High-resolution mass spectrometry strategies for the investigation of dissolved organic matter

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Motivation

High-resolution mass spectrometry (HRMS) reveals an unparalleled chemical complexity in environmental samples, and an incredible amount of data can be gathered from single samples. Unfortunately, without analytical standards, the data is not quantitative and cannot be assigned to specific structures, so the exact nature of natural mixtures like dissolved organic matter (DOM) is unknown. Current research of DOM using HRMS aims to either probe the nature of such mixtures or to explore how DOM mixtures change during environmental processing. In this chapter, we will discuss the elements of both research trends.

We begin by exploring the environmental reasons for the ubiquitous feature diversity found and then will discuss analytical strategies that aim to improve our understanding of environmental samples and the way that they are processed in nature. This work is intended as a reference for aquatic scientists who want to better understand the analytical obstacles presented by complex mixture HRMS analysis as well as some of the recent analytical strategies that have been developed to deal with these problems.

Environmental production and processing of DOM with regards to individual analytes

Due to the complexity of DOM and typically low analyte concentrations, it is often difficult to trace the origins of individual components [1]. Many analytes or signals (e.g., NMR features, fluorescence signals, HRMS peaks) seem to be interrelated and are often assumed to have a similar source, history, and fate. In aquatic systems, it is common to find references to "allochthonous," or "terrestrial," and "autochthonous," or "aquatic," organic matter, meaning produced previously and transported to the location or produced locally, respectively. Other studies take a more functional approach and define groups as "recalcitrant" and "fresh." "Terrestrial" DOM tends to have more phenolic, aromatic character than fresh DOM and is therefore highly light absorbent. Its source is generally considered to be plant structure material such as lignin, which has been processed and solubilized in soils before washing into the aquatic system through soil

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pore waters. "Autochthonous" organic matter is formed via primary production and local processing of the waste products of primary producers. Other "fresh" sources of DOM may include aerial sources to aquatic systems such as leaf litter, pollen, and dust (i.e., wet deposition), although these are clearly allochthonous.

Generally, biological processes use enzyme-mediated reactions to produce relatively large abundances of a small number of compounds (metabolites), while geochemical processes are less specific and tend to produce many more randomized structures. Consider, for example, the production of glucose via the Calvin cycle, which requires specific nucleotides/cofactors (ATP and NADH) and produces a single product (glyceraldehyde-3-phosphate) and compare with the Maillard browning reaction, which is a purely chemical process that takes a reducing sugar (e.g., glucose) and an amino acid and forms a complex mixture of products after application of heat [2]. For this reason, it can be expected that "fresh" organic matter has less molecular diversity than geochemically processed DOM.

Labile DOM may be cycled so rapidly that its steady-state concentration is low, despite participating in a large portion of the total active carbon biogeochemistry in the system. Confounding this problem are analytical issues. It is possible, even likely, that due to its more hydrophilic nature [3], fresh organic matter is harder to isolate from water [4], which means that studies which employ solubility-based isolation methods like solid-phase extraction are often biased toward processed, less reactive DOM. Further still, freshly produced compounds may be less compatible with electrospray ionization (ESI) (see "Ionization and optimizing sample transfer to the mass spectrometer" section) due to a lower abundance of readily ionized groups (e.g., more sugars and fewer organic acids), higher mass (proteins and polysaccharides), and less hydrophobic character (worse ESI spray character). ESI-HRMS and SPE thus combine with the biogeochemical fact of low concentration to form a situation where fresh DOM is less well characterized and understood than relatively inactive, stable DOM.

The few studies that investigate fresh DOM with MS find that it does indeed have a lower isobaric diversity and a less Gaussian distribution, indicating a less randomized and processed nature [4–6]. By inference, the Gaussian and highly patterned distributions found in processed DOM are controlled by combinatorial limits of molecular compositions, and the averaging of an exponentially increasing number of isomers [6–8]. Furthermore, the overall distributions may also be a factor of ionization efficiency and response based on structural functionality. Hertkorn et al. [9] conducted a comprehensive, philosophical analysis of the isobaric, isomeric, and structural diversity of DOM. They report that the number of chemically sensible isomers at 178 Da ranges from 10^4 to 10^8 depending on the molecular formula. Additionally, they note that increasing oxygen saturation at first increases the number of isomers, then eventually decreases it, as oxygen only takes two chemical bonds. As a result, the diversity of isomers is highest in the central area of the van Krevelen diagram (near to $H/C = 1$ and $O/C = 0.4$). It is probably no coincidence that this area of van Krevelen space contains the largest peak intensities as measured by ESI-HRMS—the isomeric diversity is greatest here, and so even though each isomer may have a vanishingly low concentration [1], the accumulated concentration is higher. It is debated whether functional diversity is as large as isomeric diversity—it may be that similar core structures are present with randomly arranged but similar functional groups [7, 10, 11], and similar core structures may be present in diverse environments due to their chemical stability [6, 8].

HRMS strategies for the investigation of DOM

In an idealized case, an organic analyte in a water sample would be completely extracted and concentrated from the sample without alteration, it would ionize efficiently (give a large response) with a known and reproducible sensitivity (signal/abundance) and it would have no interferences from other analytes with the same mass. Unfortunately, each of these components of the analytical pipeline has multiple complications that do not allow such confidence in analyte determination.

Sample preparation

Seldom can environmental samples be analyzed by MS directly [12], without some preparation, due to the problematic interference of salt and the typically low concentration of DOM. Recent developments in sample preparation have been reviewed comprehensively [13, 14], and here we will focus on how the most common approaches influence the results gathered by HRMS.

Most methods simply aim to maximize the total amount of organic carbon recovered [3, 15–17]. The most common techniques for isolating DOM involve acidification of the sample in order to protonate carboxylic acids, facilitating their immobilization onto hydrophobic resins, polymers or silica phases, and the most common sorbent used is Agilent PPL due to the relative ease and low cost of the method [15]. DEAE cellulose and DEAE sepharose [16, 18], along with weak anion exchange (WAX) resins are also useful, because they can be used at ambient pH, minimizing modification of the sample and also allowing long-term passive sampling [19].

A selection of 24 SPE cartridges was tested for extraction efficiency and proton environment as observed by nuclear magnetic resonance and obtained O/C and H/C ratio by FT-ICR MS using a freshwater and marine sample [20]. The study clearly demonstrated that the chemistry of DOM isolates can be tailored to suit research goals in cases where certain compound classes are required (e.g., weak anions can be extracted by weak anion exchangers; polar sorbents preferentially extract low oxygen species). The study showed that while PPL gave the highest recovery for extraction of marine DOM, different sorbents may be useful for different research questions. Studies that aim to investigate hydrophobic naphthenic acids can selectively target these compounds using hydrophobic polymers [21].

Raeke et al. [3] found that PPL extraction at pH 2 only had a minor effect on the results of ESI(−)-MS in environmental mixtures, despite only recovering 61–66% of total carbon. This suggests that PPL has similar selectivity to the available analytes as negative mode ESI. The same study found that carbohydrates and other polar compounds were poorly extracted, particularly compounds with log D (octanol/water partition coefficient at pH 2) lower than −1. Further tests found that a large portion of environmental material was extracted but not eluted (i.e., irreversibly retained) [22]. This effect can even be observed by eye in very colored samples, as the frit on top of the cartridge often stains yellow-brown during extraction. This indicates that some irreversible adsorption occurs, possibly by filtration of colloidal DOM aggregates rather than bonding.

Because carboxylic acids constitute a large proportion of aquatic DOM, hydrophobic resin techniques (e.g., PPL) are quite successful in recovering a majority of DOM but are highly selective and poor at extracting more labile and bioactive components such as amino acids (and presumably peptides) and free sugars or polysaccharides [3]. This leads to important and drastic limitations for studies that aim to investigate these types of molecules. Any purification method is inherently biased and selective to some portion of the rich diversity of DOM and this must be considered when planning the study and evaluating the results. This is especially important in the context of dissolved organic nitrogen or phosphorous, which are not selectively targeted with current protocols.

Because there is little ESI(−) ionizable material in the whole water sample that is not also found in the PPL method extract, the ESI(−) ionizable portion of DOM is likely to be quantitatively constrained at the upper end by the typical extraction efficiency on PPL (∼50%) in aquatic systems and could be somewhat lower [23, 24]. It may also be considered that methods which use methanol for elution of retained material (e.g., C18, PPL, as opposed to DEAE) can inadvertently lead to methylation (methyl esterification) of carboxylic acids in the sample, and this may explain the apparent loss of the most carboxylated analytes [17, 25, 26]. Samples prepared in methanol for MS analysis may also suffer the same fate although this is not proven to occur on the typical sample preparation timescales [27]. Sequential elution from sorbents can yield useful results similar to preparative chromatography ("Formula assignment and distinguishing isobars" section), as the material is fractioned into chemically defined portions [22, 28–31]. Sample preparation is not a solved problem and although the PPL SPE technique is cheap and convenient, its limitations should always be kept in mind.

Ionization and optimizing sample transfer to the mass spectrometer

Ionization techniques

A fundamental stage in MS is the generation of ions, and the choice of ionization technique substantially influences the resulting mass spectrum. In complex mixture analysis, especially DOM characterization, negative mode ESI dominates for several reasons. It is exceptionally well suited to ionizing polar carboxylic acids that seem to constitute a major portion of DOM, and it is a soft technique generating thousands of singly charged, deprotonated molecular ions across a large mass range [32] so the spectra are readily interpreted. Additionally, it is the standard source that is sold with commercial instruments. However, ESI—like all ionization techniques—is biased in which species it ionizes and how efficiently it ionizes them.

In ESI, the solution is vaporized, ejecting sequentially smaller droplets, and eventually desolvated ions or neutral molecules. ESI preferentially ionizes polar species [33–35]. The complex ESI mechanism leads to a challenging prediction of the extent to which gas-phase ions are produced, called the "ionization efficiency" [33, 36] or "monomer ion yield" [24]. Ionization efficiency spans orders of magnitude and is further complicated by ionization suppression in which ions "compete" for charge with each other and solvent and additive molecules. Ionization efficiency is thus affected by solvent composition and other matrix effects (protic vs aprotic, polarity, pH, other additive concentrations, and overall sample concentration) [35], meaning that it is not a well-predicted property. The effect of aspects such as solvent composition, concentration, and pH have been investigated to some extent in complex organic mixtures [37–39].

Ultimately, not every analyte in the spray is ionized and drawn into the mass spectrometer to the same extent, and the unknowable ionization efficiency for unknown analytes means that ESI-MS is not quantitative. The intensity of each peak in the spectrum is thus correlated to the final ion abundance in the mass analyzer (with ion abundances also moderated by the ion optics leading up the analyzer) but has no necessary relationship with the analyte abundance in solution (concentration). Approximate relative comparison—of the same ions—between samples requires a matched matrix. This nonquantitative property has an obvious problematic effect on the interpretation of MS data from complex mixtures, as the most intense ions can skew statistical or visualization efforts without appropriate normalization. Additionally, some analytes are so poorly ionized that their peak is below the limit of detection of the analyzer and they are not considered in the resulting data.

Adducted ions—where the charge is introduced with a, for example, sodium or potassium (positive mode) or chloride (negative) ion—can be produced in ESI, further complicating the spectra. This is typically more problematic in positive mode for DOM analysis, thus further encouraging negative mode analysis. Complementary ionization techniques, including atmospheric pressure photoionization and chemical ionization (APPI and APCI), ionize less polar species through photon or electron capture mechanisms and are less susceptible to interference from the salts or fatty acids which plague ESI spectra [40, 41]. However, APPI and APCI spectra are complicated by the formation of molecular radical ions and may require higher performance mass analyzers to resolve and correctly identify these species.

These solution state techniques can be complemented by solid-phase ionization, such as matrix-assisted laser desorption/ionization and laser desorption ionization (MALDI, LDI), where one (or more) lasers ablate and ionize molecules from a solid surface. This approach has had some recent interest [27, 42] although its advantages are more apparent for environmental samples which require extraction—such as soil—rather than DOM, which is or should be, inherently soluble. That said, analysis of DOM standards such as SRFA and SRNOM by LDI yields complementary spectra and largely different compound identification than by ESI. The mechanisms and analysis of LDI spectra of environmental samples are still being actively pursued by the community. Another technique that uses deposited material is paperspray ionization (PSI, PSCI), however, ions produced are similar to those of ESI and APCI. One reported advantage of this technique is the lowered sensitivity to salt [43], which may reduce the amount of sample clean up required.

Alternatively, a gas-phase method—proton transfer reaction (PTR)-MS—has been used to analyze the volatile and semi-volatile components of seawater DOM [44] and successfully used to fingerprint differences between DOM sources based on lower molecular weight compounds (<100 Da) than typically observed by ESI-FTMS [45].

The analytical window is limited by the use of just one ionization technique or polarity, and if more laboratories had access to a wider range of ionization sources, it is likely that more studies would have been conducted with multiple ionization techniques for increased coverage of the mixture [40, 41, 46]. Similar to multiple sample preparation techniques, studies must evaluate the benefit of extra analytical techniques vs the extra time and cost required.

Segmented acquisition

One straightforward method to boost the dynamic range of the mass spectra is to acquire multiple regions of the mass spectrum and stitch them together [47–49]. Use of a quadrupole mass filter can remove all but a narrow range of masses (10–100 *m/z*). This, coupled with an increase in the ion accumulation time, increases the signal-to-noise within that mass region. This can be repeated across the mass ranges of interest to produce a pseudo-broadband mass spectrum, but with a substantially higher dynamic range than typically achieved through normal broadband acquisition. Note that ion accumulation time has been shown to affect relative ion abundances in complex mixtures [50]. The increased sensitivity can be used to characterize DOM in samples with very low carbon concentrations [51]. This approach was recently applied, along with absorption mode processing, in petroleomics to yield over 200,000 unique molecular formula assignments in a single sample [49]. Mass window isolation can also be used to exclude high abundance contaminants like fatty acids, which ionize exceptionally well in negative mode ESI and can obscure mass spectra.

Investigating complex mixture diversity

Formula assignment and distinguishing isobars

Complex environmental mixtures have both isobaric and isomeric diversity. Isobars are defined as ions that have the same nominal mass but a different molecular formula (and a different exact mass), while isomers have the same formula and exact mass, but a different structure. Isobars can theoretically be resolved into different peaks by a mass spectrometer while isomers cannot. In complex mixtures such as DOM, both isobaric and isomeric complexity present problems to the analyst. Isobars have different masses because each atom has a slightly different "mass defect," which results from the nuclear binding energy (dependent on the specific number of protons and neutrons). Due to these slight differences, CH_4 and O deviate slightly in mass from the nominal 16 Da by 36.4 mDa. The number of isobaric peaks found at any nominal mass is dependent on two factors: the diversity of the sample (i.e., the number of ionizable isobars above the detection limit) and the resolving power of the mass spectrometer (how narrow the peaks are). The isobaric complexity of the sample is unknown and could theoretically be extremely high when an increasing number of atom possibilities are considered [9, 40]. Hundreds of peaks per nominal mass have been observed in complex natural mixtures [49], but theoretical possibilities can be constrained after considering some chemical factors like solubility, ion type, and knowledge of the sample such as heteroatom (e.g., S, P) content.

It is difficult to know in advance what resolving power is required. Usually, researchers simply use the highest resolution instrument available, but there may be reasons to decrease resolving power due to easier access to an instrument [52] or increased frequency of transients at a lower resolution, meaning more transients (better signal-to-noise) or better coupling with chromatography [53].

Resolving the CH_4 vs O mass split (36.4 mDa) and C_4 vs O_3 mass split (21.1 mDa) are essential for the analysis of aquatic samples because these substitutions are so common in the carboxylic acids that dominate the sample's ionizable material. This requires a resolving power of at least 30,000 at 200–600 Da, which is in the range of some modern time of flight analyzers and is easily achieved by all commercially produced Orbitrap and Fourier transform—ion cyclotron resonance (FT-ICR) analyzers (Fig. 4.1). The higher the resolving power, coupled with high mass measurement accuracy, the greater the confidence in formula assignment. Because CHO species dominate negative mode ESI-HRMS spectra, high resolving powers may not be necessary to find major geochemical trends [52, 54], but more diverse samples would require higher resolution, meaning that prior knowledge of the sample is important for determining the required analytical technique.

Various strategies for formula assignment have been reported [55–60]. Broadly speaking, most tools utilize exact mass accuracy, within a tolerance threshold, matching to a precompiled database of molecular formulas based on elemental constraints, coupled with homologous series and isotope peak matching. Unambiguous assignments are often resolved by a preference for simplicity with heteroatomic counts (fewer NSP atoms is deemed more likely). These tools have mostly been developed and investigated in the context of negative mode ESI and often presume CHO compounds the most prevalent. Formula assignment is not a solved problem, and new strategies that utilize the quality of the data, identify more isotopologues, and which report confidence of assignments are needed in the community.

Separation methods for investigating complex mixture diversity

Preparative chromatography

Preparative chromatography, like sequential SPE, can be used to simplify complex environmental samples [11, 61–67]. This technique has been shown to be very useful, as fraction collectors can be programmed to collect multiple replicates of the same fractions [62], greatly increasing the abundance of analytes in certain polarity fractions [65, 66]. These portions of the material can then be reconstituted for ESI-HRMS [65], or deposited for paper spray or LDI. The advantage of this strategy is that ionization suppression is greatly alleviated, allowing for observation of analytes that were previously suppressed [30, 68, 69]. This helps with gaining a more complete coverage of the sample's diversity (assuming the anticipated diversity exists), but if only one

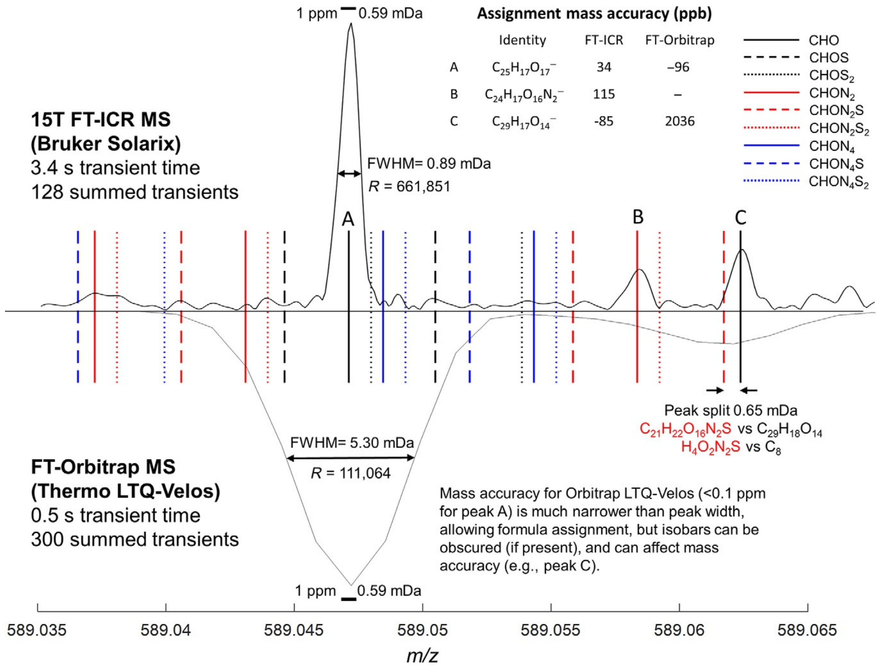

Fig. 4.1 Mass spectra generated for Elliot Soil Fulvic Acid in negative mode by two instruments with ~6× difference in resolving power. A small mass window (30 mDa) is shown, with possible formula assignment locations indicated by vertical lines, allowing for reasonable atomic ratio constraints (e.g., H/C < 2.2), up to four nitrogen atoms and two sulfur atoms, as indicated in the legend. Two CHO peaks should be assigned, and one $CHON_2$ peak was resolved by the FT-ICR and not by the Orbitrap. The possibility for false assignment increases as resolving power decreases or sample diversity increases. After calibration, FT-ICR MS can typically assign formulas to better than 200 ppb accuracy. FT-Orbitraps can also routinely achieve <500 ppb accuracy, but obscured isobars can affect the apex position and accuracy, as in Peak C of this figure.

ionization technique is used to measure the isolated fractions, there is still substantial bias in the analysis. The dissolution of fractions in the same solvent is a key benefit of fraction collection over online chromatography, as ionization methods are highly sensitive to solvent and buffer composition. Additionally, each fraction can be analyzed better by summing multiple transients, while online chromatography can lead to low signal to noise, as transients (which usually take at least 1 s) are usually slow compared with chromatographic separation [11].

The clear disadvantage of this approach is the large demand on time and materials. A lot of extra equipment is required—HPLC equipment, fraction collectors, vacuum drying apparatus, along with a requirement for a larger amount of DOM material. The time required can increase to a great extent as well, as the DOM is typically separated into at least four fractions [66], or even hundreds of fractions in some studies led by Stenson [11, 61, 67]. This tends to restrict preparative chromatography approaches to the detailed study of a small sample set, and thus is not suited to studies across large geographical regions or timescales.

Studies to date have shown that isobaric complexity is maintained after fractionation, but also that some molecular peaks were unique to one particular fraction, hinting at the possibility of isolating individual components of the mixture through further separation [66, 67, 69]. More comprehensive attempts to isolate individual molecules from complex environmental mixtures have been made by taking smaller chromatographic slices and via the second dimension of chromatography [11, 61–63]. Various studies have undertaken the detailed mass spectrometric analysis of such highly fractionated material, reporting a decrease in isobaric complexity but not usually finding that individual compounds with simple fragmentation patterns could be isolated [11, 61]. Brown et al. (2016) reported a decrease in isomeric complexity for the most polar compounds in SRFA compared to the more hydrophobic fractions [61]. They first used a long reversed-phase HPLC method to separate 100 fractions, subjecting an early and late fraction to a second, orthogonal separation based on charge and polarity. The hydrophilic compounds that eluted early on column 1 have the highest oxygen content, and therefore should have the lowest isomeric diversity (following Hertkorn et al. [9]), while the oxygen-poor, late-eluting material has higher theoretical isomeric diversity. Brown et al. seemed to confirm this theory using low resolution tandem MS on the second column's eluent, finding increased feature diversity in the chromatograms of individual fragments although the feature resolution remained poor. Capley et al. [11] found that passing the most hydrophilic material over a second column with an immobilized metal (nickel) showed very little retention of material, and suggest that this indicates that the structures are small and rigid [11]. They also used fragmentation of masses 341 and 339 in the late-eluting material to find that the core structures also had a mass difference of 2, demonstrating common neutral losses, (see "Fragmentation and structural elucidation" section) with the mass difference of two hydrogens a feature of the core structure.

Offline chromatographic fractionation has also been used in petroleomics using different techniques such as normal phase chromatography [30] and size exclusion chromatography [24]. These complex mixtures, like DOM, are inherently inseparable by chromatography, but these approaches are useful for adding functional information to the sample (e.g., how basic the nitrogen-containing compounds are and therefore whether they are pyridinic [30]) or giving access to higher molecular mass material (>1000 Da) that is otherwise not observed because of ion suppression [24, 30, 68]. These studies used the actual signal intensity and quantity of material present in each fraction to assess the "monomer ion yield" of the material in each fraction. This is a useful concept that may be greatly exploited in future chromatography studies in order to investigate which chromatographic (e.g., polarity) fractions of the material have the highest ionization potential and therefore have a great effect on HRMS results [70].

Chromatography, electrophoresis, and ion mobility to "separate" isomers

Direct online coupling of high-pressure liquid chromatography or gas chromatography to mass spectrometry (LCMS/GCMS) may seem to be an obvious strategy for DOM fingerprinting considering their widespread use in fields like metabolomics and contamination research. However, these topics often have a targeted approach, or relatively little diversity (in the case of metabolomics), due to the freshness of the material, which has not been chemically processed. The lower diversity makes coupled chromatography or electrophoresis work well because analytes give well-resolved peaks that can be easily distinguished from noise and separated from other compounds that may suppress ionization or interfere with the signal (i.e., isomers).

In very diverse, chemically processed mixtures such as DOM, this is not the case, and material elutes in humps, or "unresolved complex mixtures (UCMs)." Signal-to-noise ratio is sacrificed because the number of transients that can be summed is limited by the fact that the eluent cannot easily be stopped for analysis without causing diffusion and band broadening. Additionally, variable ion count generation through an LC run is not compatible with fixed ion accumulation time detectors (e.g., Bruker SolariX FT-ICR systems), and the result is variable ion populations in the ICR cell, which results in space charge effects, peak splitting and/or coalescence, and frequency shifts. This can be solved by the use of automated gain control (AGC) available on Orbitrap instruments (Thermo Fisher). The chromatographic timescale does not match the analytical timescale well, and the solvent cannot be optimized for ionization without diluting the eluent with an additional flow of organic solvent although this could be used to add ionization modifiers (e.g., acid/base).

Despite these drawbacks, and even though chromatographic peaks cannot be resolved, online chromatography can give important advantages for analysis. A wider coverage of material is detected due to the alleviation of ionization suppression [53, 70] and sample contaminants are conveniently removed from most transients. Additionally, the measured material has added chemical information related to the separation—for example, its octanol:water partitioning coefficient, in the case of reversed-phase chromatography [71], or its apparent size or charge density in the case of size exclusion chromatography [23, 72, 73] or electrophoresis [69]. Transient analysis frequency can be increased using quadrupolar detection (on four detector plates instead of two) in FT-ICR [53, 74], or by decreasing resolution, in cases where this can be sacrificed because the analyzer's power exceeds the mixture's diversity [61, 75]. It may also be the case that isobaric interferences are overcome by the separation itself, allowing lower resolution analysis, as in the case presented by Byer et al. [76], where molecules differing by C_3 vs SH_4 (3.4 mDa) were resolved using 2D gas chromatography rather than by mass alone. Furthermore, unlike preparative fraction collection methods, online chromatography can be conducted on a large number of samples in a short time. Hawkes et al. [77] used a 30-min reversed-phase method to analyze 74 headwater stream samples, 4 SRFA controls, and 2 blanks in less than 48 h, which would be challenging to match with a manual (user-operated) direct infusion approach.

Online chromatography of DOM has provided several recent insights to long-standing debates in biogeochemistry. Reversed-phase chromatography combined with ESI-HRMS has demonstrated that isomeric diversity exceeds tens or possibly hundreds of isomers per exact mass, by showing that extracted ion currents have almost no peak features, even after additional separation steps [8, 53, 63, 70]. Size exclusion chromatography-HRMS has cast doubt on the popular theory that apparently large molecular weight material is composed of loose aggregates of carboxylic acid monomers [23, 72, 78]. Size exclusion combined with NMR also found that larger material did not contain similar proton environments as smaller material—but was rather more alcoholic and aromatic in nature, indicating lignin and polysaccharide material, as opposed to smaller carboxylic rich alicyclic material (CRAM) and material derived from linear terpenoids (MDLT) [62]. These assignments correspond well with the expected ionization efficiency and mass to charge ratio of these components, and it is likely that larger polysaccharides and lignin are not detected by ESI-MS. It is unknown how abundant this pool of ESI-invisible material is, but it should be considered that this material is not separated or assigned in direct infusion or reversed-phase chromatography methods [23].

Another recently exploited technique is coupled ion mobility spectrometry (IMS)-mass spectrometry in which ions are separated inside the mass spectrometer based on their cross-sectional area by moving (IMS) or trapping (TIMS) them in an electric field counter to the flow of a buffer gas. These separations take place over the course of several milliseconds, so a very fast mass analyzer is typically required to detect the emerging ions. For this reason, only recently has IMS-MS been used in the analysis of complex mixtures where formula assignment is required, as time-of-flight (TOF) mass spectrometry technology has reached the point where it is possible to achieve resolving power $>30{,}000$ at hundreds of scans per second. Trapped-ion mobility separation (TIMS) can also be coupled to FT-ICR MS and this gives much improved coverage in DOM mixtures compared with current TIMS-TOF capability [79].

Complex mixtures of isomers emerge from IMS drift cells in humps [22, 79–81] as we have come to expect, but the alicyclic, carboxyl rich material that is typical for DOM can quite effectively be separated from other classes of compounds that may otherwise be difficult to separate by mass, including anthropogenic contaminants such as endocrine-disrupting hormones [82]. Furthermore, Benigni et al. [82] found that the number of mass peaks identified at a large range of nominal masses increased several times after adding this ion separation step, presumably as a result of decreased isobaric complexity after ion separation. Several papers have attempted to constrain the number of isomers that contribute to a molecular formula by fitting idealized chromatographic peaks into a mobility profile [79, 80, 83] (Fig. 4.2), and this concept has also been used to demonstrate decreasing isomeric diversity after a chemical process [84] and to demonstrate high isomeric diversity after chromatography [8]. Caution should be taken in this approach as peak resolution or capacity is low for IMS, it assumes idealized peak profiles and therefore can only constrain the lower limit of isomeric diversity as unresolved peaks are not accounted for [83]. A further benefit of IMS-MS is that it can be used to monitor the formation of doubly charged ions (which most analysts try to avoid), as these travel faster through the drift tube [85].

It is also possible to determine the collisional cross section of ions directly in Fourier transform mass spectrometry [86, 87]. As ions collide with neutral background gas, their motion is perturbed and their induced signal attenuated. Application of short-time Fourier transforms, to study the ion damping profile, allows for the calculation of CCS of ions in the analyzer cell. This method requires calibration standards and a robust model and the use of short-time Fourier transforms reduces

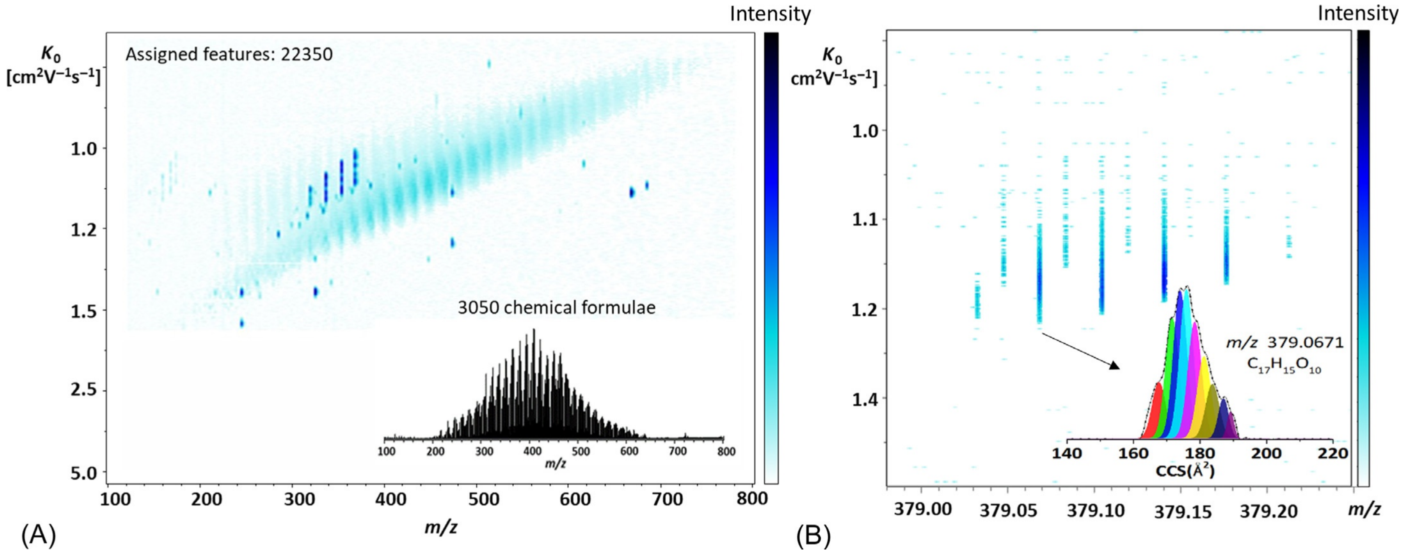

Fig. 4.2 (A) Two-dimensional TIMS-FT-ICR MS plot and MS projection (inset) of SRFA standard. (B) Expansion of (A) around *m*/*z* 379 showing IMS deconvolution of up to 9 isomers by SAME algorithm for *m*/*z* 379.0671 ($C_{17}H_{15}O_{10}$, mass error $<$ 0.1 ppm).
Credit: Dennys Leyva and Francisco Fernandez Lima (Florida International University).

the mass-domain resolution achieved. The comparability of these measurements to those made with dedicated ion mobility spectrometers requires further attention, but this approach may allow for CCS determination on existing FTICR and FT Orbitrap hardware.

MS methods for investigating complex mixture diversity

Increasing mass resolving power to increase isobaric resolution

Resolving power is key to correctly identifying species in a complex mixture; isobaric compounds which are not resolved will appear as broadened peaks with an apex between the real *m/z* of either compound, potentially leading to incorrect assignments (Fig. 4.1). Thus, any means to increase resolving power represent important advances in the field. Broadly speaking, these include hardware upgrades (larger magnets and newer instruments), signal detection changes (longer transients or quadrupolar detection of higher order harmonic signals), and signal processing developments (absorption mode processing).

Development of two ultrahigh field FT-ICR systems at 21 T at the National High Magnetic Field Laboratory (NHMFL) and Pacific Northwest National Laboratory has yielded substantial improvements in resolving powers, as well as numbers and errors of molecular formula assignments, for complex mixture characterization [75, 88]. Resolving powers in excess of 1 million (at 367 *m/z*) for 3-s transients were achieved in magnitude mode, and 1.7 million in absorption mode for SRFA, allowing for resolution of sub-miliDalton splits between chemical species across the typical mass range of DOM. Such instrumental upgrades, however, are often prohibitively expensive and require substantial planning, more cryogens, and more lab space. Furthermore, the improvements can be modest—the theoretical resolving power enhancement from a 12 to 21 T system would only be 1.75 $\times$.

Absorption mode processing has been routinely applied in NMR for decades but is only recently finding use in FT-ICR. The delay has historically been a lack of computational resources and algorithms to calculate the phase correction, and thus FTMS data is traditionally presented in magnitude mode, where absorption mode can yield a twofold increase in resolving power. Custom hardware, such as the Predator system at the NHMFL, has achieved absorption mode processing as mentioned before. However, the software has also been developed allowing for the calculation of phase correction from normally acquired data on commercial instruments [89, 90]. This allows for a "free" enhancement of resolving power, and thus also mass accuracy, either increasing the utility of lower field instruments [91] or for improved coupling to online chromatography.

Recently, commercial instrumentation has become available which includes additional detect electrodes on the ICR cell—so-called quadrupolar detection—allowing for increased resolving power in the same measurement time [92]. This is achieved through measuring the multiples of the reduced cyclotron frequency, with appropriate ICR cell design and application of tuned electric fields. These two-omega (for twice the frequency) measurements have a twofold boost in resolving power. Four-omega measurements have been reported at 21 T [93], but the very high frequencies of the low mass ions bring difficulties in digitization and signal acquisition of lower masses (below 600 m/z) and were therefore not suitable for DOM characterization. As with absorption mode processing, this method can be applied to lower field instruments to achieve results comparable to those from larger magnetic fields [94] or be used to achieve high resolution with shorter transient length, and thus apply online chromatography of NOM or petroleum to FT-ICR [53, 74].

Fragmentation and structural elucidation

Structures and molecular functionality are elucidated in fragmentation studies, most commonly with collision-induced dissociation (CID) by breaking ions apart with a buffer gas and calculating which neutral losses of molecules can occur to leave the measured stable ion behind [95]. Some mechanisms are fairly straightforward, such as loss of CO_2 from a carboxylic acid group, while others can require more complex pathways like ring closures, as in the case of loss of H_2O from two adjacent carboxylic acids, leaving a ring anhydride [11, 95]. Phenol OH groups are stable and do not fragment easily as a water loss, while aliphatic OH groups can fragment with a nearby proton as H_2O [95]. As CO_2 and H_2O losses absolutely dominate the fragmentation patterns of DOM peaks, it follows that carboxylic acid groups are very common structures [7, 8, 10, 11, 96]. Larger neutral losses indicating amide, ether or glycoside bonds are not observed, suggesting that these structures are less common in the ions produced by the sample preparation and instrumental parameters [8, 10]. Several neutral losses of CO_2 can be observed from DOM precursor ions (Fig. 4.1), so it is likely that the majority of the oxygen in typical ESI(−) ionizable DOM is, in fact, carboxylic oxygen (two per group) [10]. Alternatives to CID are photon-based fragmentation methods, such as ultraviolet photodissociation (UVPD) and infrared multiphoton dissociation (IRMPD), which may complement CID fragmentation and be more structurally selective, although their use for NOM characterization seems to be limited so far [59].

There is a well-documented problem with fragmentation in complex mixtures that if the isolation window contains several peaks, it can be difficult to elucidate which precursor peak or peaks a charged fragment originates from [7, 8, 11, 80, 95]. This can be overcome using "in-cell" isolation via selective or shaped radiofrequency pulses, which allow for the isolation of a single mass peak for fragmentation [10, 61]. Witt et al. [10] compared the fragmentation patterns of peaks that differ in CH_4 vs O at a single nominal mass, finding similar backbone masses after fragmentation of the labile carboxylic acid groups. They argue that the structural diversity of DOM is not as high as the isobaric diversity due to the common backbone structures and similar chemistry of functional groups. Further MS_n experiments in which ions from one fragmentation are trapped and fragmented again, also show that sequential losses of CO_2 and H_2O can lead to common "backbone" structures, potentially revealing the origins of the material [10, 11]. The result that carboxylic acids surround a large, alicyclic backbone with some degree of unsaturation also corresponds well with NMR data and extrapolation of the average double bond equivalence of DOM molecular formulas [97, 98].

The fragmentation pattern of DOM peaks is highly similar in different aquatic settings and biogeochemical constraints may lead to similar molecular mixtures in every environment [6]. The number of structural isomers must be very high, at least approaching hundreds per molecular formula otherwise, the fragmentation patterns would not reach such an averaged response [7]. The fact that this averaged, similar pattern remains after chromatographic separation (Fig. 4.3) suggests that structural diversity is extremely high [8]—but as pointed out by Witt et al. [10], functional diversity may be substantially lower.

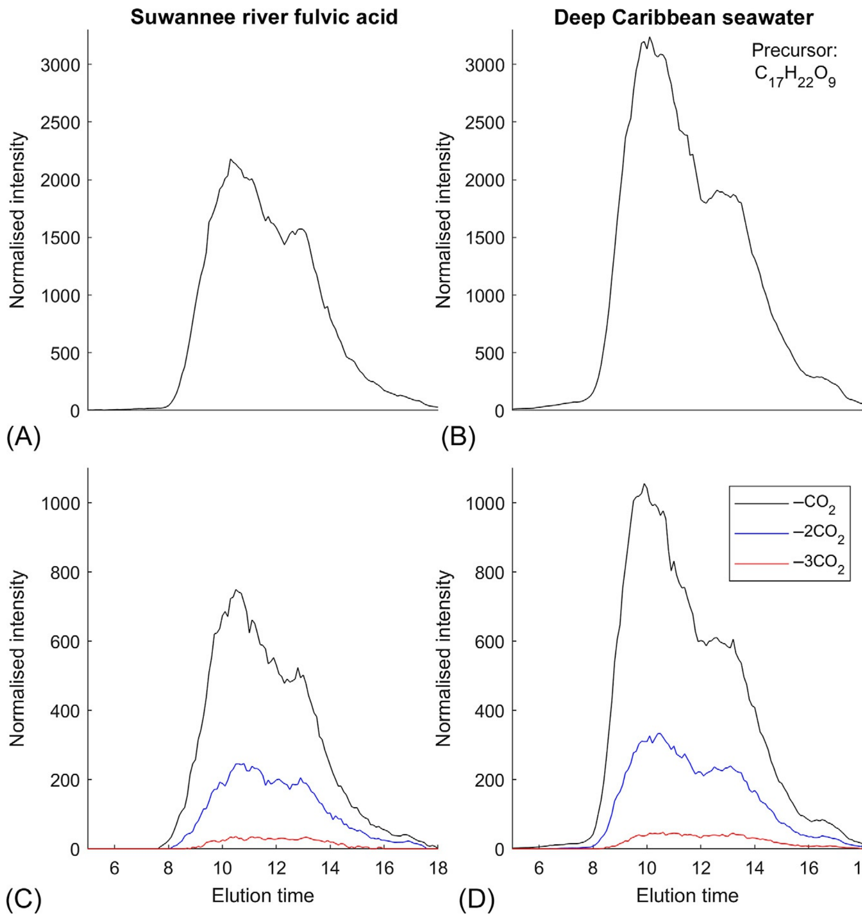

Fig. 4.3 Extracted ion currents of a reversed-phase chromatographic separation and ESI-FT-Orbitrap MS analysis of SRFA (left) and deep Caribbean seawater (right). (A and B) precursor ion $C_{17}H_{22}O_9$ with no collision-induced dissociation (CID) energy, (C and D) neutral losses of 1–3 CO_2 molecules from the precursor in (A) and (B) with 27 V normalized CID energy. The lack of feature diversity in the chromatograms (both precursors and fragments) suggests there is extremely high isomeric diversity. The similarity between the samples suggests that structures and functionality are similar across aquatic environments.

Complexation, derivatization, and exchange techniques

Along with fragmentation, there are several tools available to the analyst to probe the structural and functional chemistry of complex environmental mixtures using HRMS. Unlike NMR, or FTIR, which cannot resolve individual components of DOM directly, the information derived can be attributed to individual molecular formulas, demonstrating the nature and diversity of thousands of individual components of the mixture, rather than the bulk sample.

Derivatization methods include esterification of carboxylic acid groups to deuterated methyl esters, allowing enumeration of carboxylic acid groups [25]. Sulfur functionality has been investigated using selective derivatization methods [99] and these strategies could be extended to other atoms such as nitrogen and phosphorus. Labile oxygen atoms in carboxylic and alcohol groups naturally exchange with oxygen in solvating water, allowing determination of the number of nonlabile oxygen atoms in molecular formulas by exchanging ^{18}O for natural ^{16}O using $H_2^{18}O$ [100]. Carrying out the reaction at different temperatures and reaction times can lead to information about how labile different oxygen atoms are and how many nonlabile (e.g., ether and ketone) oxygen atoms are present. The H/D exchange is a similar process but can be carried out in the ESI spray for carboxylic and alcohol protons [100, 101], or after long reaction can be used to probe the structure of the backbone of the molecule by exchange with aromatic protons [100, 102]. H/D exchange can also be carried out inside the mass spectrometer with a deuterated reaction gas [103].

The main complication with all of these techniques is the difficulty in distinguishing incomplete reactions from isomeric diversity [11]. It was shown that a $[H_3PO_4]_4$ cluster with 11 labile hydrogens gives an almost Gaussian distribution of H/D exchanges [101] with a maximum at 11, indicating some degree of incomplete reaction. This can be confused by actual structural diversity in complex mixtures, where some isomers have more labile hydrogens than others, leading to an additional distribution of product ions. This drawback is very difficult to overcome and the results of derivatization or exchange are usually discussed in terms of the maximum number of exchanges, rather than attempting to unravel this complexity [102]. Alternatively, the techniques can be used to investigate peaks with a low number of total atoms that may be exchanged [104].

It is interesting to note that certain exchanges that are usually considered undesirable artifacts of ionization techniques such as ESI can be harnessed to gain structural information. For example, usually it is considered a concern that storing DOM samples in methanol may lead to methylation of carboxylic acid groups [17, 26], but it may actually be utilized to count the carboxylic acid groups [25]. Similarly, primary amines like the additive ammonia may form imines in the spray with ketone or aldehyde groups in positive ESI, which can be used to detect this functionality [105]. Further still, base cations are usually considered a nuisance in positive mode, particularly sodium, because of their abundance and the ease with which they form adducts with carboxylic acids [106], but they can be used to form interesting and useful complexes in certain cases using a “ligand fishing” strategy [107].

Stenson [108] used metal ions (Be^{2+}, Mn^{2+}, Cr^{3+}) to investigate the peaks that emerged in the mass spectrum of SRFA due to complexation of humic acids with these base cations, which were specifically chosen because the complexes would be placed at even nominal masses, making them easily stand out. The study showed that these ions form hundreds or thousands of complexes either in solution or during ESI spray. Waska et al. [109] have investigated the possibility of using a similar method to investigate iron- and copper-binding ligands in seawater DOM. They found that isobaric complexity in seawater DOM made the assignment of Fe and Cu complexes extremely challenging, even after further fractionation of the sample by different SPE protocols, but did successfully characterize the siderophore ferrioxamine B with this method, along with other known ligands [110]. They recommend HPLC fractionation of metal ligand complexes, and this approach was recently successfully used alongside HPLC-ICP-MS to detect iron-binding siderophores at concentrations as low as 1 pM in seawater [111]. Duncan et al. [112] demonstrated that divalent barium ions form strong complexes with carboxylic acid groups, strengthening the C—COOH bond and therefore limiting fragmentation. This is useful in the characterization of naphthenic acids, which only have one carboxylic acid group, as fragmentation can then lead to more structurally diagnostic product ions. Boron, conversely, can form complexes with diols and triols, as demonstrated for model compounds [107]. This paper demonstrated that sugar-like formulas and lignin compounds in soil fulvic acid could bind boron with a similar ligand fishing approach to Stenson's.

Visualization and data handling techniques

Visualization

HRMS data containing thousands of features is inherently difficult to visualize and it is therefore typical to present the data and compare samples in a variety of figure types that reduce dimensionality. The most common visualization is the van Krevelen (VK) diagram in which elemental ratios (typically H/C, O/C) are plotted for each formula (Fig. 4.4A). This diagram was introduced for HRMS visualization at the beginning of the millennium [114] and has been used extensively since to show how environmental trends correlate with apparent chemistry of the ionized material [26, 77, 115, 116], to compare samples [117, 118], to show how geochemical transformations affect samples [119] and as an interactive tool for exploring data [57, 120]. More common in petroleomics and HRMS analysis of naphthenic acids is the double bond equivalence (DBE) vs C or DBE vs O diagram (Fig. 4.4D; [121]). Problematically, DBE is independent from O and S, thus does not account for the common C=O double bonds in DOM. Koch and Dittmar proposed the alternative "aromaticity index" in 2006 to allow for a better characterization of the aromaticity of DOM molecules [122].

Two-dimensional scatter plots with thousands of data points suffer from overlap biasing the visualization. It can be effective to plot density, not points, to more fairly represent the underlying data or to visualize the data interactively [120]. Alternatively, additional diagrams can encode further information, such as other heteroatomic content or DBE, which is preferable to three-dimensional plots, which are impossible to effectively show in 2D space (i.e., printed) (Fig. 4.4E).

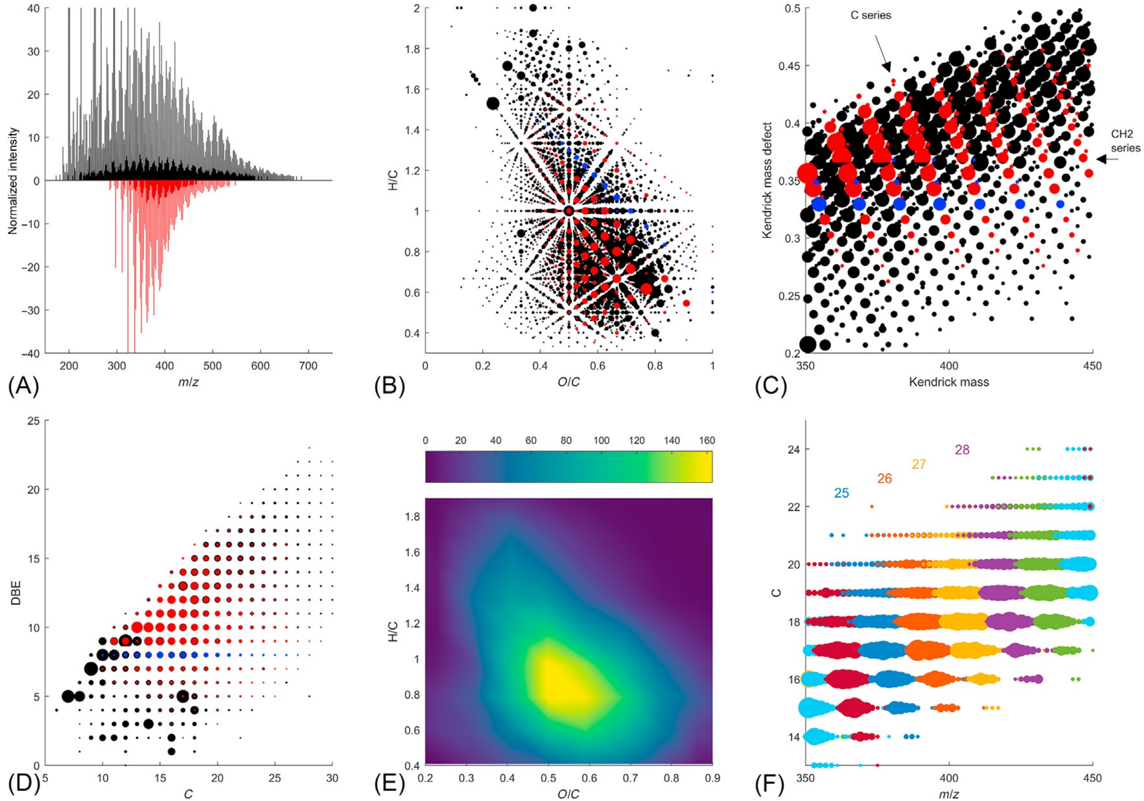

Fig. 4.4 A selection of visualization methods, displaying FT-ICR-MS data of Elliot Soil Fulvic Acid (ESFA) collected on a 15 T Bruker Solarix instrument. (A) Reconstructed mass spectrum comparison (B) van Krevelen diagram, (C) Kendrick plot, (D) DBE vs C diagram, (E) van Krevelen density plot, (F) C vs *m*/*z* diagram. In (B), (C), (D), and (F), intensity is displayed as point size. In (A), all assigned peaks are shown as black lines, and peaks with 10 oxygen atoms are shown in the negative direction in red for comparison. In (B–D), all points are colored black, overlaid in red are formulas with 10 oxygen atoms, and overlaid in blue are formulas with 10 oxygen atoms and double bond equivalence (DBE) of 8. In (E), the density (color) shows the number of peaks in each bin and intensity is not considered. In (F), the sum of carbon and oxygen are shown in color, and for some "islands" this sum is indicated as a number above the island after [113].

The Kendrick plot is an older tool that is extremely useful for identifying series of compounds that are related by some sort of homologous series [60, 123, 124]. The traditional Kendrick conversion is used to transform the mass of $^{12}CH_2$ (14.01565 Da) to be exactly 14 Da. Each homologous series of compounds that differ by a CH_2 unit (i.e., a link in an aliphatic chain), now differ by a Kendrick mass of exactly 14 and have an identical Kendrick Mass Defect (the decimal after the exact mass). Such a series can be grouped together in a Kendrick diagram (Fig. 4.4C), while they are harder to spot in a VK, and overlay on top of many other points in a DBE vs C diagram (Fig. 4.4A and B). As the main use of Kendrick plots is to aid molecular formula assignment, their utility is now limited since automatic formula assignment tools exist for DOM [55–57]. Further transformation of the data into subsequent mass defect families can allow an extreme degree of data reduction—Roach et al. [125] showed that a crude oil sample with 13,000 peaks could be reduced to just 25 families (25 points), when considered as Kendrick-like networks of CH_2, H_2, and O homologues. This type of network approach has also been used to provide impressive 3D molecular maps of HRMS data and the homologous connections that link formulas together via so-called "transformation pathways" [126–128].

There are numerous other options for displaying data that can be useful depending on the research question. Other atomic ratios can be plotted, using different heteroatoms (N, S, P, Cl, Na) and the formulas that contain specific combinations of these, or other atoms can be extracted from the data as compound classes (e.g., the SO_4 class, containing one S and four O). Reemtsma [113] examined the information gained by plotting carbon number vs *m*/*z* and grouping compounds by the sum of carbon and oxygen (Fig. 4.4F). Within each "island" there are series at the same nominal mass for which a CH_2 substitutes oxygen and a double bond, meaning that the compounds to the top are aliphatic, O limited, and the bottom is the most aromatic and carboxylic rich.

HRMS resolves thousands of peaks in DOM and displaying this data in one figure can be useful, though is challenging to achieve without compromise. In the case of the traditional H/C, O/C van Krevelen diagram, mass, intensity, and other heteroatom content (e.g., N, S, and P) information is lost. This is before chromatographic data and intersample differences are even considered. DBE vs C diagrams do indicate mass via C and hydrogen deficiency via DBE, but lack specific information about further heteroatom content (O, N, S, P), and have much more overlap of points. They are therefore often used in compound class diagrams, where the single classes are shown (e.g., O_2) in their own diagram. This limited heteroatom content information means that they are not commonly used in DOM studies but are extremely popular in petroleomics, where heteroatom content is limited.

VK diagrams are often chosen to present HRMS data in biogeochemical studies because the diagram lends itself to the division of the points into groups according to arbitrary boundaries in the atomic ratios of H/C and O/C. Because molecular classes also fall within defined boundaries (e.g., lipids are nearly saturated with hydrogen and contain few oxygen atoms), it is conceptually useful to divide the VK diagram into these defined molecular areas and therefore annotate the peaks found by HRMS according to their corresponding compound class (e.g., lipid-like peaks). This type of data analysis typically includes combinations of the following: lipids, peptides/

proteins, sugars, lignin, tannin, polyphenols, condensed aromatics, black carbon. Such "assignments" should be well constrained and accompanied with strong caveats. The removal of dimensions from the data risks poor annotations in many cases. For example, peptides should necessarily contain nitrogen (at least one per 100 Da, depending on the amino acid residues). The "peptide-like" region should, therefore, include an N/mass dimension constraint as well as H/C and O/C dimension constraints, but this is rarely discussed and is not apparent from the VK diagram [129]. Multiple N containing formulas (i.e., peptides) are also very difficult to resolve from CHO peaks (Fig. 4.1). Even after satisfying these formula constraints, it is impossible to confirm these assignments from mass spectral peaks alone, without isotopic fine structure and/or fragmentation confirmation. Visualization techniques reduce data complexity for easier consideration, but also hide important information about the signals and assignments. In the context of environmental fingerprinting, therefore, visualizations serve a very useful purpose, but their limitations must be considered when interrogating the data.

Statistical treatment of data

Many HRMS DOM studies investigate the changing nature of DOM over environmental gradients in space and time. In these cases, changing peak intensities are often correlated with environmental factors such as radiocarbon age [130], inorganic element concentrations [77], mean annual precipitation [115], or even bacterial community DNA composition [131]. This type of analysis requires large sample sets (e.g., 74 in Ref. [77], 120 in Ref. [115], and 137 in Ref. [130]) and strict criteria for correlation in order to avoid "p-hacking," as some fraction of thousands of mass peaks are likely to have random trends with at least one environmental factor. A Pearson's Rho of 0.001 was chosen in Ref. [77], and 0.01 was chosen in Ref. [130]. Kellerman et al. [115] instead used Spearman's rank correlation to acknowledge that trends may not be linear and only used peaks that were present in 90% of the samples above a certain intensity threshold. They also performed an analysis of the false discovery rate in order to assess the level of confidence required to consider significance, leading them to a value of 0.027.

These types of correlation analysis require very reliable peak intensities, as it is assumed that an increasing or decreasing ion abundance is environmentally significant, rather than due to variability (random or otherwise) in the analysis. Samples should be run in random order and ideally, replicates are analyzed to evaluate the magnitude of peak intensity change required to signify genuine change [132–134], but under the burden of constraint on instrument time, analysis of a larger number of samples is often preferred over replicate analysis. The reliability of peak intensity values is seldom discussed [132, 135, 136] and this should be addressed moving forward. Riedel and Dittmar [48] pointed out that analyzing triplicates with 1/3 the number of scans takes the same amount of time and allows an evaluation of artifact and noise peaks (those which are present in only one or two of the replicates) and this approach may be considered as a way to also evaluate peak variability without losing the capability of running a high number of samples. We found that peak intensity variability was below 5% for five sequential replicate analyses of SRFA by FT-Orbitrap MS and five analysis dispersed throughout a sample set for 15 T FT-ICR MS (Fig. 4.5A and B). This <5% variability was only found for peaks with a normalized intensity >10% of the maximum (Obitrap) and >5% of the maximum (FT-ICR). The lower intensity peaks had larger assignment errors and poorer reproducibility for both analyzers, and this should be factored into statistical analysis when considering if environmental trends are significant.

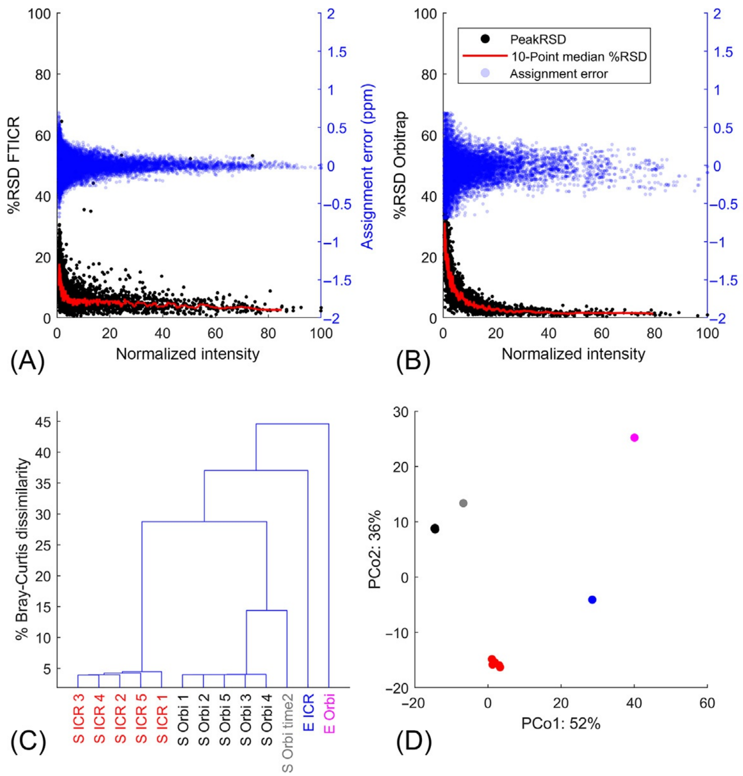

Fig. 4.5 (A and B) Relative standard deviation (%RSD) of normalized peak intensities for peaks that were present in all five replicates of SRFA measured by FT-ICR MS (A) and FT-Orbitrap MS (B), also shown are assignment errors for each replicate. (C) Hierarchical cluster analysis of Bray-Curtis dissimilarities for five replicates of SRFA (labeled S) measured by FT-ICR-MS (labeled ICR) and FT-Orbitrap MS (labeled Orbi). These are compared with SRFA measured by FT-Orbitrap MS 8 months previously and a different sample, Elliot soil fulvic acid (labeled E) measured once on each instrument. (D) Principal coordinate analysis (PCoA) diagram reproducing the dissimilarities between the samples shown in (C) (same color code). PCo1 and PCo2 are shown. Replicate analysis typically yields Bray-Curtis dissimilarities (BCD) of ~5%, while analysis of the same sample after several months may lead to BCD of 15%. Different analyzers can differ more (e.g., 30% for these examples) and different samples differ greatly (40–45% for SRFA vs ESFA).

The peaks that are found to correlate with environmental variables are usually collected together and presented with the typical visualization tools such as VK diagrams. A potential confirmation of the reliability of trends is that molecular formulas with similar chemistry (e.g., occupying a similar region of the VK diagram) follow the same biogeochemical patterns. This has been used on several occasions to show how DOM changes in character as it degrades in nature [130, 136–138], or has been used to find peaks that can be used as tracers for terrestrial input of DOM to the deep ocean [139]. The trends of individual peak intensities are therefore quite useful, but over the last decade, it has been recognized that these types of large datasets are very well suited to multivariate statistical analysis, where the data are reduced to a small number of factors, particularly because so many peaks clearly follow the same trends, indicating a great deal of redundancy in the full dataset.

A typical multivariate approach is principal component analysis (PCA), which can be conducted directly on the formula assigned and aligned sample intensity data, giving scores for the samples and loadings for each formula or mass, indicating the contribution of each formula to each principal component (PC). Often it is found that very few PCs (1–3) explain a majority (more than 50%) of all data variability, even for a fairly large number of diverse samples [77, 140, 141]. Typically, the first or second PC is found to correlate with aromaticity or oxygen:carbon ratio, leading to broad shifts (top to bottom or left to right) in VK space. These broad trends are a further result of the extreme isomeric diversity of the samples, which lead to a variety of reactivity within each molecular formula [142] and a consequent buffering of the peak intensity to the chemical change. If only half of the isomers are removed, the molecular formula's peak will decrease by $\sim$50% (disregarding isomer-specific ionization efficiency). This is the same observed result as the case where every isomer is half removed, and it bears remembering that neither case can be proven.

As an alternative to principal component analysis, sample peak intensity lists can be compared using distance metrics like Bray-Curtis Dissimilarity or Jaccard dissimilarity, which allow numerous pairwise sample distances to be reconstructed into principal coordinates via nonclassical multidimensional scaling, or to be distributed into groups using hierarchical cluster analysis (HCA; Fig. 4.5). Clustering analysis and principal coordinate modeling of intersample dissimilarities usually show that replicate analysis of the same sample are highly similar (usually more than 95% similar), while different samples can vary by 10% to almost 100% (completely different, almost no shared peaks) depending on the distance metric [52, 77, 141, 143]. This type of analysis can be conducted on presence/absence data rather than using peak intensities, which can be useful in cases where peak variability between replicates in not assessed or is unknown [5, 115, 141, 144]. Molecular formulas that correlate with hierarchical clusters, principal components (as loadings), or principal coordinate scores are often mapped onto VK diagrams to demonstrate how DOM composition can be deconstructed and clustered by the multidimensional factor analysis [135, 141], finding geochemical trends in the data and sometimes "molecular indicator species" which are indicative of certain regions or processes [5].

These multivariate approaches are almost always conducted on normalized data (where peak intensities are scaled to sum to a common value, and sometimes scaled to the standard deviation across the dataset [135]) for mathematical reasons and also to account for analytical factors that do not allow strict quantification like ionization suppression, but this often presents a problem when it comes to interpreting the data. In cases where DOC concentrations vary greatly, it can be confusing to describe trends in normalized data that go counter to the concentration gradient. For example, if a peak intensity increases in normalized data while DOC decreases, it is unclear whether this is because the sum of the analytes with that molecular formula increase are present at similar concentrations, or simply decrease less than the other analytes. The lack of understanding about the quantity of ionizable material in each sample thus stifles scientific understanding of actual (rather than relative) environmental trends. Because of variable ionization potential, great caution should be taken in correlating HRMS data with data from other techniques such as excitation emission matrices or NMR [135, 145–147], as it is possible that different analytes in the mixture are determined with the different techniques [23], making correlations coincidental, albeit related to similar geochemical trends.

Concluding remarks

There are now a wide range of HRMS techniques available for analysis of environmental samples, including tailored sample preparation techniques, ionization methods, coupling with different chromatographic phases, derivatization methods and other MS separation and structural elucidation methods. In the coming years, we hope that methods will be selected based on research questions, rather than research questions being designed around the methods and data available. The analytical window of HRMS techniques will continue to be an important concern in data interpretation, but it is equally important to remember that this window can be modified to suit the needs of the study.

An important question facing researchers is the cost/benefit analysis of running multiple samples vs multiple techniques (analytical windows) vs replicates. The conclusions drawn from results must consider the exact nature of the analytical window(s) used, including sample handling and preparation, ionization, detection limits, and data processing (e.g., formula assignment). Method validation should become commonplace and best practice should include the use of model compounds to test the analytical window(s). Additionally, studies should explicitly consider the reliability of intersample differences in comparison to analytical variability.

For environmental fingerprinting, it must be recognized that the data represents the ions in the mass analyzer, not the molecules in the raw sample, and it is of utmost importance to prepare and measure the samples as consistently as possible. Care should be taken to analyze large sample sets in one batch to minimize analytical variability and drift in signal of reference samples should always be monitored. Replicates, quality controls, and blanks remain invaluable and underused. As with all science, reproducibility and repeatability are key to ensure validity. Therefore, it should be common practice to make all raw and processed data and all data processing scripts and routines publicly available. This will also reduce the amount of reinventing performed, allowing scientists to focus their efforts on new problems.

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