

Response to Editors and Reviewers

Thanks for reviewers' careful reading and constructive comments and suggestions. We made every effort to respond to reviewers' questions point to point, and carefully revised our manuscript and Supporting Information based on their comments. To enhance clarity, we present the reviewers' comments in regular black font, while our responses are displayed in blue normal font. The modified content in both the manuscript and the Supporting Information is highlighted in red font.

Anonymous Referee #1

The manuscript by Fan et al. isolated low- and high- molecular weight HULIS from ambient samples collected at Anhui, China. The HULIS samples were measured for molecular size distribution, UV-VIS light absorption, fluorescence spectra, infrared absorption, and high-resolution mass spectra. Technical approaches of the sampling and chemical analysis procedures sound. Discussion of the data is occasionally speculative. The reviewer has a concern about the interpretation of the mass spectra, as detailed below.

The data shown in the manuscript would add some information to the existing literature of HULIS studies. The manuscript looks like a collection of data, rather than discussing novel findings in detail. The reviewer thinks that this manuscript may be more suitable to be considered as a measurement report, though the final decision should be made by the editor and authors.

[Response]: Thanks for the reviewer's suggestion. We have added some discussion on HULIS in the revised manuscript.

[Revise]: Lines 257-262, 326-340, 389-392, 436-444 in the revised manuscript:

“Many previous studies have demonstrated that fresh BB HULIS generally consist of small molecular-sized chromophores (Di Lorenzo et al., 2018; Di Lorenzo et al., 2017; Wong et al., 2017; Wong et al., 2019). However, a notable enhancement in the

formation of large molecular-sized chromophores has been found when they undergo intricate atmospheric processes (Di Lorenzo et al., 2018; Di Lorenzo et al., 2017; Wong et al., 2017; Wong et al., 2019).”

“These findings are consistent with the observation in our previous study that larger WSOM generally own higher MAE₃₆₅ but smaller AAE values than smaller WSOM (Fan et al., 2021). Generally, the results suggest that HMW HULIS exhibit stronger light-absorbing ability but with light absorption showing a weaker wavelength dependence. It is worth noting that combustion sources, such as BB and coal combustion, usually emit primary HULIS with high MAE₃₆₅ values due to the enrichment of poly-aromatic and unsaturated species (Cao et al., 2021; Fan et al., 2018; Huo et al., 2021; Zhang et al., 2021), but with small molecular weight distributions (Di Lorenzo et al., 2018; Di Lorenzo et al., 2017; Wong et al., 2017). Furthermore, subsequent pronounced photooxidation and photobleaching processes can lead to a enrichment and/or formation of large sized chromophores (Di Lorenzo et al., 2018; Di Lorenzo et al., 2017; Wong et al., 2017), concurrently resulting in a reduction in their absorption capacity and an enhancement of their spectra dependence on wavelength (Chen et al., 2021b; Fan et al., 2019; Wu et al., 2018; Wu et al., 2020; Zhang et al., 2022a).”

“It is worth noting that the ESI- HRMS could reveal molecular composition of a subset of organic molecules that are biased ionized in the negative ESI source rather than representing the entire HULIS composition (He et al., 2023; Lin et al., 2012).”

“It is noted that these values are considerably smaller than those revealed by HPSEC analysis. This difference could be explained by several factors: (1) ESI- HRMS is based towards relatively small molecules that easily protonated in negative ESI mode (He et al., 2023; Lin et al., 2012); (2) SEC can provide an apparent rather than accurate molecular size of molecules due to the lack of appropriate standards for column calibration (Fan et al., 2023; Wong et al., 2017), and (3) the potential disassembly of larger molecules stabilized by weak forces during electrospray ionization of HRMS (Fan et al., 2021; Phillips et al., 2017). Nevertheless, both HRMS and HPSEC indicate that summer HULISs exhibit larger sizes than winter HULISs.”

Major comment

The size-exclusion chromatograms demonstrated that the dominant portion of HULIS have molecular weights of larger than 1000 Da. On the other hand, the mass spectrometer (orbitrap, ESI negative mode) measured the mass ranges of $m/z = 60 \sim 900$. The authors showed the mass spectra for the range of m/z 100 ~ (approximately) 450. The reviewer is confused how to interpret the data. The reviewer only has the knowledge on analyzing relatively small molecules using the ESI mass spectrometers. The reviewer's understanding is that fragmentation of molecules is minimal in the ESI negative mode. If the idea is also applied to the current dataset, the mass spectra measured by the study would only cover a relatively small portion of HULIS. On the other hand, if the mass spectrometer were to be measuring most of components in HULIS due to fragmentation during ionization processes, the meanings of the whole discussion in section 3.3 is unclear. The reviewer suggests the authors to clarify this point in the revised manuscript, and update the descriptions in section 3.3 if necessary.

[Response]: Thanks for the reviewer's comment. The results obtained from size-exclusion chromatograms (SEC) indeed suggest that a substantial portion of HULIS have molecular weights larger than 1000 Da, while the ESI- high-resolution mass spectra (ESI-HRMS) revealed the major molecules within HULIS in the mass range of $m/z = 100\sim 450$. These results are not contradictory, primarily due to the distinct detection principles between SEC and HRMS, as well as their individual limitations.

For SEC, samples are primarily separated based on their molecular size as they pass through a stationary phase consisting of materials with varying pore sizes (Fan et al., 2023). Only molecules with a critical diameter smaller than the opening of the gel pores are allowed to enter (or be retained). Consequently, smaller solute molecules exhibit longer retention times and larger elution volumes, while larger solute molecules have shorter retention times and smaller elution volumes. Furthermore, the size of these molecules was calculated by the relationship between the retention time and the molecular weight of standard polymer molecules in solution (Wong et al.,

2017). However, due to the lack of proper standard compounds, the calculated molecular size of target samples is apparent rather than absolute (Fan et al., 2023; Wong et al., 2017).

In comparison to SEC separation processes, HRMS rarely provide signal for molecular weights above 900 Da because it is biased towards lower masses (He et al., 2023; Lin et al., 2012). In addition, HRMS with negative ESI mode is more biased towards molecules that are easily ionized by proton loss, including carboxyl, hydroxyl, carbonyl and other oxygen-containing group substances (He et al., 2023; Lin et al., 2012). Moreover, the high temperature of ESI ionization source can disrupt some intermolecular forces, leading to the detection of decomposition products of large molecules assembled with small molecules through such intermolecular forces (Fan et al., 2021; Phillips et al., 2017).

In total, the inconsistent results from SEC and HRMS might be explained by several points: (1) ESI- HRMS mainly focuses on relatively small molecules and is biased towards on those molecules easily protonated; (2) SEC can provide an apparent rather than accurate molecular size of molecules due to the lack of appropriate standards for column calibration, and (3) the potential disassembly of larger molecules stabilized by weak forces during electrospray ionization of HRMS. The distinct principles and limitations on SEC and HRMS have been added in the revised manuscript.

[Revise]: Lines 183-185, 389-392, 436-444 in the revised manuscript:

“It should be noted that the MW values estimated here are nominal rather than absolute due to the lack of appropriate standards for column calibration (Fan et al., 2023; Wong et al., 2017).”

“It is worth noting that the ESI- HRMS could reveal molecular composition of a subset of organic molecules that are biased ionized in the negative ESI source rather than representing the entire HULIS composition (He et al., 2023; Lin et al., 2012).”

“It is noted that these values are considerably smaller than those revealed by HPSEC analysis. This difference could be explained by several factors: (1) ESI- HRMS is based towards relatively small molecules that easily protonated in negative ESI mode

(He et al., 2023; Lin et al., 2012); (2) SEC can provide an apparent rather than accurate molecular size of molecules due to the lack of appropriate standards for column calibration (Fan et al., 2023; Wong et al., 2017), and (3) the potential disassembly of larger molecules stabilized by weak forces during electrospray ionization of HRMS (Fan et al., 2021; Phillips et al., 2017).”

[Reference]:

- (1) Fan, X., Cai, F., Xu, C., Yu, X., Wang, Y., Xiao, X., Ji, W., Cao, T., Song, J., Peng, P.a., 2021. Molecular weight-dependent abundance, absorption, and fluorescence characteristics of water-soluble organic matter in atmospheric aerosols. *Atmos. Environ.* 247.
- (2) Fan, X., Cheng, A., Chen, D., Cao, T., Ji, W., Song, J., Peng, P., 2023. Investigating the molecular weight distribution of atmospheric water-soluble brown carbon using high-performance size exclusion chromatography coupled with diode array and fluorescence detectors. *Chemosphere* 338, 139517.
- (3) He, T., Wu, Y., Wang, D., Cai, J., Song, J., Yu, Z., Zeng, X., Peng, P.a., 2023. Molecular compositions and optical properties of water-soluble brown carbon during the autumn and winter in Guangzhou, China. *Atmos. Environ.* 296, 119573.
- (4) Lin, P., Rincon, A.G., Kalberer, M., Yu, J.Z., 2012. Elemental composition of HULIS in the Pearl River Delta Region, China: results inferred from positive and negative electrospray high resolution mass spectrometric data. *Environ. Sci. Technol.* 46, 7454-7462.
- (5) Phillips, S.M., Bellcross, A.D., Smith, G.D., 2017. Light Absorption by Brown Carbon in the Southeastern United States is pH-dependent. *Environ. Sci. Technol.* 51, 6782-6790.

Minor comments

Abstract: ‘This observation suggests the possibility of small molecules assembling to form the HMW HULIS through intermolecular weak forces.’ To the best of my

knowledge, small molecules that are assembled by intermolecular forces are not called as high-molecular weight species. They are simply clusters or aggregates of molecules. If the authors interpretation were to be right, the reviewer is not sure if HMW HULIS should really be called as ‘HMW.’ This point is related with the above-mentioned major comment. The reviewer suggests the authors to clarify the point.

[Response]: Thanks for the reviewer’s comment. We concur with the fact that the assembly of small molecules through intermolecular forces can result in the formation of clusters or aggregates. However, it is crucial to clarify that the term of “HMW HULIS” utilized in this study represents an operational definition established through a combination of ultrafiltration (UF) and solid-phase extraction (SPE), rather than being solely based on its molecular morphology.

The UF-SPE isolation procedure for HMW HULIS is thoroughly detailed in our manuscript. In summary, we employed a stirred UF cell with a nominal-cutoff membrane of 1000 Da to isolate the water-soluble organic matters (WSOM). Consequently, the retentate solution was regarded as high-molecular-weight (HMW) WSOM, while the permeate solution was identified as low-molecular-weight (LMW) WSOM (Fan et al., 2021). Following this, both HMW and LMW WSOM fractions underwent SPE protocols, a well established method for purifying aerosol HULIS (Fan et al., 2012; Zou et al., 2020), resulting in the isolation of HMW and LMW HULIS, respectively. It is noteworthy that the term “HMW” has been consistently employed in our prior study on aerosol WSOM (Fan et al., 2021), and is prevalent in earlier research on dissolved organic matters in marine and river environments (Du et al., 2021; Simjouw et al., 2005; Yang et al., 2021).

We appreciate the opportunity to clarify the operational nature of our definition and highlight its alignment with established terminology in the broader scientific literature.

[Reference]:

(1) Du, H., Cao, Y., Li, Z., Li, L., Xu, H., 2021. Formation and mechanisms of

hydroxyl radicals during the oxygenation of sediments in Lake Poyang, China. *Water Res.* 202, 117442.

- (2) Fan, X.J., Song, J.Z., Peng, P.A., 2012. Comparison of isolation and quantification methods to measure humic-like substances (HULIS) in atmospheric particles. *Atmos. Environ.* 60, 366-374.
- (3) Fan, X., Cai, F., Xu, C., Yu, X., Wang, Y., Xiao, X., Ji, W., Cao, T., Song, J., Peng, P.a., 2021. Molecular weight-dependent abundance, absorption, and fluorescence characteristics of water-soluble organic matter in atmospheric aerosols. *Atmos. Environ.* 247.
- (4) Simjouw, J.-P., Minor, E.C., Mopper, K., 2005. Isolation and characterization of estuarine dissolved organic matter: Comparison of ultrafiltration and C18 solid-phase extraction techniques. *Mar. Chem.* 96, 219-235.
- (5) Yang, K., Zhang, Y., Dong, Y., Li, D., Li, W., 2021. Metal binding by dissolved organic matter in hypersaline water: A size fractionation study using different isolation methods. *Limnologia* 87, 125849.
- (6) Zou, C., Li, M., Cao, T., Zhu, M., Fan, X., Peng, S., Song, J., Jiang, B., Jia, W., Yu, C., Song, H., Yu, Z., Li, J., Zhang, G., Peng, P.a., 2020. Comparison of solid phase extraction methods for the measurement of humic-like substances (HULIS) in atmospheric particles. *Atmos. Environ.* 225, 117370.

L87 Previous studies have shown that aerosol WSOM, also known as brown carbon... WSOM and BrC are obviously different concepts. If WSOM and BrC are really considered as equal, please cite references to support the statement.

[Response]: Thanks for the reviewer's comment. It's acknowledged that WSOM and BrC are distinct concepts. The WSOM encompass various light-absorbing organic fractions. For the sake of studying complex BrC fractions, WSOM is often regarded as a surrogate for water-soluble brown carbon in comprehensive analyses. Consequently, the relevant statement has been revised in the manuscript.

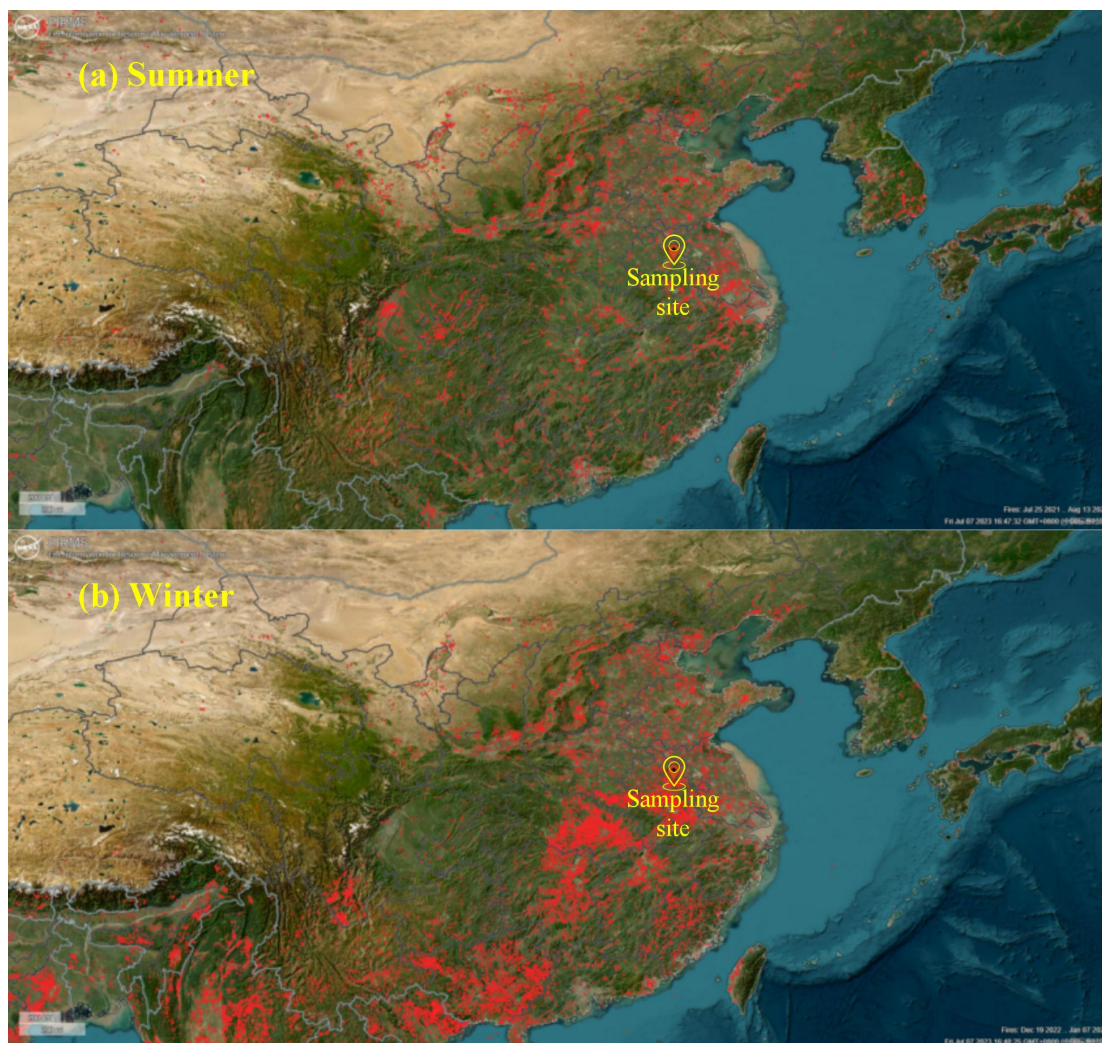
[Revise]: Line 89 in the revised manuscript:

“Previous studies have shown that aerosol WSOM, often seen as water-soluble brown

carbon (BrC), are comprised of a continuum of molecular weight (MW) species, as revealed by high-performance exclusion chromatography (HPSEC) analysis”

Figure S1: It would be better to show the location of the observation site in the map.

[Response]: Thanks for the reviewer’s suggestion. The sampling site has been labeled in Figure S1. The modified Figure S1 was shown as follow.



L258 ‘Therefore, the higher proportions of large-size chromophores and resulting larger apparent molecular size of HMW HULIS may indicate their possible secondary formation nature.’ It is not clear how this statement could be supported by discussion in other parts of the manuscript. The statement seems to be a hypothesis, rather than what the authors can convincingly tell from their results.

[Response]: Thanks for the reviewer’s comment. The relevant statements have been

modified in the revised manuscript.

[Revise]: Lines 257-265 in the revised manuscript:

“Many previous studies have demonstrated that fresh BB HULIS generally consist of small molecular-sized chromophores (Di Lorenzo et al., 2018; Di Lorenzo et al., 2017; Wong et al., 2017; Wong et al., 2019). However, a notable enhancement in the formation of large molecular-sized chromophores has been found when they undergo intricate atmospheric processes (Di Lorenzo et al., 2018; Di Lorenzo et al., 2017; Wong et al., 2017; Wong et al., 2019). Based on these limited studies, it is suggested that HMW HULIS, characterized by higher proportions of large-size chromophores and the resulting larger apparent molecular size, might be associated with the products from atmospheric aging process rather than being emitted directly by primary sources.”

L330 ‘Therefore, it can be speculated that LMW HULIS are more susceptible to enrich the by-products resulting from the degradation and oxidation of BrC during processes like photooxidation and photobleaching.’ Are there any other possibilities in differences (e.g., emission sources)? The reviewer suggests the authors to discuss potential sources and atmospheric processes in more detail.

[Response]: Thanks for the reviewer’s comment. As indicated by previous studies, combustion sources, such as biomass burning and coal combustion, can generate amounts of primary HULIS with high MAE₃₆₅ but with small molecular weight distributions (Di Lorenzo et al., 2018; Fan et al., 2018; Wong et al., 2017). Further complex atmospheric aging processes (e.g., photooxidation and photobleaching) lead to a enrichment and/or formation of large sized chromophores (Di Lorenzo et al., 2017; Wong et al., 2017), concurrently resulting in a reduction in MAE₃₆₅ and a increase of AAE for these primary HULIS (Chen et al., 2021; Fan et al., 2019). From this perspective, the secondary organic aerosol formation might induce the generation of HMW HULIS with lower light absorption capacity and weaker light absorption wavelength dependence. In contrast, LMW HULIS is more likely to represent small-sized primary HULIS and/or by-products from degradation and oxidation of

primary large-sized HULIS. Considering the complex sources of ambient HULIS, future studies should explore the MW-dependent light absorption characteristics of HULIS from different sources.

[Revise]: Lines 330-346 in the revised manuscript:

“It is worth noting that combustion sources, such as BB and coal combustion, usually emit primary HULIS with high MAE₃₆₅ values due to the enrichment of poly-aromatic and unsaturated species (Cao et al., 2021; Fan et al., 2018; Huo et al., 2021; Zhang et al., 2021), but with small molecular weight distributions (Di Lorenzo et al., 2018; Di Lorenzo et al., 2017; Wong et al., 2017). Furthermore, subsequent pronounced photooxidation and photobleaching processes can lead to an enrichment and/or formation of large sized chromophores (Di Lorenzo et al., 2018; Di Lorenzo et al., 2017; Wong et al., 2017), concurrently resulting in a reduction in their absorption capacity and an enhancement of their spectra dependence on wavelength (Chen et al., 2021b; Fan et al., 2019; Wu et al., 2018; Wu et al., 2020; Zhang et al., 2022a). From this perspective, the SOA formation might induce the generation of HMW HULIS with lower light absorption capacity and weaker light absorption wavelength dependence. In contrast, LMW HULIS is more likely to represent small-sized primary HULIS and/or by-products resulting from the degradation and oxidation of primary large-sized HULIS. Considering the complex sources of ambient HULIS, future studies should explore the MW-dependent light absorption characteristics of HULIS from different sources.”

[Reference]

- (1) Chen, Q., Hua, X., Dyussenova, A., 2021. Evolution of the chromophore aerosols and its driving factors in summertime Xi'an, Northwest China. *Chemosphere* 281, 130838.
- (2) Di Lorenzo, R.A., Place, B.K., VandenBoer, T.C., Young, C.J., 2018. Composition of Size-Resolved Aged Boreal Fire Aerosols: Brown Carbon, Biomass Burning Tracers, and Reduced Nitrogen. *ACS Earth and Space*

Chemistry 2, 278-285.

- (3) Di Lorenzo, R.A., Washenfelder, R.A., Attwood, A.R., Guo, H., Xu, L., Ng, N.L., Weber, R.J., Baumann, K., Edgerton, E., Young, C.J., 2017. Molecular-Size-Separated Brown Carbon Absorption for Biomass-Burning Aerosol at Multiple Field Sites. *Environ. Sci. Technol.* 51, 3128-3137.
- (4) Fan, X., Li, M., Cao, T., Cheng, C., Li, F., Xie, Y., Wei, S., Song, J., Peng, P.a., 2018. Optical properties and oxidative potential of water- and alkaline-soluble brown carbon in smoke particles emitted from laboratory simulated biomass burning. *Atmos. Environ.* 194, 48-57.
- (5) Fan, X., Yu, X., Wang, Y., Xiao, X., Li, F., Xie, Y., Wei, S., Song, J., Peng, P.a., 2019. The aging behaviors of chromophoric biomass burning brown carbon during dark aqueous hydroxyl radical oxidation processes in laboratory studies. *Atmos. Environ.* 205, 9-18.
- (6) Wong, J.P.S., Nenes, A., Weber, R.J., 2017. Changes in Light Absorptivity of Molecular Weight Separated Brown Carbon Due to Photolytic Aging. *Environ. Sci. Technol.* 51, 8414-8421.

L338 'humic-like fluorophores'. I am confused about this statement. All the fluorophores in humic-like substances could be called as humic-like fluorophores.

[Response]: Thanks for the reviewer's suggestion. Humic-like substances (HULIS) are atmospheric compounds resembling humic and fulvic acids found in terrestrial and aquatic systems (Graber and Rudich, 2006; Win et al., 2018). They share many functional groups, such as poly-carboxylates, carbonyls, phenols, quinones, aliphatics, and aromatics (Kumar et al., 2017; Zou et al., 2020). In practice, HULIS are typically isolated from water-soluble organic compounds (WSOM) in ambient aerosols through solid-phase extraction, making them more of an operational definition (Fan et al., 2012; Zou et al., 2020). Nevertheless, HULIS comprise complex organic compounds rather than pure humic matters.

Excitation-emission matrix (EEM) spectroscopy can provide structural information about chromophores and has been widely applied to identify the sources and chemical

nature of WSOM, including HULIS (Cao et al., 2023; Chen et al., 2016;). It is important to note that fluorescence is a radiative process occurring between two energy levels of the same multiplicity (Andrade-Eiroa et al., 2013). Generally, compounds with rigid planar structures and highly conjugated systems exhibit intrinsic fluorescence emission characteristics. Consequently, the complex composition in HULIS results in different fluorescence region (Ex/Em) in EEM spectra. The chromophores in fluorescence spectra can be considered a “fingerprinting” tool, with distinct regions representing different components. According to many previous studies (Chen et al., 2003; Qin et al., 2018), six fluorescence regions can be identified and assigned, as detailed in the following table:

	Ex (nm)	Em (nm)	Assignment
I	200–250	290–330	Aromatic proteins I
II	200–250	330–380	Aromatic proteins II
III	200–250	380–520	Fulvic acid-like substances
IV	250–400	290–380	Soluble microbial byproducts
V	250–400	380–520	Humic acid-like substances

In general, pronounced fluorescence peaks at Ex/Em = 210-235/395-410 nm could indicate a predominance of humic-like fluorophores, consistent with findings in many previous studies (Cao et al., 2023; Chen et al., 2016; Qin et al., 2018).

[Reference]

- (1) Andrade-Eiroa, Á., Canle, M., Cerdá, V., 2013. Environmental Applications of Excitation-Emission Spectrofluorimetry: An In-Depth Review I. Applied Spectroscopy Reviews 48, 1-49.
- (2) Cao, T., Li, M., Xu, C., Song, J., Fan, X., Li, J., Jia, W., Peng, P., 2023. Technical note: Chemical composition and source identification of fluorescent components in atmospheric water-soluble brown carbon by excitation – emission matrix spectroscopy with parallel factor analysis – potential limitations and applications. Atmos. Chem. Phys. 23, 2613-2625.

- (3) Chen, Q., Ikemori, F., Higo, H., Asakawa, D., Mochida, M., 2016. Chemical Structural Characteristics of HULIS and Other Fractionated Organic Matter in Urban Aerosols: Results from Mass Spectral and FT-IR Analysis. *Environ. Sci. Technol.* 50, 1721-1730.
- (4) Chen, W., Westerhoff, P., Leenheer, J.A., Booksh, K., 2003. Fluorescence Excitation-Emission Matrix Regional Integration to Quantify Spectra for Dissolved Organic Matter. *Environ. Sci. Technol.* 37, 5701-5710.
- (5) Fan, X.J., Song, J.Z., Peng, P.A., 2012. Comparison of isolation and quantification methods to measure humic-like substances (HULIS) in atmospheric particles. *Atmos. Environ.* 60, 366-374.
- (6) Graber, E.R., Rudich, Y., 2006. Atmospheric HULIS: How humic-like are they? A comprehensive and critical review. *Atmos. Chem. Phys.* 6, 729-753.
- (7) Kumar, V., Goel, A., Rajput, P., 2017. Compositional and surface characterization of HULIS by UV-Vis, FTIR, NMR and XPS: Wintertime study in Northern India. *Atmos. Environ.* 164, 468-475.
- (8) Qin, J., Zhang, L., Zhou, X., Duan, J., Mu, S., Xiao, K., Hu, J., Tan, J., 2018. Fluorescence fingerprinting properties for exploring water-soluble organic compounds in PM 2.5 in an industrial city of northwest China. *Atmos. Environ.* 184, 203-211.
- (9) Win, M.S., Tian, Z., Zhao, H., Xiao, K., Peng, J., Shang, Y., Wu, M., Xiu, G., Lu, S., Yonemochi, S., Wang, Q., 2018. Atmospheric HULIS and its ability to mediate the reactive oxygen species (ROS): A review. *J Environ Sci (China)* 71, 13-31.
- (10) Zou, C., Li, M., Cao, T., Zhu, M., Fan, X., Peng, S., Song, J., Jiang, B., Jia, W., Yu, C., Song, H., Yu, Z., Li, J., Zhang, G., Peng, P.a., 2020. Comparison of solid phase extraction methods for the measurement of humic-like substances (HULIS) in atmospheric particles. *Atmos. Environ.* 225, 117370.