Laser desorption/ionization coupled to FT-ICR mass spectrometry for studies of natural organic matter

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ABSTRACT: Laser desorption/ionization (LDI) was investigated as an ionization method for Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) studies of natural organic matter (NOM). Using International Humic Substances Society standards, Suwannee River fulvic acid (SRFA) and Suwannee River natural organic matter (SRNOM), LDI was found to ionize very similar set of compounds (> 90 % of molecular formulae identity) to the matrix assisted laser desorption/ionization (MALDI), while producing higher quality spectra. A comparison of electrospray ionization (ESI) and LDI spectra showed that different types of compounds are ionized by these methods with only 9.9 % of molecular formulae common to both. The compounds ionized by LDI/MALDI belong to low oxygen classes (maximum number of species for O7-O9), while ESI compounds belong to higher oxygen classes (maximum number of species for O14-O16). Compounds ionized by LDI can be classed as aliphatic, aromatic and condensed aromatics in approximately equal measure, while aliphatic compounds dominated the ESI spectra of SRFA. In order to maximise the coverage of molecular species, LDI, as a particularly convenient and readily deployable ionization method, should be used routinely in combination with other ionization methods, such as ESI, for FT-ICR MS studies of NOM.

Humic substances (HS), as the major component of natural organic matter (NOM), play a crucial role in a number of biogeochemical processes, ranging from water retention in soils and peats, to the binding and transportation of potentially toxic elements or nutrients in natural water systems.[1-4] They form complex mixtures composed of thousands of organic compounds, the exact chemical structures of which are largely unknown. The complexity of HS is such that they are chromatographically inseparable and therefore for molecular level analysis they require an ultra-high resolution analytical technique such as Fourier transform ion-cyclotron resonance mass spectrometry (FT-ICR MS).[5,6] The majority of MS studies of HS use electrospray ionization (ESI) due to its ability to ionize a large number of polar compounds present in these complex mixtures.[7] However, alternative ionization techniques, including atmospheric pressure photoionization (APPI), atmospheric pressure chemical ionization (APCI) and MALDI, have also been used in a few cases to provide a complementary picture of HS sample composition.[8-10] The most comprehensive comparison to date comparing ESI, APPI and APCI ionization of Suwannee River fulvic acid (SRFA) found that the choice of the ionization method had major impact on the nature of observed compounds. All methods were only able to ionize parts of the sample. Some molecules were common to all three ionization methods; some were shared between two, while some were unique to a specific ionization method.[8]

Matrix-free laser desorption/ionization (LDI) is significantly less used in complex mixture analysis.[11,12] As LDI requires increased laser power[13] to ionize analytes compared to MALDI[14], it was thought that it causes excessive fragmentation of HS,[10] and MALDI was accepted as a "soft" laser-based ionization technique suitable for analysis of HS. However, this notion originated from early works on HS, which assumed that HS were composed of large polymeric molecules[15]. When low (200 – 700 m/z) and higher (1000 – 1600 m/z) molecular weight peaks were observed in the LDI (but also ESI) MS spectra, the low mass peaks were initially interpreted as fragmentation products.[16] However, subsequent studies have shown that HS are aggregates of small molecules.[17] This breakthrough in understanding the nature of HS raised the need for assessing viability of LDI as a possible ionization method for this field. It is the purpose of this work to compare the performance of LDI with two established ionization techniques, MALDI and ESI.

EXPERIMENTAL SECTION

Chemicals and Sample Preparations. SRFA was supplied by the International Humic Substances Society (IHSS). 2, 5-Dihydroxybenzoic acid (DHB) ≥99 %, LC-MS grade methanol and LC-MS grade water were purchased from Fischer Scientific. Three samples were prepared for FT-ICR MS analysis. For MALDI-FT-ICR MS a 2 mg mL^{-1} SRFA/10 mg mL^{-1} DHB solution and for LDI-FT-ICR MS a 2 mg mL^{-1} solution in 50 % methanol:water solution were prepared. 1 μL of each solution was spotted onto a MTP 384 polished steel plate and dried at room temperature. For ESI-FT-ICR MS a 0.1 mg mL^{-1} SRFA solution in 50 % methanol:water was used. Identical conditions were used to prepare the SRNOM sample. When deuterated methanol (CD_3OH) was used in place of CH_3OH, an identical spectrum was obtained, indicating that neither CH_3 nor CD_3 methylation took place.

Instrumentation. Mass spectra were collected using a 12 T SolariX FT-ICR MS (Bruker Daltonics) coupled with ESI and MALDI, fitted with a solid-state 1 kHz smartbeamTMII laser,

sources. All analyses were performed using negative ion mode. For ESI data acquisition, a continuous flow sample was infused with a syringe flow rate of 200 μL h^{-1}. The nebuliser gas pressure was set to 2.0 bar. The drying gas was run at 180 °C and 4 L min^{-1}. The broadband spectra were acquired between 150 m/z and 1000 m/z in 2 MW FIDs and summed over 50 scans. Each scan had an ion accumulation time of 700 ms and a time of flight of 1 ms. For MALDI and LDI data acquisition the laser power was set to the minimum required to produce ions. Broadband spectra were acquired between 150 m/z and 1000 m/z in 2 MW FIDs and summed over 50 scans; selective accumulation was used excluding scans with low ion counts. Each scan had 1000 laser shots and an ion accumulation time of 500 ms. FIDs were zero filled once prior to Fourier transformation with the default processing parameters.

Data Processing and Presentation. Spectra were internally calibrated using a calibration list comprising of known manually assigned formula in Data Analysis 4.2 (Bruker Daltonics). These were identified starting with a tuning mix containing internal calibrants from which the CH_2 homologues series were propagated. Peak lists were generated using a signal-to-noise (S/N) threshold of 4 and a minimum intensity threshold of +2σ above the average noise. The peak lists were then exported to PetroOrg S-10.2 (Florida State University) for molecular formula assignment. Assignment was restricted using elemental limits of C (1-50), H (1-100), O (0-30) and an error threshold of 1 ppm. No other atom types were considered. Thereafter, formula assignments for MALDI/LDI/ESI were exported for analysis using in-house Python software. The aromaticity of the compounds was assessed by calculating a modified aromaticity index[18], AI_{mod}, defined as AI_{mod} = (1+C–0.5O–S–0.5H)/(C–0.5O–S–N–P). Venn diagrams were produced using the matplotlib venn package, other figures were produced using in-house software[19].

RESULTS AND DISCUSSION

MALDI, LDI and ESI FT-ICR MS Spectra of SRFA. SRFA, an IHSS standard, was selected as a suitable sample and the performance of three ionization techniques, MALDI, LDI and ESI was compared. Their superficial inspection highlights a high degree of similarity between the MALDI and LDI spectra (**Figure 1**), while the ESI spectrum is clearly different. LDI and ESI spectra of another IHSS standard, Suwannee River natural organic matter (SRNOM), were also acquired. As their analysis yielded very similar outcomes, only the SRFA data are discussed in the main text, and the SRNOM data are presented in the Supporting Information.

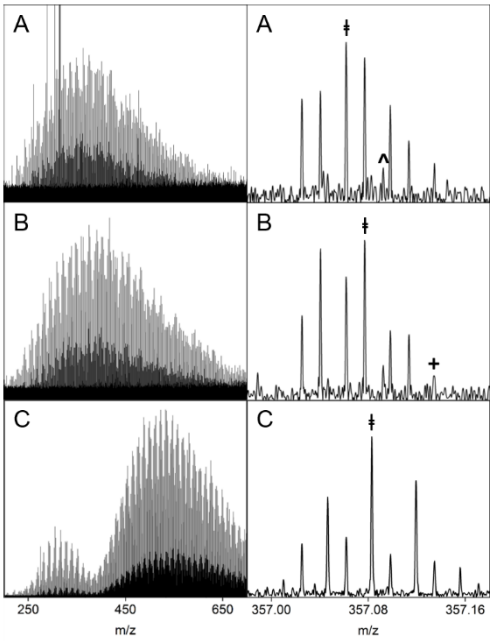

Figure 1. FT-ICR mass spectra of SRFA obtained by using MALDI (A), LDI (B) and ESI (C) ionization methods. The left hand spectra span the m/z range of 200 – 700, the right hand spectra show peaks at the nominal mass of 357 Da. The peaks labelled with a double dagger were used for the S/N comparison. The highlighted peaks are present in both MALDI and LDI spectra but below the S/N threshold for MALDI (^) or LDI (+).

For a quantitative comparison, 1396, 2209 and 5610 peaks were identified in each of the MALDI, LDI and ESI spectra, respectively, over the displayed range of m/z values using S/N threshold of 4. Out of these, it was possible to assign monoisotopic molecular formulae to 1046, 1450 and 2720 peaks, with average assignment errors of 102, 107 and 338 ppb respectively. . The average sigma value of assignments for peaks shown in the right panels of **Figure 1** was 426, 317 and 368 mσ. Altogether, 43, 221 and 1464 peaks were assigned to a molecular formulae containing one carbon-13 isotope. Cumulatively, this represents assignment of 78.0, 75.6 and 74.6 % of the peaks present in each individual spectrum. Unassigned peaks either belong to real ions outside assignment error thresholds, examples of which can be seen in **Figure 1**, secondary carbon isotope peaks, noise, or signal processing artefacts. **Figure 2** shows a distribution of 3815 unique molecular formulae identified from the MALDI, LDI and ESI spectra of the SRFA. This figure also shows the count of molecular formulae common to different combinations of ionisation methods.

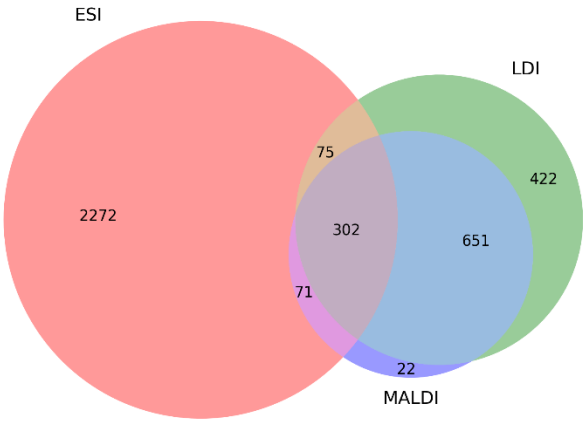

Figure 2. Venn diagram displaying the number of formulae identified that are unique to a single ionization source or present from multiple sources using MALDI, LDI and ESI.

It can be seen that MALDI and LDI spectra produced very similar data with over 90 % matching formulae relative to the total number of peaks in the MALDI spectrum. Focusing on these two spectra, manual inspection of the non-matching peaks revealed that their existence can be linked to one of three causes: (i) absence of a peak in one spectrum; (ii) the same peak is present in both spectra, although one of the peaks falls outside of the 1 ppm assignment threshold, hence a formulae is not assigned; (iii) peaks are present in both spectra, but in one data set the signal is outside the peak picking parameters. These points are illustrated in **Figure 1**, where a region showing the peak distribution at 357 Da for all three ionization methods is presented. Seven formulae were identified by all ionization methods and while visually the MALDI and LDI spectra contain the same peaks, one formula from each (labelled ^ for MALDI and + for LDI) is below the S/N threshold. This lead to a discrepancy between assignments as discussed above, and was typical for odd m/z values in the 250 – 650 m/z range.

Comparing all three methods, the S/N ratio was best for the ESI spectrum and poorest for the MALDI spectrum. For example, with reference to the double dagger labelled peaks in **Figure 1**, the S/N values were 21.8 (MALDI), 29.3 (LDI) and 39.3 (ESI). Although poorer than ESI, LDI is still substantially better than MALDI in this regard. An unavoidable problem in small molecule MALDI spectra is that the matrix peak is significantly larger than the sample peaks. This suppresses the sample peaks and reduces the average S/N in the whole spectrum; the improved quality of the LDI spectrum is the main reason why a greater number of molecular formulae could be assigned in the LDI spectrum (1450) compared to the MALDI spectrum (1046).

The analysis presented thus far (**Figures 1** and **2**) suggests that there is a significant difference in the number of matching formulae between the molecules ionized by ESI in comparison to MALDI/LDI. Hereafter we explore these differences at the molecular level.

Van Krevelen diagrams of MALDI, LDI and ESI data. ESI is understood to ionize compounds that can exist as ions in solution,[20] therefore, it is primarily suited for observing protic compounds. MALDI/LDI, however, ionize compounds through different mechanisms[21] and therefore produce spectra that include less molecules with protic functionalities. As HS are thought to be dominated by CHO compounds, it is reasonable to assume a correlation between the number of oxygens, polarity, and compounds ionized by ESI. This assumption is supported by an inspection of van Krevelen diagrams[22] produced for LDI and ESI spectra in **Figure 3**. The van Krevelen diagram for MALDI ionisation (Fig. S-2) is not included here, as it is highly similar to the one produced for the LDI data.

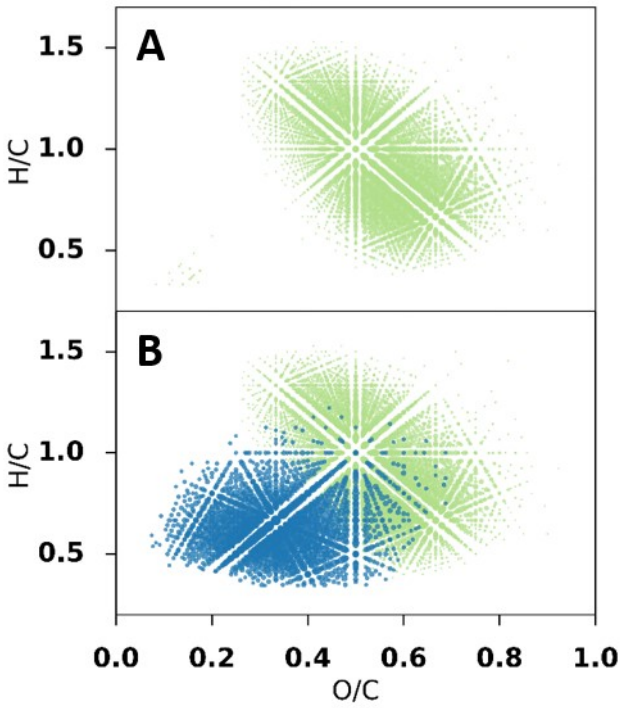

Figure 3. Van Krevelen diagrams constructed using all formulae identified in the FT-ICR MS spectra of SRFA. A displays the ESI dataset only, while B shows an overlay of the LDI (blue) and ESI (green) datasets emphasizing the significant difference in the type of compounds ionized by the two methods.

These diagrams clearly illustrate that MALDI/LDI and ESI ionize compounds with different O/C and H/C ratios. While LDI/MALDI ionize low O/C and low H/C compounds, the opposite is true for ESI. Taken together, the majority of compounds separate into the lower left (MALDI/LDI) and the upper right (ESI) regions of the van Krevelen plots, with an overlapping section in the centre.

Heteroatomic class distribution. The identified molecular formulae were further classified using their heteroatomic classes. A bar chart representing the count of O2 to O24 formulae in MALDI, LDI and ESI FT-ICR MS spectra is shown in **Figure 4**. The results reinforce a similarity between MALDI and LDI spectra, which share high abundance of low oxygen compound classes. Their bell-shaped distribution has a maximum at the O8 class. Minimal number of O2-O4 compounds were assigned in the ESI spectra, with a maximum compound count shifted towards high oxygen classes and spread over a broader range of values. Here MALDI/LDI were less effective and failed to ionize any compounds above the O16 class.

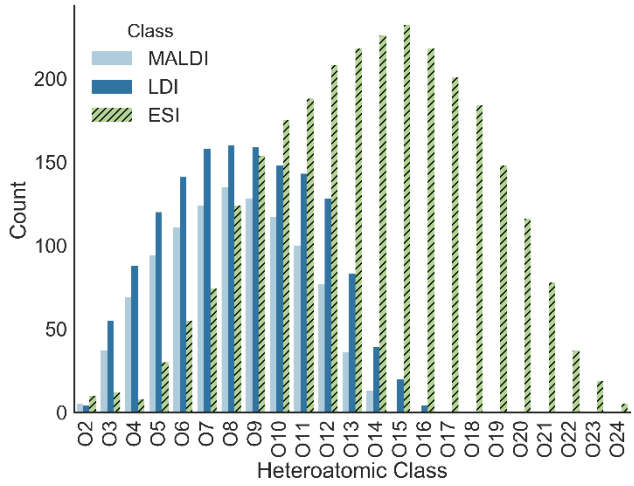

Figure 4. Bar chart of the number of formulae identified for oxygen classes from O2 to O24 for MALDI, LDI and ESI FT-ICR MS spectra of SRFA.

Comparison of Molecular formulae Assigned to LDI and ESI Spectra. Compounds with identical molecular formulae identified in both LDI and ESI spectra (377 or 9.9 % of unique formulae to ESI and LDI; see Figure 2) cluster in the middle of the van Krevelen plot in **Figure 3**. It was initially assumed that these matching peaks mostly belong to the oxygen classes where the ionization potential appears to be similar for both LDI and ESI, such as O9, where 49 of 154 (31.8 %) formulae matched (see **Figure 4**). However, oxygen classes specific comparison showed a similar percentage of matching formulae for lower oxygen classes e.g. 14 of 55 (25.5 %) for O6. Higher numbers of matches were found for higher oxygen classes e.g. 38 of 83 (45.8 %) formulae matched in the O13 class. This observation suggests that the compounds ionized by LDI are significantly different to ESI compounds across the full range of oxygen classes and the matching formulae seen in the central part of the **Figure 3** either belong to (i) identical molecules ionized by both LDI and ESI, or (ii) to structural isomers, each ionized only by one of the two methods. Due to the different ionization mechanisms of LDI and ESI, the latter explanation is more likely.

Comparison of Molecular Formulae Assigned to MALDI and LDI Spectra. A similar comparison of the MALDI and LDI spectra categorised by the oxygen classes showed that the most populated classes contain most matching formulae, relative to the number of formula identified by MALDI (O7, 121 of 124, (97.6 %) and O8, 124 of 135, (91.9 %)) whilst the least populated classes showed fewer matching formulae (O3, 33 of 37, (89.2 %) and O13, 28 of 36, (77.8 %)). This fits with the aforementioned observation that the major cause of discrepancy between the MALDI and LDI data sets is due to peaks dropping below the S/N threshold, an occurrence that is found at the extremes of the spectrum (lower m/z for low O classes and higher m/z for high O classes) where the S/N ratio is at its worst. It is therefore possible to conclude that identical compounds are ionized by both methods.

The Aromaticity Index (AI). The assigned molecular formulae were further analysed to characterise the aromaticity of molecules they represent. AI, unlike double bond equivalency (DBE), takes into account the presence of oxygen atoms in molecular formulae.[18] The amount of oxygen considered can however be reduced, assuming that not all oxygen atoms belong to carbonyl groups. As hydroxyl or ether oxygen is present in SRFA molecules alongside the carbonyl oxygen, a modified variant, AI_{mod}, which only counts half of the oxygen atoms and assumes the other half is sigma bound, was used. The following threshold limits were set for AI_{mod} to categorise the aromatic character of the molecules: $AI_{mod} \leq 0.5$ for non-aromatic molecules, $0.5 < AI_{mod} < 0.67$ for aromatic molecules and $AI_{mod} > 0.67$ for condensed aromatic molecules. **Figure 5** shows stacked bars representing normalized percentages of formula counts that fall above and below the specified AI_{mod} thresholds. This classification allows further interpretation of the character of molecules ionized by each method. It appears that LDI ionized the largest amount of compounds classified as condensed aromatics (46.8 %), more than MALDI (39.0 %). This is at the expense of non-aromatic compounds, while the relative amount of aromatic compounds ionized remained approximately constant (42.9 vs 45.4 %) between the two techniques. To the contrary, 96.6 % of compounds ionized by ESI could be classed as non-aromatic. As aromaticity has been linked to HS function within natural systems, [23] and ESI is the most frequently used technique in MS studies of HS samples, our analysis suggests that LDI should be used alongside ESI ionization to provide a more complete description of NOM.

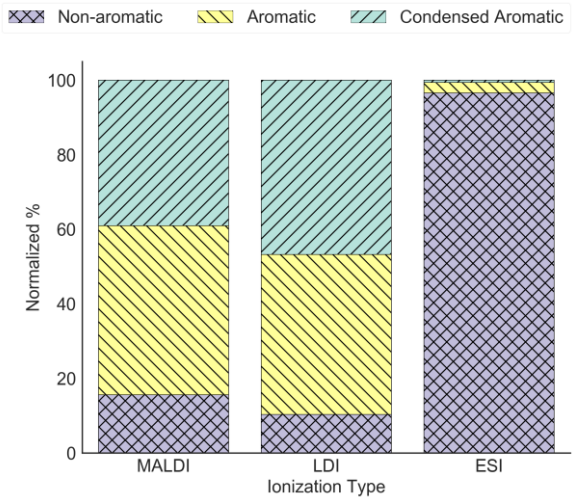

Figure 5. Stacked bar plot representing normalized % of formula count that belong to three AI_{mod} categories; i) $AI_{mod} \leq 0.5$ (non-aromatic, purple), ii) $0.5 < AI_{mod} < 0.67$ (aromatic, yellow) and iii) $AI_{mod} > 0.67$ (condensed aromatic, green) for MALDI, LDI and ESI spectra. AI_{mod} was calculated as defined in the Experimental section.

Implications for MS Studies of NOM. Hertkorn *et al*[8] compared ESI, APCI and APPI spectra of SRFA in both positive and negative modes. Due to the limited assignment capabilities available at the time of the study, molecular formulae were assigned only to 15 to 25% of the observed peaks. Amongst these only 1.7% and 3.8 %, for the positive and negative mode respectively, were identical for all three ionization techniques.

In our study, 74 to 78% of observed peaks were assigned a molecular formula and a higher percentage of identical assignments were observed between ESI and LDI (9.9 %). Analysis of our data based on van Krevelen diagrams, heteroatomic class

distributions and aromaticity indices showed that distinct types of compounds are ionized by ESI and LDI. Both studies therefore demonstrated that ESI alone does not offer a complete coverage of NOM molecules.

Importantly, a close match (> 90%) was seen in this study between the molecular formulae identified in MALDI and LDI spectra of SRFA. Whilst MALDI is an established method, it is not widely used in the investigation of NOM. As illustrated here, LDI – an even less used method – produced spectra superior to the MALDI method, ionizing identical compounds while avoiding some of the caveats of MALDI. The sample preparation for LDI is significantly simpler than for MALDI, as it does not require a trial and error process of selecting a matrix and its concentration. The complexity of HS makes the sample itself act as a matrix, enabling ionization. It is therefore a simple "spot and shoot" method.

Overall, our results challenge the dogmatic assessment of LDI as an inappropriate ionization method for MS investigation of NOM.

CONLUSIONS

FT-ICR MS spectra of NOM are today mostly acquired using an ESI as the preferred ionization method. Significant differences between the ESI and LDI FT-ICR MS spectra of SRFA observed in this study complement differences seen between other ionization techniques,[8] and endorse the view that no single method is able to ionize all NOM compounds. To maximise the coverage by FT-ICR MS of the molecular space occupied by these complex mixtures, multiple ionization methods must therefore be used. As a particularly convenient, and readily deployable ionization techniques, LDI should be included standard analytical protocols for FT-ICR MS analysis of NOM.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: containing van Krevelen diagrams for SRFA LDI and MALDI spectra, analysis of the FTICR MS spectra of the SRNOM sample and associated figures, a link to a complete assignment lists for all data sets.

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Author Contributions

The manuscript was written through contributions of all authors.

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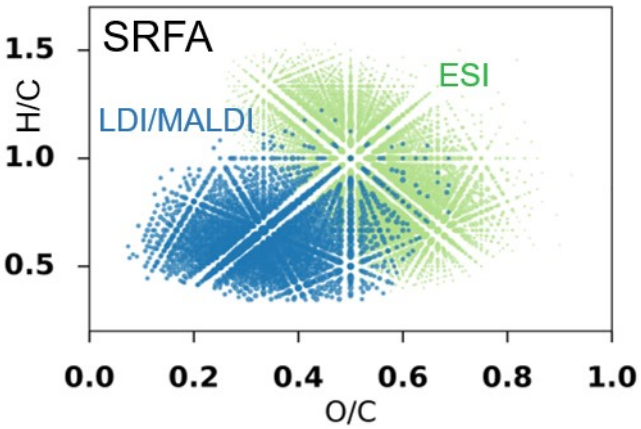

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