Multi-dimensional and multi-modal separation of dissolved organic matter.

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List of abbreviations:

Abs: absorbance

APCI: atmospheric pressure chemical ionisation

BPC: base peak chromatogram

BSTFA: bis-trimethylsilyl trifluoro acetamide

C_8: octyl bonded-silica

C_{18}: octadecyl bonded-silica

CE: capillary electrophoresis

CFC: chlorofluorocarbon

^{13}C-NMR: carbon NMR

CRAM: carboxylic rich alicyclic molecules

DAD: diode array detector

DCM: dichloromethane

DIC: dissolved inorganic carbon

DOC: dissolved organic carbon

DOM: dissolved organic matter

DON: dissolved organic nitrogen

DOP: dissolved organic phosphorous

ESI: electrospray ionisation

EtOOAc: ethyl acetate

FA: formic acid

FL: fluorescence

FID: flame ionisation detector

FIMS: field ionisation mass spectrometry

FTIR: Fourier transform infrared

FTICR-MS: Fourier transform ion cyclotron resonance mass spectrometry

GC: gas chromatography

GC-MS(/MS): gas chromatography mass spectrometry, (tandem-MS)

GF-F: glass fibre filter

HCT: high capacity trap

HILIC: hydrophilic interaction liquid chromatography

HPLC: high performance liquid chromatography

HR-MS: high resolution mass spectrometry

HR-NMR: high resolution nuclear magnetic resonance

HPCCC: high-performance counter current chromatography

HSCCC: high-speed counter current chromatography

IC: ion chromatography

IEC: ion-exchange chromatography

IRMS: isotope-ratio mass spectrometry

LC: liquid chromatography

LDOM: labile dissolved organic matter

MCP: microbial carbon pump

MDLT: molecules derived from linear terpenoids

MeCN: acetonitrile

MeOH: methanol

MS: mass spectrometry

MS-IRMS: mass spectrometry-isotope ratio mass spectrometry

NDIR: non-dispersive infrared

^{15}N-NMR: nitrogen NMR

NMR: nuclear magnetic resonance

NP-HPLC: normal-phase high performance liquid chromatography

OMP: outer membrane protein

^{31}P-NMR: phosphorous NMR

PAD: pulsed amperometric detection

POM: particulate organic matter

PPL: polypropylene

PPM: parts per million

PS-DVB: polystyrene divinylbenzene

PTFE: polytetrafluoroethylene

RI: refractive index

RP-HPLC: reversed-phase high performance liquid chromatography

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEC: size exclusion chromatography

SPE: solid phase extraction

T(gT): tonnes (gigatonnes)

T_{crit}: critical temperature

THF: tetrahydrofuran

TIC: total ion chromatogram

TOC: total organic carbon

TMAH: tetramethylammonium hydroxide

TMAAc: tetramethylammonium acetate

TOF: time of flight

UF: ultrafiltration

UV: ultraviolet

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A list of Publications and Conference Presentations

Publications:

- S. Sandron, E.P. Nesterenko, N.W. Davies, B.P. Kelleher, and B. Paull, Molecular characterisation of marine dissolved organic matter using fractionation by size exclusion chromatography and reversed-phase LC-MS analysis, Analytica Chemica Acta, *submitted*.
- S. Sandron, P.N. Nesterenko, B.P. Kelleher, M.V. McCaul, and B. Paull, Characterisation of dissolved organic matter (DOM) by high performance counter current chromatography (HPCCC) and reversed-phase chromatography-high resolution tandem mass spectrometry (RP-MS/MS), Journal of Separation Science, *submitted*.
- S. Sandron, R. Wilson, P.N. Nesterenko, B.P. Kelleher, M.V. McCaul, and B. Paull, Investigation into dissolved combined neutral sugars and their microbial conversion in naturally and artificially produced dissolved organic matter using ion chromatography with pulsed amperometric detection and reversed-phase liquid-chromatography-high resolution mass spectrometry, Analytical Methods, *submitted*.

Poster Presentations:

- S. Sandron, B.P. Kelleher, M.V. McCaul, E.P. Nesterenko, B. Paull

Application of multi-dimensional chromatography to the separation and identification of Dissolved Organic Matter components in seawater and freshwater.

ISC, 28th International Symposium on Chromatography, Valencia, Sept 2010.

- S. Sandron, B.P. Kelleher, M.V. McCaul, E.P. Nesterenko, B. Paull

Application of multi-dimensional chromatography to the separation and identification of the components of freshwater and seawater derived Dissolved Organic Matter (DOM).

IICS, 22nd International Ion Chromatography Symposium, Cincinnati, Sept 2010.

- S. Sandron, B.P. Kelleher, M.V. McCaul, E.P. Nesterenko, B. Paull

Application of two-dimensional chromatographic approaches to the separation and identification of components within Dissolved Organic Matter in freshwater and seawater samples.

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- S. Sandron, B.P. Kelleher, M.V. McCaul, E.P. Nesterenko, B. Paull

Application of multi-dimensional chromatography to the separation and identification of the components of Dissolved Organic Matter (DOM)

HPLC 2011, 36th International Symposium on High Performance Liquid Chromatography and related techniques, Budapest, June 2011.

- S. Sandron, B.P. Kelleher, M.V. McCaul, E.P. Nesterenko, B. Paull

Application of multi-dimensional chromatography to the separation and identification of the components of Dissolved Organic Matter (DOM)

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<u>Oral Presentations:</u>

- S. Sandron, B.P. Kelleher, U. Daņiļēvičs, E.P. Nesterenko, B. O'Connor, M. Fox M, D. Connolly, P.N. Nesterenko, and B. Paull

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- S. Sandron, B.P. Kelleher, U. Daņiļēvičs, E.P. Nesterenko, B. O'Connor, R. Wilson, N.W. Davies, D. Connolly, P.N. Nesterenko, and B. Paull

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Application of multi-dimensional chromatography to the separation and identification of the components of dissolved organic matter (DOM).

R&D topics, Geelong, VIC, Australia, 12-14 December 2012.

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HPLC 2013, 38th International Symposium on High Performance Liquid Chromatography and related techniques, Amsterdam, June 2013.

Other achievements

- Research Cruise on the Celtic Voyager, Irish Sea, April 2010
- Eurofleet ship-based training course, Cork, August 2010
- Research Cruise on the Celtic Explorer, Atlantic Ocean, May 2011
- Honorary Research associate, ACROSS, University of Tasmania, October 2011-February 2012
- Csaba Horváth Award Nominee at HPLC 2012, Anaheim, California
- Honorary Research associate, ACROSS, University of Tasmania, September 2012-February 2013

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Abstract:

Dissolved organic matter (DOM) in seawater and freshwater represents a carbon reservoir comparable to atmospheric CO_2 (respectively 624 and 750 gT). Despite being tightly related, as CO_2 represents a primary product of DOM mineralization, DOM still remains largely uncharacterized, containing various classes of compounds in concentrations ranging from picomolar to micromolar. Due to the complexity of DOM, conventional liquid and gas chromatographic analysis are inhibited due to extensive co-elution. In order to overcome the analytical challenges presented by DOM, this project proposes alternative multi-dimensional chromatographic approaches to fractionate and isolate single compounds from this organic pool.

Firstly, size exclusion chromatography (SEC) was coupled to reversed-phase liquid chromatography tandem mass spectrometry (RP-LC-MS/MS) to fractionate DOM constituents in terms of size and polarity. Seven fractions were collected from the first dimension, allowing the isolation of up to 142 single compounds from the second.

To fractionate DOM according to the polarity of its components, high-performance counter current chromatography (HPCCC) was chosen as an alternative first chromatographic dimension, leading to the separation of five fractions which were also further processed by RP-LC-MS/MS. High resolution MS (HR-MS) generated exact mass losses that occurred within the isolated compounds, representing a further step towards the identification of DOM constituents.Lastly, ion-exchange chromatography with amperometric detection (IEC-PAD), was applied to demonstrate the presence of analogous classes of compounds within naturally occurring and artificially produced DOM samples (ADOM), showing that the bulk of DOM is probably of microbial origin and providing the separation of various classes of neutral sugars within DOM.

Chapter 1: Chromatographic approaches for the separation and characterisation of dissolved organic matter

1.1 Dissolved organic matter

1.1.1 Definitions and description

The global water system comprises a seawater and freshwater reservoir, covering an area equal to 71% of Earth's surface. Of this system, only 2.5% is freshwater, which includes glaciers and groundwater (rivers and lakes). The remaining 97.5% is saltwater (oceans).

The organic matter held within this water system can be subgrouped into dissolved and particulate organic matter (DOM and POM). The difference between these materials is mainly methodological and was developed in the 1970s [1], when glass fibre filters (GF-F) were first used for the isolation of DOM. Such filters had a minimal pore size of 0.45 μm and this became the accepted limit for what could be considered as "dissolved": all the compounds within DOM therefore pass through these filters, while those classified as POM do not.

The total DOM pool includes three categories of compounds: dissolved organic carbon (DOC), nitrogen (DON) and phosphorous (DOP). Amongst these, DOC is present in highest percentage (97%) [1], and it is often difficult to distinguish it from the other two organic pools (DON, DOP), as many carbon-based compounds also include nitrogen and phosphorous (e.g. proteins, phospholipids and ribonucleic acids). The carbon, nitrogen, and phosphorous content within DOM are source-dependent. Indeed, a sample from the shoreline may well have a different carbon content and composition to that sourced from the open ocean.

1.1.2 Sources of dissolved organic matter

Together with DOM and POM, the oceans also contain a vast amount of dissolved gases. Their concentration is dependent on the interaction between water and the atmosphere. Gases dissolved in the ocean can be distinguished into two categories: conservative and non-conservative [1-2]. Non-conservative gases are employed and transformed during biological processes, whereas conservative gases do not undergo any transformation and remain in the environment as they are. Argon and chlorofluorocarbons (CFCs) are examples of conservative gases, while $O_{2(g)}$ and $CO_{2(g)}$ are the two main non-conservative gases dissolved within the ocean. Gas solubility is affected by both temperature and salinity; solubility increases with a decrease in temperature and/or salinity in the case of seawater.

From anthropological activity and biological respiration, $CO_{2(g)}$ is released into the atmosphere and part of this is sequestered by the oceans, where it can exist in the following forms: aqueous ($CO_{2(aq)}$), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}). The sum of these three species is defined as total dissolved inorganic carbon (DIC) [3].

Henry's law states that the amount of gas dissolved in a volume of liquid at constant temperature is proportional to the partial pressure of the gas in equilibrium with the liquid [3]. According to this, the CO_2 sequestered by the ocean is in equilibrium with atmospheric $CO_{2(g)}$. When $CO_{2(g)}$ reacts with seawater, it immediately forms carbonic acid (H_2CO_3), which dissociates to form HCO_3^- and CO_3^{2-} according to the following equilibria reactions (Eq. 1-2):

$$CO_{2(g)} + H_2O \rightleftharpoons HCO_3^- + H^+ \qquad pK_1 = 5.94 \ (Eq.\ 1)$$

$$HCO_3^- + H^+ \rightleftharpoons CO_3^{2-} + 2H^+ \qquad pK_2 = 9.13 \ (Eq.\ 2)$$

Where pK_1 and pK_2 are calculated at temperature = 15 ^{o}C, pressure = 1 atm, salinity = 35). At typical surface seawater pH (8.2), HCO_3^- is the predominant form, accounting for 89% of the total DIC pool, whereas CO_3^{2-} accounts for 10.5% and $CO_{2(aq)}$ the remaining 0.5%. [3].

HCO_3^- and CO_3^{2-} act as a buffering system against processes that can change the pH of seawater. Since acids (i.e. HCl from volcanic activity) combine with CO_3^{2-} to form HCO_3^-, such a system is therefore capable of maintaining seawater pH at 8.2. However, it is estimated that in the last century the surface ocean pH decreased from 8.2 to 8.1 as a consequence of industrialisation [4]. In fact, the rise of $CO_{2(g)}$ through anthropogenic processes affects the seawater buffering system (Eq. 1-2). If an excess of $CO_{2(g)}$ is dissolved in the water, an enhanced production of acidic species will occur to levels beyond the buffering capacity of the carbonate system.

As free Ca^{2+} ions can sequester CO_3^{2-} to produce $CaCO_3$ (calcification), ocean acidification endangers corals and other creatures, such as several phytoplankton species that use $CaCO_3$ as construction material for their cell walls. An excess of acid in the ocean reduces the availability of CO_3^{2-} which is used by organisms to build their shells or skeletons. These will become deformed, weaker and prone to external attacks from viruses and bacteria. An example of these species is the coccolithophorid (single celled algae) *Emiliana huxleyi*, which has a shell structure made from $CaCO_3$ disks (coccoliths), which provide protection to the cell.

Dissolved $CO_{2(g)}$ can also undergo fixation with $O_{2(g)}$, with concentrations varying throughout the water column. This is influenced by biological activity:

$$nCO_2 + nH_2O \xrightleftharpoons{hv} (CH_2O)_n + nO_2 \qquad (Eq.\ 3)$$

The equilibrium (Eq. 3) is shifted to the right during photosynthesis, in order to produce organic material and $O_{2(g)}$, while during respiration this reaction proceeds to the left. This process is restricted to shallow depths, where the rate of photosynthesis exceeds respiration. Below this zone, $O_{2(g)}$ is the only reactive specie and even though its concentration decreases with depth, developing an $O_{2(g)}$ minimum, it is involved in the biochemical oxidation processes.

Atmospheric CO_2 is used by plants and microbes in photo and chemo-synthesis, and eventually some can be taken up by the ocean through the aquifer system, which resides below the ocean in the basalt pores [5]. This sub-seafloor aquifer hosts many bacterial communities which are able to sequester carbon in order to synthesise DOM. [5]. McCarty *et al.* estimated that this can be a source of ancient DOM, with an age ranging from approximately 11000 to 14000 years [6]. However, the difficulty in obtaining samples from the bottom of the ocean, presents a major challenge to prove this theory [5].

Further sources of DOM to the oceans are rivers and lakes (250 T per year) [1; 7-8]. The composition of riverine and lacustrine DOM can differ from marine DOM, and is usually enriched by materials of terrestrial origin such as lignin and its breakdown products [9]. Terrestrial compounds were found at levels 2-6 times more concentrated in the Atlantic Ocean than present in the Pacific, demonstrating that the Atlantic Ocean's lower surface area has resulted in a more concentrated discharge of riverine and terrestrially-derived DOM, when compared to the Pacific [10]. Heterogeneous, high molecular weight, terrestrially derived polymeric compounds within DOM have been proved to be susceptible to both microbial and photochemical degradation [11]. Solar

radiation, especially in the UV range (100-400 nm), promotes transformation, and in particular degradation of structure, molecular weight, and optical properties of humic substances. An example for these kind of transformations is the photo-induced radical opening of double bonds, functional groups which are widely present on these kind of compounds [9; 12-13].

The transport of riverine DOM to the coastal ocean is largely conservative; the molecules characterising this complex mixture maintain their structure and are very slowly changed or metabolised by bacteria or other microbial forms before entering the oceanic environment [1]. However, it is possible to account for several losses of specific components due to flocculation (particulates aggregate together) in low salinity environments. In fact, in low salinity environments molecules are more available to interact with each other with the resulting formation of clusters. Conversely, at high salinity levels, the presence of salts prevents such interaction and flocculation is less observed [14].

1.1.3 The dissolved organic matter cycle

Dissolved organic substrates are important intermediates in the rapid cycling of bioactive elements within the ocean [15], in particular within the microbial carbon pump (MCP). The latter can be defined as a microbial system able to metabolise and transform labile forms of DOM (LDOM). The carbon demand from the MCP is about the 20-40% of the typical concentrations of DOM in seawater and freshwater [15-16] (Figure 1.1).

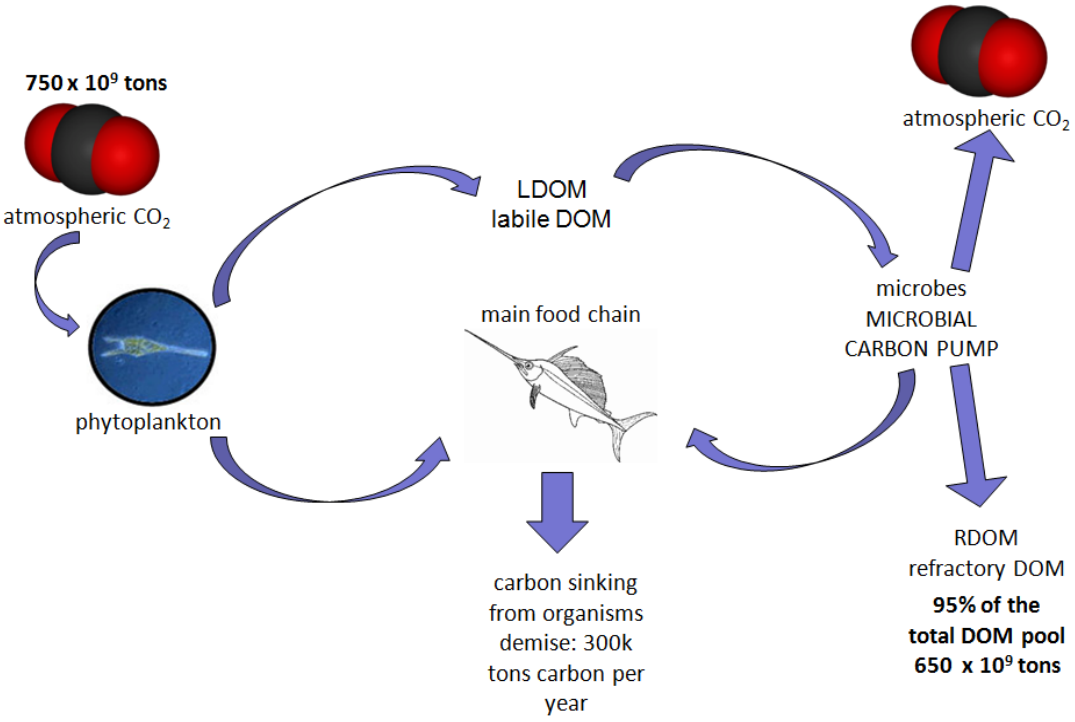

Figure 1.1: *The DOM cycle [15]. This Figure demonstrates the main source and transformations of DOM.*

The only means of supplying such a large mass of carbon-containing compounds is in the rapid cycling of DOM released from a variety of processes, including phytoplankton photosynthesis, viral lysis, and protozoan and zooplankton grazing [15; 17-18]. These classes of compounds, contributing to LDOM, are metabolised by the various classes of bacteria present in the MCP (Figure 1.1) [15], transforming them into refractory moieties (refractory DOM), which sink along the water column without further transformation or digestion [15] (Figure 1.1). Other sources of refractory DOM are materials delivered to seawater or freshwaters from oil seepages or wildfires [15].

The primary production of the MCP is 50 gT of carbon per year. Its labile and refractory products contribute to DOM by at least 10-20 gT of carbon per year [15]. The in-depth mechanisms for the formation of DOM within the MCP are poorly understood, but it is possible that nonspecific enzymatic transformations contribute to the formation of this material [1; 15; 19]. However,

it is known that such activity is also related to pollution, as in highly contaminated waters the MCP activity, and therefore the transformation of LDOM, is much lower, with a consequent decreased production of refractory DOM [20].

Considering the ages of DOM in the deep Atlantic and Pacific (4000 and 6000 years respectively), it has been estimated that 20% of deep water DOM is removed during each mixing cycle (1000 years) of the deep ocean [16; 21-22]. The slow removal of refractory DOM during deep-water circulation has been shown [10], but the mechanism is still unknown. It is estimated that refractory DOM is removed during its residence in surface waters [10]. Its radiocarbon age indicates that, on average, refractory DOM passes through surface waters several times before being removed. At such low depth, some forms of refractory DOM are susceptible to photochemical transformations that can directly remove carbon. For this reason, the spectral properties of seawater and freshwater are affected by DOM absorbance, which reduces light permeability [8; 23-24]. Furthermore, since DOM has a different composition and concentration according to its source and photodegradation history [14; 25], dissolved organic molecules can provide detailed information about the origins of the parent waters [26].

It is clear, therefore, that information on the many thousands of differing organic molecules dissolved in seawater [15] can provide a better understanding of where that water has been and what has happened within it over time.

1.1.4 Dissolved organic matter analysis: complexity and analytical challenge

DOM contains classes of compounds ranging from high to low polarity. In particular, the following functional groups can be found in different proportions within this mixture: substituted alkyl carbons, unsaturated carbons, amides, carboxylic groups, aldehydes and ketones, amino groups and phosphate esters [1; 27].

Nuclear magnetic resonance (NMR) analysis of a seawater DOM sample obtained by Hertkorn *et al.*, showed the following prominent features: aliphatic C-H and C-C bonds, C-N carbon linkages, aliphatic C-O linkages typical of alcohols, esters, ethers and anomeric carbons, aromatic and olefinic carbon linkages, carbonyl groups of amides, carboxylic acids, esters and ketones, with less significant phenol peaks coming from tannin and lignin-like materials [28]. This data is further supported by high resolution mass spectrometry (HR-MS), which highlights the presence of peaks differing by 14 m/z, with clusters, in a typical Gaussian distribution, occurring at about 1300 m/z. The low molecular weight ions (less than 300 m/z) show mostly odd masses, hence with zero or two nitrogens per molecule [28-29].

The aforementioned functional groups can be found within the following major classes of compounds: amino acids, proteins, peptides, sugars, amino-sugars, carboxylic rich alicyclic molecules (CRAM), materials derived from linear terpenoids (MDLT), lipids, phospholipids, DNA, RNA, and sterols [1; 27-29]. Each of these classes of compounds will be discussed in more depth in the following sections.

1.1.4.1 Sugars

A major fraction of characterised DOM is comprised of carbohydrates, which are defined as sugars released after the hydrolysis of polysaccharides [30]. Their presence in seawater and freshwater is due to phytoplankton photosynthetic activity, microbial degradation and cellular lysis. Carbohydrates represent a major component within LDOM pool and mainly include neutral and amino sugars. Amino sugars play a key role in metabolic pathways, and are an essential component of bacterial cell walls, whereas neutral carbohydrates are the main constituents of plant cells. Neutral sugars account for 2-6% of the DOC in surface water and 0.3-0.9% of the DOC in deep water [1]. In particular, fucose, rhamnose, arabinose, galactose, glucose, mannose and xylose have been commonly measured in seawater using anion exchange chromatography with pulsed amperometric detection (IEC-PAD) [31-33]. Typical concentrations range from 200 to 800 n*M* on surface seawaters (above 200 m depth), and from 20 to 170 n*M* in deep waters (below 200 m depth) [1].

Amino sugars account for 0.4-0.6% of the DOC in surface water and 0.04-0.07% of the DOC in deep water [1]. IEC-PAD is the most popular analytical approach, being able to identify glucosamine, galactosamine and muramic acid as prominent forms [32; 34]. Amino sugars can also be found in polymeric form. For example, acetyl amino sugars, can be found in complex structures such as chitin and peptidoglycan, major constituents of plant and bacterial cell walls, respectively.

1.1.4.2 Carboxylic rich alicyclic molecules and molecules derived from linear terpenoids

CRAM have been found to be a major constituent of DOM [28-29]. This class of materials is defined as multiple, fused non-aromatic sterol- and hopanoid-like structures, with a carboxylic to aliphatic ratio of approximately 1 to 2, and up to 1 to 7 [28]. CRAM are rich in carboxylic groups, double bonds and have a low hydrogen content. The complexity of this class of compounds also arises from the fact that several molecules belonging to CRAM are structural isomers, and this property can be only confirmed by NMR data (Figure 1.2) [28-29; 35].

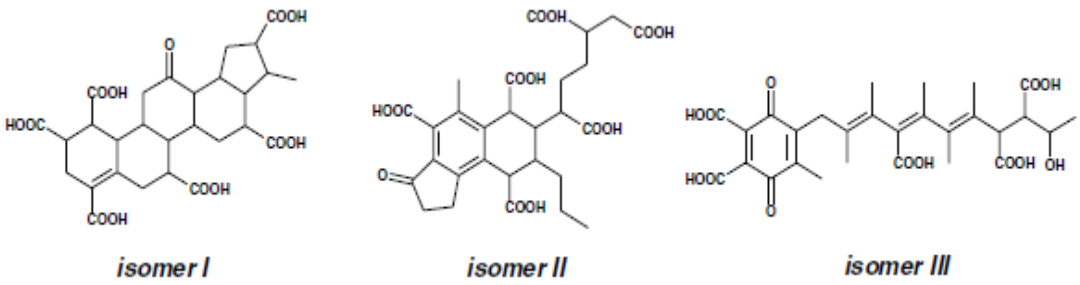

Figure 1.2: *Example for three possible CRAM isomers with molecular formula $C_{28}H_{32}O_{13}$. Structural information were gained from multi-dimensional NMR experiments performed by Hertkorn et al. in 2006 [28].*

Other complex structures such as terpenoids are also major constituents of DOM [35]. Such molecules are classified by the number of five carbon isoprene units within their structures and include more than 30000 identified compounds [36]. Their fate is poorly understood, but they are thought to produce several transformation derivatives, which are defined as MDLT. Most of them are highly oxidised sterol-type cyclic structures [35] containing a high degree of unsaturated and conjugated double bond systems, branched methyl groups, and quaternary aliphatic carbon structures [37].

1.1.4.3 Amino acids and proteins

Dissolved amino acids constitute a significant portion of DON [38], and are found in free and combined forms (proteins and peptides, proteins linked to sugars, and amino acids adsorbed to humic and fulvic acids). Free amino acids are present at low concentrations (0.04-1.06 μ*M*) and are an important source of nitrogen for autotrophic and heterotrophic organisms. Combined amino acids (i.e. amino sugars or proteins/peptides) are more concentrated, and their uptake seems to be five times higher than that of free forms [38].

The amino acid alanine is the most abundant free amino acid, followed by leucine, glycine and serine [38-39]. Approximately 60% of combined amino acids are in the form of peptides with a molecular size below 1000 Da. The highest concentration of these compounds is observed in spring time, during periods of phytoplankton blooms. In fact, high levels of such low molecular weight compounds within DOM are usually found in areas with high phytoplankton productivity, and it is thought that phytoplankton may break down proteins into smaller peptides derived from the autolysis of organisms [38; 40].

Analytical techniques such as Edman degradation in combination with MS, has permitted the sequencing of proteins such as Porins, which are outer membrane channel proteins of gram-negative bacteria [41], and outer membrane protein A (OMP A) homologues [42]. The latter, being also characterised by a beta-barrel structure, are known to be resistant to enzymatic digestion [43-44]. Further approaches employing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) coupled with MS detection [44-45] provided an increase in the number of protein classes to be sequenced.

Two main classes were found to be prominent: membrane and envelope proteins, and enzymes with conserved sequences.

SDS-PAGE was also applied in a two-dimensional capillary electrophoresis (CE) approach [42; 46], allowing the identification of a further 30 proteins. In particular, two 34 and 39 kDa classes of glycoproteins were classified as isoforms, with the same amino-acid sequence, underlining a further presence of isomers in the DOM pool [28-29]. The glycoforms of the 39 kDa protein were identified as low molecular weight alkaline phosphatase, hydrolase enzymes belonging to the Pseudomonas group, a family of aerobic bacteria which are involved in the removal of phosphate groups from proteins or nucleic acids. Such enzymes play a key role in cellular metabolic pathways and can potentially be targeted as biomarkers to assess the MCP variations within different environmental conditions (i.e. pollution or seasonal change) [42].

1.1.4.4 Lignin

A further component of DOM is lignin. This biopolymer is one of the most abundant on Earth, mainly present in plant cell walls, allowing for the transport of nutrients and water and providing the necessary strength for plants to grow [47-48]. Lignin is characterised by an aromatic structure derived from the radical-radical coupling of monolignols and other monomers.

The structural complexity of lignin and the difficulty in understanding how these monomers are linked, requires the use of MS sequencing experiments. Using such an approach, 134 species of lignin trimers to hexamers were identified, with 36 of them sequenced [47]. All of those identified were produced from the combination of compounds derived from coniferyl (Figure 1.3a) and sinapyl alcohol (Figure 1.3b), which are the most common monomers in

angiosperms (flowering and seed producing plants) and *p*-coumaryl alcohol (Figure 1.3c).

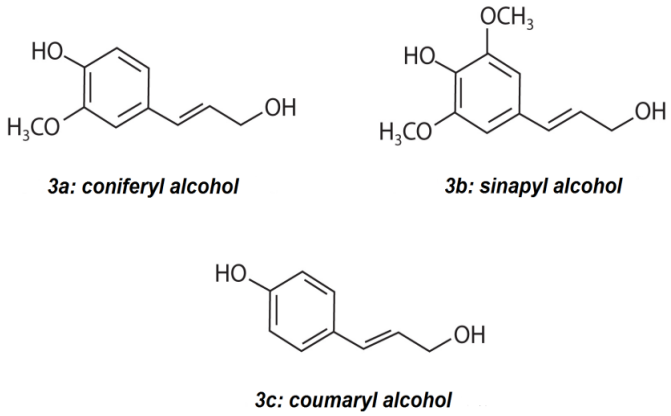

Figure 1.3: *Chemical structures for lignin building blocks: coniferyl alcohol (3a), sinapyl alcohol (3b), p-coumaryl alcohol (3c) [47].*

1.1.4.5 Lipids

Lipids and phospholipids, together with sterols, can provide valuable information on DOM origin [20; 49]. Lipids, nucleic acids, amino acids, proteins, and carbohydrates are present in all living organisms: animals, plants, fungi, protista, bacteria, archaea and viruses, all contributing to DOM. Lipids are hydrophobic or amphipathic small molecules (<1500 Da) of biological or synthetic origin, of which tens of thousands of variants are known to exist. Lipids have enormously diverse chemical structures, and are classified into eight main categories [20; 49-50], namely fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, polyketides (Figure 1.4).

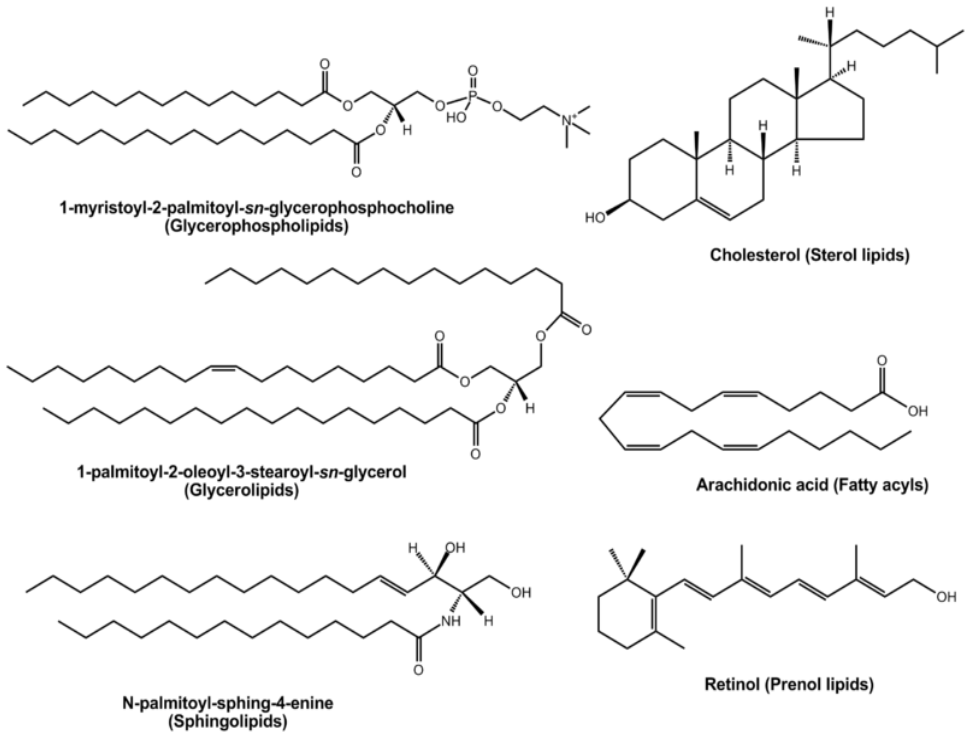

Figure 1.4: *Representative structures for major lipid categories [49].*

Lipids play pivotal roles in regulating a wide variety of cellular processes in all organisms: within each cell exist thousands of types of lipids whose composition, or lipid profile, changes in response to chemical signals from the cellular environment. In estuarine waters, the origins of lipids are different: through *in-situ* production (i.e. lysis), terrestrial material transported by river flow, and atmospheric deposition, anthropogenic sources (wastewater treatment and industrial plants), and material from the coastal ocean transported via tidal exchange. Lipids have been used extensively as biomarkers to track terrestrial and planktonic DOM through the estuary and continental shelf [20; 51]. For example, polyunsaturated fatty acids are commonly attributed to plankton, branched C_{15} and C_{17} fatty acids to bacteria, specific sterols to dinoflagellates, 16:0 n-alcohol to wax esters of zooplankton, and long-chain (C_{24} to C_{32}) saturated fatty acids, n-alkanes, and alcohols to terrestrial plants [20; 49; 52].

Furthermore, since lipids are cell membrane constituents, they can be an important biomarker to assess the MCP activity. Lipids and phospholipids are released during lysis processes, since the bacteria acting in the MCP are subject to viral and phage attacks, the concentration of such components can potentially assess the degree of functionality of the MCP itself [15].

1.1.5 Dissolved organic matter extraction

The size based definition of “dissolved” as used within the term DOM is rather general and can even be said to be inaccurate, since viruses, some bacteria, and microscopic living organisms can sit within this size range (Figure 1.5). For this reason, it is possible, as shown on Figure 1.5, to further discriminate between the size of the various DOM materials by using different filtration methods, and by employing filtration systems with different pore sizes prior to DOM extraction.

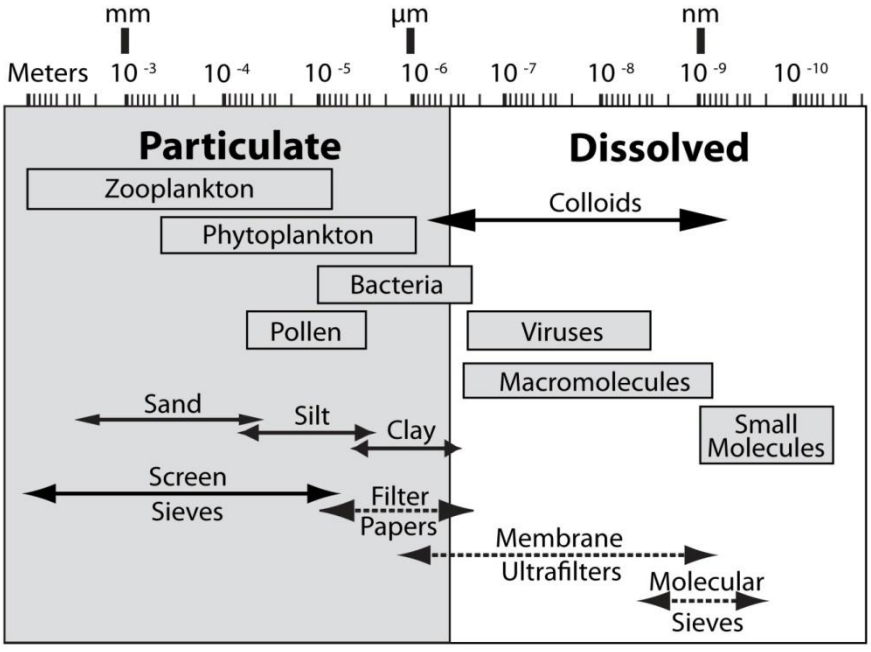

Figure 1.5: *Size and isolation methods used for DOM [1].*

The problem within DOM isolation is the absence of a protocol and of a commercially available seawater or freshwater reference sample, with which to compare the extraction efficiency or the characteristics of the obtained DOM. This is mainly due to the fact that DOM composition is dependent upon the sampling point and seasonal variability, therefore, it is virtually impossible to obtain a homogeneous reference DOM standard [27].

An ideal DOM extraction should have a 100% recovery, produce a unbiased distribution of all solutes, and chemical properties that existed in the original sample (i.e., minimise chemical or physical alteration of the sample), be able to process very large volumes of water in minimal time, and minimise the retention of inorganic salts [27].

Ultrafiltration (UF) and solid-phase extraction (SPE) are the most commonly used techniques to extract DOM. UF is a physical process, while SPE is based on the partition coefficient existing between sorbent and aqueous phases. This allows the isolation of classes of compounds according to their affinity for the stationary phase in use. The operational differences between these techniques can cause several compositional differences within the extracted DOM [27; 53-55]. UF and SPE typically yield only 10-40% of the total marine DOM, and thus it is questionable if the extracted DOM can be regarded as representative [27]. Both methods have severe limitations, such as contamination due to bleeding, side reactions with DOM functional groups, and the inability to elute strongly adsorbed DOM components from the solid support in the case of SPE [56-59].

Furthermore, when extracting seawater DOM, there are problems due to the very high concentrations of inorganic salts (20-35 g/L), compared to the very low DOM concentrations (1-3 mg/L). For this reason, efficient desalting

techniques are of primary importance prior to DOM analysis, as the presence of salts does not allow, for example, direct MS analysis, as charged salt-derived clusters obscure MS signals due to ion suppression [1].

1.1.5.1 Ultrafiltration

UF can fractionate DOM in high and low-molecular weight fractions according to selected membrane cut-offs [1; 54-55]. This extraction technique involves high flow rates and large membrane surface areas and the possibility to extract more sample. However, problems are encountered regarding contamination and adsorption [1; 27; 53-55]. Furthermore, UF extraction systems are expensive and require several optimised parameters, such as membrane nature and conditioning, and operating conditions. Variability in membrane performance and systems from different manufacturers have also been observed, as well as between laboratories using the same UF systems [55; 60-62].

DOM recoveries can range from 10 to 40% for marine and up to 66% for freshwater DOM [1]. The UF yield is reported to be tightly dependent upon salinity levels [55; 60-62]. This phenomenon is attributed to flocculation [63]; namely the higher the salinity, the lower the flocculation, therefore the lower the extraction efficiency.

1.1.5.2 Solid phase extraction methods

1.1.5.2.1 Amberlite XAD™ resins

Nonionic macroporous polymeric sorbents (Amberlite XAD™) have been widely used in the past to extract DOM from natural waters, since they are reported to provide high recoveries and the capability to process large volumes

of water [27; 54; 58; 64]. However, this approach requires major changes in sample pH, salinity, and polarity, as XAD™ resins must be thoroughly cleaned with sequences of organic solvents, and rinsed multiple times with basic and acidic solutions. This, together with altering DOM properties, renders the extraction process very time consuming and inefficient. Other recognised problems include cartridge bleeding and therefore, extensive sample contamination [1]. For these reasons, XAD™ resins are now less commonly used in DOM extraction.

1.1.5.2.2 C_{18} and polystyrene divinylbenzene-based extraction methods

SPE media such as C_{18} (octadecyl bonded-silica) and PS-DVB (polystyrene divinylbenzene) are those most commonly employed in DOM extraction, and are usually activated using polar organic solvents (e.g., MeOH or MeCN). Samples of pre-filtered water must be acidified to low pH before they are passed through such extraction cartridges. Using this method, the majority of carboxylic groups within DOM are protonated, reducing their solubility in aqueous media and enhancing their sorption onto the hydrophobic stationary phase. This increases the extraction efficiency of species such as organic acids and phenols [53]. The adsorbed hydrophobic DOM is then eluted using a strong base, in order to deprotonate the organic species bonded onto the silica support, and/or MeOH (or MeCN). In this case the fraction of DOM that adsorbs onto the sorbent is pH dependent (the maximal sorption occurs under pH 4) [53].

One of the problems of the SPE is the potential contamination due to the release of material from the sorbent [1], together with the changes that DOM can undergo during the pH change: it is not clear to what extent the treatment

modifies molecular structures and compositions [65-66]. Furthermore, there is not a “universal” SPE resin which would be able to extract all compounds within DOM, therefore the extracted DOM is only representative for certain classes of molecules.

According to the literature [53; 67-68], C_{18} based extraction methods have a lower recovery, as about 35% of the total DOM pool is adsorbed onto the stationary phase. However, using PS-DVB cartridges, Dittmar *et al.* reported a recovery increase of up to 62% [53]. The authors explain this by suggesting that PS-DVB cartridges are able to extract molecules in a range from highly polar to non-polar [53]. For this reason, the extraction method employing PS-DVB cartridges is, together with UF, the most widely used.

1.1.5.3 Combined techniques

In two publications from Vetter *et al*. and Koprivnjak *et al*. [69-70] a combination of two extraction methods was used in order to enhance the recovery of DOM. The employment of reverse-osmosis electrodialysis from Vetter *et al.* yielded a recovery of organic carbon up to 90%. However, a large amount of salts was still contained within the extracted sample, rendering any further form of characterisation more challenging. Specifically, the amount of salt existing in the extract was higher than that reported by Dittmar *et al.* [53].

An analogous technique was employed by Koprivnjak *et al*. [69] with a 75% efficiency. In this case, even though the extracted sample still contained a significant amount of salts, the isolation procedure allowed both NMR and FTICR characterisation for different seawater samples.

1.2 Characterisation of dissolved organic matter

Despite the importance of DOM, there is still a lot to be revealed regarding its exact composition, how DOM components interact between each other, and how their compositions vary between seawater and freshwater. Two approaches are used for the chemical characterisation of DOM: the direct analysis from water, and the analysis of DOM extracted from seawater or freshwater [1; 27]. The first one potentially avoids contamination and artefacts associated with the previously discussed isolation procedures, but any analysis of organic compounds at nano or picomolar level is extremely difficult, especially if dissolved in aqueous solutions with high salt contents. [27; 60].

Within their 2007 review, Mopper *et al.* [27] point out the significant limitations of many analytical techniques applied to DOM analysis. For example, commonly used methods only describe bulk properties or small fractions of the total DOM pool. TOC detection or C:N ratios [71-73] reduce DOM to an average theoretical material, whereas single class analysis is not representative of the total DOM sample [33-34; 74-79]. For these reasons, the characterisation of such a complex organic pool commonly involves “universal” forms of detection, such as MS or NMR, which can be employed in direct analysis or coupled to a chromatographic technique.

1.2.1 Simplification of dissolved organic matter

Due to the presence of thousands of compounds with potentially different chemical properties, extensive signal overlap renders any direct analysis such as MS or NMR challenging. Therefore, the use of a single or multi-dimensional chromatographic technique can allow the fractionation and simplification of the DOM pool, and thus an improved chance to obtain molecular level information.

The challenge of this type of study arises from the difficulty in finding the right chromatographic method and characterisation technique (e.g. chromatographic column and conditions).

In many cases, DOM samples are subjected to a series of harsh chemical extractions, derivatisation procedures, and chemical analysis, such that one can only obtain structural fragments indicative of specific DOM components. Attempts to make DOM more amenable to MS and gas chromatography (GC), such as pyrolysis or methylation have yielded valuable biomarker information [80-83], but only represent a small fraction of total DOM, which is able to be derivatised by using such techniques.

1.2.2 Detection of dissolved organic matter

A major obstacle to an improved understanding of DOM chemistry and composition is the low resolution of most of the affordable instrumental approaches able to detect the majority of DOM components (i.e. MS). The DOM pool contains tens of thousands of molecules of unknown complexity at trace level [27; 60], therefore, HR-MS techniques such as Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) and NMR spectroscopy have been cited as the most promising detection techniques for obtaining specific molecular information, especially if used in combined approaches [27].

1.2.3 Mass spectrometry

Even though MS is the best approach to obtain structural information, a major issue is the suitability of the ionisation source. Electrospray ionisation (ESI) is considered as a soft technique; therefore such an ionisation mode is only able to disaggregate non-covalent forces or to detect the lower molecular

weight fraction of DOM, which contains a polar region able to hold a charge. The most universal ionisation technique seems to be atmospheric pressure photoionisation (APPI), due to a charge transfer between a dopant and the molecule of interest, also allowing neutral species to be ionised. If compared to ESI, APPI is usually characterised by a greater sensitivity and less noise, with less suppression due to the presence of salts. However, protonated and deprotonated molecules, and radical species are simultaneously formed in the APPI source, further complicating the spectral interpretation.

MS is a destructive technique and does not provide the positional information on functional groups within a detected molecule. This represents a major issue considering the presence of isomers within DOM [28-29]. NMR, and in particular multi-dimensional NMR, allows this possibility in a non-destructive way.

1.2.4 Nuclear magnetic resonance spectroscopy

As a general rule, solid state NMR is four times more sensitive than liquid state NMR [27]. This is due to the fact that the sample is placed in the probe at its highest concentration, as a solid. This also means that the sample handling is minimised, decreasing the contamination risk.

Despite all the advantages that NMR can give, a limitation for such analysis is the low sensitivity, and the amount of sample and time required to complete a single analysis. If a ^{31}P or ^{15}N NMR experiment is performed, there is a need for different probes, together with high signal power due to the low abundance of such isotopes. Also, the absence of multi-dimensional databases relative to nuclei other than carbon or hydrogen renders any structural assignment very difficult.

1.2.5 Combination of mass spectrometry and nuclear magnetic resonance

Due to the limitations and advantages of both NMR and MS, in the most successful studies such techniques have been applied to complement each other. Coupled multi-dimensional NMR and FTICR-MS experiments have been able to identify the main features within DOM and to understand and qualitatively characterise its main constituents (i.e. CRAM) [27; 29].

However, even if crucial components were identified, such approaches fail to isolate single molecules within the DOM pool, or to fractionate this complicated organic mixture in classes of compounds according to a main chemical-physical property (i.e. polarity). This results in extensive signal overlap on both MS and NMR spectra. Furthermore, MS spectra are not reported with characteristic fragmentation pathways, rendering the assigned formulae purely statistical.

Due to the absence of standard materials which can reassemble the properties of compounds such as CRAM, it is difficult to confirm the identity of these unknown molecules. Despite the complexity from an operative point of view, a synthetic preparation of CRAM-like materials according to their structural features, discovered in the main multi-dimensional NMR and MS approaches [28-29], could be tested. Such artificially prepared materials could be employed as standards in MS and NMR spectrometry in order to fully confirm the identity of this class of compounds, mainly characterising DOM.

1.2.6 Chromatographic Methods

1.2.6.1 Liquid chromatographic methods

Due to their versatility, the separative methods employed in DOM fractionation are reversed-phase chromatography (RP-HPLC), hydrophilic interaction liquid

chromatography (HILIC) and, above all, size exclusion chromatography (SEC). Such chromatographic techniques can cover most compounds comprising the main molecular classes present within DOM. However, due to the complexity of DOM, none of these approaches are ideal, therefore, in an effort to enhance the fractionation of DOM prior to detection, new multi-dimensional experiments have recently been studied [35].

1.2.6.1.1 Reversed-phase high-performance liquid chromatography

RP-HPLC separates compounds according to polarity, by employing a non-polar stationary phase (i.e. C_{18} bonded silica) and a mixture of organic and aqueous mobile phase. Compounds with higher affinity towards the mobile phase (and therefore more polar) are expected to be less retained on the stationary phase. Conversely, low polarity molecules will be more retained as they exhibit an increased affinity towards the stationary phase.

Simpson's research group [84] have applied RP-HPLC coupled to a diode array detector (DAD) and NMR to the analysis of DOM collected from different freshwater sources (Table 1.1). A deuterated water/MeCN gradient was used with a reversed-phase C_{18}-functionalised column, with UV detection at 280 nm in order to detect compounds enriched in double bonds and aromatics. From the four fractions obtained during the RP-HPLC-UV run (Figure 1.6), a total of 150 NMR spectra were collected. The spectra from the first eluting fractions contained sharp aromatic peaks of highly polar species (phenols and/or aromatic acids), which elute quickly at the beginning of the gradient in a nearly pure aqueous phase. However, the NMR spectra from the

following fractions were dominated by broad signals, indicating co-eluting species.

As emphasised by the extensive co-elution of molecules within two “humps” on the RP-HPLC chromatogram (Figure 1.6), the deuterated water/MeCN gradient is probably not optimal in this analysis, causing the consequent NMR spectra to be characterised by extensive signal overlap.

In order to increase the retention of analytes, the interaction between sample and stationary phase can be enhanced, for example by decreasing the eluent pH. Such modification renders the analytes more selective towards the apolar stationary phase. However, the presence of fused alicyclic rings, and aromatic structures in DOM [28-29; 35], can result in strong interactions between sample and stationary phase, and this can cause irreversible adsorption, ending in false positives, and in sample to sample carryover. This has already been experienced for such complex mixtures [65], and renders RP-HPLC challenging to apply as the first chromatographic dimension.

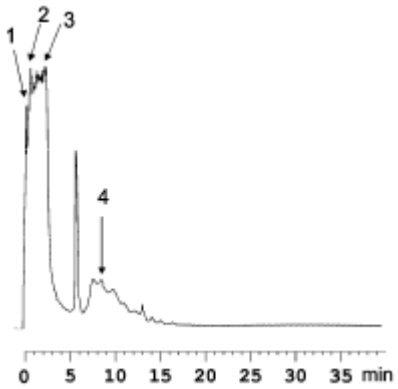

Figure 1.6: *RP-HPLC separation of DOM with a water/MeCN gradient and UV detection (280 nm). The enumerated fractions were subsequently analysed by NMR [84].*

Normal-phase chromatography could be attempted, however the organic solvents potentially involved exhibit numerous signals in NMR and are poor solvents for DOM.

To enhance the chromatographic resolution, Koch *et al*. also used RP-HPLC with a water/MeOH (adjusted to pH 7 with NaOH) gradient (Table 1.1) [85]. MeOH is more polar than MeCN and can act as both proton acceptor and donor, whereas MeCN can only be a proton acceptor. For this reason, MeOH can undergo polar or hydrogen bonding interactions with solutes. This enhances the solubility of the species in the mobile phase, rendering them less prone to irreversible interactions towards the apolar stationary phase. Obviously, such consideration applies when the pH of the mobile phase is maintained neutral, so that any secondary interaction is prevented.

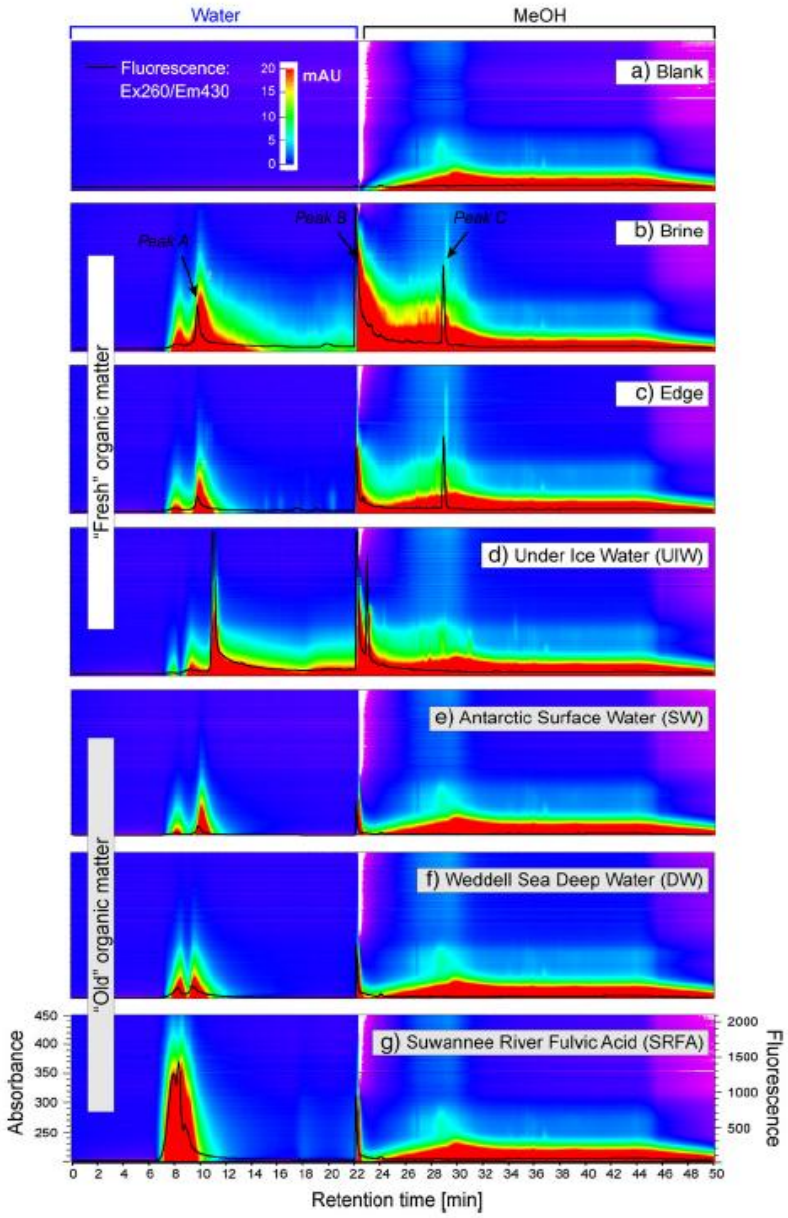

Figure 1.7: *RP-HPLC separation of different DOM samples employing a water/MeOH mobile phase at pH 7 and fluorescence detection (excitation 260 nm, emission 430 nm) as obtained by Koch et al. [85].*

The approach proved to be successful in obtaining more resolved peaks of water soluble components (Figure 1.7). In particular, the employed eluent pH was considered the key factor to the improvement of the chromatographic separation. Lower pH was tried (i.e. 2.5 and 6.8), but this caused extensive co-elution. In fact, the peaks eluting when the gradient was more aqueous were broader and shifted to higher retention times, being more selective towards the stationary phase. Despite the partial success of this approach, the authors point

out the necessity to further reduce the complexity of DOM samples and propose the use of a multi-dimensional chromatographic approach involving RP-HPLC and SEC.

1.2.6.1.2 Size exclusion chromatography

Ideally, SEC separates compounds on the basis of hydrodynamic molecular size. Samples are injected onto a column containing a porous gel stationary phase. Small molecules can access more of the internal pore volume than larger molecules, which are excluded from such pores. Large molecules elute first, followed by the smaller ones, as long as the surface of the porous material does not interact with the solutes, namely through secondary adsorption. SEC is used to obtain molecular weight distributions within samples. However, this can sometimes be problematic due to the effects of the aforementioned solute-gel interactions. Adsorption due to Van der Waals and electrostatic forces between the gel surface and analytes affects the retention time in the column and hence the calculated molecular weight ranges. Hydrophobic compounds can adsorb onto the gel surface, resulting in secondary retention, and an artificially low molecular weight. Electrostatic repulsion will result in artificially high molecular weights, as the analytes are eluted faster than expected.

Table 1.1: *Overview of the LC approaches in the study of DOM.*

PAPER	YEAR	SEPARATION METHOD	WATER SOURCE	EXTRACTION METHOD[a]	ELUENT	ELUENT PH[b]	COLUMN	PARTICLE SIZE	DETECTOR(S)[c]
Everett *et al. [86]*	1999	SEC	freshwater	UF	2 m*M* phosphate buffer, 0.1 *M* NaCl	-	Waters HPSEC	-	UV, TOC
Pelekani *et al. [87]*	1999	SEC	freshwater	UF	20 m*M* phosphate buffer	6.8	Protein Pak 125 (7.8 x 300 mm)	10 µm	UV
Muller *et al. [88]*	2000	SEC	freshwater	UF	25 m*M* phosphate buffer	6.8	Superdex 75 column (10 x 300 mm)	13 µm	CD, UV, TOC
Her *et al. [71]*	2002	SEC	freshwater	reverse osmosis, filtration	phosphate buffer	6.8	Protein Pak 125 (7.8 x 300 mm), TSK-50S (20 x250 mm), Biogel P6 (5 x 900 mm)	10 µm, 30 µm, 90-180 µm	UV,TOC
Minor *et al. [90]*	2002	SEC	freshwater seawater	UF	100 m*M* phosphate buffer	7	TSK-gel G3000 (7.8 x 300 mm)	5 µm	RI, UV, MS
Her *et al. [89]*	2003	SEC	freshwater	reverse osmosis, filtration	phosphate buffer	6.8	TSK-50S (2 x 250 mm)	30 µm	UV, FL, TOC
Brinkmann *et al. [91]*	2003	SEC	freshwater	filtration	28 m*M* phosphate buffer	6.6	TSK HW 40S (2 x 250 mm)	4 µm	UV, CD,TOC
Reemtsma *et al. [93]*	2003	SEC	freshwater	filtration	10 m*M* carbonate buffer and MeOH	-	PL-Aquagel-OH 30 (4.6 x 250 mm)	8 µm	UV,TOC, MS
Woods *et al. [94]*	2010	SEC	freshwater	reverse osmosis, filtration	30 m*M* ammonium and sodium chloride buffer	11	Ultrahydrogel 250 and 120 (7.8 x 300 mm)	6 µm	DAD, NMR
Kawasaki *et al. [73]*	2011	SEC	freshwater	filtration	20 m*M* phosphate buffer	6.8	Tosoh TSK gel (7.8 x 300 mm)	5 µm	UV, TOC, NDIR
Yan *et al. [95]*	2012	SEC	freshwater	XAD™	10 m*M* carbonate buffer	-	PL-Aquagel-OH 30 (7.5 x 200 mm)	8 µm	DAD, TOC
Landry *et al. [96]*	2012	SEC	freshwater	UF	10 m*M* ammonium bicarbonate and MeOH	-	PL-Aquagel-OH 30 (4.6 x 250 mm)	8 µm	DAD, TOC, FTIR
Woods *et al. [97]*	2011	HILIC	freshwater	reverse osmosis, filtration	100 m*M* deuterated ammonium acetate/MeCN	-	Phenomenex Luna (4.6 x 150 mm)	3 µm	DAD, FL, NMR

PAPER	YEAR	SEPARATION METHOD	WATER SOURCE	EXTRACTION METHOD[a]	ELUENT	ELUENT PH[b]	COLUMN	PARTICLE SIZE	DETECTOR(S)[c]
Woods *et al. [35]*	2012	HILICxHILIC	freshwater	reverse osmosis, filtration	100 m*M* deuterated ammonium acetate/MeCN	-	Phenomenex Luna (4.6 x 150 mm), Phenomenex Kinetex (4.6 x 150 mm)	3 µm, 2.6 µm	NMR
Simpson *et al. [84]*	2004	RP-HPLC	freshwater	XAD™	deuterated water/MeCN	-	C_{18} Supelcosil LC18 (4.6 x 150 mm)	5 µm	DAD, NMR
Koch *et al. [85]*	2008	RP-HPLC	seawater	SPE	water/MeOH	7	C_{18} Phenomenex Synergi (4 x 250 mm)	4 µm	FL, MS

a *UF: ultrafiltration, SPE: solid phase extraction, XAD™: Amberlite XAD™ resin;* ***b*** *Where a dash is reported, no data were present;* ***c*** *UV: ultraviolet detection, TOC: total organic carbon, CD: conductivity detection, RI: refractive index, MS: mass spectrometry, FL: fluorescence, DAD: diode array detector, NMR: nuclear magnetic resonance, NDIR: non-dispersive infrared.*

Everett *et al.* [86], first employed SEC for the separation of DOM (Table 1.1). This study illustrates clearly the previously mentioned challenges with this kind of sample, as chromatograms shown report extensive co-elution (a “hump” is the only detected signal across the chromatogram). This reflects the complexity of DOM itself, especially considering that 0.1 *M* NaCl was added to the 2 m*M* phosphate buffer mobile phase in order to reduce ion exchange interactions between the sample and the OH-functionalised stationary phase. In the absence of salts, analytes are usually more likely to bind strongly to the stationary phase with possible irreversible adsorption. The addition of salt suppresses such binding, usually leading to lower retention times. Such consideration indicates that the mobile phase composition needs to be optimised according to the analysed species and this appears a challenging task when considering the different classes of compounds within DOM. The task is further complicated by the fact that the molecular weight range of the different classes of compounds within DOM is probably narrow. Pelekani *et al*. [87] also proposed a further explanation for poor peak shape, as being a consequence of nonspecific interactions during the extraction procedure. During this stage, pH changes can occur, resulting in a modification of the original nature of the sample.

Considering such issues, Muller *et al*. [88] employed a similar OH-functionalised SEC column with a phosphate buffer mobile phase at higher concentration (25 m*M*, ionic strength 0.04 *M*) not enriched in NaCl (Table 1.1). This method provided an improved separation and fractions were collected and further re-injected on the same SEC column, originating broad peaks of a nearly Gaussian shape. This further proves that eluent composition, particularly pH and ionic strength, plays a key role on SEC analysis. In fact, Her *et al.* [71]

confirmed that significant ionic interactions occur when the ionic strength is low. However, at higher ionic strengths (greater than 0.2), such effects are suppressed in favour of other kind of interactions (i.e. hydrophobic). Aromatic groups within DOM had irreversible adsorption issues, with retention time shifts also observed. Such consideration led Her *et al.* to optimise their chromatographic method and one year later [89] to fractionate DOM by molecular weight classes (Figure 1.8, Table 1.1).

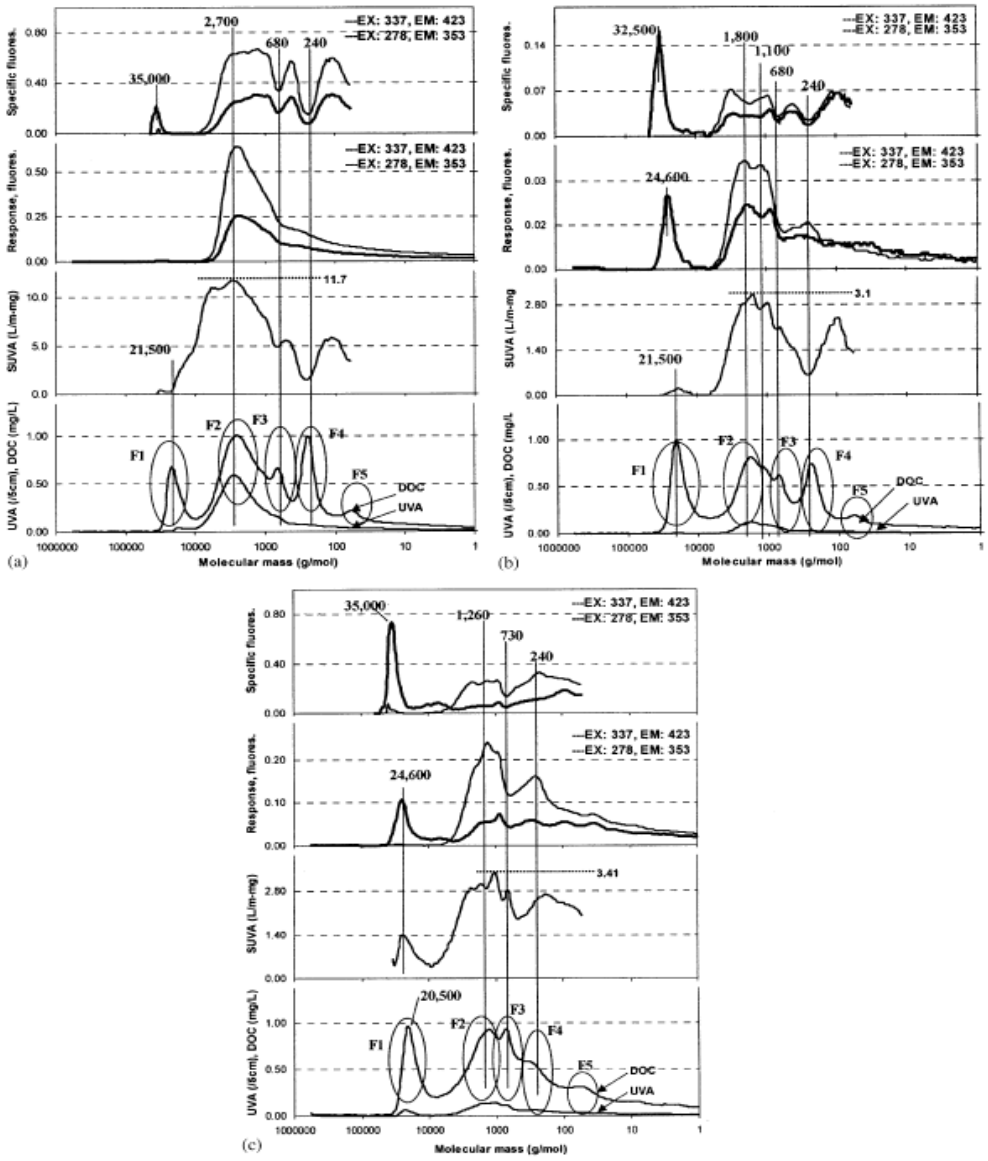

Figure 1.8: *SEC chromatograms with UV, fluorescence and TOC response obtained after method optimisation by Her et al. [71; 89].*

Even though peaks were not fully resolved in this study by Her *et al.* [89], DOM was divided into five fractions according to molecular weight. However, such molecular weight determination within DOM points out another major issue with SEC based approaches, namely that related to DOM molecular weight dispersion. It is very hard to find a suitable standard to understand the molecular distribution of the unknown DOM constituents. SEC is usually calibrated using polymeric materials as representatives of the injected solute. It is not possible to do so for DOM, as such molecules are non-representative of this organic mixture. Furthermore, even if molecules have the same molecular size, they can have different tertiary structure, i.e. a low molecular weight compound can have a linear structure, or a high molecular weight one can be cyclic. Depending on the type of solvent the sample is dissolved in, some structures can unfold or shrink, rendering the molecular weight challenging to be determined by retention times.

In the attempt to find a suitable calibrant, Minor *et al*., employed SEC with a phosphate eluent [90] to analyse two DOM portions: high (>1 kDa, found to be rich in aminosugars, deoxysugars, and methylated sugars) and low molecular weight (<1 kDa, enriched in hexose sugars) (Table 1.1). The selected SEC calibrant was a polysaccharide with molecular weight ranging from 186 to 12 kDa. Such a molecular weight range did not cover the low molecular weight fraction of DOM. Furthermore, since the sample desalting occurred after SEC, it was possible for ionic interaction to occur between the dissociated ionic species present in DOM and the OH-functionalised stationary phase. Also, considering that DOM is rich in hydrogen bonds, especially in its sugar fraction and carboxylic fraction, hydrogen interactions between analytes and stationary phase cannot be neglected. Such considerations can also be applied to a

following study from Brinkmann *et al.* [91] (Table 1.1). According to Specht *et al.*, secondary interactions take place regardless of whether the stationary phase is a polymer or silica based gel [92]. For example, hydrophobic interactions were found to be the main contributor for compounds with -OH and -COOH functionalities, reporting an increased elution volume which, contrary to the classic SEC elution mechanism, was found to be proportional to the increasing number of carbon atoms.

In a further attempt to decrease the risk of secondary interaction between sample and OH-functionalised stationary phases, Reemtsma *et al.*, [93] used a SEC eluent enriched in organic modifier (80/20 NH_4HCO_3/MeOH) to analyse a portion of DOM (fulvic and humic acids) (Table 1.1). Ammonium bicarbonate was used as the electrolyte, in order to decrease the interactions between the analytes and the stationary phase. If no MeOH was used, the elution time would have increased due to hydrophobic interactions between the sample and the stationary phase. The employed eluent was also sufficiently volatile, which was critical as SEC was coupled on-line to ESI-MS, which is not the case for many SEC papers which used phosphate based buffers.

In a recent study, Woods *et al*., coupled SEC [94] (0.1 *M* NaCl and 0.03 *M* NH_4Cl adjusted to pH 11 with NH_4OH) to NMR (Table 1.1). Two columns were used in series as to obtain two fractionations of DOM according to size, prior to NMR analysis. Three fractions were collected: the first, enriched in carbohydrate and aromatic-like structures; the second, representative of CRAM, and the third of MDLT. Even though the chromatography in this case also needed to be improved, for the first time CRAM and MDLT were partially separated from each other. This was also the first work employing a highly basic mobile phase to avoid any sample protonation. However, this again acts

to potentially cause strong interactions between sample and stationary phase: as DOM is characterised by carboxylic functionalities, such eluent composition can lead to sample deprotonation and nucleophilic interaction between the sample and the polymethyl-methacrylate stationary phase, causing irreversible adsorption.

Due to such issues, in a subsequent qualitative and quantitative multi-detector approach (UV and non-dispersive infrared (NDIR)), Kawasaki *et al.* [73], re-employed a phosphate buffer eluent with pH close to neutrality (6.8), with an OH-functionalised stationary phase (Table 1.1). Polystyrene and sulfonate standards were used as calibrants, providing the suggestion that most of DOM constituents have a molecular weight lower than 4000 Da. Although once again the calibrants did not necessarily properly represent the compounds present in DOM.

Similar considerations can be applied to the work from Yan *et al.*, [95] , where the selected calibrant was polyethylene glycol, the nature of which poorly suits the real structures within DOM. In fact, despite showing an apparent molecular weight ranging from 3 to 16 kDa, measurement errors of ±30% were encountered. However, considering that SEC fractionations usually employ a single wavelength UV detection, which by no means is comprehensive of all the chemical-physical properties within DOM, within this study for the first time it was highlighted that a universal form of detection is required for a more comprehensive study of DOM. Yan *et al.*, coupled SEC to DAD in multiple wavelength mode (200-445 nm) and such an approach allowed the detection of a wider range of UV-absorbing compounds, compared to previously reported ones.

Phosphate buffer was again used in a most recent paper showing the combination between SEC and Fourier transform infra-red spectroscopy (FTIR) [96] (Table 1.1). This method proved to be quite innovative, as the authors developed a solvent-elimination interface to deposit DOM fractions isolated from SEC onto a germanium disk, where the sample can be concentrated prior to FTIR. Despite showing lack of resolution along the SEC chromatogram, the sample was divided in fractions with low (500−900 Da) and high molecular weight (>900 Da) showing that the 500-900 Da fractions contained more carboxylic and OH groups, while the >900 Da fractions of DOM contained more carbohydrates, amides, aromatics/alkenes and aliphatics. However, all the analysed chromatograms showed peak overlap probably due to the complexity and the narrow molecular weight dispersion within the sample.

1.2.6.1.3 Hydrophilic interaction liquid chromatography

HILIC separates compounds by using a mostly organic and hydrophobic mobile phase through a hydrophilic stationary phase, allowing analytes to elute in order of increasing hydrophilicity. The aqueous portion of the mobile phase adsorbs onto the polar stationary phase, allowing the formation of a polar water layer. The separation is achieved by analyte partitioning from the eluent into this hydrophilic layer. Here, the more hydrophilic the analyte of interest is, the more will be partitioned within the water layer on the stationary phase, rendering it more retained.

Woods *et al*., explored the possibility of coupling HILIC to HR-NMR, both in single and multi-dimensional studies, with the aim of obtaining the identity of single DOM components [35; 97].

On the mono-dimensional approach, a HILIC column with silica bearing diol functional groups was employed to divide DOM into 80 fractions according to polarity [97]. The latter were then examined by means of NMR, which showed isolated peaks, in cases indicative of single components. The material eluted from the HILIC column was structurally similar to the neighbouring fractions but, with increases in retention time, more hydrophilic compounds were eluted and found to be correlated with carbohydrate distribution. Aromatics were found to be most abundant in mid-polarity fractions (where the most extensive co-elution was present), while CRAM and MDLT-type molecules were co-eluting in the earliest and latest eluting portions of the chromatogram, demonstrating their wide spectrum of chemical-physical properties. Multiple retention mechanisms were observed, with the most influential interactions being analyte partitioning between the organic-rich eluent and the water layer

near the polar stationary surface. Charge interaction, hydrogen-bonding, dipole-dipole interactions, and hydrophobic effects further affected analyte selectivity. It is therefore difficult to understand what was the leading mechanism involved in the separation of a multifunctional material such as DOM. For these reasons, the DAD and fluorescence response prior to the NMR analysis still reveal extensive peak overlap, causing the collected fractions to contain several similar compounds (Figure 1.9).

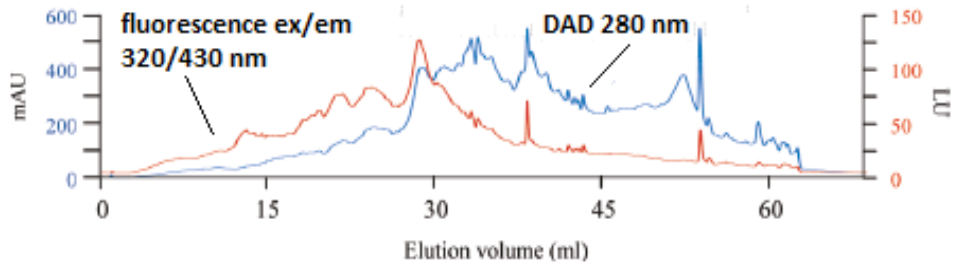

Figure 1.9: *HILIC separation of DOM with a $CD_3CO_2ND_4$/MeCN gradient. The most characteristic fractions within the chromatograms were further analysed by multi-dimensional NMR [97].*

Recently, in order to improve the chromatographic resolution and to obtain more resolved fractions from DOM, Woods *et al.*, for the first time employed a multi-dimensional chromatographic approach (HILICxHILIC) coupled to NMR [35]. The column employed on the first chromatographic dimension was the same as that used in previous mono-dimensional experiments [97], however, on the second on-line dimension a normal-phase bare silica column was used. This reduces the orthogonality of the chromatographic methods, as both the stationary phases have similar surface OH chemistry. By definition, on a multi-dimensional separation, each dimension should separate according to different chemical-physical properties to obtain a fully orthogonal approach. In the HILICxHILIC separation described by Woods *et al.*, both stationary phases are polar, with analogous mobile phases (MeCN

and deuterated ammonium acetate) even though in different compositions. Despite showing evidence for the classes of compounds mainly characterising DOM (i.e. MDLT), it was not possible to check if the chromatography had improved if compared to the previous mono-dimensional HILIC approach, as no chromatograms were shown, however, there was less extended peak overlap on the NMR spectra reported, therefore the data interpretation was less challenging.

1.2.6.2 Gas chromatography methods

In GC a carrier gas (i.e. He or N_2) allows the separation of compounds that are, or which can be made, volatile. Prior to separation by GC, compounds containing functional groups with active hydrogen atoms such as -COOH, -OH, -NH, and -SH need to be protected as they tend to form intermolecular hydrogen bonds that can reduce volatility. Furthermore, their low volatility can cause strong interactions between the compounds of interest and the stationary phase of the column, causing peak broadening.

The derivatisation reactions employed in the GC analysis of DOM fall into three general reaction types: thermal pyrolysis, alkylation, and silylation. All of which, change the chemical structure of a molecule.

Pyrolysis is suitable to materials which would otherwise not be amenable to GC analysis (i.e. powders). Chemical bonds within large macromolecular structures are broken by the application of heat. This results in the production of smaller and more volatile fragments which can be separated and further detected by means of MS. The limitation of this technique is the unintentional decomposition of sensitive classes of molecules, causing the observation of erroneous results [98-99].

Alkylation reactions replace active hydrogens in an organic acid or amine by an aliphatic or aliphatic–aromatic group. This technique is used to transform carboxylic acids into esters, which are more volatile. A common reactant is tetramethylammonium hydroxide (TMAH), which allows the production of ethers, secondary amines and esters.

In silylation, active hydrogens from acids, alcohols, thiols, amines, amides, enolisable ketones and aldehydes are replaced with a trimethylsilyl group. Both silylation reagents (e.g. bis-trimethylsilyl trifluoro acetamide (BSTFA)) and trimethylsilyl derivatives are unstable and must be protected from moisture.

In general, the analysis of DOM by GC is targeted to certain classes of molecules (i.e. lipids, lignin), or non-targeted, in an attempt to process a generic screening of the entire organic pool (Table 1.2). Considering the majority of published methods, the GC column that was mostly employed (Table 1.2) was the DB5 column. The latter is characterised by a non-polar stationary phase, which renders it generally applicable to the separation of unknown materials (mainly cyclic and aromatic moieties). A more specific column would in fact be of no use with such a complicated range of chemical functionalities within DOM, causing many compounds to be either non-retained or irreversibly absorbed.

As reviewed by Aiken *et al.* [100], one of the first attempts to use GC in the analysis of complex mixtures such as POM, was performed by Stainton [101]. This method reported a versatile yet simple sample extraction and preparation approach prior to analysis. Acidified water was extracted through a gas stripping procedure with helium flow, the latter being used as carrier to deliver the sample to GC. However, as polypropylene (PPL) syringes were

used, they can be a source of potential contamination [102-103]. Furthermore, the efficiency of the method is tightly dependent on the stripping time and on the nature of the sample as only highly volatile materials will be delivered to GC and be separated.

Since the complexity of DOM and the co-elution due to the absence of a derivatisation technique in the previously described work, Schulten *et al.* associated a pyrolysis GC-MS analysis of DOM to its computational model [104]. Carbohydrates, phenols, lignin materials, lipids, aromatics, humic building blocks, sterols and peptides were found to be the most prominent structures, and were further confirmed in a later study [105]. Such structures were related to computational data, where the contributions from bond energies, Van der Waals and electrostatic interactions were quantified, with particular interest on the hydrogen bonds occurring at intra-molecular level between the detected compounds. Since the high degree of aromaticity and the presence of functional groups such as -OH and -COOH within DOM, non-bonded interactions such as Van der Waals and hydrogen bonds were found to cover a key role in the formation of colloidal structures. However, the above proposed computational model was rather limited since the GC analysis itself was restricted by the low volatility of highly polar constituents and cross-linked clusters within DOM, limiting the obtained data to only low polarity compounds [82].

In order to perform a targeted analysis, Mannino *et al.* [106] used GC-MS (mass range 50-600 amu) to analyse lignin-like and polar lipid-like materials after organic solvent (CH_2Cl_2) extraction and TMAH derivatisation (Table 1.2). The extraction technique used by Mannino *et al.*, aimed to isolate the targeted classes of molecules from all the other compounds present in waters. However, other groups of molecules can all be extracted into this organic solvent, such as

complex sterol-like materials, e.g. CRAM and terpenoids, thus not allowing a fully targeted analysis, and consequent co-elution especially in the first and middle part of the GC chromatogram. However, using this method, the majority of lipids were detected for samples in proximity of the river estuary, and included fatty acids and sterols, which comprised 0.33% of the high molecular weight DOM (1-30 KDa), and the 1.6% of very high molecular weight DOM (30 KDa-2 µm). This confirms how lipids represent a minor percentage of DOM, and also confirms their higher presence in terrestrially influenced DOM [1; 20; 107].

Due to the solid nature of extracted DOM, Kracht *et al.* [108] also employed thermal pyrolysis to derivatise freeze-dried DOM. This study was the first to employ a combined form of detection involving elemental analysis and mass spectrometry-isotope ratio mass spectrometry (MS-IRMS), in order to correlate MS spectra to isotopic ratios and derive more comprehensive information on the origin of DOM (Table 1.2). Although using only one form of derivatisation, the authors proposed to treat the sample with different derivatisation techniques simultaneously. This approach could be used to analyse other volatile compounds present in DOM, such as silyl derivatives, to compare their elution profile and detector responses to those obtained after thermal pyrolysis.

The main issue within the aforementioned study is related to the employed extraction method. Although freeze-drying can provide a potentially uncontaminated extract (e.g. free from plastic-derived materials or artifacts), is time consuming and unsuitable for sample desalting if seawater samples are processed. Freeze-drying as a process is also dependent on the chemical characteristics of the frozen species. Every class of compounds has different freeze-drying requirements, making optimisation difficult, which can lead to

inconsistent dryness across the sample, reduced stability or rehydration. For such reasons, the sample must be cooled under its critical temperature (T_{crit}) to ensure it is fully frozen, and since every compound has a specific T_{crit}, the latter should be known before beginning freeze-drying. For example, delicate materials within DOM (i.e. proteins) have a risk of structural damage from ice crystal growth if the rate of freezing is too fast and large crystals are formed. Conversely, a slower freezing rate results in smaller crystals and a lower risk of structural damage, but the resulting crystals will cause a greater impediment to the flow of vapour and slow the drying process.

Aluwihare *et al.* [109] performed a targeted analysis on two different classes of compounds, lipids and sugars, which were analysed by GC with both flame ionisation detector (FID) and NMR. Even though FID is a destructive detection technique, its high sensitivity to many different classes of organic compounds can be beneficial. Obviously FID does not provide structural information, therefore was not able to assess the identity of the detected compounds without the availability of standards. However, such information was provided via NMR spectroscopy.

Lipids were also analysed by Jandl *et al.* [76] both from seawater and freshwater DOM. The method comprised an extraction in CH_2Cl_2/acetone and TMAH derivatisation (Table 1.2). GC-MS data was compared to that available in databanks, confirming the presence of $C_{14:0}$ to $C_{28:0}$ *n*-alkyl fatty acids series. The highest concentration was observed by employing reverse-osmosis extraction on freshwater (river) samples (309.3 μg/g), whereas in freeze-dried brown lake water the concentration was nearly halved (180.6 μg/g). This finding not only further highlights the dependence of DOM upon its source, but also the different efficiencies from various extraction methods in use. Non optimal

freeze-drying extraction is probably a major cause of low extraction efficiency if compared to reverse-osmosis.

Weishaar *et al.* [110] combined the information from carbon NMR, UV adsorption at 254 nm and TMAH derivatised GC-MS, to focus upon the aromatic portion of DOM (Table 1.2). As already seen, the combination of MS and NMR spectra, provided a more comprehensive understanding of the different classes of compounds within the DOM sample (i.e. proteins, ketones, chlorophyll pigments and aromatics). One of the highlighted issues in this study was that not all of these species seemed to be suited to the TMAH derivatisation technique used. However, such kind of compounds could be detected through NMR or UV spectroscopy. An interesting additional experiment was performed in this work, where the interference of UV-absorbing ionic species such as iron and nitrate were tested. Both of those species caused severe interference due to light scattering and adsorption, highlighting the necessity to filter the samples before analysis.

Page *et al.* [80] were the only group who treated a seawater sample with alum in order to remove color and turbidity. The filtered material was then freeze-dried and analysed by thermal pyrolysis GC-MS (Table 1.2). The alum-extracted samples were found to be rich in alkylbenzenes, alkylphenols and polycyclic hydrocarbons, whereas the fraction removed with this kind of technique was characterised by the presence of aromatics, in particular lignin-like materials. Since DOM is enriched in clusters of compounds, in particular from lignin (i.e. through π-π stacking or hydrogen bonds) [104; 111], alum treatment is likely to prevent such compounds from being analysed.

In an analogous study, Fraizer *et al.* [112] were able to quantify the main compound classes discovered in the previous study from Page *et al.* (i.e. fatty

acids, carbohydrates and lignin materials) through TMAH derivatised GC-MS. The significance of this work arises from the potential to understand the variations these compounds can undergo within different water sources.

Multiple detection approaches were also employed by Maie *et al.* [113] and Templier *et al.* [114] who compared NMR data to those obtained using TMAH GC-MS (Table 1.2). Despite the possible contamination due to the fractionation method, the novelty of the Templier *et al.* study was based upon the combined use of different XAD™ resins to extract DOM, leading to the separation of two fractions with different polarity. This technique simplified the GC-MS chromatograms to an extent that, even if with low intensity, single peaks were detected (Figure 1.10). The DOM sample was also characterised by the presence of a large, late-eluting “hump” representative of unresolved compounds. This unresolved portion of chromatogram therefore needs to be separated by different chromatographic techniques, such as HPLC, as such behavior demonstrates that these compounds exhibit poor volatility. NMR analysis was also improved by the fractionation, and even though extensive signal overlap was characteristic of such spectra, it was possible to recognise more defined signals.

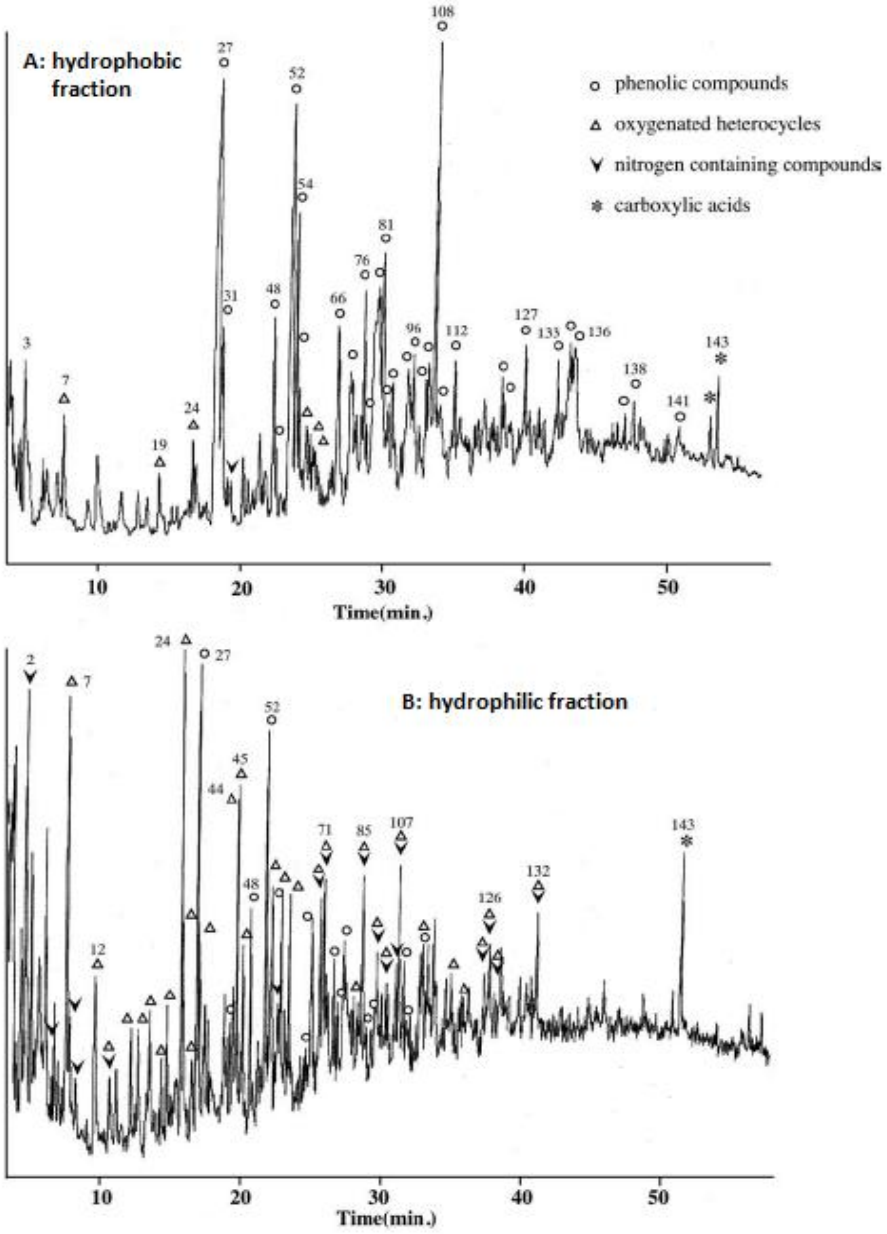

Figure 1.10: *GC-total ion chromatograms (TIC) for the hydrophobic (A) and hydrophilic (B) fractions from a freshwater DOM sample [114].*

Peuravuori *et al.* [81] employed a combined chromatographic approach, (LC and consequently GC), in order to fractionate and then characterise DOM. The DOM sample was firstly separated into eight fractions using SEC, according to decreasing molecular weight, and then subsequently analysed by means of GC-MS using two alkylating reagents: TMAH, to reveal both esterified and free carboxylic acids; and tetramethylammonium acetate (TMAAc), to determine free carboxylic acids (Table 1.2). Supramolecular associations of

smaller moieties with similar structural functionalities were found between humic-like substances, whereas, the concentrations of free aliphatic and aromatic dicarboxylic acids were so low that they were hardly detectable using MS. Since the SEC detector was UV at 254 nm, non-aromatic molecules were not detected, which can explain the absence of aliphatic dicarboxylic acids.

Due to the importance of biologically derived compounds in marine ecosystems, a targeted analysis of crucial biomarkers was conducted by Louchoarn *et al.* [115] who, on the basis of previous experiments [116], applied CuO oxidation with GC-MS/MS, with particular reference to lignin (Table 1.2). After oxidation, lignin was hydrolysed into its three building blocks: vanillyls, syringyls, and cinnamyls. Since each class of lignin oxidation product is comprised of an acid, an aldehyde and a ketone (for vanillyls and syringyls), or only acids in the specific case of cinnamyls, these could be detected by means of MS and MS/MS, allowing the isolation of such components from DOM.

More recently, Lang *et al.* [117] developed an innovative method for the isotopic analysis ($\delta^{13}C$) of organic samples which were oxidised to CO_2 using persulfate and heat (Table 1.2). The isotopic composition of the produced CO_2 was determined by GasBench, an on-line gas head-space sampling tool coupled to an IRMS. This technique offers high sensitivity (the limit of detection (LOD) was 1.2 µg of carbon), and the possibility to directly analyse seawater (before desalting). It also has the disadvantage of offering high sample throughput.

Table 1.2: *Overview of the GC approaches in the study of seawater and freshwater.*

PAPER	YEAR	WATER SOURCE	EXTRACTION METHOD[a]	DERIVATISATION[b]	TARGET COMPOUNDS[c]	COLUMN[d]	TEMPERATURE GRADIENT(°C)[e]	DETECTOR(s)[f]
Schulten *et al. [104]*	1999	freshwater	XAD™	thermal pyroloysis	DOM	DB5 30 m 0.32 mm i.d. 0.25 mm film thickness	40-250	MS
Mannino *et al.[106]*	2000	freshwater	UF	TMAH	fatty acids, lignin	DB5 30 m or 60 m 0.32 mm i.d. 0.25 mm film thickness	50-300	MS
Van Heemst et al. [105]	2000	freshwater	UF	TMAH	DOM	DB5 30 m 0.25 mm i.d. 0.25 mm film thickness	60-280	FID,MS
Kracht *et al. [108]*	2000	freshwater	freeze-drying	thermal pyroloysis	DOM	BPX 5 60 m 0.32 mm i.d. 1.0 µm film thickness	36-300	elemental, MS-IRMS
Louchoarn *et al. [116]*	2000	freshwater seawater	SPE, UF	CuO	terrigenous DOM (lignin)	DB5 30 m 0.32 mm i.d. 0.25 mm film thickness	100-270	FID, MS
Aluwihare *et al. [109]*	2002	seawater	UF	BSTFA	sugars, lipids	DB5 30 m 0.32 mm i.d. 0.25 mm film thickness and DB5 30 m 0.25 mm i.d. 0.20 µm film thickness	55-320 and 150-250	radiocarbon, FID NMR
Jandl *et al. [76]*	2002	freshwater	reverse-osmosis, freeze-drying	TMAH	lipids	BPX 5 25 m 0.32 mm i.d. 0.25 µm film thickness	150-280	MS
Weishaar *et al. [110]*	2003	freshwater	XAD™	TMAH	DOM	DB5 30 m 0.32 mm i.d. 1 µm film thickness	60-280	UV, MS, NMR
Page *et al. [80]*	2003	freshwater	freeze-drying	thermal pyroloysis	DOM	DB5 30 m 0.32 mm i.d. 0.2 µm film thickness	35-280	MS
Frazier *et al. [112]*	2003	freshwater	freeze-drying	TMAH	DOM	RTX5MS 30 m 0.25 mm i.d. 0.1 µm film thickness	40-310	MS
Templier *et al. [114]*	2005	freshwater	XAD™	TMAH	DOM	RTX5SilMS 30 m 0.25 mm i.d 0.5 mm film thickness	50-300	MS, NMR
Maie *et al. [113]*	2005	freshwater	UF	BSTFA, TMAH	sugars, neutral lipids	DB5 30 m 0.25 mm i.d. 0.25 µm film thickness	40-310	FL, MS, NMR, TOC
Peuravuori *et al.[81]*	2006	freshwater	SEC	TMAH, TMAAc	DOM	NB1701 50 m 0.32 mm i.d. 0.25 µm film thickness	30-220	MS

PAPER	YEAR	WATER SOURCE	EXTRACTION METHOD[a]	DERIVATISATION[b]	TARGET COMPOUNDS[C]	COLUMN[d]	TEMPERATURE GRADIENT(^{o}C)[e]	DETECTOR(s)[f]
Louchoarn *et al. [115]*	2010	freshwater	SPE	CuO, BSTFA	terrigenous DOM (lignin)	VF 5MS 30 m 0.25 mm i.d. 0.25 µm film thickness	65-300	elemental, MS-MS
Lang *et al. [117]*	2012	freshwater	oxidation to CO_2	persulfate, heat	DOM	Poraplot Q 25 m 0.32 mm i.d. 5 µm film thickness	60 (constant)	TOC, IR

a *extraction techniques; UF: ultrafiltration, XAD™: amberlite XAD™, SPE: solid phase extraction;*

b *derivatisation, preparation or oxidation technique employed before analysis;BSTFA: N,O-bis(trimethylsily)-trifluoroacetamide; TMAH: tetramethylammonium hydroxide; TMAAc: tetramethylammonium acetate; CuO: Copper(II) oxide;* ***c*** *compounds targeted during the analysis; DOM dissolved organic matter;* ***d*** *employed GC column;* ***e*** *temperature gradient during the separation;* ***f*** *detection technique; FID: flame ionisation detector; TOC: total organic carbon; MS: mass spectrometry; MS-MS: tandem mass spectrometry IRMS: isotope ratio mass spectrometry; NMR: nuclear magnetic resonance; FL: fluorescence; IR: isotope ratio.*

1.3 Future directions and conclusions

The main issues in DOM analysis start with sampling. The difficulties in collecting uncontaminated samples and their extraction in an efficient way, in order to collect all the different classes of compounds within DOM, are still considerable. The most efficient approaches seem to involve the combination of different extraction techniques, which have different affinity towards the thousands of DOM constituents. The most comprehensive findings on DOM were provided after SPE or chromatographic fractionation (i.e. SEC) prior to any form of LC or GC [81; 114]. This allowed the separation of compounds that might have co-eluted in a single-technique approach.

Clearly work needs to be carried out in order to obtain more resolved chromatography and clearer subsequent HR-MS or NMR spectra, in order to be able to isolate single compounds within DOM, and multi-dimensional chromatography seems to be the approach to follow. Woods *et al.* [35], illustrated this very clearly by employing HILICxHILIC separations of DOM, to deliver more resolved NMR spectra.

Another crucial issue for DOM analysis is the use of multi-detection systems. For example, two of the most widely used detectors in DOM analysis are UV and fluorescence [17; 66; 118-121]. However, not all the compounds in DOM absorb, for example, in the UV spectrum (e.g. long-chain alkenes and lipid-like materials). Therefore, it is important to use different forms of detection, or to associate the data given from UV or fluorescence to those from “universal” detectors. An example for the latter can be refractive index (RI). Considering that every single compound has a typical RI, this would theoretically allow the detection of all the molecules present in DOM. However, this detector would

show extensive peak overlap and is usually less sensitive if compared, for example, to a UV detector.

In the end, the need of structural information within DOM components renders the combination of HR-MS and NMR essential. In particular, HR-MS is crucial as DOM exists in a wide array of compounds, with analogous molecular weight but different functional groups and structural features. In order to get the most out of MS, it is essential to use both the positive and negative mode: it is known that lipids respond much better in negative mode than the positive [122-123], therefore if the focus is to obtain information on lipid biomarkers series, this can be done by preferring negative mode HR-MS to the positive mode.

A further significant issue in DOM analysis is the presence of a large amount of isomers. MS is not able to discriminate them; and so, microgram-level NMR can provide this information, proving that MS and NMR spectra can and should be used to compensate each other.

All these remarks, other than pointing out the extreme complexity of DOM, underline that much more work needs to be done before obtaining a total understanding DOM [1]. However, the guidelines to understand DOM and therefore its role in the carbon cycle have been laid down within all these pioneering results.

To increase the opportunity for individual molecular identification within DOM, SEC has been found to be the most popular chromatographic approach to fractionate and separate DOM components. However, as with many standard spectroscopic and spectrometric methods, it is the complexity of DOM which limits the usefulness of SEC when used in isolation. For example, SEC with common absorbance detectors, alone offers limited resolution and very little

detailed structural information. Indeed, neither UV absorbance nor fluorescence detection, either alone or couped with HPLC, are able to detect a large portion of DOM which contains neither a chromophore or fluorophore [73; 89]. In fact, to increase the opportunity for individual molecular identification within DOM, fluorescence or UV detection are usually coupled to HR-MS and/or NMR [35; 90].

In the work presented here, different chromatographic techniques have been employed with the aim to simplify DOM and create robust approaches which could lead to the isolation of single compounds from this intricate organic pool. SEC and HPCCC are proposed with the aim to fractionate and therefore simplify DOM. When SEC was employed as first dimension, DOM was fractionated according to both size and polarity according to a mixed mode retention mechanism, whereas when HPCCC was used as first chromatographic dimension, DOM was fractionated exclusively according to polarity.

IEC-PAD was also employed to fractionate DOM, according to an anion exchange mechanism which aimed to understand the differences between naturally occurring DOM and ADOM.

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Chapter 2: Fractionation of marine dissolved organic matter using size exclusion chromatography followed by reversed-phase liquid chromatography-mass spectrometry analysis.

Abstract

SEC was used as the primary fractionation method in a multi-step chromatographic approach to the molecular characterisation of marine DOM. Fractions of DOM sample collected from the SEC column were further resolved using RP-HPLC-MS/MS. Analysis of seven SEC fractions, allowed for detection of up to 142 individual peaks, with the isolation of several individual components possible in a number of fractions. Clean MS/MS spectra were obtained in many cases, often indicating analogous fragmentation patterns indictative of analogous structural backbones, with differing functionalisation, although HR-MS/MS would be necessary for further identification of isolated individual compounds. GC-FID of an unresolved hump common in varying amounts in each SEC fraction revealed a retention pattern indicative of a series of lipid-like materials.

The aim of this experiment was to develop a new separation method able to fractionate DOM through SEC, in order to obtain unique chromatograms on the second off-line dimension.

2.1 Introduction

As discussed within Chapter 1, the main issue with SEC is the selection of an appropriate eluent. Due to the wide range of chemical functionalities present within DOM, it is difficult to find the right ionic strength in order to minimise irreversible adsorption onto the stationary phase. Phosphate buffer is the most widely used mobile phase, however, the electrolyte concentration changes dramatically from one approach to another, with variations of even two orders of magnitude (see Chapter 1, Table 1.1). Mobile phase pH covers in fact a key role not only in the modification of DOM properties, but also in SEC retention mechanism, by changing the availability of the stationary phase pores towards the analytes and therefore by changing the apparent molecular weight distribution of the eluting molecules.

Such difficulties also affect the calculation of the apparent molecular weight distribution within DOM, which is also influenced by the selection of inappropriate calibrants, only representing few classes of compounds which are commonly not naturally occurring in DOM.

Due to such difficulties, DOM separations achieved to-date using SEC have been limited to partially resolved ‘humps’, pointing the way for SEC to be applied as a pre-fractionation step, followed by a secondary chromatographic separation, such as RP-HPLC coupled with MS detection [1-8].

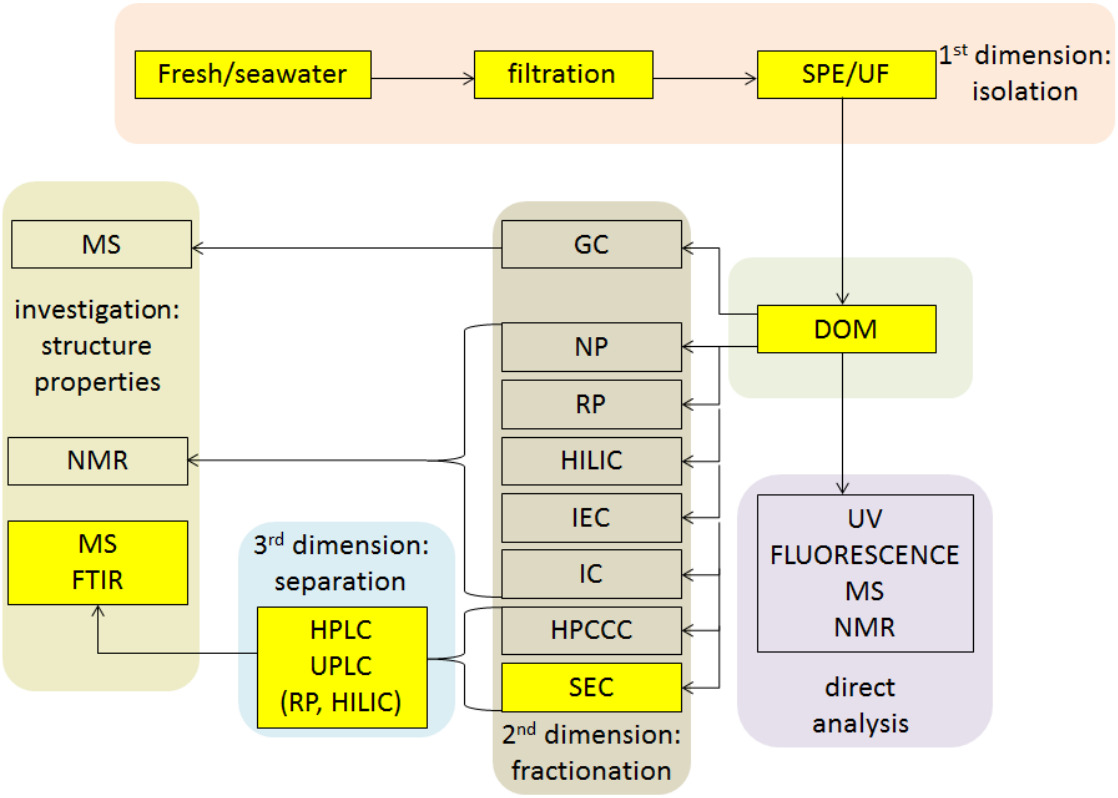


Figure 2.1: *Potential DOM analysis pathways. Highlighted is the chromatographic approach employed in this chapter.*

In particular, the application of RP-HPLC coupled to MS and NMR has proven to be partially successful [9-10] (Figure 2.1). However, even if the chromatographic profiles are different, proving that DOM had undergone a separation process, it is still clear that co-elution prevents more understandable MS or NMR profiles, which are characterised by extensive peak overlapping. For this reason, the pre-fractionation of DOM is a requirement to maximise the potential of this analytical approach.

Therefore, in the work presented here, the combination of SEC with RP-HPLC-MS/MS is examined, with the aim of determining how size-distinguished DOM fractions differ according to their polarity and molecular composition. In this initial study SEC fractions were collected off-line prior to subsequent RP-HPLC-MS/MS analysis. In addition, poorly resolved late eluting compounds

from RP-HPLC, which were not well suited to ESI-MS, were subjected to further off-line gas chromatography with GC-FID and FTIR absorption analysis.

2.2 Materials and methods

2.2.1 Reagents

The 32% HCl and MeOH employed in DOM extraction were purchased from Sigma-Aldrich (Sigma Aldrich, Dublin, Ireland). Solvents and reagents used for each of the chromatographic and FTIR analyses, namely HPLC grade tetrahydrofuran (THF), MeCN, dichloromethane (DCM), hexane and BSTFA were purchased from the same source.

2.2.2 Size exclusion cromatography

For initial DOM fractionation, SEC was employed using a Biobasic 60 SEC column (Thermo Scientific, Horsham, UK), of dimensions 300 x 7.8 mm I.D., with 5 µm particles and pore size of 60 A. The porous stationary phase particles have a silica-based core and a proprietary hydrophilic polymeric coating. The column was installed on an Agilent 1200 Quaternary HPLC system (Agilent, Santa Clara, California, USA), consisting of a 1200 Series pump, autosampler and UV detector and equipped with Chemstation software. De-ionised water with 0.1% formic acid was used as mobile phase A, with a MeCN gradient moving from 2 to 98% MeCN/0.1% formic acid (as mobile phase B) in 45 mins at 0.350 mL/min. After 45 minutes, the 98% MeCN moblie phase composition was held for 10 minutes as clean-up procedure (Table 2.1). UV absorbance detection was performed at 220 nm. All of the collected fractions, both positive and negative peaks (see Figure 2.2), were collected and concentrated. Collected fractions were subjected to MeCN evaporation under

nitrogen flow, with the remaining aqueous portion freeze-dried overnight. The DOM concentrates were then recovered in 200 µL MeCN/0.1% formic acid, which is also one of the mobile phases used during the second off-line RP-HPLC separation.

Table 2.1: *Gradient method steps employed during SEC fractionation.*

TIME (mins)	MOBILE PHASE A PERCENTAGE (v/v)	MOBILE PHASE B PERCENTAGE (v/v)	COMMENT
0 to 10	98	2	Isocratic
10 to 35	98 to 2	2 to 98	Linear gradient
35 to 45	2	98	Isocratic
45 to 50	2 to 98	98 to 2	Return to initial conditions
50 to 60	98	2	Re-equilibration

2.2.3 Liquid chromatography tandem mass spectrometry

As the second chromatographic separation step, reconsitututed DOM sample fractions were analysed using RP-HPLC-MS/MS with an ESI interface. The column used was a Dionex Acclaim C_{18} Polar Advantage II, with 3 µm particle size and 150 x 2.1 mm column dimensions. The gradient applied was from 2% to 98% MeCN/0.1% formic acid (0 to 75 min), at a flow rate of 0.300 mL/min. The HPLC-MS system was an Agilent 1220 Binary HPLC system (Agilent, Santa Clara, California, USA), consisting of a 1200 Series pump, autosampler, UV detector and connected to a Bruker high capacity trap (HCT) MS (Bruker Daltonics, Bremen, Germany) with ESI interface. The ESI-MS was operated in positive mode (capillary 4500 V, collision energy +20 V, nebulizer 10.0 psi, dry gas 5.00 L/min, dry temperature 350 ºC). From the TICs of the fractions collected from the SEC column, the base peak chromatogram (BPC) was extracted in order to minimise background ion contributions. The BPC was

then smoothed with a Gaussian algorithm (width 1.97). The MS/MS fragmentation spectra were extracted from the two most intense ions within peaks from the BPC.

2.2.4 Gas chromatography with flame ionisation detection

The GC-FID analysis was performed on an Agilent 7820 A series (Agilent, Santa Clara, California, USA), with an applied oven temperature gradient from 65 ^{o}C to 300 ^{o}C over 60 minutes. The detector temperature was set at 300 ^{o}C. The column was a Agilent J&W (Agilent, Santa Clara, California, USA) model DB-5MS with 0.50 µm film thickness, 0.25 mm ID and 30 m length. The injection port had a temperature of 280 ^{o}C and the split ratio was 2:1. The sample was diluted in hexane and injected in a volume of 10 µL. The DOM fraction was dryed and silylated with BSTFA in the presence of pyridine at 80 °C for 90 minutes before GC-FID analysis.

2.2.5 Fourier transform infrared spectroscopy

The FTIR analysis was performed using a Perkin Elmer Spectrum 100 infrared spectrometer (Waltham Massachusetts, USA), following reconstitution of the collected fraction in DCM. The sample was analysed by the conventional KBr pellet method. The scanning range was 400-4000 cm^{-1} and the resolution was 1 cm^{-1}.

2.2.6 Seawater collection and sample preparation

Seawater samples (120 L each) were collected from the Irish Sea coastline in Bray, Ireland, sourced (1) from the immediate coast, (2) at 10 m depth, and (3) at 60 m depth. They were subsequently stored in 10 L Nalgene

containers (Fisher Scientific, Dublin, Ireland) at 4 ^{o}C. The latter were washed with Milli-Q water and MeOH before sampling in order to use the same solvents used during the following extraction procedure. No HCl cleaning procedure was employed. DOM was then isolated from sea water as described by Dittmar *et al*. [11]. Briefly, the water was filtered through Nucleopore (Fisher Scientific, Dublin, Ireland) polycarbonate filter cartridges (3 µm, 1 µm and 0.20 µm pore sizes sequentially) and Whatman GF-F filters (0.20 µm pore size) (Fisher Scientific, Dublin, Ireland) and acidified drop by drop to pH 2.0 with concentrated HCl. This step was performed immediately after sampling in order to preserve the sample. The water was then passed through PS-DVB Bond Elut cartridges (1 gr, 60 mL) (Varian, Stockport, UK). Before loading the sample, the latter were previously cleaned with MeOH. Before elution of DOM, the cartridges were rinsed with two cartridge volumes of 0.01 M HCl for complete removal of salt and the DOM eluted within one cartridge volume of MeOH, and subsequently concentrated by high vacuum evaporation. The obtained DOM, the appearance of which is a fluffy brownish powder, was stored at -80 ºC prior to any analysis in order to preserve the sample from any degradation process or microbial activity. The DOM was then weighed (0.3 mg) and dissolved into 300 µL THF/MeCN, in a 2:1 ratio in order to dissolve even the most refractory and least polar components. A 1.0 mg/mL solution was hence obtained. The prepared sample was sonicated for 20 minutes to fully disperse the material in solution, and then vortexed for 15 seconds to obtain a homogeneous solution (pale yellow colour). All solutions were filtered through Swinney syringe filters (13 mm diameter and 0.22 µm pore size) (Pall Corporation, Ann Arbor, Michigan, USA) to remove any non-dissolved portion. The solution was then transferred into an amber vial in order to UV degradation, prior to SEC chromatographic analysis.

2.3 Results and discussion

In this study 120 L of coastal seawater was filtered in order to isolate DOM. The filtrate was then acidified to increase the affinity of the organic matter to the SPE PS-DVB cartridges (see Section 2.2). According to the literature, the above process is not quantitative [11-14], and it is known that the extraction method may exclude the bulk of small organic acids or uncomplexed ions (Figure 2.1, isolation).

In preliminary investigations several chromatographic methods were considered as a means to fractionate DOM. Since DOM is characterisatically high in carboxylic functionalities and charged species [15-17], IEC was initially considered. However, IEC proved unsuccessful, with a large non-retained fraction followed by a very broad unresolved 'hump'. Both normal phase and RP-HPLC [9] were also considered, but in both cases the complexity of the unfractionated sample appeared to result in irreversible absorption issues and sample carryover between runs. Related to normal phase high-performance liquid chromatography, HILIC was also considered, as previously used by Woods *et al.* in combination with multi-dimensional NMR and mass spectrometry [3; 18-19]. However, once again the DOM sample provided a large unresolved 'hump', eluting across the majority of the chromatogram, and so was not considered further.

2.3.1 Size exclusion chromatography

In previous studies employing SEC to separate or characterise DOM, phosphate buffers are often employed as the mobile phase [1-3; 20-22]. However, the composition of DOM is known to be highly sensitive to pH

changes [9; 23]. For example, Koch *et al*. [9], demonstrated how a chromatographic separation can change dramatically if the mobile phase pH is lowered below 4, with a shift to significantly higher retention times following protonation of functional groups (i.e. carboxylc acids, ethers and esters) (using RP-HPLC). A further issue specifically related to SEC of DOM, is the absence of suitable standard calibrant molecules, which limits the accuracy to which molecular mass ranges can be assigned to the eluting peaks. This issue was recently highlighted in a paper by Yan *et al.* [20], where SEC was used to identify the molecular weight distribution of DOM. The results showed a molecular weight range from 3 to 16 kDa. However, the selected calibrant was polyethylene glycol, which may not be an appropriate standard for the complex molecular variety found within DOM, this being a potential cause of the measurement errors of up to ± 30% reported (see Chapter 1).

Correct choice of standards for SEC is important as secondary solute-gel interactions within SEC are well known [24-25]. Surface adsorption, both hydrophobic and electrostatic are in many instances unavoidable, obviously influencing retention time and peak shape, and hence the apparent molecular weight. Considering the complexity of DOM, which is known to be rich in carboxyl and hydroxyl groups, and fused aliphatic and aromatic rings [15-16], possible secondary interactions with the stationary phase have to be considered as a likely cause of poor fractionation [3; 26]. However, recently the development of bonded silica and polymeric gels to minimise reactive surface sites has allowed SEC to become a more reliable tool for separations based upon molecular weight.

Herein, a surface bonded silica based SEC column was selected for DOM fractionation, containing 5 µm size particles (60 Å pore size), coated with

a hydrophilic polymer. The phase was also selected to allow greater flexibility in the mobile phase selection, as alternative polymer based SEC columns are sometimes prone to shrink or swell according to the mobile phase composition, often limiting the use of organic solvents [27-29]. In order to attempt to maintain some element of fractionation primarily based upon molecular size, and circumvent irreversible adsorption issues, the use of a buffer was avoided and an organic solvent gradient utilised. The exclusion of potentially non-volatile buffers also improved compatibility with downstream fractional analysis using RP-LC-MS/MS, and maintained the DOM sample in similar aqueous solvent systems throughout the various extraction, fractionation and analytical separation phases. However, it is likely therefore, under these conditions, the SEC fractionation stage can be more accurately described as being based upon a mixed retention mechanism, involving the size based retention between solute and exclusion pores, together with separation based upon solute polarity.

Figure 2.2 shows the SEC chromatograms for each of the three coastal seawater (Bray) DOM samples, with UV absorbance detection at 220 nm. For each DOM sample, all the observable peaks eluted from 18 minutes onwards (~ 20% MeCN), with the last peaks eluting at approximately 35 minutes (~ 75% MeCN). (Although data not included herein, the fractions from 0-18 minutes and > 45 minutes were collected, concentrated and analysed using LC-MS/MS, with no significant content identified).

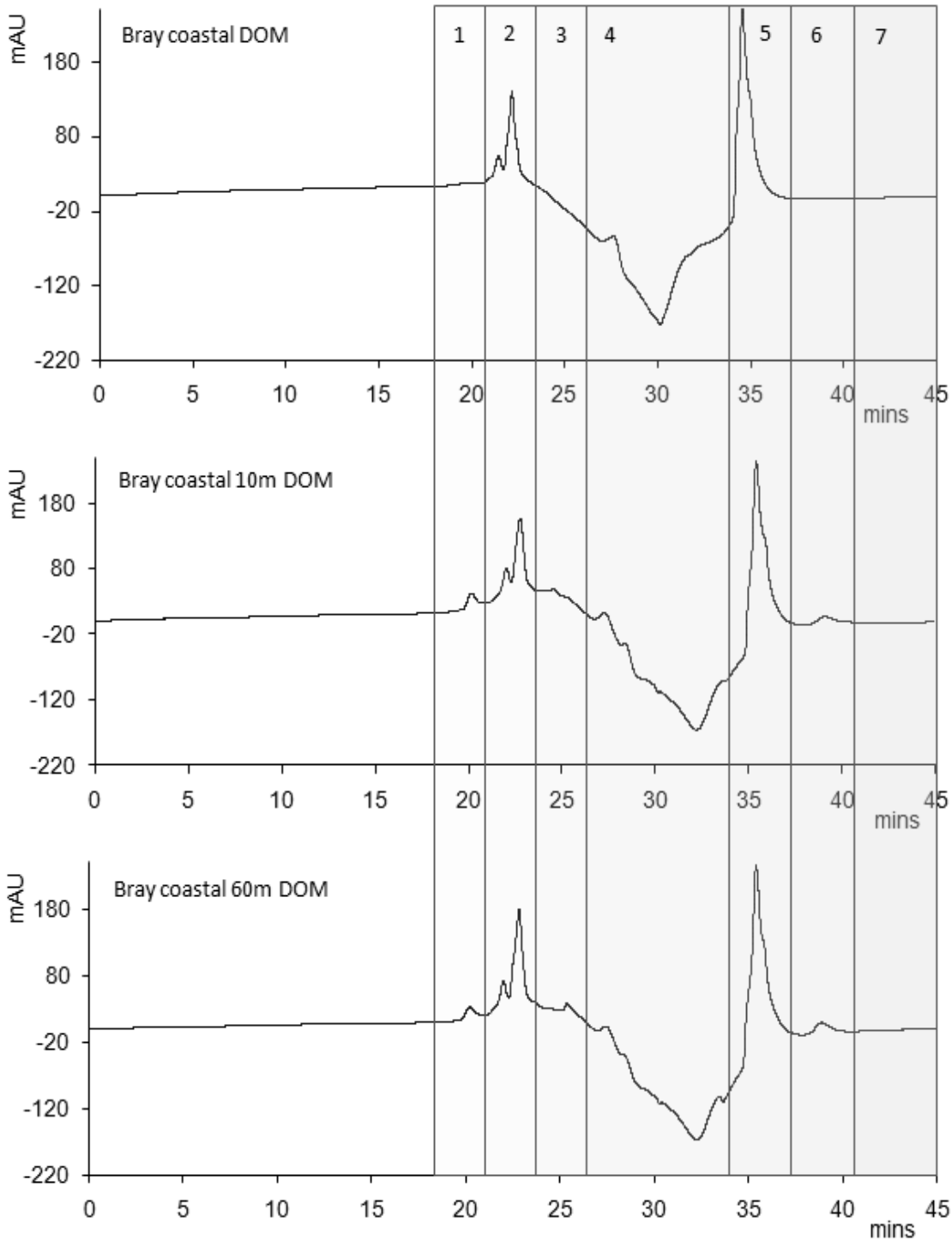


Figure 2.2: *SEC for the 1 mg/mL DOM sample collected at Bray coastline. Detection at 210 nm, gradient from 2% to 98% MeCN/0.1% formic acid at 0.350 mL/min. The dashed lines represent the times when the enumerated fractions were collected.*

The SEC chromatograms were each dominated by three obvious features, a number of peaks co-eluting at 20-25 minutes, a significant negative

unresolved broad peak eluting from 25 to 32 minutes, and a large unresolved, yet relatively sharp, peak eluting at ~ 33 minutes. It was expected that the large negative portion of the chromatograms corresponds to the class of hydrophobic compounds, including mid-sized fatty acids, sterols and lipids. The highest absorbing peak (> 33 minutes) was presumed to represent the bulk of the lowest molecular weight components.

Negative peaks in SEC arise when too strong interactions between sample and stationary phase are present. Given the mixed mode retention mechanism and the increased hydrophobicity of the gradient, compounds with a higher affinity towards the low polar stationary phase started to elute within this portion of the chromatogram, where the concentration of MeCN approached its maximum. This means that low polarity compounds are retained within this chromatographic fraction, such as lipid like materials.

2.3.2 Reversed-phase liquid chromatography

The retention mechanism in RP-HPLC can be considered orthogonal to that of the hydrophilic SEC phase used for fractionation, with large hydrophobic molecules retained longest upon the C_{18} stationary phase, with the higher concentrations of MeCN required to complete elution. Utilising this orthogonal approach provides maximum opportunity to improve resolution of the DOM sample.

Figure 2.3 shows the RP-HPLC-MS BPCs, obtained using positive mode ESI, for each of the 7 SEC fractions collected. The chromatograms clearly show a considerable number of component features spread across each gradient chromatogram, with distinct regions of partially and unresolved peak clusters. In total the BPCs indicated the presence of up to 142 resolved or partially resolved

peaks, with approximately 30 of them recurring at varying intensities on all of the chromatograms. This number of peaks obtained, although encouraging, still represents a minor fraction of the estimated total number of components within DOM, proposed to number within the several thousand [15-16; 30-31]. However, only those molecules suited to positive ionisation ESI-MS are detectable here. In addition, given the estimated molecular weight range within DOM of 3 to 16 kDa [20], the full molecular size range cannot be analysed by ESI-MS, since unless the compounds are multiply charged only molecules less than the 2 kDa on most mass spectrometers can be detected.

Despite these limitations, a number of distinct features can be identified, which appear to be spread across multiple fractions, the clearest example of which is the region of the chromatograms from 50 to 65 minutes (as highlighted within Figure 2.3), where the MeCN gradient is approaching 75-85%.

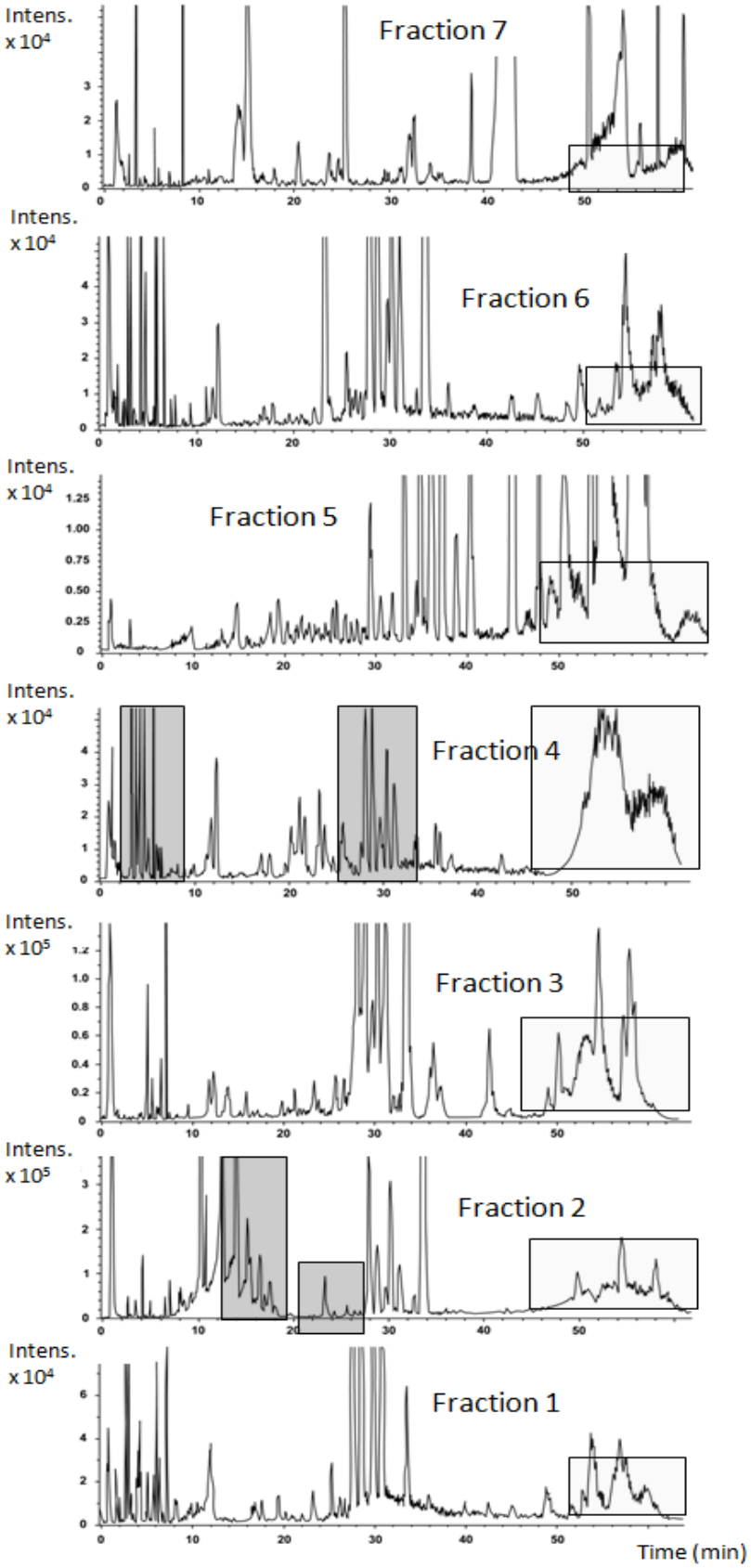


Figure 2.3: *RP-HPLC BPC (positive mode) for the SEC fractions (capillary 4500 V, nebulizer 10.0 psi, dry gas 5.00 L/min, dry temp. 350 ºC). Gradient: 2% to 98% MeCN/0.1% formic acid (0.300 mL/min). BPCs smoothed with a Gaussian algorithm (1.97 width).The highlighted areas represent the most distinguishing features on each chromatogram.*

This dominant feature is at its greatest intensity across fractions 2 to 4, corresponding to the large negative unresolved peak within the SEC chromatogram. This would support the suggestion that this feature corresponds to hydrophobic and lipid-like structures, which also generally have a relatively poor response in positive mode ESI-MS. Commonly, only species which are polar and can hold a charge are able to be detected through ESI-MS. Due to their apolar nature, lipids do not have this capability, therefore are usually difficult to analyse with this kind of ionisation mode. Other features, more characteristic to each fraction are as follows. Fraction 1 shows a cluster of early eluting polar compounds, followed by the elution of a partially resolved hump between 25-40 minutes. Fraction 2 shows an earlier eluting partially resolved region between 5 and 20 minutes. Fraction 3 shows several features similar to fraction 4, although a cluster of peaks eluting between 27 and 32 minutes are present at relatively high intensities within this fraction. Fraction 5, which corresponds to a distinctive positive peak(s) within the SEC chromatogram, is characterised by a large region of relatively well resolved peaks from 30 to 60 minutes, the latter of which co-elute with the large hump region of fractions 3 and 4. Fraction 6 shows similarity to fraction 5, in this 30-60 minute region, albeit at lower intensity, whilst a peak rich zone from 0-15 minutes reappears.

From the above observations, a large number of resolved and partially unresolved peaks can be seen to elute within each fraction in the region of 20-40 minutes, within the mid-range of the applied solvent gradient, which would be indicative of a high proportion of this DOM sample consisting of intermediate polarity species. Small polar organic molecules, such as amino acids, alcohols

and carboxylic acids should elute very early from the RP-HPLC column, within the 0-10 minute region. This region is rich in peaks within fractions 1 and 7 from the SEC chromatogram, which should correspond to very polar bulky molecules and these small polar species, respectively.

However, when considering the attempted size-based fractionation on the first chromatographic dimension, and the measured m/z ratios obtained for each of the peaks within the BPC, for each of the 7 SEC DOM fractions, as either $[M+H]^+$ or $[M+Na]^+$, it was not possible to establish a relationship between molecular weights and retention time. The mass spectrometer used had a relatively low resolution, thus it was not possible to determine if molecules were singly or multiply charged, or to obtain compositional information through isotope patterns. Due to this, in some cases it was not possible to determine the molecular weight of the compounds of interest or to assess a size exclusion trend across the BPCs of the analysed fractions.

Table 2.2 shows data for the most intense m/z ratio for each of the first 20 peaks present on the BPC of two representative SEC fractions, namely fraction 2 and fraction 4. Those peaks highlighted are unique to each fraction, whilst unhighlighted peaks also appear in most other fractions, to varying degrees, with no observable correlation between fraction number and peak intensity. These highlighted unique peaks appear to occur in distinct clusters, (as also highlighted within Figure 2.3), which clearly indicates groups of closely related compounds and homogenous series, successfully fractionated by SEC. The peak rich region from 25 to 40 minutes, evident in each of the fractions 1 to 6, could reflect the presence of mid-range polarity compounds, which respond in positive mode MS, representing the most predominant features in this analysis. However, it must be emphasised that under these peaks a wide range

of structural isomers can co-elute, which cannot be isolated with this kind of chromatographic approach and which are commonly present in DOM samples [15-16].

Table 2.2: *Peak number, retention times, peak area and most intense m/z ratio for the first 20 peaks* from fractions 2 and 4 collected from SEC. The MS conditions were: Capillary 4500 V, nebulizer 10.0 psi, dry gas 5.00 L/min, dry temperature 350 ºC.*

FRACTION 2				FRACTION 4			
#[a]	RT (mins)[b]	AREA[c]	m/z	#[a]	RT (mins)[b]	AREA[c]	m/z
1	0.9	1140941	56.9	1	1.4	176158	56.9
2	11.8	248287	496.2	**2**	**4.5**	**691147**	**391.2**
3	**12.3**	**279947**	**570.3**	**3**	**5.1**	**250852**	**536.3**
4	**13.9**	**367820**	**811.6**	**4**	**5.5**	**257496**	**417.4**
5	**15.2**	**40643**	**699.5**	**5**	**6.5**	**218677**	**345.7**
6	**15.9**	**139436**	**965.7**	**6**	**6.7**	**532470**	**789.3**
7	**16.6**	**27846**	**609.4**	7	11.8	248287	496.2
8	17.1	70118	543.3	**8**	**12.4**	**257891**	**736.2**
9	**18**	**32335**	**501.3**	9	18.9	87987	675.5
10	19.9	167777	675.5	10	20.3	147987	316.6
11	20.3	82413	316.6	11	21.1	147123	333.1
12	21.1	230048	333.1	**12**	**21.4**	**124713**	**321.6**
13	**21.7**	**115490**	**333**	13	22.2	40474	522.9
14	22.1	40447	522.9	14	23.3	159871	505.8
15	23.3	267317	505.8	**15**	**24.3**	**94523**	**351.1**
16	**23.8**	**166545**	**365.2**	**16**	**27.8**	**166471**	**463.7**
17	**24.3**	**49627**	**365.1**	**17**	**28.1**	**1878824**	**508**
18	**25**	**34033**	**562.4**	**18**	**28.9**	**92504**	**541**
19	**25.8**	**241430**	**475.5**	**19**	**30.1**	**85506**	**585.6**
20	26.6	281837	463.7	**20**	**31.8**	**89547**	**571.6**

a: peak number; b: retention time; c: peak area

**The peaks highlighted in bold are those which are unique to each fraction.*

2.3.3 Mass spectrometry data

As the current study was carried out on a relatively low resolution spectrometer, it was not possible to determine the exact formulae of either the protonated molecules or the neutral losses (structural determination of isolated peaks would require high mass resolution and accurate mass measurements).

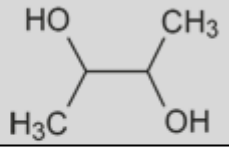
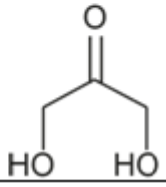
Nevertheless, herein, the two most intense ions for each chromatographic peak from each of the SEC fractions were subjected to MS/MS. These are labelled as red diamonds on the MS spectrum and as blue diamonds on the MS/MS spectra (Figures 2.4-2.7).

It was not possible to obtain clear fragmentation patterns for all the BPC peaks observed within the collected fractions, and in most cases the intensity of the chosen ions did not correspond to information rich fragmentation patterns. For example, Figure 2.4 shows typical MS and MS/MS spectra for a peak eluting at 36.4 minutes from SEC fraction 3. The m/z 523 ion (from m/z 541 parent) is due to the loss of water, which only indicates the present of an oxygen containing functional group. Within the MS/MS spectrum for the m/z 487 ion (Figure 2.4), it is possible to recognise a peak with higher molecular weight than the precursor ion. This is probably due to the presence of solvent clusters within the analysed specie. Figure 2.5 shows MS spectra from the peak eluting at 38.3 minutes from the SEC fraction 5. Here once again the only easily recognisable loss was 18 (m/z 596 – m/z 578), due to the loss of water. Figure 2.6 (spectra for the peak at 40.2 minutes from SEC fraction 5) and 2.7 (peak at 39.2 minutes from SEC fraction 7) also show only losses of water. However, interestingly, Figures 2.5 to 2.7 also all show losses of 90 in their MS/MS spectra (i.e. Figure 2.7: m/z 740-650 m/z). These can be assigned to a loss of a neutral molecule, such as $C_4H_{10}O_2$ or $C_3H_6O_3$ (Table 2.3). Similar fragmentation can be observed in Figures 2.5 and 2.6, indicating that the compounds present are likely to have analogous structural backbones.

Further to the above, in Figures 2.5-2.7 it is possible to recognise a lack of fragmentation occurring within the MS/MS spectra for ions with m/z 596 (Figure 2.5), m/z 688 (Figure 2.6), m/z 688 and 740 (Figure 2.7). This is

possibly due to the stable nature of the fragmented ions, which might be related to a policyclic aromatic or aliphatic nature.

Table 2.3: *Proposed structures for the most common neutral losses occurring within the BPCs.*

NEUTRAL LOSS	PROPOSED STRUCTURE
$C_4H_{10}O_2$	
$C_4H_8O_3$	

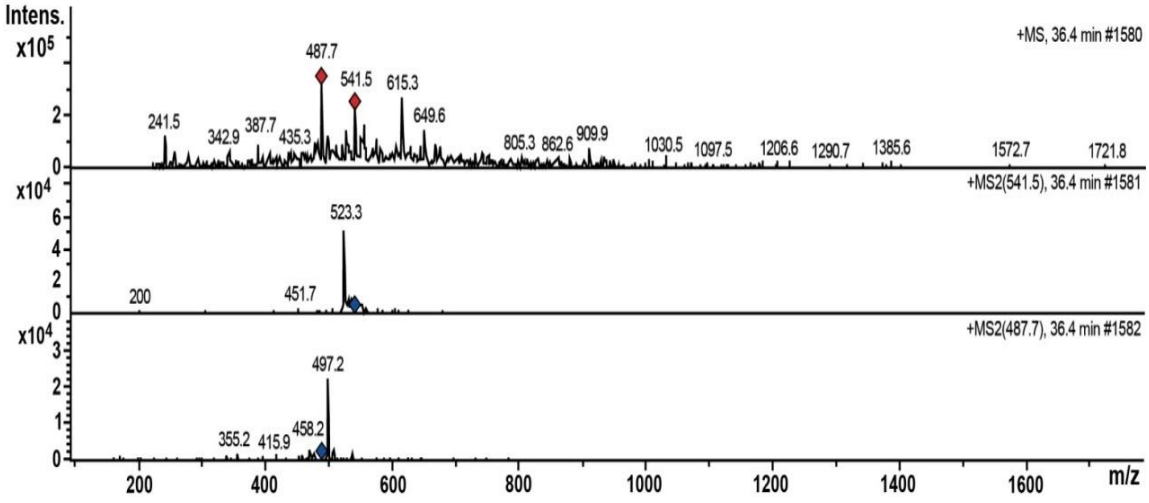


Figure 2.4: *MS and MS/MS spectra from the two most intense ions on the peak eluting at 36.4 mins in the BPC from SEC fraction number 3. Capillary 4500 V, nebulizer 10.0 psi, dry gas 5.00 L/min, dry temperature 350 ºC.*

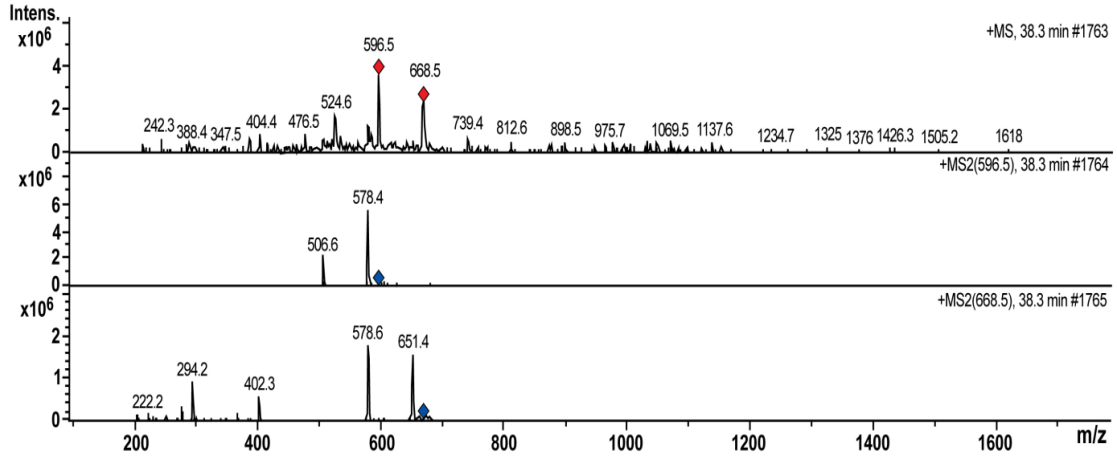


Figure 2.5: *MS and MS/MS spectra from the two most intense ions on the peak eluting at 38.3 mins in the BPC from SEC fraction number 5. Capillary 4500 V, nebulizer 10.0 psi, dry gas 5.00 L/min, dry temperature 350 ºC.*

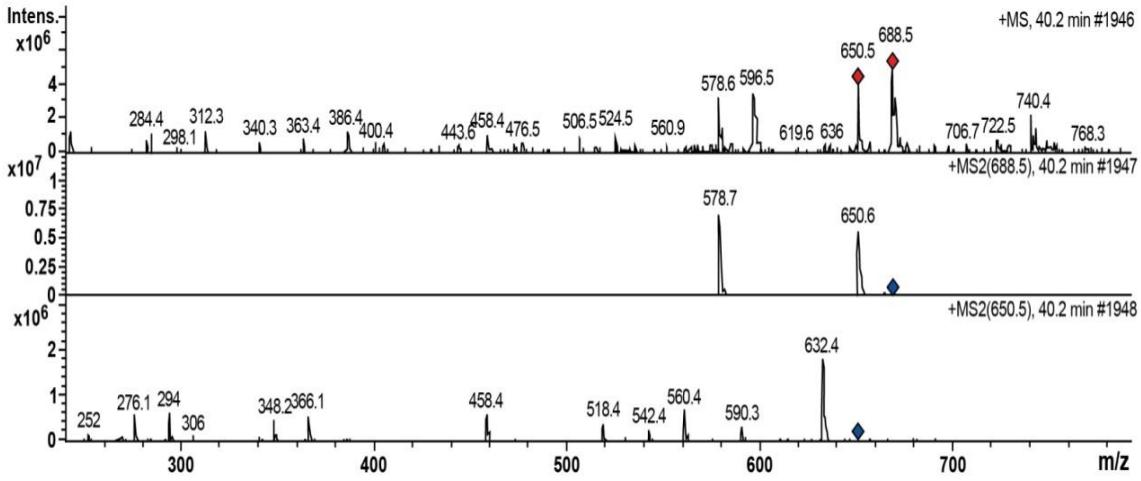


Figure 2.6: *MS and MS/MS spectra from the two most intense ions on the peak eluting at 40.2 mins in the BPC from SEC fraction number 5. Capillary 4500 V, nebulizer 10.0 psi, dry gas 5.00 L/min, dry temperature 350 ºC.*

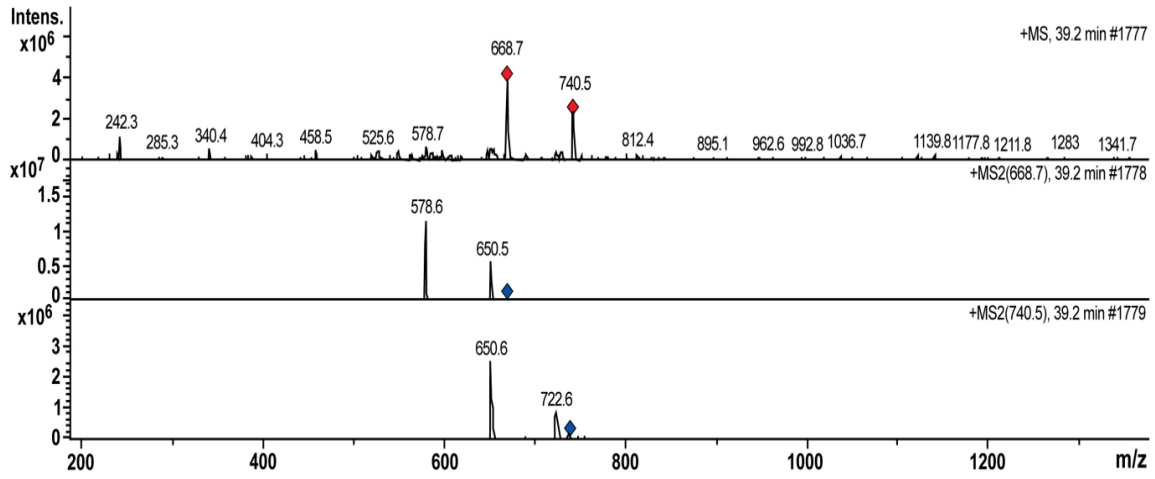


Figure 2.7: *MS and MS/MS spectra from the two most intense ions on the peak eluting at 39.2 mins in the BPC from SEC fraction number 7. Capillary 4500 V, nebulizer 10.0 psi, dry gas 5.00 L/min, dry temperature 350 ºC.*

Given the mixed elution mode of the SEC column and the fact that these molecules have different retention times along the BPCs, despite the fact they have analogous cores, they would appear to have different polarities (i.e. those eluting later are less polar) and therefore contain different functional groups. According to Hertkorn *et al.* [30], MS and NMR analysis of DOM reveal the presence of classes of compounds varying only in the number of methylene groups, and mass differences corresponding to variation in unsaturation and exchange of oxygen versus CH_2 units. It is therefore reasonable to conclude that the spectra shown in Figures 2.5 through 2.7, are potentially representative of an homologous series of molecules, bearing analogous structural features, but differing degrees (and nature) of functionalisation.

The spectrum on Figure 2.8(a) (peak at 12.4 in the BPC of SEC fraction 4) is dominated by m/z 736 as the most intense ion. The latter loses 155 to produce the product ion at m/z 581(Figure 2.8(b)), and also a product ion at m/z 298. In the absence of accurate mass data no obvious interpretation could be made from these losses. The MS in Figure 2.8(a) also indicates possible co-

elution of other compounds (i.e. m/z 824), which were not intense enough to be selected for data-dependent further MS/MS fragmentation. The most characteristic ions reported on Figure 2.8(a) and 2.8(b) were searched on Metlin databank [32] but no match was found. The MS/MS fragmentation obtained from the m/z 282 ion (Figure 2.8c) for the major peak with retention time 33.5 minutes (Figure 2.2, fraction 2), shows a fragmentation pattern which can be related to either a compound characterised by the presence of alkyl chains, a major structural feature of DOM [35-36, 49]. However, its even $[M+H]^+$ ion implies an odd number of nitrogens in the molecule, which is consistent with oleamide. The latter showed a generally similar fragmentation pattern in the Metlin databank [32] when a collision energy of +20 V was chosen. This can be an artefact from the sample preparation or a plasticiser existing in seawater, particularly as the seawater DOM sample analysed here was obtained close to the shoreline, making it more likely to contain some contamination with compounds coming from human activity [33].

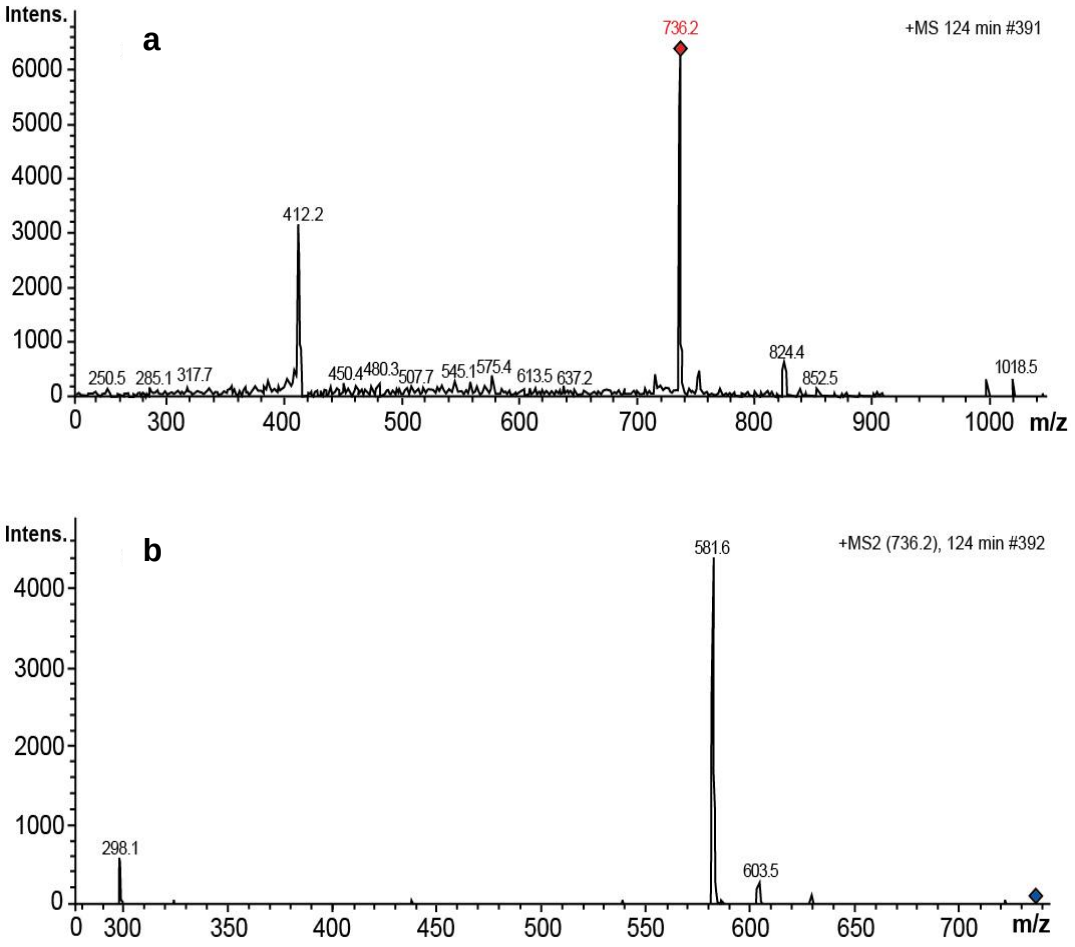


Figure 2.8(a) and 2.8(b): (a) *MS spectrum for the peak at 12.4 in the BPC of section 4 from SEC (Figure 2.2).* ***(b)*** *MS/MS fragmentation from the most intense ion on Figure 2.7a (736.2 m/z). Capillary 4500 V, nebulizer 10.0 psi, dry gas 5.00 L/min, dry temperature 350 ºC.*

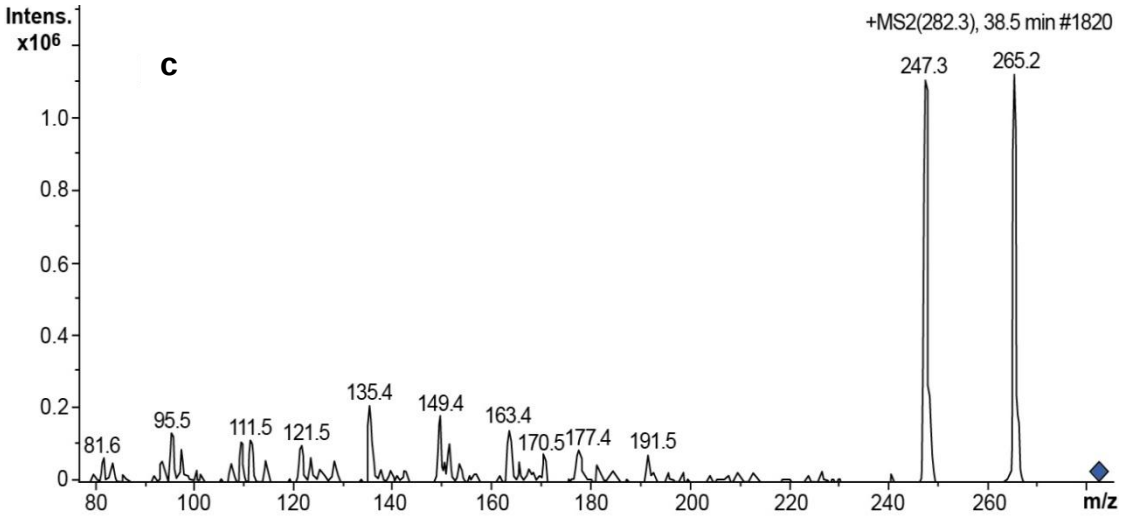


Figure 2.8(c): *MS/MS spectrum for the peak with mass 282.3 m/z at 33.5 mins in the BPC of section 2 from SEC (Figure 2.2).*

The examples of the reported molecules indicate that these structures, despite having similar core structures, have been successfully isolated within the SEC fractions and could potentially be further characterised via MS^n experiments, HR-MS, and NMR in order to achieve a structural identification of the isolated compounds.

2.3.4 Investigations of the unresolved 'hump': gas chromatography with flame ionisation detection and Fourier transform infrared spectroscopy

Where the BPCs lacked in resolution (Figure 2.2, fraction 4), an alternative chromatographic technique was applied, namely GC-FID. Clearly, given the diversity of material in DOM, not all components are suitable for separation using LC, or indeed detection using ESI-MS, such as for example, non-polar lipid materials [34-35]. On the BPC for fraction 4 (Figure 2.2), an unresolved hump can be noticed when the reversed-phase gradient achieves its maximum (98% MeCN 0.1% formic acid), from 50 to 58 minutes, within which the same MS ions could be observed for the whole period. The components of this unresolved hump were not detectable using HPLC with UV absorbance detection (data not shown), and their reversed-phase retention indicates a very non-polar class of compounds. Additionally, these compounds eluted within the middle portion of the SEC chromatograms, which appeared as a large negative unresolved group of peaks. It is therefore reasonable to assume these may well be lipid-like materials.

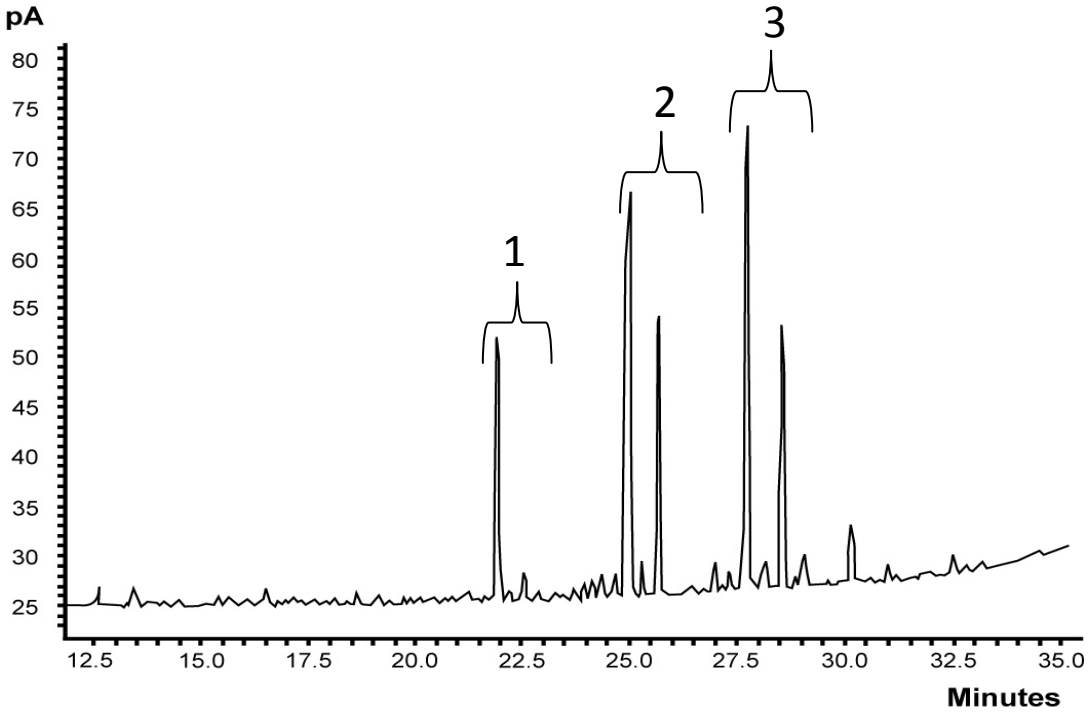


Figure 2.9: *GC-FID for the "hump" on fraction 4 (Figure 2.2) showing three series of compounds. Temperature gradient from 65 ^{o}C to 300 ^{o}C in 60 minutes, inlet heater 280 ^{o}C, split ratio 2:1. Detector temperature 300 ^{o}C.*

The unresolved hump was therefore collected and analysed by GC-FID, following (BSTFA)-pyridine derivatisation. The resultant gas chromatogram (Figure 2.9), shows after 20 minutes, a trend that, although not conclusive without the use of MS confirmation, is typical of a series of lipid-like molecules (usually, the higher the retention time, the longer the lipid chains, members of homologous fatty acids emerge at approximately equal time intervals, giving symmetrical peaks of approximately equal width on the chromatogram) [36-37].

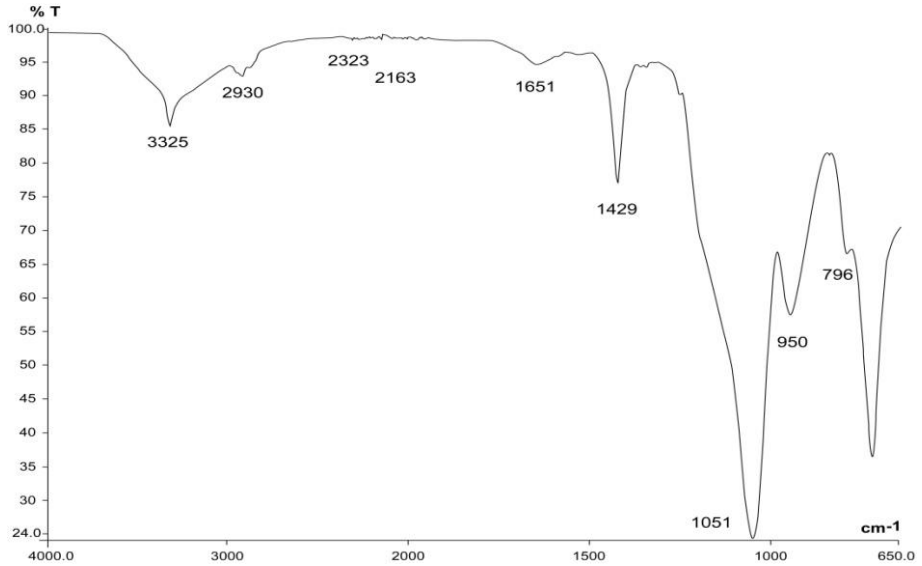


Figure 2.10: *FTIR for the hump on fraction 4 (Figure 2.2). Sample analysed on a KBr pellet with a scanning range from 400 to 4000 cm^{-1} and resolution 1 cm^{-1}.*

Finally, the same collected 'hump' was investigated using FTIR (Figure 2.10). FTIR analysis revealed the following main peaks, which can also be related to lipidic compounds: 3325 cm^{-1}: O-H stretching; 2930 cm^{-1}: C-H stretching; 1651 cm^{-1}: C=C stretching; 1429 cm^{-1}: C-H bending; 950 cm^{-1}: C=C bending; 723 cm^{-1}: C=C bending.

2.4 Conclusions

SEC has been applied successfully fractionate DOM components, prior to their subsequent further separation and detection using RP-HPLC-MS. Despite limited UV absorbance and ESI-MS sensitivity for many compounds known to be present within DOM, each BPC from the second separation dimension exhibited a significant number of unique features and information rich chromatograms. Some compounds with the same m/z precursor (m/z 280) were seen to elute within every BPC, indicating their lack of separation within the SEC dimension, and an unresolved late eluting 'hump' also occurs on the

BPCs of a number of the collected SEC fractions, assumed to be lipid-like material, as further supported by GC-FID and FTIR analysis.

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Chapter 3: Normal phase high-performance counter current chromatography for the fractionation of dissolved organic matter from a freshwater source

Abstract

A normal phase HPCCC method was optimised to obtain a preliminary fractionation of components in DOM from a freshwater source. In general terms, HPCCC is a form of automated liquid-liquid extraction and is ideally suited as an initial fractionation tool for complex assemblages such as DOM. The HPCCC solvent system involved a normal-phase approach with water-MeOH (1:1) as stationary phase and hexane-EtOOAc (1:1) as mobile phase. The critical experiment parameters were optimised as following: revolution speed 1800 rpm and flow rate 0.15 mL/min. Under these conditions 50 µl of a 0.50 mg/mL DOM solution was loaded. The instrument response was monitored at 330 nm, in order to consider the main portion of DOM, which includes substances such as CRAM with a different degree of functionalisation.

By optimising this system it was therefore possible to isolate materials which, according to GC-MS analysis, can be related to molecules with analogous structural background. Where fraction analysis was not suitable for GC-MS, RP-HPLC with UV absorbance detection was used, showing unique chromatograms for each fraction at both 210 and 330 nm.

The aim of this study was to employ HPCCC as a new approach to fractionate DOM according to polarity and to further investigate the so obtained fractions by different chromatographic approaches (GC and LC).

3.1 Introduction

HPCCC is a form of automated liquid-liquid extraction, which has proven to be an excellent way to fractionate natural extracts, primarily as a first step in the isolation of specific compound categories or single molecules [1-3]. An HPCCC system employs a rotating coil consisting of a polytetrafluoroethylene (PTFE) tubing connected to a rotating bobbin. When in motion, the column rotates about its own axis and revolves around the centrifuge axis. This kind of movement provides a continuous elution of mobile phase through the rotating separation column in order to provide a hydrodynamic motion of two solvent phases within the rotating column due to the Archimedean screw effect. When two immiscible solvents are introduced into the column, the rotation separates the two phases along the column length (Figure 3.1). The lighter phase will occupy the head of the column, the heavier the tail. Hence, other components present inside the coil with different densities (like an injected solute) are driven toward the head of the column.

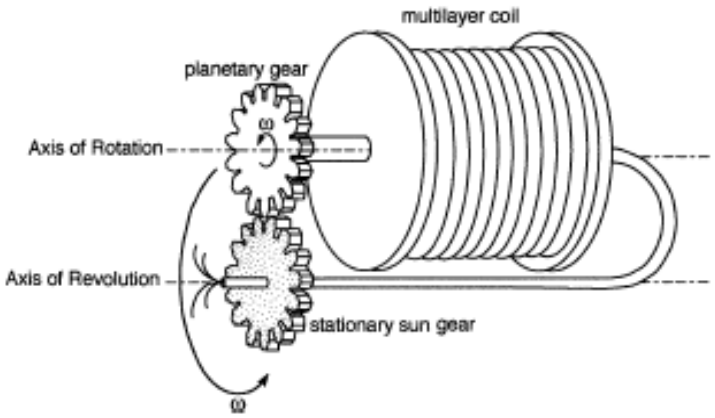

Figure 3.1: *Diagram for an HPCCC apparatus [4].*

Operatively, HPCCC can be operated in either normal or reversed-phase mode. In the first instance, the stationary phase is an aqueous solution, while the mobile phase must be an organic solvent immiscible with water. Conversely,

in reversed-phase HPCCC, an aqueous medium is used as mobile phase and an organic solvent, immiscible in water, as stationary phase. Since HPCCC uses no solid support matrix, irreversible adsorption and loss of samples onto the solid stationary phase of conventional column chromatography is minimised.

The main challenges of HPCCC are in the optimisation of the separation procedure, which is very time consuming, especially if attempting to fractionate a mixture of unknown materials.

The first and most important parameter to be optimised is the solvent system, which can be chosen from a large number of combinations. In particular, as Ito *et al.* suggest [4], if the search for an appropriate solvent system within the literature is unsuccessful, then “trial and error” is the only way to find the right stationary and mobile phases where the analyte is stable and soluble. The solvent system should provide a suitable partition coefficient to the analytes (between 0.5 and 1.0) and stationary phase retention (S_f), which is directly proportional to the peak resolution) [4-5].

Another key parameter in HPCCC is the flow rate. This determines the separation time, the amount of stationary phase retention and therefore the peak resolution. A low flow rate elongates the separation time but gives higher retention of the stationary phase and a better peak resolution [4-5].

The stationary phase retention is also related to the revolution speed (ω): a lower speed will reduce the volume of the stationary phase in the column leading to lower peak resolution, while higher speeds may produce sample band broadening due to an increased column pulsation [4].

HPCCC can be used in an off-line or on-line configuration, coupled with GC or LC, allowing the collection of any eluting fraction of interest, which can then be further separated/characterised.

Given the difficulty in dividing DOM into classes of compounds according to a characteristic chemical-physical property [6-8], and the wide spectrum of polarity characterising this intricate organic pool, here HPCCC was for the first time investigated as a method to fractionate SPE-DOM from a freshwater source. The aim of the study is to investigate the different classes of compounds within DOM according to their polarity [9-11]. Samples fractionated using HPCCC were then amenable to further analysis using GC-MS, and subsequently with RP-HPLC with UV detection. The optimisation of DOM fractionation by HPCCC and the ability to obtain characteristic and distinguishing features from each fraction of the HPCCC chromatogram represents a significant step towards the isolation and identification of single components from this naturally occurring organic pool.

3.2 Materials and methods

3.2.1 Instrumentation

3.2.1.1 High performance counter current chromatography separation

The HPCCC system was a Dynamic Extractions Mini model system (Dynamic Extractions, Slough, Berkshire, U.K.). The Mini system is hydrodynamic type of CCC including the coil planet centrifuge arrangement and a coiled column consisted of 35 meter long PTFE tubing of 0.8 mm I.D., with total volume of 17.9 mL [12]. The external diameter was 1.6 mm, revolution radius 5 cm and the β value 0.5-0.76 (β = r/R, where r is the distance from the coil to the holder shaft and R the revolution radius). The centrifuge was tested at different rotation speeds and was connected to a Shimadzu LC10 pump (Shimadzu, Melbourne, Victoria, Australia) in order to deliver the organic phase and a Knauer HPLC pump dedicated to the lower stationary phase (Knauer,

Berlin, Germany). A Waters 485 (Waters, Milford, USA) UV detector was employed in single wavelength mode at 330 nm. The normal phase HPCCC was performed using de-ionised water/MeOH (1:1) as the lower stationary phase and hexane/EtOOAc (1:1) as upper mobile phase. The solvent system was investigated at different flow rates.

3.2.1.2 Gas chromatography with mass spectrometry detection

Five fractions were collected from the HPCCC, concentrated and recovered in 500 µL hexane and injected (2 µL) into an Agilent GC-MS (Agilent, Santa Clara, California) composed of the following modules: 5975C VL MSD, 6850 Network GC system and a 7683B series injector. The injection was in split (1:2) mode and the column was an Alltech capillary column with EC-WAX stationary phase. The column length was 15 m, the internal diameter 0.53 mm and the film thickness 1.20 µm. The gradient temperature went from 50 to 300 ^{o}C in 90 minutes, with a collection window from 50 to 800 m/z. The MS parameters were: emission voltage 1612 ABS, solvent delay 2.5 minutes, acquisition mode: scan. The GC-MS databanks which were employed included: Terpenlib, Mainlib, Replib, Nist_RI and Nist_MSMS. Only the compounds showing at least 80% matching level were considered and were verified by studying fragmentation patterns and consulting the literature. The calibration mixture was from Sigma Aldrich (Sigma Aldrich, Sydney, Australia) and included alkanes with chains from 8 to 20 carbon units.

Blanks were run before each analysis in order to confirm the absence of any contamination from solvents or sample preparation.

3.2.1.3 Reversed-phase high-performance liquid chromatography

For those fractions obtained from HPCCC which did not show any significant peaks or differences within the GC chromatogram, further analysis via RP-HPLC with dual wavelength detection (210 and 330 nm) was performed. The system used was a Waters 2695 (Waters, Millford, USA) Separations Module equipped with a Waters 2487 dual absorbance detector. The column was a C_{18} Novapak (Waters, Millford, USA) with dimensions 150 x 3.9 mm and 4 µm particle size. The flow rate was 0.8 mL/min in a 15 minutes long isocratic mode (2.5% MeCN in water); the injected volume 100 µL.

3.2.2 Reagents

The MeOH and HCl used in DOM extraction were both obtained from Sigma Aldrich (Dorset, U.K.). For HPCCC separations, hexane was purchased from Emsure (Merck, Kilsylth, Victoria, Australia), while EtOOAc and MeOH from Sigma Aldrich (Sigma Aldrich, Sidney, Australia). For RP-HPLC and GC analysis, respectively, MeOH and hexane were both purchased from Sigma Aldrich (Sigma Aldrich, Sidney, Australia). De-ionised water was obtained using a Milli-Q system (Millipore, Watford, U.K.).

3.2.3 Preparation of the solvent system

A two-phase solvent system composed of hexane-EtOOAc-MeOH-water (1:1:1:1, v/v/v/v) was prepared. The solvent mixture was equilibrated in a separating funnel at room temperature and the two phases were divided before use. The sample solution was prepared by dissolving 1.00 mL of a 1.00 mg/mL solution of sample in 1.00 mL lower stationary phase (1 to 2 dilution) of the solvent system for isolation and purification.

3.2.4 Freshwater collection and sample preparation

The freshwater sample (10 L) was collected from the source of river Shannon (54^{o} 14'05''N, 7^{o}55'08''W, collected in May 2011), in Ireland. They were stored in 10 L Nalgene containers (Fisher Scientific, Dublin, Ireland) at 4 ^{o}C. The containers were washed with de-ionised water before sampling. The samples were extracted as described in Chapter 2 by following the method proposed by Dittmar *et al.* [13].

3.3 Results and discussion

3.3.1 Selection of the biphasic solvent system

It should be first noted that the method employed to isolate DOM after the filtration step was SPE [13], which eliminates the presence of highly polar compounds (e.g. small organic acids) and ions (unless complexed), which are non-retained. However, a dominant presence in DOM, are the quinone-like materials which form the basis of CRAM, and thus a particular target for this investigation. These compounds are characterised by low polarity indexes, due to their steroid and hopanoid-like structures and carboxyl functionalities conjugated with double bonds. For this reason, UV absorption at 330 nm was the detection technique employed with the HPCCC system.

Initial HPCCC fractionations were attempted using a biphasic solvent system composed simply of water (relative polarity 1.000 [14]) as stationary phase and hexane (relative polarity 0.009 [14]) as mobile phase. Different flow rates, rotation speeds and loadings were tested but this system showed little separation or retention for any class of compounds visible at 330 nm. Due to the big difference in polarity between the two, and given this lack of retention, it was

clear that the target compounds were not interacting sufficiently with the water stationary phase and a lower polarity system was necessary.

Therefore, a solvent system with a lower difference in polarity index between phases was investigated. A 1:1 composition of hexane and EtOOAc (relative polarity 0.228), with a stationary phase of water and MeOH (relative polarity 0.762 [14]) in a 1:1 ratio, was chosen. The development of such a solvent system allowed the widest range of polarities to be covered, whilst mobile and stationary phase remained immiscible. This approach showed promising results, which prompted further investigation of the chromatographic conditions. However, as DOM contains thousands of unknown structures [9; 15], it was not possible to calculate a specific partition coefficient and separation factors for these chromatographic conditions.

3.3.2 Variation of mobile phase flow rate

Flow rate (*F*) is one of the key parameters for the optimisation of separation in HPCCC. The chromatographic performance of a column in HPCCC is defined by the stationary phase retention ratio, S_f,

$$S_f = V_S/V_C \qquad (Eq.\ 1)$$

Where, V_S and V_C are stationary phase and total column volumes, respectively. According to Sutherland *et al.,* [12; 16] S_f can be expressed by formula:

$$S_f = 100 - \frac{800}{\omega d_C^2}\sqrt{\frac{2\eta_M LF}{K\pi\Delta P}} \qquad S_f = 100 - \frac{800}{\omega d_C^2}\sqrt{\frac{2\eta_M LF}{K\pi\Delta P}}$$

Where, *K* is a constant, d_C and *L* are internal diameter and length of tubing, respectively, ω is rotation speed, *R* is the rotor radius, η_M is the dynamic

viscosity of a mobile phase and ΔP is a pressure drop in the column. Clearly, both the stationary phase retention ratio, S_f, and the thickness of the stationary layer at the internal surface of PTFE tubing affect both separation efficiency and maximum loading capacity of the column, and are dependent on flow rate F and rotation speed ω. The linear dependence of S_f from $\sqrt{F}$ in CCC at constant ω was observed by Du *et. al.* [17] for various two phase solvent systems.

In this set of the experiments a full loop injection (50 µL) was performed at 1800 rpm with a 0.50 mg/mL DOM solution (1:2 dilution from the 1.0 mg/mL mother solution). As can be seen from Figure 3.2 at a flow rate of 1.0 mL/min, no separation occurs between the sample constituents. However, at the slower rate of 0.5 mL/min, several sample fractions start to appear. Further investigation revealed the best separation could be found at a flow rate of 0.15 mL/min, where 3 distinct zones of the chromatogram can be distinguished over the elution period. In HPCCC, lowering the flow rate provides more time for the solutes present to partition between stationary and mobile phase, providing better resolution [18-19]. However, this obviously lengthens the chromatographic run time.

The S_f values were evaluated at different flow rates according to the Eq. 1. Where V_C is the column volume (17.9 mL) and V_M the volume of mobile phase used during the separation (i.e. 2.25 mL for a 0.15 mL/min flow rate, 7.50 mL for 0.50 mL/min and 15.0 mL for 1.00 mL/min). The following S_f (%) were calculated for each flow rate: 16.2% at 1.00 mL/min, 58.1% at 0.50 mL/min and 87.4% at 0.15 mL/min. Obviously, at 0.15 mL/min there is a higher column capacity available, consequently increasing the resolution.

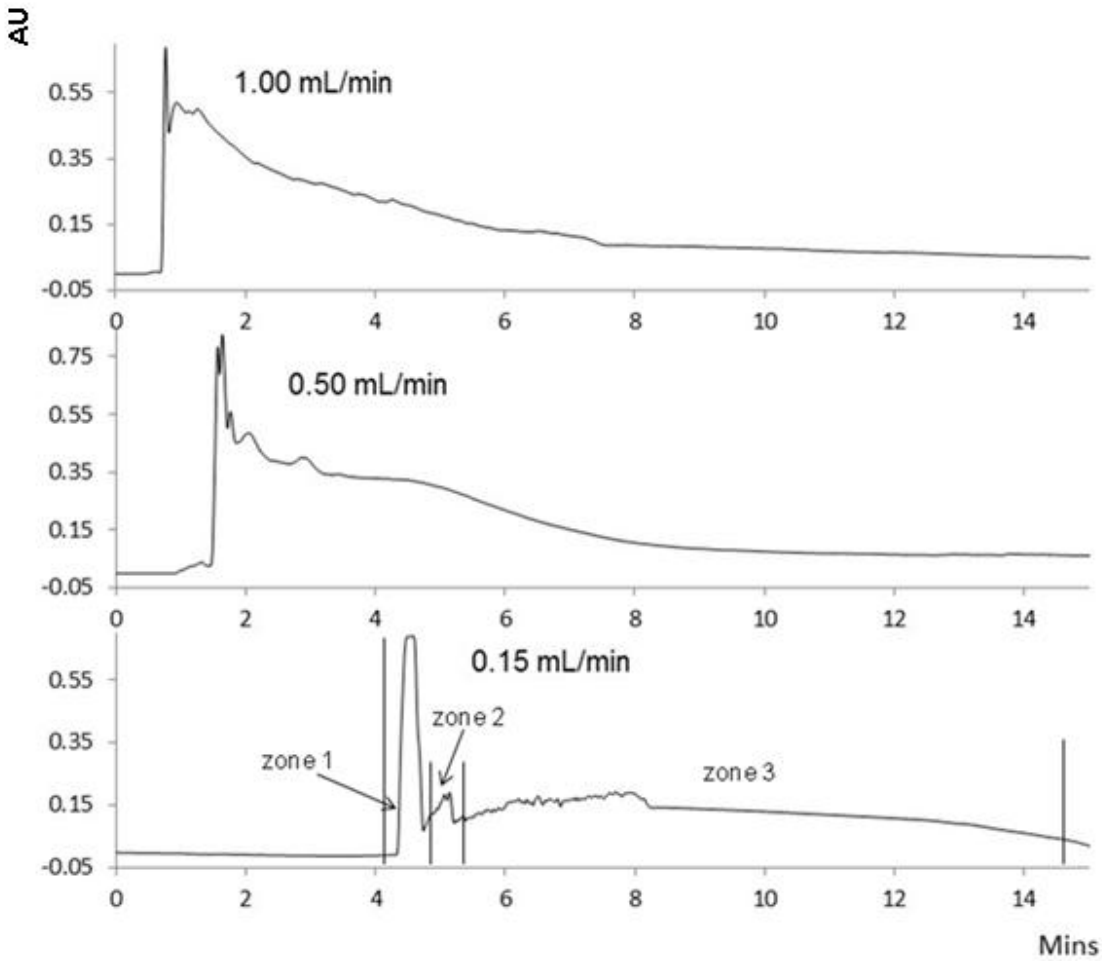

Figure 3.2: *HPCCC separations of DOM sample at different mobile phase flow rates. Conditions: normal phase hexane/EtOOAc/water/MeOH (1:1:1:1) solvent system, at 0.15 mL/min to 1.00 mL/min flow rate, rotation speed 1800 rpm, with UV detection at 330 nm.*

At 0.15 mL/min, three distinct zones on the chromatogram are apparent. The first zone/fraction exhibits significant UV absorbance at 330 nm (4.3 to 4.5 min) and likely contains molecules with a complex systems of double bonds conjugated with carboxylic groups or aromatic functionalities, which represent a main component of DOM [9; 20]. This fraction contains those compounds with the lowest affinity towards the stationary phase (water and MeOH 1:1), therefore with the lowest polarity within the DOM mixture. Fraction 1 is therefore potentially the richest in those hopanoid-like molecules which constitute the DOM core [20].

Solutes with an intermediate degree of polarity can be seen to be separating from the first major peak at flow rates of 0.5 mL/min and below, evident as smaller peaks between 2 and 4 minutes at 0.5 mL/min, and as a relatively isolated group between 4.8 and 5.1 minutes, at a flow rate of 0.15 mL/min. A large unresolved 'hump' of more polar solutes elutes between 3 and 8 minutes at 0.5 mL/min, and over an extended window of 5.1 to 14 minutes at the 0.15 mL/min flow rate (zone 3, Figure 3.2). The limited UV response for the later eluting fraction, suggests the presence of compounds with higher polarity, particularly if compared to those eluting at the beginning of the chromatogram. This fraction likely includes short alkylic chains with poor conjugation between carboxylic groups and double bonds.

3.3.3 Revolution speed

For the same instrument and column having constant values of d_C, L, ΔP, and with the eluent of constant composition, which also means constant value of η_M, the simplified version of the equation 2 is correct:

$$S_f = 100 - \frac{K\sqrt{F}}{\omega} \qquad S_f = 100 - \frac{K\sqrt{F}}{\omega}$$

Showing that the stationary phase retention ratio (S_f) depends on flow rate and ω. At the same time, the resolution, R_S, in HPCCC can be expressed by the following formula [12]:

$$R_S = \frac{\sqrt{N}}{4} \times \frac{S_f(K_{D2} - K_{D1})}{1 - S_f[1 - (K_{D2} + K_{D1})/2]} \qquad R_S = \frac{\sqrt{N}}{4} \times \frac{S_f(K_{D2} - K_{D1})}{1 - S_f[1 - (K_{D2} + K_{D1})/2]}$$

Therefore, the resolution should be higher with increased S_f, or at lower flow rates, *F,* and higher rotational speed, *ω.* Theoretically the column efficiency, *N*, increases with column length, *L,* and with decrease of tubing diameter, d_C, and with decrease in thickness of the layer of the stationary phase. Also the thickness of the layer is proportional to S_f and viscosity of the stationary phase. Usually, the column efficiency is not evaluated as important parameter in CCC, but according to the conditions reported by Berthod *et al.* the column efficiency for the standard column (35 m x 0.8 mm I.D.) of the Mini HPCCC system is approximately 3500 theoretical plates [12].

Herein DOM fractionation was investigated over speeds of 800 to 2300 rpm. The resultant chromatograms are shown within Figure 3.3. It is clear that when operating at low speeds, starting at 800 rpm, that only two clear fractions can be seen, which are only partially resolved. At 1300 rpm this remains the case, with little difference in the retention of these two fractions. However, while operating at speeds of 1800 rpm, the DOM sample separation was returned to that obtained earlier (Figure 3.2), with the separation of 3 distinct fractions. At 2300 rpm the first zone/fraction, can be seen to be further separated into three to four partially separated peaks, eluting prior to the broad unresolved 'hump', which now elutes over the extended period of 6 to 12 minutes. This partial separation of the previously non-retained peak was significant as the bulk of the DOM sample is supposed to be contained within this fraction [20]. However, unfortunately it was not possible to operate at 2300 rpm for extended periods of time due to the instrument overheating. Therefore, a compromise was found at 1800 rpm and fraction collection was carried out under these final conditions.

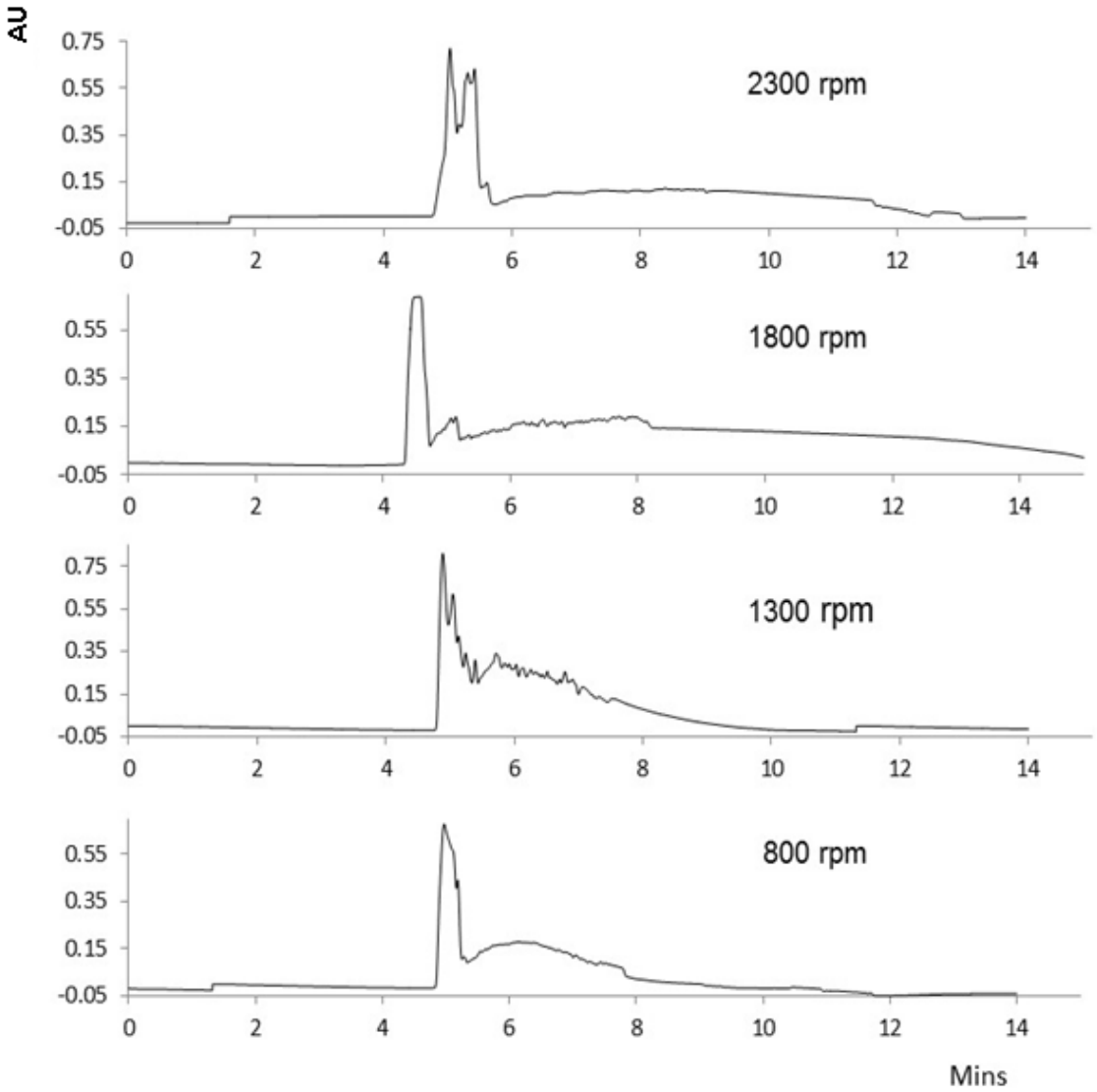

Figure 3.3: *HPCCC separations of DOM sample at different sample concentrations. Conditions: normal phase hexane/EtOOAc/water/MeOH (1:1:1:1) solvent system, at 0.15 mL/min flow rate, rotation speed 1800 rpm, with UV detection at 330 nm.*

In order to calculate column efficiency, benzoic acid was used at the same operating conditions as the DOM separation. Benzoic acid is a known component of DOM [21]. However, considering the retention time of this compound and its peak width at half height, only 127 theoretical plates were calculated for these conditions, with a plate height of 1.33 cm.

3.3.4 Concentration of the sample

As can be seen from Figure 3.4, with a full loop injection of 50 µL, the sample concentration and system capacity play a key role (see discussion in the section 3.1) in chromatographic separation (other conditions as in Figure 3.2, flow rate= 0.15 mL/min, 1800 rpm). As the aim of the separation was to facilitate sample fractionation and collection, it was desirable to inject as high a concentration of sample as possible within conditions providing highest S_f. According to the Eq.1 and 2, the higher S_f can be achieved with a lower F and a higher ω. The dynamic viscosity of mobile phase is included in expression for the coefficient K in Eq. 2 and can influence on loading capacity but it is difficult to manipulate this parameter in such complex system. Finally, the loading capacity depends on the solubility of DOM components in the mobile phase.

At sample concentrations of 1.0 mg/mL and above, more evidence of co-elution was seen, which was less prominent in the case at concentrations of 0.5 mg/mL and below. In the case of the 1.0 mg/mL injection (Figure 3.4), the zones/fractions as discussed in relation to Figure 3.2, were less resolved from each other, therefore subsequent fraction collection was compromised. Thus the 0.5 mg/mL injection represented the best compromise in terms of sample concentration and chromatographic resolution.

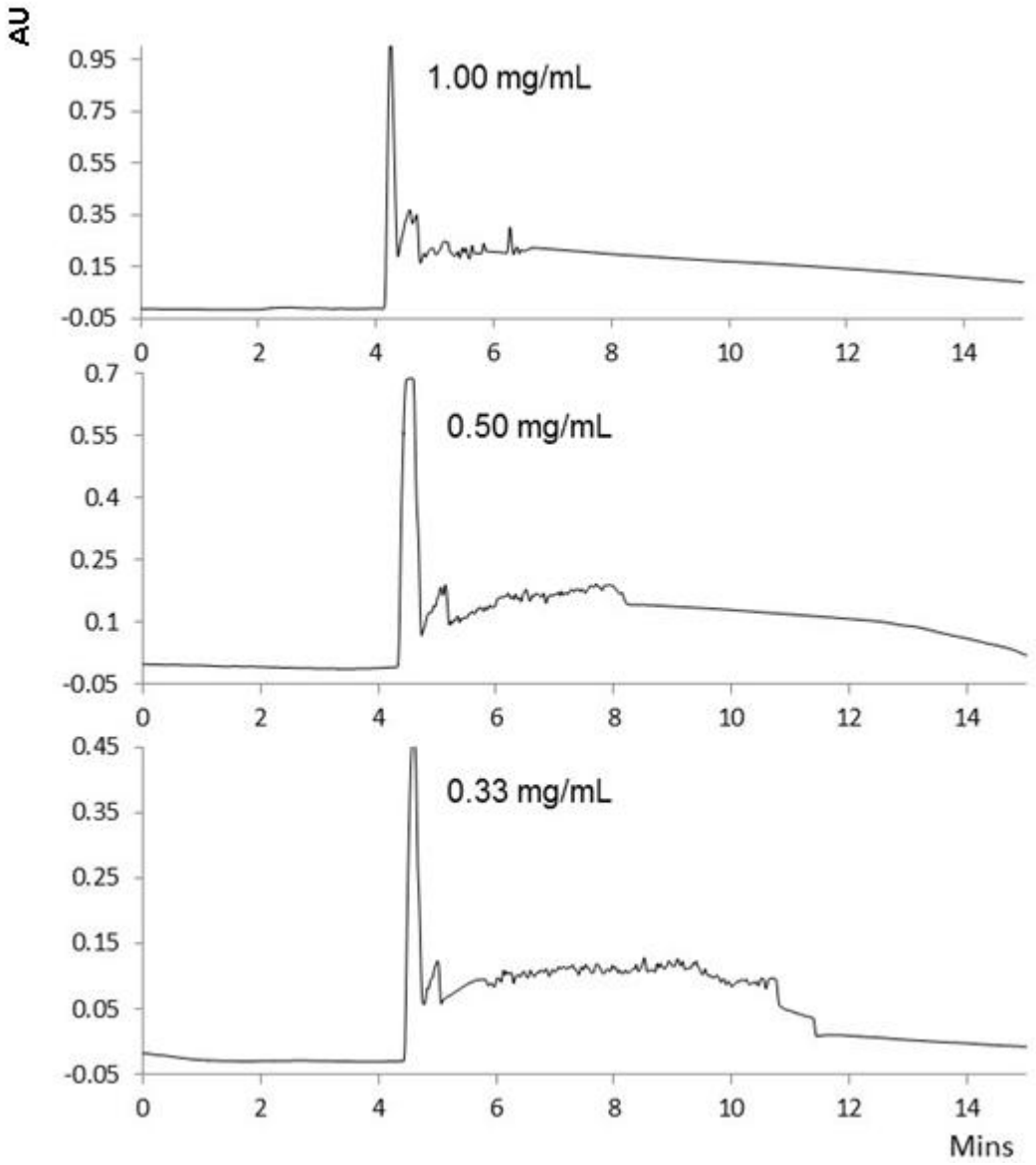

Figure 3.4: *HPCCC separations of DOM sample at different sample concentrations. Conditions: normal phase hexane/EtOOAc/water/MeOH (1:1:1:1) solvent system, at 0.15 mL/min flow rate, with UV detection at 330 nm.*

3.3.5 Fraction analysis: gas chromatography with mass spectrometry detection

By applying the optimised conditions as outlined above, five fractions were collected from the HPCCC separation of the freshwater DOM sample. These fractions are listed in Table 3.1. To check for variation along the length of the broad unresolved hump, three fractions were collected (fractions 3 to 5) for

separate analysis, together with the non-retained fraction 1 and the series of small peaks constituting fraction 2.

Table 3.1: *HPCCC fractions of freshwater DOM sample.*

FRACTION NUMBER	COLLECTION WINDOW, mins
1	4 to 4.7
2	4.7 to 5.6
3	5.6 to 8.2
4	8.2 to 11
5	11 to 14.5

The GC column used was a EC-WAX capillary column, with a poly(ethylene oxide) backbone stationary phase. This stationary phase has intermediate-high polarity and was therefore suited to the separation of compounds such as thiols, ketones, ethers, esters, alcohols, amines, carboxylic acids and diols. Given the uncertainty in the nature and content of each fraction, the versatility of this stationary phase was deemed an appropriate option. In addition to dispersive interactions, interactions between polar compounds and the stationary phase can include dipole, π-π stacking and acid-base interactions.

With the GC-MS chromatograms obtained for the five collected fractions (see Figure 3.5 for fractions 1 to 3) very clear compositional differences could be seen through fractions 1 to 3, with a homologous series of peaks very clearly evident in fraction 1, leading into a small unresolved hump. This homologous series is likely either alkylic chains or condensed aliphatic rings. Fraction 2 shows lower traces of this series leading into a higher concentration of unresolved components forming a dominant "hump". The stable semi-volatile nature of the bulk of the material present in fractions 1 and 2 is typical for DOM samples [9], and the reason why pyrolysis is often applied in DOM analysis

using GC [22-24]. Such molecules can include lignin-like materials which are characterised by conjugated double bonds (therefore visible in the HPCCC chromatogram at 330 nm), lack a primary structure, and are usually heterogeneous in their chemical funtionalities [22; 25]. These complex multi-functional molecules exhibit a low degree of volatility, rendering non-derivitised GC analysis difficult. The analysis of the CRAM and MDLT [26-27] components of DOM are also challenging, as both include compounds with multiple functional groups.

GC chromatograms from the fractions 3 to 5 contained one peak and an absence of any carryover of materials from fractions 1 and 2. The solitary peak, highlighted in Figure 3.5 (retention time: 38.8 mins), also appears to be present in fractions 1 and 2, but is identified from its mass spectra to be of the phthalate family, and likely therefore to be an introduced plasticiser.

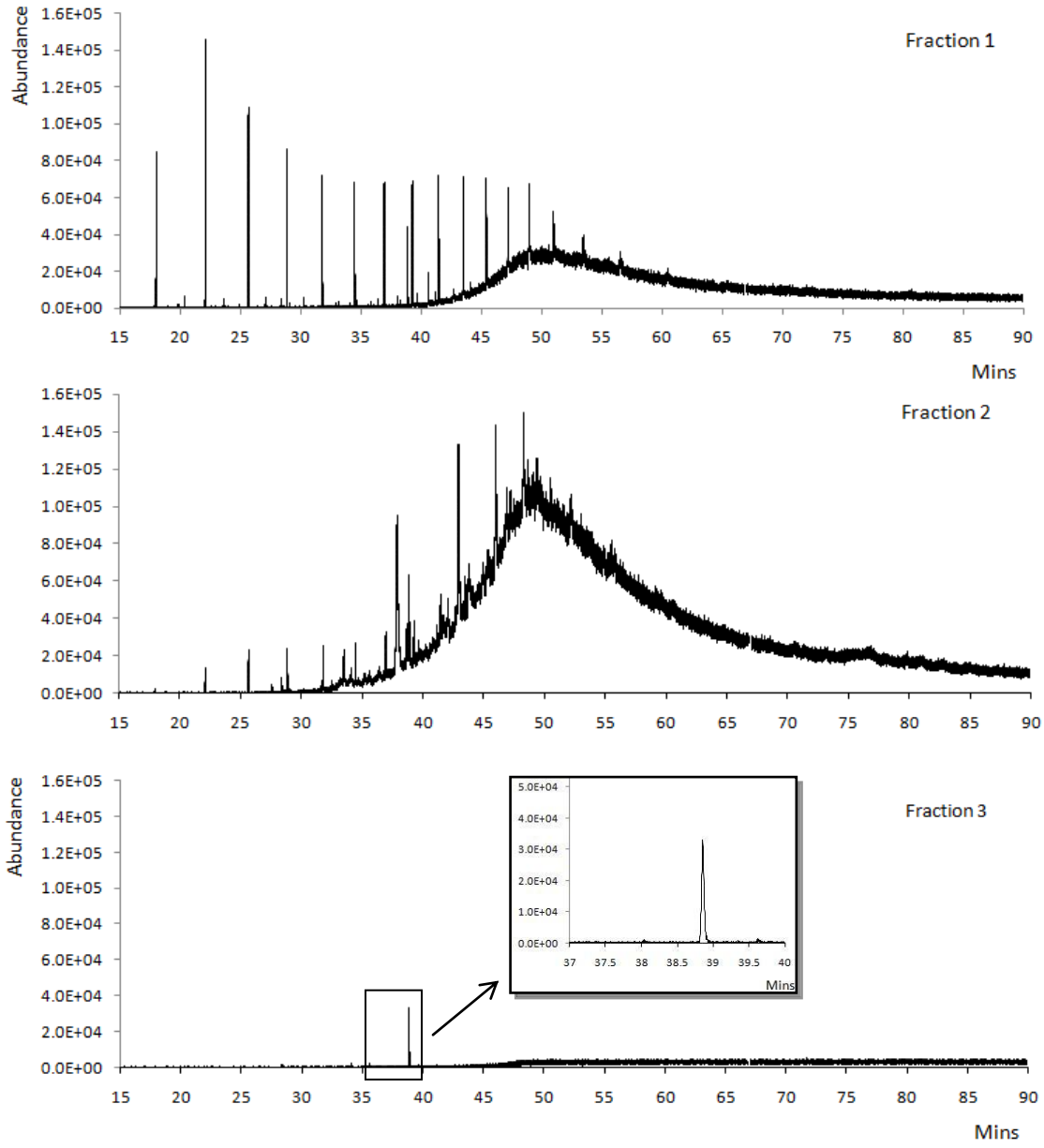

Figure 3.5: *GC separations obtained for each of the HPCCC fractions 1 to 3. HPCCC conditions: normal phase hexane/EtOOAc/water/MeOH (1:1:1:1) solvent system, at 0.15 mL/min flow rate, UV detection at 330 nm. GC conditions: column: EC-WAX capillary column, with temperature gradient from 50 to 300 ^{o}C in 90 minutes, MS collection window from 50 to 800 m/z. MS parameters: emission voltage 1612 ABS, solvent delay 2.5 minutes, acquisition mode: scan.*

Consideration of the MS spectra for the GC peaks seen within fractions 1 and 2 indicate that the majority of the compounds share the same structural background. For example, m/z values of 73 and 147 are seen for the vast majority of GC peaks. A typical spectrum is shown for the peak eluting at 34.45

minutes in Figure 3.6. The fragmentation from m/z 192 to 147 can be related to a loss of an amidic functionality, while the m/z 74 losses between m/z 221 to 147, and m/z 147 to 73, can be attributed to the loss of a $C_2H_2O_3$ functionality, which is typical of esters. However, it must be noted that in this preliminary study the MS spectra was relatively low resolution, failing to provide confirmatory losses.

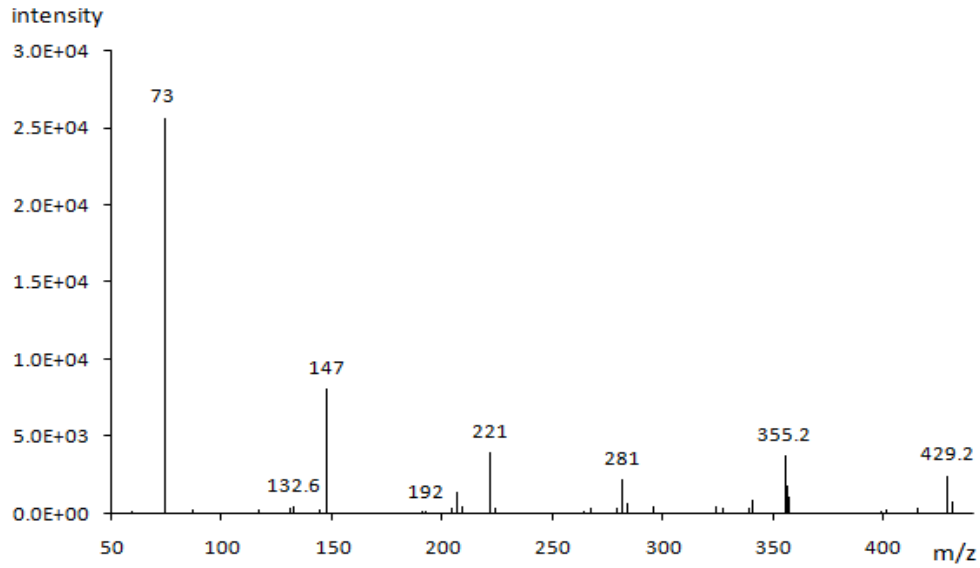

Figure 3.6: *MS spectrum from the peak at 34.45 minutes from HPCCC fraction 1. MS parameters: emission voltage 1612 ABS, solvent delay 2.5 minutes, acquisition mode: scan.*

3.3.6 Fraction analysis: reversed-phase high-performance liquid chromatography

As expected, the three last eluting fractions of HPCCC contained the highest polarity compounds when compared to the two previous fractions, which were likely to contain species rich in carboxylic acid and oxydryl groups, such as organic acids and peptides [21]. To prove these fractions did indeed contain such polar organic species the three fractions were subjected to analysis using RP-HPLC, with UV detection at both 210 nm and 330 nm.

HPCCC fractions 3 to 5 were re-suspended in 95:5 water:MeCN and injected onto a Novapak C_{18} column. Initially a 5% to 80% MeCN gradient was

applied to separate these fractions, but it was noticed that the majority of peaks exhibited very little retention under such conditions, confirming the polar nature of the bulk of the material present. Therefore, the fractions were separated in isocratic mode using only 2.5% MeCN in water as the mobile phase, and applying dual wavelength monitoring at 210 and 330 nm (shown as Figure 3.7 and 3.8, respectively).

The 210 nm chromatogram shows the bulk of the material present in fractions 3 to 5 remains relatively weakly retained, indicative of compounds with very high polarity, possibly small organic acids or ionic species, but the chromatograms also clearly show the further separation of each fraction into 10 or more fractions or indeed individual peaks. The bulk unresolved peaks appearing at the beginning of each chromatogram differed in concentration, with a higher response for fraction 3, which corresponds with the most concentrated region of the polar "hump" from the HPCCC separation.

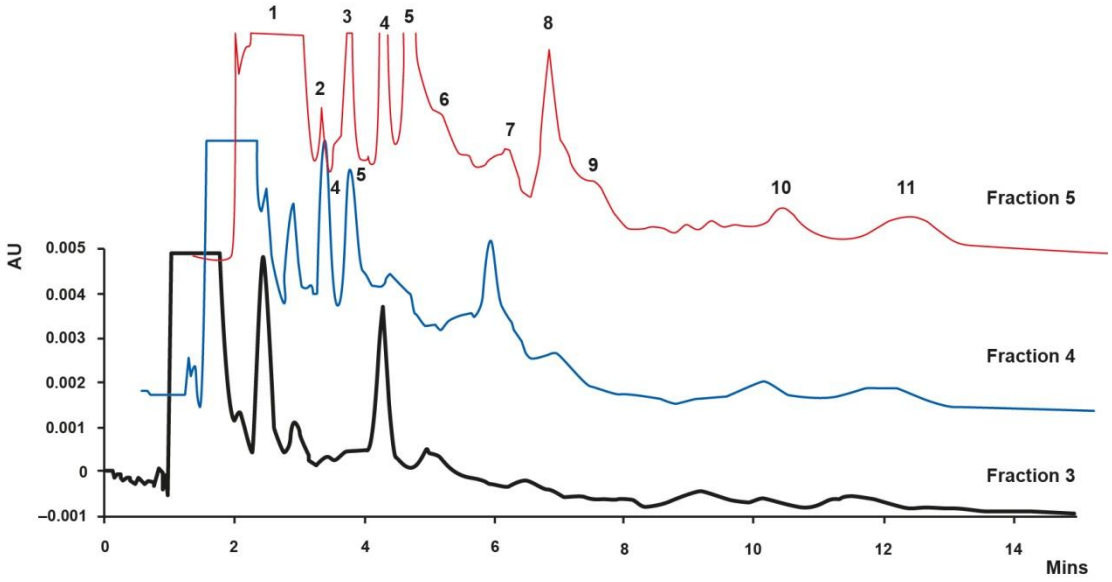

Figure 3.7: *RP-HPLC chromatograms at 210 nm for HPCCC fractions 3 to 5. Column: Waters C_{18} Novapak (150 x 3.9 mm, 4 µm particle size), flow rate 0.80 mL/min in isocratic mode (2.5% MeCN in water), injected volume 100 µL. UV detection at 210 nm.*

At 330 nm any compound with an aromatic and conjugated-aliphatic structure should be detectable, which in this case should target the main constituents of the DOM core. Figure 3.8 very clearly shows the presence of multiple peaks, but perhaps more significantly shows the differences between each fraction of the large unresolved HPCCC hump. Peaks 4 and 5 are evident in fractions 4 and 5, with the first likely individual compound and the second characterised by the presence of a shoulder (Figure 3.7). These can represent complex aromatic structures characterised by the presence of polar functional groups, which were amongst those most strongly retained within the normal phase HPCCC separation.

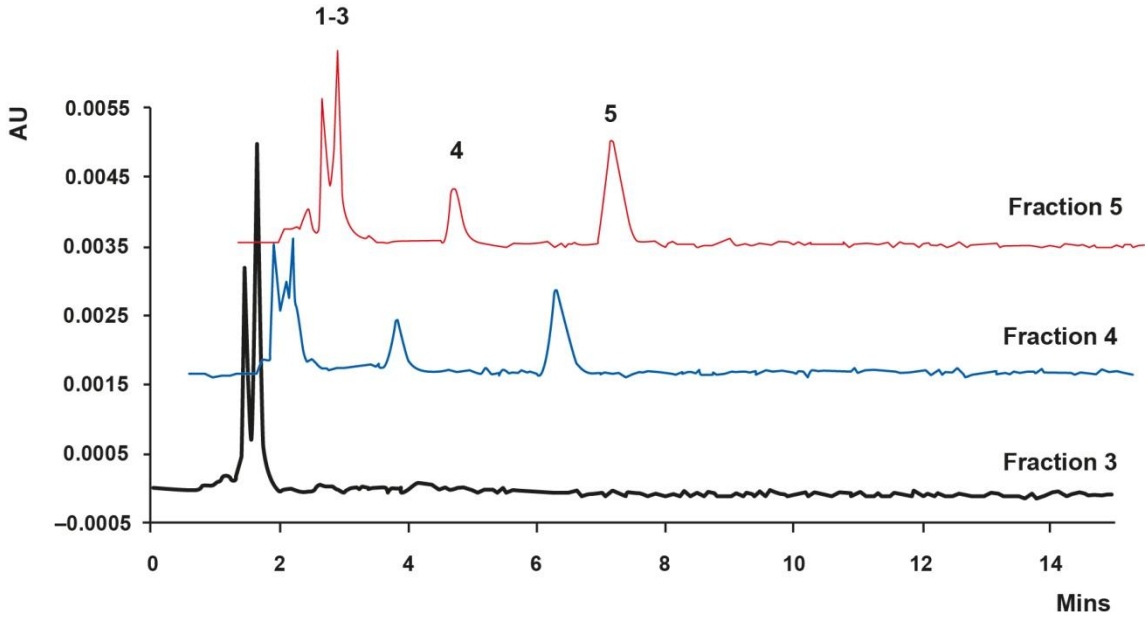

Figure 3.8: *RP-HPLC chromatograms at 330 nm for HPCCC fractions 3 to 5. Column: Waters C_{18} Novapak (150x3.9 mm, 4 µm particle size), flow rate 0.80 mL/min in isocratic mode (2.5% MeCN in water), injected volume 100 µL. UV detection at 330 nm.*

3.4 Conclusions

An HPCCC method was applied to the fractionation of freshwater DOM samples, and a reproducible and consistent fractionation of DOM components was obtained. The HPCCC separation was divided into five identified fractions,

which were subsequently analysed by means of GC-MS and RP-HPLC-UV to clarify fractional composition. The GC chromatograms clearly showed varying composition for the first three fractions and in each case it was possible to detect a number of molecular features by their MS spectra. The GC-MS fragmentation suggested an analogous molecular skeleton for the vast majority of the eluting compounds, which can potentially be related to fused cyclic structures mainly characterising DOM. RP-HPLC with UV detection isolated a number of polar species, which could not be detected using GC-MS. Once again, clear chromatographic differences were seen between fractions using LC, highlighting the significant presence of both non-volatile and polar compounds within DOM. Further work (see Chapter 4) utilised RP-HPLC coupled with HR-MS/MS detection to aid identification of these unknown polar species.

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Chapter 4: Characterisation of dissolved organic matter by high-performance counter current chromatography and reversed-phase chromatography-high resolution tandem mass spectrometry.

Abstract

Normal phase HPCCC was employed to separate semi-polar and apolar components within DOM in an increasing order of polarity. Fractions were subsequently collected and analysed by means of RP-HPLC with HR-MS/MS detection. This allowed the identification of the structural properties and molecular formulae of the main molecular classes present within the fractionated DOM sample. Further to the above, the use of RP-HPLC allowed to confirm the selectivity of HPCCC as compounds with an increasing polarity were found from the first to the last eluting fractions from HPCCC. The HR-MS analysis showed that the DOM sample was rich in classes of compounds such as: alkenes, ethers and acetals, with singly charged ionic species ranging from m/z 150 to 350. This study presented an important basis for the application of further analytical techniques in order to perform a more target-oriented analysis aimed at the determination of source and process biomarkers within DOM.

In the work presented within this Chapter the optimised HPCCC separation obtained as described in Chapter 3 was employed to isolate single components from the DOM pool.

4.1 Introduction

Previously reported studies on DOM analysis underline how challenging it is to find an analytical approach that allows the identification of single compounds within this complex organic pool (see Chapter 1).

Within Chapter 3, the challenges encountered in optimising a robust chromatographic method that allowed the collection of fractions eluting from HPCCC were presented. The heterogeneous response obtained in the second chromatographic dimension from GC and LC demanded further investigation, in particular on the nature of the compounds giving the most characteristic peaks.

As already seen, if working with HPCCC and unknown mixtures such as DOM, the best choice is to develop a multi-solvent system, in order to cover a broad range of polarities and to make the method compatible with further chromatographic dimensions and characterisation techniques, such as HR-MS and NMR [1].

Prior to this study, HPCCC or high speed counter current chromatography (HSCCC) had never been used for the fractionation of DOM. However, it has been shown to be a more reliable approach to isolate and purify natural extracts present in complex matrixes (i.e. seawater or marine organisms) [2-4]. HSCCC is also often coupled to other chromatographic techniques such as RP-HPLC, coupled with various forms of detection [2-6].

Long *et al.* [4] employed normal phase HSCCC to extract chlorophyll a from seawater. The two-phase solvent system consisted of hexane-EtOOAc-MeOH-water. The collected fraction in hexane-EtOOAc were further analysed by means of RP-HPLC, showing only one peak with the chromatogram. The extracted compound was characterised by both MS and NMR and was reported to have a purity of 99%.

An analogous solvent system was more recently employed by Kim *et al.* [7] to isolate a carotenoid from marine algae. HSCCC was employed as an alternative approach to conventional solid-phase chromatography, to selectively fractionate the molecule of interest from all the other biologically active compounds present within the sample. The applied technique proved to be successful to an extent that the identity of the carotenoid could be confirmed through NMR and MS, and was subsequently quantified by means of RP-HPLC, further highlighting the compatibility of HSCCC to other orthogonal chromatographic approaches and detection techniques. In two most recent approaches, HSCCC has been coupled to MS with both ESI and time of flight (TOF) detection [8-9].

In the work described within this chapter, the use of the optimised HPCCC fractionation (see Chapter 3) of SPE-DOM from a freshwater source (Shannon Pot, Ireland) is described (Figure 4.1-4.2). The fractions collected from this first off-line dimension were further analysed by means of RP-HPLC-HR-MS/MS (Figure 4.1-4.2), both in positive and negative mode, proving that a fractionation according to polarity occurred on the first chromatographic dimension.

The aim of which study was to further improve the characterisation of DOM to isolate single components and justify molecular formulae assignments through the characteristic MS and MS/MS fragmentation pathways on the BPCs obtained from the fractions isolated from HPCCC.

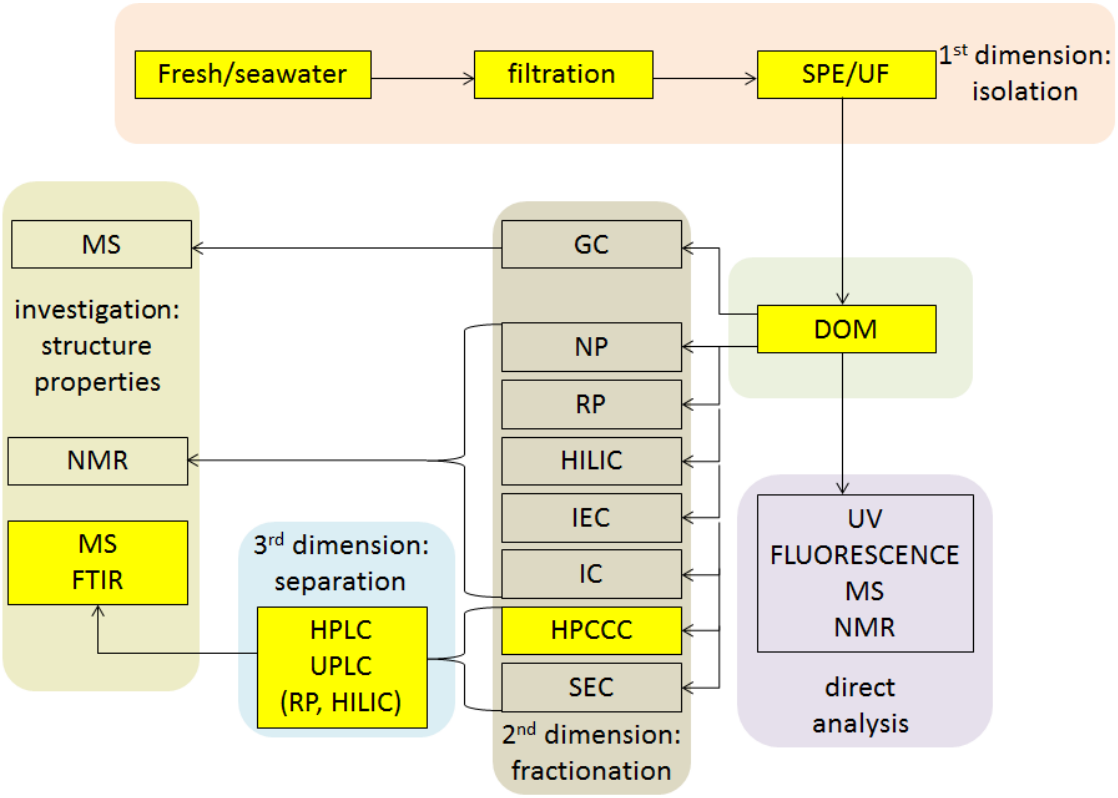


Figure 4.1: *Analytical procedures for the isolation and further characterisation of DOM. Highlighted is the pathway employed for the analysis presented here.*

4.2 Materials and methods

4.2.1 Reagents

The HCl and MeOH used in DOM extraction were obtained from Sigma Aldrich (Dorset, U.K.).

When using HPCCC, hexane, EtOOAc and MeOH were purchased from Emsure (Merck, Kilsyth, Victoria, Australia), while the water was Milli-Q grade (Millipore, Watford, U.K.). For RP-HPLC-HR-MS/MS, MS grade MeOH and water were purchased from Sigma Aldrich (Sigma Aldrich, Sidney, Australia).

4.2.2 Instrumentation

4.2.2.1 High performance counter current chromatography separation

The normal phase HPCCC separation was run after optimisation as described in Chapter 3. Briefly, the normal phase HPCCC was performed using

de-ionised water/MeOH (1:1) as the lower stationary phase and hexane/EtOOAc (1:1) as upper mobile phase. The mobile phase flow rate was 0.15 mL/min and the revolution speed 1800 rpm.

4.2.2.2 Reversed-phase high-performance liquid chromatography coupled to high resolution tandem mass spectrometry

The fractions collected from HPCCC were concentrated by nitrogen flow, recovered in 150 µL MeOH 0.1% formic acid and further separated using a Waters 2690 separations module. A 30 µL aliquot of each sample was injected onto a Waters Nova-Pak C_{18} column with dimensions 3.91 x 150 mm and 4 µm particle size (Waters, Milford, USA). The flow rate was 0.800 mL/min. MS-grade solvents (0.1% formic acid in water and 0.1% formic acid in MeOH) were employed during a 30 minute step gradient of 10-100% MeOH 0.1% formic acid. The column operated at ambient temperature (20 °C).

HR-MS analysis was conducted by collaborators (Dr. Richard Wilson and Prof. Noel Davies) using a hybrid Linear Trap Quadrupole/Orbitrap (Thermo Fisher Scientific, Bremen, Germany). Centroid mass spectra were acquired in the m/z range of 50-1000 at a target resolution of 30.000 operating according to the following parameters: capillary temperature of 300°C; sheath gas and auxiliary gas flow rates were set to 30 au and 5 au, respectively. A capillary voltage of 7 V was used for both positive and negative ion acquisition.

Along the BPCs features were searched and associated to statistically assigned molecular formulae on Quall browser operating on Xcalibur software (Thermo Fisher Scientific, Bremen, Germany) and following public database (METLIN) [10] search by accurate m/z alone. The chosen parameters in positive mode were: degree of tolerance ±2 ppm and $[M+H]^+$ and $[M+Na]^+$ as

possible adducts. When negative mode data was examined, the $[M-H]^+$ and $[M+Na]^+$ adducts were considered at the same degree of tolerance. Any nonsensical match or non coherence with the nitrogen rule was not considered further. Once candidates were selected, data such as isotopic trends and retention times were used together with MS/MS fragmentation for either exclusion or increased confidence. Both positive and negative mode MS/MS data were automatically collected by fragmentation of the 4 most intense ions along the mass spectrum.

4.2.3 Freshwater collection and sample preparation

The freshwater sample (10 L) was collected from the source of river Shannon (54^o 14'05''N, 7^o55'08''W, collected in May 2011), in Ireland. The latter was stored in 10 L Nalgene containers (Fisher Scientific, Dublin, Ireland) at 4 ^{o}C and extracted as described by Dittmar *et al.* (2008) in Chapter 2 [11].

4.3 Results and discussion

4.3.1 High-performance counter current chromatography

As seen in Chapters 2 and 3, the use of a multi-dimensional chromatographic approach simplified the analysis of this complex mixture. In particular, in HPCCC, the fractionation of DOM according to polarity appeared to be a more reliable means to separate DOM components into groups which could be further analysed. In the optimised HPCCC separation of Shannon Pot DOM (see Chapter 3 and here in Figure 4.2), the compounds started to elute at very low retention times, indicating a high affinity towards the mobile phase (hexane/EtOOAc), hence nonpolar analytes elute first and polar species last. Such a mobile phase is not expected to separate compounds like proteins,

which are denaturated in organic solvents, but to isolate the molecules representing the main core of DOM: for example CRAM, MDLT and lipid-like materials. Such compounds are likely to possess a low degree of polarity and therefore to be suitable for normal-phase counter current chromatography. CRAM, MDLT and lipid-like materials are all characterised by conjugated double bonds, aromatic and carboxylic functionalities, which are UV responsive at 330 nm. Therefore this wavelength was chosen to separate the most characteristic fractions of DOM [12-13].

On the obtained HPCCC chromatogram (Figure 4.2) it was possible to recognise a very unique trend, reflecting three major fractions: a main peak eluting at the beginning of the chromatogram, immediately followed by a less intense peak, which is a shoulder of the previous one and by an unresolved "hump". The first fraction, which is the most UV-responsive, was collected from 4 to 4.5 minutes and was expected to contain the lowest polarity compounds within DOM, therefore possibly characterised by a strong π-conjugation or to obey the aromaticity rule. The collecting time for fraction 2 was chosen between 4.5 and 5.5 minutes. According to the elution profile of the sample, compounds eluting at this stage of chromatogram probably contain a low-intermediate range of polarity, with possible co-elution from the previous fraction. The last portion of counter current chromatogram was divided into three portions (fraction 3 from 5.5 to 8.3 minutes, fraction 4 from 8.3 to 11 minutes and fraction 5 from 11 to 14.5 minutes). Here, the peak is much broader if compared to the first eluting fractions and the more polar molecules are expected to be retained.

After all the fractions were collected from the HPCCC, they were concentrated and further separated by means of RP-HPLC with HR-MS detection. As retention mechanism in RP-HPLC is also regulated by polarity,

with high polarity compounds eluting earlier within the chromatogram, RP-HPLC can be considered orthogonal to normal phase HPCCC, where low polarity species elute first.

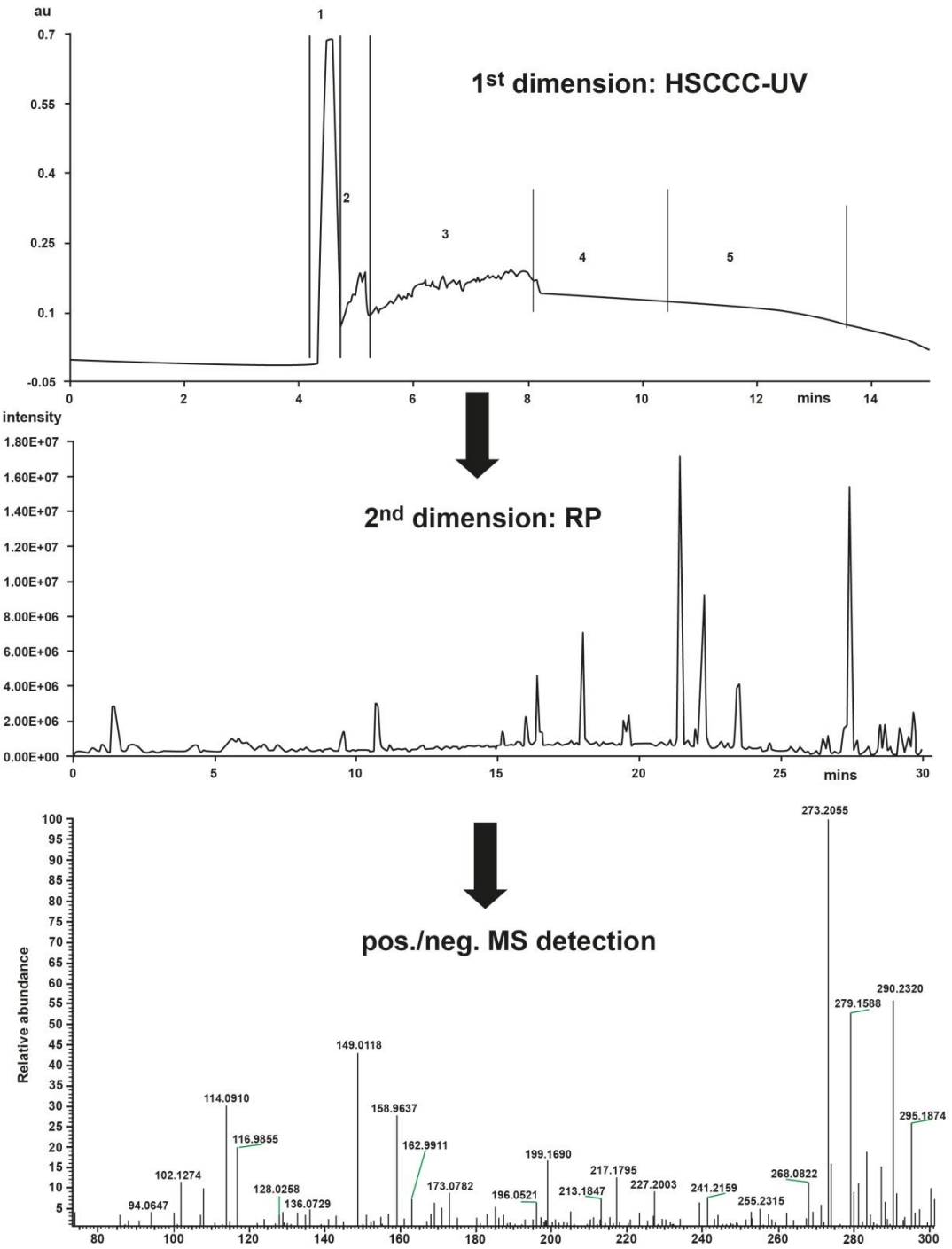
By means of RP-HPLC with HR-MS detection, characteristic BPCs were obtained on every injected sample, showing the most distinguishing peaks from the middle until the end of the gradient, therefore supporting the hypothesis that the compounds belonging to the fractions collected from HPCCC have an intermediate to low degree of polarity.

Since not all the DOM components are UV responsive (i.e. long alkylic chains), MS was used as detection form. This allowed verification of the efficiency of the normal phase HPCCC fractionation as, as already mentioned, compounds eluting at the beginning of the counter current chromatogram should exhibit a lower polarity than those being more retained on the last eluting fractions. It was therefore expected to find lower polarity moieties and functional groups on the MS/MS spectra obtained from the most intense signal along the BPC from the earlier eluting fractions.

Since a HR mass spectrometer was used, most of the signals due to the analytes were hidden by those derived from the solvents used during the gradient, which were in higher concentrations than the analytes [14-15]. Features (ions with a unique m/z) were present at different retention times. Therefore the aim was to determine which of these were characteristic of one fraction from HPCCC if compared to another.

For the ions detected on the most intense peaks along the BPCs, the empirical formula of the molecules was calculated through Quall Browser. This software provides a test of how close the calculated formula is to the real one through a confidence value. The closer the value is to zero, the closer the

estimated formula to the real one. The tolerance (ppm) set in the software was 2. If the m/z range for a selected compound was between 200 and 300 m/z, the maximum number of allowed carbon atoms was 30, 50 for hydrogen, 10 for oxygen, 5 for nitrogen, 2 for sulfur and 2 for phosphorous. The charge on the molecule parameter was set as +1 as the analysis were at first run in positive mode.



(Figure continues on the following page)

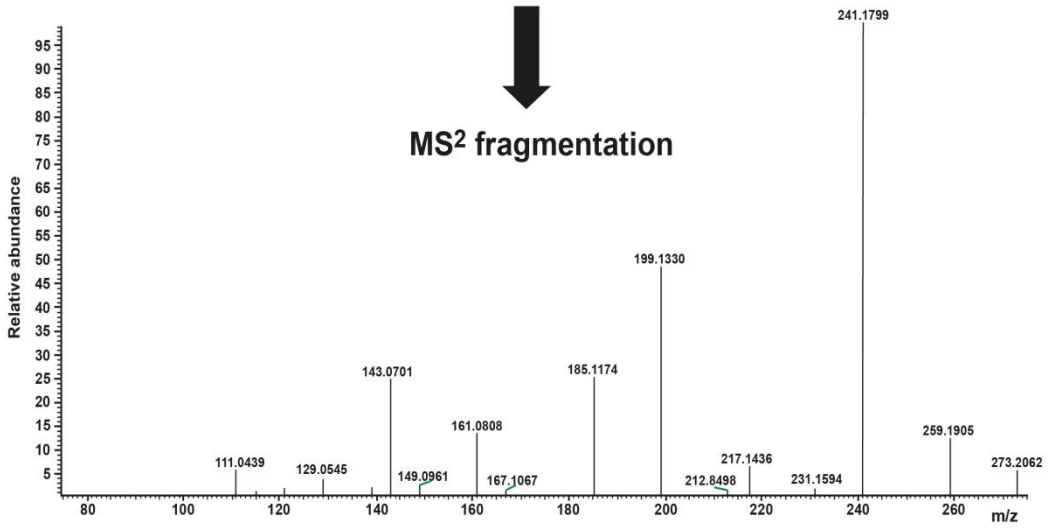


Figure 4.2: *DOM fractionation via HPCCC, followed by RP-HPLC-HR-MS/MS. HPCCC conditions: normal phase hexane/EtOOAc/water/methanol (1:1:1:1) solvent system, at 0.15 mL/min flow rate, UV detection at 330 nm. RP-HPLC conditions: flow rate 0.800 mL/min with a 10% to 100% MeOH 0.1% formic acid gradient in 30 minutes. MS conditions: positive mode, capillary temperature of 300°C; sheath gas and auxiliary gas flow rates 30 au and 5 au, capillary voltage 7 V.*

4.3.2 Reversed-phase high-performance liquid chromatography coupled to positive mode high resolution mass spectrometry

Within the following sections, examples for representative compounds retained by analogous gradient conditions within the BPCs from early (fraction 1), intermediate (fraction 3) and late (fraction 5) eluting fractions are reported (Figures 4.2 to 4.4). All these were obtained in positive mode.

4.3.2.1 Fraction 1

Figure 4.3 shows the mass spectrum for one of the most predominant peaks on the BPC from fraction 1. In this spectrum, m/z 425.2137 represents $M+Na^+$ and 403.2322 $M+H^+$. Beside $M+H^+$, its corresponding ^{13}C peak can be noticed at m/z 404.2353. Since the peak height for this isotopic signal is 20 intensity units, it is clear that the number of carbons for this compound is also 20. Other information that can be obtained from this positive mass spectrum is

that according to the nitrogen rule, as the molecular ion is an even number, this compound has 0 or an even number of nitrogens.

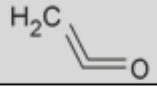
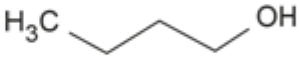
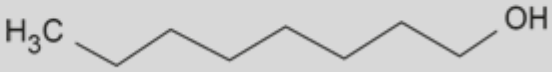
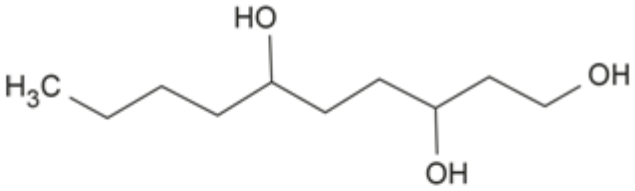
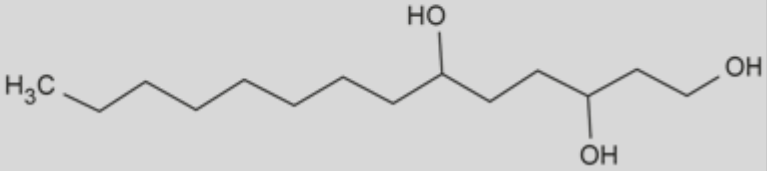
m/z 403.2322 was selected to be fragmented via MS/MS as being one of the four most intense ions along the mass spectrum. m/z 361.2220 is indicative of a C_2H_2O loss, which is typical of acetals (Table 4.1). According to the neutral losses reported on Figure 4.3, the signal at m/z 329.1594 is representative of a $C_{16}H_{25}O_7$ unit, due to the loss of $C_4H_{10}O$, whereas m/z 273.0968 derives from $C_{12}H_{17}O_7$ (Table 4.1). According to the fact that a saturated molecule has the formula C_nH_{2n+2} and since the only possible formula for the spectrum here presented is $C_{20}H_{25}O_8$, the number of rings and double bonds equivalents (RDE) is 4. One double bond or a ring is equivalent to a loss of 2 protons from a saturated system. For a generic formula $C_nH_xO_z$ this value is represented by the relationship:

$$RDE = \frac{(2n+2)-x}{2} \qquad RDE = \frac{(2n+2)-x}{2}$$

Whereas, if nitrogen is contained (i.e. $C_nH_xN_yO_z$):

$$RDE = n - \frac{x}{2} + \frac{y}{2} + 1$$

Table 4.1: *Proposed structures for the most common neutral losses occurring within the MS spectrum for the considered compound on fraction 1.*

NEUTRAL LOSS	PROPOSED STRUCTURE
C_2H_2O	
$C_4H_{10}O$	
$C_8H_{18}O$	
$C_{10}H_{22}O_3$	
$C_{14}H_{30}O_3$	

$$RDE = n - \frac{x}{2} + \frac{y}{2} + 1$$

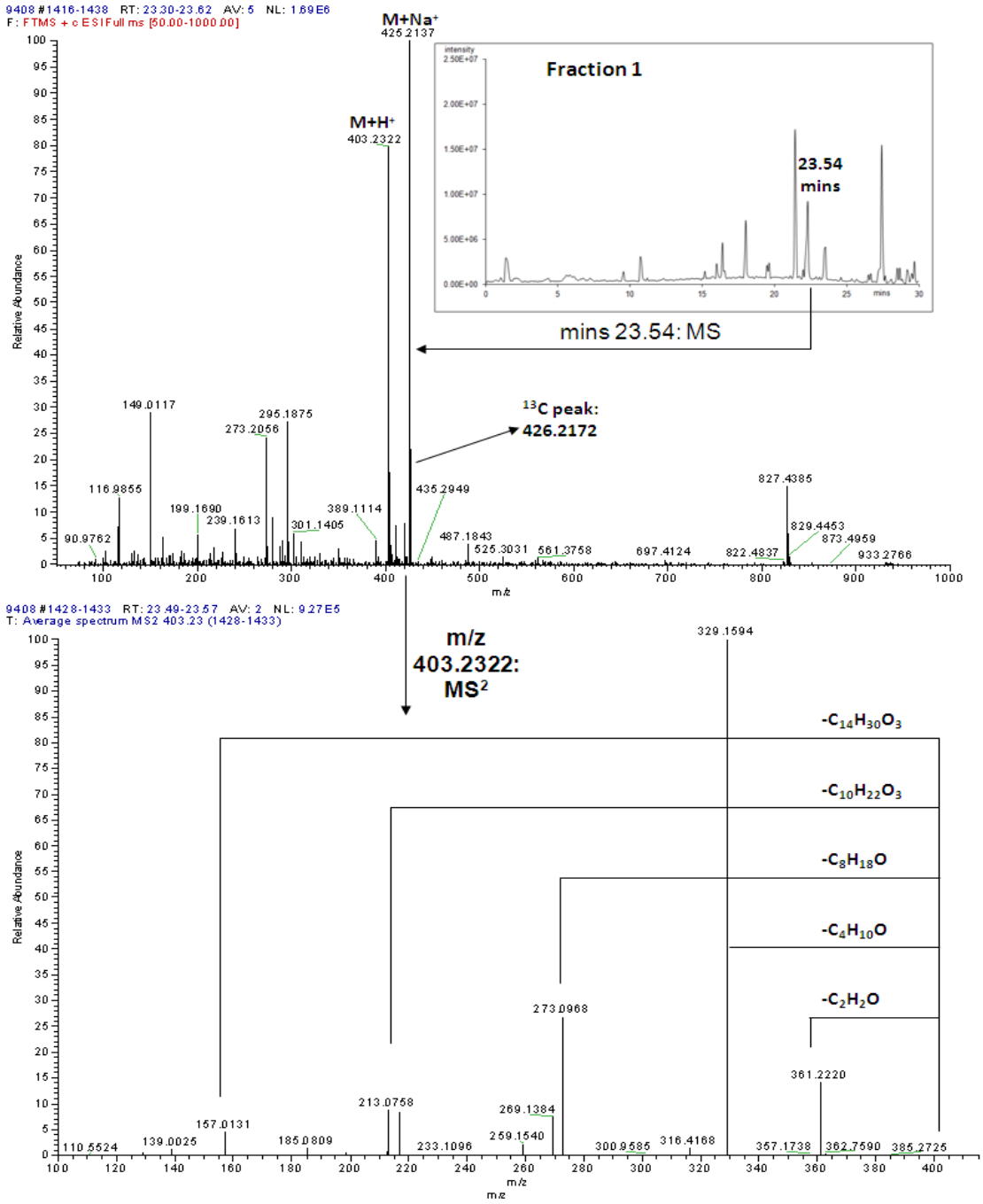


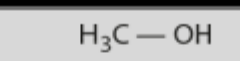
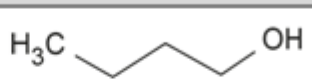
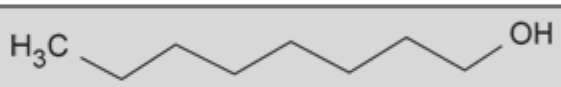
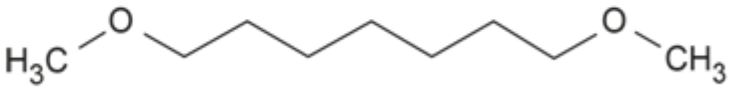
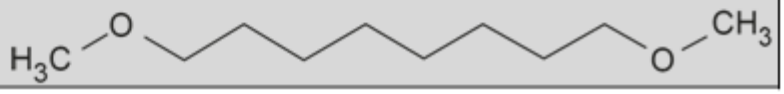
Figure 4.3: *RP-HPLC chromatogram from fraction 1 highlighting the peak analysed by MS and MS/MS (retention time 21.34 mins). MS/MS was performed on the four most intense ions along the MS spectrum. RP-HPLC conditions: flow rate 0.800 mL/min with a 10% to 100% MeOH 0.1% formic acid gradient in 30 minutes. MS conditions: positive mode, capillary temperature of 300°C; sheath gas and auxiliary gas flow rates 30 au and 5 au, capillary voltage 7 V.*

4.3.2.2 Fraction 3

From the chromatogram originating from fraction 3 (Figure 4.4), since 273.2055 is $M+H^+$ and 295.1874 $M+Na^+$, the isotopic relative abundance at m/z 274.2089 suggests that this structure contains 15-16 carbons.

The MS/MS spectrum indicates that the molecule loses MeOH (m/z 361.2220). Therefore, the structure can have ether or alcohol functional groups. The m/z at 199.1330 represents the unit $C_4H_{10}O$ (Table 4.2); the m/z at 161.0808 corresponds to $C_7H_{13}O_4$, m/z 143.0701 to $C_7H_{11}O_3$, m/z 129.0545 to $C_6H_9O_3$ and m/z 83.0490 to C_5H_7O (Table 4.2). The formula for this compound is $C_{15}H_{28}O_4$, with 2 RDE.

Table 4.2: *Proposed structures for the most common neutral losses occurring within the MS spectrum for the considered compound on fraction 3.*

NEUTRAL LOSS	PROPOSED STRUCTURE
CH_3OH	
$C_4H_{10}O$	
$C_8H_{18}O$	
$C_9H_{22}O_2$	
$C_{10}H_{22}O_3$	

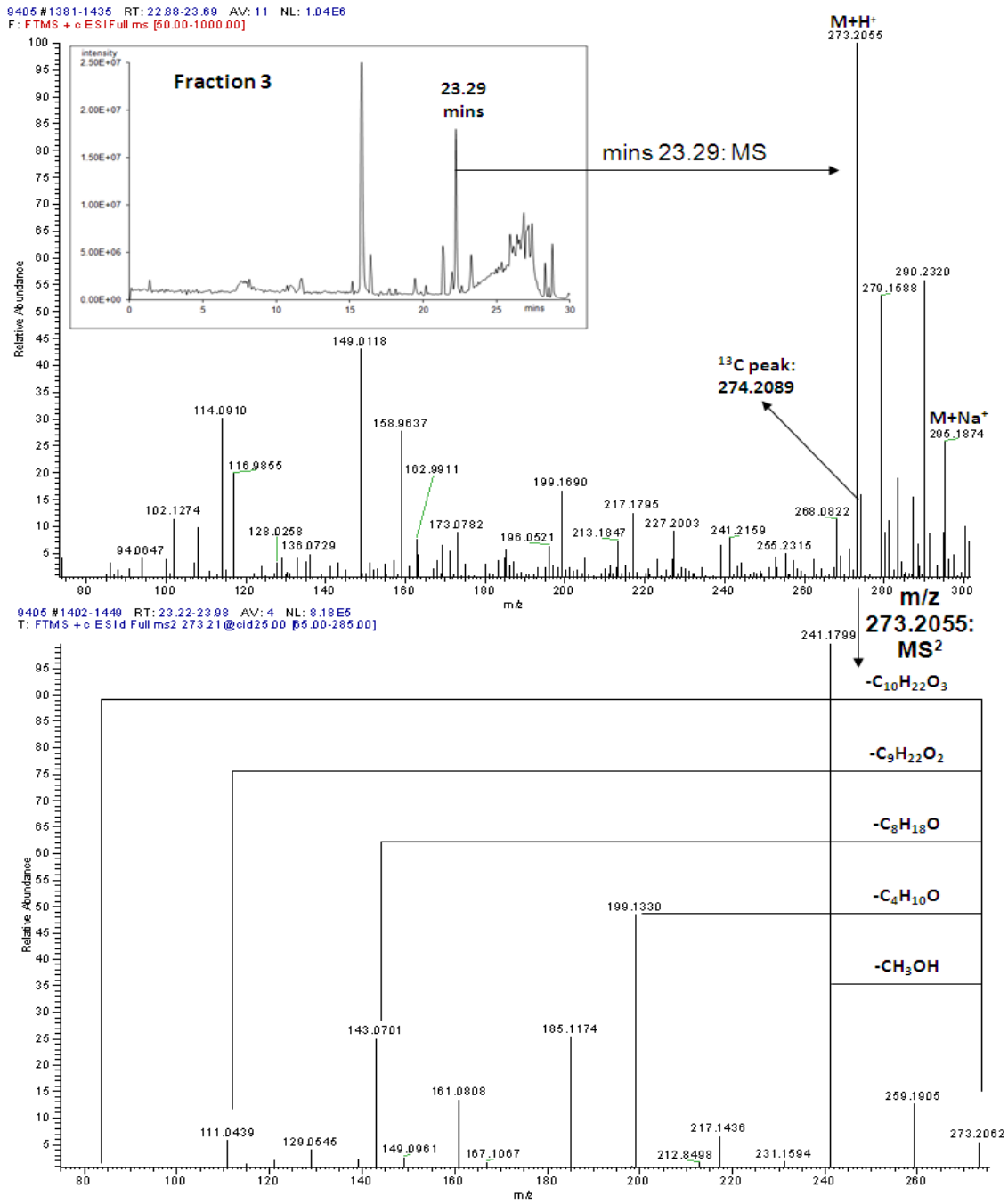
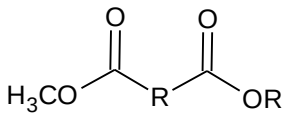


Figure 4.4: *RP-HPLC chromatogram from fraction 3 highlighting the peak analysed by MS and MS/MS (retention time 23.29 mins). MS/MS was performed on the four most intense ions along the MS spectrum. RP-HPLC conditions: flow rate 0.800 mL/min with a 10% to 100% MeOH 0.1% formic acid gradient in 30 minutes. MS conditions: positive mode, capillary temperature of 300°C; sheath gas and auxiliary gas flow rates 30 au and 5 au, capillary voltage 7 V.*

According to the data search performed on the databank NIST MS 2.0, this structure seems to be an open chain diester with a structure:



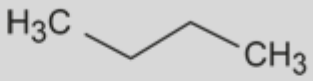
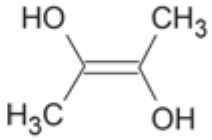
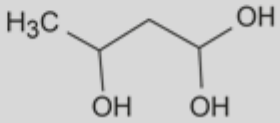
Such structure justifies the losses of MeOH from m/z 273.2062 to m/z 241.1799 and from 143.0701 to m/z 111.0439, which might be at the beginning of the alkylic chain or as branching units.

If compared to the previously examined compound from fraction 1 (Figure 4.3), this molecule appears to be smaller in size, indicating that the dipolar moment due to the presence of oxygens is less dispersed along the structure. This indicates a higher degree of polarity.

4.3.2.3 Fraction 5

Considerations analogous to those made for the previously described molecule from fraction 3 can be applied to the compound eluting from fraction 5 (Figure 4.5). Here, the peak eluting at 19.53 minutes is considered, showing m/z 239.1610 as $[M+Na]^+$ and m/z 217.1792 as $[M+H]^+$. As for the isotopic trend the molecule contains 12 carbon atoms and, according to the MS/MS spectrum, m/z 199.1691 accounts for $C_{12}H_{23}O_2$, m/z 185.1171 for $C_{10}H_{17}O_3$ (representing a loss of water if compared to the previous signal), m/z 129.1271 for $C_8H_{17}O$, whereas m/z 111.1166 for C_8H_{15} (Table 4.3). The formula for this molecule is $C_{12}H_{25}O_3$, with 1 RDE. This underlines an even smaller molecule if compared to fraction 3 (Figure 4.4), with a more concentrated charge.

Table 4.3: *Proposed structures for the most common neutral losses occurring within the MS spectrum for the considered compound on fraction 5.*

NEUTRAL LOSS	PROPOSED STRUCTURE
C_4H_{12}	
$C_4H_8O_2$	
$C_4H_{10}O_3$	

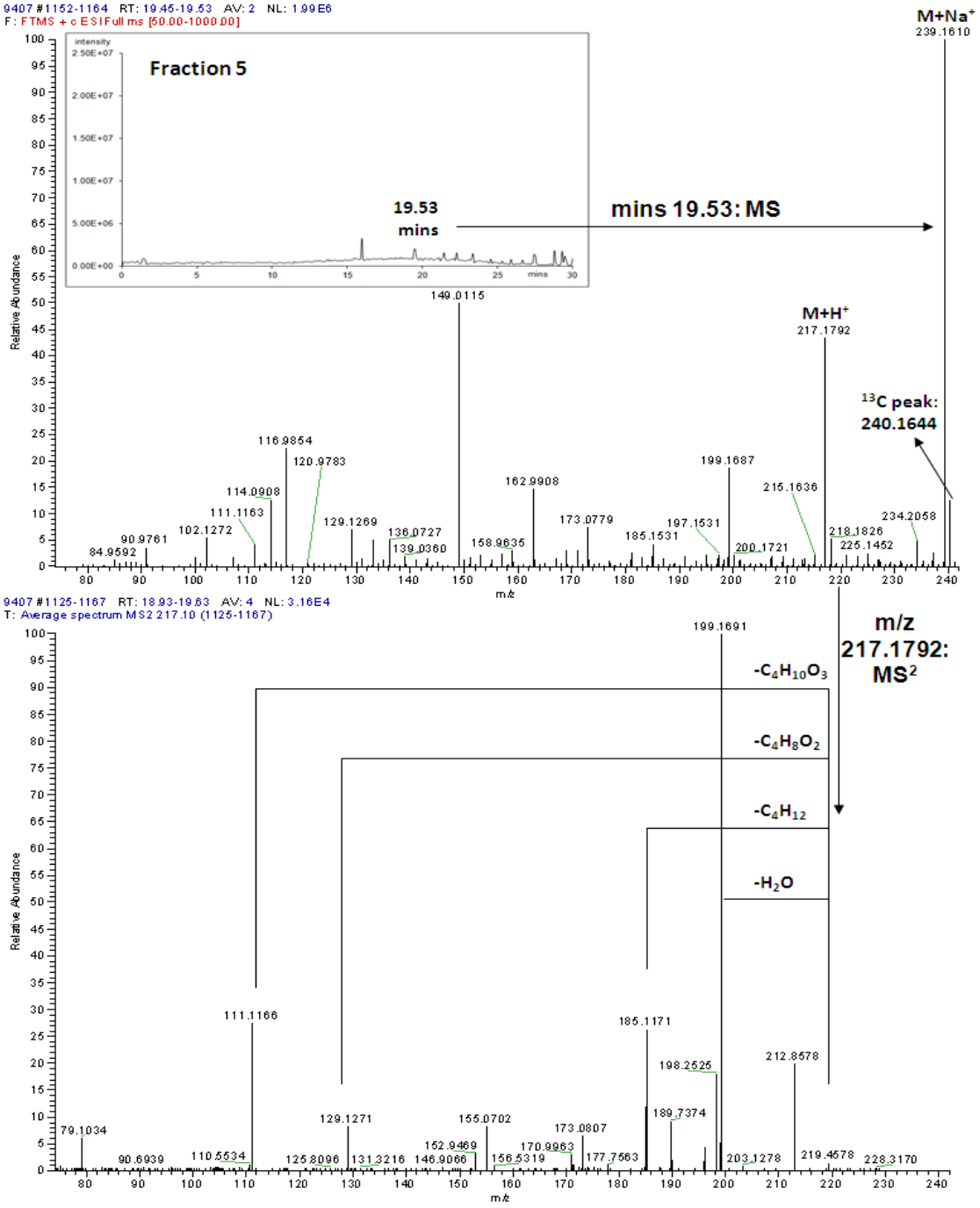


Figure 4.5: *RP-HPLC chromatogram from fraction 5 highlighting the peak analysed by MS and MS/MS (retention time 19.53 mins). MS/MS was performed on the four most intense ions along the MS spectrum. RP-HPLC conditions: flow rate 0.800 mL/min with a 10% to 100% MeOH 0.1% formic acid gradient in 30 minutes. MS conditions: positive mode, capillary temperature of 300°C; sheath gas and auxiliary gas flow rates 30 au and 5 au, capillary voltage 7 V.*

4.3.2.4 General considerations on positive mode high resolution mass spectrometry data

Globally, the MS and MS/MS spectra for the BPCs from fractions 1, 3 and 5 showed evidence for characteristic functional groups within the analysed DOM sample. Fragments generated from peaks in fraction 1 were richer in losses due to long alkylic chains. Fragments generated from fraction 3 were rich in double bonds and keto-groups. In fraction 5, fragment losses of OH groups were predominant, together with long alkylic chains enriched in double bonds. Such behaviour further underlines that fractionation due to relative polarity occurred within the first chromatographic dimension.

All the molecules detected from positive mode MS were enriched in compounds with C,H,O composition. Given the fact that DOM samples are usually processed in negative mode MS, underlining the presence of low polarity steroid-like moieties [16-18], positive mode MS is in this case able to focus the attention on more basic compounds (which easily accept protons) such as esters, diols and acetals (Figure 4.3 to 4.5), which would be less effectively detected in negative mode MS.

According to the spectral behaviour here described, a trend is shown which underlines the fact that the fractions collected from HPCCC were eluting according to their polarity order, indicating the reliability of this chromatographic approach as a preparative means to help simplifying DOM characterisation. The fragmentation spectra obtained in positive mode underlines the consistent presence, in this mixture, of alkanes, alkenes, acetals, alcohols and ethers as main functional groups. However, as already mentioned on Chapter 1, this MS approach alone fails in defining where the functionalities are located along each molecule.

4.3.3 Reversed-phase high-performance liquid chromatography coupled to negative mode high resolution mass spectrometry

After concentration in MeOH, the fractions obtained from HPCCC (Figure 4.2), were also analysed by means of RP-HPLC-HR-MS/MS in negative mode.

The signal intensity for the features along the BPCs was much lower if compared to positive mode data. Therefore to understand the compounds retention time and study the MS and MS/MS spectra, the extracted ion chromatograms (EICs) were examined on m/z windows of 20 m/z from 150 to 910 m/z (Figure 4.6). This allowed highlighting of the most intense features along the chromatograms and to further understand their structural characteristics by means of MS/MS (Table 4.4). Similarly with positive mode data, the most characteristic EICs were obtained from fraction 1, which appeared to be the most interesting and rich in terms of unique features. On Figure 4.6 are represented those molecules appearing in the m/z windows from m/z 150 to 170 and from m/z 240 to 260, which clearly highlight different profiles that would not be appreciated if a full BPC was considered.

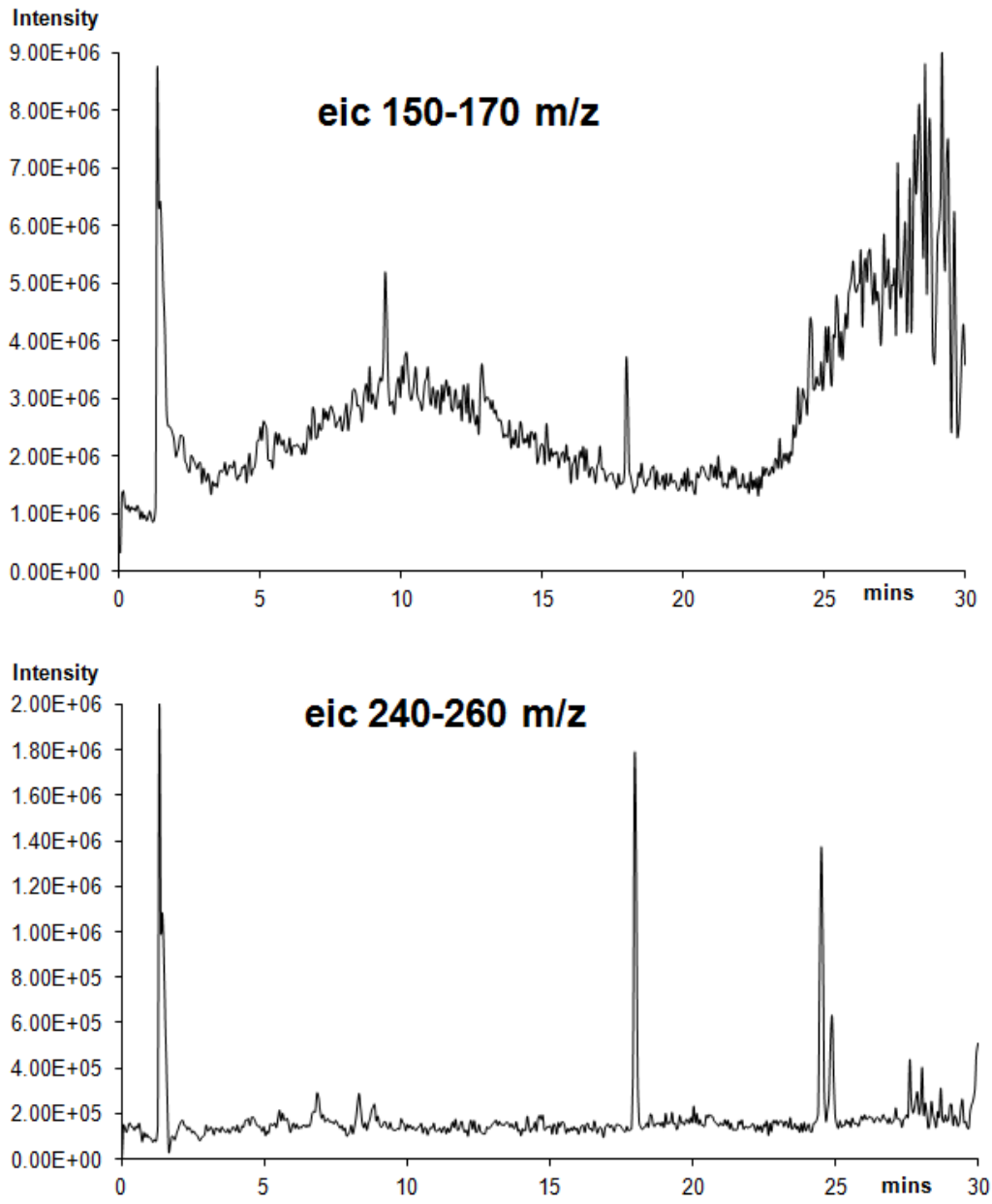


Figure 4.6: *EICs for m/z windows 150-170 and 240-260 from fraction 1. RP-HPLC conditions: flow rate 0.800 mL/min with a 10% to 100% MeOH 0.1% formic acid gradient in 30 minutes. MS conditions: negative mode, capillary temperature of 300°C; sheath gas and auxiliary gas flow rates 30 au and 5 au, capillary voltage 7 V.*

Fractions 3 to 5 presented a co-elution of compounds already eluting on fractions 1 to 2, with signals mainly under the LOD. This is the reason why the features reported in Table 4.4 refer to fractions 1 and 2 only.

Most of the detected ions were singly charged and this can again be proved by the fact that in all of their MS spectra, the sodium adduct is reported at +22 m/z from the deprotonated molecular ion ($M\text{-}H^+$). The m/z for the ions in Table 4.4 ranged from 170 to 470 m/z, confirming the previously reported finding of a DOM sample characterised by small molecules. A paper by D'Andrilli *et al.* [19] reports that DOM analysed by ESI and APCI exhibit a Gaussian distribution with m/z values ranging from 300 to 800, typical of small molecules [17-21].

The main losses noticed along the MS/MS spectra from fraction 1 are due to CO_2 and water, indicating the presence of diols, esters ethers and carboxylic acids as main functional groups, reporting a RDE from 2.5 to 4.5.

As can be seen (Table 4.4), molecules with higher RDE are eluting at higher retention times along the HPCCC. Considering that the bond energy for a single bond between two carbons is 347 kJ/mol and 612 kJ/mol for a double bond, the later eluting molecules on fraction 2 appear to be more stable if compared to those characterising fraction 1. Furthermore, the ions detected from fraction 2 are richer in CO_2 losses, potentially underlining that this portion of HPCCC chromatogram is characterised by carboxylic acids, ketones or esters, functional groups that confirm previous HR-MS experiments in negative mode [17-18].

The DOM isolated from SPE extraction is known to preferentially extract semi-polar and apolar compounds, hence the occurrence of unsaturated and low polarity molecules is consistent with this extraction method. Also, as reported in the literature, terrestrially influenced samples, such as the one here analysed, are usually rich in aromatic and olefinic functionalities [12-13].

Due to lack of space it is not possible to show all of the compounds which were associated with a matching formula; here are just reported those characterised by the most intense peaks on the EICs.

All fractions collected from HPCCC have different characteristics and elution profiles when compared to each other. Also, the assigned formulae indicate that this DOM sample is mainly characterised by compounds bearing carbon, hydrogen and oxygen, as just few examples in Table 4.4 are nitrogen-containing molecules [22].

Table 4.4: *Main MS features matching for the EICs on negative mode MS.*

FRACTION	RT[a]	M-H$^+$	PUTATIVE FORMULA	Δppm[b]	RDB[c]	PRODUCT ION	COMMENT
1	4.04	201.0768	$C_9H_{13}O_5$	1.01	3.5	139.0763	loss of CO_2 and water
1	5.35	215.0924	$C_{10}H_{15}O_5$	1.00	3.5	153.0920	loss of CO_2 and water
1	7.43	303.1446	$C_{14}H_{23}O_7$	0.78	3.5	285.1335	loss of water
1	9.40	186.1136	$C_9H_{16}O_3N$	0.18	2.5	125.0971	product ion for $C_8H_{13}O$
1	10.32	173.0820	$C_8H_{13}O_4$	1.16	2.5	111.0815	loss of CO_2 and water
1	12.83	187.0976	$C_9H_{15}O_4$	0.09	2.5	125.0971and 79.1243	product ion for $C_8H_{13}O$ possible nonadioic acid according to Metlin MS^2
1	13.38	301.1652	$C_{15}H_{25}O_6$	0.80	3.5	N/A	-
1	16.59	415.2335	$C_{21}H_{35}O_8$	0.85	4.5	369.2274	loss of CO_2 and water
1	15.12	217.1079	$C_{10}H_{17}O_5$	0.89	2.5	199.0973	sequential losses of water
1	17.96	242.1761	$C_{13}H_{24}O_3N$	-0.28	2.5	225.1491 and 181.11595	aminoacid-like: sequential loss of the ammino group and CO_2
1	18.88	293.1754	$C_{17}H_{25}O_4$	0.64	5.5	236.1048	loss of C_4H_9
1	19.37	323.2224	$C_{19}H_{31}O_4$	0.74	4.5	305.2113	loss of water
1	25.04	423.2603	$C_{20}H_{39}O_9$	1.45	1.5	N/A	-
1	25.14	465.3069	$C_{23}H_{45}O_9$	1.07	1.5	419.3009	loss of CO_2 and water
1	25.47	451.2914	$C_{22}H_{43}O_9$	1.26	1.5	405.2851	loss of CO_2 and water
1	26.39	271.2277	$C_{16}H_{31}O_3$	0.93	1.5	225.2219	loss of CO_2 and water
1	24.49	249.1494	$C_{15}H_{21}O_3$	-0.88	5.5	N/A	^{13}C peak 14.88%
1	28.57	333.2433	$C_{21}H_{33}O_3$	0.89	5.5	289.2530	loss of CO_2, unique fragment on MS^2
2	8.30	243.1234	$C_{12}H_{19}O_5$	0.70	3.5	N/A	-
2	12.88	277.1289	$C_{12}H_{21}O_7$	0.73	2.5	213.0190	-
2	9.44	317.1600	$C_{15}H_{25}O_7$	0.52	3.5	N/A	analogous pattern as 8.3
2	21.65	322.1658	$C_{17}H_{24}O_5N$	0.86	6.5	278.1757	loss of CO_2
2	26.38	389.3060	$C_{25}H_{41}O_3$	0.94	5.5	345.3156	loss of CO_2
2	27.68	389.3060	$C_{25}H_{41}O_3$	0.94	5.5	345.3156	loss of CO_2

a: retention time; b:confidence degree; c: rings and double bonds equivalent

4.4 Conclusions

The method presented herein reports a selective and robust chromatographic separation both on the first and second off-line dimensions. All of the fractions from HPCCC show unique features on the RP-HPLC-HR-MS analysis, highlighting the specificity of HPCCC towards a mixture containing molecules with different degrees of polarity and chemical functionalities. Compounds with a lower polarity eluted on the first HPCCC fraction, having higher affinity towards the mobile phase (hexane/EtOOAc). Consistently, compounds such as those detected in the longer retained fractions, displayed a higher degree of polarity, hence greater affinity towards the stationary phase (water/MeOH).

The technique here described represents a further improvement in the separation and identification of DOM components, showing the isolation of single compounds according to their chemical-physical properties. The constituents of the DOM sample here discussed could be characterised not only by their molecular formulae but also according to its structural matching on a pre-existing databank (Quall browser).

HR-MS allowed the resolution of several molecular formulae; however is strongly dependent on the detection method on the first chromatographic dimension and on the ionisation method applied. In fact, by using normal-phase HPCCC and UV detection at 330 nm, it was not possible to detect the full DOM pool, but only the compounds with a rather low degree of polarity. It would therefore be of great interest to couple different forms of detection on the first dimension, like RI or fluorimetry, which were employed in previous works [12; 23-24].

In addition, HPCCC provided a first step fractionation in the isolation of single components in a relatively fast and cheap way. The flow rate involved was very low (0.150 mL/min) and after the initial purchase of the instrument, the costs are small, involving only the necessary amount of mobile and stationary phase. By using a liquid stationary phase, there is no need for expensive HPLC columns; and there is also the possibility to reverse this kind of technique, by moving, on the same piece of equipment from normal to reversed-phase. The latter approach will be the subject of future work.

For the first time, individual components were isolated from DOM by a polarity and partition-based off-line multi-dimensional chromatographic approach, allowing a better understanding of structural characteristics typical of single components from this DOM mixture and providing information related not only to their molecular formulae but also on their polarity and structural features.

This MS study represents a preliminary work which aims to target compounds of interest which will be further processed by multiple MS fragmentation (MS^n) and microgram-level NMR experiments in order to fulfil more comprehensive structural information and identification.

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Chapter 5: Investigation into dissolved combined neutral sugars and their microbial conversion in naturally and artificially produced dissolved organic matter using ion chromatography with pulsed amperometric detection and reversed-phase liquid-chromatography-high resolution mass spectrometry

Abstract

IEC-PAD was employed to investigate dissolved combined neutral sugars and their microbial conversion in artificially prepared dissolved organic matter (ADOM) and naturally occurring DOM obtained from seawater and freshwater sources. The analysis of ADOM and naturally occurring DOM samples using IEC-PAD resulted in chromatograms suggesting very similar composition, each characterised by three early eluting peaks, the latter of which being a broad, yet retained, co-elution of multiple compounds. For naturally occurring DOM, several sugars, including arabinose, glucose, galactose, xylose and ribose, could also be identified.

The three distinctive peaks obtained from IEC-PAD of ADOM were collected and further analysed by means of RP-HPLC-HR-MS, the latter showing that glucose was totally consumed during microbial production of ADOM and potentially transformed into higher molecular weight materials.

The aim of the study here presented was to compare the IEC-PAD chromatograms from different DOM sources in order to understand their sugar content and their similarities to the IEC-PAD chromatogram obtained from ADOM.

5.1 Introduction

Due to the complexity of DOM and the various classes of compounds within it, it is difficult to find a suitable analytical approach able to investigate the MCP activity. The important role of sugars in this major source of DOM has been recognised [1], as carbohydrates represent a percentage of the phytoplankton photosynthetic production comprising LDOM (See Chapter 1). Ogawa *et al.* were one of the first groups to assess the microbial origin of the refractory portion of DOM and to kinetically monitor glucose and glutamate microbial consumption in seawater using TOC analysis [2]. Thus, the relationship between the chemical characteristics of DOM and microbial activity was proved, as microbes can alter the molecular structure of DOM, transforming it into refractory materials.

Sugars within sea and freshwater are commonly analysed using GC. However, poorly volatile carbohydrates, such as terrestrially derived polymeric materials are not only difficult to detect, but can also cause extensive unresolved “humps” along the GC chromatogram [3-4].

IEC, and in particular anion exchange chromatography with PAD is commonly used in carbohydrate analysis, even applied to a complex matrix such as seawater [5-8]. In this technique, species are negatively charged by the highly basic eluent used to ensure retention on the positively charged stationary phase; the interaction strength is commonly dependent on the number of the charges on the molecule. Molecules with the weakest ionic interactions elute from the column first; conversely, those that have a stronger ionic interaction have higher retention times.

However, such a chromatographic approach has frequently been challenging, mainly because of the difficulty in determining low concentrations

of sugars dissolved into such a complex environmental matrix such as seawater. For example, Mopper *et al.* [9] were one of the first groups to employ IEC-PAD to directly analyse seawater. The reported chromatograms show the presence of unresolved “humps” which are possibly due to the complexity of the sample matrix. Furthermore, because salt anions were found to interfere with the analysis, a cation exchange resin was used to desalt the samples. These resins, however, also remove acidic sugars, causing them not to be detected. Wicks *et al.* [10] tried different desalting resins such as amino, cyano, diol, aromatic sulfonic, quaternary amine and polyethyleneimine to optimise the desalting procedure. However, the average recovery of neutral, amino and acidic sugars ranged from 6.5 to 19.4%, with considerable differences in the sample composition.

In 2000, Kaiser *et al.* [5] processed a seawater sample without employing any extraction technique and further improved the LOD to 4 n*M*. This approach targeted the analysis of amino sugars only, though it is not clear to what extent the sample preparation (3 *M* HCl for 5 h at 100 °C) affected individual sample components. Such issues, as highlighted by Cheng *et al.* [11], can lead to an overestimation of the sugar content within the sample.

To overcome the problems associated with the presence of salt anions, Engel *et al.*[12] employed a membrane dialysis desalting method prior to IEC-PAD. The authors were able to target neutral, amino and acidic sugars in a single chromatographic run, and highlighted that hydrolysis conditions needed to be optimised according to the different classes of sugars. The acid hydrolysis of combined carbohydrates was reported to change with the kind and source of analysed sample, with the strength, type of acid and the hydrolysis conditions

(i.e. duration and temperature). For example, strong acids were more effective in breaking stable bonds, but can destroy sensitive sugars.

In the work here presented, IEC-PAD was applied with the aim to evaluate the chromatograms obtained from ADOM with those obtained from naturally occurring DOM samples, from sea and freshwater. Further to this, ADOM was also compared to seawater DOM at different depths. The ADOM was fractionated using the IEC method, and peaks subsequently further analysed by means of RP-HPLC-HR-MS to understand if the nutrients employed to prepare ADOM had been totally consumed and potentially transformed into different materials.

5.2 Materials and methods

5.2.1 Reagents

The 32% HCl and MeOH employed in DOM extractions were purchased from Sigma-Aldrich (Sigma Aldrich, Dublin, Ireland).

For the preparation of ADOM, glucose, Na_2HPO_4 and NH_4Cl were also obtained from Sigma-Aldrich (Sigma Aldrich, Dublin, Ireland).

In the IEC-PAD analysis, the water was Milli-Q grade (Millipore, Watford, U.K.). For RP-HPLC-HR-MS, MS grade formic acid, MeOH and water were purchased from Sigma Aldrich (Sigma Aldrich, Sidney, Australia).

5.2.2 Seawater and freshwater collection and sample preparation

Seawater and freshwater samples were collected in August 2011 respectively from the Irish Sea coastline in Bray, (53°12'04''N, 6°06'41''W) and from the source of river Shannon (54° 14'05''N, 7°55'08''W, collected in May 2011), in Ireland. The 10 and 60 m Irish Sea samples (54°10'08''N, 5°45'47''W)

were collected on a research cruise (CV10_028) in June 2010. The samples were extracted as described in Chapter 2 by following the method proposed by Dittmar *et al.*[13].

5.2.3 Artificially prepared dissolved organic matter

The experimental conditions proposed by Ogawa *et al.* [2] were applied to assess the difference between a seawater microbial culture (Bray, 53°12'04''N, 6°06'41''W) and a blank culture, constituted of Milli-Q water instead of seawater. The culture media was prepared in duplicate in an inoculum containing 2 L of Bray seawater (or Milli-Q water in the case of the blank), 4 g/L of glucose as carbon source, 6 g/L Na_2HPO_4 as phosphorous source and 1 g/L NH_4CI as nitrogen source. After two weeks incubation, both blank and ADOM from the inocula were also extracted according to the method from Dittmar *et al.*[13]

5.2.4 Ion exchange chromatography with pulsed amperometric detection

The IEC-PAD was performed on a Dionex 500 system (Dionex Corp., Sunnyvale, CA) equipped with a Dionex GP50 quaternary pump, an ED40 electrochemical detector module, a Dionex ED40 compartment with a KOH eluent generator and a column compartment (Dionex LC30). The separations were performed on a CarboPac-PA1 column (4 x 250 mm, particle size 10 µm) equipped with a Carbopak PA1 guard column, with dimensions 4 x 50 mm and particle size 10 µm (Dionex Corp., Sunnyvale, CA) in a gradient mode (50 to 100 m*M* KOH in 50 minutes). The flow rate was 1.0 mL/min and the injected volume 25 µL.

PAD was performed with an Au working electrode (ca. 0.15 cm^2), and an Ag/AgCl reference electrode (Dionex Corp., Sunnyvale, CA). The PAD reference electrode parameters were set according to Dionex optimised parameters for the analysis of carbohydrates (Table 5.1).

Table 5.1: *Time intervals and voltages applied on the Ag/AgCl working electrode .*

TIME (sec)	VOLTAGE (V)
0-0.1	0.1
0.1	0.1 (begin)
0.2	0.1 (end)
0.21	-2
0.22	-2
0.23	0.6
0.24	-0.1
0.27	-0.1

5.2.5 Reversed-phase high-performance liquid chromatography with high resolution mass spectrometry

The three characteristic peaks which were collected from the IEC-PAD separation were concentrated by compressed air flow to a final volume of 150 µL and processed by collaborators through RP-HPLC-HR-MS. The column used was a Nova Pak C_{18} (3.9 x 150 mm, 4 µm pore size, Thermo Scientific Horsham,UK), on a Waters Acquity (Waters, Milford, USA) system connected to the Thermo Finnegan LTQ Orbitrap in negative mode (Thermo Scientific, Scoresby, Australia). Mobile phase A was H2O 0.1 % formic acid, while mobile phase B MeOH 0.1% formic acid. The flow rate was 0.800 mL/min and the gradient ran from 10% to 100% MeOH 0.1% formic acid in 20 minutes. The mass spectra were analysed in negative mode (capillary 4500 V, collision energy 20 eV, nebulizer 10.0 psi, dry gas 5.00 L/min, dry temperature 350 ºC).

5.3 Results and discussion

5.3.1 Separation of a standard mixture for the identification of sugars within dissolved organic matter

A standard mixture of sugars was firstly separated by IEC-PAD with a 50 to 100 m*M* KOH gradient to obtain the retention factors for each sugar. The column employed was a PA1, packed with a nonporous resin based on PS-DVB and bearing anion exchange functionalities (alkyl quaternary ammonium groups) [14].

The separation mechanism on IEC is based on charge density, with the strength of the interaction determined by the number and location of the charges on the molecule. By increasing the KOH concentration, the molecules with the weakest ionic interactions start to elute from the column first, whereas those with a stronger ionic interaction require a higher KOH concentration and elute later. Polarisation and hydrated ionic radius also play a key role in the retention mechanism. Ions which can be polarised have a longer retention time as the charge concentrates in one portion of the molecule, the latter being more strongly retained onto the stationary phase. Conversely, if an ion has a high charge density, it can attract more solvent molecules and this results in a greater hydrated radius and a consequent lower retention time if compared to those with a narrower hydrated radius.

A mixture of 10 sugars commonly found in DOM [2; 4-6; 15] was prepared at a concentration of 50 µg/mL in order to compare the retention times appearing on the chromatograms from DOM samples to those of known standards.

Table 5.2: *Retention times for the 50 µg/mL standard mixture prepared to optimise the IEC-PAD method. Flow rate 1.0 mL/min of a 50 to 100 mM KOH gradient, injected volume 25 µL.*

#	RT (mins)[a]	SUGAR	K'[b]	PEAK HEIGHT (nC)	N[c]
1	2.61	fructose	0.63	1.59	2233
2	3.49	mannitol	1.18	0.33	3993
4	10.0	arabinose	5.25	0.59	1539
5	11.9	glucose	6.44	0.57	2179
6	14.2	galactose	7.88	0.73	2280
7	17.3	xylose	9.81	0.59	2047
3	19.26	sorbitol	11.03	0.19	2055
8	25.6	sucrose	15.00	0.23	1985
9	34.5	ribose	20.56	0.30	1362
10	39.5	maltose	23.69	0.22	2667

a: retention time; b: retention factor; c:number of theoretical plates

5.3.2 Separation of artificially produced dissolved organic matter

Two litres of seawater from Bray were placed in a conical flask and nutrients added in order to stimulate the activity of microbes naturally present within seawater. After two weeks, the ADOM formed was extracted using SPE [13] and a brown powder obtained. Due to the employed extraction method, the presence of anionic species, which can affect the chromatographic resolution, was eliminated. In parallel, the same procedure was adopted for two litres of Milli-Q water, in order to obtain a blank sample, where no microbes were present to metabolise the nutrients. This time a white powder indicating the presence of unconsumed glucose was obtained after the extraction procedure. A 0.1 mg/mL solution of each sample was prepared in water and analysed by IEC-PAD.

On the ADOM chromatogram, it is possible to notice an important difference if compared to the blank (Figure 5.1). If the latter only shows a broad signal relative to unconsumed glucose (identified by comparison with a standard), the ADOM chromatogram shows no glucose signal, highlighting that this sugar had been transformed into other products.

Another interesting feature along the ADOM chromatogram is the presence of a tailed peak eluting for 6 minutes (from minute 4 to 10), which possibly represents coeluting materials characterising a consistent portion of molecules derived from the microbial consumption of glucose.

According to literature [2], one of the main microbial by-products is glutamate, which was detected neither by PAD nor by the following RP-HPLC-HR-MS analysis.

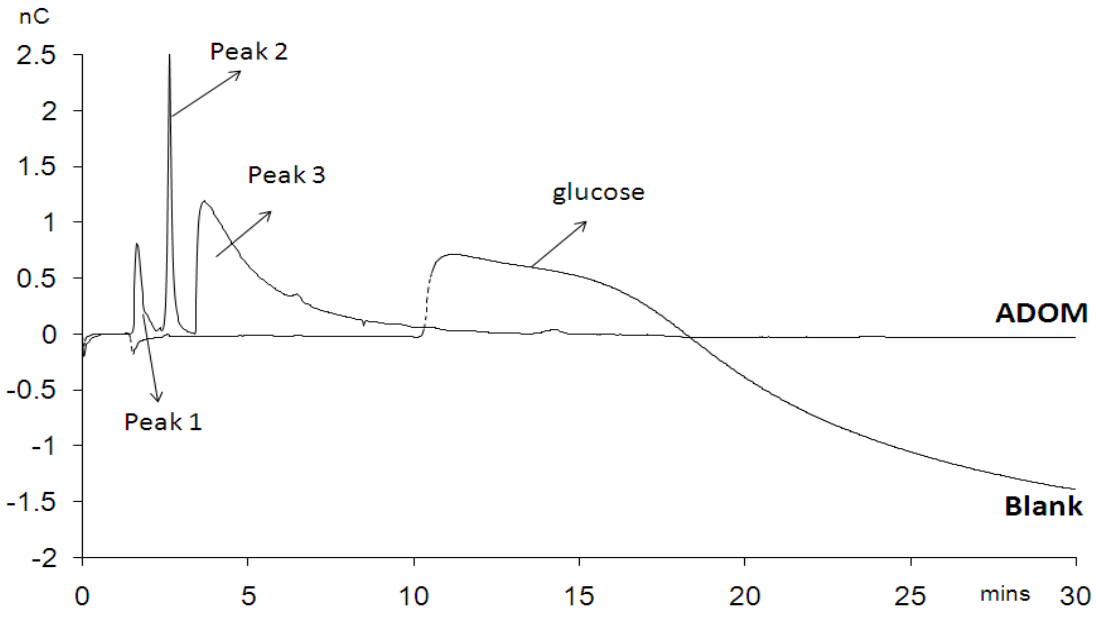


Figure 5.1: *Comparison between ADOM and the correspondent Milli-Q water blank. Reported are the peaks which were collected and further analysed by RP-HPLC-HR-MS. Flow rate 1.0 mL/min of a 50 to 100 mM KOH gradient, injected volume 25 µL.*

5.3.3 Bray and Shannon Pot dissolved organic matter samples

DOM from seawater and freshwater sources were also analysed by IEC-PAD in order to compare their profiles with that obtained from ADOM. As can be seen from comparing Figure 5.1 to Figure 5.2, the chromatograms are similar, underlining that ADOM possibly has similar components with naturally occurring DOM. Furthermore, as can be seen from Figure 5.2, the bulk of seawater and freshwater DOM probably also share similar composition. In fact, these two different DOM sources have been found to have in common the following classes of compounds: CRAM, heteropolysaccharides, lipid-like materials, aromatic compounds and MDLT [1-3].

Within the chromatograms on Figure 5.2, by comparison with the retention times of the standards (Table 5.2), the freshwater sample showed potential peaks for glucose (11.2 mins) and galactose (14.2 mins). No sugars were detected on the coastal seawater sample.

Since the Bray sample was collected from the shore line and the Shannon Pot is a pool surrounded by trees, it is reasonable to think that these water sources are probably heavily affected by materials of terrestrial origin and are characterised by a high microbial activity. As emphasised by Hedges *et al*., low sugar contents are usually related to an enhanced presence of humic-like substances as products of microbially degraded leaf materials solubilised in water [16]. Also, considering the absence of detected sugars, in particular of glucose, on the Bray sample, it must be considered that this nutrient is usually difficult to accumulate as is readily used not only by microbes naturally occurring in seawater and freshwater, but also by species such as algae, which commonly accumulate on the shore line [17].

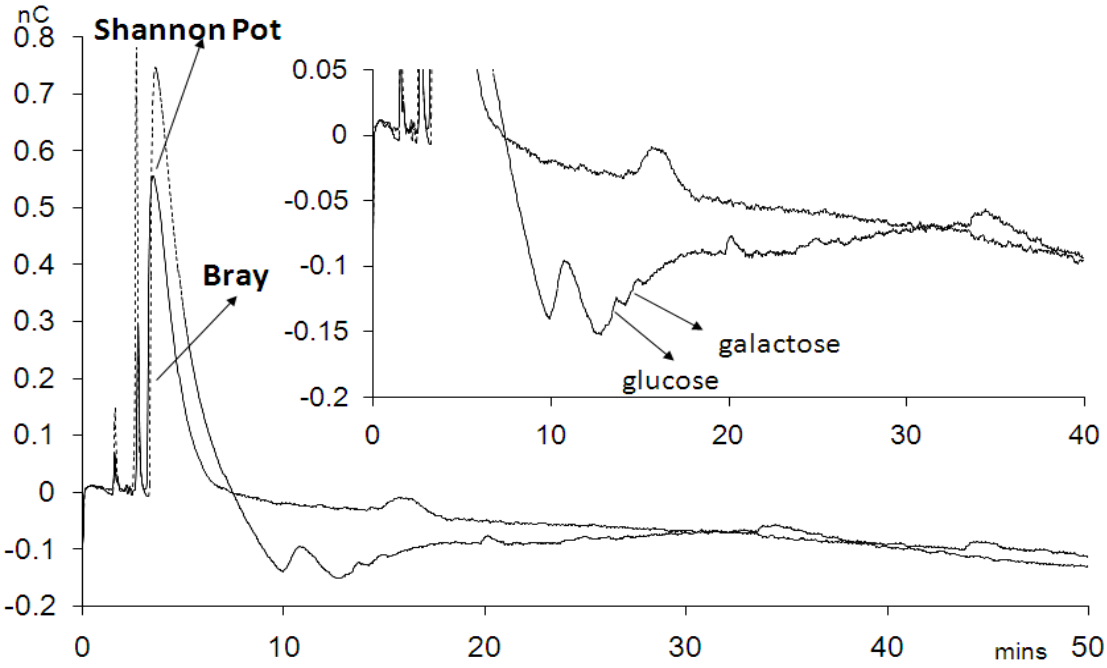


Figure 5.2: *Comparison between Bray (seawater) and Shannon Pot (freshwater) samples. Flow rate 1.0 mL/min of a 50 to 100 mM KOH gradient, injected volume 25 µL.*

The presence of galactose can also be related to the occurrence of materials of terrestrial origin within the Shannon Pot sample. This sugar is in fact a constituent and the hydrolysis product of galactan, a polymer present in hemicellulose, the main constituent of plant cell walls [18].

5.3.4 Irish Sea dissolved organic matter samples from the depth of 10 m and 60 m

In order to compare the depth profile for sugars, two samples from the same seawater column (10 and 60 m depth from the Irish Sea) were analysed (Figure 5.3). According to the separation obtained for the sugar standards (Table 5.2), at 10 m depth can be noticed the possible presence of glucose (11.2 minutes), together with arabinose (10.3 minutes), galactose (14.5 minutes), xylose (17.5 minutes) and ribose (34.4 mins). Galactose is present at lower concentration in the 60 m depth sample, which probably means a higher

influence from plant derived materials (i.e. hemicellulose). This is reasonable as this sample is at the top of the water column. Xylose and arabinose also suggest the terrestrial influence of the sample as these sugars can also be found as hemicellulose building blocks.

Ribose (Figure 5.3) can be related to the presence of RNA and other derivatives (i.e. ATP), which cover a key role in cellular metabolism. As described by Cowie *et al.* [19], the distribution of microbially derived aldoses are often related to the ribose content within seawater. According to this study, ribose concentration is directly proportional to the content of aldoses. Microbes within seawater are prone to phage attacks, which can rip their host causing the release of organic compounds [1]. The latter can include genetic material present in the cell. Therefore, lysis processes could have caused the release of ribose within this water source.

As can be seen by comparing Figure 5.2 to Figure 5.3, both the 10 m and 60 m samples are richer in sugar content than the Bray and Shannon Pot samples, which is probably due to a lower microbial activity on the samples collected from the same water column. This can be consistent with the higher concentration of glucose on the 10 m and 60 m samples, not only if compared to the Bray and Shannon Pot DOM, but also to the ADOM (Figure 5.1).

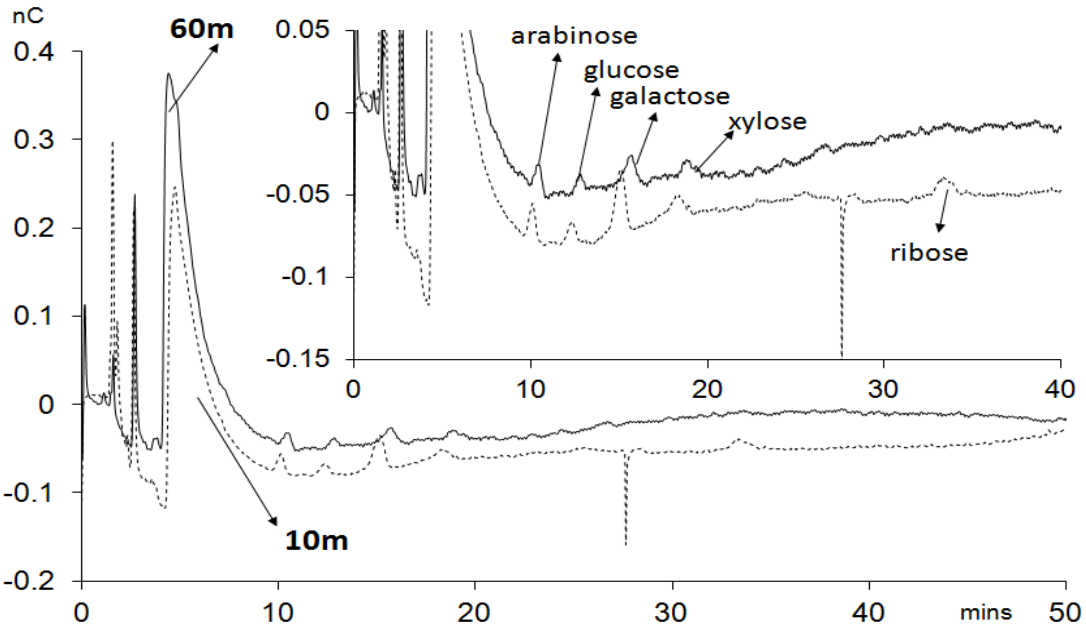


Figure 5.3: *Comparison between 10 m and 60 m depth Irish Sea samples. Flow rate 1.0 mL/min of a 50 to 100 mM KOH gradient, injected volume 25 µL.*

To understand if the peak at 11.2 minutes was really glucose, the sample was spiked with different concentrations of a glucose solution: 3.75 µg/mL and 7.5 µg/mL. As can be seen from Figure 5.4, the peak intensity increases with an increasing concentration of added glucose. This confirms that the signal is due to the presence of glucose in the sample. Considering the peak height for the samples at a concentration of 3.75 µg/mL (0.3871 nC) and 7.5 µg/mL (0.9132 nC) and that for the unspiked sample the peak height is 0.03 nC, a concentration of glucose of 3.6 µM was calculated from the extracted DOM powder, which is in line with those reported in previous studies [11-12; 20].

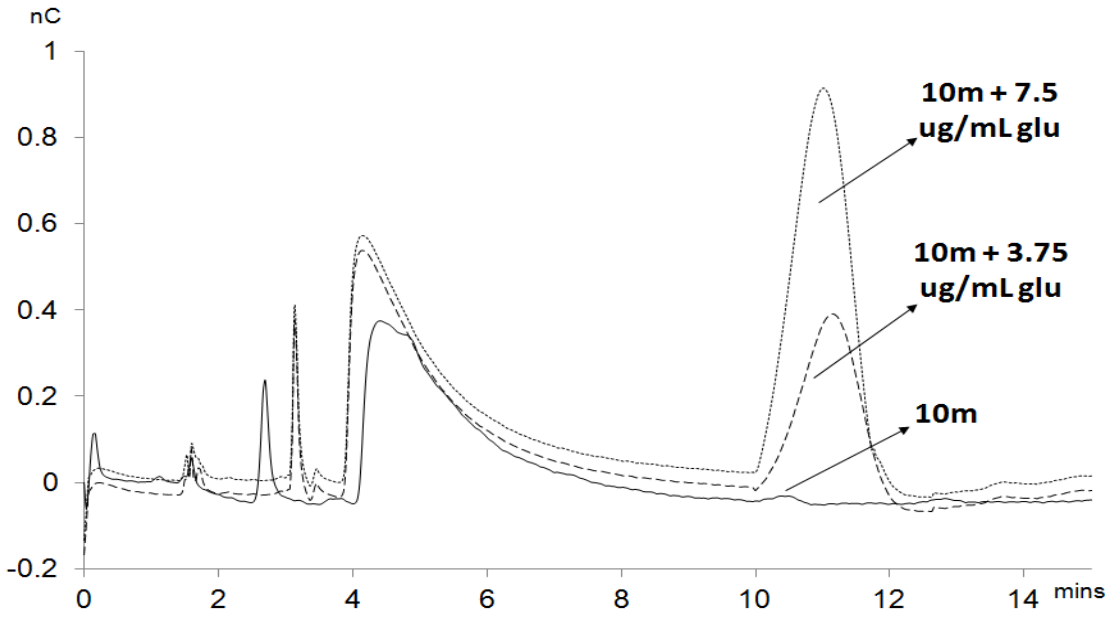


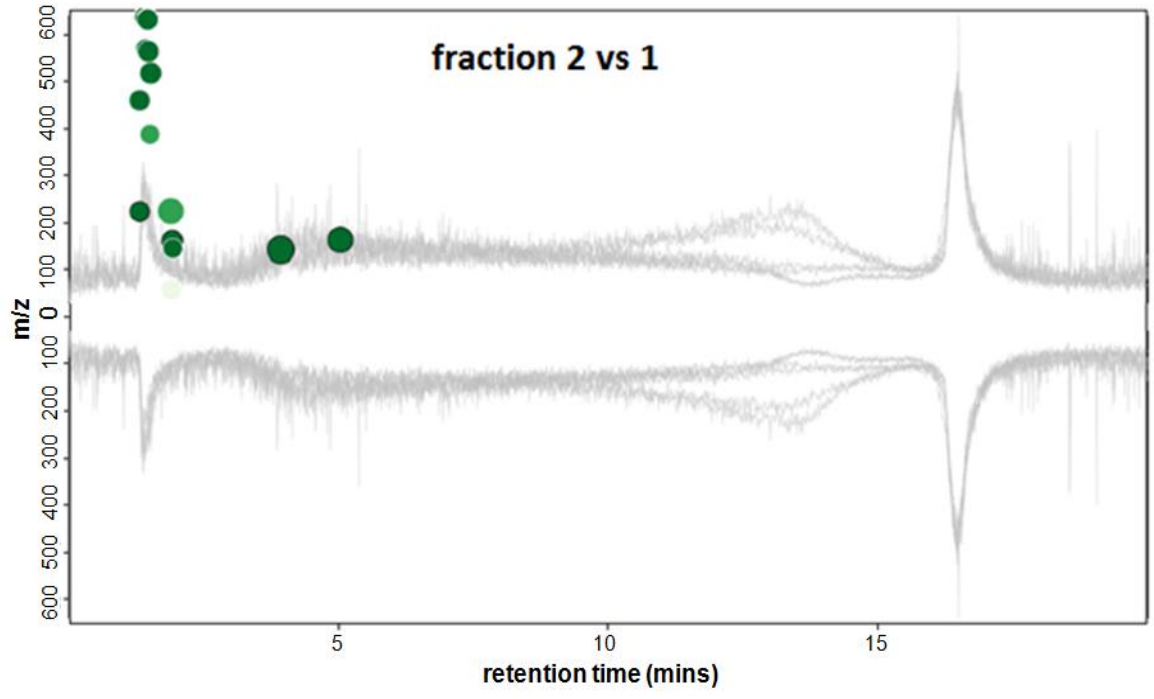
Figure 5.4: *10 m Irish Sea sample spiked with different concentrations of glucose. Flow rate 1.0 mL/min of a 50 to 100 mM KOH gradient, injected volume 25 µL.*

5.3.5 Analysis of the three peaks from artificially produced dissolved organic matter through reversed-phase high resolution mass spectrometry

Since the chromatograms from the naturally occurring DOM samples were analogous to those obtained for the artificially obtained one, three peaks from the ADOM chromatogram (retention times: 1.6 mins, 3.6 mins and the "hump" going from 4 to 9.5 minutes, Figure 5.1) were collected, concentrated by compressed air flow and injected into RP-HPLC-HR-MS in negative mode. Such ionisation mode was chosen because KOH was used as mobile phase in IEC. Therefore, the high concentration of KOH present in the collected peaks after concentration caused the deprotonation of the eluting species. Each of the three RP-HPLC-HR-MS analyses was performed in triplicate to assess the intra-sample variability. On each collected peak, 153 to 180 distinctive features (signals three times more intense than the baseline) were identified through HR-MS. Due to the complexity and the number of data that were due to be treated,

XCMS on-line was employed in order to simplify the data processing [21-22]. This web-based platform is able to identify features whose relative intensity varies between two different samples and to highlight the most distinguishing ones.

The mirror plots (Figure 5.5) display ions in which the intensities are altered between two different samples according to statistical thresholds set by the user (in this analysis a ≤0.005 p-value). Features that are down-regulated (represented in red colour) are marked as circles on the bottom of the plot, whereas features that are up-regulated (in green colour) are represented as circles on the top. The size of each circle corresponds to the logarithm of the fold change of the feature: larger circles correspond to peaks with greater fold changes. The colour intensity represents the p-value: the brighter the circles, the lower the p-value [21-22]. Furthermore, if the features match those present on Metlin databank, the circles are underlined in black [21].



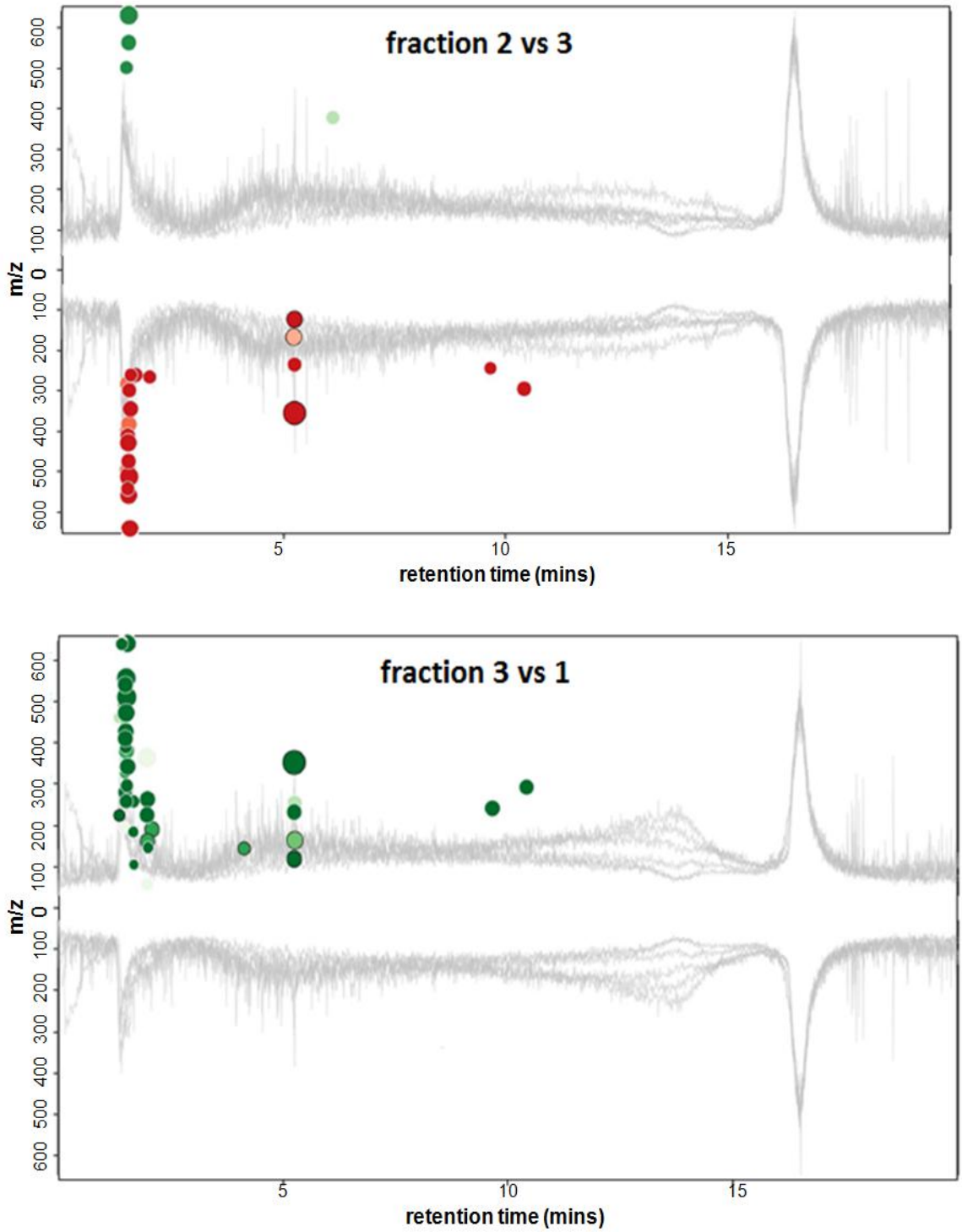


Figure 5.5: *Mirror plots representing the comparison between the three collected peaks and the markings for their unique features. Flow rate 0.8 mL/min, capillary 4500 V, collision energy 20 eV, nebulizer 10.0 psi, dry gas 5.00 L/min, dry temperature 350 ^{o}C.*

On the Figures reporting the comparison between the three chromatographic peaks (Figure 5.5) can be seen that all the chromatograms show a similar behaviour. This is not only due to co-elution issues, but mainly to

the fact that in HR-MS most of the signals are hidden by the baseline noise, which is due to the solvent contribution. As the most predominant species are solvents, the chromatographic features have to be searched through by subtracting any interference from contaminants present along the chromatogram. For example, on Figure 5.6, the ions at m/z 91.0032 and 112.9850 are due to solvents and always present from the start to the end of the analysis. As can be seen, they have the highest intensity (Figure 5.6). As these ions do not represent unique features along the analysis, they were not considered any further in the data processing.

The most distinctive features detected from peaks 1 to 3 can be found at the beginning of the chromatogram (Figure 5.5 and 5.6), where the gradient is dominated by the presence of water. It can be concluded that such compounds have polar functional groups which render them poorly retained on the C_{18} stationary phase (i.e. -OH and -COOH).

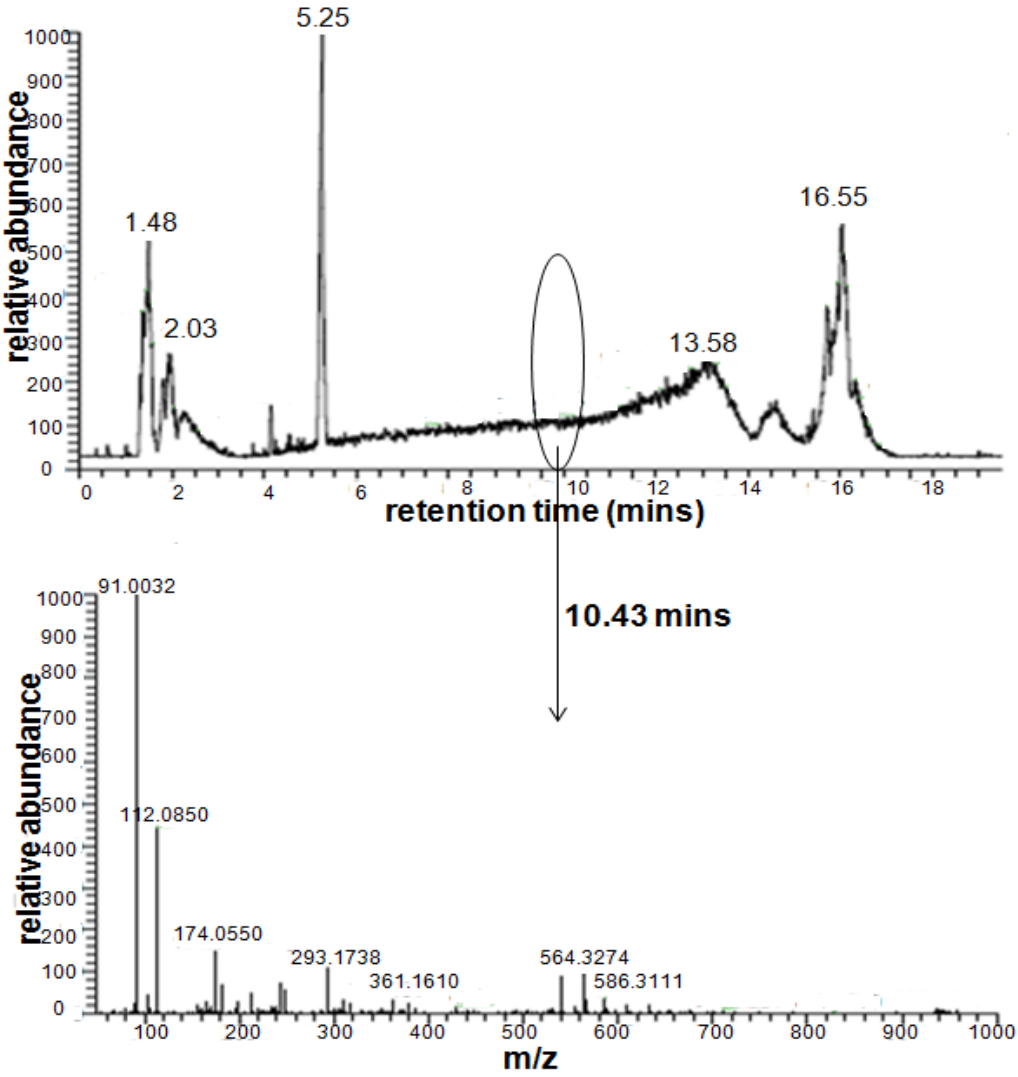


Figure 5.6: *BPC for peak 3 with mass spectrum for a compound eluting at 10.43 minutes. Flow rate 0.8 mL/min, capillary 4500 V, collision energy 20 eV, nebulizer 10.0 psi, dry gas 5.00 L/min, dry temperature 350 ^{o}C.*

Between all, the most dominant features which differentiate the three peaks collected from IEC, just 5 matched Metlin databank, none of them appearing from peak 1 (Table 5.3).

Table 5.3: *Compounds from peaks 2 and 3 with a match on Metlin Databank. Flow rate 0.8 mL/min, capillary 4500 V, collision energy 20 eV, nebulizer 10.0 psi, dry gas 5.00 L/min, dry temperature 350 ^{o}C.*

PEAK	RT (mins)	m/z	METLIN MATCH
2	1.3	225.0606	hexahydroxyheptanoic acid
2	3.9	145.0500	adipic acid
3	2.1	191.0190	citric acid
3	4.1	145.0500	adipic acid
3	5.2	121.0289	benzoic acid

Although the identity of the compounds present in Table 5.3 needs to be confirmed by the injection of standards on the RP-HPLC-HR-MS and MS/MS experiments, the match for hexahydroxyheptanoic acid and adipic acid (present both on peaks 1 and 2, see Table 5.3) support the theory that the compounds eluting on these poorly retained peaks are alkylic materials with polar functional groups. Such compounds are usually not naturally occurring and might have been present from the sampling. The same considerations apply for benzoic acid. Previous experience with protocol blanks did not reveal such species. However, this does not preclude subsequent potential contamination.

This remark highlights the need to obtain a DOM sample without anthropogenic materials and the need for a more targeted extraction procedure. This could be achieved by using different SPE cartridges in series, with different affinities for the different classes of compounds present within DOM.

The match for citric acid is quite interesting as this molecule is a naturally occurring compound, therefore further investigation needs to be carried out to confirm its identity and role within DOM.

According to the m/z detected within the three peaks from IEC, all the molecules are in a low molecular weight range (none of the m/z exceeds 530, see Table 5.4). The peak characterised by the highest number of distinctive

features was number 3 (Figure 5.5 and 5.6), the most representative being showed in Table 5.4.

Given the m/z range of the compounds detected from RP-HPLC-HR-MS, it can be concluded that most of the products of microbial activity have a higher molecular weight if compared to the starting products (i.e. glucose), underlining the occurrence of a transformation to more complex materials.

Table 5.4: *Most characterising m/z detected from peak 3. Flow rate 0.8 mL/min, capillary 4500 V, collision energy 20 eV, nebulizer 10.0 psi, dry gas 5.00 L/min, dry temperature 350 ^{o}C.*

#	m/z	RT(mins)
1	451.1201	1.35
2	225.0606	1.36
3	461.149	1.36
4	529.1357	1.38
5	89.0242	1.67
6	128.0347	1.88
7	191.019	2.05
8	145.05	4.12
9	165.0185	5.23
10	166.0219	5.25
11	121.0289	5.25
12	233.0054	5.25
13	353.026	5.25
14	173.081	5.71
15	187.0967	6.57
16	242.175	9.66
17	91.0035	12.01
18	311.1674	14.38
19	339.1986	14.48
20	325.183	15.1
21	339.1987	16.08
22	311.1674	16.11

5.4 Conclusions

In this approach it is shown a unique off-line multi-dimensional method which aimed to investigate the influence of glucose in the production of DOM

and the presence of neutral sugars within different DOM samples. To do so, the chromatogram obtained through IEC-PAD from ADOM was compared to those from naturally occurring DOM. All the analysed samples from different water sources or depths show very similar chromatograms, allowing the consideration that the bulk of DOM is analogous in nature and probably from microbial origin, as shown by the chromatogram of the ADOM sample. Since most of the ions detected in the HR-MS experiment have higher m/z than the molecular weight of the starting compounds used to produce ADOM (i.e. glucose) and since on IEC-PAD there is no trace of glucose, it can be reasonable to conclude that such nutrient possibly underwent a transformation into heavier and unknown materials.

This can be verified by constantly monitoring the products derived from microbial activity during the preparation of an ADOM sample. A progressive decrease in glucose level and the formation of new compounds should be observed during different time scales.

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Chapter 6: Conclusions and future work

The purpose of this work was to fractionate DOM and isolate single compounds in order to understand their chemical structure. This represents a

first step to a better understanding of the properties within the main constituents of this complex organic mixture.

The use of SEC played a key role in fractionating DOM as each section of the size exclusion chromatogram which was collected and further analysed on the second dimension, showed a different BPC, highlighting the presence of characteristic compounds on each fraction. A low resolution mass spectrometer was employed as the form of detection, providing qualitative information on the fragmentation pathway the isolated compounds underwent.

Due to the mixed-mode retention mechanism occurring during the SEC fractionation, HPCCC was proposed as an alternative first off-line dimension. In this case, the fractionation occurred according to polarity, allowing the generation of characteristic chromatograms on the second dimension both by means of GC-MS and RP-LC when compounds may not have been volatile enough for detection by GC-MS. These preliminary results represented a first crucial step in the isolation of single compounds from DOM by employing RP-HPLC with HR-MS. The latter was not only able to show that characteristic features along the BPCs were eluting from the different fractions collected from HPCCC, but also provided their molecular formulae together with their fragmentation pathway. This further underlines that a HR detector must be used in DOM analysis. Low resolution MS cannot provide an exact formula of compounds or class of compounds, therefore cannot be employed to obtain any kind of structural information from unknown materials. For instance, an m/z 45 loss can be mistaken both for a CO_2 or a $CONH_2$ functionality, therefore not providing enough information for the clear identification of characteristic functional groups within DOM. Alternatively, HR-MS Orbitrap technology can recognise fragmentation pathways.

Within these experiments, major areas were highlighted where further improvements are needed to have a more comprehensive isolation and characterisation of DOM.

An important issue in this project has been the isolation of DOM itself. By definition, the PS-DVB stationary phase employed in the SPE method did not guarantee a total recovery for all the existing molecular species within DOM, but mainly molecules with semi-polar to apolar properties. A more efficient method must be found, possibly exploiting the different properties which SPE resins can have. Different and more specific SPE cartridges can be employed in series, allowing a more targeted extraction which could also simplify the following chromatographic analysis. For example, a phenyl-bonded silica support would allow the extraction of aromatic compounds via π-π stacking between the stationary phase and the analytes of interest, or a dyol-bonded silica support would be otherwise specific for highly polar compounds.

The choice of the chromatographic technique is a very important component in the analysis of DOM. Both chromatograms obtained from the first off-line dimension SEC or HPCCC showed very low resolution, with extensive co-elution. A further chromatographic dimension in the analysis of such a complicated mixture is therefore crucial and possibly the only way to isolate single compounds. RP-HPLC was employed as second dimension in order to provide an orthogonal approach. However, in the future, it will be important to investigate other forms of liquid chromatography as second or third off-line dimensions, as it appears evident that only the combination of orthogonal chromatographic techniques is able to untangle such a complex mixture. For example, cyano-bonded silica stationary phases (Si-CN) could be tested. The latter are commonly employed to isolate moderately polar compounds such as

steroid-like materials. This could potentially allow the specific isolation of CRAM. Another promising technique might be silver chromatography, which allows the separation of isomeric compounds with different number of double bonds, which have been found to be prominent features within DOM.

The use of multiple and “universal” forms of detection on the first chromatographic dimension is also of primary importance as UV detection cannot offer comprehensive information on the whole DOM sample. RI was tried but the sensitivity was too low to provide any sort of informative signal. An option for future analysis could be evaporative light scattering (ELS) or charged aerosol detection (CAD), wich might discriminate compounds that usually cannot be detected by classical detectors such as UV and fluorescence (i.e. lipids).

Despite HR-MS being a valuable source of information, the identity of single compounds could not be confirmed. For this reason, and because of the absence of standard materials matching DOM components, the analysis through microgram-level multi-dimensional ^{1}H and ^{13}C nuclear magnetic resonance (NMR) will be performed in the near future.

The comparison between naturally occurring DOM and ADOM showed that the bulk of DOM is probably of microbial origin and also highlighted that ion exchange chromatograms of DOM from different water sources (i.e. seawater and freshwater) are analogous. Such experiments can provide a better understanding of the MCP and its role in the production of refractory forms of DOM. For example, kinetic studies together with the use of ^{14}C labelled substrates (i.e. glucose) might be helpful to better understand the reactions that occur within such complicated biological systems. For example, the processes

that yield ADOM can be studied at different time intervals by means of both NMR and HR-MS, in order to potentially identify crucial reaction intermediates.

This project underlines how off-line multidimensional chromatography with HR detection represents an important step in DOM fractionation in an attempt to overcome the analytical challenge presented by the complexity of DOM. However, the optimization of the extraction and chromatographic conditions still presents a major challenge to the isolation and consequent identification of DOM components. Until this is achieved, it will be very difficult to understand the sources, fate and contribution to carbon cycling of DOM.