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Benefits of multidimensional fractionation for the study and characterization of natural organic matter

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ABSTRACT

The belief that chromatographic separation of complex environmental mixtures or natural organic matter (NOM) produces featureless humps from which little, if anything, can be learned is still pervasive. Meanwhile improvements in chromatography and the use of information-rich detection methods have led to meaningful fractionation and revealed consistent data. Here, we build on this work and developed a robust, facile two-dimensional separation with high orthogonality between dimensions. We illustrate that re-injections of fractions (both in the first and in the second dimension) leads to individual peaks at the expected retention times and use information-rich detection to investigate the basis on which NOM is fractionated. We demonstrate unprecedentedly feature-rich chromatograms are observed even with standard UV detection for polar NOM fractions. The second stage of fractionation is demonstrated to separate isomers, providing a direct look at isomeric complexity in NOM as well as a tool to reduce it. Consistent with expectation, but confirmed for the first time through mass spectral data, radicals were detected for NOM components that were generally nonpolar and grouped in the condensed aromatic structure – like region of van Krevelen plots. High-resolution tandem mass spectral data, furthermore, suggests that many higher-MW components of fulvic acids (especially the highly oxidized ones) have formulas that do not match any known compounds in the literature, supporting the hypothesis that fulvic acids are a unique compound-class. Combined, the data illustrate that meaningful reduction in complexity reveals new compositional and structural detail and avails new avenues of investigation.

1. Introduction

Complex environmental mixtures from crude oil, tar balls, marine and freshwater dissolved organic matter (DOM) to humic like substances (HULIS) in aerosols and all subgroups (e.g., humic substances (HS), humics acids (HA), fulvic acids (FA)) are a great challenge to analytical chemistry. Here, the umbrella term natural organic matter (NOM) is used to describe all such mixtures. NOM is of environmental, agricultural, industrial, and biomedical importance. For example, NOM, is studied for its effect on limiting nutrients in open oceans [1], its own role as organic nutrient [2,3], carbon cycling/storage [4,5], formation of harmful disinfection byproducts in drinking- and waste-water [6,7], sorption/transport/bioavailability of metals and radioactive waste [8–10], and aerosol composition [11]. Moreover, humics and "humic-mimetics" (synthetically made HULIS) are studied for their antiviral, antibacterial, and cancer-related properties [12–14]. In short, against the backdrop of a rising global population with increasing needs for food/high-yield agricultural soil, safe drinking water, new pharmaceuticals, fuel, clean air and climate normalization, the ability to characterize structure-activity relationship of NOM would constitute an enormous advancement.

Separation is the obvious first step in the study of any complex mixture. Recent reviews which address NOM fractionation include: Nebbioso et al. [15], Minor et al. [16], and Sandron et al. [17]. Early chromatographic results of NOM were not encouraging with the approach characterized by poor recoveries, poor reproducibility, and irreversible retention. Over the last decade, however, the combination of information-rich/selective detectors, improved column technology, and NOM-specific method development, has led to robust fractionation of NOM into physiochemically distinct components, revealing previously unobtainable compositional detail [15–22]. Differences between humic substances from distinct origins and stages of transformation

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can readily be determined [18–24]. These experiments have shown that fractionation increases the number of detectible constituents. In addition, a positive correlation between polarity and carboxylic content and a negative correlation between polarity, saturation/aliphatic character, and relative size has been established [18,21–23,25]. The general consensus is that early/polar/hydrophilic fractions are highly oxygenated and unsaturated, whereas late/nonpolar/hydrophobic fractions are the opposite [18,25,26].

To some, a remaining deterring aspect of chromatographic separation of NOM is the absence of features or general appearance of NOM-"humpograms". In the past, some peaks have been induced through the use of two-dimensionalseparation and/or information-rich detection [20,21,27,28]. For example, Mawhinney et al. [27] cleverly introduced features by using mass defect information from high-resolution (time-of-flight) mass spectrometry. Similarly, Li et al. [28] obtained striking features with selective protein-like emission/absorbance wavelength pairings in fluorescence detection. Features can also be artificially induced through abrupt mobile phase changes which usually also change the pH (dissociation decreases in organic solvent), pressure, optical density in UV–vis detection, and/or ionization potential in electrospray (ESI) sources [29,30]. However, chromatographic separation of NOM is unlikely to ever produce individual peaks for each analyte. To use the chromatography of NOM to its full potential, it is important to abandon the expectation of feature-rich chromatograms, and focus on the true value of separation to the study and characterization of NOM. Prefractionation of NOM not only makes most analyses more meaningful but also adds additional information (e.g., polarity, size, electrophoretic mobility, K_{ow}) which enhances interpretability. For example Li et al. coupled reversed phase chromatography (RP-HPLC) and size-exclusion chromatography (SEC) with multi-excitation/emission scan fluorescence to identify linked fluorescences and improve structural fingerprinting of NOM [28]. Liu et al. combined RP-HPLC with high-resolution tandem MS (MS/MS) to see more NOM components and obtain more meaningful MS/MS results. They were able to compare fragmentation patterns for isomers from different NOM samples and link the differences to expected transformations of NOM from swamp to ocean [21]. Gaspar et al. discovered a new structural type (carboxyl-rich alicyclic molecules – CRAMs) in NOM based on electrophoretic mobility coupled to high-resolution MS [22]. Woods et al. combined one-dimensional (1D) and two-dimensional (2D) hydrophilic interaction chromatography (HILIC) with NMR and fluorescence to examine correlation between polarity, structural features in NMR, and structural information from parallel factor analysis components (PARFAC). The HILIC separation reduced heterogeneity, decreased interaction with microenvironments and improved the NMR results to the point where individual small acid components could be identified [31,32]. In previous work, we combined RP-HPLC, hydrogen/deuterium exchange (HDX), metal-complexation, low-resolution multistage fragmentation (MS^n) and high-resolution MS to obtain structural and compositional information on RP-HPLC fractions (fractions which are further fractionated in the second dimension here) [18]. We also demonstrated that fractionating NOM prior to investigating NOM's interaction with other chemicals (i.e., NaOCl added during water treatment) provides far more definitive results than analyzing bulk NOM [33]. In short, combined chromatographic pre-fractionation and multiple information-rich analyses have the potential to provide unprecedented structural insight. Structural insight allows more accurate predictions of structure-activity relationships in environmental, pharmacological, toxicological and industrial settings.

As noted above, combining two LC steps has previously been shown to further reduce HS complexity [20,25,29,32] (as well as possibly reduce isomeric complexity [25]), although significant complexity is still observed. Previous work combined SEC with RP-HPLC, RP-HPLC with RP-HPLC, or HILIC with HILIC. Unfortunately, none of these combinations provide high orthogonality. Even size and polarity are generally related: charge-exclusion effects in SEC and size-exclusion effects in RP-HPLC decrease orthogonality. Few investigators attempt to optimize or test orthogonality. Among the exceptions are Góra et al. who used wide-pore RP-HPLC in the first dimension to avoid size-discrimination effects and narrow-bore SEC in the second step [29]. They were able to show statistically that the separations were not redundant and that injection-to-injection reproducibility was good in the SEC dimension. Here, we improve on such efforts by (1) individually optimizing both dimensions on analytical standards, (2) developing different second dimension gradients for polar and nonpolar first dimension fractions, and (3) evaluating orthogonality of each 2D pairing. The goal was to obtain multidimensional fractionation methods: that were as orthogonal to each other – and the dispersion mechanism within the mass spectrometer – as experimental compatibilities allow.

Previous results (e.g., those described above) have shown that the more information-rich the detection, the easier it is to detect physiochemical differences between chromatographic fractions. Here, ultrahigh resolution electrospray ionization Fourier transform ion cyclotron resonance MS (ESI-FTMS) was chosen. It has been shown to resolve individual humic ions at the milli-Dalton level and provide molecular formulas [25,34–40] but has some major shortcomings. The fact that FTMS is not quantitative and does not provide detailed structural information is usually overcome by marrying FTMS to other techniques (e.g., NMR, IR, pyrolysis-GCMS, and fluorescence data) and by matching compositional signatures in van Krevelen space to known biomolecules as reviewed by Mopper et al. [41]. The remaining shortcoming that is scarcely addressed in the literature is that even FTMS cannot distinguish between isomers. A major goal here is to specifically tailor multistage chromatographic separation for isomeric fractionation prior to FTMS analysis.

To our knowledge, no other group has focused specifically on the isomeric fractionation of NOM although previous LC-FTMS results have shown ions of identical molecular formula spread over a wide range of retention times (t_r), indicative evidence of isomeric separation [21,22,25,26]. Verifying isomeric fractionation is challenging because no technique is capable of analyzing individual analytes without some level of prior separation or differentiation. FTMS comes the closest by distinguishing between analytes so long as they differ in mass. MS/MS has long been used to distinguish between isomers for less complex samples. As mentioned above, Liu et al. also used FTMS/MS to distinguish between isomers from different samples [21]. To our knowledge, no group has used MS/MS to examine isomeric separation within a NOM sample. In the past, we have used H/D exchange and recommended it be coupled to MS/MS to provide reliable results [42]. Here, online LCMS/MS is evaluated as a facile and speedy comparison technique. Complimentary HDX analysis, which requires specialized equipment and FTMS analysis to be meaningful, will be examined in future work.

The final impediment to the universal incorporation of chromatography into NOM analysis is that meaningful fractionation of NOM is non-trivial and time-consuming. Although each dimension presented here did require extensive optimization and characterization, the resultant methods are now routinely and reproducibly used by undergraduates after minimal training. Furthermore, neither dimension requires specialized equipment, complex mobile phases, or solvents that are incompatible with HPLC instrument components or sample evaporation to dryness. In short, the proposed methods are facile, robust, and broadly applicable to investigators across the broad swath of NOM-related fields.