ASYMMETRIC FLOW FIELD FLOW FRACTIONATION WITH LIGHT SCATTERING DETECTION – AN ORTHOGONAL SENSITIVITY ANALYSIS

*Anne A. Galyean^{α^}, James J. Filliben^β, R. David Holbrook^γ, Wyatt N. Vreeland^δ, and Howard S.
Weinberg^{α*}*

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ABSTRACT

Asymmetric flow field flow fractionation (AF⁴) has several instrumental factors that may have a direct effect on separation performance. A sensitivity analysis was applied to ascertain the relative importance of AF⁴ primary instrument factor settings for the separation of a complex environmental sample. The analysis evaluated the impact of instrumental factors namely, cross flow, ramp time, focus flow, injection volume, and run buffer concentration on the multi-angle light scattering measurement of natural organic matter (NOM) molar mass (MM). A 2⁽⁵⁻¹⁾ orthogonal fractional factorial design was used to minimize analysis time while preserving the accuracy and robustness in the determination of the main effects and interactions between any two instrumental factors. By assuming that separations resulting in smaller MM measurements would be more accurate, the analysis produced a ranked list of effects estimates for factors and interactions of factors based on their relative importance in minimizing the MM. The most important and statistically significant AF⁴ instrumental factors were buffer concentration and cross flow. The least important was ramp time. A parallel 2⁽⁵⁻²⁾ orthogonal fractional factorial design was also employed on five environmental factors for synthetic natural water samples containing silver nanoparticles (NPs), namely: NP concentration, NP size, NOM concentration, specific conductance, and pH. None of the water quality characteristic effects or interactions were found to be significant in minimizing the measured MM; however, the interaction between NP concentration and NP size was an important effect when considering NOM recovery. This work presents a structured approach for the rigorous assessment of AF⁴ instrument factors and optimal settings for the separation of complex samples utilizing efficient orthogonal fractional factorial design and appropriate graphical analysis.

1. INTRODUCTION

Field flow fractionation, and specifically asymmetric flow field flow fractionation (AF⁴) has become one of the most favored and common methods for separating target analytes in complex matrices including those of environmental aquatic origin. Various online detection methods, such as multi-angle light scattering (MALS), quasi-elastic light scattering (QELS), inductively coupled plasma – mass spectrometry (ICP-MS), or Ultraviolet-Visible spectrophotometry (UV-Vis) attached downstream of the AF⁴ system detect and characterize the target analytes. However, there appears to be no comprehensive understanding of the relationship between various environmental and instrumental factors and how they affect the ability of AF⁴ to separate analytes in complex systems prior to characterization.

AF⁴ is a chromatography-like technique that does not require the use of a stationary phase but instead relies on hydrodynamic principles to separate a wide size range of particles by the ratio of Stokes drag to translational diffusion coefficient. AF⁴ has been described elsewhere in detail [1-4]. Most notably, it can be used to separate complex samples that may damage, or be damaged by, typical chromatography columns [5, 6]. AF⁴ is compatible with a wide range of matrix compositions and is capable of rapid on-line pre-concentration, which is particularly useful for highly complex matrices containing analytes of low concentration, including engineered nanoparticles (ENPs) in environmental samples [7, 8]. **Fig. 1** presents a schematic of a typical AF⁴ separation whose mechanism causes small particles to move through the channel more rapidly than larger particles.

Little is known about the separation of particles suspended in complex, non-reproducible matrices containing a continuum of similar species that differ from each other by an infinitesimal amount, as is encountered in environmental samples. Natural organic matter (NOM) is a particularly complex environmental material containing supramolecular assemblies of a wide range of molecular masses and chemical properties and is the major source of organic carbon in natural waters [9].

While the optimization of AF⁴ separation parameters based on instrument theory and fractogram observation has been extensively studied [12-18], it is important to consider that a major difficulty in comparing or combining conclusions of ENP interactions in the environment is the lack of standardization. Therefore, any optimization conclusions directly depend on the instrument as well as the sample

characteristics, all of which vary between laboratories and their methodologies. Further, it is very important that the technique itself does not impart changes to the sample. Finally, a significant challenge to using optimization schemes for developing methods for separating ENPs in complex natural systems is the limited understanding of the relationship between various instrumental operational parameters and sample characteristics and how they affect the ability of AF⁴ to separate nanoparticles.

We investigated AF⁴ optimization using a sensitivity analysis by applying statistical design of experiment (DEX) in concert with appropriate exploratory data analysis (EDA). This approach aims to better understand how the AF⁴ separation output is affected by changing inputs, specifically AF⁴ instrument parameters. Compared to trial-and-error method development, DEX is a cost-effective and systematic approach to system optimization, using both instrument knowledge and experimental data to determine the effects of multiple factors and factor interactions on a given response. In the past, Kuklenyik and co-workers [10] used multivariate DEX to optimize AF⁴ instrumental parameters for maximum channel recovery and size resolution of lipoproteins in human serum. Racamonde and co-workers [11] demonstrated the applicability of DEX analysis for environmental samples by using a mixed factorial design to identify significant parameters affecting the fabric-phase sorptive extraction of pollutants in natural water samples.

There are several commonly used experimental designs for sensitivity analysis, which aim to identify which experimental factors are most important (in this case, AF⁴ instrumental factors), relative to the selected response output. The traditional approach is “one-factor-at-a-time” (1FAT), where all k factors are fixed and runs are made in which each factor is changed successively to another value. Though simple, logical, and inexpensive, the 1FAT design approach yields biased effect estimates and fails to correctly estimate potential factor interactions. In the case of a dynamic system, such as complex environmental sample analysis where the response surface might contain significant peaks or valleys, the bias can be particularly extreme as some of these topographical features may be missed without multiple factors being adjusted together [19-21]. A randomization Monte Carlo design randomly selects a combination of factors and settings from the complete population. Due to random sampling, the resulting conclusions will be unbiased; however, the necessary sample size must be very large to achieve a desired precision [19].

In this work, an orthogonal fractional factorial design was identified as a highly efficient and robust alternative. A “two-level” design was used so that two settings were explored for each factor. The terminology from the statistics and analytical chemistry communities are both used to represent the same entities – specifically, primary and robustness factors. Statisticians use the term, “primary factors,” to describe independent variables in the experiment that can be directly controlled or changed over a particular range and are what the experimentalist would call “instrument settings” (e.g. AF⁴ cross flow or injection volume). In contrast, statisticians refer to “robustness factors” as experimental conditions that are determined by the inherent nature of the sample itself and are, in general, not controllable in the experiment - what an experimentalist would call, “sample characteristics” (e.g. the pH or composition of an environmental sample, or the aggregation state of ENPs).

There are many primary and robustness factors that can affect a chosen AF⁴ system output, each with varying degrees of importance and with a continuum of settings, depending on optimization goals. However, the objective of this work is to demonstrate the use of a statistical sensitivity analysis with both primary and robustness factors towards the separation of complex environmental samples. An initial screening study was performed here using a fractional factorial design and select factors. In contrast to traditional 1FAT and Monte Carlo approaches, the novelty of this work is that this method is a statistically rigorous way to assess the effects of any and all experimentally relevant parameters and results in graphical data that can be efficiently evaluated. Therefore, the overall objective of this work was to validate the use of this approach by reaching conclusions that are in agreement with the AF⁴ literature as determined by the less-efficient and statistically bias 1FAT optimization method traditionally employed, specifically, that cross-flow rate and mobile phase composition are important factors in successful AF⁴ separation. Although this work represents only a subset of the possible primary and robustness factors relevant to complex environmental samples, the application of this approach to a more rigorous full optimization using all relevant factors would result in fine-tuned conclusions that are unique to each individual sample type. With the correct design, the factorial dependencies of a complex system can be determined, subsequent conclusions can be validated, and the scientific experimental cost can be minimized [19, 22].

In order to demonstrate the applicability of this approach to complex systems, the AF⁴-MALS optimization methodology was applied towards the separation of ENPs in laboratory-prepared water samples that approximate environmental waters. The development of robust analytical characterization methods to investigate the occurrence and fate of ENPs in the environment is one of the highest priority goals for the ecologically responsible use of nanomaterials [23]. These methods have been difficult to achieve as they require a combination of analytical techniques in order to characterize target analyte behavior in complex environmental matrices [24, 25]. Complex samples such as natural waters yield fractograms that are less defined and contain a higher level of variability [26-28] when compared to the separations of mixtures of monodisperse or distinct particle sizes. Loeschner and co-workers [16] identified that run-buffer composition, membrane type, cross flow rates, and channel spacer thickness all significantly influenced nanoparticle recoveries and retention times in simple aqueous suspensions. However, to our knowledge, there have been no attempts to explore the relationships among primary and robustness factors and AF⁴ separation of ENPs in natural waters. In a complex environmental sample, baseline resolution between distinct particle species is unlikely. Further, when a broad size distribution of particles is inadequately separated and sized by optical techniques, the highly non-linear response of light scattering causes an over representation of the larger particles in the measured sample (light scattering intensity scales with particle radius to the 6th power [29-32]). Identifying a separation protocol that is of sufficient selectivity allows for both the large and small particles to be detected separately, resulting in an overall lower average measurement and yielding the best possible separation considering the sample polydispersity and instruments' operational limits [33].

The most desired target response, or output, for this study is defined by minimizing the average molar mass (MM) of NOM in the sample following AF⁴ separation. In this paper, MM is measured inline by MALS and UV-Vis concentration detection. Other AF⁴ system outputs include analyte recoveries and retention times [16].

In the case of environmentally relevant concentrations of ENPs, it is unlikely that they would be present above the detection limits for MALS and UV-Vis. However, since ENPs have been shown to interact with NOM in environmental samples [9, 34], this work assumes that adequately separating NOM

will also result in effective separation of any ENPs present, though additional detectors suitable for characterizing ENPs would be required for future quantitative studies (e.g. ICP-MS).

Use of a controlled synthetic matrix that simulates natural surface waters rather than using a naturally sourced surface water, allows for evaluating a wide range of water characteristics. Similarly, silver nanoparticles (AgNPs) were selected as an additional water sample analyte to ensure that the methodology developed might be applicable for use in occurrence and quantification studies of ENPs in natural waters. AgNPs are an environmentally relevant target ENP because silver is the most abundantly incorporated material in consumer products containing ENPs [35] and AgNPs have at least one transformation product with observed persistence in natural waters [36, 37]. Therefore, these samples serve as a timely and sufficiently complex example to demonstrate this AF⁴-MALS optimization approach.

An orthogonal fractional factorial design is applied towards the sensitivity analysis of AF⁴ separations of synthetic, environmentally relevant mixtures containing AgNPs to explore which primary factors and interactions have the greatest effect on separation. To broaden the scope of the instrumental factor conclusions, a parallel analysis of both primary and robustness factors is explored. This is the first application of orthogonal fractional factorial design and sensitivity analysis to AF⁴ separations of complex environmental samples. Using this sensitivity analysis approach, the AF⁴ system will be better understood, with respect to environmental sample characteristics, and the ranked importance and robustness of separation parameters can be identified.

2. MATERIALS AND METHODS¹

AF⁴ separation was performed using a Wyatt Technologies Corp. (Santa Barbara, CA, USA) Eclipse DualTec module whose separation protocol settings were controlled with OpenLab CDS Chem Station edition software (Agilent Technologies, Santa Clara, CA, USA). Separation was performed using a Wyatt Technologies short channel containing a regenerated cellulose membrane with a 10-kDa molecular weight cutoff and a detector flow of 1 mL min⁻¹. The short channel dimensions were 21 mm

¹ Certain commercial equipment, instruments, or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

width at the inlet, 2.5 mm width at the outlet, nominally 250 μM in height, and 20 cm in length. Sample injections were made with an Agilent (Santa Clara, CA, USA) 1260 Infinity series isopump and autosampler. The AF⁴ run buffer was prepared by dissolving ammonium nitrate (ACS Reagent $\geq 98\%$, Sigma-Aldrich, St. Louis, MO, USA) in laboratory-grade 18.2 M Ω -cm water (LGW) with 0.01% (w/v) sodium azide (Ricca Chemical Company, Arlington, TX, USA) as an antimicrobial. The concentration of sodium azide remained constant for each of the three ammonium nitrate concentrations (Table 1), or “run buffer composition” levels. Run buffer used for all experiments at target concentrations was degassed with a Gastorr TG-14 unit (FLOM USA, San Diego, CA, USA) at 100 hPa directly from solvent bottles, and then filtered first by a polytetrafluoroethylene frit (RESTEK Corp., Bellefonte, PA, USA) and then a 0.1 μm pore Durapore membrane filter (Millipore Inc., Billerica, MA, USA). Focus flow and cross flow were varied based on the experimental design. Bovine serum albumin (Fraction V, Sigma-Aldrich, St. Louis, MO, USA) at 1 mg mL⁻¹ was used as an internal standard reference periodically throughout the study to assess instrument drift and to ensure accuracy of the MALS detector. Water samples were analyzed from 1.5 mL amber glass autosampler vials with PTFE/silicone septa caps (Chemglass Life Sciences, Vineland, NJ, USA) that were rinsed with LGW before use. Samples were analyzed immediately by AF⁴-MALS-UV and randomized to reveal any effects of variable wait times prior to analysis or of sample hold-up from prior runs.

To assess the quality of a given separation relative to another, average MM of the NOM sample distribution was calculated for each separation protocol using standard online MALS techniques [29]. UV absorbance measurements were made at 254 nm (hereafter referred to as UV₂₅₄), a wavelength specific to NOM (with no interference from AgNPs) using an online Agilent 1260 Infinity diode array detector (model # LV G1315D) following AF⁴. MALS and UV data were collected and analyzed with ASTRA Software version 6.1.1.17. MALS data were plotted with a Debye formalism and fit with a 2nd order polynomial. The details of how ASTRA Software calculates the weight average MM (MM_w) by relating excess scattering intensity to the NOM concentration from UV absorbance measurements are described in the literature [32]. Although the Suwannee River NOM standard MM was not reported by the manufacturer, the accuracy of MM measurements via light scattering is directly dependent on accuracy of knowledge of optical properties of the NOM (i.e. variability in the refractive index (RI)). In this work, the

absolute MM measurement was not as important as the relative MM measurements between separation protocols, and therefore the differential refractive index of NOM was estimated to be $dn/dc = 0.151 \text{ g mL}^{-1}$, based on measurements for NOM in phosphate buffers from the literature [30, 31].

Specific conductance measurements were made with a Wyatt Technologies Möbiuζ instrument using Dynamics Software version 7.2.4. EDA plots were generated with the NIST-developed analysis software, DATAPLOT [38]. The factors chosen for this sensitivity analysis are listed in **Table 1**.

Water samples, as well as their DEX factors and levels, were designed according to U.S. average freshwater measurements [39] as a guide for matrix composition. For example, the conductivities of Little Rock Lake, Vilas, CO; Harriet Lake, Minneapolis, MN; and Colorado River, AZ. were used as the basis for low (0.22 mS cm^{-1}), medium (0.40 mS cm^{-1}), and high (0.59 mS cm^{-1}) ionic strength waters, respectively [39]. Hence, waters of corresponding character were prepared in LGW at concentrations of $1029 \mu\text{eq L}^{-1}$, $2992 \mu\text{eq L}^{-1}$, and $4781 \mu\text{eq L}^{-1}$, respectively, from a combination of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NaHCO_3 , KHCO_3 , MgCl_2 , CaCl_2 , CaCO_3 , and NaCl (all analytical grade; Sigma-Aldrich, St. Louis, MO, USA), as described in the Supplementary Information (**Table S.1**). These solutions were allowed to equilibrate for 24 hours before adding a Suwannee River NOM (International Humics Substances Society, St. Paul, MN, USA) solution, prepared in LGW pH-adjusted to 6, 7, or 8 by HCl (1 mol L^{-1} , Sigma Life Sciences, St. Louis, MO, USA) or NaOH (1 mol L^{-1} , Fluka Analytical, St. Louis, MO, USA) according to sample requirements, and allowed to equilibrate for an additional 24 hours. Organic carbon in NOM stock solutions was quantified as dissolved organic carbon (DOC) concentration [40] after filtration through $0.45 \mu\text{m}$ pore size nylon membranes (EMD Millipore, Billerica, MA, USA). UV_{254} absorbance measurements (Nanodrop, Thermo Scientific, Wilmington, DE, USA) were then used to estimate NOM concentration for sample preparation from a calibration of absorbance versus NOM concentration in a series of diluted NOM solutions. This estimate was not used for MM calculations but only for estimating the NOM in the sample as prepared and before AF^4 -MALS analysis. NanoXact citrate-stabilized AgNPs were diluted from a manufacturer supplied 0.02 mg mL^{-1} stock in 2 mmol L^{-1} sodium citrate solution (Nanocomposix, San Diego, CA, USA) and were introduced to each sample 1 to 12 h prior to AF^4 separation, depending on sample analysis randomization. Stock AgNP solution stability was monitored with UV-Vis spectrophotometry at 400 nm. AgNP size range was confirmed by quasi-elastic light scattering (QELS) with a WyattQELS™ detector at

a nominal angle of 140° , and was in good agreement with that reported by the manufacturer [41]. All solutions and NOM samples were stored in the dark at 4°C prior to analysis and held no longer than one week or 48 hours, respectively.

2.1 Experimental Design Methodology

Statistical design of experiment (DEX) methodology is a rigorous problem-solving approach to gaining detailed insight on the characteristics of the complex and unknown response surface of a given system containing a number of defined factors, k . With the correct design, factorial dependencies of this system can be determined; the subsequent conclusions can be robustly validated, while simultaneously minimizing analysis time [19, 22]. A full factorial two-level design to test every combination of the primary and robustness factors would have resulted in an untenable 43 days of continuous instrument operation. Therefore, a two-level orthogonal fractional factorial design ($2^{5-1} \times 2^{5-2}$) was identified as a highly efficient and robust alternative. The settings for each individual separation protocol (primary factors) and sample (robustness factors) are given in the Supplementary Information (**Tables S.2 and S.3**, respectively). The design details and a discussion of balancing experimental rigor with analysis time are presented in the Supplementary Information (**Discussion S1**).

In particular, sensitivity analysis involves varying input parameter settings and assessing the subsequent changes in selected response variables in order to identify which input parameters have the most significant influence on response behavior [42]. The primary sensitivity analysis results in a ranked list of factors (and interactions), according to least-squares-based estimation of their effects [19]. To reinforce and to illustrate the degree of confidence in the sensitivity analysis conclusions, a heavily statistical graphics-intensive EDA approach was applied.

The number of factors, k , and the reasonable/affordable number of sample runs, n , are the key pair of values that quantitatively describe a chosen experimental design. The class of fractional designs defined as “orthogonal”, balanced in every factor and every pair of factors, have known superior qualities in that they adequately sample the k dimensions, reduce the bias in the factor estimates, and reduce the variation in the effect estimates [43]. Orthogonal fractional factorial designs for both the primary

instrument factors and the robustness environmental factors are referred to as Taguchi parameter designs with “inner and outer arrays” [44] and were used in the present work.

3. RESULTS AND DISCUSSION

3.1 Analysis of primary and robustness factors on measured molar mass

Of the various factor design combinations considered in the design, the “most optimal” settings which yield the smallest mean response, (*i.e.* the smallest measured MM) are (- + - -), where (-) represents “low” and (+) represents “high” for each of the five factors described in **Table 1**. This corresponds to low cross flow = 0.5 mL min⁻¹, high ramp time = 40 minutes, low focus flow = 1 mL min⁻¹, low injection volume = 200 µL, and low buffer concentration = 0.1 mmol L⁻¹ NH₄NO₃. In contrast, the “least optimal” settings which yield the largest mean response (*i.e.* the largest measured MM) are (+ - + + +), corresponding to high cross flow = 2.5 mL min⁻¹, short ramp time = 20 minutes, high focus flow = 3 mL min⁻¹, high injection volume = 500 µL, and high buffer concentration = 5 mmol L⁻¹ NH₄NO₃. **Fig. 2** shows the MALS fractogram and UV₂₅₄ response for both the most optimal (**Fig. 2a**) and least optimal (**Fig. 2b**) factor settings according to the results of the statistical sensitivity analysis.

The most optimal AF⁴ instrument settings yielded a fractogram (**Fig. 2a**) showing the majority of organic material, as represented by UV₂₅₄ absorbance, eluting near the beginning of the elution period as small NOM particles or aggregates. In the plot representing the least optimal settings (**Fig. 2b**), most material detected by MALS and absorbing at UV₂₅₄ is co-eluting at the end of the separation, suggesting larger material. It is important to note that light scattering detects all material in the sample with a refractive index different from the buffer, regardless of its chemical composition, in contrast to UV₂₅₄ absorbance, which in this system, is specific to NOM. The samples in this study have two major components: NOM and AgNPs. It is unlikely that individual AgNPs are contributing any significant light scattering signal since the concentration of AgNP is on the order of 0.01% of the total mass of NOM in each sample. For investigations into AgNP fate and transport in the presence of NOM, an additional detector, such as ICP-MS, would need to be interfaced with AF⁴ in order to adequately monitor AgNP elution.

The main effects plot [45] for primary factors, presented in **Fig. 3**, is a series of k mean plots (associated with k factors), all on the same scale, so each factor can be compared for relative importance. The effects (in this case, the mean MM measured under a specific combination of factors and settings) are the result of adjusting each primary factor from low to high. Buffer concentration and cross flow are the factors considered statistically significant ($p < 0.05$ for a one-way ANOVA F-test). The ranked order of factor importance, according to decreasing magnitude of effect, is buffer concentration, cross flow, focus flow, injection volume and ramp time. The orthogonality of the underlying experimental design ensures that the main effects' estimates are identical to the least-squares effects' estimates. One potential outlier was identified (not shown; primary factor settings (+--+)) corresponding to high cross flow, low ramp time, high focus flow, high injection volume, and low buffer concentration) which would have greatly reduced the importance of buffer concentration while simultaneously increasing the importance of focus flow and injection volume beyond that of buffer concentration. Since this work models the MM response over a very large experimental space with only high, mid, and low factor settings, it is possible that the MM behavior at the edge of the parameter space no longer reflects the behavior either over the broad majority of the space, or, over the region of smaller MM that is of most interest. While the outlier occurred for all replicates of that condition, its inclusion in the model skews it inappropriately, based on experimental and instrumental experience, in the region of most interest. It is unknown why this outlier occurred and how this particular combination of settings caused consistently spurious results. Therefore, in order to retain experimental design balance, the response to this design setting was replaced with the global average for all mean MM values with the assumption that this would minimize any bias of local means.

A main effects plot using response means for the five robustness factors (*i.e.* the varied sample characteristics) is shown in **Fig. 4** and reveals the ranked order of factor importance as AgNP concentration, AgNP size, NOM concentration, ionic strength, and finally, pH. This main effects plot provides insight into the relative importance of robustness factors (sample characteristics), independent of primary factors (instrument settings), and agrees with the primary factor conclusions. The robustness factors effects, as measured by the mean MM range, are all very small compared to those of the primary factors, and none were identified as significant by the ANOVA F-test ($p > 0.05$). This suggests that the primary factor settings are valid across the range of water quality characteristics explored. Although not

shown, the robustness factor main effects were also explored using medians, rather than means, as the estimators for a “typical” response of a factor at a certain level. An analysis using medians is useful for confirming effects conclusions because they are less affected by outliers. The median main effects were in good agreement with the mean main effects and emphasize the strength of the experimental design.

The ordered data plot [46] of **Fig. 5** presents the response data (measured MM) primarily to identify the best primary factor (instrument settings) for optimization analysis and, secondarily, the most important primary factor settings (instrument settings) for sensitivity analysis. That is, it shows the best combination of settings (yielding the lowest measured MM) and which settings are most impactful to the response (measured MM). The vertical axis shows the mean of replicated average MM measurements for $n=15$ experiments, ordered from smallest to largest. The horizontal axis lists the factor settings defined in **Table 1**. **Fig. 5** shows that the best-observed settings are low cross flow, low ramp time, low focus flow, high injection volume, and low buffer concentration. These settings correspond to (- - - + -), respectively. The second most optimal combination is nearly as good or even equivalent by observation, (- + - - -). The least optimal settings are (+ + + + +). The robustness factor (sample characteristics) ordered data plot is given in the Supplementary Information (**Fig. S.1**).

The main effects plot of primary factors (instrumental settings) in **Fig. 3** identifies the ranked order of factor importance, according to decreasing magnitude effect on measured MM, as buffer concentration, cross flow, focus flow, injection volume and ramp time. From the sign of the response in **Fig. 3** for each factor, the predicted optimal state is (-+---) if cross terms are negligible. This conclusion differs slightly from the observations in **Fig. 5**, which predicted (---+-). However, ramp time was found to be a non-significant factor, so the difference between low and high ramp time has negligible effect on the response value (measured MM). The main effects plot in **Fig. 3** takes all trends across all factor settings into consideration to display the average best settings, whereas the ordered data plot in **Fig. 5** only lists factor settings and individually ranked responses. Note (see highlighting, **Fig. 5**) that buffer concentration and cross flow are generally low at the smaller MM end of **Fig. 5**’s ordered data plot and generally high at the larger MM end of the plot. Unfortunately, the experimental design does not differentiate between the sources of these effects, but can conclude which combination of AF^4 settings results in the lowest

average MM over all samples tested—thus demonstrating a robustness regardless of sample characteristics.

Finally, the contour plot [47] shown in **Fig. 6** is used to determine whether the response could, perhaps, have been better optimized beyond the experimental input and, specifically, which settings would improve the response. The vertical axis represents the second most important factor, cross flow, while the horizontal axis contains the most important factor, buffer concentration. Only the main effects are used in the contour plot, as they can be directly related to primary factor settings, whereas interactions cannot be controlled. The four corners represent the combinations of the two most important factors, each with an average response value (MM). The remaining factors are fixed at their best settings based on conclusions made in the main effects plots and ordered data plots for both primary (instrument settings) (**Figs 3 and 5**, respectively) and robustness (sample characteristics shown in **Fig. 4**) factors. The contour curves produce a visual projection of the response surface and allow for the determination of the best theoretical response value [42, 48]. Improved optimal theoretical settings can be found from the interaction of the steepest descent direction with the optimal curve [47, 48], demonstrating that further optimizing the AF⁴ system towards a minimized average MM would require (in this case) decreasing both the buffer concentration and the cross flow.

Decreasing to zero buffer concentration and zero crossflow would not be experimentally feasible and further decreasing these parameters would result in either failure of the measurement, practical instrumental reasons, or an eventual increase in the response (measured MM). It is likely that further exploration into this suggested zone would identify a saddle point as the factor setting combination effects neared the asymptote at zero.

An interaction effects matrix is provided in Supplementary Information (**Fig. S.2**) and reveals that the cross flow/buffer concentration interaction was the fourth most important effect when ranking the combined primary, robustness, and interaction effect factors. The remaining 2-factor interactions were insignificant, illustrating the quality of the fractional factorial design utilized. Further exploration of the effects and interactions is also included in Supplementary Information (**Fig. S.3**) with an absolute effects plot. The investigated primary factors and their associated settings were chosen for this study because they were simple to adjust between separation protocols. There are several other factors that were not

considered such as membrane type, buffer composition, focus time, channel flow rate, and spacer thickness. Therefore, this work does not identify a complete optimization of all possible primary factors but, instead, the best possible separation conditions for the factors and settings tested.

A replication analysis matrix is provided in Supplementary Information (**Fig. S.4**) and explores the most consistent and precise primary and robustness factor settings across each of the two replicates relative to the experiment day. Of interest, there does not appear to be any significant instrument drift as variability between replicates is a consequence of factor settings and noise.

3.2 Analysis of primary and robustness factors on NOM recovery

A sensitivity analysis was performed on NOM recovery based on the estimated NOM concentrations and main effects plots were generated for both the established primary/instrumental (**Fig. 7**) and robustness/sample (**Fig. 8**) factors. Neither main effects plots identified any statistically significant factors for NOM recovery. However, further analysis revealed several 2-term interactions (not shown), the most important being between NP concentration and NP size.

The 2-term interaction between NP concentration and NP size is in agreement with NOM-ENP interactions that have been studied in the literature, which include the adsorption of dissolved NOM to the particle surface as well as cation bridging and other flocculation mechanisms that induce ENP aggregation into NOM-ENP complexes [34]. Alterations in primary and robustness factors may effect changes in the chemical and physical states of the ENP and NOM mixtures, thereby altering the MM distribution of the mixture, though not significantly affecting the AF⁴ separation.

3.3 Target response: Measured molar mass (MM) and application to future work

It is important to note that although the goal of this work was to minimize the average sample MM, the average MM measurements reported here are on the order of 10⁶ g mol⁻¹ and were approximately two or three orders of magnitude higher than many of those reported in the literature for different NOMs [14, 30, 51, 52]. However, there is a large degree of variability in measurements depending on NOM type, optical properties defined, and analytical approaches. Light scattering studies, compared to traditional humics-sizing methods such as atomic force microscopy, transmission electron

microscopy, and fluorescence correlation spectroscopy, typically result in the detection of larger molecules above $10,000 \text{ g mol}^{-1}$ [30, 31]. For example, Caceci and Billon [53] reported evidence of large organic scatterers (50 to 200) nm in humic acid samples. This suggests that NOM complex molecules may be extensively associated into large, light-scattering aggregates. The presence of AgNP, resulting in potential NOM-Ag and NOM-AgNP complexes, may also contribute to larger aggregates.

For the purposes of this screening study, the average NOM MM of was selected to represent the separation of the entire sample, containing both NOM and AgNP, as the concentration of AgNP is on the order of 0.01% that of the NOM concentration. Neubauer et. al [54] suggested that obtaining accurate MM determinations and optimizing separation were distinct analytical tasks, and that optimizing separation will likely occur at the expense of accurate MM determination. However, in the case of [this current work](#), the absolute accuracy of MM was less important and instead the relative MM measurements revealed conclusions about the separation. In the case of applying this method to real waters, decisions must be made about the desired analytical goal, and if separation optimization is favored (such as for subsequent characterization of ENPs by ICP-MS), then an alternative separation metric to measured MM is recommended.

The chemistry and character of an environmental matrix directly control potential NOM-ENP behavior if they co-occur in the environment. While the most likely NOM-ENP interaction is adsorption of dissolved NOM to the particle surface, cation bridging and other flocculation mechanisms can induce ENP aggregation into NOM-ENP complexes [34]. Louie and coworkers [9] investigated how different MM components of NOM interact with metal or metal oxide ENPs. They concluded that higher MM fractions, primarily comprised of $>100,000 \text{ g mol}^{-1}$ humic acid aggregates, disproportionately dominate aggregation behavior of sample components compared to lower MM fractions that tend to enhance ENP stability. Therefore, small differences in NOM MM distribution and an increase in NP size or concentration might allow more dissolved NOM to reach the detector as part of a NOM-ENP complex or as NOM-stabilized ENPs, rather than pass through the AF^4 membrane in the dissolved fraction. However, Bae et al. [55] demonstrated that potential NOM-ENP interactions are less likely to be based on adsorption of NOM to ENPs, but, instead, on the adsorption of NOM-ENP complexes to humic acids or NOM aggregates. Despite both theoretically- and experimentally-based predictions, the implication is that larger and

potentially unstable aggregates will be formed in waters containing ENPs at environmental concentrations and heterogeneous mixtures of NOM.

The response model obtained from the measured MM as a function of the primary and robustness factors does not give a minimum for MM within the investigated range of the factors, even though the ranges of the most important factors, cross flow and buffer strength, bracketed the values used in studies reported in the literature. As discussed above, alterations in primary and robustness factors may give changes in the chemical and physical states of the ENP + NOM mixtures, thereby altering the MM distribution of the mixture.

Additional studies are needed to obtain a separation metric that optimizes AF^4 for particles in the size range of greatest interest in ENP studies. Future work should investigate whether an alternative separation metric would be superior to the average MM. For example, the average MM of a fixed fraction of the largest-sized species may be less sensitive to changes in MM due to variable passage of NOM through the filter and more sensitive to particles in the size range of interest for environmental samples.

To advance the field and remain experimentally relevant, future studies for improved modeling and prediction should include a comprehensive investigation into the primary and robustness factors that are most relevant to the chosen separation metric and sample type. As this work represents an initial screening design, only selected factors and settings were explored and it is possible that several interactions may be obscuring a more accurate conclusion. In an industrial optimization, the initial screening design described in this paper should be followed by a central composite design approach [49, 50] centered on the best setting to estimate a quadratic response surface and better optimize multifactor problems associated with complex samples. An investigation into classical AF^4 evaluation parameters, such as selectivity, band broadening, particle-particle interaction, etc., and additional AF^4 primary factors, including ultrafiltration membrane type, buffer ionic strength, focus time, channel flow rate or spacer thickness, etc., would also contribute to a complete industrial optimization, as many of these parameters depend on both the operator and sample requirements.

4. CONCLUSIONS

This work describes a systematic investigation of AF⁴ instrumental parameters to examine separation performance in the analysis of complex aquatic matrices. A sensitivity analysis was applied to ascertain the relative importance of AF⁴ primary factors (instrument settings) in the separation of synthetic natural waters containing AgNPs. The analysis targeted five instrumental parameters: cross flow, ramp time, focus flow, injection volume, and run buffer concentration on the multi-angle light scattering measurement of NOM molar mass. A 2⁽⁵⁻¹⁾ orthogonal fractional factorial design was used to minimize analysis time while preserving the accuracy and robustness of the main effects and 2-way interactions. The most important and significant AF⁴ instrumental factors were buffer concentration and cross flow. A parallel 2⁽⁵⁻²⁾ orthogonal fractional factorial design was also employed on five robustness factors (sample characteristics) for synthetic natural water samples containing AgNPs namely, AgNP concentration, AgNP size, NOM concentration, specific conductance, and pH. None of the water quality characteristic effects or interactions was found to be significant in minimizing the measured MM. The approach involves the combination of orthogonal fractional factorial experiment designs, for both primary factors (instrument settings) and robustness factors (sample characteristics), in concert with graphical data analysis methodology. Any AF⁴ separation and subsequent analysis directly depends on the primary factors (instrument settings) and their settings, which vary between instruments and sample requirements. Therefore, a structured methodology of statistical design of experiment (DEX) and sensitivity analysis is useful to better understand how the system output is affected by the system inputs. In order to obtain consistent, robust, and reproducible results, it is critically important to explore and fully understand the combined instrumental system as a whole, relative to the environmental matrix characteristics, an approach not often explored.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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LIST OF FIGURE CAPTIONS

Fig. 1. Schematic showing large and small particles being separated in an AF⁴ channel by hydrodynamic forces.

Fig. 2. AF⁴-MALS-UV fractogram representing the (a, blue) most optimal and (b, red) least optimal combinations of primary and robustness factor settings.

Fig. 3. Main effects plot of primary factors (instrument settings) on the measured MM across all separation protocols. Significant effects, buffer and cross flow, are highlighted in red and correspond to ANOVA F-test p-values of 1.35% and 3.42%, respectively. Factor units: Cross flow (mL min⁻¹), ramp time (min), focus flow (mL min⁻¹), injection volume (μL), and buffer concentration (mmol L⁻¹ NH₄NO₃).

Fig. 4. Main Effects Plot of robustness factors (sample characteristics) on measured MM across all separation protocols for robustness factors. Factor units: [NP] (μg L⁻¹), [NOM] (mg L⁻¹ C), ionic strength (μeq L⁻¹), and NP size (nm).

Fig. 5. Ordered data plot of primary factors (instrument settings) and settings. Error bars represent the 95% confidence interval for the mean value across eight replicated samples. Buffer concentration (green box) and cross flow (red box) are highlighted as the most important factors as discussed in the text.

Fig. 6. Contour Plot. The red arrow signifies the direction of steepest descent. Contour lines represent intervals of $3.00 \times 10^6 \text{ g mol}^{-1}$.

Fig. 7. Main Effects Plot of primary/instrument factors on NOM recovery across all separation protocols. Factor units: Cross flow (mL min⁻¹), ramp time (min), focus flow (mL min⁻¹), injection volume (μL), and buffer concentration (mmol L⁻¹ NH₄NO₃).

533 **Fig. 8.** Main Effects Plot for robustness/sample factors on NOM recovery across all separation protocols
534 for robustness factors. Factor units: [NP] ($\mu\text{g L}^{-1}$), [NOM] ($\text{mg L}^{-1} \text{ C}$), ionic strength ($\mu\text{eq L}^{-1}$), and NP size
535 (nm).
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Figure 1

Figure 2a

Figure 2b

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Table 1

Table 1. Chosen primary factors (instrument settings), robustness factors (sample characteristics), and their respective settings. [NP] and [NOM] refers to nanoparticle and NOM concentration, respectively. mg L⁻¹ C refers to milligrams carbon per liter, as estimated by UV₂₅₄ absorbance.

<i>Primary Factors (instrument settings)</i>				
Factor	Low (-)	Midpoint	High (+)	(units)
Cross Flow	0.5	1.5	2.5	mL min ⁻¹
Ramp	20	30	40	min
Focus	1	2	3	min
Injection volume	200	350	500	µL
Buffer	0.1	1	5	mmol L ⁻¹ NH ₄ NO ₃
<i>Robustness Factors (sample characteristics)</i>				
Factor	Low (-)	Midpoint	High (+)	(units)
[NP]	3	50	100	µg L ⁻¹
[NOM]	1	5	10	mg L ⁻¹ C
Ionic Strength	1029	2992	4781	µeq L ⁻¹
pH	6	7	8	
NP Size	10	50	100	nm