation of the type of nitrogen content. There is evidence to support each of these possibilities. Firstly, McKnight (1994) has proposed that the higher nitrogen level found in PLFA are simply due to the absence of lignin monomers from higher order plants, whose high oxygen levels effectively dilute the contribution of nitrogenous components. This is backed by the relatively lower oxygen levels found in PLFA. Therefore, it is likely that the nitrogenous compounds found in PLFA are similar to those found in nearly all fulvic acids, but with different contribution to total body mass. Alternately, a study of PLFA using $^{13}C\{^{14}N\}$ SPIDER NMR showed that 48% of all nitrogen is heterocyclic in nature (Fang et al., 2011). This result differs significantly from the results of CP/MAS ^{15}N NMR reported above, which indicated only a 29% contribution by heterocyclics. To obtain more information regarding the nature of the nitrogenous component of PLFA more analyses at the molecular-level is needed.

2.1.2 Derivatisation methods for GC analysis of fulvic acid

Derivatisation consists of chemically modifying a compound to produce a derivative of the compound that has properties more appropriate for analysis by GC. When it comes to fulvic acid analysis, the derivatisation typically includes the conversion of polar functional groups such as carboxylic acid, alcohols, phenols, amides and amines to less polar groups or to eliminate those functional groups producing alkanes. The following sections briefly describe each derivatisation employed in this study.

2.1.2.1 Trimethylsilylation

In this method, active hydrogens such as those on carboxylic acids, phenols, alcohols and amines are replaced with a trimethylsilyl (TMS) group via a nucleophilic substitution reaction (S_N2) with the silylating reagent. The trimethylsilylated compound thus formed is significantly less polar, more volatile and thermally stable compared to the underivatised compound. Being a nucleophilic substitution reaction, the silylation reaction is driven by a good leaving group, with the ability to stabilise a negative charge in the transitional state of the S_N2 reaction. There are several trimethylsilylation reagents commercially available for different purposes, differing in their leaving group such as BSA (N,O-bis(trimethylsilyl)acetamide), HMDS (hexamethyldisilazane), TMSI (N-trimethylsilylimidazole) MSTFA (N-methyl-trimethylsilyltrifluoroacetamide) and BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide). The last reagent, BSTFA, has been our choice throughout this study as it is highly volatile, produces by-products that are more volatile than other reagents, and can also act as its own solvent. For derivatisation of amides, many secondary amines and hindered hydroxyls, BSTFA alone is often insufficient to affect derivatisation. However, when a catalyst such as TMCS is added, many of these compounds can be readily derivatized. Therefore BSTFA+ 1% TMCS was used for all trimethylsilylation in this study.

2.1.2.2 Methylation

Another method for derivatisation of fulvic acid by GC is by alkylation, typically methylation, of its polar functional groups; the main targets of alkylation in fulvic acid are usually carboxylic acids, phenols and sometimes alcohols. Methylation of humic substances has previously been undertaken using a variety of reagents. However, most often methylation is used in conjunction with other modes of derivatisation or chemical degradation (Schnitzer and Calderoni, 1985) to assist with the obtaining DCM or chloroform-soluble material (Fujitake et al., 2003). The most common methylation reagents for humic substances have been diazomethane (Arsenie et al., 1992, Sachs et al., 2002, Schnitzer and Calderoni, 1985), methyl iodide in basic media and methyl iodide catalyzed by methylsulfinyl carbanion (Fujitake et al., 2003, Ikeya et al., 2006); the latter has recently been examined on fulvic acids after its successful application on carbohydrate methylation (Hakamori method). In this study, we have examined all of these methods on PLFA and have used the best method in combination with reduction (discussed later) to obtain a higher yield of GC-amenable material.

2.1.2.3 Alkyl Chloroformate derivatisation

Derivatisation using alkyl chloroformate has been most widely used for amino acid analysis (Qiu et al., 2007, Smart et al., 2010, Villas-Bôas et al., 2011). Nonetheless, alkyl chloroformates are versatile reagents due to the fact that they can convert carboxylic acids, phenols and amines to volatile esters, ethers and carbamates according to the mechanism shown in Figure 2.1. These functional groups are abundant in fulvic acid, making this reagent an appropriate choice for characterisation purposes; moreover, unlike many derivatisation agents which only work in non-aqueous media, alkyl chloroformates are reactive in an aqueous medium. To date, there have been no reported studies of humic substances using alkyl chloroformate derivatisation. Its application for the characterisation of PLFA, however, was explored in this study.

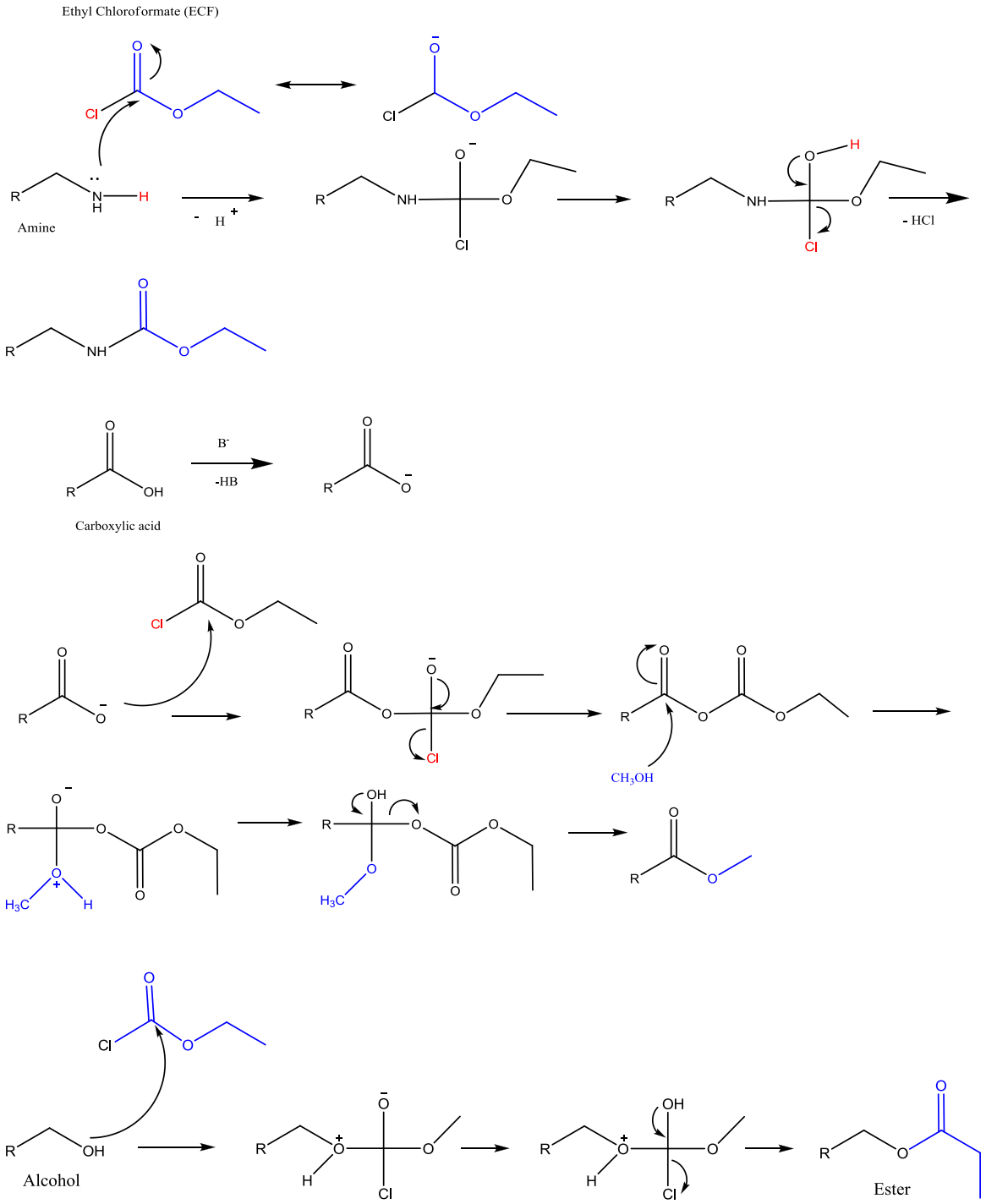

Figure 2.1 Scheme of chemical derivatisation of amines, carboxylic acids and alcohols using ethyl chloroformate (ECF) (Villas-Bôas et al., 2011)

2.1.2.4 Reduction

An alternate approach to derivatisation for the conversion of polar functional groups to less polar and thus more GC-amenable forms is reductive hydrogenation. A recent novel approach to reductive hydrogenation has been the use of alkyl silanes as the reducing agents.

Alkyl silanes have been shown capable of the simultaneous reduction of various functional groups such as carboxylic acids, alcohols, aldehydes and ketone to alkanes (Nimmagadda and McRae, 2006a;2006b). Using this method of reduction, employing n-butylsilane (n-BS) and tris (pentafluorophenyl) borane ($B(C_6F_5)_3$) as a catalyst, numerous backbone structures were identified from Suwannee River fulvic acid (Nimmagadda and McRae, 2007). This method has been applied in the present study for PLFA reduction and its subsequent analysis using GC/MS.

2.1.2.5 Combination of derivatisation methods

The combination of two or three derivatisation methods for the analysis of materials containing various functional groups is common in analytical chemistry especially when it comes to analysis of precious samples, normally available in limited quantities. This approach has also been considered for derivatisation of Antarctic fulvic acid in this study. The main advantage is the possibility of simultaneous analysis of functional groups with dissimilar reactivity toward specific reagents; these functional groups include amino acids, carboxylic acids, phenols, alcohols etc. Here, based on the approximate structure of our fulvic acid we have considered several reagents for combined derivatisations including combination of methylation and reduction, then methylation and trimethylsilylation. The result of these new approaches will be discussed later in this Chapter.

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2.2 Results

2.2.1 Analysis of bulk (unfractionated) PLFA

2.2.1.1 Amino acid analysis

As amino acids are an important family of nitrogen-containing compounds normally found in humic substances, a qualitative and quantitative analysis of total amino acids (AA) in PLFA was performed using UPLC after gas phase hydrolysis (Table 2.4).

Table 2.4 Amino acid (AA) distribution in Pony Lake Fulvic Acid (PLFA)

Peak[1]	Amino acid	Concentration [2] (mg/g)	(µmol/g)
H	Histidine	0.26	1.9
S	Serine	3.09	35.5
R	Arginine	1.25	8.0
G	Glycine	6.45	113.1
D	Aspartic acid[3]	9.97	86.6
E	Glutamic acid[3]	7.40	57.3
T	Threonine	3.69	36.5
A	Alanine	3.61	50.8
P	Proline	3.21	33.1
K	Lysine	2.24	17.4
Y	Tyrosine	1.45	8.9
M	Methionine	0.95	7.2
V	Valine	3.43	34.6
I	Isoleucine	2.25	19.9
L	Leucine	3.22	28.5
F	phenylalanine	2.29	15.5
Total		**54.73**	**554.6**

[1] peaks correspond to Figure 2.2, [2] mg of amino acid per g of PLFA, [3]because Asparagine is hydrolysed to Aspartic acid and Glutamine to Glutamic acid, the reported amount of these acids is the sum of those respective components

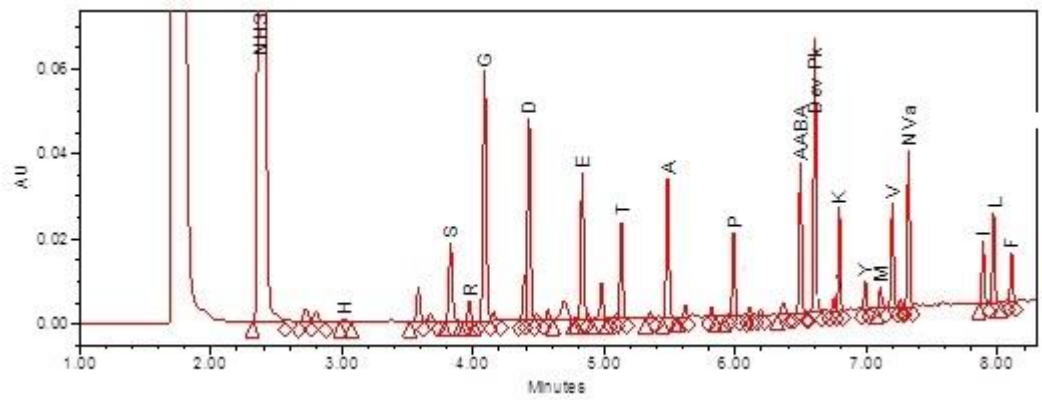


Figure 2.2 Chromatogram of separated amino acids in PLFA (NVa, AABA= internal standard, Dev Pk= side product of the derivatisation)

The result of analysis shows that of the amino acids produced after hydrolysis, glycine, aspartic acid, glutamic acid, threonine and alanine account for about 50 % of total amino acid (AA) distribution. Serine, proline, valine and leucine contribute to 13 % of AA detected. There are lower amounts of other AA such as lysine, phenylalanine, arginine, tyrosine etc. Similar results have been reported for fulvic acid isolated from two other Antarctic lakes, Lakes Fryxell and Hoare (Mcknight et al., 1991); in these two lakes around 70 % of total amino acids were glycine, aspartic acid, glutamic acid and alanine. The high contribution of these types of AA is in agreement with values reported for aquatic fulvic acids (40 to 65% contribution from major amino acids) (Thurman, 1985). An atypical feature in the AA profile for PLFA, however, is the relative abundance of alanine. Typically, alanine is among the most abundant reported AA in aquatic humic substances, however in PLFA it is only the fifth most abundant after threonine.

On the other hand, the total amino acid content for PLFA is very high (554.6 µmol/g). High total amino acid content is expectable for eutrophic lakes, however, the value for PLFA is more than 5 times greater than the largest value (98 µmol/mg) reported for samples from eutrophic lakes; this is notable as this high amount of total AA has been only previously been reported for soil humic acids (Thurman, 1985). The contribution of amino acid nitrogen to

total nitrogen of samples is not known, however, it can be estimated based on the value presented in Table 2.3. If we consider the average nitrogen percentage of the most abundant amino acids in PLFA (glycine, glutamic acid, aspartic acid, threonine and alanine) is roughly 11.17 % and with respect to the amino acid percentage in PLFA which is about 5.47 %, we will have around 0.61 % amino acid nitrogen in PLFA. Compared to its 6.5% total nitrogen (Table 2.1), the amino acid nitrogen is low and comprises less than 10.0% of total nitrogen. This suggests that nitrogen-containing compounds in PLFA are mostly non-peptidyl, and are possibly in the form of heterocyclic or amino-quinone structures.

Looking at individual amino acids, the abundance of glutamic acid confirms the presence of significant bacterial activity (Manning, 1991). The abundance of glycine, aspartic acid, glutamic acid is believed to be related to the material from the cell-wall of microorganisms (Stevenson, 1994). Sulfur-containing amino acids are also scarce and limited to methionine (0.95 % of total amino acids).

2.2.1.2 Molecular weight determination using Multi-Angle Laser Light Scattering (MALS)

An important bulk analytical technique for the measurement of absolute molecular mass and dimension of particles (e.g. radius of gyration) in solution is Multi Angle Laser Light Scattering (MALS). The recent development of the MALS detector and its combination with size exclusion chromatography (SEC) has been widely exploited for protein purification and structural studies. A few studies have also used this technique for the determination of size and molar mass of humic substances (von Wandruszkaa et al., 1999, Wagoner and Christman, 1998, Wagoner et al., 1997).

Based on the two basic principles of the light scattering, the degree to which light is scattered by the particle in the sample solution is related to its size or radius. According to the first principle the amount of light scattered is directly proportional to the product of the molar

mass and the molecular concentration, R(θ), in limit as θ→0, ∝ Mc]. The second principle states the variation of light scattered with respect to $\sin^2\theta/2$, in the limit as θ→0, is directly proportional to the average molecular mean square radius. $dR(\theta)/d\sin^2\theta/2 \propto \langle r_g^2 \rangle$ where M and c are the molar mass and concentration, respectively. r_g is particle radius of gyration (Wyatt Technology, 2014) .

The combination of two light scattering principles in one equation will be:

$$R(\theta) = K^* McP(\theta)[1 - 2A_2 McP(\theta)] \quad (1)$$

A_2 = Second virial coefficient

Also: $$K^* = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4}\left(\frac{dn}{dc}\right)^2 \quad (2)$$

n_0 – solvent refractive index

N_A – Avogadro's number

λ_0, vacuum wavelength of incident light

dn/*dc*, spec. refractive index increment

The instrument is required to measure the intensity of light scattered at zero angle. This is practically impossible, therefore as an alternative, light scattering is measured at many angles and extrapolated using a model to obtain scattering at a zero degree angle. The current instruments and their associated software normally perform all of these calculations and report the desired values as long as other parameters such as refractive index increment values (*dn/dc*) and concentrations are accurately calculated and set. *dn/dc* is representative of the change in solution refractive index (*dn*) as a function of the concentration (*dc*) , this value is in turn used for the calculation of instrumental optical constant, K* (equation (2)).

Ideally, for a better estimation of the mass and dimension of all fractions and particles in the solution, a chromatographic step such as SEC is combined with the MALS detection system in order to separate possible fractions (e.g. monomer, dimer for polymeric samples)

and then analyse the separated components with MALLS. However, due to the fact that SEC analysis of PLFA is beyond the scope of this study, we merely used light scattering technique to examine PLFA behaviour in the solution and obtain information about its mass and size.

In the first place, calculation of the refractive index increment (*dn/dc*) for PLFA was performed and yielded a value of 0.122. This value is critical for the calculation of MW by ASTRA software and was obtained by the construction of two calibration curves using solutions of a polymer with known *dn/dc* and solutions of PLFA under the same conditions. The ratio of slopes of two calibration plots is equal to the ratio of the *dn/dc* of the two substances.

Table 2.5 summarises the values calculated by ASTRA software as molar masses of PLFA, Ovalbumine as a reference protein and Suwannee River NOM (SWNOM). For comparison purposes, data previously reported for SWNOM using similar conditions is presented. At first glance, the molar mass calculated for PLFA is very high and close to mass of proteins or polymers; that is also the case for SWNOM. Regarding the high value, the primary explanation that can be suggested, would be the presence of aggregation in samples which leads to the formation of supramolecular aggregates (Piccolo, 2001; Piccolo et al., 2001; Piccolo, 2002).

Table 2.5 Molar mass (weight, number and average) of PLFA, Suwannee River NOM and ovalbumin as a sample protein analysed in similar condition

	PLFA[1]	**Ovalbumine[2]**	**SRNOM[3]**
Sample conc. (mg/mL)[4]	10.50	5.06	9.481
***dn/dc* (mL/g)**	0.122	0.188	0.135
Molar mass (g/mol)			
M_n	16770	50090	15050
M_w	29180	50900	20185
M (avg)	11630	46650	28390
polydispersity (M_w/M_n)	1.740	1.016	1.342

[1]Pony Lake FA analysed in current study, [2] Reference egg protein with mw of 43 KDa analysed using conditions similar to those used for PLFA, [3] Suwannee River NOM previously analysed (Wagoner et al., 1997). [4] all samples were prepared in 0.1 M phosphate buffer

The impact of high concentration in solution on aggregation has to be considered because light scattering detection normally needs concentrated samples which may encourage aggregation; moreover, the molar mass of humic substances is affected by different parameters in the solution such as pH, concentration and finally aggregation that can result in formation of large particles which adds to the value reported as molar mass. What was immediately observable during sample preparation was the formation of micelles-like components in the solution; when PLFA is simply dissolved in water, a large amount of foam appeared on the solution surface (Figure 2.3); this seems interesting and the reason for such behaviour, which is a function of high concentration, may be related to the composition of PLFA. The difference between values obtained for two NOM is notable; given that PLFA is a fulvic acid and Suwannee River NOM is an unfractionated mixture of fulvic and humic acid, the measured difference in molar mass would have expected to be greater than that found.

Therefore, it can be concluded that PLFA is potentially more prone to micelle formation than Suwannee River NOM. This behaviour of PLFA can be traced to old studies performed on physicaland chemical state of aggregated humic materials in solutions which will allow us to better understand the interactions that take place between humic materials and other components in natural water systems. For instance, Wershaw et al. (1977) proposed that humic acids are made up of a network of structural elements. At the lowest level in this network are simplefunctional groups such as phenolic, quinoid and benzene carboxylic acid groups which are covalently bonded together into small particles. Particles of similar chemical structure may be linked together by weak bonds to form "homogeneous" aggregates which may be linked in turn by weak bonding to other types of "homogeneous" aggregates to form mixed aggregates or micelles.

Overall, although the absolute molar mass obtained by this method is not very representative due to the batch analysis and absence of individual fractions to be studied precisely, it highlights some specific properties of this fulvic acid and implies that the technique can be useful for the studies of size and shape as a function of solution composition.

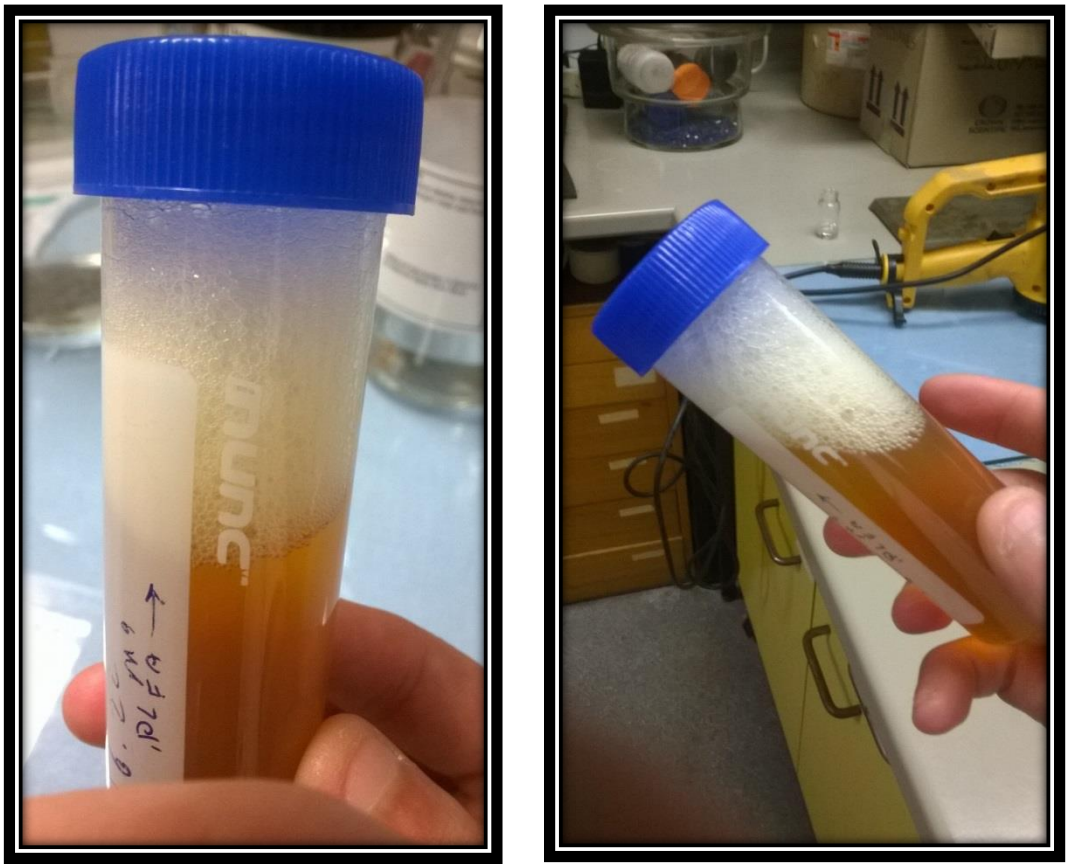


Figure 2.3 The aqueous solution of PLFA, foams above the solution can be a sign of micelle formation

2.2.1.3 Molecular weight determination using Vapour Pressure Osmometry

Vapour Pressure Osmometry (VPO) is a well-known technique for the determination of the number average molecular weight (M_n) of polymers. It has also found applications for fulvic acid molar mass determination as an unbiased and convenient technique. However, it is not without its limitations. When water is used as a solvent for VPO measurement, dissociation of any acidic groups can be a source of error for M_n measurement because the dissociated protons are counted as solute molecules, resulting in an incorrect value for the number of solute molecules present in the solution (Aiken and Malcolm, 1987, Gillam and Riley, 1981). Therefore, the majority of previous studies have used solvents other than water but compatible with the VPO procedure, such as THF, 1,4 dioxane etc. Unfortunately, PLFA is not dissolvable in common pure organic solvents and a mixture of those solvent with water is needed to prepare the solutions and to measure M_n. In order to obtain accurate results, a

correction method for measurements using binary solvents was suggested by Gilam and Riely (1981) and was employed in this study for PLFA MW measurements.

In order to obtain accurate results, here we used a mixture of a 1,4 dioxane and water (81.66% dioxane) as the measurement solvent and for ordinary VPO. Afterwards a correction method as described by Gilam and Riely (1981) was applied and M_n values were calculated using equations 3 to 5.

In fact, due to the presence of water, the pH values of the samples can be used for the calculation of value of a^* which is the slope of corrected osmolality (θ^*) versus concentration of sample and is calculated as follows:

$$\theta^* = \theta/(1 + [H^+]/C_a) \quad (3)$$

Where C_a is calculated as:

$$C_a = C\,a/K_{app} - [H^+] \quad (4)$$

(C= sample concentration, a = slope of osmolality vs. conc. curve, K_{app} =apparatus constant)

As K_{app} is calculable and equals the slope of concentration versus Osmolality for a non-dissociable standard compound; M_n value of analyte can be achieved:

$$M_n = \frac{K_{apparatus}}{a^*} \quad (5)$$

According to this method K_{app} for our instrument was found to be 2332.9 using glucose solutions in 1,4 dioxane: water mixture. Values for a, corrected a (a*) and M_n are presented in Table 2.6 for PLFA.

Table 2.6 VPO data and M_n for PLFA in 1,4 dioxane:water mixture

Conc. (gr/L)	θ	a	pH	θ*	a*	M_n(Da)
4.0	38.0	3.225	4.09	37.44	3.249	718.03
6.0	47.0		4.03	46.47		
8.0	51.5		4.00	51.03		
10.0	58.0		3.96	57.58		

In comparison to other fulvic acids, the value obtained for M_n of PLFA (718 Da) is lower than that of Suwannee River Fulvic Acid (829 Da) and consistent with the general idea that fulvic acids from marine environments are smaller than their freshwater counterparts (Gillam and Riley, 1981), however it is significantly higher than M_n values previously reported for Vestfold Hills Antarctic fulvic acids (Nimmagadda, 2008) which ranged from 483-567 Da.

2.2.1.4 Chemical analysis of bulk PLFA

2.2.1.4.1 Trimethylsilylation

The chromatogram of TMS-derivatised PLFA and the compounds identified are presented in Figure 2.4 and Table 2.7, respectively.

Surprisingly, a significant number of compounds are identifiable using this simple method of derivatisation; the method has enabled the detection of the following families of organic compounds: short and long chain fatty acids including unsaturated chains, sugars including mono and disaccharides as well as amino sugars, a few nitrogenous heterocyclic compounds and finally aromatic acids.

Almost the first half of whole the chromatogram (Fig 2.4) is dedicated to the volatile short mono and di alkanoic acid (straight and branched forms). Longer straight chain molecules up to C10 (peak 41) are also detected, the longest chain fatty acid detected was hexadecanoic acid. C12 to C16 n-alkanoic acids can be indicative of lipid present in algae (Cranwell, 1987).The nitrogen-containing compounds observed are small aliphatic amines (peak 13, 25) and amino sugars (peaks 49, 50). The abundance of these structures supports the aliphatic nature of Antarctic fulvic acid as predicted by NMR, specifically when aromatic molecules are quite rare relative to other types of fulvic acids and are limited to a few benzoic acid derivatives (peaks 16, 34, 42).

Table 2.7 List of Identified compounds in TMS-derivatised PLFA

Peak no[1]	Compounds Identified	Identification method[2]
1	Trimethylsilyl 4-(trimethylsilyl)butyl ether	a,d
2	Trimethylsilyl 2-trimethylsilyloxyacetate.	a,d
3	Pyruvic acid ditms	a,d
4	Propane, 2-methyl-1,2-bis(trimethylsiloxy)	b,d
5	2-trimethylsilyloxy-acrylic acid trimethylsilyl ester	a,d
6	Oxalic acid di tms	a,d
7	Propanedioic acid, bis(trimethylsilyl) ester	a,d
8	Pentanoic acid, 4-oxo-, trimethylsilyl ester	a,d
9	β-hydroxybutyric acid-ditms	a,d
10	2-butenoic acid, 2-[(trimethylsilyl)oxy], trimethylsilyl ester	a,d
11	Pentane, 1,5-bis[(trimethylsilyl)oxy]	b,d
12	β-hydroxy pyruvic acid, trimethylsilyl ether, trimethylsilyl ester	a,d
13	Glyoxime, bis(trimethylsilyl)	a,d
14	4-oxohexanoic acid, trimethylsilyl ester	a,d
15	Trimethylsilyl 3-[(trimethylsilyl)oxy]-3-butenoate	a,d
16	Benzoic acid trimethylsilyl ester	a,d
17	Trimethylsilyl ether of glycerol	a,d
18	Octanoic acid trimethylsilyl ester	a,d
19	Silanol, trimethyl-, phosphate	a,d
20	Malonic acid di tms	a,d
21	Maleic acid di tms	a,d
22	Succinic acid, ditms	a,d
23	Glyceric acid, tritms	a,d
24	Pyrotartaric acid-ditms	a,d
25	4-N,N-Dimethylamino(trimethylsilyl)butyrate	a,d
26	3-ethyl-4-methyl-1-(trimethylsilyl)-1h-pyrrole-2,5-dione	a,d
27	Bis(trimethylsilyl) fumarate	a,d
28	Hexandioic acid di tms	a,d
29	2-pentenedioic acid, 3-methyl-, bis(trimethylsilyl) ester	a,d

30	Trans-3 (cis)-5-bis[(trimethylsilyl)oxy]-(trans)-1-methyl, (cis)-2-t-butylcyclopentane	a,d
31	D-Eythro-pentofuranose, 2-deoxy-1,3,5-tris-o-(trimethylsilyl)	a,d
32	β -D-arabinose, tetrakis(trimethylsilyl	a,d
33	α- D-arabinose, tetrakis(trimethylsilyl	b,d
34	Benzeneacetic acid, α,4-bis[(trimethylsilyl)oxy]-, methyl ester	a,d
35	D-ribofuranose, 1,2,3,5-tetrakis-o-(trimethylsilyl)	c,d
36	D-xylopyranose, 1,2,3,4-tetrakis-o-(trimethylsilyl	a,d
37	Octanedioic acid, bis(trimethylsilyl) ester	a,d
38	Xylose tetrakis(trimethylsilyl)- open chain	a,d
39	α-D-mannotopyranose, 1,2,3,4,6-pentakis-o-(trimethylsilyl)-	a,d
40	β-D-galactofuranose, 1,2,3,5,6-pentakis-o-(trimethylsilyl)-	a,d
41	Azelaic acid, bis(trimethylsilyl) ester	a,d
42	Benzoic acid, 3,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	a,d
43	α-D-galactopyranose, 1,2,3,4,6-pentakis-o-(trimethylsilyl)	a,d
44	α-D-glucopyranose, 1,2,3,4,6-pentakis-o-(trimethylsilyl)	a,d
45	Mannose,-tetrakis-(trimethylsilyl)- open chain	a,d
46	Galactose,tetrakis(trimethylsilyl) open chain	a,d
47	α-D-glucose, -tetrakis-(trimethylsilyl)- open chain	a,d
48	Hexadecanoic acid, trimethylsilyl ester	a,d
49	α-D-glycopyranose, 2-(acetylamino)-2-deoxy-1,3,4,6-tetrakis-o-(trimethylsilyl)	b,d
50	α-D-mannopyranose, 2-(acetylamino)-2-deoxy-1,3,4,6-tetrakis-o-(trimethylsilyl)	b,d
51	α-D-glucopyranoside, 1,3,4,6-tetrakis-o-(trimethylsilyl)-β-d-fructofuranosyl 2,3,4,6-tetrakis-o-(trimethylsilyl)- (disaccharide)= (tms-disaccharide)	a,d

[1]Numbers correspond to the peaks in Fig 2.4, [2] identification was based on three methods (a) matching compound suggested by NIST Mass Spectral Search Program with above 90 % match probability (b) using NIST MS Interpreter with quality indices reported as above 85 % (c) tentative identification (d) suggested structures were checked against retention data reported in the literature or in (Medeiros and Simoneit, 2007)

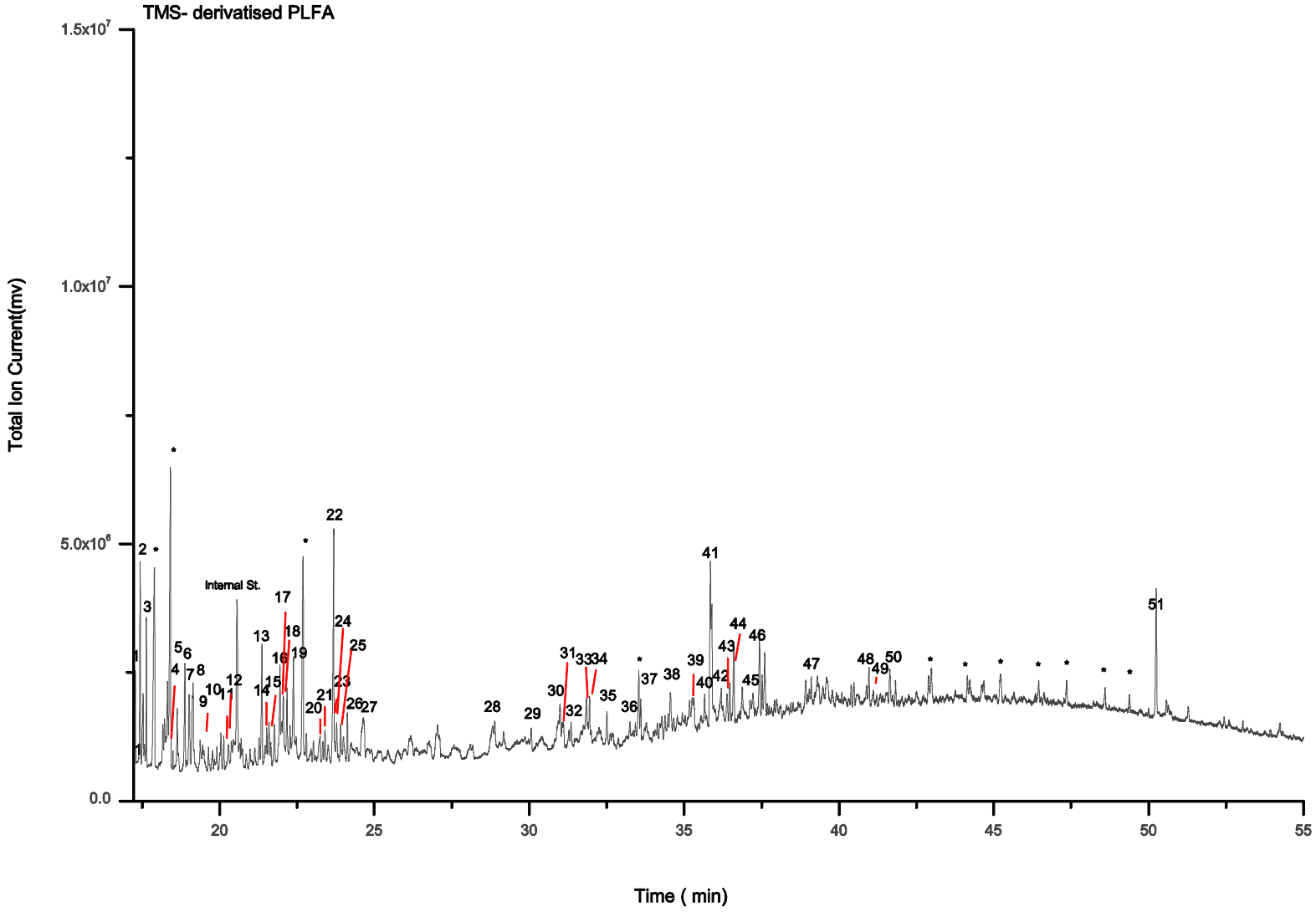


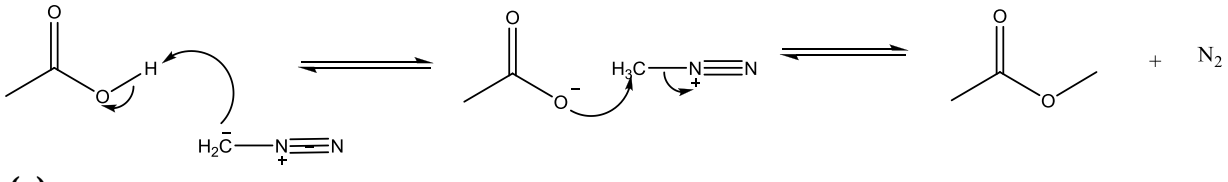
Figure 2.4 Total ion chromatogram of PLFA obtained using TMS- derivatisation, ** indicates siloxane or non-identified peaks

BSTFA is a strong reagent especially useful for alcohols TMS-derivatisation; herein the detection of sugars has been noteworthy and highlights the reagent efficiency. As a result some hexose and pentose monosaccharides including arabinose, xylose, glucose and amino glucose and mannose, also a disaccharide as peak 51(possibly sucrose) were detected. Moreover, it seems that these sugar molecules were accessible by the reagent, resulting in more efficient derivatisation and detection by GC.

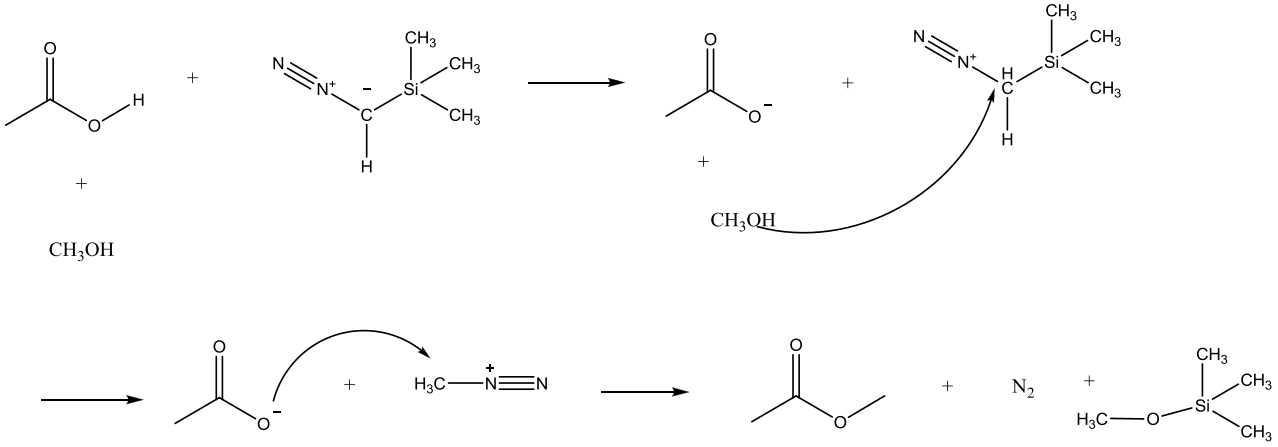
The reaction yield is around 14% calculated based on the sum of peak area of all detected products relative to the peak area of internal standard on GC chromatogram assuming response factor of 1 for the analytes relative to the internal standard.The low yield suggests that only parts of material that were accessible by the reagent became detected using this approach. It is not far-fetched to assume that they formed an external layer surrounding the inaccessible refractory core that is either sterically hindered or not GC-amenable. Given that only a very limited number of nitrogenous compounds were recovered from this nitrogen-rich material during TMS-derivatisation, the difficulties normally associated with the TMS-derivatisation of amino compounds, may be a limiting factor.

2.2.1.4.2 Methylation

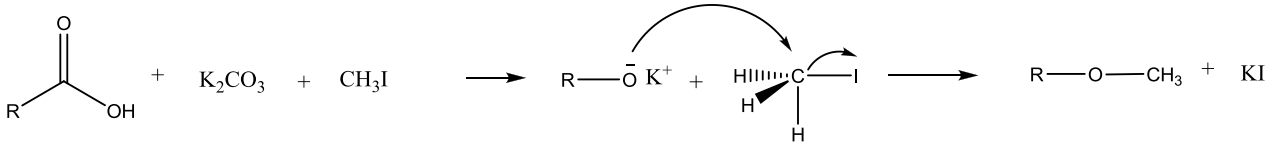
Another strategy for conversion of polar functional groups to the less polar ones is methylation. Various methylation procedure including classical diazomethane, trimethylsilyl diazomethane (TMSD), and methyl iodide were employed for methylation of PLFA (Figure 2.5).



(a)



(b) (Seyferth et al., 1972)



(c)

Figure 2.5 Mechanisms of mehtylation of carboxylic acid (or alcohols) using (a) diazomethane (b) trimethylsilyldiazomethane and (c) methyl Iodide

In the early stage of methylation approach diazomethane methylation was attempted, however did not yield methylated products (Benzoic acid as a model compound was tested with this reagent and completely methylated), this is interesting, but inconsistent with previous studies using diazomethane for humic substances acid methylation, for instance,

weathered coal fulvic acid (Zhoua and Zhang, 1994) was characterised using this methylation method, also Schnitzer and Calderoni (1985) used diazomethane for structural study of humic acids. As an alternative, trimethylsilyldiazomethane (TMSD) was investigated. TMSD is easier to use (less-explosive), also able to methylate alcohols where present. However, despite the fact that it was combined with TMS-derivatisation, it too appeared inadequate to form GC-amenable products (section 2.2.1.4.5).

The failure to become methylated was assumed, to some extent, to be due to the presence of non-protonated carboxylic acid groups, resulting in incomplete reaction. Yet, the reaction of cation-exchanged sample was also unsuccessful. Therefore, we switched to methylation using methylsulfinyl carbanion; the result of this methylation appeared as unreliable due to the harsh reaction condition and rearrangements occurred to products. The last approach seemingly more compatible to the nature of hydrophilic PLFA was methylation using strong electophile, methyl iodide, we combined this methylation with reduction; the correspondent data is presented in section 2.2.1.4.4.

2.2.1.4.3 Reduction

The result of reduction of PLFA using n-BS and $B(C_6F_5)_3$ is shown in Figure 2.6 and compounds identified as a result of reaction are presented in Table 2.8. As shown in that Table, there are very few compounds detected as a result of reduction of PLFA; as the final products of the reduction are ideally supposed to be hydrocarbons (without any functional group).The majority of products reported are aliphatic including both saturated and unsaturated compounds. Nevertheless, there are non-reduced products (peak 3). The difficulty involved in the deconvolution of peaks due to the presence of a large number of silyl ethers as reaction by-products and siloxanes (formed due to the presence of moisture during the course of the reaction) has also contributed to the inadequate products identification.

The results clearly indicate that a major part of fulvic material has not been recovered as GC-amenable materials; this could be due tho the fact that either the reductive hydrogenation was not successful, or, that the reduction products were very volatile and lost. The former may happen due to the presence of amino compounds with the basic properties that could destroy the lewis acid catalyst of the reaction. On the other hand, the volatility of the reduced products (reduced forms of small molecules in Table 2.7) could impede their detection.The majority of compounds detected belong to the saturated alkane family; among them some interesting alicyclic compounds are observed (peaks 5, 14).

Table 2.8 Identified compound in the reduced PLFA

Peak No	Compounds identified	Identification method
1	3-octanone	a,d
2	Decane	a,d
3	4,4-Dimethyl-3-hexanol	a,d
4	2-methyl nonane	a,d
5	2,4-Diethyl-3,3-dimethycyclolopentene	a,d
6	Undecane	a,d
7	1H-Pyrrole, 2,3,4,5-tetramethyl	a,d
8	Decane, 2,4-dimethyl	a,d
9	Dodecane	a,d
10	4-Ethyl-2,2-dimethyl-3-octene	b,d
11	Cyclohexane, hexyl-	a,d
12	Dodecane, 2,6,11-trimethyl	a,d
13	Tridecane	a,d
14	Ethane-1,1-diymethyldicyclohexane	c,d
15	Tetradecane	a,d
16	Cyclohexane, n-octyl-	a,d
17	Hexadecane	a,d
18	Heptadecane	a,d
19	Eicosane	a,d

[1]Numbers correspond to the peaks in Fig 2.6, [2] identification was based on three methods (a) matching compound suggested by NIST Mass Spectral Search Program with above 90 % match probability (b) using NIST MS Interpreter with quality indices reported as above 85 % (c) tentative identification (d) suggested structures were checked against retention data reported in the literature

Saturated long chain alkanes which are likely products of fatty acids reduction are expectedly observed. Overall, the number of compounds detected is significantly less than those observed using TMS-derivatisation. In comparison to the previous study undertaken on

reduction of fulvic material (Nimmagadda and McRae, 2007), it became evident that PLFA has not been reduced as efficiently as Suwannee River FA or soil and peat FAs.

The results of previous study investigating reduction of another nitrogen-rich Antarctic fulvic acid form Pendant Lake Vestfold Hills (Nimmagadda, 2008) showed some similarities with current reduction results in terms of compounds detected such as cyclohexane derivatives, C8-C11 linear and branched chain alkanes as well as non-reduced alcohols. However, terpenes (linear and cyclic) and alkyl benzenes are not very abundant in PLFA while several of them were previously reported in the reduced Pendant Lake Fulvic Acid (PNFA) by Nimmagadda (2008). Moreover, larger hydrocarbons up to C20 are more found in PLFA rather than PNFA; these structures could be the reduced form of the long chain fatty acids that were detected after trimethylsilylation of PLFA.

In summary, reduction of bulk PLFA seems unable to identify additional building blocks using GC/MS, mainly because the major part of it would become volatile after reduction and, therefore non-detectable. In order to improve the yield and performance of the reduction, its combination with other derivatisation methods will be examined and discussed in the following sections.

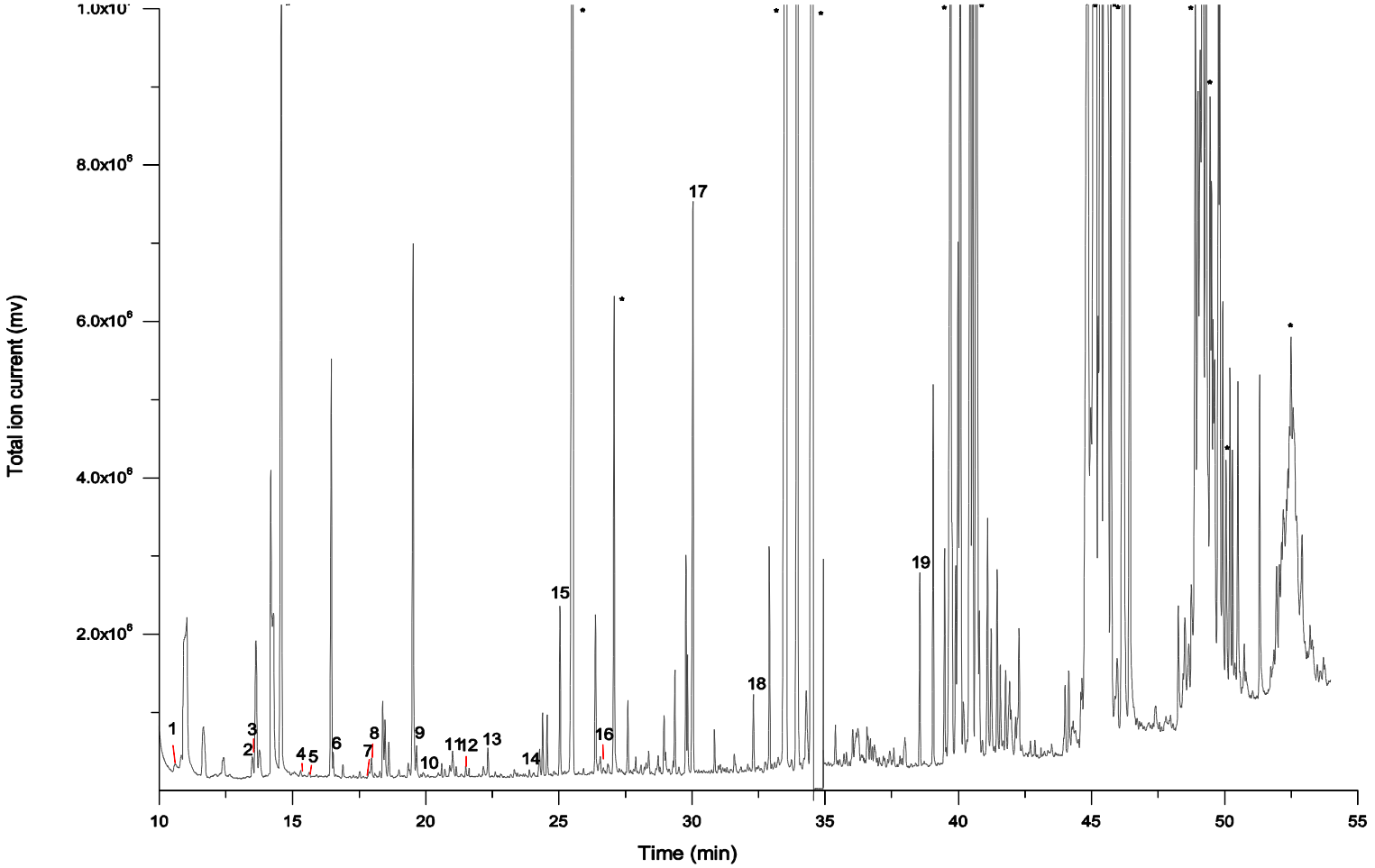


Figure 2.6 Total ion chromatogram of PLFA products obtained after reduction, * indicates siloxanes or silyl ether peaks (byproducts of the reaction)

2.2.1.4.4 Methylation combined with Reduction

In an attempt to enhance the performance of the reduction reaction, the combination of O-methylation and subsequent reduction of functional groups were performed. The reasons behind this approach were firstly to derivatise functional groups such as phenols which are not fully reduced (n-BS reduces phenols to silyl ethers), also to decrease intramolecular hydrogen bonding which limits the access to the substrate reaction sites, another reason was to assist the material to become more soluble in DCM after reduction. Furthermore, an effective methylation could facilitate the reduction of remaining material because the only part that needs reduction would be methyl esters rather than free acid and amine. Figure 2.7 summarises the steps to perform methylation combined with reduction for PLFA. The attempts to reduce the material in the supernatant (acetonitrile extracted) did not yield any products, indicating fulvic material remained in the aqueous phase.The results of the reduction of aqueous phase are presented in Figure 2.8 and Table 2.9.

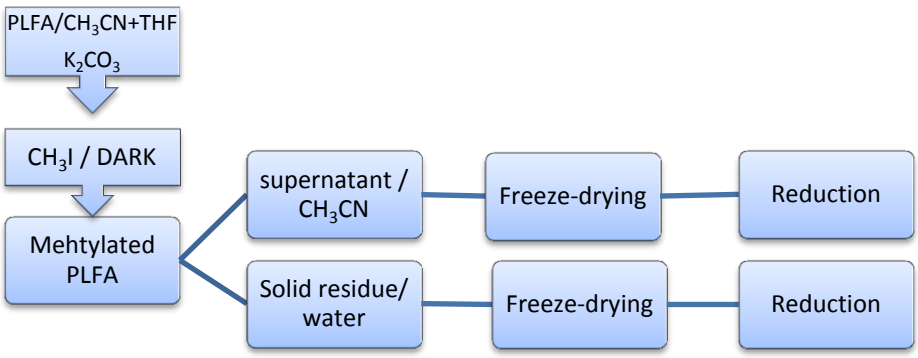


Figure 2.7 Flow chart of the scheme yielding methylated reduced PLFA

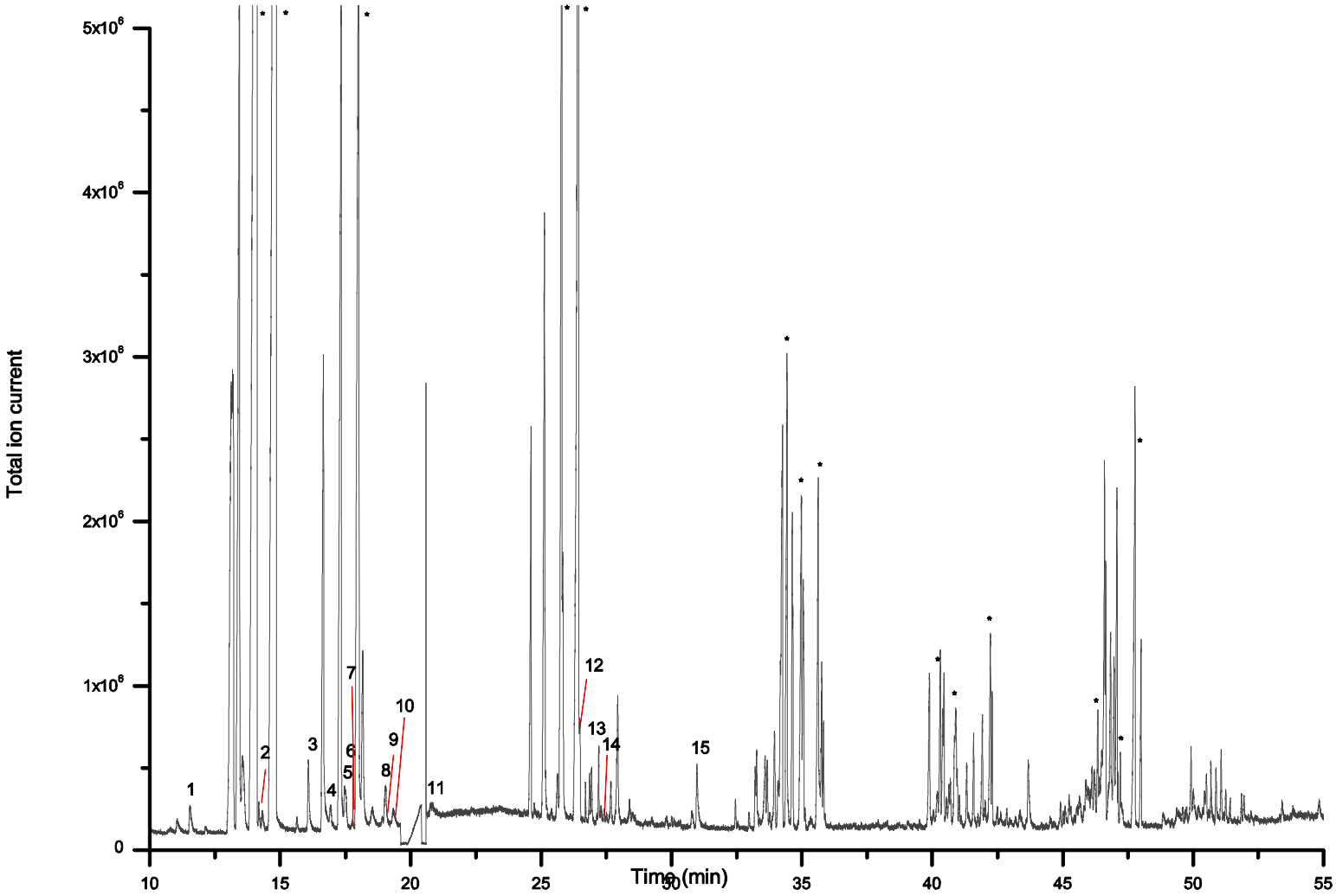


Figure 2.8 Total ion chromatogram of PLFA products obtained after methylation and reduction, * indicates siloxanes or silyl ether (as by products of reaction) peaks

Table 2.9 Identified compound PLFA that methylated and reduced

Peak no	Compounds identified	Identification method
1	1,1-Dimethoxy-Octane	a,d
2	2-Acetoxy-5,5-Dimethylcyclohexa-1,3-Diene	a,d
3	1,3-Dioxolane-2-Ethanol, 2-Methyl	a,d
4	Acetic acid, 2-propyltetrahydropyran-3-yl ester	c,d
5	Cyclohexene, 1-acetyl-2-(1-hydroxyethyl	c,d
6	Cyclohexene, 1-acetyl-2-(1-Hydroxyethyl	c,d
7	2,7-Dimethyloctane	a,d
8	2-Pentene-5-butoxy	a,d
9	Methyl (Z)-4-[(2-oxopropyl)oxy]-2-butenoate	a,d
10	2,5-Norbornanedione monoethylene acetal	c,d
11	2-Methyl-1,3-oxathiolane-2-acetic acid Methyl Ester	a,d
12	3-Butyl-1H-Indole	c,d
13	Octadecane	a,d
14	1H-Pyrazole, 3,5-bis(1,1-dimethylethyl)-4-ethyl-	b,d
15	Ethyl 3-methyl-4-oxononanoate	b,d
16	3-methyl- tridecane	a,d

[1]Numbers correspond to the peaks in Fig 2.8, [2] identification was based on three methods (a) matching compound suggested by NIST Mass Spectral Search Program with above 90 % match probability (b) using NIST MS Interpreter with quality indices reported as above 85 % (c) tentative identification (d) suggested structures were checked against retention data reported in the literature.

According to the results, the combination of methylation and derivatisation yielded very few recognisable structures; in some cases (peaks 4, 15) unreduced functional groups still exist. Cyclic ethers (peaks 4, 9, 10) are also detected which were not observed in the reduced PLFA. Alkanes are also among main products as was detected for reduced PLFA.

Apparently, methylation did not complete due to the fact that acetonitrile-extractable material was trace so that it did not show any product peak in the next reduction step. Obviously, the approach could not help to improve the reduction efficiency as ultimately very few peaks were identified as reaction products. The possible reasons for incomplete methylation have to be associated with the state and position of functional groups in the structure. Incomplete reaction can be due to severe steric hindrance of carboxylic acid groups, preventing the methylation reagent to approach substrate. This explanation does not seem convincing because reagents used such as methyl iodide are theoretically open to nucleophile attack by functional groups. Another possibility is that there might be a reaction between functional group in sample and reagent, but not with sufficient yield to make the entire

material GC-amenable and that is why no or very limited derivatised components are observable by GC/MS analysis.

2.2.1.4.5 Methylation combined with trimethylsilylation

In a separate experiment methylation using trimethylsilyldiazomethane (TMSD) combined with trimethylsilylation using BSTFA/TMCS was examined. The initial idea was methylation of carboxylic acids and phenols then trimethylsilylation of alcohols. However, the final result did not show any improvement relative to single step TMS-derivatisation both qualitatively and quantitatively (Figure 2.9).

As the sample became fully protonated before methylation, the methylation should have been taken place at least for more reactive groups, however it seems that its combination with TMS -derivatisation did not improve the GC-analysable parts of the material. In other words, the degree to which methylation contributes to the detection of PLFA by GC/MS is not substantial, trimethylsilylation, however, plays a more important role for the derivatisation rather than methylation.

The reason why methylation is not successful can be associated with the type of functional groups that become methylated with this reagent. As mentioned earlier, carboxylic acids and phenols are the best candidates, but it is not the case for alcohols and that is why TMSD is sometimes combined with trimethylsilylation (Kowalewski and Gierczak, 2011); TMSD has also found applications for the analysis of multifunctional substrates (Kowalewski and Gierczak, 2011), natural products and herbicides (Ranz;Eberl; et al., 2008, Ranz;Korpecka; et al., 2008). Applications of TMSD on humic substances, however, have been limited so far and to the best of our knowledge the only study related to the methylation of humic acid was to investigate its metal transport properties (Klučáková et al., 2013). Nevertheless, due to the fact that TMSD methylation is a mild method, its ability to react with

the complicated materials such as fulvic acids, with high possibility of engaging carboxylic acid in intramolecular hydrogen bonding, remains uncertain.

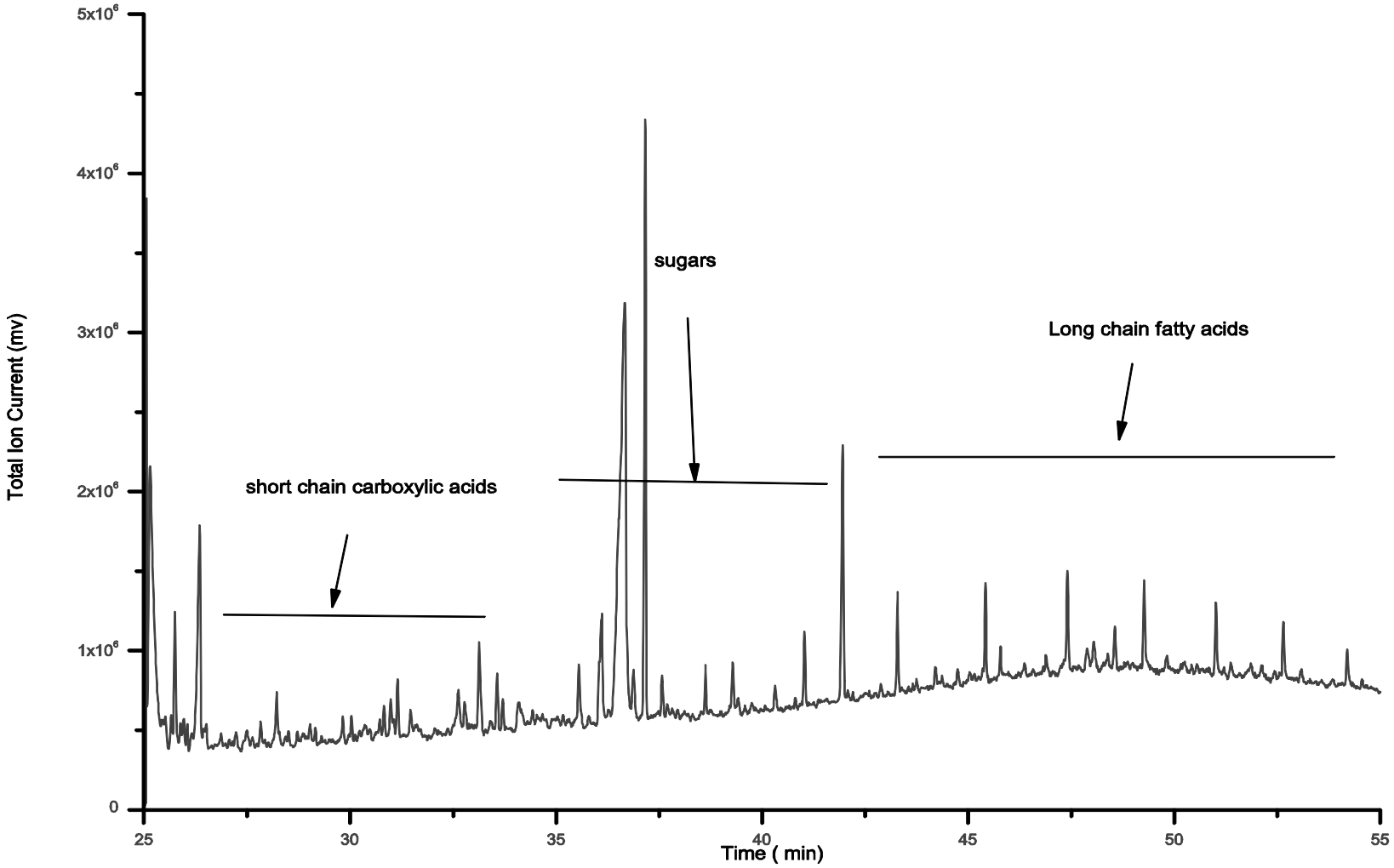


Figure 2.9 Total ion Chromatogram of PLFA exposed to combined TMSD and TMS derivatisation

2.2.1.4.6 Alkyl chloroformate derivatisation

The result of derivatisation of a model compound (4-(4-Aminophenyl) butyric acid) using ethyl chloroformate (ECF)-GC/MS and its derivatisation scheme are presented in Figures 2.10 and 2.11, respectively.

As demonstrated in Figure 2.10, ECF derivatisation has been able to convert the model compound containing amino and carboxylic acid to their carbamate and methylester derivatives (the mechanism of conversion of model molecule to the derivatised product is displayed in Fig 2.11) with good yield (86% based on the peak areas of GC/MS and assuming response factor of 1 for analyte versus internal standard).

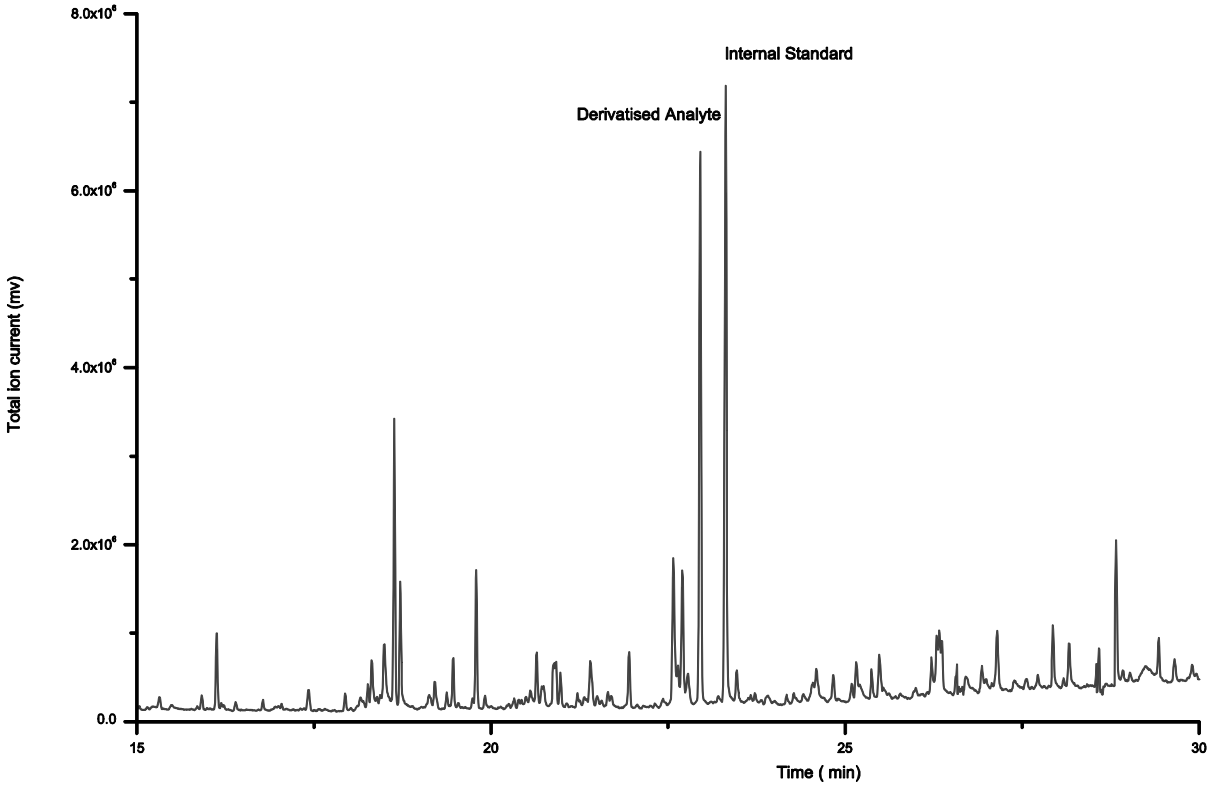


Figure 2.10 Derivatisation of 4-(4-Aminophenyl) butyric acid with ECF - GC/MS (reaction yield= 86.0 %)

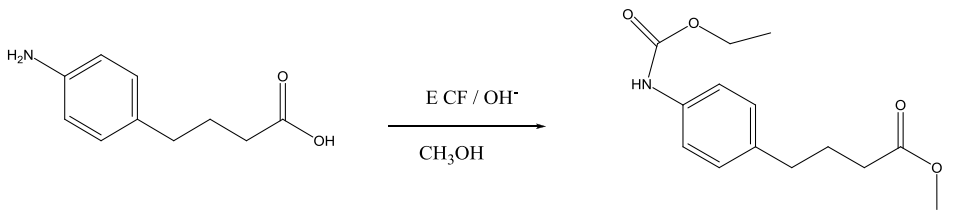


Figure 2.11 scheme of chemical derivatization of 4-(4-Aminophenyl) butyric acid using ethyl chloroformate (ECF)

After successful completion of ECF-derivatisation on a model, it was also performed on PLFA. The compound identified and chromatogram of PLFA derivatised using ECF is presented in Table 2.10 and Figure 2.12, respectively.

As shown in the results, this derivatisation has been more effective than reduction as more compounds have been identified, although the nature of components detected are similar in the two methods. For example, terpenoids (peaks 6), long chain ethers (peak 18) as well as alkandioic acids (peak 23) and long chain fatty acids which were also detected as TMS-derivatised products (peaks 27 to 32). The aromatics are not abundant; however nitrogenous compounds are predictably detected (peaks 5, 13, 14, 16) in line with the goal of this derivatisation that was derivatisation of amino compounds.

Table 2.10 Identified compound PLFA derivatised using ECF-GC/MS

Peak no[1]	Compounds Identified	Identification Mehtod[2]
1	Benzoic Acid, methyl ester	a,d
2	1-(ethoxyethoxy)-2-propanol	a,d
3	2-t-Butyl-5,5,6-trimethyl-[1,3]dioxan-4-one	a,d
4	Hexanoic acid, 2-athyl-, methyl Ester	a,d
5	N-carboethoxyimidazole	a,d
6	5-octene, 2,7-dimethyl-3-vinyl-	a,d
7	Benzoic acid	a,d
8	Nonanoic acid	a,d
9	2-acetoxy-2-methyl-4-phenyl-3-butanone	c,d
10	1-decene	a,d
11	Decanal	a,d
12	Dodecane	a,d
13	1-Piperidinecarboxylic acid, ethyl ester	b,d
14	Ethyl 3-methylcyclopent-1-enylcarbamate	b,d
15	1-undecene, 9-methyl-	a,d
16	2,5-pyrrolidinedione, 3-ethyl-4-methyl	b,d
17	(E)-hept-3-enoic acid	a,d
18	Cis-1-acetoxy-1-heptene	a,d
19	2-Propenoic Acid, 1,4-butanediyl ester	a,d
20	Tetradecane	a,d
21	1-decene, 3,4-dimethyl-	a,d
22	Dimethyl β-Isopropenyl-α-Methylenesuccinate	a,d
23	Nonanedioic acid, dimethyl ester	a,d
24	3-hexenoic acid, 3-hexenyl ester	a,d
25	Pentadecanoic acid, methyl Ester	a,d
26	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	a,d
27	Hexadecanoic acid, methyl ester	a,d
28	Hexadecanoic acid	a,d
29	Hexadecanoic acid, ethyl ester	a,d
30	Hexadecane, 2,6,10,14-tetramethyl	a,d
31	Octadecanoic acid	a,d
32	Octadecanoic acid, ethyl ester	a,d

[1]Numbers correspond to the peaks in Fig 2.12, [2] identification was based on three methods (a) matching compound suggested by NIST Mass Spectral Search Program with above 90 % match probability (b) using NIST MS Interpreter with quality indices reported as above 85 % (c) tentative identification (d) suggested structures were checked against retention data reported in the literature.

There are also some non-derivatised carboxylic acids (peaks 7, 8, 17, 28) their presence may be due to incomplete derivatisation or possibly they are contamination entered the sample after reaction and during extraction process.

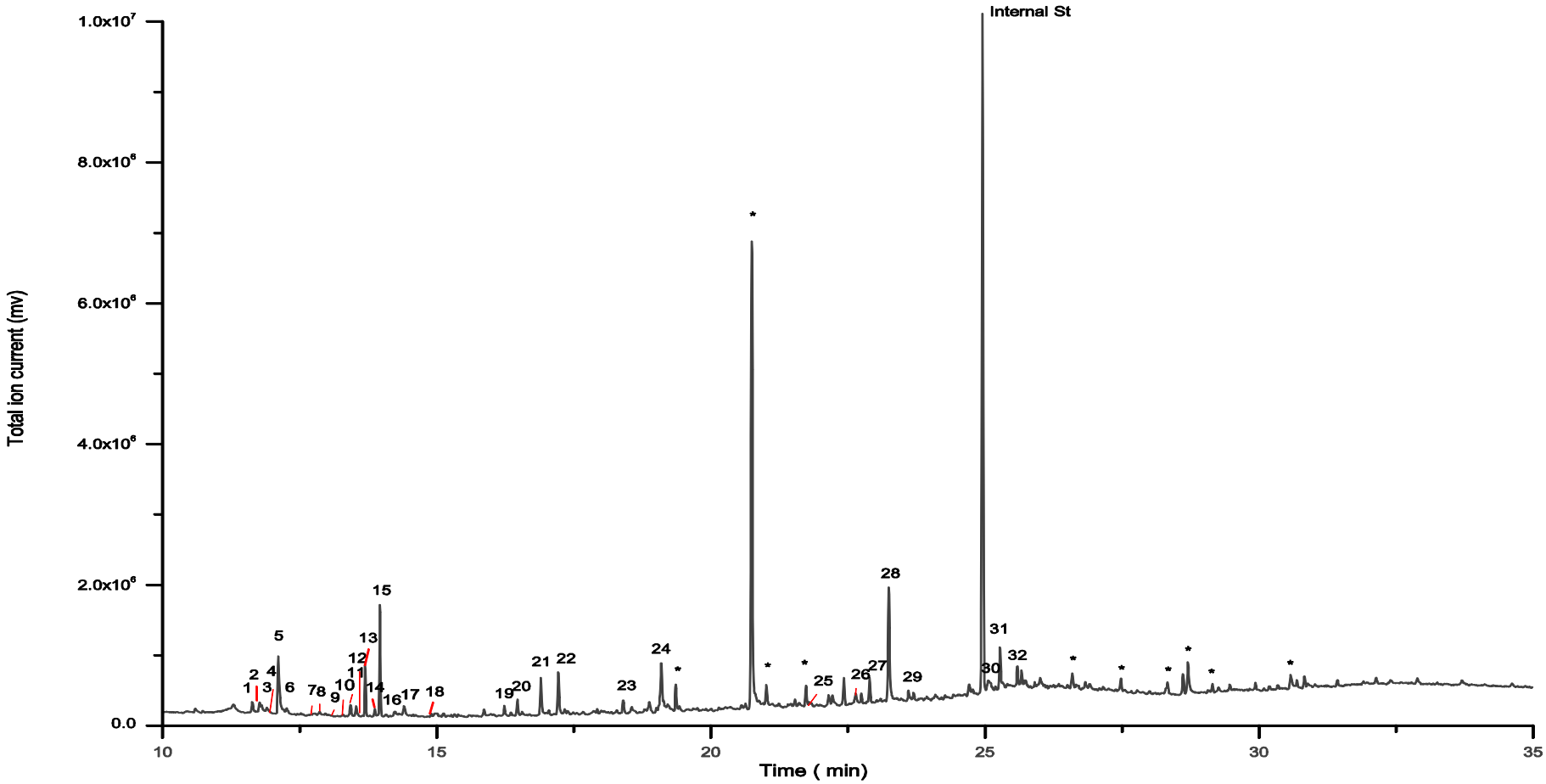


Figure 2.12 Total ion chromatogram of PLFA products obtained after derivatisation with ECF, * indicates impurities in reagents or solvents ** indicates non-identified peaks

2.3 Discussion

PLFA, as a reference Antarctic fulvic acid and a pure microbially derived fulvic acid, displays some distinct characteristics relative to its counterparts from other environments. Apart from its isolated environment and confined source material, Antarctic fulvic acid differs due to its high nitrogen content that has raised questions about its origin and formation pathways. The analysis of bulk sample of PLFA revealed some interesting features of this microbial DOM. The formation of the micelle structure is of great importance as it evidently supports the presence of supramolecule fulvic material suggested in previous works (Piccolo, 2001; Piccolo, 2002). The information that we obtained through our chemical analysis in this chapter indicated that the part of fulvic material that is extractable by organic solvents and analyzable using GC/MS is quite limited, or in other words it is less than what has been obtained for other kind of fulvic acids such as freshwater and soil fulvic acid under similar conditions (Nimmagadda and McRae, 2007). Even so, it seems that the quantity of this GC-analysable material is improvable by using appropriate methods; because as we observed in section 2.2.1.4.1 trimethylsilylation was able to derivatise many compounds in PLFA and was more efficient than reduction. Almost the same principle applies to chloroformate derivatisation of PLFA. Furthermore, some difficulties were encountered for identification of components due to the peak overlaps and possibly incomplete deconvolution of signals. Basically, the results obtained from the methylation methods showed the lowest efficiency which is surprising for well-known reagents (methyliodide and diazomethane) under S_N2 mechanism causing less rowding at the reaction center. Overall, these methods, under the mild conditions used, can not be sufficient to obtain complete methylation. In contrast, the trimethylsilylation had better recovery values compared to the other methods. It presents the best balance combination between detection of a range of compounds, and consistent identificationof components. It is likely that using NaOH along with methanol in

chloroformate derivatisation could compete in reaction with PLFA and explains the low yield of the reaction. Accordingly, the combination of derivatisations could be appropriate for the routine analysis and compositional study of simpler samples, whereas for fulvic acid it makes the analysis more complicated.

One method to conquer these types of problems in complex matrices such as PLFA could be performing fractionation of original material into more analysable and separable fractions. This is not only effective to enhance the chemical separation, but can be intrinsically interesting because it enables the study of PLFA behaviour during fractionation and quality of its breakdown from the original network to small fractions. Also, as highlighted throughout this chapter, PLFA contains a significant amount of nitrogenous compounds that were not completely identified using the techniques employed here and there is a need to conduct further analyses. Therefore, in the next chapter we will firstly focus on fractionation, then analysis of the resulted fractions. At the next step, we will try to carefully identify the compounds especially nitrogen-containing compounds detected.

2.4 Materials and Methods

2.4.1 PLFA Sample

The Pony Lake was purchased from IHSS (International Humic Substance Society) and in all experiments was used as obtained unless otherwise stated; The isolation was performed as follows: the acidified Pony Lake water was processed in parallel through two 4 L XAD-8 columns. The acidified lake water was applied to these columns and back eluted with 0.1 N NaOH. The eluent was acidified to pH 2 with HCl immediately and stored at 4°C until re-concentration. The eluate was applied to a 2 L column and was rinsed with deionised water to remove Cl^- and back eluted with 0.1 N NaOH. The NaOH eluent from the 2 L column was instantly applied to a cation exchange column to remove the sodium and then freeze-dried as PLFA sample (Cawley et al., 2013).

2.4.2 Amino acid analysis

PLFA, as supplied by IHSS, was resuspended in MilliQ water. Duplicate aliquots were dried for hydrolysis and further aliquots dried for free amino acid analysis. For High sensitivity amino acid analysis samples underwent gas phase hydrolysis in 6 M HCl at 110°C for 24 hours. After hydrolysis all amino acids were analysed using the Waters AccQTag Ultra chemistry on a Waters Acquity UPLC. For Free amino acid analysis, aliquots of prepared sample in MilliQ water were dried and derivatised without hydrolysis using the Waters AccQTag Ultra chemistry. The sample was analysed in duplicate and results were expressed as the average.

2.4.3 Molecular weight determination using Multi-Angle Laser Light Scattering (MALS)

PLFA sample was used as obtained to prepare 10.00, 5.00, 2.50, 1.25 mg/mL solutions in 0.1 M phosphate buffer (0.05 M Na_2HPO_4 and 0.05 M NaH_2PO_4) with pH 6.8. This pH ensured solubility of unfractionated PLFA as it does not have high solubility in MilliQ water. No chromatographic separation was performed for sample and only on-line (batch) analysis was accomplished with a Wyatt Technology miniDAWN laser light scattering photometer, using a 658 nm laser, and a Shimadzu RID-10 refractive index (RI) detector. Bovine serum albumin (BSA) was used for normalization of the miniDAWN detectors at 41.5° and 138.5°, relative to the 90° detector. The calculation of the refractive index increment (*dn/dc*) which is used for the calculation of the light scattering instrumental optical constant, K*, and of the mass-based concentration of the analyte was performed as follows: Peak areas obtained by RI detector for samples (unfractionated PLFA and PLFR 1) with concentration ranging 10.00, 5.00, 2.50, 1.25 mg/mL, were used for construction of an area vs. concentration plot and the slope was obtained. Similar plot was constructed for ovalbumine. The ratio of the slopes of two plots was calculated to be 1.523. As this ratio is proportional to the ratio of refractive index increments and given that for ovalbumin, *dn/dc* equals to 0.188, *dn/dc* of PLFA will be 0.122. This value was given to the ASTRA 5.1 software for size and molecular weight calculations.

2.4.4 Molecular weight determination using Vapour Pressure Osmometry

A Westcor Vapro 5520 vapour pressure osmometer was used to measure the number average molecular weight (M_n) of PLFA according to the method of Gillam and Riley (1981) except for the solvent which was replaced by water:1,4 dioxane mixture (81.66% dioxane). The reason behind this selection was that 1,4 dioxane is able to dissolve all the PLFA fractions (except PLFR1), also 81.66: 18.34 dioxanen: water solution is an azeotropic micture, and therefore appropriate for VPO measurement. A set of solutions of a non-dissociable

compound such as glucose in the solvent ranging 4-10 g/L was prepared for construction of concentration versus osmolality plot and the slope calculated; then apparatus constant (K_{app}) was measured.

PLFA solutions ranging 4-10 g/L in water:1,4 dioxane mixture (81.66% dioxane) were also prepared and pH of all solutions were accurately measured using Beckman pH meter, then a plot similar to glucose plot was built and the slop that equals value of "a" was obtained. Afterwards, using the pH values of solutions, the corrected value of "a" obtained and M_n was calculated using this corrected value and K_{app}.

2.4.5 Trimethylsilylation of PLFA

2.4.5.1 Samples and chemicals

PLFA was purchased from IHSS, BSTFA:TMCS (99:1) and anhydrous pyridine was obtained from Sigma-Aldrich and kept away from any exposure to air and moisture during handling using sealed bottles. Dodecane was purchased form Sigma-aldrich and was used to prepare internal standard solutions.

2.4.5.2 Derivatisation procedure

Sample preparation

PLFA (1.0 mg) was accurately weighed to 0.01mg and placed in a 1 mL oven-dried glass reaction vial containing small magnet (the glass reaction vial was left in the oven at 110^0C for 24 h before use. It was also equipped with mininert valve to prevent any exposure to air). The vial containing sample was purged with nitrogen and set aside.

Derivatisation

20 µL anhydrous pyridine was added by syringe through the mininert valve to the reaction vial and mixed well, then 5 µL of 100 ppm dodecane in anhydrous pyridine solution was added as internal standard. At this stage 50 µL BSTFA:TMCS (99:1) was added dropwise,

after a few seconds the stirring solution was placed in a ultrasonic bath at 60 ^{0}C for 30 minutes. The reaction was then transferred to a hotplate at 60 ^{0}C and stirred for a further 15 hours. 0.5 µL of prepared sample was directly injected to GC/MS for analysis. A similar procedure was performed to prepare the blank sample without adding PLFA.

2.4.5.3 GC/MS analysis

A Shimadzu Gas chromatograph-mass spectrometer (QP-2010) was used for separation and identification of relevant samples equipped with a SGE BPX-5 capillary column (30m × 0.15 mmID × 0.15 µm film thickness). 0.5 µL of sample was injected while splitless injection was performed with a sampling time of 1.5 minutes. Injector temperature was 270 ºC and MS interface temperature was 300 ºC. The temperature program ran as 60 ºC for 4 minutes, then ramping to 320 ºC at 5 ºC/ minute and holding at 320 ^{0}C for 5 minutes. Carrier gas was ultra high purity helium (BOC) flowing with linear velocity of 39 cm/sec. The mass spectrometer was operated with a source temperature of 200 ^{0}C and 70 eVelectron ionisation energy. The scan range was from 50 to 800 (m/z) ratios in 0.4 second. AMDIS (Automated Mass spectral Deconvolution and Identification System) version 2.68 software was used to acquire and process data. Identification of products was undertaken by matching with the NIST mass spectral search program (version 2.0) with match factors of higher than 90 %. In cases with lower match, identification was confirmed using NIST MS interpreter (version 2.0) when the quality indices reported by interpreter were greater than 85 %. For peaks with no match in the library, structures were determined by examination of mass spectra and based on the detection of molecular ion and fragmentation patterns, this was mentioned as tentative identification in the identification method.

2.4.6 Reduction of PLFA

2.4.6.1 Samples and chemicals

PLFA was obtained from the IHSS and used as, tris(pentafluorophenyl)borane $B(C_6F_5)_3$, n-Butylsilane (n-BS) 97% and anhydrous CH_2Cl_2 (DCM) were purchased from Sigma-Aldrich, DCM was dried over $CaCl_2$ and distilled from CaH_2 to ensure that any trace of water was removed.

2.4.6.2 Reduction procedure

Sample preparation

Due to the necessity of establishing extremely dry and inert condition, all reactions were undertaken in a glove bag filled with Argon. Reaction vials and syringes were always oven-dried for 24 h at 110°C prior to use for the reaction. $B(C_6F_5)_3$ was always kept in an Argon-filled desiccator, n-BS and DCM were kept in sealed vials in the freezer before use.

Reduction

The reduction reaction was always performed inside the glove bag according to the procedure of Nimmagadda and McRae (2007), to the glass reaction vial equipped with mininert valve, 1.0 mg of PLFA was added followed by 0.5 mg $B(C_6F_5)_3$ as catalyst, then 100 µL of dry DCM. After stirring for 5 minutes, 100 µL of n-BS was added followed by 25 µL octadecane 500 ppm solution in DCM as an internal standard. An Argon atmosphere was established during the course of the reaction and the reaction mixture stirred for 24 hours before GC analysis. The similar procedure was performed for the blank sample without adding the PLFA.

2.4.6.3 GC/MS analysis

The instrumental analysis was carried out as mentioned in 2.4.5.3

2.4.7 Methylation (using methyl iodide) combined with reduction of PLFA

2.4.7.1 Samples and chemicals

PLFA was purchased from IHSS, Acetonitrile (HiPerSolv), THF and CH_3I was obtained from VWR International, Sigma-Aldrich and Omnilab, respectively. tris(pentafluorophenyl)borane $B(C_6F_5)_3$, n-Butylsilane (n-BS) 97 % and anhydrous CH_2Cl_2 (DCM) were purchased from Sigma–Aldrich. K_2CO_3 was obtained from Merck.

2.4.7.2 Derivatisation procedure

10 mg of PLFA sample was suspended in 3 mL 20% v/v acetonitrile /THF in a flask and stirred under N_2 atmosphere for 10 minutes. Then, 1.5 mL of 0.15 M K_2CO_3 was added and stirring continued. In order to prevent from oxidation of fulvic material N_2 atmosphere was stablished during the course of reaction. After 20 hours, 200 μL methyl iodide (CH_3I) was added to the reaction mixture and flask was sealed with glass stopper to prevent the evaporation of the reagent. The flask outer surface was covered by aluminium foil to prevent any exposure to light and decomposition of methyl iodide. The reaction was left for 24 hours, afterwards, solvents including methy iodide were evaporated in the rotary evaporator and remaining water was removed by freeze-drying. At this stage, first DCM was added but as it was not able to dissolve any material, removed in the rotary and acetonitrile was added and the supernatant was evaporated in rotary evaporator; to ensure drying, residue was freeze-dryed then dried in the vacuum oven for 24 hours. The remaining solid was separately dissolved in the water and was also dried as supernatant. Both dried samples were reduced as described in 2.4.6.2.

2.4.7.3 GC/MS analysis

The instrumental analysis was carried out as mentioned in 2.4.5.3

2.4.8 Methylation (using TMSD) combined with Trimethyl silylation of PLFA

2.4.8.1 Samples and chemicals

PLFA was purchased from IHSS, trimethylsilyldiazomethane (TMSD) solution (2M in diethylether) was obtained from Sigma-Aldrich. Methanol and dichloromethane and hexane (ACS grade) were obtained from BDH. Methyl octadecanoate was an analytical standard from Poly Science Corporation and was used to prepare internal standard solutions. Strata X-C cation exchange SPE was obtained from Phenomenex.

2.4.8.2. Derivatisation procedure

Due to the fact that methylation using TMSD requires PLFA to be in its fully protonated form, it was first dissolved in Milli-Q water and passed through a cation-exchange cartridge, the effluent of SPE was collected and freeze-dried before use. The methylation of fully protonated PLFA was conducted according to a method proposed for methylation of polyphenols (Actis-Goretta et al., 2012) in two steps. The first step was carried out according to the Kowalewski's method (Kowalewski and Gierczak, 2011). 2.0 mg of sample was suspended in 500 µL methanol:DCM 50:50 v/v, then 40 µL of 200 ppm methyl octadecanoate in hexane solution as internal standard and 100 µL of TMSD solution was added, the reaction vial was left at room temperature for 20 hours. After this stage, sample was dried under nitrogen stream and to ensure drying, the semi-dried sample was redried in a freeze-dryer for 12 hours. At the second step, dry sample was dissolved in 30 µL pyridine and mixed, then 100 µL BSTFA:TMCS (99:1) was added, sonicated for 30 minutes at 60 ^{0}C, then left under the same temperature while stirring for 15 hours.

2.4.8.2 GC/MS analysis

The instrumental analysis was carried out as mentioned in 2.4.5.3.

2.4.9 Choloroformate derivatisation of PLFA

2.4.9.1 Samples and chemicals

PLFA was purchased from IHSS and used as supplied. Ethyl chloroformate (ECF) was obtained from Merck. Pyridine and methanol (Hipersolve) was purchased from Sigma-Aldrich and BDH, respectively. Chloroform from RCI Labscan and NaOH was from Sigma-Aldrich.

2.4.9.2 Derivatisation procedure

Derivatisation was performed according to the method suggested by Villas-Bôas et al. (2011). Briefly, 1.0 mg PLFA was placed in a reaction vial and 50 µL of 0.1 M NaOH solution was added, then 150 µL methanol and 20 µL pyridine was added and mixed well. Afterwards, 30 µL of 250 ppm methyl octadecanoate in pyridine solution was added as internal standard. At this stage 10 µL ECF solution was added and vortexed for 3 minutes, this step was repeated once more. The reaction was left for 1-2 hours at room temperature while stirring and it was quenched by adding brine and extracted three times with chloroform. The chloroform extract was concentrated under N_2 and 0.5 µL injected to GC/MS.

2.4.9.3 GC/MS analysis

The instrumental analysis was carried out as mentioned in 2.4.5.3

2.5 References

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Chapter 3
The Characterisation of Fractionated Pony Lake Fulvic Acid

3.1 Introduction

The bulk analyses of PLFA revealed that the detection and identification of individual components are not readily achievable via chemical analysis due to the overlapping of peaks and the incomplete separation of compounds closely incorporated inside a complex assembly of organic material. In addition, given the natural polydispersity of fulvic acid structure and the complexity of the physical and chemical properties of the components present, there is a need to simplify the original material so that it becomes more homogenous and easier to detect and identify.

In order to obtain simpler subsamples from a DOM complex matrix including humic substances fractionation seems to be the best approach. A perfect fractionation is difficult to achieve, mainly due to the strong intramolecular interaction and the lack of separable components. Nevertheless, fractionation has been the primary choice of many researchers studying natural organic matter, for two main reasons. Firstly, fractionation or isolation normally involves non-invasive techniques which do not affect the chemical nature of samples and merely unfold the structure by a physical separation of the components. Secondly, fractionation enables examination of less complex and more homogenous materials compared to complicated original structure. This not only assists the selection of strategies involving fractionsin subsequent analyses, but also facilitates the understanding and interpretation of results obtained. Further fractionation, therefore, seems necessary for gaining insight into the structure of PLFA, due both to the reasons mentioned above, and to the

difficulties in obtaining useful information from unfractionated material (extensively described in Chapter 2).

One of the most commonly-used methods for DOM fractionation is molecular size-based fractionation. This type of fractionation is usually accomplished by either size exclusion chromatography (SEC) and/ or ultrafiltration (UF).The former method sorts the molecules in the sample by size via passing them through a porous gel, measuring the molecular mass distribution of the compound. Several studies have combined this technique with various detection systems to obtain extra information about the DOM fractions isolated (D'Orazio and Senesi, 2009). However, the necessity for calibration using a standard material brings about some limitations for DOM analysis using SEC An attempt was made to overcome this problem by absolute molecular weight determination using SEC combined to the light scattering detector (von Wandruszk et al., 1999, Wagoner and Christman, 1997), the data obtained were mostly in poor agreement with the results of using other SEC techniques. UF methods may also have some disadvantages, such as the interaction of analytes with membrane surfaces (Aiken et al., 1992, Thurman and Malcolm, 1981).

Another useful technique for the fractionation of DOM including fulvic acid, is polarity-based fractionation. Solid-Phase Extraction (SPE) is a method very commonly used for this purpose. It is usually based on intermolecular interactions between the organic material in solution and the stationary phase bonded onto a silica or polymeric surface of a sorbent available as XAD-8, XAD-4 resins or commercially available SPE cartridges.

Fractionation using XAD resins has been widely used for fractionation of DOM and specifically for the separation of fulvic acid from humic acid or other DOM; however, some overlaps between hydrophobic and hydrophilic compounds are expected because there is a possibility of trapping low MW hydrophilic compounds as neutral acids (at pH = 2) within the pores of the XAD-8 resin and eluting as hydrophobic fractions (Li et al., 2009).

The XAD scheme has also been approved by the International Humic Substances Society (IHSS) as a standard method for the separation of DOM based on the polarity (relatively hydrophobic or hydrophilic) using pH gradient elution combined with ion exchange resins. This scheme, however, employs extreme conditions such as a strong acidic or basic environment which may alter the nature of the fractions.

A stepwise fractionation approach has also been recently introduced by Piccolo and his group based on the separation of molecules from humic suprastructures by progressively breaking intermolecular bonds and using advanced analytical techniques to characterise their structures (Nebbioso and Piccolo, 2011; Nebbioso and Piccolo, 2012). The approach is named“Humeomics” and it involves characterisation by means ofseparation of molecules released from complex humin matrices as (i) unbound, (ii) weakly ester-bound, (iii) strongly ester-bound, and (iv) ether-bound. The quantitative analysis of the fractions is the key step in this method (Nebbioso et al., 2015).

RP-SPE, on the other hand, seems to be an unbiased method for fulvic acid fractionation (Schwede-Thomas et al., 2005). This method normally employs the cartridges filled with silica or polymeric stationary phase derivatised with hydrocarbon chains; these materials are designed to physically interact with hydrophobic material in the aqueous solution. The solutes molecule will be separated based on their hydrophobic affinity during their elution by a mobile phase containing a mixture of water and miscible organic solvents. RP-SPE does not have the complexity of RP-HPLC, and is faster and more preparative. It is also simpler to execute than XAD chromatography, and there is a low probability of alteration or loss of samples during preparation.

In this chapter, we present a detailed characterisation of IHSS’ PLFA subjected to further fractionation. RP-SPE was employed to isolate the fractions and the fractions were studied using various spectral and chemical methods.

The major aims of this chapter are:

(1) To explore the impact of fractionation on the ease of characterisation by comparing the results obtained in this chapter with those of Chapter 2.

(2) To draw a conclusion regarding the building blocks of Pony Lake fulvic acid (PLFA) and its molecular features.

3.2 Results

The following sections describes the outcomes of fractionation of PLFA and the results of experiments performed on isolated fractions in order to determine their structures and the compounds present. The spectral methods applied involved common spectroscopic techniques such as FTIR, FTNMR, and fluorescence spectroscopy which provide a general picture of the materials present in the fractions. Component-level analysis methods were selected based on the techniques which were shown to be most effective for bulk material characterisation (described in Chapter 2) besides extra experiments, such as depolymerisation to investigate the linkage between different parts of the molecule and gaining detailed structural information.

3.2.1 Fractionation of Pony Lake Fulvic Acid using Reverse Phase Solid Phase Extraction (RP-SPE)

The classical RP- SPE fractionation was carried out on IHSS PLFA with a polarity-based approach. Four distinct fractions eluting by water, 5% acetonitrile, 20% acetonitrile and 50% acetonitrile in water, were isolated. Additionally, to examine the effect of working condition on the fractionation some variables such as heating, sonication and solvent composition were checked. These variables will be discussed in more detail in the following sections.

3.2.1.1 Comparison of different fractionation conditions using RP- SPE

Five separate conditions were used for fractionation of PLFA. Condition 1 or simple fractionation was carried out by fractionation of a sample dissolved in Milli-Q water using water and acetonitrile (as described above). Condition 2 used sonication to aid solubility of the sample prior to fractionation. Under Condition 3, the PLFA aqueous solution was warmed (40 °C) before starting the fractionation step to improve the solubility.In the Condition 4,

organic solvent (acetonitrile) was added to fully dissolve the sample and finally in Condition 5, a diluted sample was fractionated.

The distribution of fractions resulting from various fractionation conditions is shown in Figure 3.1. At first glance, it is evident that recoveries of fractions were greatly affected by the changes applied during fractionation, indicating the variability of the original structure and its instability when subjected to even mild physical changes such as moderate heating and ultrasonication.

3.2.1.2 Impact of heating, sonication and organic solvent on fractionation

According to the results, warming to 50 °C dramatically affected quantities of all the fractions. As expected, PLFR 5 (which stands for undissolved material) was reduced due to a general increase in solubility upon heating in Condition 3. The mass of PLFR 1(hydrophilic material) showed a decrease, whereas amounts of PLFR 2 and PLFR 3 increased. Interestingly, almost the same trends were observed in the results of Condition 2, in which ultrasonication was used to assist solubility. PLFR 2 did not change much in this case. PLFR 3 (eluted by 20% acetonitrile) significantly increased as per Condition 3. PLFR 4, however, decreased under heating, but not using sonication. Moreover, sonication appeared more effective in terms of solubility as PLFR 5 (undissolved material) was not observed.

A possible explanation for these changes in distribution of the fractions recovery could be the presence of aggregation or conjugation between the hydrophilic and hydrophobic parts of fulvic acid or in other words, the formation of micelle-like structures. This is also in line with the supramolecular theory of humic substanes suggested by Piccolo and his colleagues indicating that humic substances are associations of self-assembling heterogeneous and relatively small molecules stabilised by Hydrophobic and hydrogen bonds (Piccolo, 2001; Piccolo et al, 2001;Piccolo, 2002). Although studies concerning micelles formation by humic substances are scarce (Piccolo et al., 1996), the influence of temperature and sonication on

micelles of many compounds such as proteins and liposomes have been well-documented (Dua, 2013).With respect to the available data, the increase of hydrophobic fractions obtained under Conditions 2 and 3, could be explained as a consequence of the rupture of micelle-like aggregates upon heating and sonication, resulting in the release of the hydrophobic parts from hydrophilic–hydrophobic conjugates.

The results obtained from Condition 4 using an organic-rich solvent mixture, disaggregating possible micelles, yielded similar quantities for PLFR 1 and 2 (Fig.3.1). These results are comparable to those of Condition 3 (heating). However, the yields for PLFR 3 and 4 appeared different under this condition compared to Condition 2 and 3, since the contribution of these two fractions is equal. These values are comparable to those of Condition 1, in which no heating or sonication was applied. This suggests that the organic solvent was not able to fully break the micelles, or its effect was less pronounced than those of temperature or of sonication.

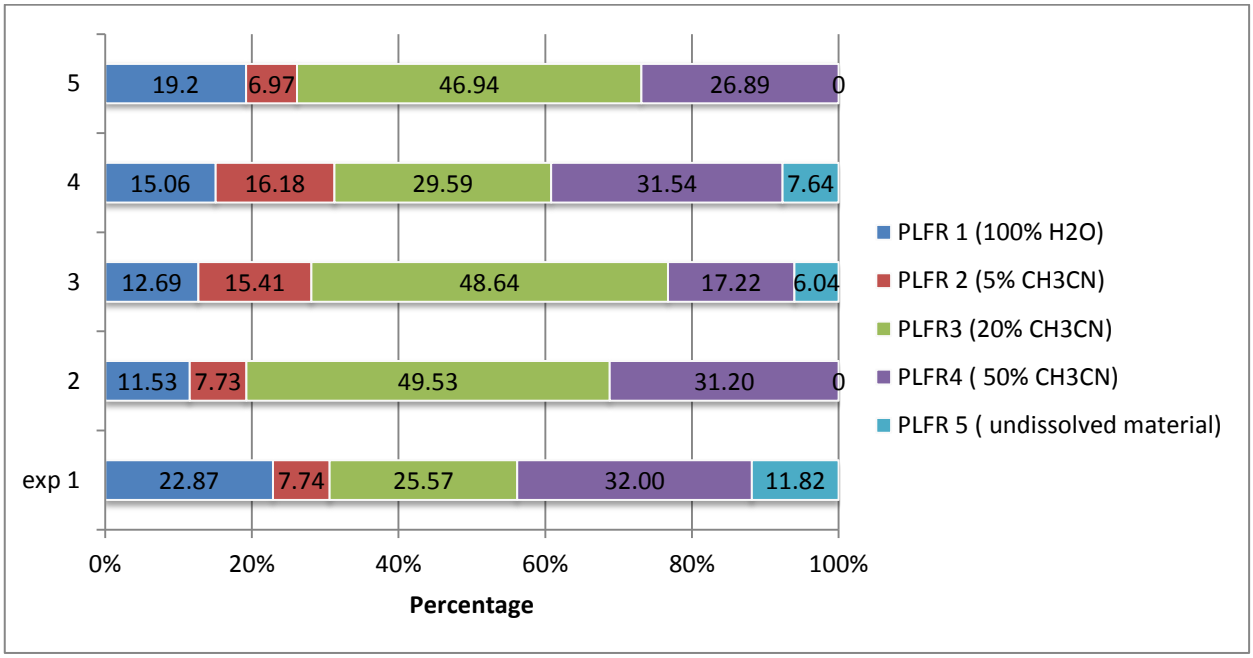


Figure 3.1 Pony Lake fulvic fractions distribution obtained under different fractionation conditions
Cond 1 = simple fractionation, Cond 2 = sonication – fractionation, Cond 3 = heating-fractionation,
Cond 4 = organic - rich solvent – fractionation, Cond 5 = diluted sample- fractionation

3.2.1.3 Impact of concentration of sample on fractionation

Another factor requiring consideration is the impact of the fulvic acid concentration on fractions distribution. In order to investigate this, a diluted sample of PLFA was prepared and fractionated as described in Section 3.4.1.5. The recovery of fractions prepared under this condition (Condition 5) shown in Figure 3.1 was similar to that achieved by sonication and heating (Condition 2 and 3). Although PLFR 1 showed an increase under diluted conditions, the general trend seems similar. These results indicate that the micelles were disrupted due to dilution and probable deviation from critical micelle concentration (CMC). Fractions obtained under the conditions of Condition 2 were chosen for further characterisation studies.

3.2.2 Elemental composition of fractions

The elemental composition of the fractions is summarised in Table 3.1. The carbon content ranged from 50.84 to 31.21% the highest and lowest values were found in the PLFR 4 and PLFR 1, respectively. The nitrogen and hydrogen contents of the four fractions ranged from 4.92 to 6.99% and 3.44 to 5.44%, respectively. Interestingly, there was a similar nitrogen distribution in the fractions, especially in PLFR 1, 2 and 3, in which the content ranged from 6.35 to 6.99%, PLFR 4 had a little less nitrogen, around 4.92%. This implies that nitrogen-containing compounds were almost equally present in all the fractions. This is surprising given that the fractions were separated by a polarity-based method, and could be expected to differ in terms of their components. In addition, the nitrogen content is significantly higher than that of other kinds of fulvic acids of Antarctic water, soil, peat and freshwater origin (Table 3.2). The C/N ratios are also notable. For example C/N ratio in PLFR 1 (5.4) is approximately two times lower than in PLFR 4 (12.1), showing obvious dissimilarity in their whole structures and probably in nitrogenous material. Generally, the common nitrogen-containing materials present in nature and possibly in humic substances are from three main families: peptides, amino sugars and heterocyclic nitrogenous compounds (McCarthy et al., 1997, Schulten and Schnitzer, 1998). The C/N value of the fractions does not point to peptides being the major constituent. It seems that two other families are more likely to contribute to the structure. Further study is needed to more precisely investigate this idea.

Table 3.1 Elemental composition of fractions of PLFA

Fractions	% Carbon	% Oxygen*	% Hydrogen	% Nitrogen	% Sulfur	H/C	C/N	O/C
PLFR1	31.21	56.03	3.44	6.81	2.51	1.30	5.4	1.35
PLFR2	40.85	45.64	4.22	6.99	2.30	1.23	6.9	0.84
PLFR3	48.98	37.37	5.07	6.35	2.23	1.25	8.9	0.57
PLFR4	50.84	37.16	5.44	4.92	1.64	1.28	12.1	0.93

* Calculated by difference

The H/C ratios did not vary significantly within the fractions, ranging from 1.30 to 1.23 in the four fractions, comparable values are generally seen in aliphatic structures, (Conte et al., 2003, Essington, 2004). The O/C ratio, indicating the presence of carbohydrates and carbonyls (or carboxyls) was significantly higher in PLFR1, being the most hydrophilic fraction.

The sulfur content was also very high in the PLFA fractions as compared to values observed in other Antarctic and non-Antarctic fulvic acids (Table 3.2), being an indicator of the microbial origin of this fulvic material. Sulfur was distributed equally among the fractions, making the identification of sulfur-containing compounds difficult due to the very low concentration in all fractions.

Table 3.2 Elemental composition of IHSS' fulvic acids

Fulvic acids	% Carbon	% Oxygen	% Hydrogen	% Nitrogen	% Sulfu
Pony Lake[1]	52.47	5.39	31.38	6.51	3.03
Lake Fryxell[2]	55.0	34.9	5.50	3.10	nd[3]
Lake Hoare [2]	54.9	35.7	5.50	2.90	nd[3]
Nordic Lake [4]	52.31	3.98	45.12	0.68	0.46
Suwannee River [5]	52.34	4.36	42.98	0.67	0.46
Waskish Peat [6]	53.63	4.24	41.81	1.07	0.29
Elliott Soil [7]	50.12	4.28	42.61	3.75	0.89

[1] IHSS standard sample (1R109F),[2] McKnight et al. (1994), [3]not determined,[4] IHSS reference sample (1R105F), [6] IHSS standard sample (2S101F),[6] IHSS reference sample (1R107F),[7] IHSS standard sample (2S102F)

3.2.3 Molecular weight determination of the fractions using Vapour Pressure Osmometry

The values obtained for each fraction is presented in Table 3.3. For PLFR 1 azeotropic mixture of the water: 1,4 dioxane was replaced by water.

Table 3.3 M_n values for PLFA fractions

Fraction	**PLFR 1**	**PLFR 2**	**PLFR 3**	**PLFR 4**
$\mathbf{M_n}$	217.47	317.18	656.62	545.07

As shown in the Table 3.3 the molecular mass of the fractions ranged from more than 217 Da for PLFR 1 to around 656 DA for PLFR 2. The PLFR 1 and PLFR 2 have the lower values while value for PLFR 3 is the largest, even larger than PLFR 4. It is notable that molar mass of PLFR 3 is slightly lower than the value obtained for PLFA (718 Da), indicating it is probably has a similar structure to the Bulk PLFA. Nevertheless, it is difficult to speculate about the fractions structure based on these values because as mentioned earlier, the high possibility of micelle formation might affect the distribution of components in each fraction.

3.2.4 Spectral characterisation of fractions

3.2.4.1 Fluorescence spectroscopy

Fluorescence spectroscopy has been recently recognised as one of the useful analytical methods for the charaterisation of dissolved organic matter and humic substances. This technique is able to show characteristic maxima, representative of the sample fluorophores that can be attributed to the sample structure and its surrounding environment (Coble, 1996, Coble et al., 1990, Green and V., 1994, Zhao et al., 2009). Excitation Emission Matrix (EEM) or three dimensional fluorescence spectroscopy has recently provided useful information about the presence of two fluorophores in Antarctic fulvic acid including PLFA (Pony Lake fulvic acid) (D'Andrilli et al., 2013, McKnight et al., 2001).

EEM profiles of solutions of the PLFA fractions are shown in Figure 3.2. The contour lines show the distribution of fluorescence intensity at different excitation-emission wavelengths. Two fluorophores are observable; the fluorophore at low excitation named fluorophore 1 and the fluorophore at high excitation energy named fluorophore 2. Former Spectrofluorometric studies of Bulk PLFA showed the presence of additional fluorophore at fluorophore Ex_{max}/Em_{max} = 230/412 nm due to amide-like structure (McKnight et al., 2001). However, it is surprisingly absent from spectra of the fractions, possibly due to the low concentration of chromophores appearing in this area.

As shown, fluorescence spectra of PLFR 1 and PLFR 2 are very similar in terms of fluorophores' positions, both showing Ex_{max}/Em_{max}= 350/430 nm for fluorophore 1 and Ex_{max}/Em_{max} = 499/450 nm for fluorophore 2. PLFR 2, however, contains peaks with higher intensities than those of PLFR 1, indicating the presence of higher degree of aromatic components as well as conjugated chromophores.

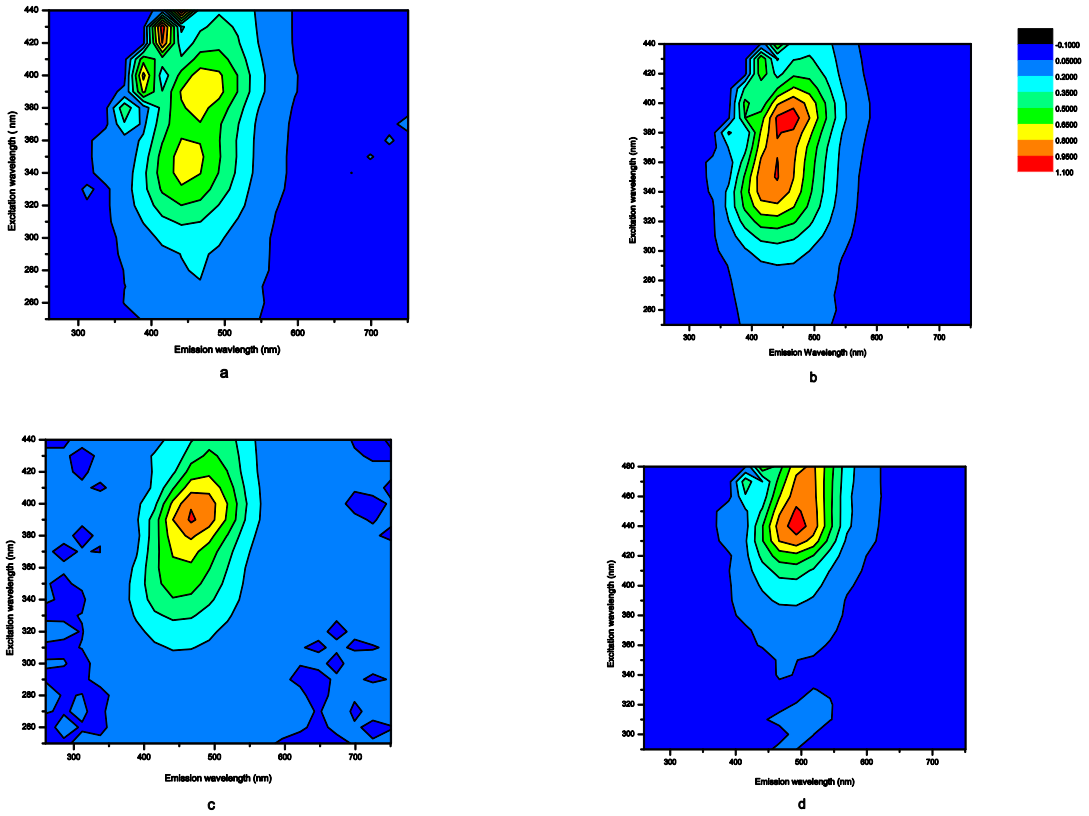


Figure 3.2 Three dimensional excitation- emission matrices (EEMs) of four fractions of Pony Lake fulvic acid (a) PLFR1 (b) PLFR2 (c) PLFR3 (d) PLFR4. The line at the up left corner represents Rayleigh scatter

In PLFR 3 and PLFR 4, only fluorophore 2 was observed, this fluorophore also shifted to the higher excitation wavelengths compared to PLFR 1 and 2. The EEM at higher excitation wavelengths can be due to highly substituted aromatic nuclei such as electron-donating groups or conjugated arrangements (Senesi et al., 1989). As these two fractions have high hydrophobicity these types of structures are highly likely to be observed. The absence of fluorophore 1 is notable and indicates the difference in nature of the fraction's components. It can also be concluded that the absence of fluorophore 1 in PLFR 3 and 4 is due to a decrease in aromatic structures resulting from photochemical degradation (Miller et al., 2009) during the fractions preparation and storage. Alternatively, it may be simply due to the lower concentration of the chromophores forming this fluorophore in these two fractions.

In general, fluorescence demonstrated dissimilarities in the character of the fractions and differences in the possible fluorophores present in them, highlighting the suitability of the

fractionation procedure. However, the technique provided little information in terms of molecular character of the fractions which necessitates employing chemical analysis methods alongside spectral techniques.

3.2.4.2 IR Spectroscopy

FTIR analysis was performed on all four fractions. To better examine the character of the isolated fractions, compound-class assignments were carried out on the fractions by comparing the spectra shown in Figure 3.3 with characteristic absorption patterns from the literature (Leenheer and Rostad, 2004, Movasaghi et al., 2008, Shang et al., 2013, Zhang et al., 2011). At first glance, the IR spectra of PLFR 2, 3, 4 and unfractionated PLFA seem very similar, but PLFR 1 is in fact significantly different on some parts of the spectrum. Comparison of PLFR 2, 3 and 4 indicates the presence of aliphatic structure by clear CH_2 stretching absorbance at 3000-2900 cm^{-1} for PLFR 3 and 4.This peak is less pronounced in PLFR 2, which is possibly an indicator of less aliphatic moieties in the structure. Other parts of the spectra for three fractions contain similar absorbance at 3500-3000 cm^{-1}, 1700 cm^{-1} and 1400 cm^{-1}, assigned and O-H stretching, C=O stretching, CH_3 umbrella deformation, respectively. They originate from fatty acids, phenols and alicyclic structures. A weak absorbance is present in these three fractions around 1650 cm^{-1} which could be due to the presence of carbonyls of amides.

In the finger printing area of the aforementioned fractions, 1150-1280 cm^{-1} broad bands and 1040 cm^{-1} absorbance are predominant; the former may indicate the presence of aromatic carboxylic acid while the latter is associated with C-O-C stretching of polysaccharides (Movasaghi et al., 2008) which appeared as very weak peaks in all three fractions. Absorbance around 760 cm^{-1} could be due to C-H aromatic out of plane bending. As mentioned above, PLFR 1 shows dissimilarity from the other fractions. Absorbance around 2960 cm^{-1} in PLFR 1, also observed in PLFR 3 and 4 can be attributed to methyl stretching.

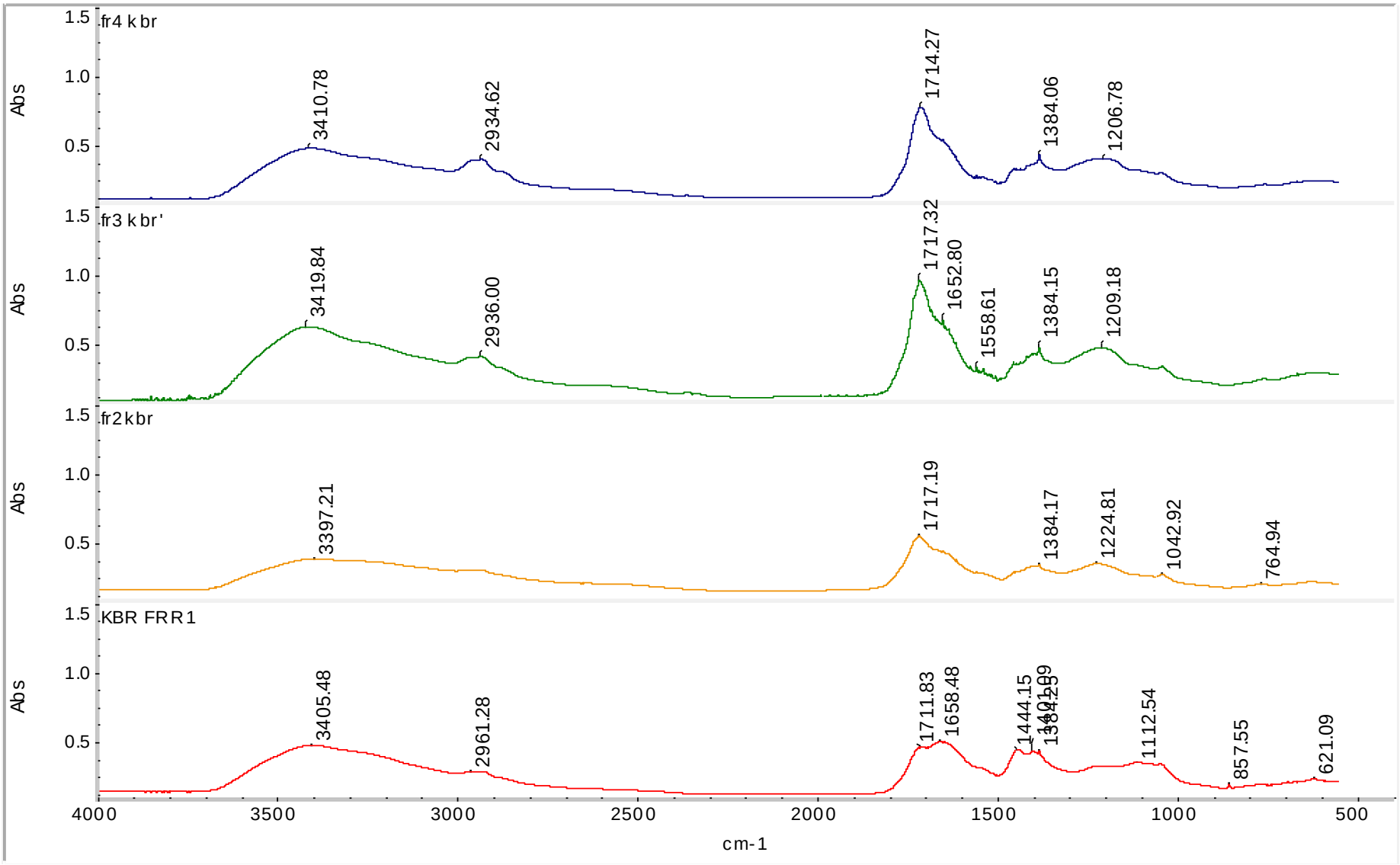


Figure 3.3 Absorbance IR spectra of four fractions of PLFA

In the carbonyl region two peaks are observable for PLFR 1. The first one appearing at 1711 cm^{-1} is assigned to the carbonyl group of carboxylic acid, or carboxylates, while the second one at 1649 cm^{-1}could be due to C=O stretching of amide known as amide 1 band. Amide 2 band is also observed around 1550 cm^{-1} (Muruganantham et al., 2009, Schmitt and Flemming, 1998). Noticeable bands at 1444 and 1380 cm^{-1}could have resulted from CH_2, CH_3 bending vibrations in sugars (well-known in polysaccharides) (Kurjatko, 2007). Another distinct feature in the IR spectrum of PLFR 1 is the peak at 1112 cm^{-1} which is absent from the other fractions. This peak indicates C-O stretching of carbohydrates (Enev et al., 2014, Hladký et al., 2013). A summary of the peaks assigned to different compound classes in the four fractions is presented in Table 3.4.

Table 3.4 Summary of bands assigned to different compounds present in four fractions

Infrared frequency bands (cm^{-1})	Compound class	PLFR 1	PLFR 2	PLFR 3	PLFR 4
3400-3300 (O-H), 1100-1000 (C-O)	Carbohydrates	✓✓	×	×	×
2960 (CH_3), 2940 (CH_2), 1460 (CH_2), 1380 (CH_3)	Aliphatic hydrocarbons	✓	✓	✓	✓
700-900, Ar-H, 1660-1630 (Ar-C=O), 1280-1150 (Ar-O, COOH)	Aromatic hydrocarbons	-	✓	✓	✓
1660 (amide 1 band, N-C=O), 1540 (amide 2 band, N=C-O)	Proteins	×	×	×	×
1660 (amide 1 band, N-C=O), 1550 (amide 2 band, N=C-O) 1380 (CH_3)	N-acetyl amino sugars	✓✓	×	×	×
1760 (COOR), 1720 (COOH), 2960 (CH_3), 2940 (CH_2), 1460 (CH_2), 1380(CH_3)	Lipids	✓	×	✓✓	✓✓
2700-2500 (COOH), 1760 (COOR), 1720 (COOH)	Humic-like structure	×	✓	✓✓	✓

Although, fraction structure cannot be concluded based on IR spectra alone, an approximate assessment according to the data shown in Table 3.4 suggests the following interferences: PLFR 1 contains considerable carbohydrate, including N-acetylated amino sugar; due to the presence of strong amide 1, 2 and methyl absorbance along with strong sugar C-O peaks. The presence of these moieties is well-matched with the presence of chitin, a linear polysaccharide composed of (β1→4) linked N-acetyl-D-glucosamine units widely found in fungi , algae and bacterial cell walls (Amir et al., 2006). It is more difficult to speculate based on PLFR2's IR spectrum; it looks to be similar to PLFR 3 and PLFR 4, but at the same time resembles PLFR1at the carbonyl region. It is apparently not of aliphatic nature.

PLFR 3 and 4 exhibit similar patterns and probably contain larger lipids and have phenolic or quinone structures.

3.2.4.3 NMR Spectroscopy

^{1}H and ^{13}C NMR spectra of all four fractions are presented in Figures 3.4 and 3.5, respectively.^{1}H NMR spectra of the fractions exhibit some interesting features; at first glance, the spectra of PLFR 1 and PLFR 2 resemble each other. On the other hand, PLFR 3 and PLFR4 look similar. The majority of the peaks present in PLFR 1 and 2 are observed within 0 to 3 ppm, where aliphatic, benzilic, carbonyl, ester and acidic (not carboxylic) hydrogen normally appear. The next region containing peaks is 6.5 to 9 ppm. These peaks are due to aromatic and olefinic hydrogens. A peak around 7 ppm is outstanding in in both the PLFR 1 and 2 spectra; this peak must be due to hydrogens in conjugated double bonds because the significant aromatic structures in these two fractions are highly unlikely.

In general, PLFR 3 and 4 show almost the same pattern as PLFR 1and 2 except extra peak at 12 to13 ppm. These peaks are from carboxylic groups which were to be expected in almost every fraction. Their absence from PLFR 1 and 2 (hydrophilic fractions) was surprising. Allied with this is the sharp peak at 2.5 ppm that was detected in all fractions, but is very pronounced in PLFR3. This peak is the hydrogen of the carbon connected to carboxylic group. Further, peaks at the aromatic region (6-9 ppm) are sharper and more pronounced in these two fractions compared to PLFR 1and PLFR 2.

Looking at the ^{13}C NMR spectra, the spectra can be separated into five regions, 0 to 60 ppm (aliphatic C-H, C-C, C-N), 60 to 90 ppm (aliphatic C-O such as alcohols, esters, and ethers),90 to110 ppm (O-C-O), 110 to 160 ppm (aromatic and olefinic carbon),160 to190 ppm (carbonyl groups of amides, carboxylic acids, and esters) and 190 to 220 ppm (carbonyl group of ketone). In general, the fractions exhibited similar spectroscopic features especially for major spectral bands. However, some differences are observable in minor bands. PLFR 1 and

2 look similar in terms of showing peaks at 20-30 ppm indicating methyl group (Leenheer et al., 2004). Peaks at 70 ppm are due to ether, alcohols or acetals while peaks at 116 ppm are an indicator aromatic structure. Peaks at 120-140 ppm are due to aromatics, and170-175 ppm are due to amides or carboxylic acids carbonyls (Thorn and Cox, 2009). However, PLFR 2 shows a peak at 150 ppm which is not present in PLFR 1.This could also be due to aromatic amino acids (Zhang et al., 2011). In general, there are more peaks in the aliphatic region in PLFR 2 than PLFR 1, confirming the more hydrophobic nature of PLFR 2.

PLFR 3 and PLFR 4 also showed comparable ^{13}C NMR spectra with main peaks at aliphatic (20-30ppm), O-alkyl (70ppm), aromatic (120-140ppm) and carboxylic and amide (160-190ppm). The bands at the aromatic region of these two fractions are more significant than for PLFR 1 and 2. Especially notable is the absence of a quinone peak, usually appearing around 180ppm and previously reported for bulk PLFA (Thorn and Cox, 2009) (Figure 3.6). This may be an indicator of limited quinone structure that could not be detected in individual fractions, or it may happen due to different instrumental settings, preventing comprehensive detection of peaks. PLFR 3 and 4 show more peaks in the aliphatic region especially in 30-40 ppm, consistent with their hydrophobic structures.

Overall, it seems that simple ^{1}H and ^{13}C NMR cannot distinguish between structures of fractions as the spectra show very similar patterns and peak distributions. Therefore, it is hard to speculate detailed structural information based on the current NMR data. Application of sophisticated NMR techniques in combination with other analytical methods can assist with demonstrating a more precise picture of PLFA fractions.

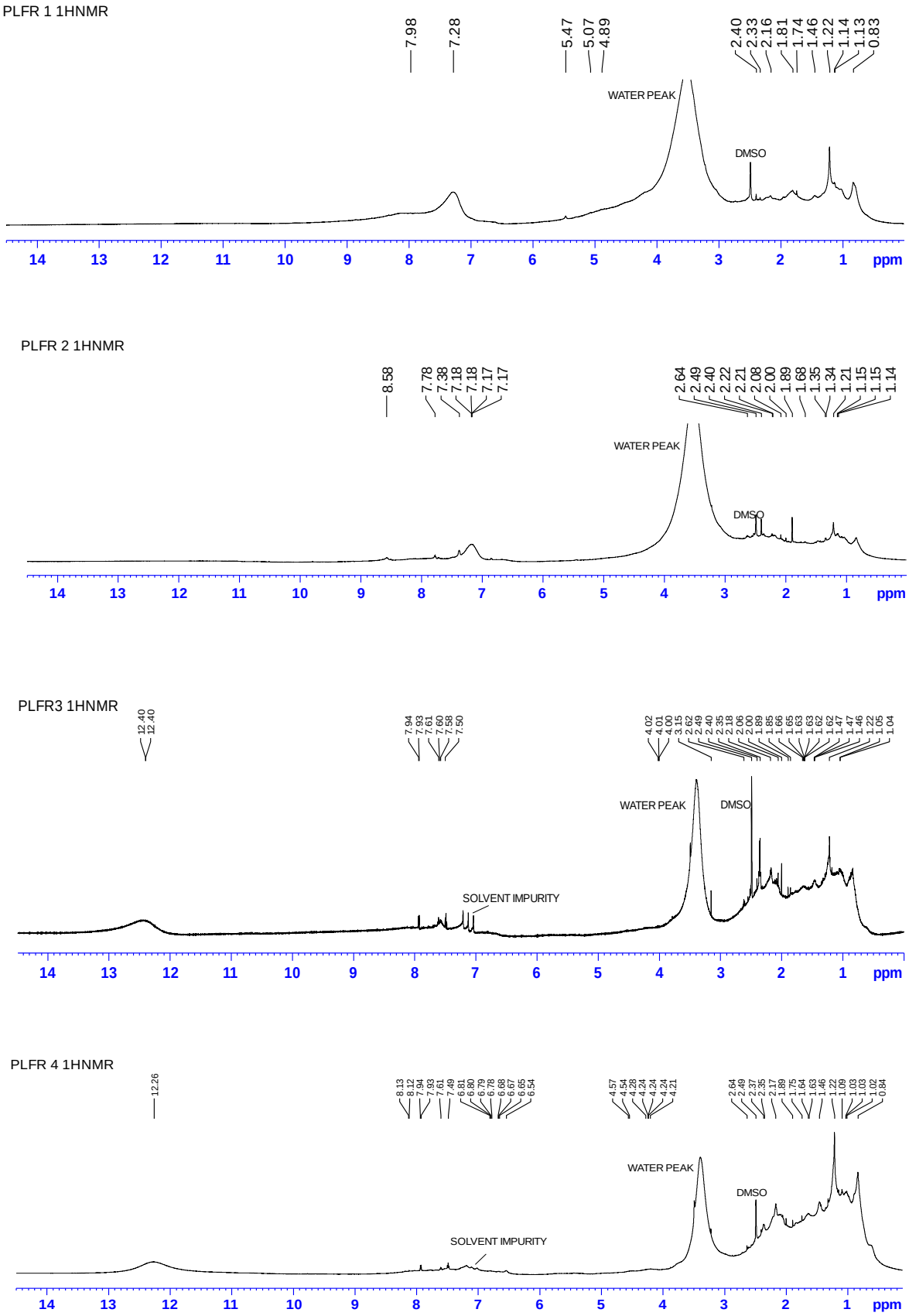


Figure 3.4 ^{1}H NMR spectra of fractions

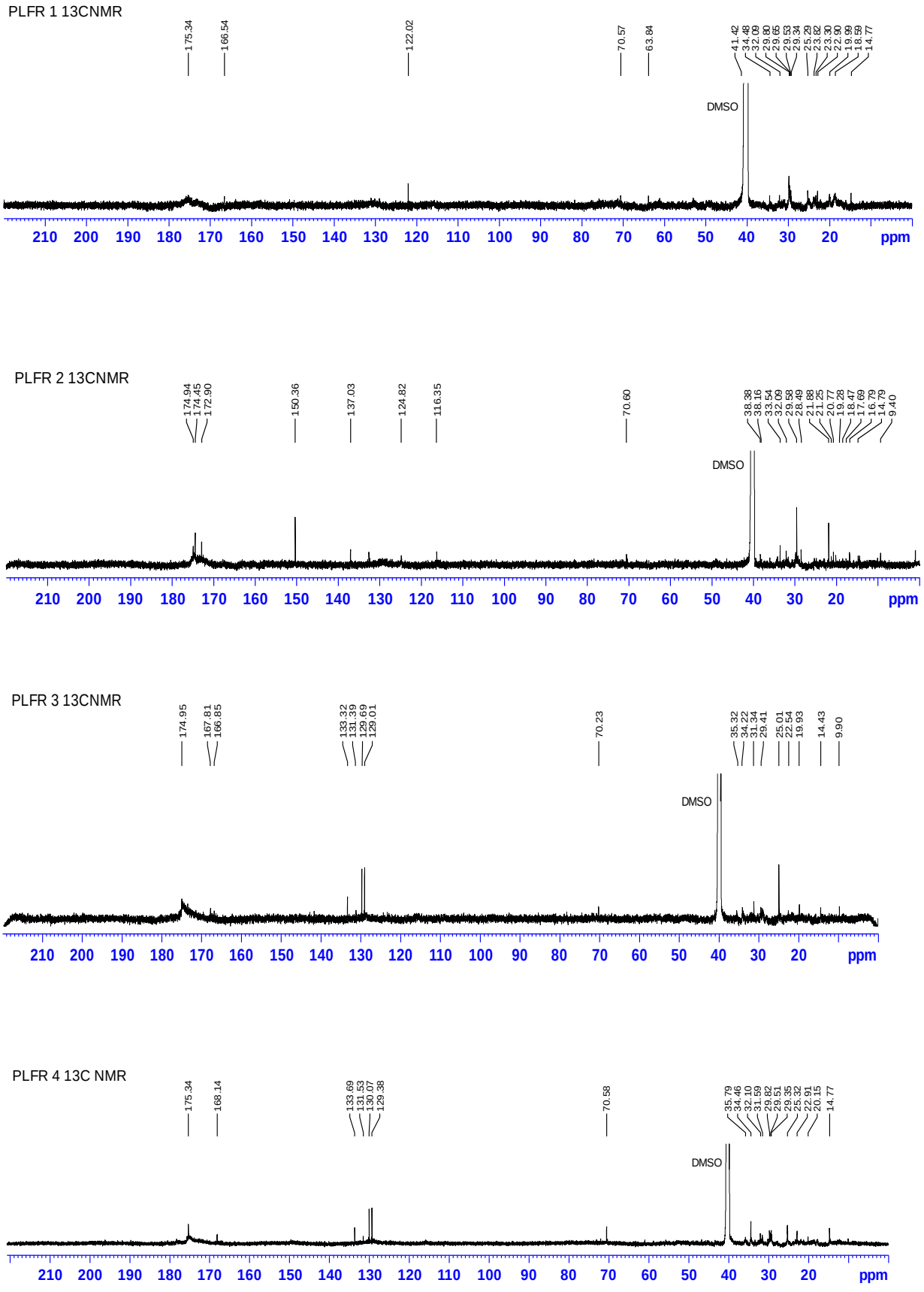


Figure 3.5 ^{13}C NMR spectra of fractions

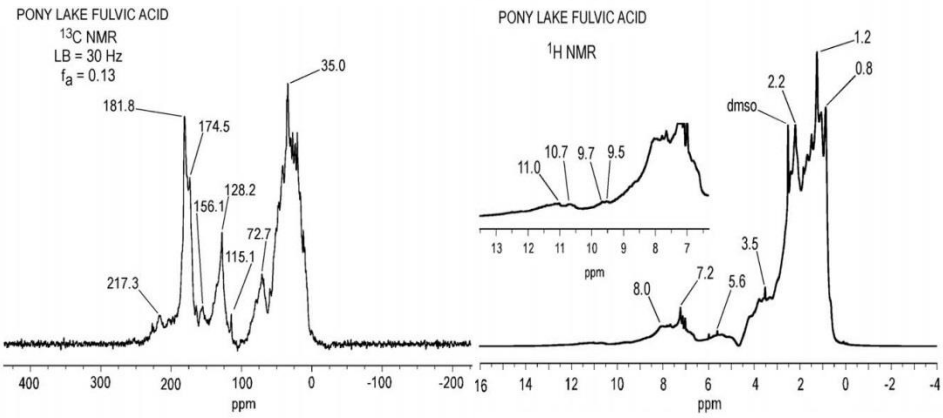


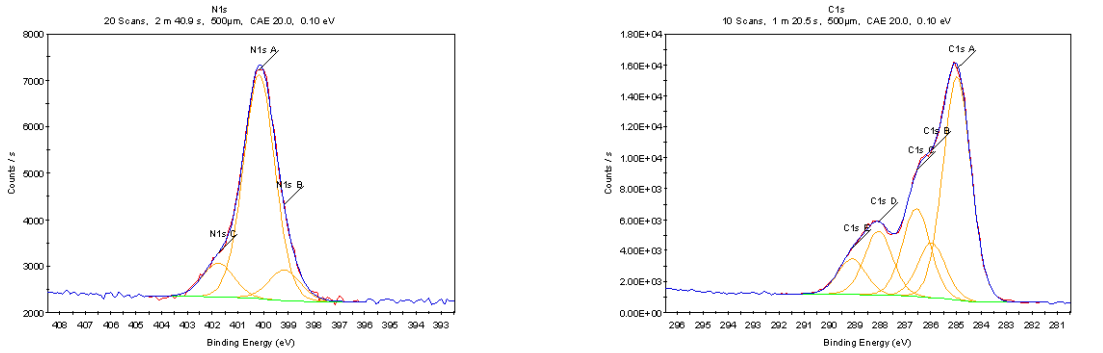
Figure 3.6 Liquid state ^{13}C NMR spectrum and liquid state ^{1}H NMR spectrum of Pony Lake fulvic acid (Thorn and Cox, 2009)

3.2.4.4 X ray photoelectron spectroscopy (XPS)

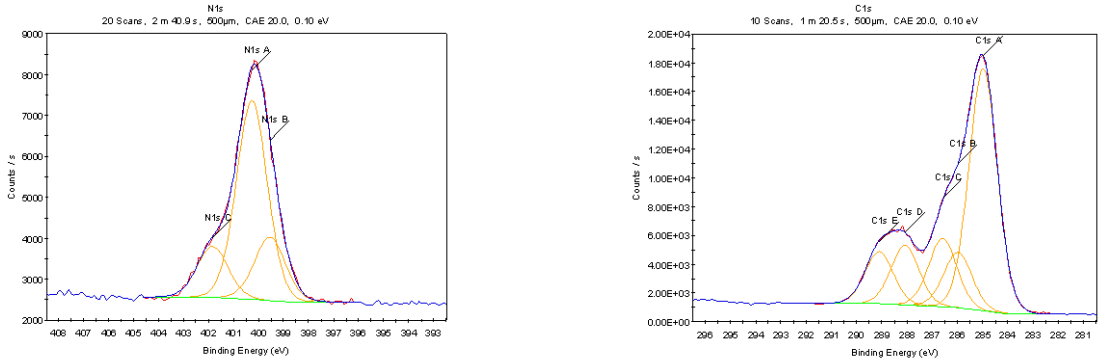
The study of chemical structure of DOM using XPS has recently attracted more attention after its successful application in surface and particle analysis in polymer, wood and soil science. XPS capability in deleting different elements based on chemical shifts of the binding energy of electrons present in the atoms can be an advantage to the study of unknown materials. XPS provides information about the relative abundance of elements in solid samples as well as the different chemical forms of the bound elements. It is also a non-destructive technique using very small amounts of sample, and is practical to complete within a short run time. The recognition of different elemental species by XPS has assisted the emergence of valuable information about the structures of DOM and humic substances not attainable by other spectroscopic techniques such as NMR or IR. For instance, Monteil-Rivera and her colleagues using as a series of XPS analyses, have been able to reveal an overestimation in detection of carboxylic acids in humic acid appearing in ^{13}C NMR spectra, apparently attributed to the amides group (Monteil-Rivera et al., 2000). A few XPS studies were recently performed to elucidate the structural features of humic acids from aquatic and soil environment (Abe and Watanabe, 2004, Bubert et al., 2000), Interesting findings with

respect to nitrogen functionalities in humic substances have also been reported in previous studies which have highlighted the utility of XPS analyses in both qualitative and quantitative terms.

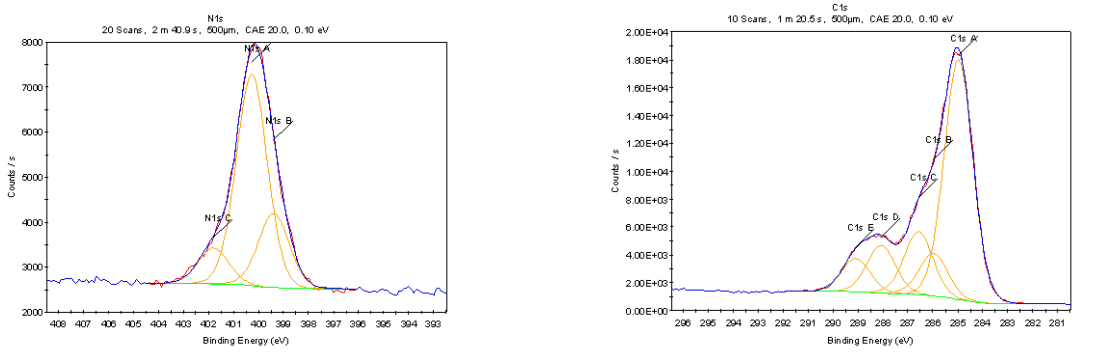
In this section, we describe how XPS analysis was performed on fractions of PLFA in order to better understand the composition and distribution of various functional groups especially nitrogen and sulfur-containing ones in fulvic material structure. Figure 3.7 represents the XP spectra of C1s and N1s lines in PLFA fractions. The notation of the lines and different bonds assignments are summarised in Table 3.5. The assignments were obtained based on the binding energy of each line and comparison of those values with the assignments reported in the literature.



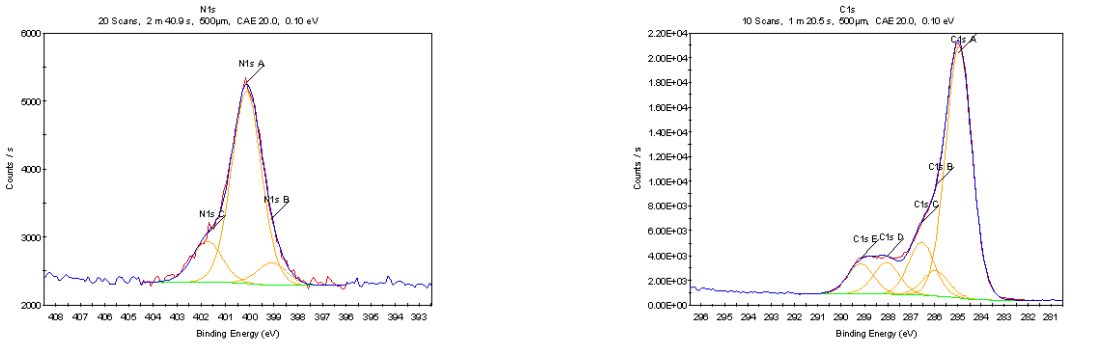
a



b



c



d

Figure 3.7 X-ray photoelectron spectra (XPS) of nitrogen and carbon functionalities in PLFA fractions (a) PLFR 1, (b) PLFR2. (C) PLFR 3 and (d) PLFR 4

Table 3.5 Atomic concentrations in composition of PLFA fractions extracted from XPS spectra

				PLFR 1	PLFR 2	PLFR 3	PLFR 4
Line	Notation	Position /BE (eV)	Bond/ Compound	Concentration (At%)[1]	Concentration (At%)	Concentration (At%)	Concentration (At%)
C1s	A	284.96	C-C/C-H	29.59	31.34	37.00	45.9
C1s	B	285.96	C-N	7.27	7.21	6.59	4.56
C1s	C	286.56	C-O	11.68	8.82	9.71	9.70
C1s	D	288.06	C=O	8.52	7.75	7.35	5.85
C1s	E	289.11	O=C-O	4.71	6.75	5.12	5.47
Cl2p3	A	197.9	nd[2]	0.49	0.12	0.22	0.10
Cl2p3	B	200.22	nd	0.06	0.03	0.03	0.04
N1s	A	400.16	Amide N	**6.23**	**5.64**	**6.11**	**3.98**
N1s	B	399.17	Aromatic N	**0.85**	**1.81**	**2.14**	**0.46**
N1s	C	401.77	Primary amine N	**0.94**	**1.45**	**1.03**	**0.85**
Na1s	-	1071.75	nd	1.99[3]	0.97	0.38	0.24
O1s	-	532.16	nd	25.85	26.43	22.58	21.77
S2p3	A	163.94	thiol/ thiophene	**0.74**	**1.00**	**1.18**	**0.75**
S2p3	B	168.33	Sulfone	**1.09**	**0.68**	**0.57**	**0.34**

[1] The values are different from those obtained from elemental analysis due to possible inhomogeneity in XPS samples, [2]nd : not detected, [3] high Na content comes from glass slides being used as sample base

The atomic concentration of carbon functional groups in all four fractions indicates the dominance of aliphatic structure. The contribution of oxygen- containing groups C-O, C=O and O=C-O, is also notable, as these groups are seen in the building blocks of carbohydrates and carboxylic acids as well as amides. However, the values reported may have been affected by the presence of Na originating from the glass sample holder. While these values differ from the elemental composition results, the whole data set provides a good general map of elemental distribution and contribution in different functional groups within this complex matrix. N1s lines can be fitted to three chemical forms of nitrogen bonded to carbon as follows: binding energies at 399.0 ± 0.1 eV for aromatic N; 400.4 ± 0.1 eV for peptide bond N; and 402.4 ± 0.2 eV for primary amine N (Abe and Watanabe, 2004, Fimmen et al., 2007). Considering this distribution, as shown in Table 3.5, there is a common trend in all fractions, indicating the dominance of amide-like structure as nitrogen-containing moiety, whereas primary amine and aromatic nitrogen are observed as minor contributors in all fractions. This is in agreement with the ^{15}N NMR of PLFA, indicating the presence of around 40 % (~2/5 of all N) as peptide (amide) structure (Mao et al., 2007).The peptide-like structure was later proven as non-aromatic heterocyclic components such as hydantoin and dihydrouracil (Fang et al., 2011). Therefore, it is reasonable to assume that a large amount of what appeared as amide N in N1s signal of PLFA fraction belongs to the aforementioned structures; the rest of the amides may originate from small peptides present in fulvic material.

S2p lines analyses for sulfur-containing components in fractions are also given in Table 3.5. Previous characterisation of XPS of organic sulfur functional groups indicated that sulfur 2 p electrons with binding energy around 163.7–164.0 eV are characteristics of reduced functional groups such as thiophenes, thioesters, and thiols, while 2p electrons with binding energies >165 eV are characteristic of oxidised sulfur functionalities (166.0 eV sulfoxides, 168.0 eV sulfones, 169.2 eV sulfonates, and 174.8–175.8 eV sulfates) (Fimmen et al., 2007, Lowe, 1992, Olivella et al., 2002). With respect to the appearance of 168.3 eV binding energy in S2p3 B lines, it is highly likely that sulfones as the oxidised form of sulfur are present in PLFA and as such in its fractions.

3.2.5 Chemical Analysis of Fractions

Although bulk analysis of PLFA fractions has provided useful information as described in the previous sections, it still seems inadequate for gaining deep insight into the whole concept of fulvic material. Hence, there is a need to combine current information with the results of other types of analysis especially those which have not been attempted on PLFA (or only in a limited way). We, therefore, extended our characterisation approach to the individual compound level. This will not only be of great scientific value, but can also be potentially beneficial for detailed structural analysis of PLFA as the main focus of this study.

Gas chromatography- mass spectrometry (GC/MS) combined with a range of derivatisation methods was our choice for component- level analysis. Selection of methods was based on three strategies to either simplify or modify fractions, enabling formation of GC-amenable materials. The first strategy involved reduction or derivatisation of polar functional groups including carboxylic acids, alcohol, phenols, amines and amides with minimum damage to the original structure. Methods used to fulfil this approach were methylation, trimethylsilylation, chloroformate derivatisation and reduction which have been extensively described in Chapter 2. The second strategy involved using the well-known

tetramethylammonium hydroxide (TMAH) thermochemolysis able to hydrolyse and derivatise, simultaneously. TMAH cleaves ester and some ether linkages instantly under high temperature. This can liberate every molecule linked to the neighbour group by means of these sorts of linkages and facilitates its detection. The last scheme, being more invasive, follows the classical polysaccharide analysis technique which cleaves glycosidic bonds by hydrolysis or methanolysis, releasing monosaccharaides and other components from a heteropolysaccharide body. This strategy disintegrates the original fulvic material, but usually does not lead to further degradation of monomers. It also provides a good opportunity to detect compounds other than monosaccharides liberated from the whole skeleton.

3.2.5.1 Trimethylsilylation

Trimethyl silylation derivatisation was performed on freeze-dried fractions and GC/MS analysis was undertaken to identify derivatised compounds. Products of TMS derivatisation of PLFR 1 to 4 are displayed in Table 3.6.

Generally, the range of compounds observed in the fractions was similar to that observed for unfractionated PLFA, albeit distributed within four fractions. However, as a general trend, one can see sugar moieties mostly in PLFR 1 including mono and disaccharides, expected for this hydrophilic fraction. Saccharides are obvious by the presence of pyrano ring of TMS derivatised hexose and furano ring of TMS-derivatised pentose among the product of this fraction as shown in Table 3.6. A limited number of small carboxylic acids and alkandioic acids were also detected. PLFR 2 contained more short-chain carboxylic acids and alkandioic acids (ranging C2-C6) than PLFR 1, but very limited sugars. Aromatic acids and fatty acids (saturated and unsaturated) up to C18 carbon appeared in PLFR 3 and PLFR 4, reflecting their more hydrophobic nature. Ironically, PLFR 4 as the most hydrophobic fraction contained sugar in the form of mono and disaccharide (Fig 3.8).

Table 3.6 Identified compound in TMS-derivatised fractions of PLFA

Peak no [1]	CompoundsIdentified	Identification method [2]	PLFR 1	PLFR 2	PLFR 3	PLFR 4
1	Silane, (cyclohexyloxy)trimethyl	a, d	-	+	-	-
2	2-Hydroxypropanoic acid (D-lactic acid-ditms)	a, d	+	-	-	-
3	2,5-Furandione, 3-ethyl-4-methyl	b, d	-	-	+	-
4	2-Hydroxyethanoic acid (Glycolic acid-ditms)	a, d	+	-	-	-
5	N-Formylpiperidine	b, d	-	-	+	+
6	cis-6-ethyl-2,5,6-trimethyl-2-cyclohexen-1-on,	a, d	-	-	-	+
7	(3H)-Furanone, dihydro-4,4-dimethyl-3-[(trimethylsilyl)oxy]-	a, d	-	+	-	+
8	tris(trimethylsilyl) phosphate	a, d	+	+	-	
9	Butanedioic acid, bis(trimethylsilyl) ester	a, d	+	+	-	-
10	Propanedioic acid, ethyl-, bis(trimethylsilyl) ester	a, d	-	+	-	-
11	2-Butenedioic acid (E)-, bis(trimethylsilyl) ester	a, d	-	+	-	-
12	1-Dodecanol, trimethylsilyl ether	a d	-	+	-	-
13	4-Trimethylsilyl-6,7-dihydro-5H-2-pyrimidine	a, d	-	-	-	+
14	Hydroquinone di-tms	a, d	-	-	+	+
15	3-Methoxybenzoic acid, trimethylsilylester	a, d	-	-	-	+
16	Hexanedioic acid, bis(trimethylsilyl) ester	a, d	-	-	-	+
17	1-(1'-((Trimethylsilyl)oxy)-2'-methylcycloheptyl)-2-butene-1-one	a, d	-	-	-	+
18	Benzoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester	a, d	-	-	+	+
19	Octanedioic acid, bis(trimethylsilyl) ester	a, d	-	-	-	+
20	aldopentose tms ether	c ,d	+	-	-	-
21	aldohexose -tmsether	c, d	+	+	-	+
22	aldohexose -tmsether	c, d	+	-	-	+
23	Decandioic acid, bis(trimethylsilyl) ester	a, d	-	-	-	+
24	α-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-4-O-methyl-3,6-bis-O-(trimethylsilyl	b, d	-	-	-	+
25	aldohexose -tmsether	c, d	+	+	-	-
26	aldohexose -tmsether	c, d	+	-	-	-
27	aldohexose -tmsether	c, d	+	-	-	-

28	Trimethylsilyl (9Z)-9-octadecenoate (oleic acid trimethylsilylmethylester)	a, d	-	-	-	+
29	Methyl stearic acid methylester	a, d	-	-	-	+
30	Sucrose 8-tms (α-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-β-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)	a, d	+	+	+	+

[1]Numbers correspond to the peaks in Fig 3.8, [2]dentification was based on three methods (a) matching compound suggested by NIST Mass Spectral Search Program with above 90 % match probability (b)using NIST MS Interpreter with quality indices reported as above 85 % (c) tentative identification (d) suggested structures were checked against retention data in the literature

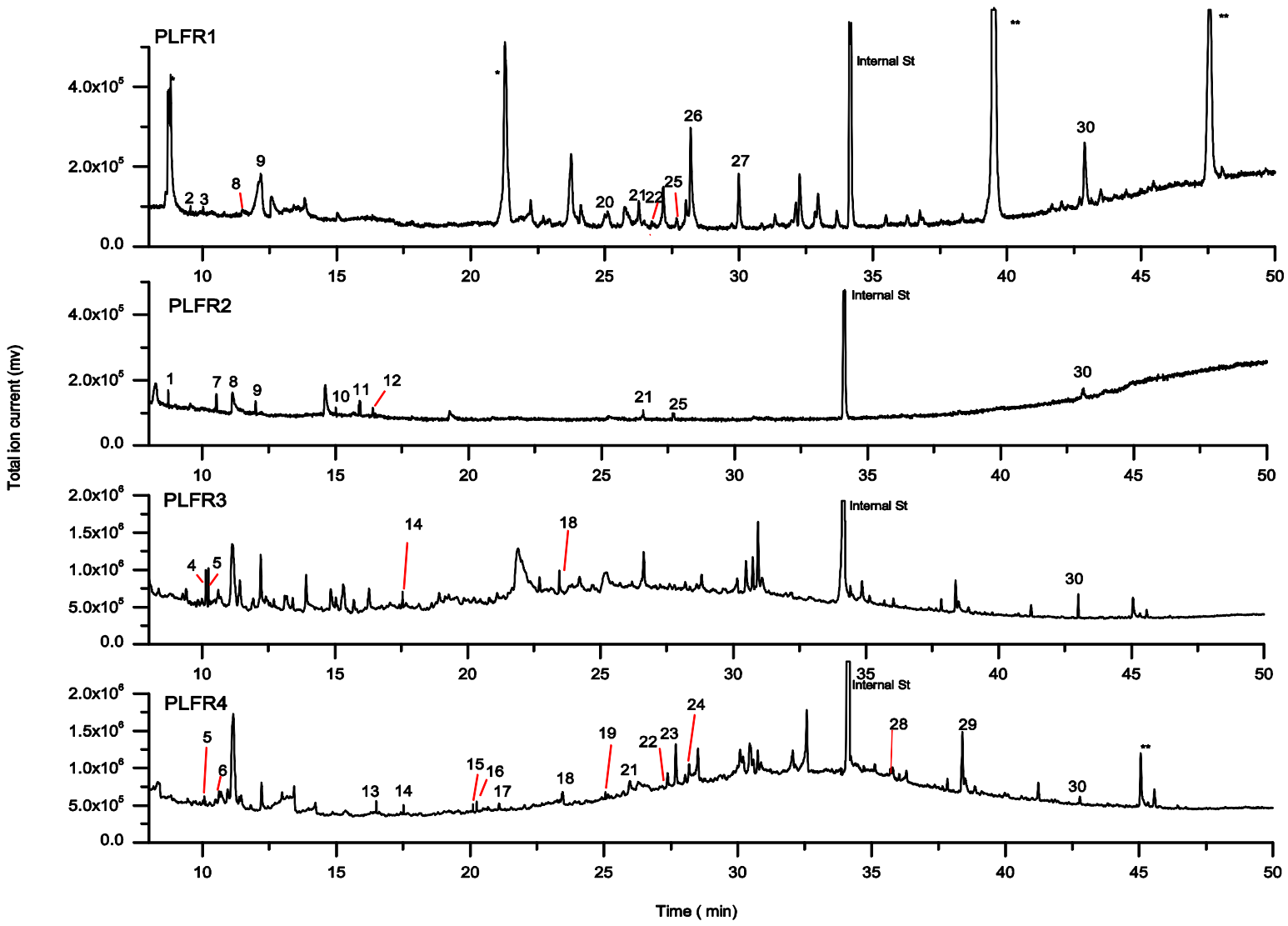


Figure 3.8 Total ion chromatogram of TMS -derivatisation products from of PLFA fractions.* indicates siloxane peaks, ** indicates contaminations

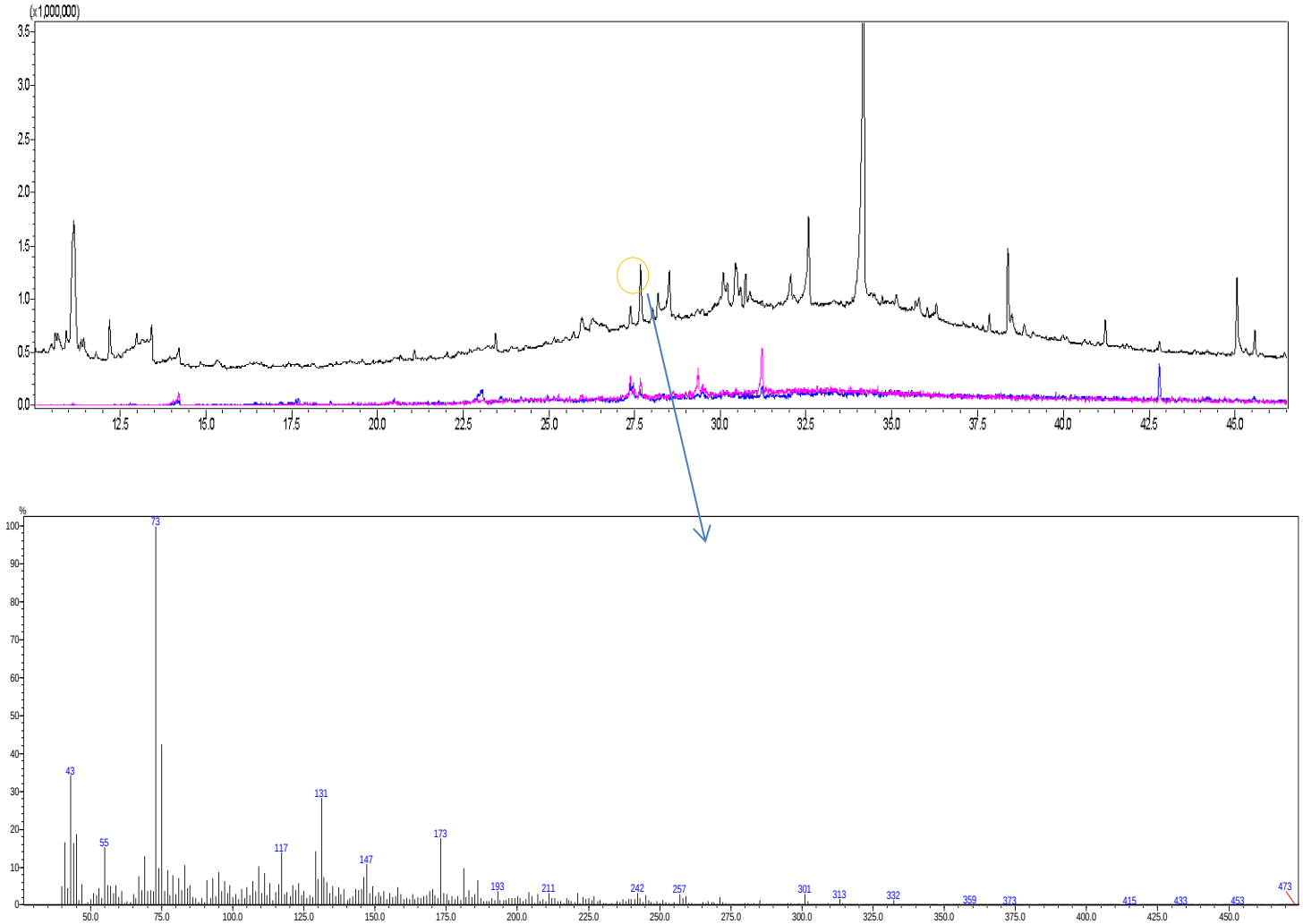


Figure 3.9 (a) Total ion chromatogram of TMS-derivatised PLFR 4 scanned for m/z 204 (purple) and 217 (blue) (b) mass spectrum of peak in 27.7 min identified as N-acetyl glucosamine

The departure of sugars, including mono and disaccharides, from the original sample to PLFR 1 is indicative of the presence of non-covalently bonded sugars in PLFA structure. However, the contribution of these kinds of sugars to the whole structure is ambiguous, as there may be non-bonded sugars strongly associated with the structure, but not separable via fractionation. There are some evidences from other studies consistent with such behaviour. For example, based on the result obtained by DOSY NMR (Simpson, 2002), fractionated peat humic acids show sugar residue that is not covalently bonded to the structure, but are associated with the core structures glycoconjugates. The presence of sugars in the hydrophobic PLFR 4 (Fig 3.9) possibly due to hydrophobic effect is in line with this theory and more importantly with the supramolecular theory of formation of humic substances (Piccolo, 2001). Also, it indicates that these sugar molecules are quite accessible by the reagent and possibly enriched at the surface, resulting in easier detection by GC. Especially important is the detection of a compound with mass spectrum containing m/z 131, 173, 259 (peak 24 in Fig 3.8) similar to mass spectrum of N-acetyl glucosamine (Medeiros and Simoneit, 2007). This observation may be a sign of the presence of acetylated amino sugars polymers in PLFA, as discussed earlier.

On the other hand, with respect to the result of NMR studies on PLFA, which suggest the presence of about 8-12 % carbohydrate (Mao et al., 2007, Thorn et al., 1989), a large number of sugars in PLFA should be covalently bonded (glycosidic bonding) to the structure, or as mentioned above heavily interacted with the main body and are not released by partitioning during SPE. Detailed analysis of the carbohydrates in PLFA fractions will be given later in this chapter.

Other detectable groups of compounds were lipids which are limited to saturated and unsaturated mono and dicarboxylic acids, apparently present as their “free” forms, whereas

bound lipids exist as ester or amide form (Wakeham et al., 2003). The di carboxylic acids were detected as short chain acids (C4-C6). C12 to C16 n-alkanoic acids appear among the products, indicative of lipid present in algae (Cranwell, 1987) cited by (Peters et al., 2005). Absence of hydroxyl fatty acids characteristics of bacterial lipopolysaccharides (LPS) (Cranwell, 1981) was expected due to the lack of ester and amide bond cleavage in the procedure.

In summary, the range of compounds identified in the four fractions was very similar to that detected in unfractionated PLFA, but identification was carried out more easily due to the improved signal to noise ratio afforded by fractionation and less unresolved complex mixture (UCM) occurrence (Fig 3.10). Furthermore, the distribution of compounds among the fractions clearly indicates the presence of micelles or other forms of hydrophobic-hydrophilic conjugated structure.

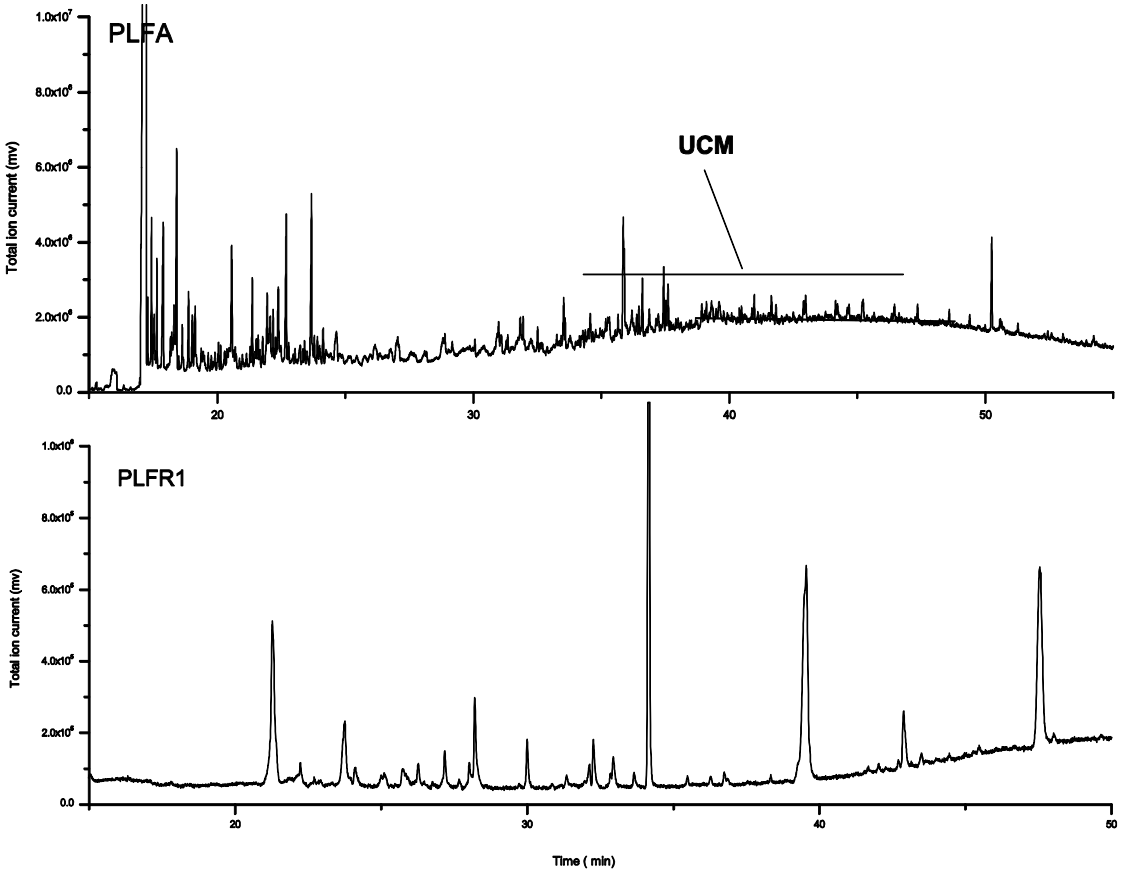


Figure 3.10 Comparison of UCM in TIC of TMS -derivatised unfractionated PLFA and PLFR 4 under similar reaction condition

3.2.5.2 Trimethyl Silyl Diazomethane (TMSD) combined with Trimethyl silylation

Methylation of carboxyl groups using trimethylsilyldiazomethane is an additional derivatisation strategy for carboxyl compounds. It was used in conjunction with trimethyl silylation for characterisation of unfractionated PLFA in Chapter 2. Here, we used the same method in order to examine the fractions of PLFA isolated in 3.2.1.

Similar to unfractionated PLFA, this additional methylation step did not yield significant derivatised components for fractions and did not appear as operative even as trimethylsilylation. This behaviour is in line with results of other attempts (see Chapter 2) including methyl-8, diazomethane, methyl iodide) for methylation of carboxylic acid groups in PLFA.

Based on the results obtained in this section, fractionation did not improve the methylation process of fulvic acid. The possible reasons for this inadequate methylation were discussed in Chapter 2. However, similar outcomes for fractions could indicate that carboxylic acids as the main target of aforementioned derivatisation did not become accessible to the reaction upon fractionation, or that they did react, but connected to other non-volatile parts, preventing them from being detected by gas chromatography.

3.2.5.3 Ethyl Chloroformate (ECF) derivatisation

The four fractions similarly to unfractionated PLFA, were subjected to ECF derivatisation. The reaction products were isolated using solid phase extraction prior to analysis, and subsequently identified using GC/MS. Figure 3.11depicts the scheme yielding ECF derivatised fractions of PLFA. Fig 3.12 and Fig 3.13 display the chromatogram of derivatised fractions. The list of compounds identified in fractions using ECF in combination with GC/MS analysis is also presented in Table 3.7.

According to the results obtained**,** derivatisation of fractions using chloroformate enabled detection of a number of heterocyclic nitrogen- containing compounds, mostly with derivatised uracil, pyrrolidindione and indole structures, considered as the target compounds of this derivatisation. Interestingly, these kinds of compounds were barely detected using the other derivatisation methods attempted in this study. Most importantly, they were obtained without much alteration of the structure as this reagent only attacks the functional groups and not the skeleton.

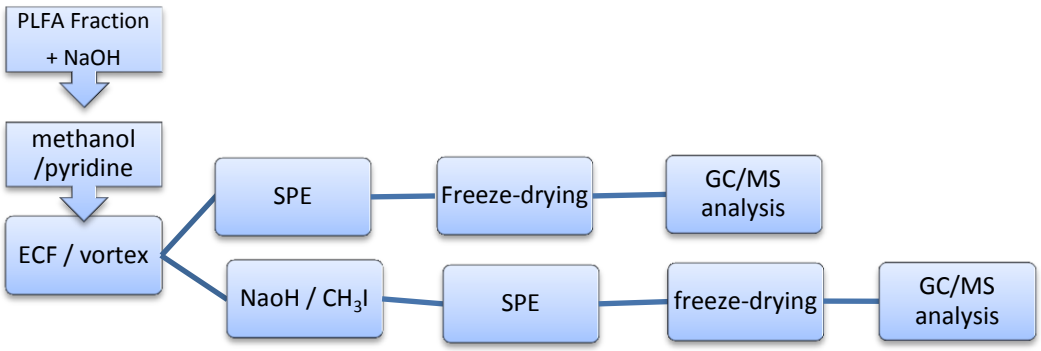


Figure 3.11 Flow chart of the scheme yielding ECF derivatised samples

Table 3.7 peak identification of compounds in PLFA frctions using ECF- GC/MS

Peak no[1]	Compounds Identified	Identification Method[2]	PLFR1	PLFR2	PLFR3	PLFR4
1	N-Formylpiperidine	a,d	-	-	+	+
2	Benzoic acid	a,d	-	-	+	+
3	Dimethyl β-isopropenyl-α-methylenesuccinate	a,d	+	+	-	-
4	dimethyl 4-oxoheptanedioate	a,d	-	+	-	-
5	(E)-methyl 7-methylnon-4-enoate	b,d	-	+	-	-
6	Nonanoic acid	a,d	-	-	+	+
7	Z-3-hexenyl methoxyformate	a,d	+	-	-	-
8	ethyl cyclohexenyl (ethyl)carbamate	b,d	-	+	-	-
9	Butanoic acid, 1H-indol-3-yl ester	a,d	-	+	-	-
10	3-(3-Methyl-2,4-dioxopyrrolidin-3-yl)propionic acid, methyl ester	b,d	-	+	-	-
11	N-decanoic acid	b,d	-	-	+	+
12	2,4,7,9-Tetramethyl-5-decyne-4,7-diol	b,d	-	-	+	-
13	Diethyl 2-oxopyrimidine- 1,3 (2H,4H)-dicarboxylate	b,d	+	+		
14	methyl 4-(ethoxycarbonylamino)-3-(methoxymethyl)cyclohexa-1,3-dienecarboxylate	b.d	-	-	+	+
15	(Z)-3-(2-methoxy-2,6,6-trimethylcyclohexylidene)oxiran-2-one	b,d	-	-	+	+
16	Nonanedioic acid, dimethyl ester	a,d	-	-	+	+
17	ethyl 2-(N-methyldecanamido)acetate	b,d	-	-	+	+
18	dimethyl 4,5,6,7-tetrahydrobenzofuran-2,3-dicarboxylate	a,b,d	+	+	-	-
19	ethyl 2-ethoxy-5-methoxynicotinoylcarbamate	b,d	+	+	-	-
20	Naphthalene, 1-(phenylmethoxy)	a,b,d	-	+	-	-
21	Pyridine-2,6-dicarboxylic acid, 4-(dimethylamino)-, diethyl ester	b,d	+	-	-	-

[1]Numbers correspond to the peaks in Fig 3.12 and 3.13, [2]dentification was based on three methods (a) matching compound suggested by NIST Mass Spectral Search Program with above 90 % match probability (b) using NIST MS Interpreter with quality indices reported as above 85 % (c) tentative identification (d) suggested structures were checked against retention data in the literature

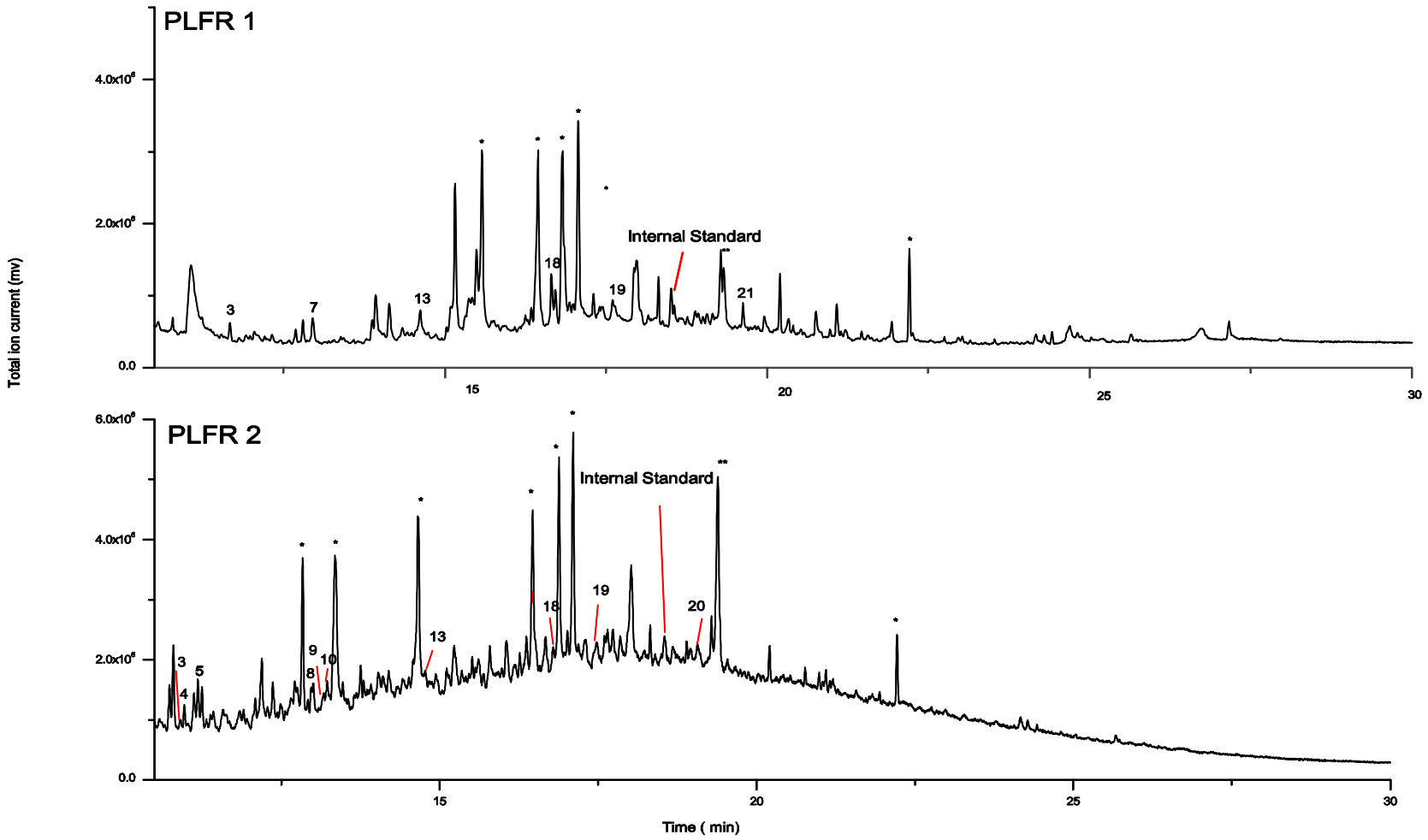


Figure 3.12 Total ion chromatogram of ECF -CH_3I derivatisation products of PLFR 1 and PLFR 2 fractions .* indicates siloxane peaks, ** indicates reagent interferences

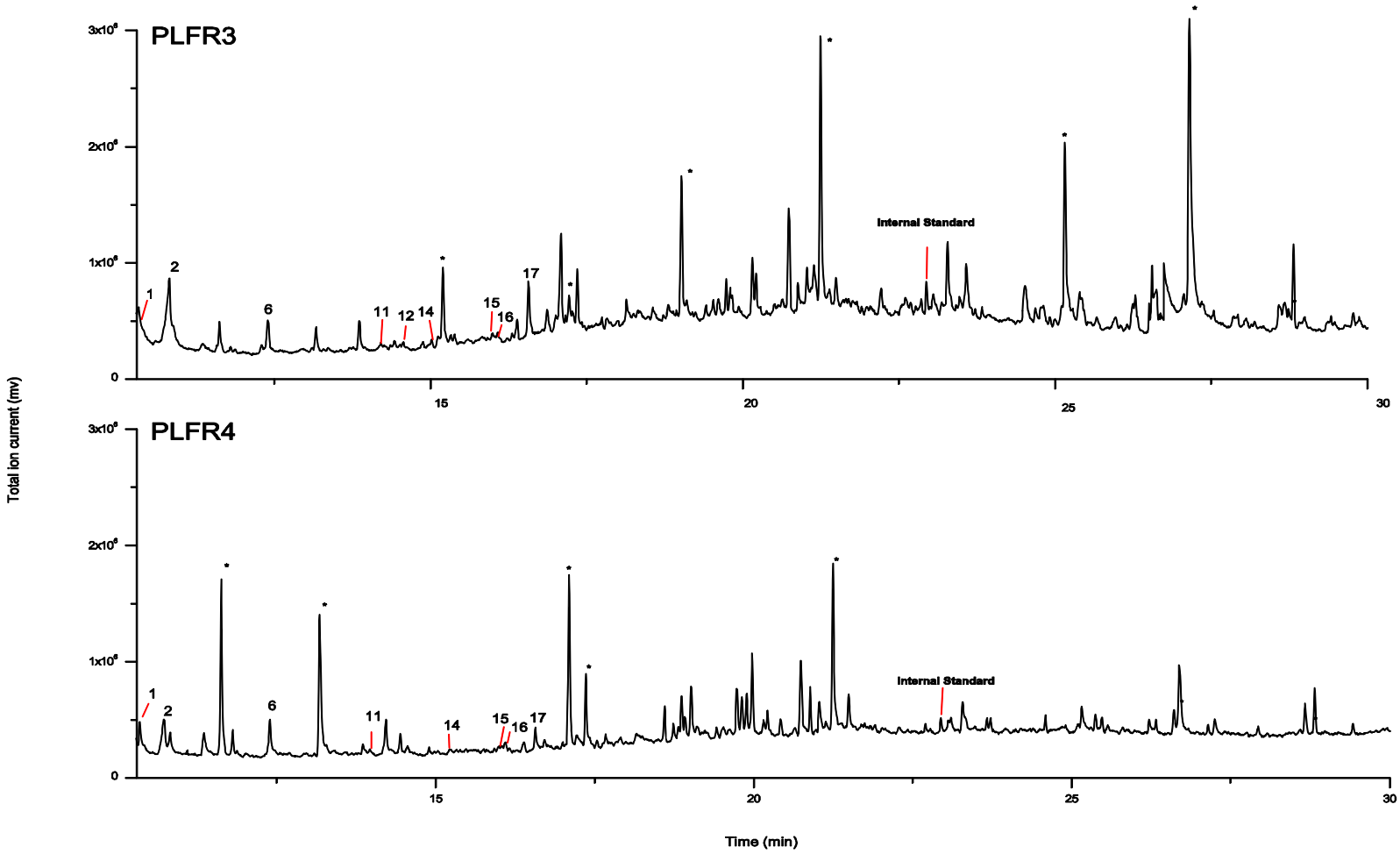


Figure 3.13 Total ion chromatogram of ECF-derivatisation products of PLFR3 and PLFR 4,*indicates siloxane peaks, ** indicates reagent interferences or contamination

The distribution of components among the fractions shows that the majority of identified compounds were detected in PLFR 2, 3 and not in PLFR 1. This could be due to the presence of more carbohydrate in PLFR 1 as compared to the other fractions, preventing PLFR 1 from satisfactory amenability and detection to GC/MS. Carbohydrates are present as free or bound forms in humic substances, and, as mentioned earlier in Section 3.2.5.1, in PLFA. In both cases (i.e. free or bound forms) sugar monomers are unlikely to become derivatised and detected by ECF, because monosaccharides are not target compounds for alkyl chlorofomate derivatisation and normally remain untouched (Husek, 1998). Moreover, carbohydrates are prone to react with amino acids (Maillard Reaction) to form complex compounds which would be difficult to detect by GC-MS. Other fractions show a range of components, For example, one can see heterocyclic structures bearing acidic or amino groups in PLFR2, while alkanoic acid (mostly di acids) are found in this fraction as well as in PLFR 3 and 4. Aromatic compounds appear in the hydrophobic fractions (PLFR 3 and 4).

Generally, ECF combined with GC/MS enabled detection of a range of compounds, although in limited quantity. It seems to be more effective for heavier constituents than volatiles. The average reaction yields for fractions based on comparison of the peak areas of known amount added internal standard (methyl stearate) to the sum of the peak areas of detected compounds is low (around 3%).This could be because there was a SPE extraction step (to isolate derivatised material and to improve resolution), which totally removed underivatised material, producing peaks in the chromatogram. This is probably why the chromatograms do not show much UCM (except for PLFR 2) and peaks are relatively well-resolved. Interestingly, comparing the results with the unfractionated PLFA, fractions derivatisation shows a better derivatisation in terms of detection of significantly more constituents. Therefore, it can be concluded that fractionation has assisted ECF, as a mild reagent to better penetrate the structure and reach hindered functional groups.

To the best of our knowledge, there is no study regarding chloroformate reaction with humic substances, therefore it is not possible to compare the current results with former works. However, alkyl chloroformtae reagents have been used for the study of phytohormones and metabolites (Villas-Bôas et al., 2011) and are able to produce simultaneous carbamates and alkyl esters on one substrate with good yield under optimum conditions.

3.2.5.4 TMAH methylation-GC/MS

The compounds identified as products of TMAH methylation are listed in Table 3.8. As observed, a wide variety of organic compounds were detected by this method. Especially notable is the abundance of nitrogen-containing compounds covering a wide range of functional groups. While many of these compounds were not (or barely detectable) in previous GC-based analyses of PLFA, their occurrence, here is significant. This is due to extensive cleavage of ester, amide and certain ether bonds (such as B–O–4aliphatic-aryl bonds) by TMAH, enabling the release of a distribution of monomers entrapped in macromolecular network (Grasset and Amblès, 1998). This confirms the efficiency of peptide bond cleavage under strong basic conditions precluding the need to apply elevated temperature. Since peptides and proteins usually undergo transformation at high temperatures (Knicker et al., 2001), the relatively lower temperature used in TMAH methylation (290 ^{0}C), as compared to Pyrolysis is an advantage. Furthermore methylation of active sites of amino acids after depolymerisation prevents alteration and facilitates the assignment of identified products to the original structure of PLFA.

Table 3.8 Peak identification of TMAH-GC/MS products in four fractions of PLFA

Peak no[1]	Compound identified	Identification method[2]	PLFR1	PLFR 2	PLFR3	PLFR 4
1	Pentanedioic acid, dimethyl ester	a,d	-	+	+	+
2	Butanedioic acid, ethyl-, dimethyl ester	a,d	-	+	+	-
3	2-Butenedioic acid, 2,3-dimethyl-, dimethyl ester	a,d	-	+	-	-
4	Pentanedioic acid, 2-methyl-, dimethyl ester	a,d	-	+	+	+
5	Benzeneacetic acid, methyl ester	a,d	-	+	+	+
6	Benzoic acid, 3-methyl-, methyl ester	a,d	-	+	+	+
7	2,5-Pyrrolidinedione, 3-ethyl-1,3-dimethyl	b,d	-	+	+	-
8	Pentanedioic acid, 3-ethyl-3-methyl-, dimethyl ester	b,d	-	+	+	-
9	1,3 dimethyl-4,5-imidazolidinedione	b,d	-	+	+	-
10	4,5 dimethyl uracil	a,d	+	+	+	+
11	2,4-Imidazolidinedione, 3,5,5-trimethyl-	a,d	+	+	+	-
12	Hexanedioic acid, dimethyl ester	a,d	-	+	+	-
13	Dimethyl-2-methoxypent-2-enedioate	b,d	-	+	+	-
14	1-(8-methyl-8-azabicyclo[3.2.1]octan-2-yl)ethanone	c,d	-	+	+	-
15	Benzoic acid, 2-amino-, methyl ester	a,d	-	+	+	-
16	1,3,5-Triazine-2,4,6(1H,3H,5H)-trione, 1,3,5-trimethyl-	a,d	+	+	+	-
17	benzoic acid 3-methoxy methylestermethylester	a,d	+	+	+	+
18	4,6(1H)-Pyrimidinedione, 3,4,5,6-tetrahydro-2-imino-	b,d	-	+	-	-
19	2-Propenoic acid, 3-phenyl-, methylester,	a,d	-	+	+	-
20	Trimethyl 1,2,3-propanetricarboxylate	a,d	-	+	+	-
21	1H-Quinolin-4-one, 8-methoxy	b,d	-	+	+	+
22	methyl 2-(4-propoxyphenyl)acetate	b,d	-	+	+	-
23	2-Butyl-5-(6-heptenyl)pyrrolidine	b,d	-	+	+	-
24	5-Methoxycarbonyl-2-thiophenecarboxylic Acid Hydrazide	b,d	-	+	+	-
25	Undecanoic acid, methyl ester	c,d	-	-	+	-

Table 3.8 continued

Peak no[1]	Compound identified	Identification method[2]	PLFR1	PLFR2	PLFR3	PLFR4
26	6-methoxy-5,6-dihydrothiazolo[2,3-c][1,2,4]triazole-3-carboxamide	b,d	-	+	+	-
27	Octanedioic acid, 3-methyl-, dimethyl ester	a,d	-	-	+	+
28	Dimethyl 2-[(pyrrole-2-yl) methylidene]malonate	b,d	-	+	+	+
29	Carbamic acid, 2,5-dimethoxyphenyl-, methyl ester	a,d	-	+	+	+
30	2-(p-Methoxyphenyl)-N-methyl-2-oxoacetamide	b,d	-	+	+	+
31	4-(N',N'-Dimethylamino)-N-(3-methylphenyl)aniline	b,d	-	+	+	+
32	ethyl 1-methyl-3-phenylpyrrolidin-2-carboxylate	a,d	-	+	+	+
33	Pentadecanoic acid, methyl ester	a,d	-	-	+	+
34	2-Benzylthio-5-methyl-2,4-hexadiene	b,d	-	+	+	-
35	2-(benzyloxy)-2-oxoethyl 5-methoxypicolinate	b,d	-	+	+	-
36	(E)-1-(2-methoxyethyl)-3-(6-phenylhex-5-en-2-yl)benzene	b,d	-	-	+	-
37	3-(4-Methylphenyl)-5,5-Dimethyl-4-Phenyl-5h-Pyrazole	b,d	-	-	+	-
38	Methyl 8-Isopimaren-18-Oate	b,d	-	-	+	-
39	4-methoxy-9-phenyl-3a,4-dihydronaphtho[2,3-c]furan-1(3H)-one	b,d	-	+	+	-

[1]Numbers correspond to the peaks in Fig 3.14, [2] Identification was based on three methods (a) matching compound suggested by NIST Mass Spectral Search Program with above 90 % match probability b) using NIST MS Interpreter with quality indices reported as above 85 % (c) tentative identification (d) suggested structures were checked against retention data in the literature.

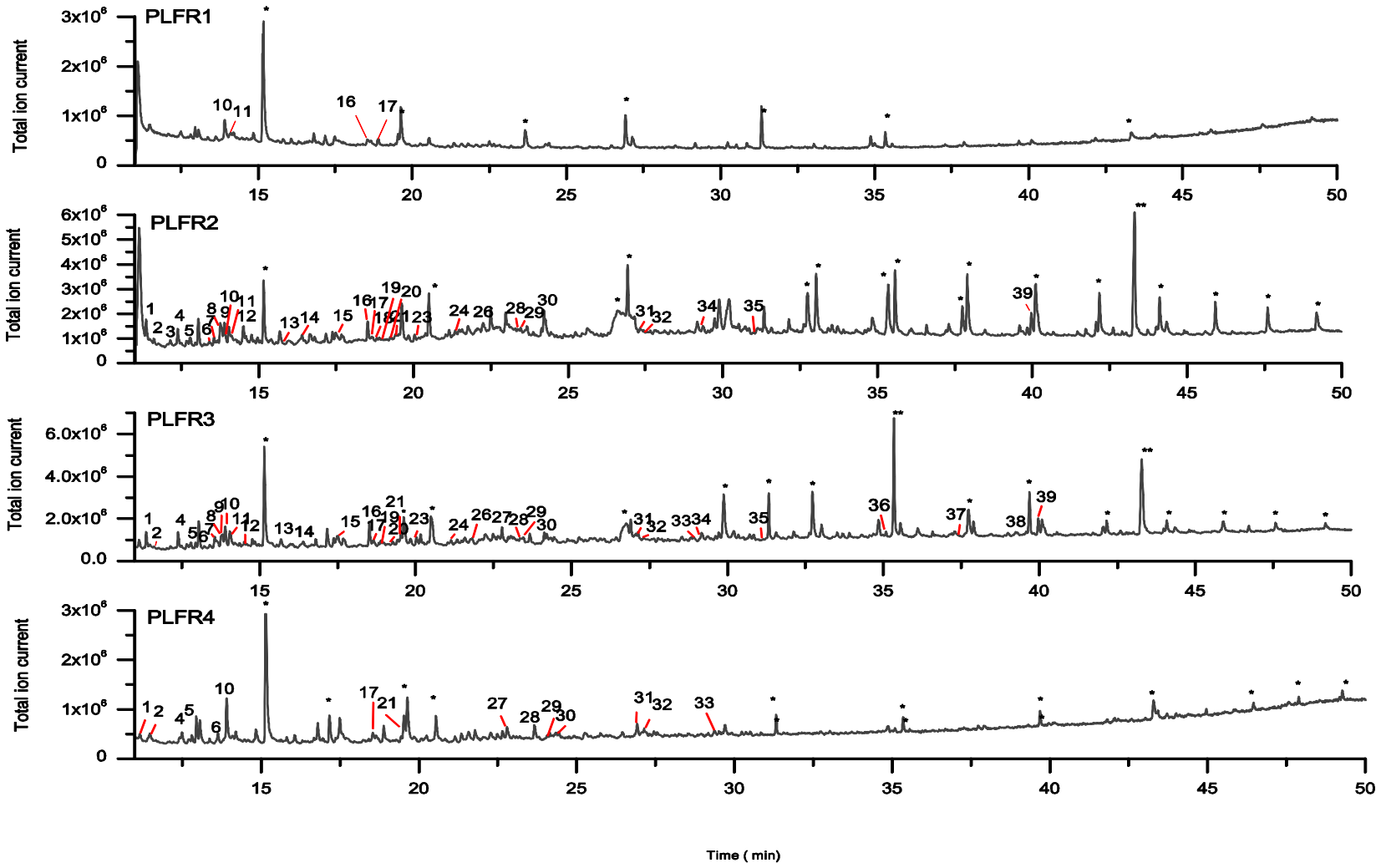


Figure 3.14 Total ion chromatograms of PLFA fractions products obtained under derivatisation with TMAH, * indicates siloxane peaks, ** indicates contaminations

3.2.5.4.1 Nitrogen-containing compounds

The abundance of heterocyclic nitrogen-containing structures such as pyrroles, pyrimidines, imidazoles and indoles is remarkable in this experiment. The majority of these compounds are believed to be protein biomarkers, for example, uracil (Pyrimidine-2,4 (1H,3H)-dione) can be produced from RNA (Iwaia et al., 2013), while pyrrolidones may be derived from proline-containing peptides (Madigan et al., 2000). Pyrrolidine and pyrrolidine-diones are usually derived from amino acids bound to phenolic or quinone group as detected in soil organic matter by TMAH-GC/MS (Stevenson, 1994). Indole may have originated from tryptophan-containing peptide (Chefetz et al., 2002 a). Pyridine, pyrazine and primary amide are structures formed by the incorporation of ammonia into NOM (Thorn and Cox, 2009). The presence of pyrrole and pyridine derivatives can be due to the condensation of amino acids with carbohydrate (Knicker et al., 2001).With respect to low abundance of these kinds of products, such condensations is not likely to occur.

Heterocyclic N-compounds in Antarctic fulvic acid have been previously examined very briefly using TMAH-GC/MS. The presence of heterocyclic nitrogenous compounds, including proline, pyrrolidinedione, piperidine and indole structures was indicated (Fimmen et al., 2007) in agreement with our identified compounds. The number of compounds reported was fewer compared to what was derived here from PLFA fractions. Heterocyclic compounds occurrence has been reported using spectroscopic techniques in PLFA using ^{15}N and $^{13}C\{^{14}N\}$ NMR for nitrogenous materials (Fang et al., 2011). Several nitrogen-containing model molecules were investigated via dipolar dephasing and their spectra compared to PLFA under similar condition. The attempts yielded compelling results, suggesting the presence of hydantoin and alatonin in PLFA, which has been confirmed by molecular-level analysis in this study.

Admittedly, identification of organic nitrogen in an environmental sample is tremendously perplexing. The reasons should be sought in different areas. From a spectroscopic perspective, the large chemical shift anisotropy is a complicating factor in the detection of pyridine- type nitrogens by NMR (Kelemen et al., 2002) cited by (Thorn and Cox, 2012). Additionally, problems of chemical exchange may hamper the detection of nitrogens in enamino-imino structures (Thorn and Cox, 2012).

When it comes to chromatography, in many cases peptides as the primary source of dissolved organic nitrogen, are resistant to depolymerisation by common hydrolysis methods (Cheshire et al., 1992).This makes the detection and estimation of amino acid moieties uncertain. Moreover, it is difficult to detect nitrogenous organics due to their adsorption and subsequent tailing on GC columns (Kataoka, 1996), appropriate derivatisation can thus be problematic. Nevertheless, the real challenge lies in the former issue. Previous studies (Knicker and Hatcher, 1997, Knicker et al., 1996 b)have indicated the presence of amide functionalities in the HCl-hydrolysis residue of humin fraction of sapropel in terrestrial-based lake. In a separate study, Cheshire (Cheshire et al., 1992) by comparison of curie-point pyrolysis mass spectra of acid- hydrolysed and non- hydrolysed soil fulvic acid, indicated that the difference between the two sample may be attributed to a glycoprotein structure.

3.2.5.4.2 Fatty acids

There are various short and long chain fatty acidsin the four fractions. The main source of fatty acids in natural organic matter is lipid membrane in eubacteria as glycolipids and phospholipids (Peters et al., 2005). Short alkanoic acids are normally of microbial origin (Allard, 2006). Likewise, long fatty acids such as hexadecanoic and octadecanoic acids, as components of lecithin in phospholipids are to be expected in dissolved organic matter with microbial origin. They are observable in the PLFA fractions as presented in Table 3.8. These fatty acids and their unsaturated counterparts can also come from lipids in algae(de Leeuw

and Baas, 1993, Gallois et al., 2007). Marine sediments were also reported as the origin of even carbon-numbered fatty acids methyl esters (Iwaia et al., 2013). Odd carbon –numbered and branch chained fatty acids are well-known bacterial biomarkers (Deshmukh et al., 2001).

3.2.5.4.3 Aromatic compounds

Aromatic moieties were found mostly in PLFR 2 and 3 which contain larger overall numbers of compounds relative to PLFR 1 and 4.There are small aromatic molecules bearing benzene, benzoic acid or phenol with a short branch attached. Interestingly, the majority of them comprise at least one nitrogenous functional group which means nitrogen atoms are significantly connected to aromatic structure. This behavior has already been reported by Mao and his colleagues using ^{15}N NMR (Mao et al., 2007). Other aromatic compounds include 4-methoxy phenyl derivatives, possibly originating from amino acid tyrosine. However, tryosine is not very abundant (1.83 %) in PLFA, with methoxy phenyls being relatively high, the origin must be something other than tyrosine. Methoxy phenols are believed to be indicators of lignin, originating from vascular plants (Chefetz et al., 2002, Estournel-Pelardy et al., 2013, Nierop and Filley, 2008, Tan, 2014).Therefore, their presence is surprising in microbial-based natural organic matter. Purdue and Benner (Perdue, 2009) have previously highlighted the fact that marine DOM and fresh water DOM sometimes show similar composition in their extracted humic substances, but, it is not clear at this stage how reasonable it is to observe terrestrially-derived molecules in autochthonous DOM. Apart from lignin monomers there is a compound named Methyl 8-isopimaren-18-oate (peak 38 in Table 3.8) among products of PLFR3 thermochemolysis, this compound has a structure similar to resin acids which normally originate from plant decomposition, this also implies that there may be terrestrially-derived material in Pony Lake fulvic acid.

3.2.5.4.4 Carbohydrates

Carbohydrate-derived compounds were not observed among the identified compounds, probably because TMAH methylation is not able to fully break glycosidic bonds and release sugar monomers, (del Rioa et al., 1998).As previously shown, this technique allows the analysis of free (or terminal) carbohydrates. As such, amounts of non-cellulosic carbohydrates obtained by TMAH methylation are reported to be lower than those obtained by classical acid hydrolysis(Estournel-Pelardy et al., 2011). In a study comparing classical TMAH methylation of humic substances with a two-step analysis using hexamethyl disilizane prior to TMAH methylation, it was revealed that classical TMAH similar to what was used here, is biased against the detection of sugar moieties (Estournel-Pelardy et al., 2013). This necessitates the application of hydrolysis on PLFA fractions in order to cleave the intermolecular bonds, enabling a better carbohydrate identification and detection.

Generally, taking all the results of TMAH together, it appears more effective for structural analysis of PLFA fraction using gas chromatography compared to the previous methods presented in this chapter, specifically for nitrogenous components. In terms of distribution of compounds among the fractions, given that similar compounds were detected in fraction 2 and 3, these two fractions seem to be similar in composition and possibly not very well-resolved during SPE fractionation. PLFR 1 did not show many compounds, which could be due to the presence of sugars which do not derivatise as mentioned above. PLFR 4 had apparently different composition and its components were limited to the higher molecular weight moieties.

3.2.5.5 C-O bond cleavage of PLFA fractions using acid digestion

Various acid hydrolysis methods for cleaving glycosidic bonds in carbohydrates including naturally occurring poly and oligosaccharides, have been described in the literature The most commonly used hydrolytic agents are 2.0 M trifluoroacetic acid (TFA), 3.0- 6.0 M methanolic hydrochloric acid and concentrated sulfuric acid. The last which is harsher than the other two methods normally involves a two-step procedure including digestion with 12 M sulfuric acid solution followed by dilution of the resulting solution to around 0.1 M for cleavage completion (Templeton et al., 2012). However, there is no universal method for the study of natural carbohydrates and the applications of acidic hydrolysis are different depending on the nature of the polysaccharides, or in other words, the type of monosaccharides present in the polysaccharide structure. Analysis of carbohydrates in DOM is also wide-ranging, covering all of the above methods, Hydrolysis efficiency also appears questionable when it comes to DOM applications; this is because DOM usually comprises hetero polysaccharides (Aluwihare and Repeta, 1999) unpredictably connected via different α and β (1-6, 1-4, 1-3 and 1-2) linkages. This linkage variety in turn affects the hydrolysis feasibility and the yield of the carbohydrates involved (Panagiotopoulos and Sempéré, 2005). Aligned with this is the quality of the linkage to peptides as a common concomitant material present in DOM , forming O and N- linked glycol peptides which cleave differently under acidic digestions (Mega and Ikenaka, 1982). Another issue is associated with the hydrolysis of uronic acid-containing polysaccharides; this kind of polysaccharides is proving challenging to analyse as carboxyl groups prevent glycosidic bond cleavage via its stabilization (Lindberg et al., 1975). Uronic acids are quite abundant in nature as part of extracellular polysaccharides in bacteria or plants (Hung et al., 2001, Mopper, 1977, Walters and Hedges, 1988) and therefore are expected to be present in DOM.

The origin of marine-based polysaccharidesis believed to be marine organisms such as bacteria, fungi and microalgae, mostly in the form of extracellular polysaccharides (known as EPS). For monosaccharide analysis of bacterial polysaccharides both HCl methanolysis and TFA hydrolysis were employed. For marine cyanobacteria's sugar moieties, for instance, methanolysis using HCl 4M has been used (Jensen et al., 2013). EPS of cyanobacteria was also characterised using TFA 2M (Nicolaus et al., 1999, Parikh and Madamwar, 2006).There are other studies which have employed a combination of HCl methanolysis and TFA hydrolysis to improve the recovery(De Ruiter et al., 1992, Muldoon et al., 2001).

Remarkably, one major category of marine polysaccharides are found in particulate organic matter (POM) and HMWDOM, this type of organic matter was extensively studied for their sugar constituents(Amon et al., 2003, Benner and Kaiser, 2003, Hernes et al., 1996) . Although the origin of marine humic substances is still unknown to geochemists, it can be attractive to assume that oceanic HMWDOM and humic substances have similar sugars in their structure. Aluwihare and her colleagues' key finding (Aluwihare et al., 2005) about acetylated amnio polysaccharides in HMWDOM of surface oceanic water were associated with using methanolic HCl 4M, although they reported an incomplete cleavage using that reagent. They also reported application of TFA 2M for the analysis of polysaccharides in HMWDOM isolated from Atlantic waters (Aluwihare et al., 2002).

Overall, it seems inappropriate to suggest a specific hydrolysis procedure especially for ill-defined materials such as fulvic acids. Also, no direct comparison of TFA, H_2SO_4 and HCl methanolysis has been made to date. To the best of our knowledge, there is no previous study on carbohydrates present in PLFA, and IHSS has not provided any information in this regard. Herein, hydrolysis of PLFA fractions using four hydrolytic agent including trifluoroacetic acid (TFA), sulfuric acid, methanolic HCl and a combination of methanolic HCl and TFA will be discussed. The subsequent molecular-level analysis of hydrolysates will be presented.

3.2.5.5.1 Recovery of monosaccharides after acidic digestion methods in PLFA fractions

Table 3.9 represents the amount of monosaccharides recovered using different acid digestion methods. The maximum amount of sugar released is around 2.8 % released from PLFR1 which was achieved via HCl methanolysis. Other methods yielded lower sugar monomers of this fraction as well as other fractions. Generally, the total amount of carbohydrates obtained using gas chromatography after acidic digestions is lower than that reported by NMR studies. Possible reasons are that firstly, many glycosidic bonds do not cleave, resulting in an underestimation of the quantity of monosaccharides. Secondly hydrolysates are not GC amenable or in other words their analysis is totally infeasible using chromatographic methods including gas and liquid chromatography (Panagiotopoulos et al., 2007). In the current study, however, we recovered around 2.8 % sugar at the maximum (in PLFR 1). This is in agreement with past studies indicating that only 5-20 % carbon in HMWDOM is detected as sugar monomers using hydrolysis-based methods and subsequent gas chromatographic detection (Panagiotopoulos et al., 2007, Quan and Repeta, 2007).

Table 3.9 Monosaccharides percentage(mg/100 mg dry substance) of PLFA 's fractions and chitin obtained under different acidic hydrolysis method, (percentages calculated based on peak areas of acetylated monosaccharide compared to acetylated inositol as internal standard analysed by GC/FID). For comparison purpose, only PLFR1 was hydrolysed by sulfuric acid

Fractions	CF_3COOH 2 M	H_2SO_4 12 M	HCl 4M (8 h)	HCl 4M (24 h)
PLFR 1	1.66	1.89	1.10	2.80
PLFR 2	0.16	nd[1]	0.11	1.10
PLFR 3	trace	nd	trace	1.03
PLFR 4	trace	nd	trace	2.04
Chitin[2]	nd	70.76	40.15	70.45

1 = not determined, 2 = Sigma cat. No C7170

3.2.5.2 Hydrolysis using trifluoroacetic acid (TFA)

Hydrolysis using TFA followed by derivatisation to alditol acetates (Fig 3.15) showed low recovery of monosaccharides (Table 3.9). Low recovery of sugars for PLFR 3 and PLFR 4 or even PLFR 2 is not surprising because they are not quite hydrophilic, and supposedly would not contain much sugar. For PLFR 1, the recovery was higher, at around 1.66 %. In comparison to the 8-12 % carbohydrate reported by NMR (Mao et al., 2007) for bulk PLFA, the aforementioned value implies that we had experienced ineffective glycosidic bonds cleavage within polysaccharide units, yielding an overall recovery of around 2% for unfractionated PLFA. De Ruiter (De Ruiter et al., 1992) has also reported incomplete depolymerisation of fungal poly saccharides and glucosamine-containing pectin materials using TFA as hydrolysis agent, However, TFA is a common reagent for analysis of carbohydrates in different types of polysaccharides and oligosaccharides (Sassaki et al., 2005) as well as for DOM derived from soil and marine HMWDOM (Allard, 2006, Aluwihare et al., 2002). This suggests that carbohydrates present in PLFA can be different from those of in its soil or even marine originated counterparts.

3.2.5.5.3 Hydrolysis using sulfuric acid

Fig 3.16 represents the total ion chromatogram of PLFR 1 exposed to hydrolysis by concentrated sulfuric acid and the resulting alditol acetate of the monosaccharides. It is immediately evident that the sulfuric acid was more effective in cleaving glycoside bonds than TFA and released higher amount of sugar monomers, although the total yield was still not satisfactory (Table 3.9). One reason for the unsatisfactory recovery might be due to the missinghydrolysate during sulfate ion removal using ion pair agent (dimethyl octylamine) which could remove anionic sugars, such as uronic acids. Another problem is the potential failure of sulfuric acid in cleaving beta(l-4) linkages in polyglucuronic acid (Biermann, 1988) cited by (De Ruiter et al., 1992). This is also in agreement with previous studies. For example

incomplete cleavage of polysaccharides by acid hydrolysis using sulfuric acid has necessitated the need to use enzymatic cleavage for marine particulates(Leskovkk et al., 1994). In order to test the efficiency of the procedure to disintegrate polysaccharide structure the same acidic digestion and derivatisation was performed on commercial chitin. As presented in Table 3.9 more than 70 % recovery of monosaccharide (acetylated glucosamine) was obtained using sulfuric acid two-step digestion, which shows the applicability of the method for C-O bond cleavage.

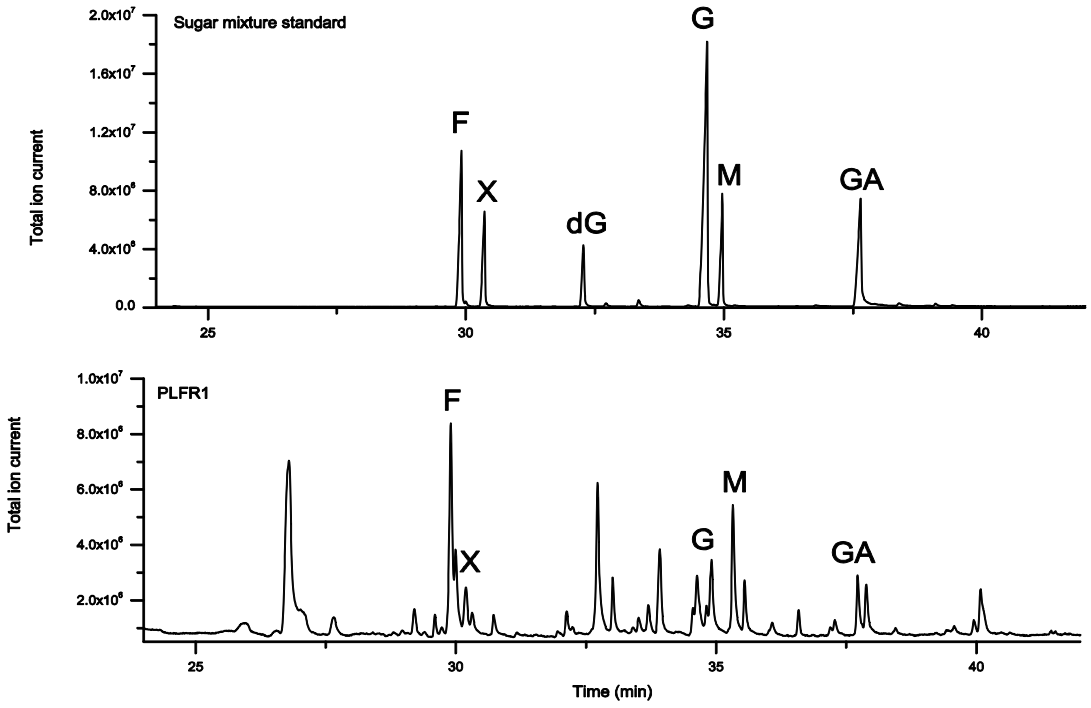


Figure 3.15 Total ion chromatograms of (a) PLFR1 hydrolysed by TFA and (b) standard sugar mixture containing alditol acetate s of Fucose, Xylose, Mannose, Galactose, 2- Deoxy glucose, Glucosamine

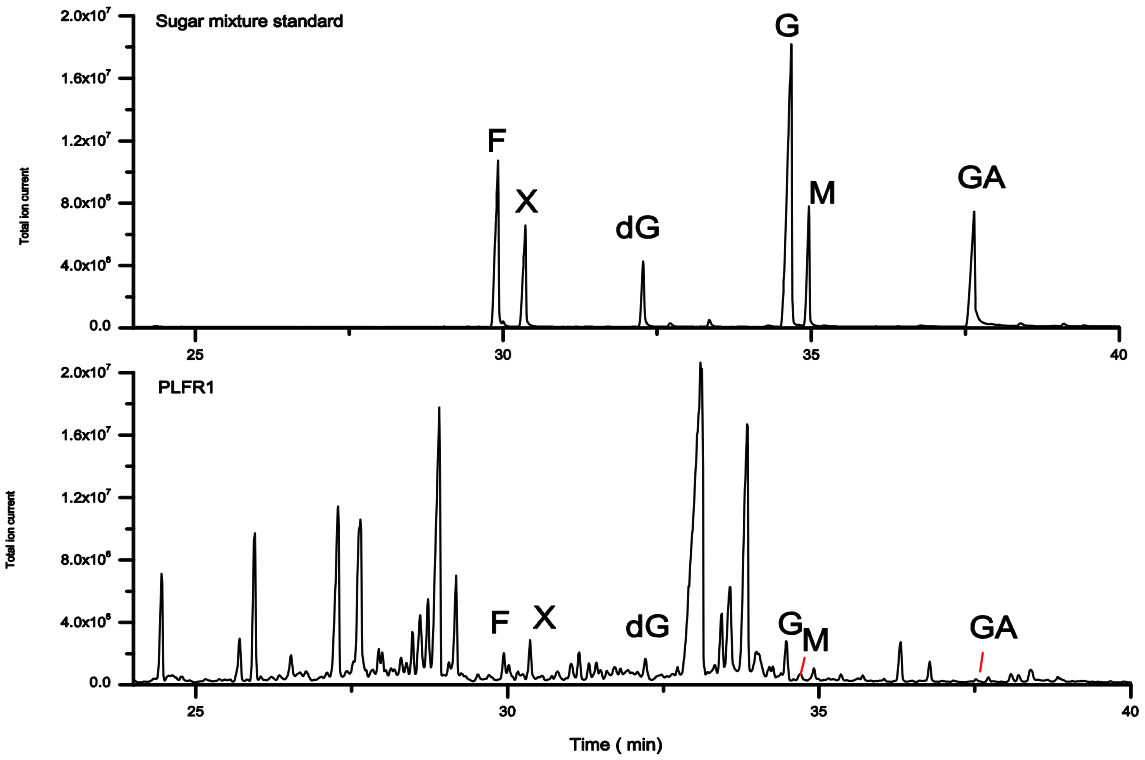


Figure 3.16 Total ion chromatograms of (a) Fraction1 hydrolysed by sulFuric acid and (b) standard sugar mixture containing alditol acetate s of Fucose, Xylose, Mannose, Galactose, 2- Deoxy glucose, Glucosamine

3.2.5.5.4 Methanolysis using hydrochloric acid

Recovery of monosaccharides after methanolysis of four fractions and chitin using HCl under two reaction periods (8 and 24 h) is shown in Table. 3.9. As shown the recovery of glucosamine in chitin was around 40 % after 8 hours reaction time , whereas after 24 hours it significantly improved to more than 70 % comparable with the amount reported previously for chitin hydrolysis using HCl (Benner and Kaiser, 2003).The same trend is observable for fractions, furthermore, recovery of monosaccharides by HCl after 24 h is the highest among the three acidic depolymerisation methods. Although HCl 4M is a weaker acidic solution than sulfuric acid 12 M, it eventually acted more effective with the aid of heating and extended reaction period (24 h). The necessity of using hot acid in longer period to fully depolymerize fulvic material is consistent with the previous finding about close association or protection of

proteinaceous amino acids by humic substances (Cheshire et al., 1992, Schulten and Schnitzer, 1998). Interestingly, while PLFR 2 and 3 showed comparable values, PLFR 4 showed higher sugar content than PLFR 2 and 3. Given that this fraction containing hydrophobic material, the only explanation can be the presence of hydrophilic moieties such as sugar due to the micelle formation.

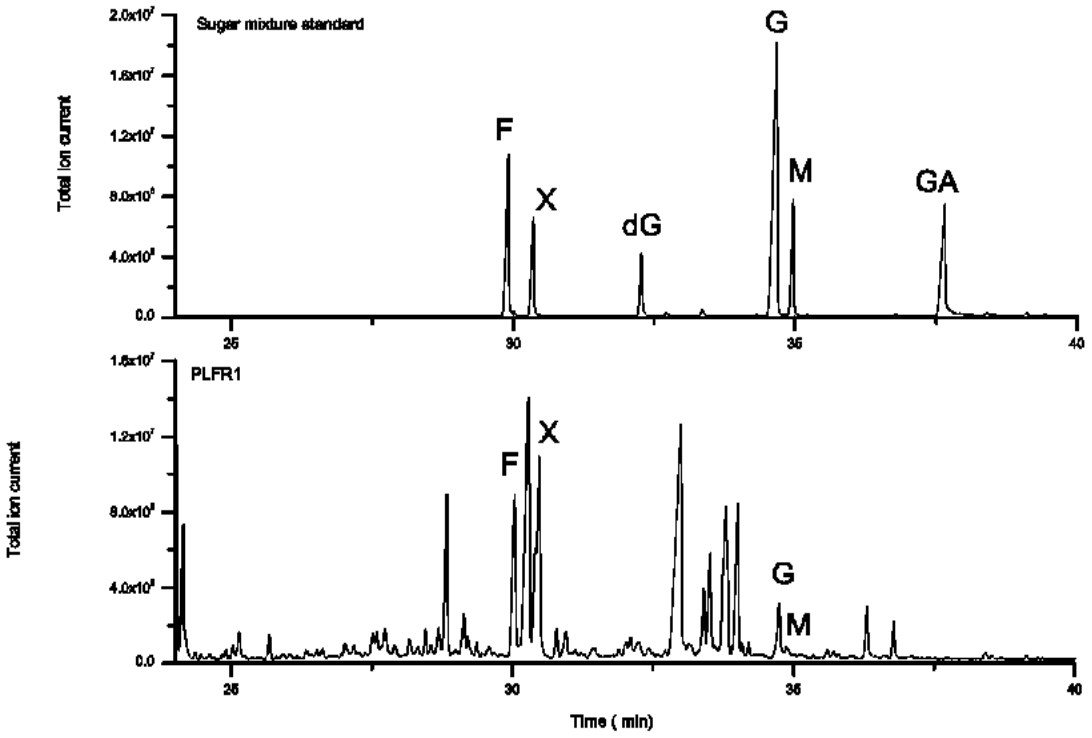


Figure 3.17 Total ion chromatograms of (a) Fraction1 hydrolysed by methanolic HCl and (b) standard sugar mixture containing alditol acetate s of Fucose, Xylose, Mannose, Galactose, 2- Deoxy glucose, Glucosamine

Correspondingly, Aluwihare (2005) indicated that acid hydrolysis of MMWDON from surface seawater recovered only low amount of N-acetyl glucosamine from a sample believed to contain significant chitin structure; their procedure was based on overnight exposure to methanolic HCl 4M which is a common method for polysaccharide analysis. Our results also showed low recoveries of monosaccharides for the fractions (Table 3.9) using the aforementioned method. Application of an extended methanolysis time (24 h), however, appeared to be more successful for both chitin and the fulvic acid fractions.

3.2.5.5 De-lactonisation prior to reduction to improve the detection of sugar acids

Due to the importance of sugar acids in naturally-occurring polysaccharides, and in view of the failure to detect sugar acids after various hydrolysis methods were applied on the fractions, in a separate experiment a procedure especially designed for release and detection of sugar acids by gas chromatography (Sassaki et al., 2005) was investigated. This procedure which adds an alkaline hydrolysis after hydrolysis (methanolysis) and prior to the reduction step, breaks the possible lactone formed by neighbour acidic and alcohol group on sugar rings, yielding carboxylate anions instead of carboxylic acid. By subsequent methylation of these anions, they become detectable by GC analysis. Our attempt to recover possible sugar acids using this method was not successful and almost no new structure including sugar acids were detected after this experiment. This can imply that all hydrolysable bonds were cleaved using the previous hydrolysis, and that further hydrolysis is not achievable. Sugar acids, may be present in very trace amounts, possibly missed during multiple derivatisations and extractions or are in positions which are not easily exposes to acidic cleavage. There are previous report about the incomplete hydrolysis of the glycosidic linkage of aldobiuronic acids types (acidic disaccharide in which one of the two sugar constituents is a uronic acid joined as a glycoside to a hexose or pentose) (Garleb et al., 1991). Assuming such a linkage is present in Pony Lake fulvic acid, this observations can empower the possibility of sugar acids contribution in the hydrolysis-resistant part of Pony Lake fulvic acid.

3.2.5.6 Distribution of monosaccharides released by sulfuric acid and hydrochloric acid digestion

The monosaccharides (acetylated products) detected in the four fractions using sulfuric acid and hydrochloric acid digestions are presented in Table 3.10 (peak no. 25 afterwards). As expected, mainly neutral sugars and amino sugars such as amino glucose and amino galactose were detected in the fractions (not detected after TFA hydrolysis).The presence of amino sugar could be a sign of acyl heteropolysaccharides (APS), or in other words N-acetyl aminopolysaccharide (NAAPs) in the fractions. N-AAPs appears as peptidoglycan or chitin widely found in DOM from aquatic environments, including surface and deep ocean (Gogou and Repeta, 2010, Repeta et al., 2002); its origin is believed to be microorganisms such as microalgae or microbial communities (Hedges, 1997). Products of HCl methanolysis are mostly O-methylated monosaccharides, their formation is due to the exposure of substrate to hot acid in methanol solution which encourages the ring formation in monosaccharides (Mega and Ikenaka, 1982). This is why some extra products (peaks) in the case of methanolysis are observed in contrast to sulfuric acid digestion. Majority of monosaccharides were detected in PLFR 1 (the most hydrophilic fraction) using methanolysis. Identified monosaccharides cover a wide range of sugars, including neutral sugars, deoxy sugars, anhydrous sugar, methylated sugar (methylated hexsose and pentoses) as well as amino sugars. They are mostly hexsoses such as glucose, galactose, mannose, or pentoses such as xylose, arabinose, ribose, also fucose, rhamnose as deoxy hexoses which are all present in PLFR 1. Glucosamine is, interestingly observed in all four fractions while galactosamine was present in all fractions except PLFR 3.

The studies of carbohydrate composition of NOM are mostly limited to HMWDOM or UDOM. Thereby, there are not many paper dedicated to analysis of sugar moieties in humic substances in natural ecosystems. One of the few examples that focused on humic substances is the study by Cheshire (Cheshire et al., 1992), which extensively examined carbohydrates

from soil fulvic acid qualitatively and quantitatively using various hydrolysis methods, and was able to detect only reducing sugars. In another study humic acid neutral sugar was analysed after TFA hydrolysis and the quantities of neutral monosaccharides were calculated in order to prove the microbial origin of the material (Allard, 2006). One important shortage in the detection of sugar compounds is related to sugar acids and most importantly uronic acids, found in natural ecosystems. As previously discussed, this type of sugar because of their linkage and combination to reducing sugars, are difficult to hydrolise and this has negatively affected their structural studies.

In conclusion, the presence of various deoxy sugars, methylated sugars and anhydrosugars such as galactosane previously reported in marine HMWDOM (Panagiotopoulos et al., 2013) indicates that refractory marine DOM, consisting high level of fulvic acids are structurally related or indirectly originated form heteropolysaccharides in HMWDOM.

Table 3.10 The list of monosacharides detected by methanolysis in four PLFA fractions

peak no	Compounds identified	Identification method [2]	PLFR 1	PLFR2	PLFR3	PLFR4
1	Butanedioic acid, monomethyl ester	a,d	+	+	+	-
2	Methyl 2-acetamidoacetate	a,d	+	+	+	-
3	N-acetyl 2- pyrrolidone	a,d	-	-	-	-
4	1-(2,3-dihydro-4-methyl-1H-pyrrol-1-yl)ethanone	a,d	-	-	+	+
5	2-acetamidobutyric acid	b,d	+	-	-	-
6	N,N'-Diacetylethylenediamine	a,d	+	-	-	-
7	Methyl N-acetyl-L-valine	a,d	+	+	+	+
8	Methyl N-acety l,leucinate	a,d	+	+	+	+
9	Methyl N-acety l,Isoleucinate	b,d	+	+	+	+
10	Glycerol triacetate	a,d	+	+	+	+
11	Acetyl N-acetyl threonine	a,d	+	+	+	+
12	Acetyl N-acety leucinate	a,d	+	+	+	+
13	Dimethyl-N-acetyl-L-aspartat	a,d	+	+	+	+
14	Methyl 4-O-acetyl-2,3-di-O-methyl-6-deoxy-α-D-mannopyranoside	a,d	+	-	-	-
15	Dodecane, 4,6-dimethyl-	b,d	-	+	+	+
16	Benzoic acid, 4-(acetyloxy)-, methyl ester	a,d	-	+	+	+
17	2-Piperidinecarboxylic acid, 1-acetyl, ethyl ester	a,d	-	-	+	+
18	6,7-Dimethoxy-4-methyl-4H-chromene	b,d	-	+	+	+
19	1,4-O-methyl-α-D-xylopyranoside (cyclic)	a,e	+	-	-	-
20	Dimethyl-N-acetylglutamate	a,d	-	+	+	+
21	Ethyl-N-Acetyl-L-methionine	b,d	+	+	+	+
22	Trimethyl(1R,2R,3S)-cyclopentane-1,2,3-tricarboxylate	b,d	-	-	+	-
23	Hexadecane	a,d	-	+	+	+
24	Butane-1,2,3,4-tetraol (open chain)	a,e	+	-	+	-
25	3-deoxy rhamnose triacetate	c	+	-	-	-
26	Methyl -β-D-ribopyranoside (cyclic)	e,d	+	-	-	-
27	Methyl β-D-xylopyranoside (cyclic)	e,d	+	-	-	-

Table 3.10 continued

28	methyl 4-acetoxy-5-methoxy-2-(pentan-2-yl)benzoate	b,d	-	-	+	+
29	Methyl D-arabinopyranoside (cyclic)	e,d	+	-	-	-
30	5(4H)-Oxazolone, 2-methyl-4-(phenylmethylene)-	b,d	-	+	+	+
31	MethylN-acetyl-L-Phenylalanine,	a,d	+	+	+	+
32	Galactosane(1,6-Anhydro-hexoses)	e,d	+	-	-	-
33	Rhamnose (open chain)	e,d	+	-	-	-
34	Ribose (open chain)	e,d	+	-	-	-
35	Fucose (open chain)	e,d	+	-	-	-
36	Arabinose (open chain)	e,d	+	-	+	+
37	Xylose (open chain)	e,d	+	-	-	-
38	O-Methyl β-L-mannopyranoside (cyclic)	a,e	+	-	+	+
39	O-Methyl β-D-glucopyranoside(cyclic)	a,f	+	-	-	
40	O-Methyl β-D-galactopyranoside (cyclic)	e,d	+	+	-	-
41	6-(acetoxymethyl)-4-methoxypiperidine-2,3,5-triyl triacetate	c	-	-	+	+
42	n-Hexadecanoic acid underivatised	a,d	-	+	-	+
41	6-O Methyl Glucose/6-O Methyl Galactose (open chain)	e,d	+	-	-	-
42	Quinovosamine	e,d	+	-	-	-
43	Fucosamine	e,d	+	-	-	-
44	3-O Me Galactose (open chain)	e,d	+	-	-	-
45	Mannose (open chain)	e,d	+	-	-	+
46	Glucose (open chain)	f	+	-	-	+
47	Galactose (open chain)	e,d	+	-	-	-
48	O-Methyl galactosamide (cyclic)	a,e	+	+	+	+
49	O-Methyl glucosamide (cyclic)	a,f	+	+	+	+
50	Pentaacetyl glucosamine (not reduced) (open chain)	f	+	+	+	+
51	Galactosamine (open chain)	a,e	+	-	+	+
52	Glucosamine (open chain)	a,f	+	-	-	-

[1]Numbers correspond to the peaks in Fig 3.18, [2]Identification was based on three methods (a) matching compound suggested by NIST Mass Spectral Search Program with above 90 % match probability (b) using NIST MS Interpreter with quality indices reported as above 85 % (c) tentative identification (d) suggested structures were checked against retention data in the literature (e) Characteristic fragments (m/z) of peaks were matched to the reported values for the same components in selected papers (Leskovkk et al., 1994, Panagiotopoulos et al., 2013, Sassaki et al., 2008)(f) mass spectra and retention times were matched with the glucose peak obtained from chitin methanolysis and hydrolysis under same circumstances

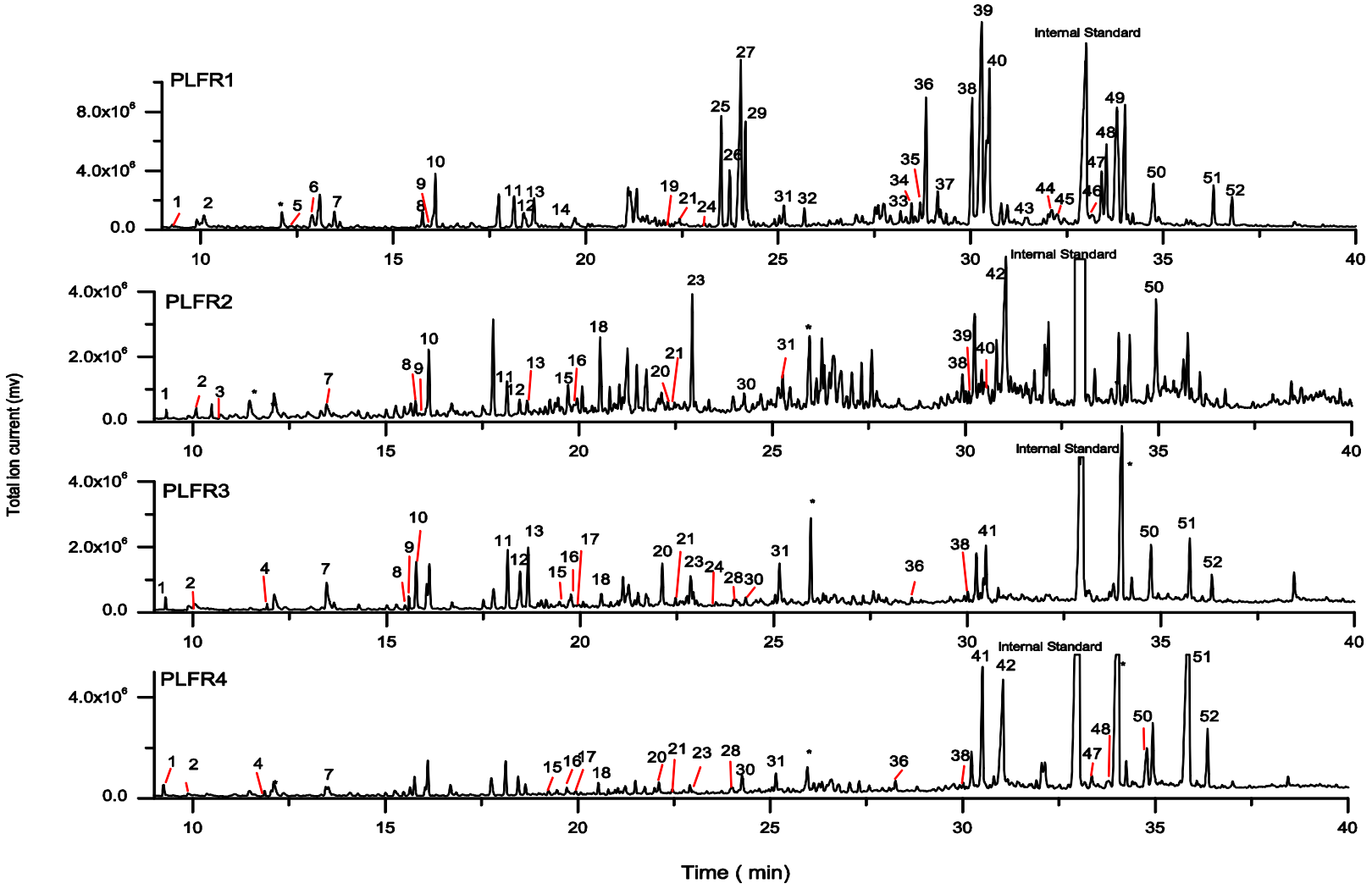


Figure 3.18 Total ion chromatogram of methanolysis products of PLFA fractions, * indicates reagent interferences or contamination

.3.2.5.5.6 Non-sugar compounds released after hydrolysis

Digestion of PLFA fractions with acids not only disintegrates oligosaccharide structure in PLFA, but releases other component connected via ester and amide linkages in the fulvic acid network. A good example of this is the release of amino acids linked in the form of peptides. Compounds other than monosaccharides were also identified in hydrolysates of all four fractions. Table 3.10 also represents the identified non-sugar components in PLFA fractions exposed to methanolysis (peaks 1 to 24) as the most effective method for C-O bond cleavage. Amino acids (in their acetylated forms) were easily detectable, obviously indicating the presence of peptides. Interestingly, these amino acids were observable in all four fractions, suggesting peptides as a part of all the fractions. This can also be a sign of the presence of glycopeptides in the fractions, as peptide-sugar linkage is quite common in DOM. Another possibility is that proteins are linked to the specific oligosaccharides that almost equally contributes to all the fractions, but did not disintegrate very well during methanolysis. Looking at Table 3.10, the only sugar component similarly detected in the fractions is glucosamine. It is attractive to relate the unknown carbohydrate to acetylated amino sugars, however, proving this hypothesis needs further investigation and evidence.

Apart from amino acids, other low molecular-weight carboxylic acid such as dialkanoic acid and, benzoic acid derivatives, as well as alkanes, were observed (Table 3.10). Overall, it seems reasonable to assume dichloromethane-extractable material after methanolysis and acetylation is a small part of the whole material and there it might be required to examine the rest of the molecule remained in aqueous phase after digestion.

3.3 Discussion

The fractionation of a heterogeneous and inseparable consolidation of organic molecules such as Pony Lake Fulvic Acid, although not perfectly accomplished, has added a new perspective to the structural investigation of this material. The fractions of PLFA were examined using different physical and chemical methods including spectral and chromatographic techniques. These spectral methods assisted with obtaining a general picture of the fractions and their possible differences and resemblances in terms of quality and quantity of components. Clearly, without fractionation many of these dissimilarities and similarities would not become identifiable.

One of the major finding from this fractionation study is the evidence supporting the presence of micelle in the aqueous solution of PLFA, change of temperature or the addition of organic solvent lead to a decreases in the average molecular weight of the hydrophilic fractions (Section 3.2.3) which can only be explained by disintegration of "micelle-like" components affected by these physical parameters. This means that these changes disrupted PLFA conformational arrangements stabilized by only weak hydrophobic bonds into smaller-size aggregates of greater conformational stability. These results confirms that humic molecules in solution were organised in supramolecular associations of relatively small molecules loosely bound together by dispersive interactions and hydrogen bonds, and here PLFA specifically responded to chemical changes brought about by factors such as increasing the temperature as well as changing the ratio of water to acetonitrile. Confromatonal changes in humic substances was previously reported by Piccolo and his group (2002) using acetic acid treatment of humic solution exposed to high performance size exclusion chromatography (HPSEC) ,the study revealed that acetic acid treatment altered the distribution of humic molecular components in the size-fractions. Another study (Nuzzo et al., 2013) demonstrated

that iron complexation induced major changes in molecular size distribution determined by HPSEC with UV–vis and refractive index detectors for both humic and fulvic acid.

The overlaps of components detected and identified in the four fractions using chemical analysis (various derivatisation and GC/MS analysis) is interesting, again indicating the carryover of materials in the fractions apparently as a result of micelle formation. The presence and occurrence of micelle has been previously observed in humic substances (Piccolo et al., 1996) consistent with our finding for Pony Lake fulvic acid. This also underscores the importance of theory of supramolecular structures for Humic substances.

The nearly equally-distribution of nitrogen content amongst the fractions is also notable, suggesting the presence of a common nitrogen-containing body in nearly all the fractions (PLFR 4 showed lower nitrogen). One part of this nitrogenous body could be amino sugar as acidic hydrolysis revealed that this sugar is found in all fractions even in PLFR 4, however, the quantity of nitrogen extracted from this sugar does not explain the high nitrogen content. Consequently, this nitrogen-rich structure not only is not sugar, but also is mysteriously non-hydrolysable. This would probably exclude peptides from the possible candidates, as we attempted varied and extended acidic digestion approaches. The only structure remaining to be nominated is heterocylic nitrogenous structure. The high abundance of nitrogen in heterocyclic nitrogen-containing molecules such as uracil and hydantoin, however, does not match the whole nitrogen content of the fractions. A possible answer to this would be the presence of nitrogen-free molecules such as fatty acid and alkanes that could dilute the nitrogen content. Finally, in spite of our knowledge about the presence of these structures, their exact assemblage and the mechanism by which they contribute to the integrated structure of fulvic acid is still unclear.

Another notable observation is the feasibility of detection of some higher molecular weight structure (around 300-400 Da) using TMAH-GC/MS analysis of fractions. Similar

work on unfractionated PLFA (Fimmen et al., 2007) only reported a few volatile and medium-molecular weight moieties. The results achieved also suggest that there are some lignin-derived compounds among the identified compounds in fractions, not supposed to be present in Antarctic deserts. This could challenge the general belief of the microbial origin of Pony Lake fulvic acid.

In summary, these findings illustrate the important role that fractionation can play in improving the detection and identification of fulvic acids. The results also highlight the importance of nitrogen-containing compounds in the formation and preserving of refractory dissolved organic matter that naturally occur in Antarctica.

3.4 Materials and Methods

3.4.1 Fractionation of Pony Lake fulvic acid using RP-SPE

3.4.1.1 Samples and chemicals

Fulvic acid from Pony Lake (1R109F) was obtained from the International Humic Substance Society (IHSS, St. Paul MN, USA) and used as supplied. Acetonitrile (HiPerSolv) was obtained from VWR International. SPE cartridges (Strata Polymeric RP 500 mg/ 6mL) were purchased from Phenomenex and stored in acetonitrile before use. The isolation of PLFA was performed as follows: the acidified Pony Lake water was processed in parallel through two 4 L XAD-8 columns. The acidified lake water was applied to these columns and back eluted with 0.1 N NaOH. The eluent was acidified to pH 2 with HCl immediately and stored at 4°C until re-concentration. The eluate was applied to a 2 L column and was rinsed with deionised water to remove Cl- and back eluted with 0.1 N NaOH. The NaOH eluent from the 2 L column was instantly applied to a cation exchange column to remove the sodium and then freeze-dried as PLFA sample (Cawley et al., 2013).

3.4.1.2 Simple fractionation

15 mg Pony Lake fulvic acid obtained from IHSS was dissolved in 8 mL of Milli-Q water without any further treatment. The Sample was stirred for 15 minutes to assist solubility and the yellow-coloured solution obtained was used for fractionation. SPE C-18 fractionation were performed in the following manner: C-18 cartridges (500 mg/ 6 mL) previously stored in acetonitrile were further washed with 20-30 mL of acetonitrile and rinsed with approximately 20 mL of Milli-Q water. The Sample was then loaded on the cartridge and the effluent was collected as PLFR 1 (highly hydrophilic material). The Sample was also eluted with 20 mL of Milli-Q water and combined with PLFR 1. Next, the sample was eluted using 20 mL of 5 % acetonitrile in water and the eluent was collected as PLFR 2. The PLFR 3 and PLFR 4 were

collected using 20 mL of 20 % and 50 % acetonitrile aqueous solutions, respectively. Acetonitrile present in PLFR 2, 3 and 4 was evaporated in rotary evaporator. All the fractions along with PLFR 1; were frozen, then lyophilised.

3.4.1.3 Ultrasonication prior to fractionation

Pony Lake fulvic acid solution was prepared as above, and then subjected to ultrasonication for 15 min (Condition 2). It was then was left for 3-4 hours, when fractionation was performed as above.

3.4.1.4 Heating prior to fractionation

In Condition 3, after the dissolution of PLFA in water as above, the solution was heated at 40 ^{0}C for 30 minutes while stirring to dissolve completely. The obtained sample was cooled down to room temperature and filtered using a glass fibre filter prior to fractionation.

3.4.1.5 Organic rich solution prior to fractionation

In Condition4, PLFA (11 mg) was dissolved in 70% acetonitrile; undissolved material was filtered with a GF/F glass fiber filter. Acetonitrile present in the filtrate was evaporated using a rotary evaporator and the remaining aqueous solution was frozen and then lyophilised. The dried sample were loaded on to a C-18 SPE cartridge and fractionated as described above.

3.4.1.6 Fractionation of diluted solution of PLFA

In a separate experiment named Condition 5, a diluted solution of PLFA was prepared by dissolving 15 mg IHSS sample in 80 mL Milli-Q water and exposed to fractionation as previously indicated.

3.4.2 Elemental analysis

The elemental composition of the freeze-dried fractions was determined using a Model PE2400 CHNS/O elemental analyser (PerkinElmer, Shelton, CT, USA) for carbon, hydrogen, nitrogen and sulfur. Oxygen was determined by difference. The sample was analysed in triplcate and results were expressed as the average. Blank cups and standards were run to correct for the C and N associated with tin cups.

3.4.3 Molecular weight determination using Vapour Pressure Osmometry

The Molecular weights of the fractions were calculated based on the procedure described for Bulk PLFA (Section 2.4.4). The same solvent mixture (azeotropic water: 1,4 dioxane) was used except for PLFR 1 which was dissolved in water due to the low solubility in the water : 1,4 dioxane mixture. Aqueous solution of glucose, therefore, was used as an standard for PLFR1 measurements.

3.4.4 Fluorescence spectroscopy

Solutions of the PLFA fractions were prepared according to the procedure of McKnight (2001). Briefly, 500 mg/L aqueous solutions of the fractions were prepared using Milli-Q water except PLFR4 which was dissolved in 30 % acetonitrile solution. Then, samples were acidified with concentrated HCl to pH=2 to ensure the presence of fully protonated fulvic acid during the analysis.

Fluorescence was measured using a Perkin Elmer LS 50B Fluorescence Spectrometer with a xenon discharge lamp. The EEM (Emission Excitation Matrix) spectra were obtained from the emission spectra collected at 20 excitation wavelengths . The fluorescence intensity was measured at excitation wavelengths ranging from 250 to 450 nm at 10-nm increments and at emission wavelength ranging between 260-750 nm with a step width of 0.5 and slit width of 4 nm , and scan speed of 250 nm/min.3.4.5 IR Spectroscopy. IR spectra of the samples were recorded using a Perkin Elmer Spectrum 100 FT-IR spectrometer. Spectra were acquired from 4000 to 600 cm^{-1} at 2 cm^{-1} resolution. Base line correction was performed before each run.The freeze-dried samples were homogeneously mixed with KBr (2 % w/w) and prepared as transparent discs before analysis.

3.4.5 NMR spectroscopy

^{1}H and ^{13}C NMR Spectra were recorded on Bruker DRX600K (600 MHz) spectrometer and reported in ppm using DMSO-d_6 as internal standard (DMSO-d_6 at 2.54 and 4.45 ppm for

proton and carbon MNR, respectively). 25 mg of each fraction were dissolved in 450 μL of DMSO-d_6. The samples solutions were ran for at least 24 hours to obtain ^{1}HNMR spectra. Due to lower carbon content PLFR1 and 2 were run over 72 hours to obtain ^{13}C NMR while for PLFR 3 and 4, a 36-hours run were sufficient to observe the peaks.

3.4.6 X ray photoelectron spectroscopy (XPS)

Photoelectron spectra were recorded on an X-ray photoelectron spectrometer (ESCALAB250Xi, Thermo Scientific, UK) using mono-chromated Al K alpha radiation (energy 1486.68 eV), an electric current of 12 mA, and a voltage of 13 kV. The background vacuum pressure was around 2E-9 mbar and binding energy reference was C1s = 285.0 eV for adventitious hydrocarbons. To prepare a homogeneous sample, freeze-dried samples were dissolved according to the following procedure and placed on the absolutely clean glass slides before drying in a desiccator. To prepare the sample solutions, 1.0 mg of the fractions (weighed to 0.01mg) were dissolved in 200 μl acetonitrile/Milli-Q water solutions (PLFR 1 was dissolved in water, PLFR 2 was dissolved in 5:95 acetonitrile : water, PLFR 3 in 20 : 80 acetonitrile: water and PLFR 4 in 50 :50 acetonitrile: water). Then 100 μl of prepared solution was placed on the slides using micropipette before analysis. The sildes were transferred carefully to the vacuum oven and kept for 36 hours at 30^{0}C to dry before analysis. The XPS data were analysed using software provided with the spectrometer. Elemental composition was estimated using the area of C 1s, O 1s, S 2p and N 1s peaks.

3.4.7 Trimethylsilylation

3.4.7.1 Samples and chemicals

PLFA fractions obtained under the conditions of Condition 2 (section 3.4.1.2) were freeze-dried for 24 hours and kept in sealed bottles at 4°C before derivatisation. BSTFA:TMCS (99:1) and anhydrous pyridine was obtained from Sigma-Aldrich and kept

away from exposure to air and moisture during handling using sealed bottles. Methyl octadecanoate was an analytical standard from Poly Science Corporation and was used to prepare internal standard solutions.

3.4.7.2 Derivatisation procedure

0.80 mg of each of the freeze-dried fractions (weighed to 0.01mg) was placed in a 1mLoven-dried glass vial equipped with a mininert valve to prevent leakage and purged with nitrogen. Then 20 µL of anhydrous pyridine was added by syringe through the mininert valve and mixed well, then 5 µL of 1000 ppm methyl octadecanoate in anhydrous pyridine solution was added as internal standard. At this stage 50 µL BSTFA:TMCS (99:1) was added dropwise, After a few seconds stirring, the solution was placed in a ultrasonicator bath at 60 ^{0}C for 30 minutes, and afterwards on a hotplate at the same temperature while stirring for 15 hours.0.5 µL of prepared sample was directly injected to GC/MS for analysis.

3.4.7.3 GC/MS analysis

A Shimadzu Gas chromatograph -mass spectrometer (QP-2010) was used for separation and identification of relevant samples. A SGE BPX-5 capillary column (30m × 0.15 mmID × 0.15 µm film thickness) was used. 0.5 µL of sample was injected using splitless with a sampling time of 1.5 minutes. Injector temperature was 270 ºC and MS interface temperature was 300 ºC. The temperature program ran as 60 ºC for 4 minutes. Then increased to 320 ºC at 5 ºC/ minute, holding at 320 ^{0}C for 5 minutes. The carrier gas was ultrahigh purity helium (BOC) flowing with linear velocity of 39 cm/sec. The mass spectrometer was operated with a source temperature of 200 ^{0}C and 70 eV electron ionisation energy. The scan range was from 50 to 800 (m/z) ratio in 0.4 seconds.

AMDIS (Automated Mass spectral Deconvolution and Identification System) version 2.68 software was used to acquire and process data. The identification of products was undertaken by matching with the NIST mass spectral search program version 2.0 (with a

match factor of higher than 90 %). In cases with lower match factors, identification was performed using NIST MS interpreter version 2.0 and structure confirmed when the quality indices reported by interpreter were greater than 85 %. Alternatively, structures were identified by examination of mass spectra and based on the presence of molecular ion and fragmentation patterns; this was indicated as tentative identification in identified compounds list.

3.4.8 Trimethyl Silyl Diazomethane (TMSD) combined with Trimethyl silylation

3.4.8.1 Samples and chemicals

PLFA fractions obtained under the conditions described for Experiment 2 (section 3.4.1.2), were freeze-dried for 24 hours and kept in sealed bottles at 4 ^{0}C before derivatisation. (Trimethylsilyl) diazomethane solution (2M in diethylether) was obtained from Sigma-Aldrich. Methanol and dichloromthane and hexane (ACS grade) were obtained from BDH. Methyl octadecanoate was an analytical standard from Poly Science Corporation and was used to prepare internal standard solutions.

3.4.8.2 Derivatisation procedure

The freeze-dried fractions were derivatised in two steps, The first step was carried outin accordance with Kowalewski's paper (Kowalewski and Gierczak, 2011). 2.0 mg samples were suspended in 500 µL methanol: DCM 50:50 v/v, then 40 µL of 200 ppm methyl octadecanoate in hexane solution as internal standard and 100 µL of (trimethylsilyl) diazomethane solution was added. The reaction vial was left at room temperature for 20 hours. At this stage, the sample was dried under a nitrogen stream, and for better drying the semi-dried sample was kept in freeze-dryer for 12 hours. In the second step, the dried sample was dissolved in 30 µL pyridine and mixed. Then 100 µL BSTFA:TMCS (99:1) was added, and ultrasonicated for 30 minutes at 60 ^{0}C, then under the same temperature while stirring for 15 hours.

3.4.8.3 GC/MS analysis

The instrumental analysis was carried out as mentioned in 3.4.7.3

3.4.9 Ethyl Chloroformate (ECF) Derivatisation

3.4.9.1 Samples and chemicals

PLFA fractions obtained under conditions described for Experiment 2 (Section 3.4.1.2), were freeze-dried for 24 hours and kept in sealed bottles at 4 ^{0}C before derivatisation. Ethyl Chloroformate (ECF) and methyl iodide was obtained from Merck. Methanol (Hipersolve) was from BDH. and pyridine from Sigma Aldrich. Acetonitrile (HiperSolv) was obtained from VWR International. Sodium hydroxide pellets were used to prepare 0.1 M aqueous solution. Strata Polymeric RP-SPE cartridges (10 mg /1 mL) were purchased from Phenomenex. Methyl octadecanoate was an analytical standard from Poly Science Corporation and was used to prepare internal standard solutions.

3.4.9.2 Derivatisation procedure

Derivatisation was performed in accordance with a method previously used for metabolite analysis (Villas-Bôas et al., 2011)with some modifications such as using more diluted sodium hydroxide solution and using ethyl instead of methyl chloroformate in order to distinguish between the naturally methylated components and the methylation products of carboxylic groups. Briefly, 100 µL NaOH (0.1 M) solution was added to 2.0 mg of samples in an ordinary reaction vial to ensure the deprotonation of acidic group and the formation of anions to attack chloroformate. Then, 60 µL of 250 ppm methyl octadecanoate in pyridine solution was added as internal standard. After that, 240 µL methanol was added (to maintain the methanol: pyridine ratio around 4 times). Then15 µL ECF was added and vortexed for 1 minute. The same amount was then re-added to ensure the reaction efficiency, vortexed again and left stirring for 30 minutes. After that, 1 mL water was added to quench the reaction. For PLFR 1 and PLFR 2 a different procedure was conducted from that for PLFR 3 and PLFR 4.

For PLFR 1 and PLFR 2, at this stage 100 µL NaOH (0.1 M) was added to ensure the basic pH of the sample. Then 100µL methyl iodide was added as a methylating agent to complete the methylation of functional groups may have failed to become derivatised initially due to steric hindrance. These types of functional groups such as amides in uracil or other amides or amino structures could be more abundant in hydrophilic fractions including PLFR1 and PLFR 2. After 12 hours reaction time, in darkness, methyl iodide was removed using a rotary evaporator and the remaining solution set aside. In the cases of PLFR 3 and PLFR 4 aqueous solutions, no extra methylation was applied. After quenching the reaction, the solution was gently purged with nitrogen to remove organic solvents from the water as much as possible. All samples extracted using RP-SPE cartridge. Solid phase extraction was preferred to liquid-liquid extraction (LLE) due to the fact that phase separation between water and organic solvent was difficult to achieve. Each sample was then loaded on one small C18 cartridge (10mg/1 mL), after multiple washing with 4-5 mL Milli-Q water to remove sodium ions and salts, products were then extracted by elution of cartridge using acetonitrile: water 75% : 25 % v/v. The eluents were evaporated in rotary and subsequently freeze-dryer, and then redissolved in NMP (N-methyl pyrrolidone). 0.5 µL of the sample was injected to GC/MS

3.4.9.3 GC/MS analysis

The instrumental analysis was carried out as mentioned in Section 3.4.7.3, except temperature programming which was as follows: 100 ^{0}C for 4 minutes, then increased to 320 ^{0}C at 10^{0}C / minute and holding at 320 ^{0}C for 5 minutes.

3.4.10 TMAH methylation

3.4.10.1 Samples and chemicals

The PLFA fractions obtained under condition described for Condition 2 (Section 3.4.1.2), were freeze-dried for 24 hours and kept in sealed bottles at 4 ^{0}C before

derivatisation. TMAH (25% wt in methanol) was obtained from Aldrich and used as supplied.

3.4.10.2. Derivatisation procedure

The methylation using tetramethyl ammonium hydroxide (TMAH) commonly known as "flash methylation" was performed according to the method of Orata (Orata, 2012) .40 µL of TMAH solution was added to 1.0 mg of accurately weighed sample in a conical reaction vial and mixed well after 15 minutes, 0.5 µL of the sample was injected to the hot injector port (300 ^{0}C).

3.4.10.3 GC/MS analysis

The instrumental analysis was carried out as mentioned in 3.4.7.3, except using split injection with split ratio of 1:20.

3.4.11 C-O bond cleavage using acid digestion

3.4.11.1 Samples and chemicals

Samples were prepared as stated above. TFA (99%) from Fluka, HCl (pro analysis 37%) from Merck, H_2SO_4 (98%) from BDH, CH_3COOH (glacial) from Univar, and NH_4OH (25-0%) from Sigma-Aldrich was used for preparing solutions. Sodium borohydride (98%) from Merck, and acetic anhydride (98%) from SAFC was used. N,N-dimethyloctylamine (95%)and chitin (practical grade) were both obtained from Sigma-Aldrich.

Pyridine, methanol and DCM were obtained as mentioned above. Inositol was from Hopkin & Williams and used to prepare internal standard solution. RP-SPE cartridges (200 mg/3 mL) were from Phenomenex.

3.4.11.2 GC/MS analysis

The instrumental analysis was carried out for all hydrolysis products as mentioned in Section 3.4.7.3.

3.4.11.3 Procedure for hydrolysis using trifluoroacetic acid (TFA)

The hydrolysis using TFA was conducted according to the method of Aluwihare (2002) with some minor changes. 2.0 mg of dry fractions was accurately weighed and placed in a 1.0 mL oven-dried reaction vial equipped with mininert valve, 20 µL of 1000 ppm inositol aqueous solution was added as internal standard and mixed, then 500 µL of TFA 2M aqueous solution was added. The vial was completely sealed and left for 2 hours at 121°C while stirring. The samples were then dried under a gentle nitrogen stream. At this stage obtained monosaccharides were reduced using 500 µL of sodium borohydride solution (0.3M in 1M NH_4OH) and incubated at room temperature for 4 hours. The reduction was quenched by the addition of 250 µL of CH_3COOH solution (10 % in methanol), then evaporated using nitrogen gas. This was repeated to ensure the conversion of borohydride to boric acid. Then 500 µL methanol was added and dried again. This was also repeated to remove converted boric acid to its methyl ester from the reaction mixture. The dry reduced sugars were (alditols) were subjected to per acetylation using 200 µL acetic anhydride and 200 µL pyridine as a catalyst at 100 ^{0}C for 30 minutes. The reaction was quenched with the addition of water, mixed, and then DCM was added to extract the acetylated alditols, after 1 minute vortex, centrifuged, the organic phase was separated and dried over magnesium sulphate, then sample transferred to a separate vial, DCM removed under nitrogen, and 50 µL methanol was added, 0.5 µL of the sample was injected to GC/MS.

3.4.11.4 Procedure for hydrolysis using sulfuric acid

To hydrolyse the samples using H_2SO_4, a two -step digestion process was performed (Templeton et al., 2012) as per literature. Briefly, 2.0 mg of sample (fractions) or 0.5 mg of chitin was placed in a clean glass reaction vial , then 20 µL of 1000 ppm inositol aqueous solution (internal standard), then 400 µL H_2SO_4 12 N and incubated at room temperature for 16 hours. After this period, the reaction mixture was diluted with MiliQ water to reach a 0.5 N concentration (around 10 mL) and heated for 8 hours at 100 ^{0}C . In order to eliminate

sulfuric acid, which is not volatile enough to be removed by nitrogen purge , an extraction method was performed as per Fox (Fox, 2002). A 50% v/v solution of N,N-dimethyloctylamine in chloroform was prepared to be used as the ion pair agent to transfer sulfate from water to organic phase. Therefore, 7 mL of this solution was added to hydrolysate and mixed vigorously then centrifuged and organic phase removed. The procedure was repeated 2-3 times until the pH of the aqueous phase became neutral to ensure the elimination of acid. After that aqueous phase was loaded onto a RP-SPE cartridge (200 mg/ 3 mL) for adsorption of the trace of organic bases possibly remained in the solution. The effluent from cartridge was freeze-dried. The reduction and acetylation was undertaken as described in 3.4.11.2 for the fractions. For chitin the reagent amount was adjusted and added accordingly.

3.4.11.5 Procedure for methanolysis using hydrochloric acid

Methanolysis was carried out according to the procedures recommended by Aluwihare (Aluwihare et al., 2002) and Jensen (Jensen et al., 2013) with some modifications. Firstly, in order to prevent hydrolysis of esters by hot aqueous acid, anhydrous methanolic HCl was prepared. To prepare this solution (4M HCl in methanol) in a designated glass apparatus, to the concentrated sulfuric acid, a known volume of commercial HCl (36%) solution was added through the dropping funnel. This produced HCl gas with no water which bubbles up and goes through the sidearm into the receiving container containing anhydrous methanol. Secondly, using the first procedure suggested for methanolysis of amino sugars in DOM, an 8-hour methanloysis time was conducted. The second procedure suggesting a 24- hour reaction time (reported for cleavage of exopolysaccharides (EPSs) was applied. The general procedure, therefore was as follows: 2.0 of mg sample (fractions) or 0.5 mg chitin was accurately weighed into an oven-dried clean reaction vial equipped with a mininert valve, then 1000 µL of freshly prepared anhydrous methanolic HCl was added, Inositol was not added at this time, as the solution contained water, then the sample was exposed to 80 ^{0}C for

24 hours. Afterwards, the hydrolysate evaporated using a gentle nitrogen gas stream for drying. At this stage 20 µL of inositol 1000 ppm aqueous solution was added as internal standard, then reduction and acetylation procedures were undertaken as described in Section 3.4.11.2. For chitin, the reagents amount were adjusted and added accordingly.

3.4.11.6 Procedure for De-lactonisation prior to reduction

In a separate experiment a de-lactonisation step was undertaken before reduction to convert possible sugar acids to their salt forms, according to the method of Sassaki and his colleagues (Sassaki et al., 2005). According to the procedure, samples subjected to HCl methanolys is and subsequent drying by nitrogen as described in 3.4.11.5, were dissolved in 200 µL of 0.5 N NH_4OH solution and held at room temperature for 15 minutes. Reduction was then performed as described in Section 3.4.11.2 which did not affect possible carboxylates ion from sugar acids, Then 300 µL of 0.5 M methanolic HCl was added and the reaction heated for 15 minutes at 100 ^{0}C to form methyl esters of anions. Subsequently, acetylation was carried out as previously mentioned.

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Chapter 4
The Characterisation of Antarctic Fulvic Acids from Vestfold Hills

4.1 Introduction

Study of fulvic materials of various geochemical environment and origin (source material and generation process) is crucial for gaining insight into the fulvic acid chemistry. As previously reported in the literature, there are obvious differences between fulvic acid of microbial and terrestrial origins. As presented in previous chapters, Pony Lake Antarctic fulvic acid as a microbial end-member reference material showed notable characteristics such as low aromaticity and low GC amenability, distinguishing it from its terrestrial counterparts. However, the question is whether all microbially-derived fulvic materials show comparable properties and have similar structures or not. This question can be even more attractive if the area of interest is a geographically and climatically isolated region such as the Antarctic.

In order to find an answer to the above question, in this chapter we examined the fulvic material isolated from other Antarctic lakes located in the Vestfold Hills of Eastern Antarctica; the results of the various chemical analysis were compared with those obtained from PLFA characterization (previous chapters), this comparison helped to test the hypothesis suggested in the previous chapters regarding structure and properties of microbial fulvic acids. Further, possible dissimilarities observed in the structures of different microbial fulvic acids were recognised and explained based on the intrinsic differences in the lake's environmental properties.

The area of study, Vestfold Hills, is a vast ice-free region with several meromictic lakes believed to be a very unique characteristics of the region (Burton, 1981). This area has

received much attention in recent years for geological and ecological studies (Leishman and Wild, 2001;Van Trappen, 2004), however, there has been little study of the nature and properties of the dissolved organic matter in the lakes of the area. Therefore, the information obtained from the current study not only provides additional data important for better understanding of the aqueous geochemistry of Vestfold Hills lakes, but also presents a wide-ranging and comparative work about one of the purest microbially-derived humic substances in Antarctica.

In this chapter, first some background information about the Vestfold Hills and its lakes will be provided, then a general comparison between the situations and condition of lakes systems based on the previous studies found in literature will be described. Lastly, more detailed discussion about the structure of DOM (fulvic material) isolated from the lakes and its relation to PLFA will be provided.

4.1.1 Study area

Vestfold Hills is located at Eastern Antarctica (near the Australian Antarctic station, Davis) and has several lakes spread over an area of 413 km^2 of ice-free rock, the lakes are surrounded by Prydz Bay on the north and west, while their eastern side is restricted by the continental ice sheet, and the south by the Sørsdal Glacier (Leishman and Wild, 2001). There are 150 lakes ranging from freshwater to hypersaline in the Vestfold Hills (Gilbert, 2004); these lakes are approximately 8000-10000 years old (Adamson and Pickard, 1986;Bowman et al., 2000). The detailed characteristics of all lakes are not within the scope of this study and only information and data regarding the three lakes named Pendant Lake, Mossel Lake and Organic Lake are discussed here. The fulvic acids isolated from water samples of the three lakes are also discussed. The reason for the selection of these three lakes is mainly related to the nitrogen content of the DOM in lake water. Pendant Lake and Mossel Lake have both nitrogen-rich DOM and are good choice for comparison with PLFA. Organic Lake fulvic

material, on the other hand has low nitrogen content and was chosen as a complementary sample, enabling the study of the possible differences and dissimilarities due to its low nitrogen. Moreover Organic Lake system is geochemically interesting due to its meromictic status and very high DOC.

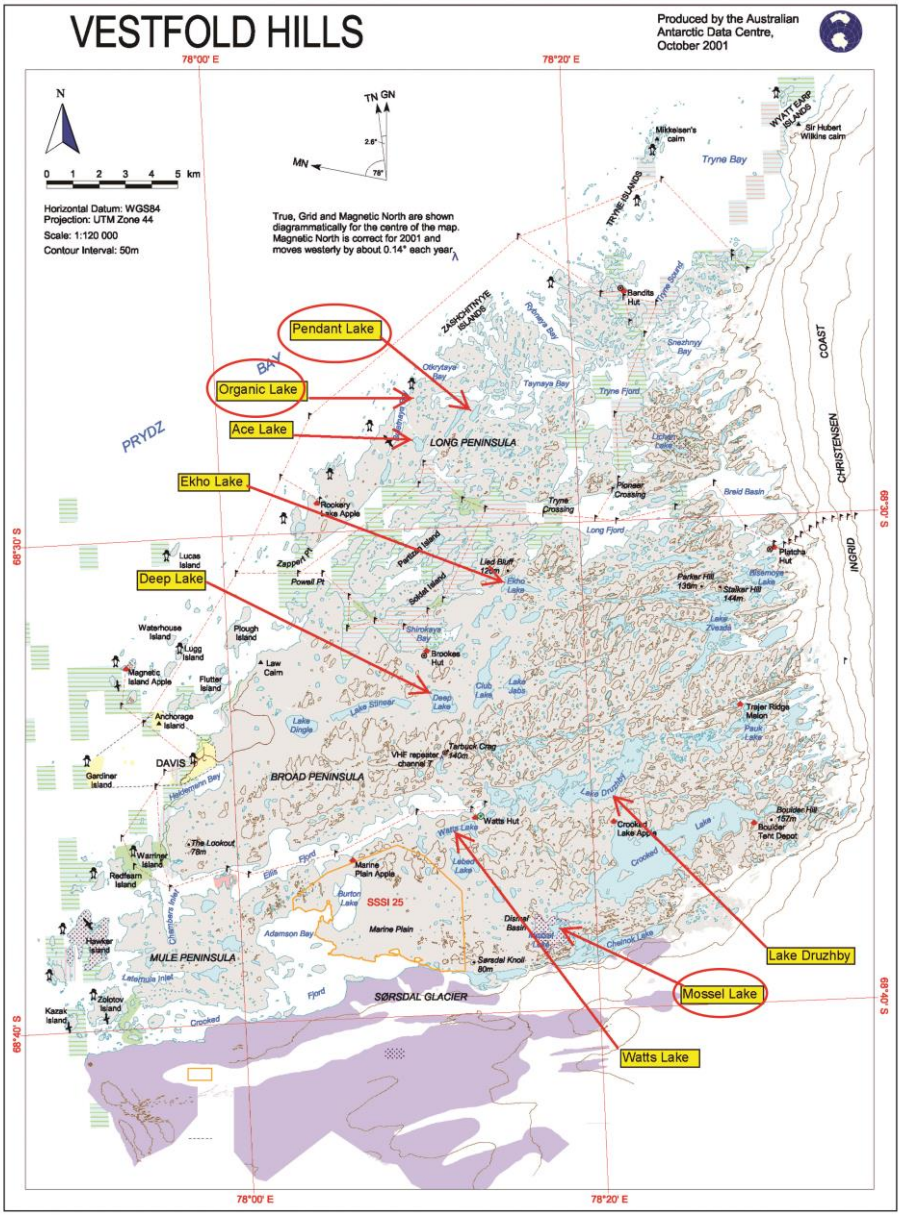


Figure4.1 Location of Pendant, Organic and Mossel Lake as well as other lakes in the Vestfold Hills, Antarctica (Australian Antarctic Data Centre, 2015)

4.1.2 Background information about Pendant, Mossel and Organic Lake and their DOM nature

The first lake to be studied is Pendant Lake, a hyper-saline (100-120 g/L) marine-derived lake with a low DOC concentration (3.6 mg/L) (Nimmagadda, 2008). As mentioned earlier, it is located on the northern part of the Vestfold Hills where the lake system was believed to have been initially formed through isostatic glacial marine uplift (Bowman et al., 2000) in which fjords were detached and seawater was trapped in basins.

The second Lake is Mossel Lake located at 68° 38.358' S 78° 17.623'E, it is a fresh water lake with low DOC (0.4 mg/L) (Nimmagadda, 2008). NOM source is mainly snowmelt from nearby Sørsdal Glacier located to south of the lake providing ornithogenic, algal and lichen inputs. Due to the presence of nesting sites of sea birds such as snow petrels and south polar skuas in the soil near the Sørsdal Glacier , the inputs sources to this lake are rich in nutrients (N and P) (Leishman and Wild, 2001).

A remarkable feature of the Vestfold Hills involves its meromictic lakes which are considered as very unique in the world (Gibson, 1999). These specific features have inspired many geological and biological studies in this area in recent years. An important lake in this category is Organic Lake which is also studied in this chapter; it is a hypersaline, shallow meromictic lake with an oxycline at 4-5 m depth (Volkman, 1986); There is considerable ornithogenic, and algal inputs to this lake and DOC level is high (69.2 mg/L) (Nimmagadda 2008).

4.1.3 Comparison of Pony Lake and Vestfold Hills Lakes and their DOM properties

While Pendant, Mossel and Organic Lake are located in the Vestfold Hills in the eastern part of Anatrctica, Pony Lake is located on Cape Royds in McMurdo Sound area (on the western side of Antarctica). Basically, microbial communities are the dominant source

material in Antarctic Lakes, because few or no zooplankton and fish are found in the lakes (Laybourn-Parry et al., 2002). Both regions are without higher order plants, therefore they are classified as dessert. The main species found in the region are algae, fungi, lichen and planktons. Although, vegetation and climate are similar in two areas, each area has understandably its own ecological features. Table 4.1 compares the physiochemical properties of the lakes.

Figure 4.2 Locations of the McMurdo and the Vestfold Hills in Antarctica

Table 4.1 Physico-chemical properties for the investigated lakes of the Vestfold Hills and McMurdo

Lake	Latitude	Longitude	Max Depth (m)[1]	Anatarctic region	Salinity (ppt)	DOC (ppm)
Pendant	68^0 27	78^0 14	18.4	Vestfold Hills	100-120[2]	3.6[2]
Mossel	68° 38	78° 17	-	Vestfold Hills	-	0.4[2]
Organic	68° 27	78° 11	7.0	Vestfold Hills	180-225	69.2[2]
Pony	77° 33	166° 9	2.0	McMurdo	5.5[3]	110.4[3]

[1] (Volkman et al., 1989) , [2] Nimmagadda(2008), [3] High DOC related to the dense blooms of chlorophyte (Brown et al., 2004)

Regarding the source of organic carbon, in Pony Lake, considering its very high DOC, degradation of algal material is believed to be the major source of dissolved organic carbon due to the fact that chlorophyte bloom occurs in the lake (Mcknight et al., 1994;Brown et al., 2004). The fulvic acid, however, has a small contribution to this organic carbon (Mcknight et al., 1994).

Pendant Lake is a lake with significant biological activity, unlike Pony Lake it exists as a meromictic lake so that it has freshwater on top and saline water near the floor (Bowman et al., 2000). The DOC content in this lake is low as a result of the flushing of marine input with fresh water.

On the other hand, Organic Lake is a stratified lake in terms of density and oxygen concentration, there is no marine connection, and the only influx is snow bank melt streams The main difference of Organic Lake from other lakes in the area is the abundance of dimethyl sulfide (DMS) in its sediment (Rogerson and Johns, 1996). DMS production seems to be related to bacterial degradation of sulfur-containing compounds such as amino acids, dimethyl sulfoxide, dimethyl-β-propiothetin originated from algal cells (Franzrnann et al., 1987).

Mossel Lake differs from other lakes in that it has more than 7.0 % nitrogen in its DOM, probably because the sources of input mainly from the nearby Sørsdal Glacier are rich in nutrients (Leishman and Wild, 2001).

Previous studies have also indicated different distribution of oligotrophic bacteria in the lake systems of the McMurdo area and the Vestfold Hills, although the differences were shown to be correlated to the salinity of lakes (Van Trappen, 2004).

4.1.3.1 Comparison of the properties of fulvic acids isolated from Vestfold Hills lakes and Pony Lake

In order to compare the nature of DOM in Pony Lake and Pendant Lake, we briefly refer to the analysis of hydrophobic acid, equivalent to "fulvic acid" isolated from three Vestfold Hills Lakes performed by Nimmagadda (2008) (we named them Pendant Lake Fulvic Acid (PNFA), Mossel Lake Fulvic Acid (MLFA) and Organic Lake Fulvic Acid (OLFA)) and compare their properties studies with what previously carried out on Pony Lake Fulvic Acid (PLFA).

The elemental composition of Antarctic fulvic acids are presented in Table 4.2. The elemental distributions show some notable differences, for instance nitrogen and specifically sulfur content are higher in PLFA and MLFA than in other two fulvic acids. OLFA contains very low nitrogen in contrast to PLFA while both Lakes contain a high level of organic material (Table 4.1). The reason for lower organic nitrogen in Organic Lake can be due to the lower occurrence of organic material including nitrogenous compounds in the oxic layer (sampled in this study) relative to anoxic layer. A study of the concentrations of dissolved free amino acids (DFAA) in Organic Lake's water below and above oxycline showed an increase in concentration versus depth (Gibson et al., 1994). This is believed to be due to the lower accumulation of DFAA as a result of higher bacterial activity in the oxic condition.

The reason for this high nitrogen is not fully recognised; some believe that it may be due to the lower contribution of aromatic compounds in the algal and microbial-derived DOM which renders a higher total nitrogen concentration, others point to the non-humification of polymers such as peptides in DOM as a possible reason (Piccolo et al., 1992). Regardless of the reason for this property, it is attractive to assume that the composition and structure of nitrogen-rich fulvic acid can be interestingly different even when their nitrogen content are comparable.

Table 4.2. Elemental composition of fulvic acid from Antarctic Lakes

Lake	% Carbon	% Hydrogen	% Oxygen	% Nitrogen	% Sulphur	Phosphorus
Pony [1]	52.47	5.39	31.38	6.51	3.03	0.55
Pendant [2]	49.82	6.30	38.39	4.66	0.83	nd[3]
Mossel [2]	51.36	6.48	33.93	7.42	0.81	nd
Organic [2]	49.75	6.17	40.57	2.76	0.75	nd

[1] results obtained from IHSS website (IHSS, 2013)
[2] Nimmagadda(2008)
[3] nd= not determined

To better understand the nature of fulvic acids being studied, the carbon distribution in various Antarctic fulvic material based on their NMR spectra is summarized in Table 4.3. Comparing the values, some interesting points can be obtained; for example, generally peaks between 60-90 ppm are due to O-alkyl resonances, from carbon bonded to oxygen normally occurring in esters, ethers, alkoxy and alcohols (Leenheer et al., 2004). PNFA, MLFA and OLFA show significant peaks in this area (19-27%), whereas only 8-12 % of PLFA according to its ^{13}C NMR is attributed to this type of carbon possibly associated with sugars. Vestfold Hills FAs have also notable anomeric carbon (110-90 ppm) compared to PLFA. Combining these with high O-alkyl carbon, it can be concluded that Vestfold Hills FAs contain significant amount of carbohydrate and that their carbohydrate amount are higher than that of PLFA. If we assume that carbohydrate degradation increases over time, the results imply that DOM in Vestfold Hills Lakes is immature and much younger than DOM from Pony Lake.

NMR data of all fulvic materials shows between 12-19 % contribution from carbonyl and carboxyl groups, while ketone or quinone carbonyl are various; PLFA and MLFA contain 6.0 % of this type of carbon, whereas values for PNFA and OLFA are 1.0 and 2.0%, respectively.

In summary, Vestfold Hills FAs showed obvious differences from PLFA mainly in terms of carbohydrate content, however, differences are observable among their own structure as well, this is observed even between two nitrogen-rich MLFA and PNFA. Based on the ^{13}C NMR data PNFA seems to be structurally more similar to OLFA rather than MLFA.

Table 4.3 Distribution of carbon percentage in PNFA, MLFA and OLFA obtained from CPMAS-^{13}C NMR[1] and PLFA obtained from quantitative solution NMR[2]

Type of Carbon	PLFA	PNFA	MLFA	OLFA
Ketone/quinone 230–190 ppm	6.0	1.0	6.0	2.0
Carboxyl/amide/ quinone 190–165 ppm	19.0	12.0	15.0	14.0
Aromatic/Olefinic 165–110 ppm	13.0	7.0	12.0	7.0
Acetal/Ketal 110–90 ppm	0.3	4.0	10.0	6.0
O-Alkyl 90–60 ppm	8.0	27.0	19.0	25.0
Aliphatic 60–0 ppm	53.0	49.0	38.0	46.0

[1] Nimmagadda (2008), [2]results obtained from IHSS Website (IHSS, 2013)

Although the information obtained from previous bulk analysis are of significant importance in understanding the structure of Antarctic fulvic acids, component level analyses are required to give a more detailed picture of Antarctic fulvic materials structure. The following sections of this chapter, therefore, are dedicated to the substantial bulk and component-level analyses of these precious samples.

4.2 Results

4.2.1 XPS analysis of PNFA, MLFA and OLFA

XPS analysis was performed on Antarctic fulvic acids in order to examine the distribution of nitrogen and sulfur-containing various functional groups. Figure 4.3 represents the XP spectra of C 1s and N 1s lines in these fulvic materials.

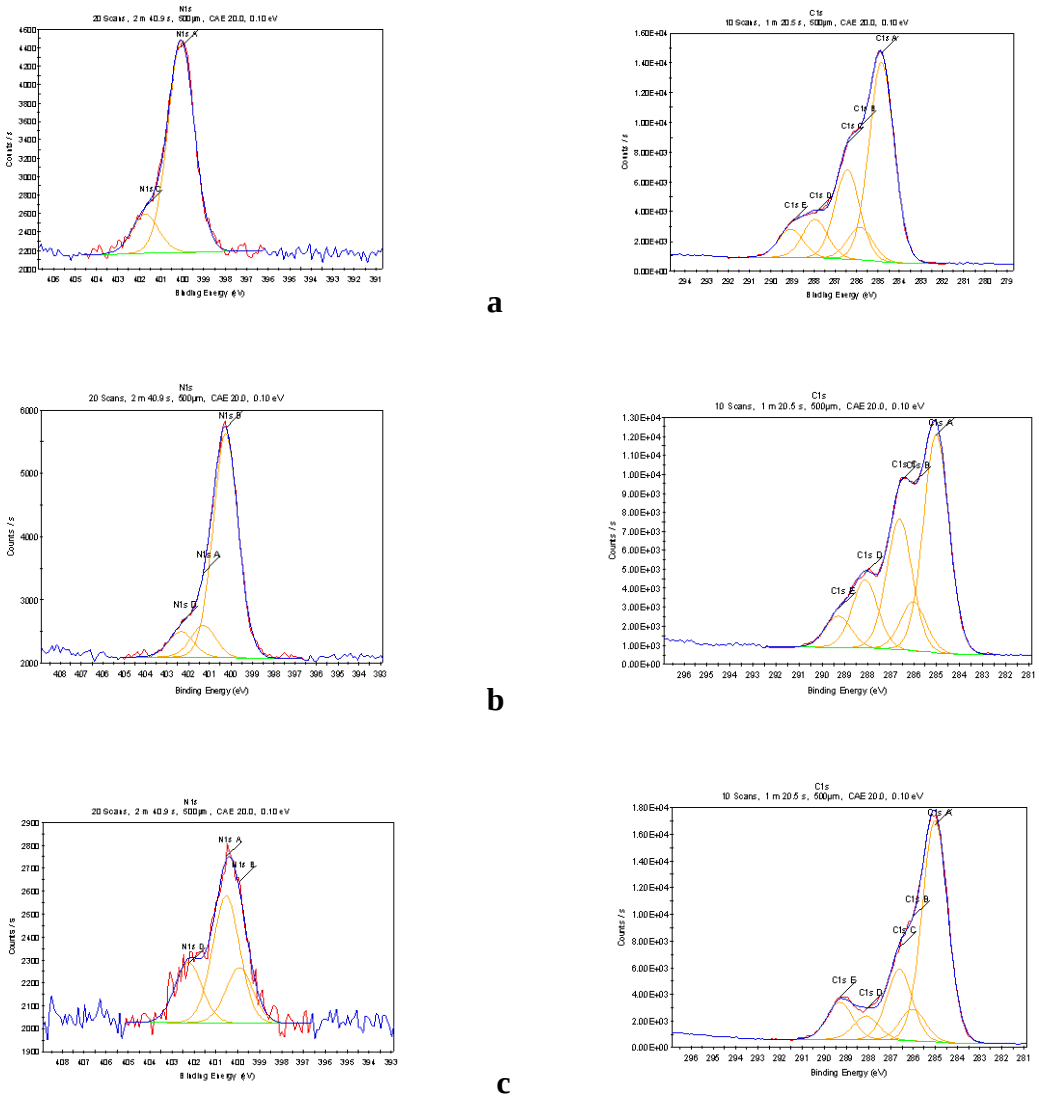


Figure 4.3 X-ray photoelectron spectra (XPS) of nitrogen and carbon functionalities in (a) PNFA, (b) MLFA and (c) OLFA

Table 4.4 Comparison of atomic concentrations in composition of Antarctic fulvic acids extracted from XPS spectra

Line	Notation	Bond/ Compound	PNFA Conc. (At%)[1]	MLFA Conc. (At%)	OLFA Conc. (At%)	PLFA Conc. (At%)
C1s	A	C-C/C-H	34.48	27.88	41.11	36.22
C1s	B	C-N	5.7	6.22	5.88	6.36
C1s	C	C-O	15.44	16.72	13.17	12.52
C1s	D	C=O	6.65	8.7	4.4	6.94
C1s	E	O=C-O	4.93	4.02	6.92	5.55
Cl2p3	A	chloride	1.14	1.22	0.24	0.07
Cl2p3	B	chloride	0.53	0.29	0.07	0.04
N1s	A	Amide (pyrimidine / peptide N)	3.51	0.73	0.83	1.94
N1s	B	Amide/ pyrrole	nd[1]	5.08	0.36	3.2
N1s	C	Primary amine/ protonated amine	0.65	nd	nd	0.67
N1s	D	Quaternary amine	nd	0.58	0.39	nd
Na 1s	-	alkali	0.99	0.9	nd	0.45
O1s	-	carbonyl	24.36	26.18	26.19	24.37
S2p3	A	thiol/ thiophene	0.21	0.13	0.18	0.75
S2p3	B	Sulfone	0.25	0.4	0.26	0.52
Si2p	A	Siloxane	0.81	0.63	nd	0.29
Si2p	B	Siloxane	nd	0.34	nd	nd
P2p	-	phosphorus oxynitride	nd	nd	nd	0.1

[1]nd = not detected

According to the table, carbon functionalities in the three fulvic materials show some similarities and differences; C1s A peak which indicates the contribution of aliphatic structure is significantly higher in OLFA compared to MLFA and PNFA. The oxygen-containing groups such as C-O, C=O (C1s C, D), on the other hand show lower contribution to OLFA than MLFA and PNFA, probably an indicator of less carbohydrates. C1s E corresponds to

O=C-O, (or carboxylic acid) is, however, present at a higher abundance in OLFA. Sulfur distribution in MLFA is different from PNFA and OLFA showing more S 2p3 A than S 2p3 B similar to PLFA.

N1s peaks are also notable, it was fitted to four chemical forms of nitrogen bonded to carbon as follows: N1s A, N1s B, N1s C and N1s D, reflecting the presence of amides/ heterocyclic nitrogen, aromatics/ heterocyclic nitrogen, primary amine/ amide and primary amines nitrogen, respectively. Due to the complex structure of fulvic acids, however, similar functional groups show similar chemical shifts of the photoelectron line and, therefore, deteriorate the resolution, resulting in overlapping of peaks. Nevertheless, the N1s line can be used to compare the nitrogen functionalities in Antarctic fulvic acids.

OLFA appears to contain all types of nitrogen functionalities, with more contribution in line N1s A. On the other hand, in nitrogen-rich PNFA and MLFA the majority of nitrogen was also detected in N1s A and B belonging to the categories of heterocyclic, amide nitrogen. It can be concluded, therefore, that the nitrogen in these Antarctic fulvic acids mostly comes from the amide in the form of peptides or possibly cyclic amides structure such as uracil and hydantoin as previously reported for PLFA (Fang et al., 2011). Comparing the XPS results with that obtained from PLFA in Chapter 2, the composition of nitrogenous compounds in PNFA is seemingly more similar to PLFA than other Antarctic fulvic acids.

In conclusion, XPS provides a fairly reliable assessment of the composition of organic matter including organic nitrogen functionalities in Antarctic fulvic acids. However, it is not feasible to precisely determine the character of nitrogen-containing compounds based on XP spectra alone, additional experiments are needed to gain insight to the possible similarities and differences between the nitrogenous compounds of Antarctic fulvic acids.

4.2.2 Fractionation of PNFA using C18 Solid Phase Extraction

Similar to PLFA, the fractionation of PNFA was performed and results were compared for the two fulvic acids. OLFA fractionation was also attempted, but was not successful due to the fact that almost the whole material was recovered as PNFR 4 (eluted by 50:50 acetonitrile: water), indicating DOM is predominately hydrophobic in nature. In the case of MLFA fractionation was not tried due to the limited sample available.

Fractionation of PNFA was carried using the same experimental conditions used for PLFA and described in Chapter 3. The initial solubilisation of PNFA (prior to loading on the column) was undertaken using 2 methods. The first method, Method 1, was performed according to the method used for PLFA and described in section 3.2.1, namely ultrasonication in water. The second method of solubilisation, Method 2, utilised an azeotropic mixture of acetonitrile:water to aid solubility (see experimental section for details). The results of fractionation based on these two solubilisation methods as well as the fractions distribution are shown in Fig. 4.4.

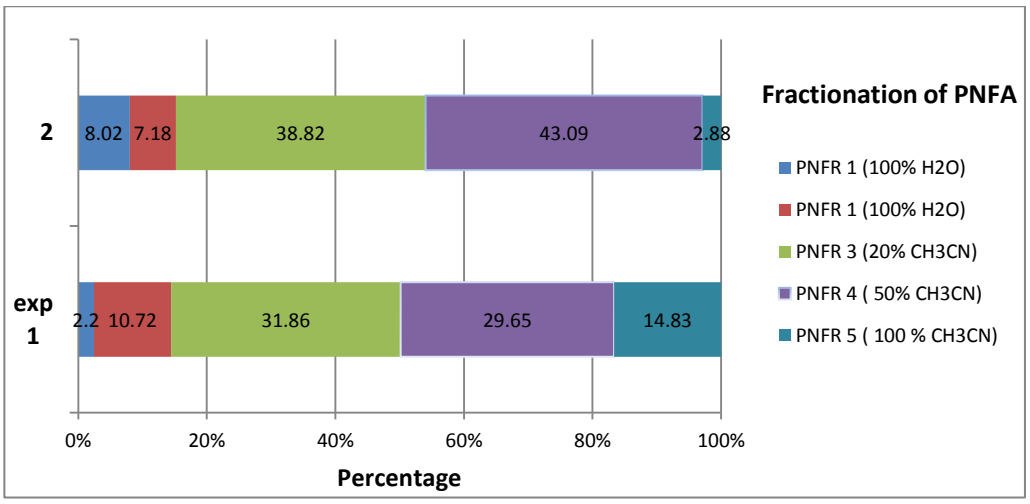


Figure 4.4 Pendant Lake fulvic acid fractions distribution obtained during Method 1 (sonication – fractionation), Method 2 (azeotropic mixture dissolution - fractionation)

As expected, the hydrophobic fractions have a greater contribution than hydrophilic fractions of this fulvic acid. Moreover, the results obtained from the two initial solubilisation methods show the impact of solvent on the solubility of unfractionated fulvic acid and in turn on the distribution of fractions. In other words, it suggests that the use of acetonitrile:water mixture instead of pure water to dissolve the sample, has encouraged the hydrophobic effect and assisted with the solubility of less polar and naturally-hydrophobic compounds. This highlights the potential formation of micelles in PNFA similar to the behaviour observed for PLFA (Chapter 3). The fractions obtained under the conditions of Method 2 were chosen for further characterisation studies.

Comparing the distribution of fractions obtained for PLFA and PNFA, it seems that a greater mass of hydrophobic material is present in PNFA compared to PLFA (Figure 4.5). The presence of an extra fraction (PNFR 5) as a result of elution by pure acetonitrile during PNFA fractionation, albeit in trace amount, supports this conclusion.

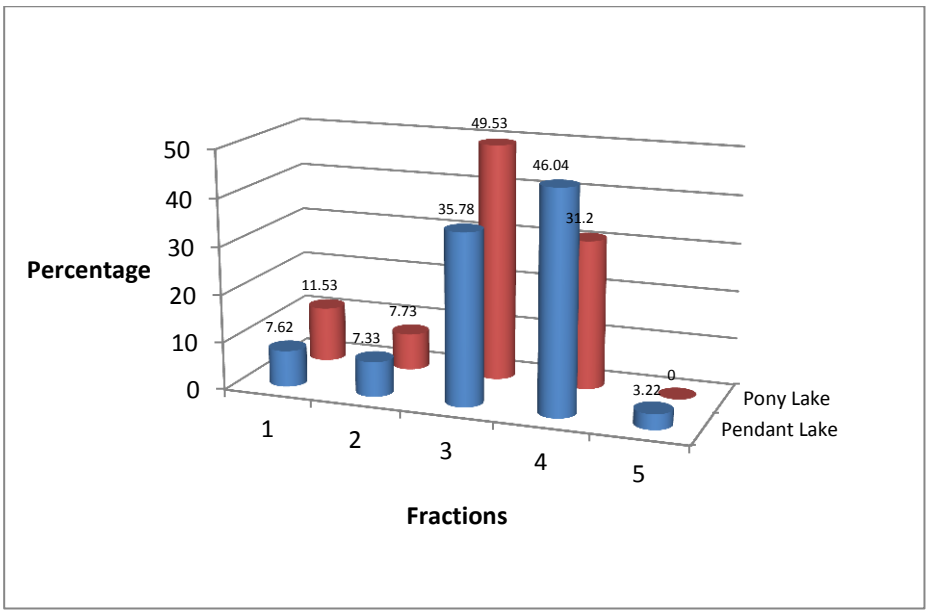


Figure 4.5 Fractions distribution in Pony Lake (Condition 2) and Pendant Lake fulvic acids (Method 2) obtained as follows: PNFR 1 (100% H2O), PNFR 2 (5 % CH_3CN), PNFR 3 (20%CH_3CN), PNFR 4 (50 % CH_3CN),PNFR 5(100% CH_3CN)

4.2.3 Elemental analysis of fractions

The elemental composition of PNFA and its fractions are reported in Table 4.1. Carbon content ranges from 43.51 to 53.16 % and the highest value is in the PNFR 4. Nitrogen and hydrogen content of the four fractions range from 3.28 to 5.11 % and 4.32 to 6.42 %, respectively. The distribution of C, H and O reflects the properties of the fractions isolated based on the polarity-based fractionation. Looking at nitrogen values, there is an equal distribution between fractions for this element, likewise in PLFA (Chapter 3), however sulfur was found to be accumulated in PNFR 1 and PNFR 2; knowledge about the nature of sulfur-containing compounds is required to discover the reason for such distribution.

Table 4.5 Elemental composition of PNFA fraction

Fractions	% Carbon	% Oxygen	% Hydrogen	% Nitrogen	% Sulphur	H/C	C/N	O/C
PNFR 1	43.51	46.63	4.32	4.63	0.91	1.19	10.96	0.80
PNFR 2	49.01	41.08	5.62	3.28	1.03	1.38	17.43	0.63
PNFR 3	50.24	38.12	6.07	5.11	0.46	1.45	11.47	0.57
PNFR 4	53.16	34.98	6.42	4.92	0.52	1.45	12.61	0.49
Unfractionated PNFA[1]	49.82	38.39	6.30	4.66	0.83	1.51	12.48	0.59

[1] Nimmagadda (2008)

4.2.4 Chemical analysis of PNFA, MLFA and OLFA

The chemical analysis of Vestfold Hills' DOM has not been performed to date and the only work done is related to the study of unfractionated Pendant and Organic Lake hydrophobic acid by Nimmagadda (2008) including reduction using n-Butylsilane (n-BS) and analysis of the reduced material by GC/MS. According to the results of that study, very limited aromatic structures were detected in fulvic acids of these two lakes and compounds identified were believed to be the result of degradation of isoprenoids and terpenes unit; for example, in Pendant Lake Fulvic Acid the presence of compounds such as camphene, cymene, limonene, dimethyl-nonene and 3,7-dimethyl-1,6-octadien-3-ol among the products could be the reduced products of terpenes degraded by microorganisms into alcohols and carboxylic acids. Apart from these constituents, a significant number of unsaturated alkanes and alicyclic hydrocarbons support the presence of terpenes. The chromatographic analysis of Pendant and Organic Lake fulvic material was limited to the aforementioned study and did not go further. This has inspired us for further study using selected number of chemical methods on fulvic acid isolated from Pendant and Organic Lake (PNFA, OLFA) as well as Mossel Lake (MLFA) all containing high nitrogen content.

In this section, selected methods that were applied on PLFA in Chapters 2 and 3 and showed acceptable results in terms of structural identification were also tested on PNFA, MLFA and OLFA. These methods involve Trimethylsilylaion, TMAH thermochemolysis as well as HCl-methanolysis and will be discussed in detail in the following sections.

4.2.4.1 Trimethylsilylation of PNFA (PNFR 3 and 4), MLFA, OLFA

Based on the result obtained in the previous sections, PNFR 3 and PNFR 4 contain the bulk of hydrophobic material in this fulvic acid and as such are more hydrophobic in nature relative to PNFR 1 and PNFR 2. For this reason and due to the limited sample available to test all fractions, this derivatisation method was only performed on PNFR 3 and PNFR 4.

Unfractionated MLFA and OLFA were also examined using only Trimethylsilylation due to the limitation in quantity of sample available precluding fractionation. Table 4.6 summarises the compounds identified in PNFA (including PNFR 3 and PNFR 4), MLFA and OLFA using TMS-derivatisation.

4.2.4.1.1 PNFA products

According to the data TMS-derivatisation has effectively derivatised PNFA fractions; a UCM is not very significant after derivatisation and also numerous components were identified; generally, the numbers of compounds identified are greater than those obtained via TMS-derivatisation of PLFA fractions, indicating more effective derivatisation. The compounds identified in PNFA fractions (PNFR 3 and PNFR 4) are mostly shared in the two fractions. Moreover, as expected the majority of components have an aliphatic structure and aromatic moieties (peaks 10, 33, 37, 41) are limited.

Apart from short and medium chain mono and di carboxylic acid (C3-C9), longer chain fatty acids ranging from C10 to C18 (peaks 28, 29, 34, 38-40, 42,43, 45, 46) in their saturated and unsaturated forms (oleic and palimitoleic acid) are also identified in PNFA fractions; these structures were similarly detected in TMS-derivatives of PLFA fractions (Chapter 3) and were in agreement with the previously reported components (in the form of linear alkanes) obtained using alkylsilane reduction by Nimmagadda (2008).

Amino acid derivatives and heterocyclic nitrogenous components (Peak 13, 16, 28) are also present, but are not being readily determined and thus made GC-amenable. This was also observed for PLFA, therefore it can be concluded that nitrogenous structures are not derivatised or accessible by derivatising reagents, so that their detection and identification becomes challenging.

Sugars are not detected in PNFR 3 and PNFR 4 due to the fact that they are the hydrophobic fractions of PNFA; the only sugar-related moiety is peak 44 in the

chromatogram (Figure 4.7), indicating the presence of disaccharide structure. Interestingly, this structure was also detected in PLFA hydrophobic fractions; however not in OLFA and MLFA. Monosaccharides are not detected in the PNFR 3 and PNFR 4, and as mentioned earlier they would supposedly be present in PNFR 1 and PNFR 2 which were not analysed due to the low amount of sample. However, TMS-derivatised monosaccharides were detected in MLFA and OLFA as shown in Figure 4.8 (likewise in PLFA). This implies that the position of sugars in all Antarctic fulvic acids in this study is relatively similar and very accessible by the reagent.

4.2.4.1.2 MLFA and OLFA products

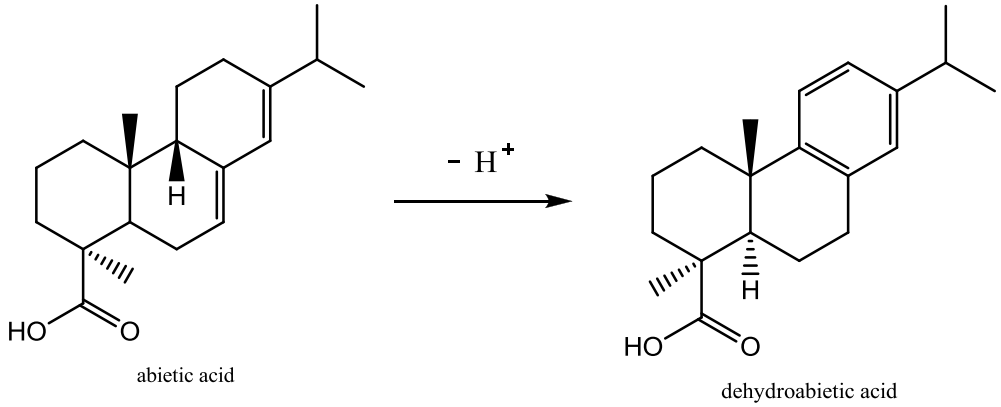
The TICs of TMS-derivatised MLFA and OLFA are presented in the Figure 4.8. At first glance, TMS-derivatised products for these two Antarctic FAs appear similar to those detected in both PNFA and PLFA (Chapter 3). However, there are some notable differences worth highlighting. As shown in Figure 4.8, the UCM (Unresolved Complex Mixture) is enormous in the TMS-dervatised OLFA. In contrast, MLFA surprisingly did not show any UCM or any unresolved material, derivatisation products are also ample and some interesting compounds were identified.

Hydroxy fatty acids in both saturated and unsaturated form (peak 35, 36) were present among products of OLFA, but not in MLFA or PNFA. As previously mentioned, their presence can due to the presence of bacterial lipopolysaccharides (LPS) (Cranwell, 1981), different families of fatty acids including poly-unsaturated FAs were extensively studied in Antarctic lakes (Matsumoto and Kanda, 1985;Matsumoto, 1989;Mancuso et al., 1990) as well as in the benthic sediments of Organic Lake (Rogerson and Johns, 1996) and was correlated to autochthonous, microbial and algal input to the lake (Volkman et al., 1989). The authors found that the lipid content in Organic Lake is significantly higher than other Antarctic lakes with similar salinity, this is probably why in OLFA we detected more fatty acids structures

(hydroxyl fatty acids) than its other Antarctic counterparts. The result of reduction of OLFA reported by Nimmagadda (2008) shows several acyclic terpenes possibly reduced products of unsaturated short hydroxy acids.

The products obtained after trimethylsilylation of MLFA are similar to those identified in its counterparts from other Antarctic lakes mostly consisting of short chain linear and branched carboxylic acids, C4-C10 alkandioic acids and their branched isomers, monosaccharides and, disaccharide (peak 48) were also detected. An interesting compound was also detected (peak 47), identified as dehydroabietic acid trimethylsily ester; dehydroabietic acid (DHAA) is a tricyclic diterpenoid which belongs to the family of resin acids. Resin acids are naturally found in the tree resins and their formation is via diagenesis of old wood and bark of vascular plants (Saiz-Jimenez et al., 1996;Adamczyk et al., 2011). The main resin acid is abietic acid that converts to DHAA via dehydrogenation and isomerisation reactions (Hjulstrom et al., 2006); it is more stable than abietic acid to degradation and oxidation (Martin and Saiz-Jimenez, 1978). DHAA was previously reported in a fossil wood sample dated back to Middle Jurassic from Polish clay-pits and of conifers origin (Marynowski et al., 2007), it was also found in humic acids (Martin and Saiz-Jimenez, 1978). Recently, terpenoid structures were also suggested as the main building blocks of PNFA and OLFA based on the small reduced analogues of terpenes and their degradation products detected by GC/MS analysis (Nimmagadda, 2008). However, terpenoid acids (resin acids) such as DHAA are expected to be a part of terrestrial DOM; to the best of our knowledge, there is no report of their occurrence in microbially-derived DOM. Herein, the effortless detection of this undegraded, large terpenoid (Figure 4.6) in fulvic material of Antarctic lakes is remarkable and it reveals the usefulness of the direct derivatisation, however, the origin of this terpenoid remains arguable. Since there is no higher order plant resin in the area of study, DHAA may come from an external source such as atmospheric particles produced from plant or coal combustion transferring to the area. There are some evidence that demonstrate the

parallel presence of resin acid and their thermal degraded products in biomass combustion aerosols and sedimentary records (Standley and Simoneit, 1994). This implies that DHAA and its similar terrestrial-originated components could deposit from atmosphere to Antarctic lakes and subsequently enter to the aquatic carbon cycles.



abietic acid

dehydroabietic acid

Figure 4.6 structures of abietic and dehydroabietic acid (DHAA)

In general, there is little background information about Mossel Lake and its DOM; studies performed to date were limited to geological and mineralogical studies (Sheraton and Collerson, 1984;Black et al., 1991). Based on our results, MLFA composition is slightly different from the other two Vestfold Hills Lakes FAs as well as from PLFA, we assume that it is more GC-analysable and possibly less mature or complex because larger constituents such as DHAA has been detected using simple derivatisation coupled to GC/MS.

Table 4.6 Identified compound in TMS-derivatised PNFA (PNFR 3 and 4), MLFA and OLFA

Peak no[1]	Compounds Identified	PNFR3	PNFR4	MLFA	OLFA	Identification method [2]
1	Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	-	+	+	+	a,d
2	Acetic acid, [(trimethylsilyl)oxy]-, trimethylsilyl ester	+	+	+	+	a,d
3	Pentanoic acid, 4-oxo-, trimethylsilyl ester	-	+	-	-	a,d
4	Ethanedioic acid, bis(trimethylsilyl) ester	+	+	+	+	a,d
5	2-Butenoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	-	+	-	-	a,d
6	Butanoic acid, 3-methyl-3-[(trimethylsilyl)oxy]-, trimethylsilyl ester	-	+	+	+	a,d
7	(4-methoxy-3,3-dimethylpent-4-enyloxy)trimethylsilane	-	+	-	-	b,d
8	2-Propenoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	+	+	+	-	a,d
9	Silane, trimethyl(3,5-xylyloxy)-	-	-	-	+	a,d
10	Benzoic acid trimethylsilyl ester	+	+	+	+	a,d
11	Silanol, trimethyl-, phosphate	+	+	-	-	a,d
12	Glycerol-tri-trimethylsilylether	+	+	-	+	a,d
13	3,5-dimethyl-4-(trimethylsilyloxy)dihydrofuran-2(3H)-one	+	+	-	-	b,d
14	Glycine, N,N-bis(trimethylsilyl)-, trimethylsilyl ester	+	+	-	-	a,d
15	Butanedioic acid, bis(trimethylsilyl) ester (succinic acid bis tms)	+	+	+	+	a,d
16	bis(trimethylsilyl) 2-methoxysuccinate	-	-	-	+	b.d
17	Glyceric acid tris(trimethylsilylester)	-	-	+	-	a,d
18	Fumaric acid, bis (trimethylsilylester)	-	-	+	-	a,d
19	trimethylsilyl 2-(trimethylsilyloxy)heptanoate	-	+	+	-	b,d
20	Propanoic acid, 2-methyl-2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	+	+	-	+	b,d
21	3-ethyl-4-methyl-1-(trimethylsilyl)-1H-pyrrole-2,5-dione	+	+	-	-	b,d
22	3,6,9-trioxa-2,10-disilaundecane, 2,2,10,10-tetramethyl	-	+	-	-	b,d
23	α,β,β-Trimethylstyrene	-	-	-	+	a,d
24	Nonanoic acid trimethylsilylester	+	+	+	-	a,d
25	Pentanedioic acid, bis(trimethylsilyl) ester	-	+	+	+	a,d
26	Hydroquinone bis(trimethylsilyl) ether	-	-	+	+	a,d
27	Hexanedioic acid, bis(trimethylsilyl) ester	+	+	+	+	a,d
28	Decanoic acid, trimethylsilyl ester	-	+	-	+	a,d

29	Hexanedioic acid, 3-methyl-, bis(trimethylsilyl) ester	-	+	-	-	a,d
30	L-Proline, 5-oxo-1-(trimethylsilyl)-, trimethylsilyl ester	-	+	-	-	a,d
31	(E)-2,2,4,6,10,10-hexamethyl-7-(trimethylsilyloxy)-3,9-dioxa-2,10-disilaundec-4-ene	-	+	-	-	b,d
32	trimethylsilyl 1,2-dimethyl-6-propoxycyclohexanecarboxylate	-	-	-	+	b,d
33	Benzoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester	+	+	+	+	a,d
34	Octanedioic acid, bis(trimethylsilyl) ester	+	+	-	-	a,d
35	trimethylsilyl 9-(trimethylsilyloxy)decanoate	-	-	-	+	b,d
36	(Z)-trimethylsilyl 2-(trimethylsilyloxy)undec-2-enoate	-	-	-	+	b,d
37	Hydrocinnamic acid, p-(trimethylsiloxy)-, trimethylsilyl ester	+	+	-	-	a,d
38	Azelaic acid, bis(trimethylsilyl) ester	+	+	-	-	a,d
39	n-Tetradecanoic acid, trimethylsilyl ester	+	+	+	+	a,d
40	n-Petadecanoicacid, trimethylsilylester	+	+	+	-	a,d
41	2-(5-tert-Butyl-2-hydroxyphenyl)-1,4-benzoquinone	+	+	-	-	c,d
42	Trimethylsilyl (9E)-9-hexadecenoate (palmitoleic acid trimethylsilylester)	+	+	-	-	a,d
43	Hexadecanoic acid, trimethylsilyl ester	+	+	+	+	a,d
44	(Z)-trimethyl(3-methyl-4-styrylphenoxy)silane	+	+	-	-	b,d
45	Oleic acid, trimethylsilyl ester	+	+	+	+	a,d
46	Octadecanoic acid trimethysilylester	+	+	-	-	a,d
47	Dehydroabietic acid trimethylsilylester	-	-	+	-	b,d
48	D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-β-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	+	+	+	+	a,d

[1] Numbers correspond to the peaks in Fig 4.7 and 4.8. [2] Identification was based on three methods (a) matching compound suggested by NIST Mass Spectral Search Program with above 90 % match probability (b) using NIST MS Interpreter with quality indices reported as above 85 % (c) tentative identification (d) suggested structures were checked against retention data in the literature

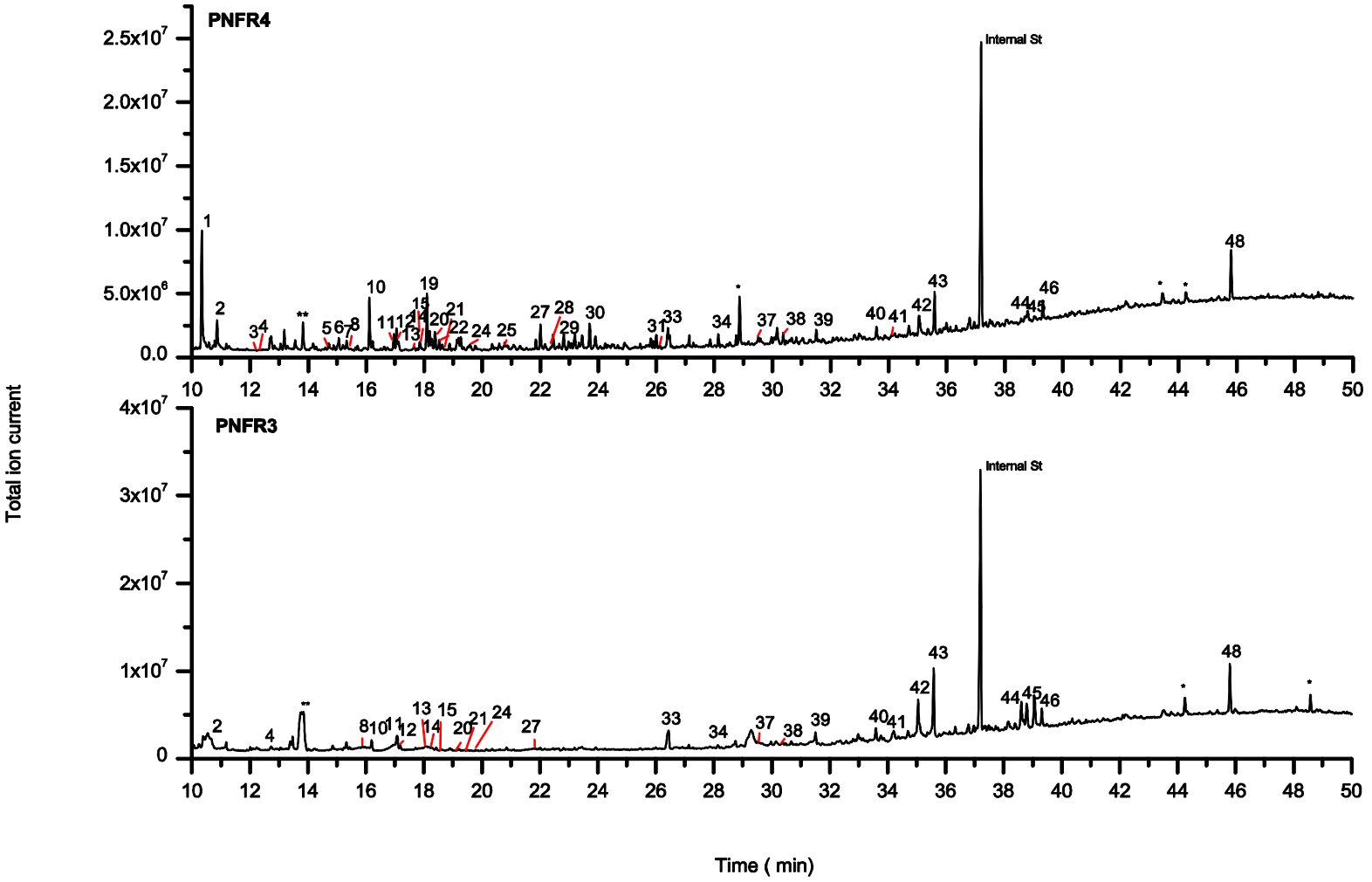


Figure 4.7 Total ion chromatogram of TMS –derivatisation products of PNFA fractions .* indicates non-identified peaks, ** indicates siloxane peaks

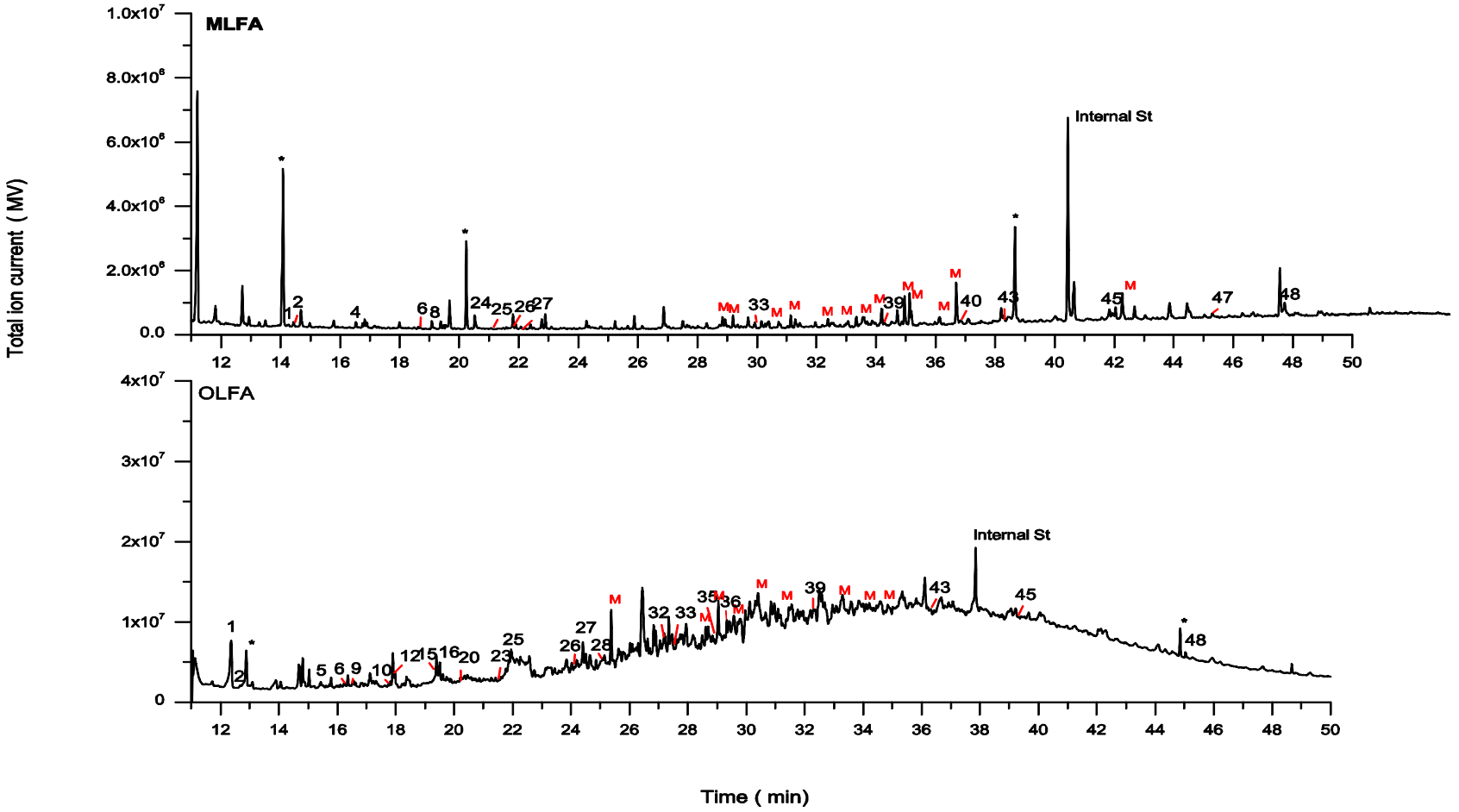


Figure 4.8 Total ion chromatogram of TMS-derivatisation products of MLFA and OLFA , **M** and * indicates derivatised monosaccharides and siloxae peaks, respectively.

4.2.4.2 TMAH thermochemolysis

The products of TMAH thermochemolysis of Vestfold Hills fulvic acids including PNFA, MLFA and OLFA are compared in Table 4.7.

In general, it seems that thermochemolysis was more effective on the two nitrogen rich PNFA and MLFA compared to OLFA (with very limited products). The derivatisaton was not ,however, perfect with the presence of underivatised compounds (e.g. peak 30) evident. It did, however, enabled the separation and detection of nitrogenous structures such as pyrrole, pyrimidine and indole derivatives.

As expected, the volatile products including short chain carboxylic acids and alkandioic acid are common among all three fulvic materials. However, when it comes to small nitrogenous compounds, the trend is different. PNFA and MLFA as nitrogen-rich fulvic acids show some heterocyclic nitrogen-containing structures (peaks 10 to 15), while OLFA does not contain many of these products possibly due to the low overall nitrogen. Furthermore, nitrogen-containing units are apparently connected to other structures such as sugars (peak 36) or aromatic structures (peak 35).

Referring back to the products of thermochemolysis of PLFA fractions (Chapter 3), many of those structures are observed here in Vestfold Hills FAs. Aromatic moieties are limited to benzoic acid derivatives (peaks 3, 9, 24-26), however, products of methylated sugars are observable (peaks 29, 32, 33), this implies that sugars were accessible by the reagents because TMAH is not a selective reagent for sugar derivatisation. Another interesting feature is the presence of dehydroabietic acid (DHAA) methyl ester (peak 37) that was previously detected in TMS-derivatised MLFA as its trimethysilylester. DHAA was also reported as major precursors of DOC in the previous studies (Leenheer et al., 2003). To ensure its presence in our sample, we separately derivatised DHAA using TMAH and

compared the retention times of peak 37 (in MLFA TIC) with pure methyl dehydroabiatate; as demonstrated in Figure 4.9 the peak positions and mass spectrum of peak 37 matches with those of DHAA that confirms DHAA presence in Mossel Lake FA.

Due to the fact that a similar compound was also observed in PLFA (peak 38 in Table 3.7, Chapter 3), it may be reasonable to suggest that these terpenoid structures are frequently found in Antarctic Lakes DOM. As previously mentioned a possible origin of such structures may be the result of atmospheric deposits to the Antarctic ecosystem.

Table 4.7 Peak identification of TMAH-GC/MS products of PNFA, MLFA and OLFA

Peak no[1]	Compounds Identified	PNFA	MLFA	OLFA	Identification method [2]
1	Butanedioic acid, dimethyl ester	+	+	+	a,d
2	Butanedioic acid, methyl-, dimethyl ester	+	+	+	a,d
3	Benzoic acid, methyl ester	+	+	+	a,d
4	dimethyl mesaconate	+	+	-	a,d
5	2-Butanone, diethylhydrazone-	+	+	+	a,d
6	2,3,5,6-tetramethyl-1,2,3,4-tetrahydropyrazine	+	+	+	b,d
7	Pentanedioic acid, dimethyl ester	+	+	-	a,d
8	Pentanedioic acid, 2-methyl-, dimethyl ester	+	+	-	a,d
9	Benzeneacetic acid, methyl ester	+	-	-	a,d
10	1H-Pyrrole-2,5-dione, 3,4-diethyl-	-	+	+	a,d
11	2,5-Pyrrolidinedione, 3-ethyl-1,3-dimethyl-	+	+	+	a,d
12	Dimethyl 2-methyl-3-methylenesuccinate	+	-	-	a,d
13	1,3-Dimethyl-4,5-imidazolidinedione	+	+	+	b.d
14	N,N'-Dimethyluracill	+	+	-	a,d
15	2,4-Imidazolidinedione, 3,5,5-trimethyl-	+	+	+	a,d
16	2-Furancarboxylic acid, tetrahydro-3-methyl-5-oxo-, methyl ester	+	-	+	a,d
17	Hexanedioic acid, dimethyl ester	+	+	+	a,d
18	2,5-Dimethoxytoluene	+	-		a,d
19	Hexenedioic acid, dimethyl ester	+	+	+	a,d
20	methyl 2,6-dimethoxy-2,5-cyclohexadiene-1-cardoxylate	+	-	-	a,d
21	2,3,4-Trimethylbenzaldehyde	+	-	+	a,d
22	Heptanedioic acid, dimethyl ester	+	-	-	a,d
23	Pyrrolid-2-one-5-carboxylic acid, N-methyl-, ethyl ester	+	+	+	a,d
24	Benzoic acid, 2-amino-, methyl ester	+	+	-	a,d
25	Benzoic acid, 4-methoxy-, methyl ester	+	+	+	b.d
26	Cinnamic acid, methyl ester	+	+	+	a,d
27	1H-Isoindole-1,3(2H)-dione, 2-methyl	+	+	-	a,d
28	dodacanoic acid methyl ester	+	-	-	a,d

29	Methylated pentose	+	-	-	c,d
30	4-(4-Methoxyphenyl)butyric acid	+	+	-	a,d
31	Nonanedioic acid, dimethyl ester	+	+	-	c,d
32	Methylated hexose	+	-	-	c,d
33	6-deoxy methylated hexose	+	-	-	c,d
34	5-(4-methylhexyl)dihydropyrimidine-2,4(1H,3H)-dione	+	-	-	b.d
35	4-Benzylidene-2-methyl-5(4H)-oxazolone	+	-	-	a,d
36	(6R)-6-methoxy-2,2-dimethyl-5-oxotetrahydro-2H-pyran-4-yl 5-methyl-1H-pyrrole-2-carboxylate	+	+	-	b.d
37	Dehydroabietic acid methyl ester	+	+	-	a,d

[1]Numbers correspond to the peaks in Fig 4.9, [2] identification was based on three methods (a) matching compound suggested by NIST Mass Spectral Search Program with above 90 % match probability b) using NIST MS Interpreter with quality indices reported as above 85 % (c) tentative identification (d) suggested structures were checked against retention data in the literature.

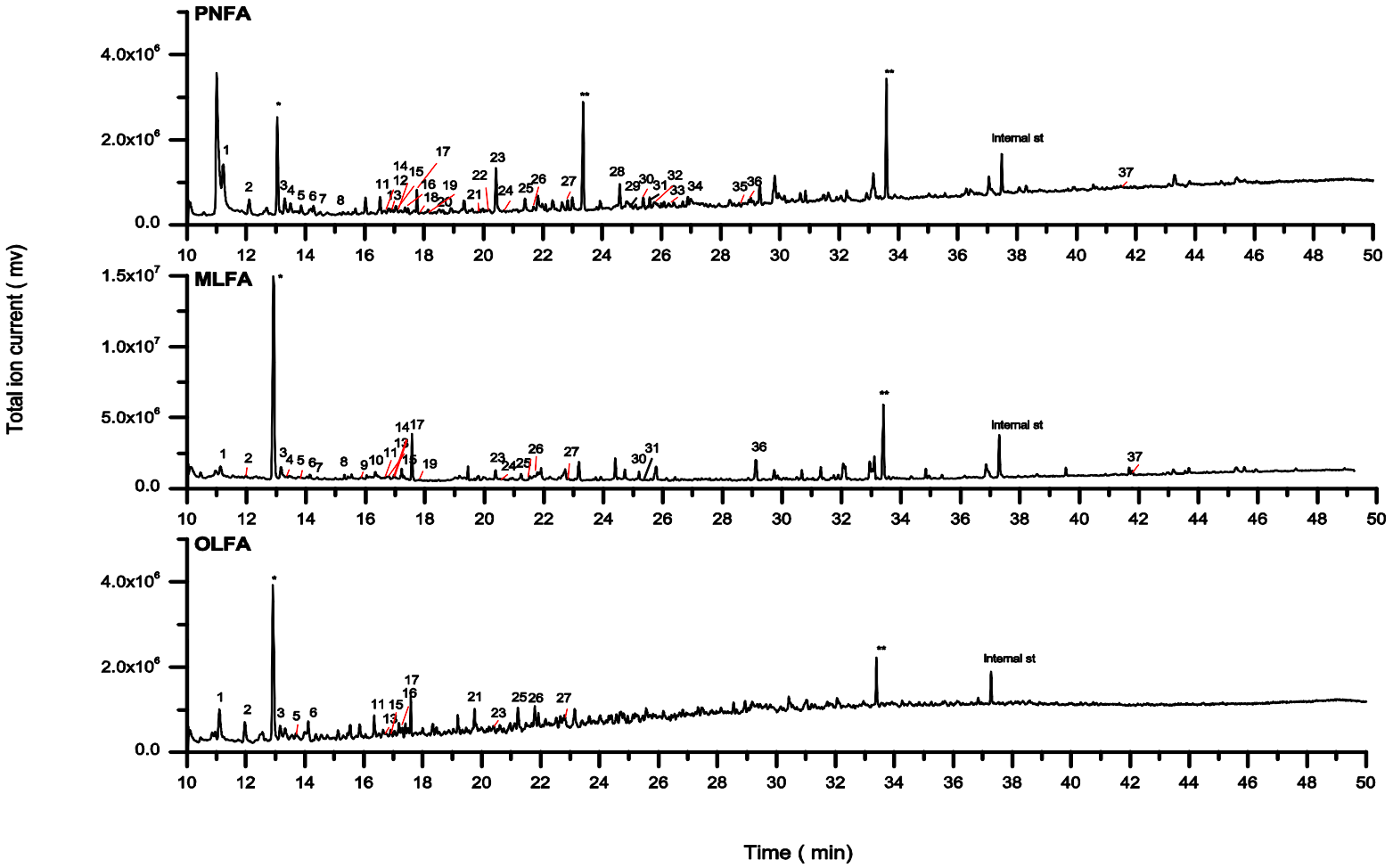


Figure 4.9 Total ion chromatograms of products obtained under derivatisation of PNFA, MLFA and OLFA with TMAH, * indicates siloxane peaks, ** indicates contaminations

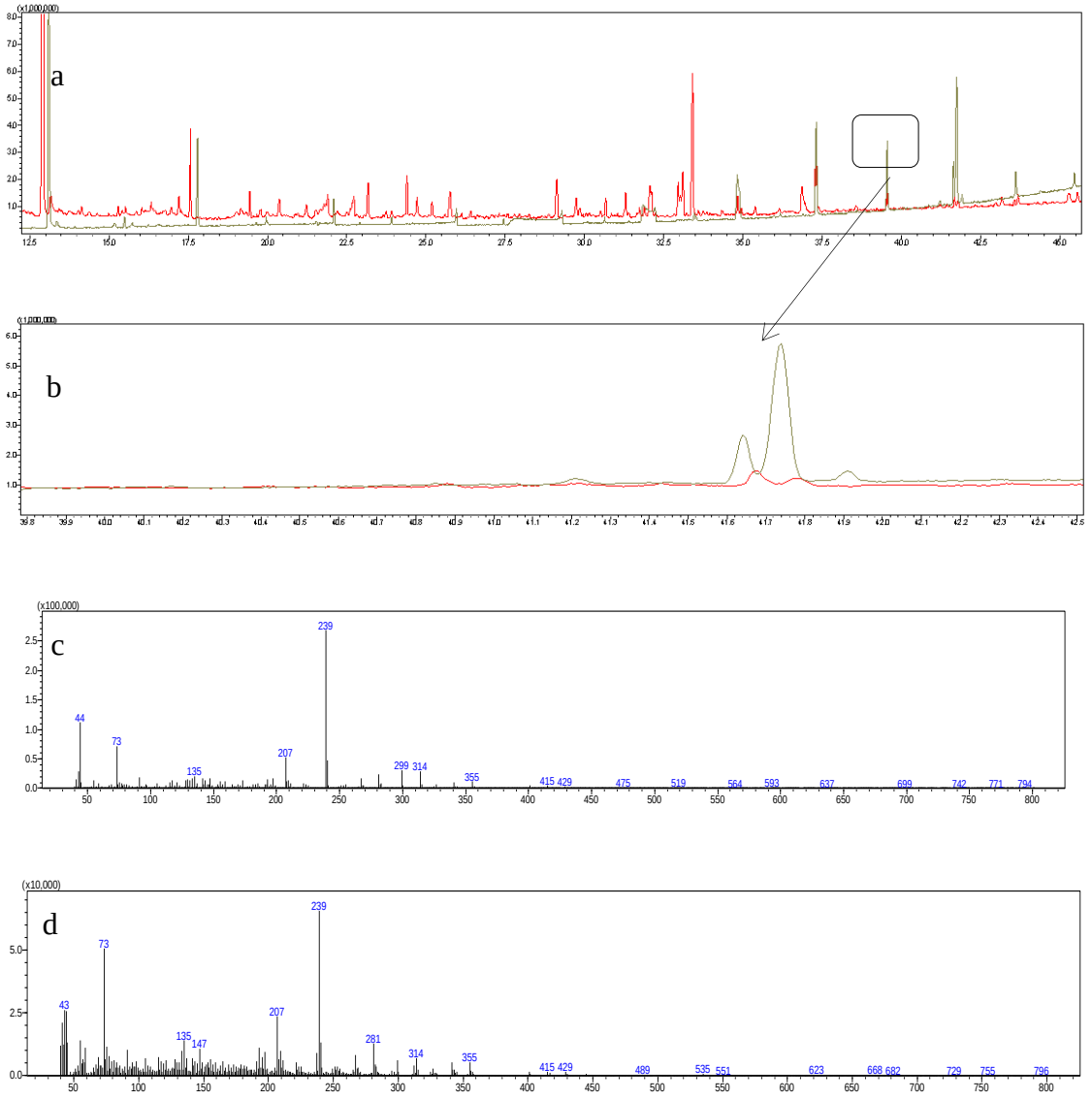


Figure 4.10 DHAA peak position in (a ,b) overlaid TIC of methylated DHAA and methylated MLFA, (c) mass spectrum of methylated DHAA, (d) mass spectrum of peak representing DHAA in methylated MLFA.

4.3 Discussion

Turning back to the question raised at the beginning of this chapter regarding the possible similarities and differences among Antarctic fulvic acids, it would be reasonable to consider the following points: first of all, the fractionation of PNFA showed micelle formation was observed for this Vestfold Hills fulvic acid similar to PLFA. As far as chemical analysis is concerned the part of all Antarctic fulvic material that separated as GC-amenable were nearly similar. The two nitrogen-rich fulvic acids, PNFA and MLFA, showed similar components to each other and to PLFA, seemingly, MLFA was derivatised easier and more effectively than two other nitrogen-rich FAs.

OLFA proved to be compositionally different based on the elemental analysis. It was often the case that the whole GC-analysable matter was lower and the UCM was significant for this fulvic acid. However, a couple of compounds especially lipids are identified. One obvious similarity among all of these four fulvic materials (PNFA, MLFA, OLFA and PLFA) is the effortless sugar detection by simple derivatisation. However, detection and identification of nitrogen-containing components has been difficult in nitrogen-rich PLFA, PNFA and MLFA as well as nitrogen-poor OLFA. This implies that the core of refractory material in Antarctic fulvic acids may be nitrogen-rich. This difficulty was to some extent overcome by thermochemolysis using TMAH, but did not appear able to improve the detection of many nitrogenous moieties.

There are other differences in the composition of fulvic acids isolated from different Antarctic lakes. These differences are more observable during the spectral analyses before the application of component-level analysis. For instance, XPS provides sensitive and reliable information about the differences in nitrogen functionalities in different fulvic acids which is overlooked by GC/MS. XPS shows that OLFA contain all types of nitrogen functionalities,

with more contribution in line N1s B (pyrrole nitrogen). It also did not show a peak as N1s C. On the other hand, in nitrogen-rich MLFA the majority of nitrogen was detected in N1s C belonging to the category of primary amine/amide. This is an interesting feature of this FA which highlights the difference of its nitrogen-containing compounds compared to its counterparts.The significant peak area of N1s A in PNFA similar to PLFA shows that the nitrogen in these Antarctic fulvic acids mostly comes from the amide in the form of peptides or possibly cyclic amides structure such as uracil and hydantoin as previously reported for PLFA (Fang et al., 2011). Thesea are interesting structural features in these FAs especially in terms of dissolved organic nitrogen. The nature of and reasoning behind these differences can be, however, related to the physiochemical properties of each lake such as salinity or microbial activity and most importantly their possible inputs. Therefore, chemical alone analysis cannot explain the reason for distinction or resemblance of DOM in the lakes and comprehensive biogeochemical investigation is required.

Regarding the information obtained during this project, the detection of structures as large and complex as DHAA using a simple derivatisation is noteworthy. Its formation in the pure microbial DOMs can also be questionable. On the other hand, the unbroken disaccharide structure in all Antarctic FAs alongside many monosaccharides which successfully derivatised, are an indicator of immaturity of Antarctic fulvic acids.

In summary, we assume that current results as preliminary data related to the area with very limited background information and restricted chemical exploration assist to open new avenues for further research on this fascinating pool of microbial DOM.

4.4 Materials and Methods

4.4.1 Sampling and preparation of Pendant Lake fulvic acid

DOM of Pendant, Organic and Mossel Lakes was isolated from around 300 L of water from the lake using mechanical pumping (peristaltic pump).Where the lake was ice-covered, a hole was drilled through the ice to collect the water samples. The sampling depths were 5 m, 2.5 m and 5 m for Pendant, Organic and Mossel Lake, respectively. In the case of Organic Lake, the sample was collected above the oxycline layer. Sample drums were transported to Davis station by helicopter for further processing. The samples were processed using the procedures given by Leenheer et al., (2000) into two fractions; a hydrophobic acid fraction, and a transphilic acid fraction. The hydrophobic fractions was considered as equivalent to fulvic acid and used for subsequent structural analyses. The fractions were freeze-dried after isolation and kept at -25 ^{0}C in the dark before use.

4.4.2 X ray photoelectron spectroscopy (XPS)

Photoelectron spectra were recorded according to the condition mentioned in 3.4.6 for PLFA for all samples except using 1mm x 1 mm clean silicon wafers as sample holders.

4.4.3 Fractionation of Pendant Lake Fulvic acid using RP-SPE

4.4.3.2 Chemicals

Acetonitrile (HiPerSolv) was obtained from VWR International. SPE cartridges (Strata Polymeric RP 500 mg/6 mL) were purchased from Phenomenex and stored in acetonitrile before use

4.4.3.3 Sonication-Fractionation

The fractionation with a sonication step was performed on Pendant Lake Fulvic Acid (PNFA) according to the section 3.4.1.3 (Method 2). However, full dissolution was not achieved; the fractionation was similar to fractionation of PLFA described in 3.4.1.2

4.4.3.4 Use of azeotropic mixture prior to fractionation

To aid the solubility, 15 mg PNFA was dissolved in 10 mL of an azeotropic mixture of acetonitrile:water (83.7 % w/w acetonitrile in water) yielding a clear solution, then the solvent was evaporated in a rotary evaporator to obtain around 2-3 mL solution, at this stage MilliQ water was added to reach the volume of 8 mL. The solution obtained in this way showed a better solubility, then the fractionation procedure was performed as previously described.

4.4.4 Elemental analysis

Elemental composition of the freeze dried fractions was determined using a Model PE2400 CHNS/O elemental analyser (PerkinElmer, Shelton, CT, USA) for carbon, hydrogen, nitrogen and sulfur. Oxygen was determined by difference. The values reported for H, C, N and S were average of three replicates. Blank cups and standards were run to correct for the C and N associated with tin cups.

4.4.5 Chemical analysis

4.4.5.1 Trimethylsilylation

4.4.5.1.1 Samples and chemicals

PNFA fractions obtained using Method 2 (Section 4.4.2.4), were freeze-dried for 24 hours and kept inside dark bottles at 4^0C before derivatisation. MLFA and OLFA were kept in the fridge before use. BSTFA:TMCS (99:1) and anhydrous pyridine was obtained from Sigma-Aldrich and kept away from exposure to air and moisture during handling using sealed bottles. Methyl octadecanoate was an analytical standard from Poly Science Corporation and was used to prepare internal standard solution.

4.4.5.1.2 Derivatisation procedure

0.5 mg of freeze-dried PNFA fractions, 1.0 mg of MLFA and OLFA (weighed to 0.01 mg) were derivatised using BSTFA:TMCS according to the procedure outlined in section 3.4.8.2.

4.4.5.1.3 GC/MS analysis

Analysis using GC/MS was carried out according to the procedure previously described in 3.4.8.3.

4.4.5.2 TMAH methylation

4.4.5.2.1 Samples and chemicals

Freeze-dried PNFA, MLA and OLFA samples were used without any treatment. TMAH (25% wt in methanol) was obtained from Aldrich and used as supplied.

4.4.5.2.2 Derivatisation procedure

The procedure was undertaken as previously stated for PLFA fractions (Section 3.4.10.2).

4.4.5.2.3 GC/MS analysis

The instrumental analysis was carried out as detailed in section 3.4.10.3

4.8 References

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Conclusions

This thesis is the first comparative study of Antarctic fulvic acids at the molecular-level using various GC derivatisations. The employment of different methods and strategies on a comparative basis was an important part of the thesis. They provide insight into the identification of building blocks of complicated fulvic acids and highlighted certain features of the structure that had a crucial impact on the development of this research. The use of multiple methods was useful to compare data in a more systematic way, so as to better interpret similarities and discrepancies.

The molecular characterisation of fulvic acids is challenging due to the presence of polar functional groups inducing strong inter-molecular interactions, making the material very complex. In order to reduce this complexity, the molecular characterisation using gas chromatography normally needs a derivatisation step to simplify the structure and reduce inter and intra molecular interactions to render GC-amenable material; the employment of derivatisations targeting functional groups such as carboxylic acids, alcohols, amines and amides in the structure is usually performed to fulfill this goal.

At the beginning of our chemical characterisation in Chapter 2, trimethylsilylation, methylation, reduction and their combination were carried out on the bulk Pony Lake Fulvic Acid (PLFA). Among them trimethylsilylation was able to derivatise many compounds in the PLFA structure and appeared more efficient than other methods. Nevertheless, the low reaction yield revealed that only a sub-set of the total sample was being detected and analysed. This inspired the next step namely fractionation prior to derivatisation, the derivatisation of the fractions were performed separately afterwards. The amino acid analysis of PLFA in this chapter also revealed that nitrogen-containing structures in PLFA are not of peptide origin, this implied that they can be possibly heterocyclic structures such as pyrimidine or pyrrole.This was the initial step to recognise the dominant type of nitrogeneous

materials in PLFA. Further experiments in Chapters 3 and 4 enabled more detailed component-level analysis for these nitrogeneous building blocks.

In Chapter 3, the careful investigation of the fractionation process as well as individual fractions revealed interesting results. Firstly, using spectral analyses such as IR, NMR and Fluorescence, the fractions were studied and showed to be similar and comparable in structure. XPS also highlighted the fact that nitrogen-containing compounds are similarly present composed of amide-like structure, whereas primary amine and aromatic nitrogen are observed as minor contributors in all fractions. This was in agreement with the previous ^{15}N NMR study of PLFA, indicating the presence of around 40 % (~2/5 of all N) as peptide (amide) structure (Mao et al., 2007). Moreover, chemical analysis using TMAH-GC/MS showed that the majority of these compounds are protein biomarkers, for example, uracil (Pyrimidine-2,4 (1H,3H)-dione) can be produced from RNA, while pyrrolidones may be derived from proline-containing peptides. Pyrrolidine and pyrrolidine-diones are usually derived from amino acids bound to phenolic or quinone group. Indole may havebeen originated from tryptophan-containing peptide Pyridine, pyrazine and primary amide are structures formed by the incorporation of ammonia into NOM.

Secondly, the molecular analysis of fraction demonstrated overlaps of components in all four fractions, indicating the carryover of materials in them. This was strong evidence in support of formation of micelle in the aqueous solution of fulvic acids that was also supported by subsequent experiments. For example, the change of temperature or the addition of organic solvent into the aqueous solution of fulvic acids caused a decreases in the average molecular weight of the hydrophilic fractions which could only be explained by disintegration of ‘’micelle-like’’ components affected by these physical parameters. This means that these changes disrupted PLFA conformational arrangements stabilized by only weak hydrophobic bonds into smaller-size aggregates of greater conformational stability. These results confirms

that humic molecules in solution were organised in supramolecular associations of relatively small molecules loosely bound together by dispersive interactions and hydrogen bonds, and here PLFA specifically responded to chemical changes brought about by factors such as increasing the temperature as well as changing the ratio of water to acetonitrile The multi angle light scattering (MALS) analysis presented in Chapter 2 also indirectly revealed the possibility of micelle formation. The presence of micelle structures in Antarctic fulvic materials importantly supports the theory of supramolecular structures for Humic substances.

Moreover, the identification of various compounds after fractionation appeared easier and more reliable. Especially notable was the detection of large molecules such as tricyclic diterpenoides in the fractions using TMAH themochemolysis that was not detectable in unfractionated PLFA. Further, in contrast to the result obtained for unfractionated PLFA, almost all derivatisation methods employed for the fractions were successful in detection of fulvic acid building blocks.

The hydrolysis of the fractions also enabled the estimation of carbohydrate content and identification of the products releasable upon hydrolysis as a complementary attempt to the charaectrisation. Interestingly, some nitrogen-containing compounds such as amino acids and amino sugars were effortlessly attained and identified.

The work in Chapter 4 was dedicated to the characterisation of Vestfold Hills FAs using selected methods described in the preceding chapters and comparison of result with PLFA results to interpret similarities and differences.

The fulvic materials from three lakes named Mosel, Organic and Pendant were strategically selected based on the nitrogen content. MLFA and PNFA were nitrogen rich similar to PLFA, however, OLFA was low in nitrogen. Their characterisations using Trimethylsilylation showed that MLFA and PNFA reacted similarly to PLFA, however OLFA Trimethylsilylation appeared different due to high UCM and low detectable components.

Among nitrogen-rich MLFA, PNFA and PLFA, the MLFA showed to become derivatised easier and more effective (based on the quantity and quality of components identified) rather than two other nitrogen-rich FAs. It even showed unbroken dehydroabietic acid (DHAA) as a tricyclic diterpenoid that belongs to the family of resin acid using simple trimethylsilylation. On the other hand, in all of these four fulvic materials (PNFA, MLFA, OLFA and PLFA) effortless sugar detection was achieved using trimethylsilylation. However, detection and identification of nitrogen-containing components has been difficult in nitrogen-rich PLFA, PNFA and MLFA as well as nitrogen-poor OLFA. This implies that the core of refractory material in Antarctic fulvic acids may be nitrogen-rich.

The ability of TMAH thermochemolysis was also shown by the detection of nitrogenous structures such as pyrrole, pyrimidine and indole derivatives. In fact, majority of nitrogenous components detected in this study obtained using this characterisation. More importantly, the presence of dehydroabietic acid (DHAA) in both MLFA and PNFA highlights this derivatisation efficiency. Terpenoid acids (resin acids) such as DHAA are expected to be a found in terrestrial DOM, but its detection in microbially-derived fulvic material of Antarctic lakes is remarkable in terms of structural studies.

The fact that terpenoids similar to resin acids occur in a region with no higher order plants raises interesting questions about its common origin. Resin acid are typically found in fossil woods and sedimentary records in the areas exposed to terrestrial inputs. However, their thermal degraded products such DHAA were previously reported in biomass combustion aerosols (Standley and Simoneit, 1994) produced from coal or plant combustion. The possible origin of DHAA in Antarctica can, therefore, be related to the transformation of atmospheric particles containing terpenoid acids to the area. This implies that DHAA and its similar terrestrial-originated components could deposit from atmosphere to Antarctic lakes and subsequently enter to the aquatic DOM.

In conclusion, this thesis has reported the structural and functional characterisation of fulvic acids from Antarctic regions, McMurdo Sound Area and Vestfold Hills. The results indicated that these fulvic acids show some similarities to each other in that they are more aliphatic and are more immature than FAs from other environments. In fact, while fulvic material formed in various aquatic environments are exposed to many chemical and thermal alterations; Antarctica FAs appear to be untouched and fresh due to the cold climate and limited interference. The slight differences among Antarctic FAs can, however, be related to the sources of input to the lake waters, providing more nutrients and formation of refractory nitrogen-rich DOM. for example, Pendant Lake is located on the northern part of the Vestfold Hills where the lake system was believed to have been initially formed through isostatic glacial marine uplift while Mossel Lake is a fresh water lake with its NOM source mainly from snowmelt from nearby Sørsdal Glacier located to south of the lake providing ornithogenic, algal and lichen inputs due to the presence of nesting sites of sea birds. Organic Lake, however, is is a hypersaline, shallow meromictic lake with considerable ornithogenic and algal input. All of these differences in geographical and geochemical properties can play roles in creating different NOM profiling in the lakes. Detailed geochemical study of these lakes and their possible relations to fulvic material structure is not within the scope of this study; however, the current data provided ample background information about DOM from the area in which chemical exploration has been restricted to date. This can assist to further research on microbial DOM and their formation pathways in aquatic environments.

Appendix

Conference Abstracts

1- 17th Australian Organic Geochemistry Conference

2 – 5 December 2012, Macquarie University, Sydney, Australia

Abstract For Poster Presentation

A non-invasive technique for characterisation of volatile and semi-volatile constituents of fulvic acid by gas chromatography- mass spectrometry

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Humic substances are complex heterogeneous organic material and major components of marine and terrestrial natural organic matter (NOM). Fulvic acid is the smaller molecular weight fraction of humic substances found in water and sediment. It plays an important role in soil fertility and bioavailability because of its ability to attach metal ions and organic pollutants and transfer them through organic and aqueous phase.

Molecular -level characterisation of fulvic acid is difficult due to heterogeneity and complexity of the molecule. Traditionally, GC analysis of fulvic materials were based on hydrolysis followed by derivatisation to obtain volatile components prior to GC/MS analysis (Allard, 2006). Thermochemolysis followed by methylation has been recently considered as an alternative because of its ease and low cost (Lehtonen *et al*. 2004). However, application of aforementioned methods inevitably leads to a major alteration of the original structure which can negatively affect characterisation studies. Moreover, the pathways that starting material spends to form the products remains ambiguous and not fully understandable.

In the present study, we employed a non-invasive, one-step derivatisation techniques using only BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide) as a robust trimethylsilylation reagent to reach the reasonable level of volatility without interruption of original structure. Trimethylsilylation has been applied before on warkish peat fulvic acid with the similar methodology but with limited success in terms of component level analysis, the compounds identified were mostly of volatile organic acids and fully characterisation of all of the detected compounds was not considered.

Here, we conducted the TMS- derivatisation without using a solvent but with higher amount of BSTFA in a single and shorter step. A comprehensive component level characterisation was also performed which covers all of the volatile constituents to even larger molecules like disaccharides. Moreover because of non-invasive nature of the reaction, constituents detected are supposed to be related to the original structure of fulvic acid.

In order to investigate the applicability of the method on different fulvic acids, Five fulvic acid standards including Warkish Peat, Elliot Soil, Suwannee River, Nordic Aquatic

and Pony Lake were derivatised by BSTFA containing 1 % TMCS as a catalyst in a sealed glass vial with teflon-cap .The reaction mixture was exposed to 45 min sonication in 60˚C and subsequent 15 -hours heating at 80˚C in a conventional oven. Figure1 shows a typical total ion chromatogram of TMS-derivatised Antarctic fulvic acid obtained by current method.

As expected, compounds identified in five soil and aqueous fulvic acid standard belongs to different families of organic compounds but mainly consisted of fatty acids and carbohydrates. All of the monosaccharides which has already been reported in fulvic acids by other methods were also detected by our technique. Generally, the nature of monosaccharides seemed to be independent of the origin of fulvic acid; however, the difference between soil, peat and aqueous fulvic acids appeared to be quantitative rather than qualitative which is in agreement with quantitative ^{13}C NMR reported data of fulvic acid standards (Thorn *et al*., 1989). A broad range of long and short-chain fatty acids were also identified in all five fulvic acid standards mostly of straight-chain and branched mono and di- carboxylic acid. Additionally, aromatic acids like hydroxyl benzoic acid, di hydroxyl benzene, di hydroxyl benzoic acid and hydroxyl methoxy benzoic acid (vanillic acid) were observed in five fulvic acids, the last compound has been used previously as a model molecule in fulvic acid structural analysis and proved to be a part of Suwannee river fulvic acid (Saleh *et al.*,1989).

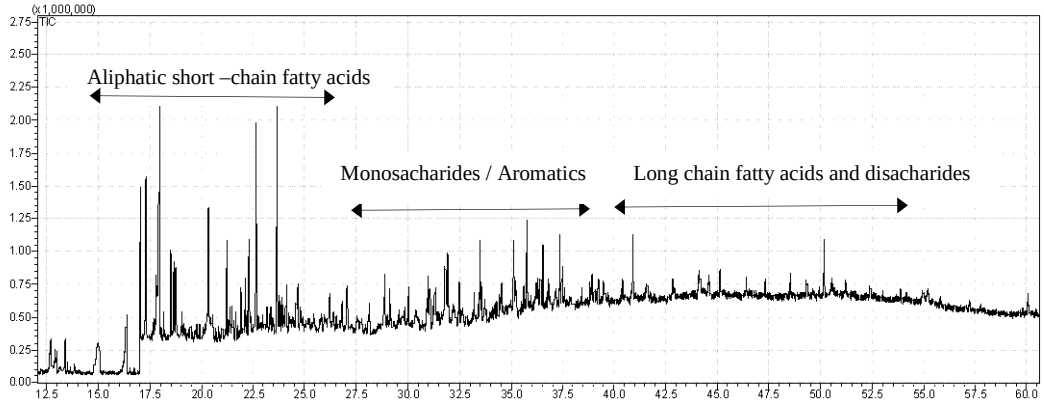

Figure1. Total ion chromatogram of TMS-derivatised Antarctic fulvic acid

Together these results suggest that current methodology not only enables detection of a wide range of fulvic acid constituents in a single step but can be potentially beneficial to molecular-level structural studies consistent with other approaches based on model molecular analysis.

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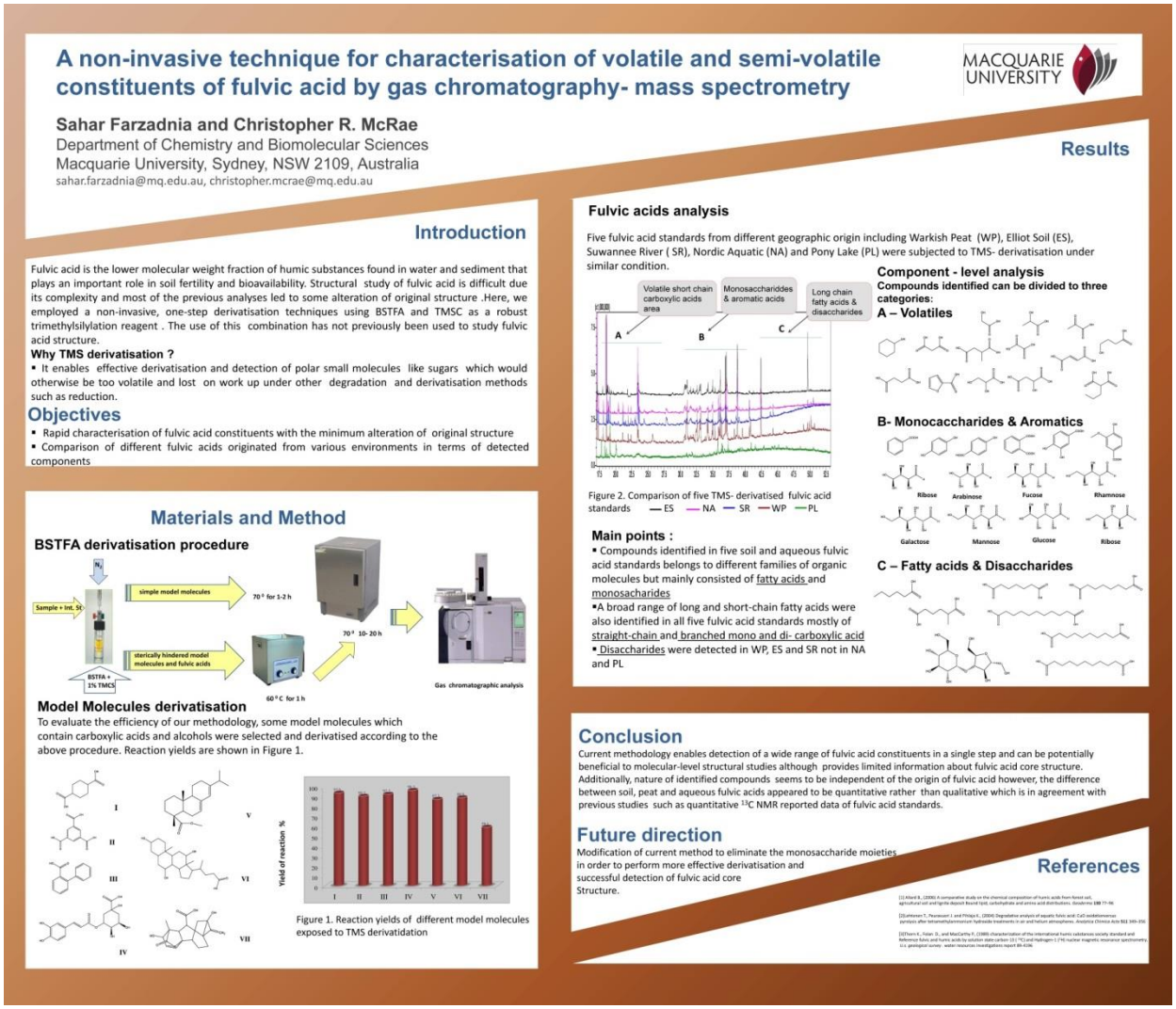

2- 20th RACI Research and Development Topics Conference in Analytical and Environmental Chemistry

11-14 December 2012, Deakin University, Geelong, Australia

Abstract For Poster Presentation

Characterisation of Antarctic aqueous fulvic acid by a new simplification approach prior to chromatographic analysis

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Fulvic acids are complicated mixture of organic compounds that accounts for about half of the Natural Organic Matter (NOM) in fresh water and 15-20 % of the NOM in marine waters. Because of the relatively small size of fulvic acids molecules they can readily enter plant roots, stems, and leaves. As they enter these plant parts they carry trace minerals from plant surfaces into plant tissues. Fulvic acid composition and structure are quite variable depending on the geographic location but it is believed consist of weak aliphatic and aromatic organic acids which are soluble in water at all pH conditions. Study of fulvic acid structure is difficult due to complexity and heterogeneity, so despite of many decades of research their chemical structures is still ill-defined. However, chemical characterisation of fulvic material from Antarctic region where cold climate and absence of higher order plants has the advantage of simpler mixture contribution, can assist with the interpretation of more complex fulvic acid found in non-Antarctic waters. Generally, microbial degradation of biomolecules like proteins, carbohydrates and lipids is the natural process responsible for the occurrence of humic substances including fulvic acids in the environment. Carbohydrates as an important family of biomolecules has been widely qualitatively and quantitatively studied in dissolved organic matter and especially in humic substances because of their significant contribution.

Based on the useful information available from previous studies and after preliminary identification of carbohydrate moieties in standard Antarctic fulvic acid by GC/MS, we exposed the sample under periodate oxidation to selectively oxidise and eliminate carbohydrates. The low-molecular weight oxidation products (mostly formaldehyde, formic and acetic acid) were simply separated from components of interest by solid phase extraction using a C18 cartridge. The extract obtained is a simplified fulvic material being investigated with both gas and liquid chromatography techniques.

Characterisation of Antarctic aqueous fulvic acid using a new simplification approach prior to chromatographic analysis

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Introduction

Fulvic acid and its role in the environment

Fulvic acids are complicated mixture of organic compounds derived from breakdown of biogenic matter. It is believed that fulvic acids are a mixture of weak aliphatic and aromatic organic acids soluble in water in all pH, so they :

- Influence water chemistry due to protonating and complexing characteristics
- Act as pH bufferes binding and tranporting metals
- Enter plant roots, stems, and leave to carry trace minerals

Why Antarctic fulvic acid ?

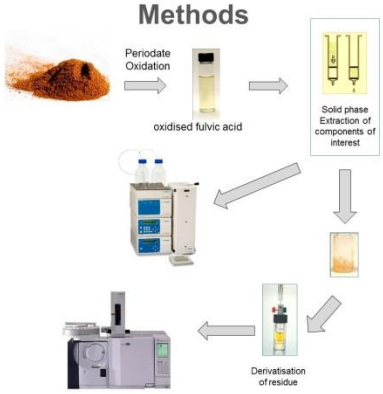
Study of fulvic acid structure is difficult due to its complexity and heterogeneity, so despite many decades of research their chemical structure is still ill-defined. However, chemical characterisation of fulvic material from Antarctic regions where cold climate and absence of higher order plants has the advantage of simpler and more defined contributions to the natural organic matter (NOM) can assist to build a general picture of aqueous NOM structure and function.

What are we doing ?

We are trying to develop a methods to simplify the fulvic acid structure prior to GC analysis, our main focus will be on the strategies which result in minimum alteration of the original structure thereby enabling effective characterisation .

Here, we employed periodate oxidation to eliminate carbohydrates in Antarctic fulvic acid, then derivatised fulvic acid was analysed by GC/MS. As a complementary investigation, HPLC-ELSD /UV analyses were also performed on underivatised fulvic material upon oxidation.

Methods



Results

Gas chromatographic analysis

A. Short- chain carboxylic acids
B. Carbohydrates
C. Limited no. of Aromatics and fatty acids

Less peaks assists with more accurate identification

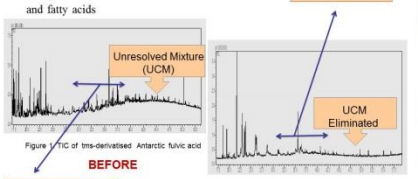


Figure 1. TIC of tms-derivatised Antarctic fulvic acid

BEFORE

Figure 2. TIC of oxidised & tms- derivatised Antarctic fulvic acid

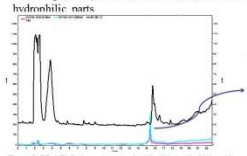
AFTER

Too many peaks due to carbohydrates

A. Short- chain carboxylic acids
B. Aromatic acids
C. Long-chain fatty acids

HPLC analysis

RP- HPLC (water/ acetonitrile gradient) with Evaporative light scattering (ELS) shows six fractions , but with minimum separation of hydrophilic parts



UV absorbance in 212, 230 nm confirms domination of aliphatic structure in hydrophobic fractions

Figure 3. RP- HPLC chromatogram of oxidised Antarctic fulvic acid with gradient program

Conclusions

- The main constituents of Antarctic fulvic acid recognised as aliphatic carboxylic acids, monosacharides , fatty acids and aromatic acids
- Periodate oxidation can effectively improve analysis of fulvic acid by simplifying gas chromatography profile
- Aromatic acids , straight chain and branched fatty acids identified in the absence of carbohyrdrates while they were not recognised before, oxidation due to peaks overlapping
- RP-HPLC analysis of sample exposed to periodate oxidation had limited success to separate all fractions.

Future direction

Development a NP- HPLC method to analyse oxidised fulvic acid will be useful to obtain more information on fulvic acid constituents which remains after periodate oxidation.

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3- Gordon Research Conference: Organic Geochemistry

3-8 Aug 2014 Holderness, NH United States

Abstract For Poster Presentation

Molecular characterisation of nitrogen-rich dissolved organic matter from Antarctica using gas chromatography/ mass spectrometry

Study of the chemical structure of dissolved organic matter (DOM) has always been of great interest because of its contribution to global carbon cycle and its recognition as source marker [1]. Nitrogen- rich DOM has proved to be extremely refractory due to the formation of non-hydrolysable amide structures; this makes its characterisation a challenging task even when employing sophisticated analytical techniques [3]. The majority of studies, therefore, were based on bulk analysis using spectroscopic methods [4].

In this study, we have extensively examined DOM samples from two different areas in Antarctica where very limited input from anthropogenic activity and sole microbial origin allow a more homogeneous material. Pony Lake fulvic acid (PLFA) and Pendant Lake hydrophobic acid (PNFA), both with high nitrogen content were fractionated and analysed by several gas chromatographic – based methods. These methods complement each other to cover a broad range of molecules present in complex fulvic acids.

Our findings indicate the presence of various heterocyclic nitrogenous material, many of them are GC amenable without the need for depolymerisation using hydrolysis. This could be indicative of that these hydrophilic molecules exist as discrete moieties, apparently not encapsulated in the core structure. Furthermore, it may be concluded that their association to the structure is non-bonded and governed by hydrophobic effect or micelle formation.

In summary, it seems that fractionation combined with gas chromatography is able to provide us with additional information regarding molecular-level analysis of Antarctic fulvic acid, this undisclosed microbial end-member DOM.

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[3] D'Andrilli et al. (2013) Organic Geochemistry 65,19–28.
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Molecular characterisation of nitrogen-rich dissolved organic matter from Antarctica using gas chromatography/mass spectrometry

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BACKGROUND

Study of the chemical structure of dissolved organic matter (DOM) has always been of great interest because of its contribution to global carbon cycle and its recognition as source marker [1]. Nitrogen-rich DOM has proved to be extremely refractory due to the non-hydrolysable amide structures [2]; this makes its characterisation a challenging task even using sophisticated analytical techniques [3]. However, GC/MS can be a good choice when it comes to molecular level analysis, enabling better separation and detection.

AIMS

- Molecular level characterisation of nitrogenous compounds in fractionated Antarctic DOM
- Comparison of structure and constituents of two nitrogen- rich Antarctic DOM of different origin

MATERIALS AND METHODS

Site and sample descriptions

DOM samples are from two Antarctic Lakes

- Pony Lake fulvic acid obtained from IHSS (International Humic Substances Society) isolated from DOM samples [4] of Pony Lake on Ross Island, Cape Royds, Antarctica.
- Pendant Lake hydrophobic acid (fulvic acid) from Pendant Lake Vestfold Hills, Antarctica.

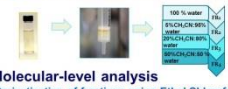
Isolation

- Samples acidified (pH=2)
- passed through XAD-8 column
- desorbed by NaOH (Pony Lake fulvic acid) or 75% CH_3CN:25% water (Pendant Lake fulvic acid)
- neutralisation & freeze-drying

Fig. 1. Location of Pony and Pendant Lakes in Antarctica

Fractionation of isolated fulvic acids

Using RP- SPE cartridge and water:CH_3CN elution



Molecular-level analysis

- **Derivatisation of fractions using Ethyl Chloroformate (ECF) and BSTFA/TMCS combined with GC/MS**

Amines, carboxylic $\xrightarrow{ECF}$ carbamate , ester

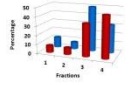
Amines, carboxylic $\xrightarrow{BSTFA/TMC}$ trimethylsilylester

- **Methanolysis of fractions followed by Acetylation combined with GC/MS**

Glycosidic bonds cleavage $\xrightarrow{Ac_2O}$ Acetate

RESULTS

Fractionation of Pony Lake and Pendant Lake fulvic acid by RP-SPE



The mass distribution of the fractions isolated by RP-SPE of Pony Lake and Pendant Lake fulvic acids show similar trends with both showing the majority of material in fractions 3 and 4.

Fig. 2. Fractions distribution in Pony Lake and Pendant Lake fulvic acids obtained as follows: Fr 1 (100% H_2O), Fr 2 (5 % CH_3CN), Fr 3 (25% CH_3CN), Fr 4 (50 % CH_3CN)

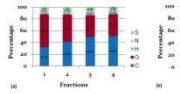


Fig. 3. Elemental composition of four fractions of (a) Pony Lake and (b) Pendant Lake fulvic acids isolated by RP-SPE

The nitrogen content of fractions of Pendant Lake fulvic acids ranges from 4.0 to 5.0%, while for Pony Lake it appears higher, ranging from 5.0 to 7.0%

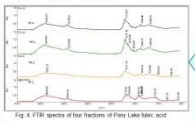


Fig. 4. FTIR spectra of four fractions of Pony Lake fulvic acid

Fraction 1 contains strong 1°, 2° amide and methyl absorbance along with strong sugar C-O peaks, well-matched with the presence of chitin (polysaccharide composed of (β1→4) linked N-acetyl-D-glucosamine units) widely found in marine DOM. Fraction 2 is similar to fraction 1. Fraction 3 and 4 lack this kind of nitrogenous structure

Molecular-level analysis of nitrogenous components in Pony Lake and Pendant Lake Fulvic acid fractions

A) Detection of heterocyclic nitrogenous compounds in Pony Lake fulvic fractions using ethyl chloroformate-GC/MS

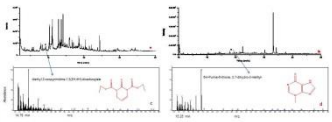


Fig. 5. TIC of (a) fraction 1 and (b) fraction 3 derivatised by chloroformate and mass spectra of detected compounds (c) and (d)

B) Detection of amino sugar and amino acids in Pony Lake fulvic fractions using methanolysis-acetylation-GC/MS

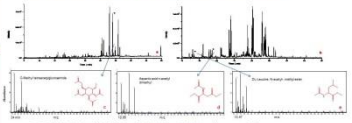


Fig. 6. TIC of (a) fraction 4 and (b) fraction 1 subjected to methanolisis and monosaccharide analysis, mass spectra of nitrogenous compounds detected (c), (d) and (e)

A) Detection of heterocyclic nitrogenous compounds in Pendant fulvic acid fractions using TMS -GC/MS

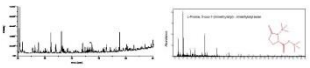


Fig. 7. TIC of fraction 3 derivatised by BSTFA / TMCS and mass spectrum of derivatised nitrogen-containing compound detected at 23.76 min

B) Detection of amino sugar and amino acids in Pendant Lake fulvic acid fractions using methanolysis-acetylation-GC/MS

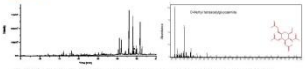


Fig. 8. TIC of fraction 2 subjected to methanolisis and monosaccharide analysis and mass spectrum of aminosugar detected at 33.94 min

- Fulvic acid from Pony Lake and Pendant Lake contain heterocyclic nitrogen-containing group that can be detected using direct derivatisation combined with GC/MS, they are mostly pyrimidine, purine and pyrrolidone structures
- There are other sources of nitrogen in this fulvic acid such as amino acids and amino sugars. These structure need acid catalysed methanolysis to be detected by GC and their presence were confirmed in both fulvic acids after additional acetylation of released monomers

CONCLUSION

- Gas chromatography/mass spectrometry with the aid of appropriate derivatisation techniques offer a promising technique for detection of nitrogen-containing compounds in Antarctic DOM.
- The presence of various heterocyclic nitrogenous material, many of which are GC amenable without the need for depolymerisation using methanolysis could be indicative of that these hydrophilic molecules exist as discrete moieties, apparently not encapsulated in the core structure.
- Nitrogen-rich DOM originated from different Antarctic waters (Pony Lake and Pendant Lake) show similar composition in their fractions, although their GC amenable structure can be quite different.

FUTURE DIRECTION

The study of methanolysis residue can provide useful information about the non-hydrolysed material especially nitrogen functionalities in fulvic acid and generally in marine DOM. Extensive NMR study such as 2D NMR can be helpful in characterising the nitrogen chemical environment in the structure of these fulvic acids.

Other non-GC based techniques such as LC/MS techniques are worthwhile to gain detailed information about the nature of this non-hydrolysed materials and complete the current conclusions.

REFERENCES

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