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Response to "Comment on the Molecular Composition of Natural Organic Matter Analyzed by Electrospray FTICR MS"

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ABSTRACT

We have recently illustrated that the LDI and MALDI spectra of the Suwannee River fulvic acid (SRFA) and Suwannee River natural organic matter (NOM) contain complementary signals to spectra obtained by negative electrospray (Blackburn, J. W. *et al.*, *Anal. Chem.* **2017**, *89* (8), 4382–4386). In that work we presented an ESI-(-) FTICR MS spectrum of SRFA that showed bimodal distribution of peaks (200-400 *m/z* and 400-800 *m/z*). He *et al*. Anal. Chem., 2018, 90 (9), pp 5965–5967, DOI: 10.1021/acs.analchem.7b05023 commented on this unusual appearance and suggested that the higher m/z values are due to dimerization. In our response we demonstrate that (i) a monomodal spectrum, acquired using different experimental parameters, produced 86% identical molecular formulae assignment to that obtained for the bimodal distribution spectrum; (ii) the heteroatom class distribution was practically identical for both spectra; (iii) taking several different experimental approaches, we did not find any evidence of oligomerisation in our spectra (iv) we showed that different experimental parameters enhance peak intensities in different *m/z* regions of the FTICR MS spectra and produce spectra of SRFA containing peaks outside of the narrow 200-700 *m/z* window (iv) we showed that it is beneficial to explore these different experimental settings to obtain a fuller coverage of the molecular space of NOM and that one should always treat the peak intensities with caution. This analysis allowed us to reaffirm the conclusions of our original paper: ESI and LDI are complementary techniques and both should be used for a more complete characterisation of complex NOM.

In our recent paper, we described the acquisition and analysis of high-resolution Fourier transform ion cyclotron resonance (FTICR) mass spectra of Natural Organic Matter (NOM) by three ionization techniques – negative electrospray, ESI-(-), negative matrix assisted laser desorption ionization, MALDI, and negative laser desorption ionization, LDI.[1] In that work, we found that (i) MALDI and LDI ionize similar classes of compounds; (ii) MALDI, and by implication LDI, does not appear to cause undue fragmentation of NOM compounds; (iii) ESI and LDI ionize largely different classes of compounds. Based on these observations, we concluded that LDI is a useful additional ionization technique when attempting to fully characterise the molecular diversity of complex NOM samples. We demonstrated this by using two International Humic Substances Society (IHSS) standards, Suwannee River fulvic acid (SRFA) and Suwannee River NOM (SR-NOM).

In their response, He *et al.* have investigated one side-aspect of our original paper. They noted that the presented ESI-(-) FTICR spectrum of SRFA had a bimodal distribution (200-400 *m/z* and 400-800 *m/z*), and that neither part of the distribution was centred at around 350 *m/z*, where SRFA is expected to display a normal distribution, extending between 200 and 700 *m/z*.[2–4] He *et al.* commented that they had not seen this bimodal distribution before. They hypothesised that the higher mass distribution we observed may be due to dimerization of compounds, and suggested that we had not optimised our experimental conditions. They further noted that the large range of oxygen class

species, from O_2-O_{24} in our ESI spectrum, did not match their own results. These are interesting points worth commenting upon.

In order to address the issues raised, we have acquired a further ESI-(-) spectrum for SRFA using the same conditions as before, but tuned the spectrometer to produce a "normal" distribution. We have also acquired additional spectra aiming to induce dimerization, as well as spectra optimised for alternative, low mass ranges.

The newly acquired FTICR MS spectrum of SRFA (**Figure 1a**; see Supporting Information, SI, for experimental parameters) extends from 200 to 1000 *m/z*, with a maximum around 380 *m/z*, and is thus more similar to the spectrum presented by He *et al.* that spanned the 220-650 *m/z* range with a maximum at 350-400 *m/z*. To test the validity of the previously acquired bimodal data (**Figure 1b**), a comparison of the molecular formulae obtained from the bimodal and "normally distributed" spectra was made. A direct comparison of the entirety of both data sets would be affected by differences in the signal-to-noise ratios (SNR), influencing peak picking and therefore assignments made. Thus, the subset region of 250-600 *m/z* was chosen to compare assignments, as this region was not at the limits of *m/z* values for either dataset. Within this region, the new spectrum yielded 1493 monoisotopic assignments (the bimodal spectrum had 1474) with 1282 common to both. This 86% commonality of molecular formulae shows that both data sets are broadly the same. The differences observed are primarily due to the different SNR, especially at around 420 *m/z*, where one spectrum has a maximum intensity and the other a local minimum. To put these results into perspective, Kido Soule *et al*. found that technical replicates of ESI-(-) FTICR MS spectra of SRFA achieved a maximum of 87% peak commonality in their methodology.[5] Additionally, Sleighter *et al*. investigated intra-day variations by acquiring duplicate and triplicate of complex samples, achieving peak commonality of 75% on average for a SNR threshold of 5.[6] It is therefore reassuring to observe this level of agreement between our two spectra across months.

To further emphasise the similarities between these two data sets, heteroatomic class distributions are shown for the same subset mass range (250-600 *m/z*) for both spectra in **Figure 2**. Here, the distribution is effectively identical. It is worth noting that both distributions show a continuous nature with no gaps, indicating that despite the reduced signal intensity around 420 *m/z* in the bimodal data set, practically identical ions within this mass range were detected by both experiments. The most populated oxygen class species recorded for the new data set is O11, the same result which He *et al* obtained for a comparable *m/z* range on a SolariX XR instrument. In our original paper, the heteroatomic distribution extends higher (maximum at O15 for the ESI spectrum). As the bimodal data had higher SNR at higher masses, and therefore more assignments in this part of the spectrum, this is to be expected: higher oxygen numbers are inherently more probable for higher molecular weight compounds.

Contrary to claims by He *et al*., bimodal spectra of SRFA have been presented by others previously. [7,8] Also, neither are we the only group to have observed ions > 700 *m/z* in these samples. Two recent 21 Tesla SRFA ESI-(-) FTICR spectra show a broad range of peaks from 200 to 1000 *m/z*, with maximum intensity between 300-500.[4,9] It would therefore be incorrect to assume that the SRFA ESI-(-) FTICR spectra should always and only present a normal distribution between 200-700 *m/z* with a maximum at about 350-400 *m/z*. Inspection of the available spectra[3,4,10,11] suggests that these are better described as a skew normal distribution with a significant tail extending to 1000 *m/z* (or potentially above) depending on the SNR.

It is insteresting to note that if one looks more closely at the published SRFA spectra,[3,10,12] including those obtained in our laboratory,[1] these are comprised of a number of normal distributions. This can

be seen in both the bimodial and normally distributed datasets at each 14 *m/z* (**Figure 1c,d**), and within each *m/z* (**Figure 1e,f**). These distributions seen across the spectra presumably reflect the inherent chemical stability, prevalence, and ionisability of certain compound classes present in SRFA. Each 14 *m/z* represents an additional CH_2 and thus extends the molecular size of very similar structures. Arguably, when trying to understand the nature of SRFA compounds as seen by FTICR MS, it is the per-*m/z* distributions that are chemically more interesting and relevant than an overarching distribution across the entire mass range. The latter is much more liable to be biased by instrumental parameters.

The question nevertheless remains, whether the appearance of peaks at higher *m/z* values in *any* SRFA FTICR spectra is caused by oligomerization. Indeed, He *et al.* suggested that the bimodal distribution we obtained was due to dimerization. To investigate this possibility, we first compare our published bimodal data with the newly acquired normally distributed data. As an example, in **Figure 3** we show the 701 *m/z* region from each of the spectra; both contain the same dominant peaks. This was typical for the entire high-end *m/z* part of the spectra. Therefore, if our bimodal data reflects aggregation, this is true of the normally distributed data as well. Interestingly, the bimodal data also contains several minor signals, as illustrated within the 701 *m/z* region; these are mostly at the noise level, though some are also evident in the normally distributed data. These peaks could also be interpreted and represent additional information about the sample. Having a greater SNR at a higher mass is therefore beneficial and a direct consequence of the instrumental setting that produced the bimodal dataset. Similarly, by focusing on the other end of the *m/z* range, we can acquire FTICR spectra with sufficient SNR at *m/z* of around 100 (see **Figure S1**) – peaks clearly not visible in the bimodal or normal distribution spectra discussed above.

To further address the question of oligomerization, we also performed additional experiments: (i) we attempted to induce aggregation and increased concentration from 0.1 mg/ml, as shown in the normal and published bimodal data, to 1, 2.5, and finally 5 mg/ml. We observed no significant differences between these low and high concentration FTICR MS spectra (see **Figure S2**). At 5 mg/ml, a far greater concentration than typically used nowadays, we observe no apparent oligomerization in the mass spectrum. However, at this concentration, spray stability begins to suffer, and we do not believe higher concentration samples would be possible to spray on our instrument. (ii) We also experimented with a variety of collision cell voltages at 2.5 mg/ml, but found no evidence of aggregation. (iii) We further investigated the effect of varying the ESI capillary temperature – aggregation should be less favourable at higher temperatures – and observed no evidence of aggregation (**Figure S3)**. (iv) Finally, we performed semi-selective MS/MS experiments at higher masses (650 and 750 *m/z*, **Figures S4-S7**) in an attempt to break non-covalently bonded aggregates into their 'monomers', but only observed covalent fragmentation. In conclusion, these results confirm that the spectra of SRFA we acquired do not contain any significant level of aggregation. A more detailed description of these experiments is available in the SI.

Looking at historic related MS data of SRFA, Stenson *et al*. presented positive ion ESI of SRFA with two bimodal distributions; the first between 300 and 1200 *m/z* and the second, lower intensity one approximately from 1400 to 2200 *m/z*.[10] This second distribution is likely a result of aggregation as they used a high concentration of 5.7 mg/ml.[10] Fievre *et al*. also presented apparently aggregated ESI spectra of SRFA, but did not report the concentration of SRFA used.[13] To the best of our knowledge, no recent paper has presented ESI spectra of aggregated SRFA. Typically, ESI spectra of SRFA are now acquired with low concentrations, < 1 mg/ml[3] not causing aggregation. Aggregation identified in older reports was probably caused by high concentration and lower quality electrospray sources used in the past. Modern sources and ion optics should break apart any aggregates that do form. We therefore

argue that it was the instrumental conditions and not oligomerization that lead us to obtain a bimodal distribution emphasising higher molecular weight species. The exact cause for the bimodal appearance of the spectra remains unknown to us.

To allow the scrutiny of the data by a wider community, several SRFA spectra acquired at various conditions in our lab have been made available in raw form online.[15] These include the bimodal dataset, a 4MW "normal" distribution dataset acquired pre-publication, the new 2MW "normal" distribution dataset, spectra tuned for high and low masses, spectra acquired at high concentrations, and spectra acquired with varying collision cell voltages. Their full details are presented in the SI. We do not have access to data acquired by He *et al.* and cannot therefore carry out a more thorough comparison of their data set with our own, neither can we comment on the N-assignments made by He *et al.* We did not identify nitrogen–containing compounds in our spectra.

Based on a classification of aliphatic compounds as having close to zero double bond equivalent (which is problematic, as it misclassifies cyclic aliphatic structure), He *et al.* commented that our published data for ESI-(-) of SRFA do not contain aliphatic compounds. In our paper, we classified molecular formulae by their Aromaticity Index,[14] a metric that describes the nature of a molecule as aromatic, condensed aromatic, or non-aromatic. By this metric, > 95% of species identified in the ESI spectra of SRFA were non-aromatic, whilst >85 % those in MALDI and LDI were classified as aromatic or condensed aromatic.

As argued in our paper, and in agreement with the comment by He *et al.,* LDI works because SRFA inherently contains matrix-like chemistry, and avoids the contamination and signal suppression of MALDI. We disagree that LDI is 'much more straightforward', however. LDI requires a more difficult sample preparation than ESI – the required spot homogeneity is an issue – and LDI can result in unstable ion numbers in the ICR cell. Summing sufficient scans, when using an ion count threshold, for an acceptable SNR can take far longer with LDI than ESI. Without such a threshold, LDI spectra are more prone to signal splitting and low SNR.

In conclusion, we hope to have addressed the concerns expressed by He *et al.* and like to thank them for their comments. The experiments we carried out subsequently and their analysis allowed us to reaffirm the conclusions of our original paper: ESI and LDI are complementary techniques and both should be used for fuller characterization of a complex NOM. This conclusion was based entirely on the presence/absence of peaks, and not their relative intensities. We have shown that the molecular formula identified in our bimodal and a "regular" dataset, were essentially the same. Therefore, at the most basic level, the fact that our ESI spectrum had a bimodal distribution rather than the typically presented normal distribution is insignificant in the context of our original paper.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: **XXXX** containing: experimental parameters and spectra acquired at different concentrations of SRFA, detail and figures for effects of varying capillary temperature, MS/MS experiments focusing at higher mass ions and discussion on the range of *m/z* values observed.

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Author Contributions

The manuscript was written through contributions of all authors.

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FIGURES

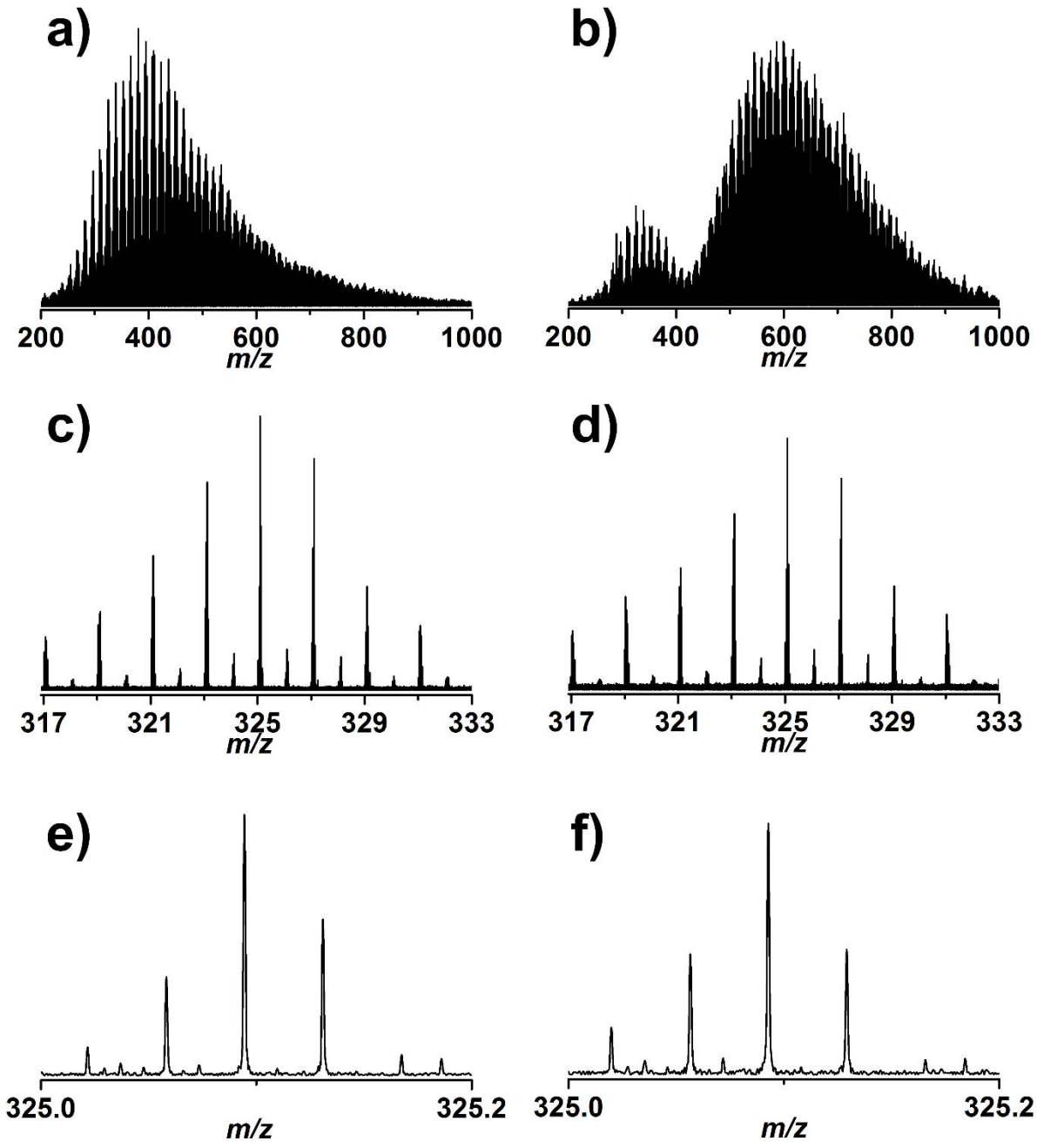

Figure 1. Negative mode ESI FTICR mass spectra of SRFA. Left column shows the broadband normally distributed data (a), a per-14 *m/z* expansion for this data (c), and a single nominal mass, 325 *m/z*, for this data (e). Right column shows the broadband bimodal data (b), the per-14 m/z expansion for the bimodal dataset (d), and a single nominal mass for the bimodal data (f).

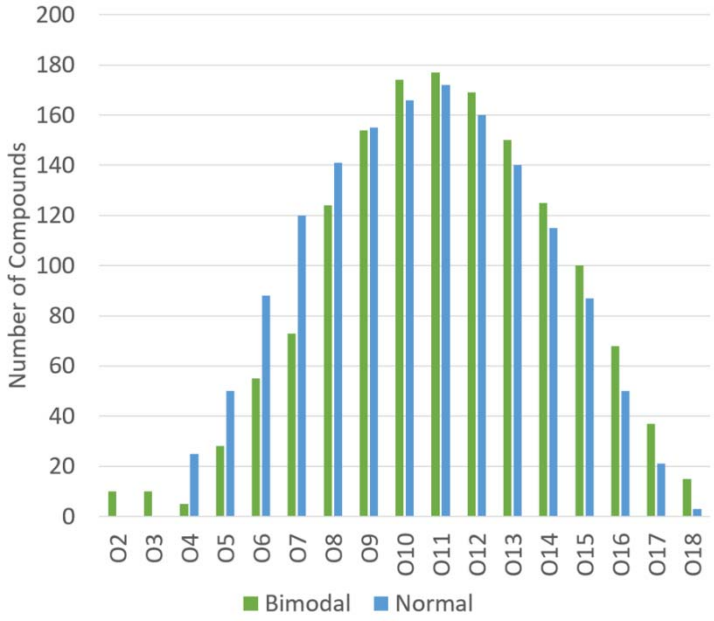

Figure 2. Heteroatomic class distributions for the bimodal and normally distributed datasets between 250-600 *m/z*.

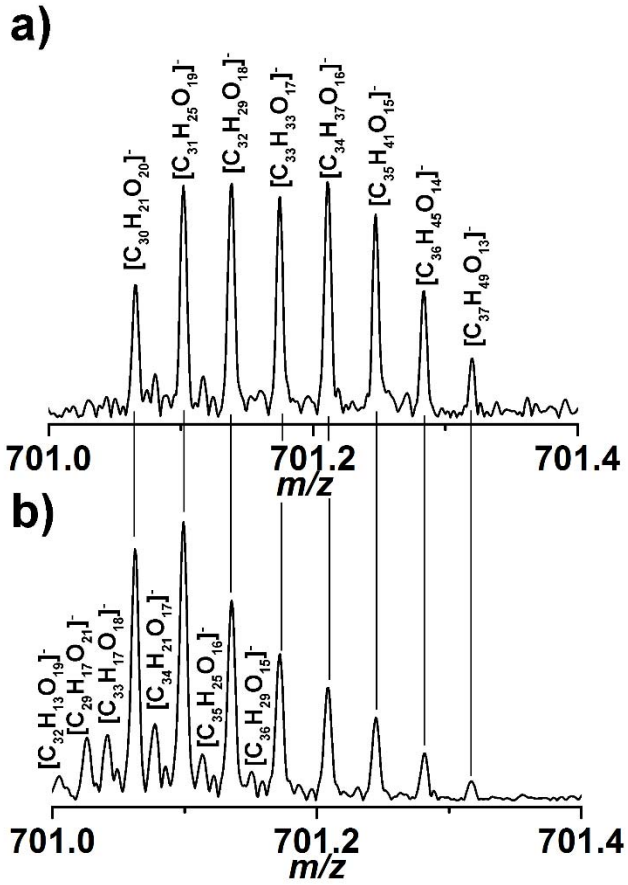

Figure 3. A single nominal mass (701 *m/z*) for the normally distributed (a) and bimodal (b) datasets. Assigned ion formula have been annotated. Median error of assignment in this window was 0.72 ppm. Vertical lines have been added to aid comparison.