

# Quantitative Analysis in Field-Flow Fractionation Using Ultraviolet–Visible Detectors: an Experimental Design for Absolute Measurements

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## Abstract

In previous works, it has been shown that a standard ultraviolet–visible detection system can be used for quantitative analysis of heterogeneous systems (dispersed supermicron particles) in field-flow fractionation (FFF) by single peak area measurements. Such an analysis method was shown to require either experimental measurements (standardless analysis) or an accurate model (absolute analysis) to determine the extinction efficiency of the particulate samples. In this work, an experimental design to assess absolute analysis in FFF through prediction of particles' optical extinction is presented. Prediction derives from the semiempirical approach by van de Hulst and Walstra. Special emphasis is given to the restriction of the experimental domain of instrumental conditions within which absolute analysis is allowed. Validation by statistical analysis and a practical application to real sample recovery studies are also given.

## Introduction

Field-flow fractionation (FFF), the most versatile of macromolecule and particle separation methodologies, comprises a family of techniques that have demonstrated for more than two decades the ability to characterize supramolecular species and particles (1). Quantitative measurements, however, have only been recently investigated in FFF practice. For instance, quantitative distribution of particle size and sample amount (PSAD) (2) has shown its advantages in terms of a valuable gain in analytical information with respect to classic, qualitative particle size distribution (PSD) studies. A fully quantitative approach to FFF has been mostly hindered because of the use of commercial ultraviolet–visible (UV–vis) spectrometers that act as turbidimeters. The extraction of quantitative information from turbidity measurements generally should, in fact, require

knowledge of the optical properties of the particles and the application of the Mie corrections for light-scattering effects. However, with supermicron particles, it has been demonstrated that forward scattering dominates, and a simplified expression for turbidity allows for an easier approach to quantitative analysis (3).

In previous works (4,5), it was shown that a derivation of the Beer–Lambert (BL) law could be applied to heterogeneous, micron-sized samples for quantitative analysis in gravitational FFF (GrFFF). GrFFF is a low-cost subset of sedimentation FFF particularly suited to the fractionation of micron-sized particles, either of inorganic (6) or biological origin (7). Moreover, such a BL-based model was demonstrated to be applicable in absolute mode. An absolute method of analysis is defined as a method through which a signal can be related to the concentration or quantity of the analyte by an exact equation that is reliable enough to allow for direct calculation of the desired quantity from a single measurement. In absolute mode, no previous experimental measurements are required except for the evaluation of instrumental constants. As a result, quantitative analysis was attained by means of single peak area measurements and extinction data taken from the literature (8) without previous measurements. However, it was observed that the accuracy of the proposed absolute method strictly depended on the accuracy with which theoretical extinction coefficients can be predicted.

In this work, an experimental design for the assessment of absolute analysis in FFF is proposed through the modelization, validation, limitation, and application of a semiempirical approach based on BL law and prediction of the extinction coefficients.

The model for extinction coefficient prediction is proposed here as a tool to choose the experimental boundaries (i.e., particle size, relative refractive index, incident wavelength, detector optics) within which it is possible to assess absolute measurements with UV–vis detectors. Once such experimental limits are found, a validation of the proposed method is carried out. The method is then applied to recovery studies. A quick

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approach to sample recovery evaluation is actually a valuable tool for the optimization of FFF procedures.

## Experimental

The GrFFF systems employed here were built as described in previous papers (2,4,9,10). The channel dimensions were 30 cm tip to tip, either 2 or 1 cm in breadth and 0.0197 or 0.0175 cm in thickness, respectively. The channel outlet was fed to a UV-vis model 2550 detector (Varian, Walnut Creek, CA) operating at variable wavelength. The detector signal was recorded on a strip-chart integrator model Mega 2 (Carlo Erba Strumentazione, Milan, Italy) and captured through a 12-bit I/O data acquisition board model Lab PC+ (National Instruments, Austin, TX) from the INT1 voltage output port.

Samples were spherical polystyrene (PS) Polybead particles with a radius of  $2.15 \pm 0.15$  and  $3.05 \pm 0.25$   $\mu\text{m}$  (Polysciences, Warrington, PA). PS particles, when compared with the silica spheres employed in our previous work, offer simpler optical properties (4). They were supplied as a concentrated suspension and then diluted to 0.002–2.63% in Milli-Q water (Millipore, Bedford, MA). The density of PS samples was given by the manufacturer as  $\rho_a = 1.05$   $\text{g cm}^{-3}$ . The mobile phase was Milli-Q water added to either different quantities of  $\text{NaN}_3$  (commonly used in FFF practice as a bactericide) or a surfactant such as sodium dodecylsulphate (SDS) (Aldrich, Steinheim, Germany). Flow was generated through a Varian model 2510 HPLC pump. The ionic strength of the mobile phase was maintained constant at  $3.08 \times 10^{-3}\text{M}$  by adding either the salt or the surfactant. Experimental evaluation of the effective extinction efficiency as a function of instrumental parameters was performed at different wavelengths by injection into the UV cell of dispersions of particulate samples, at various concentrations, with a syringe. Readings were immediately registered in order to avoid inaccurate measurements as a consequence of particles settling. Dispersions were prepared in Milli-Q water, and the same ionic strength value ( $3.08 \times 10^{-3}\text{M}$ ) was obtained in these cases by adding NaCl (Aldrich).

The solution and plotting of van de Hulst–Walstra's expressions as a function of different experimental parameters were performed by Mathematica 2.2 (Wolfram Research, Champaign, IL) and Matlab 4.2c.1 (The Mathworks, Natick, MA).

## Results and Discussion

### Modelization

It was previously demonstrated that for the absolute analysis of particles by UV-vis detectors, the following expression can be applied (4):

$$\bar{A}F/Kb = N_0 \quad \text{Eq. 1}$$

where  $\bar{A}$  (min) is the peak area,  $N_0$  (g) is the sample amount exiting the system,  $F$  ( $\text{cm}^3/\text{min}$ ) is the flow rate,  $b$  (cm) is the

cell pathlength, and  $K$  ( $\text{cm}^2/\text{g}$ ) is the total extinction coefficient. This expression directly derives from a similar relationship based on rigorous modeling that was validated for absorbing species in HPLC (5).

The absolute method for quantitative analysis of particles requires the extinction coefficient to be known from the samples' specifications and extinction efficiency. Furthermore, it was shown that for large spherical particles for which  $a > 10\lambda$ , where  $a$  (cm) is the particle radius and  $\lambda$  (cm) is the incident wavelength, forward scattering (Fraunhofer diffraction) dominates. In this case, the general expression for the extinction coefficient is given:

$$K = (0.33/\rho_a a)Q_{ext} \quad \text{Eq. 2}$$

where  $Q_{ext}$  is the extinction efficiency and  $\rho_a$  is the particle density ( $\text{g cm}^{-3}$ ).

It is known that most of the diffracted light at low angle ( $0-5^\circ$ ) can be almost completely captured within the reception cone of commercial UV-vis detectors (11). In this case, the effective extinction efficiency  $Q'_{ext}$  decreases to 1 if all of the diffracted light is captured within the reception cone (12).

In the extinction model for supermicron particles, the extinction efficiency can be generally obtained by solving van de Hulst's expression (3,13):

$$Q_{ext} = 2 - [16m^2(\sin \rho)/(m+1)^2\rho] + [4(1-m \cos \rho)/\rho^2] + 7.53[(z-m)/(z+m)]x^{-0.772} \quad \text{Eq. 3}$$

$$\text{with } z = [(m^2 - 1)(6x/\pi)^{2/3} + 1]^{1/2}$$

where  $x = 2\pi a/\lambda$ ,  $m$  is the relative refractive index of the particles (i.e.,  $m = m_1/m_2$  with  $m_2$  = refractive index of the dispersing fluid),  $\lambda$  is the incident wavelength in the dispersing fluid, and the phase coefficient  $\rho = 2x(m-1)$  is a dimensionless parameter that includes all of the optical parameters. Equation 3 is applicable when  $\rho > 2.5$  and  $m$  is slightly above 1 (anomalous diffraction).

The estimation of the actual value of the effective extinction efficiency  $Q'_{ext}$  with conventional UV-vis detectors is possible by applying a derivation of van de Hulst's expression (Equation 3) given by Walstra (14):

$$Q'_{ext} = Q_{ext} - 1/4(P^2 + Q_{ext}^2)R \quad \text{Eq. 4}$$

$$\text{with } P = -[16m^2(\cos \rho)/(m+1)^2\rho] + [4m \cos \rho/\rho^2] - 4.2[(z-m)/(z+m)]x^{-0.772}$$

$$\text{and } R = 1 - J_0^2(\theta x) - J_1^2(\theta x)$$

where  $\theta$  is the acceptance angle, defined as the sum of half the angle of divergence and convergence of the incident beam and half the angle subtended by the detector.

The extinction efficiency  $Q_{ext}$  (Equation 3) and the  $P$  term are proportional to the real and imaginary part of the forward scattering function amplitude, respectively. The term  $R$  expresses the dependence of the intensity of the diffracted light on the diffraction angle. Such a dependence is derived from the

classical Rayleigh theory for scattering.  $J_0$  and  $J_1$  are the Bessel functions of the first kind, of 0th and 1st order, respectively.

### Validation

#### *Applicability of the model for absolute analysis in FFF–UV–vis systems*

Even though the present design deals with absolute analysis, an initial instrumental calibration is always needed in order to check whether the employed FFF–UV–vis system behaves as an ideal, flow-through, homogeneous system and whether assumptions for the application of the BL law in flow-through systems hold true. Therefore, the model was first checked by means of a spectroscopic standard (15), as described in our previous paper (4). By this calibration, the needed constancy of the ratio between detector response and cell pathlength was also verified. The ratio between the detector conversion factor (from millivolts to absorbance units) and the cell pathlength ( $b$ ) was determined and found to be 1030 (mV/cm)/(1 AU).

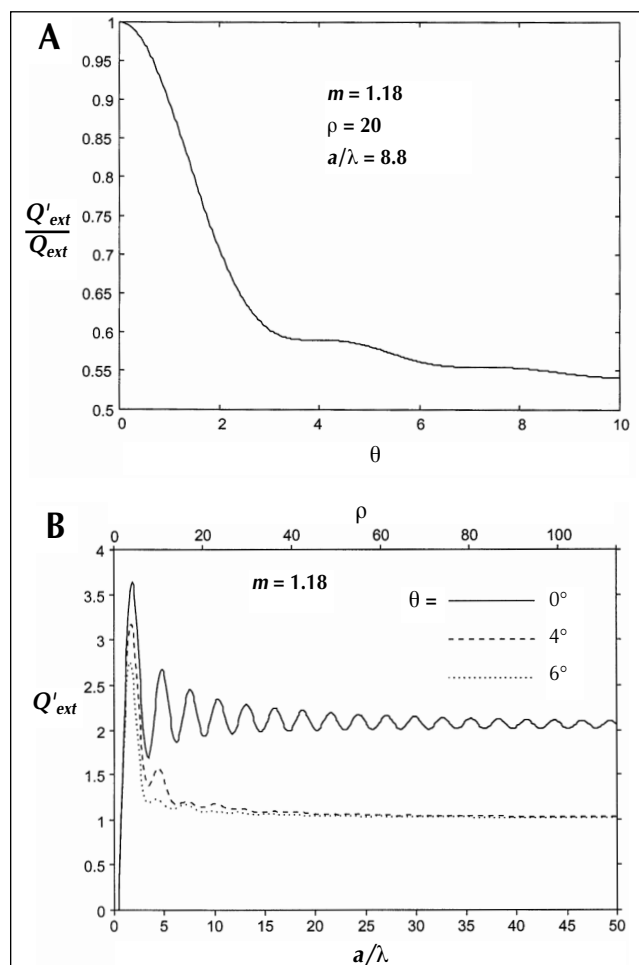
The constancy of the extinction coefficient  $K$  with changing sample amount had to be further verified. In this work, the same UV–vis detector employed in the previous studies was used for extinction efficiency determinations. With this type of

flow-through spectrophotometer, it was already demonstrated that, with silica particles, values of the extinction coefficient were always constant and independent of flow rate over a broad sample concentration range (2,4,9). As discussed later, this finding is also confirmed for PS particles.

The predictive ability of the proposed model based on the van de Hulst–Walstra expression was tested by analyzing the dependence of the effective extinction efficiency ( $Q'_{ext}$ ) on the experimental parameters that can be set in a GrFFF–UV–vis experiment. In Figure 1A, the ratio between the effective extinction efficiency ( $Q'_{ext}$ ) (Equation 4) and the extinction efficiency at  $\theta = 0^\circ$  ( $Q_{ext}$ ) (Equation 3) for a given value of  $a/\lambda$  is shown as a function of  $\theta$ . The curve is calculated for PS particles. From this curve, it is evident that the effective extinction efficiency rapidly decreases with increasing acceptance angle. In fact, at a  $\theta$  value of only  $3^\circ$ , the effective extinction efficiency ( $Q'_{ext}$ ) is nearly half of the extinction efficiency ( $Q_{ext}$ ) at  $\theta = 0^\circ$ . Moreover, in Figure 1B, it is shown that the rippling nature of the effective extinction efficiency function ( $Q'_{ext}$ ) is dampened with increasing  $a/\lambda$  and a constant unit value is rapidly approached with higher acceptance angles. From these findings, it can be confirmed that the acceptance angle is one of the most critical parameters for turbidity measurements with UV–vis detectors (11). It is evident that the best choice for a given radius ( $a$ ) is the use of a detector with the highest acceptance angle ( $\theta$ ) at the lowest possible wavelength ( $\lambda$ ).

**Predictive ability.** In Figure 2, the variation of  $Q'_{ext}$  with changing relative refractive index ( $m$ ) (Figure 2A) and incident wavelength ( $\lambda$ ) (Figure 2B) is reported. Figure 2A shows that with  $m$  decreasing to unity (that is, with particles refractive index close to the refractive index of the dispersing medium), the effective extinction efficiency ( $Q'_{ext}$ ) tends more rapidly to a constant unit value. This finding agrees with the classic theory of diffraction. In fact, when particles' have refractive indexes close to that of the surrounding medium, light is diffracted at the smallest angles. Figure 2B shows that at a given relative refractive index, the rippling nature of the  $Q'_{ext}$  function is dampened with decreasing wavelength. Here  $m = 1.18$  for PS in water (16), and its dependence on the incident wavelength is not taken into account. The effect of decreasing  $\lambda$  is qualitatively similar to that shown in Figure 1B, which is due to an increase in the acceptance angle  $\theta$ . Therefore, as a practical consequence, it can also be deduced that the calculation of  $Q'_{ext}$  values for the evaluation of the extinction coefficient  $K$  is easier for particles of given dimension and refractive index under the same conditions reported previously: detectors with the highest acceptance angle and the lowest wavelength.

**Experimental validation.** The proposed van de Hulst–Walstra approach had to be experimentally verified by comparing theoretically predicted and experimental values of  $Q'_{ext}$ . The predicted profiles for the extinction efficiency function at  $\theta = 0^\circ$  ( $Q_{ext}$ ) (Equation 3) and for the effective extinction efficiency function ( $Q'_{ext}$ ) (Equation 4) are reported in Figure 3 as a function of  $a/\lambda$  for the relative refractive index of PS particles ( $m = 1.18$ ). The experimental points of  $Q'_{ext}$  relative to PS particles of two different diameters are also plotted. Predicted  $Q'_{ext}$  pro-



**Figure 1.** Dependence of the effective extinction efficiency ( $Q'_{ext}$ ) on experimental variables: functions calculated for PS particles. Ratio between  $Q'_{ext}$  (Equation 4) and  $Q_{ext}$  (Equation 3) versus acceptance angle  $\theta$  (A);  $Q'_{ext}$  versus  $\rho$  and  $a/\lambda$  on changing  $\theta$  (B).

files were calculated for the acceptance angle value of the employed UV-vis detector. This instrumental constant was accurately determined on the detector optics by measuring the diameter of the photodiode and its distance from the cell. The acceptance angle was  $\theta = 4.6^\circ$ . Evaluation of the experimental  $Q'_{ext}$  values as a function of  $a/\lambda$  was performed by plotting turbidity values (at different  $\lambda$ ) for a set of PS samples of given diameter at different concentrations. It was shown that from such measurements, the experimental extinction coefficient  $K$  values can be obtained from the slope of linear regression analyses (2,4). Subsequently, by applying Equation 2, effective extinction efficiency values can be determined.

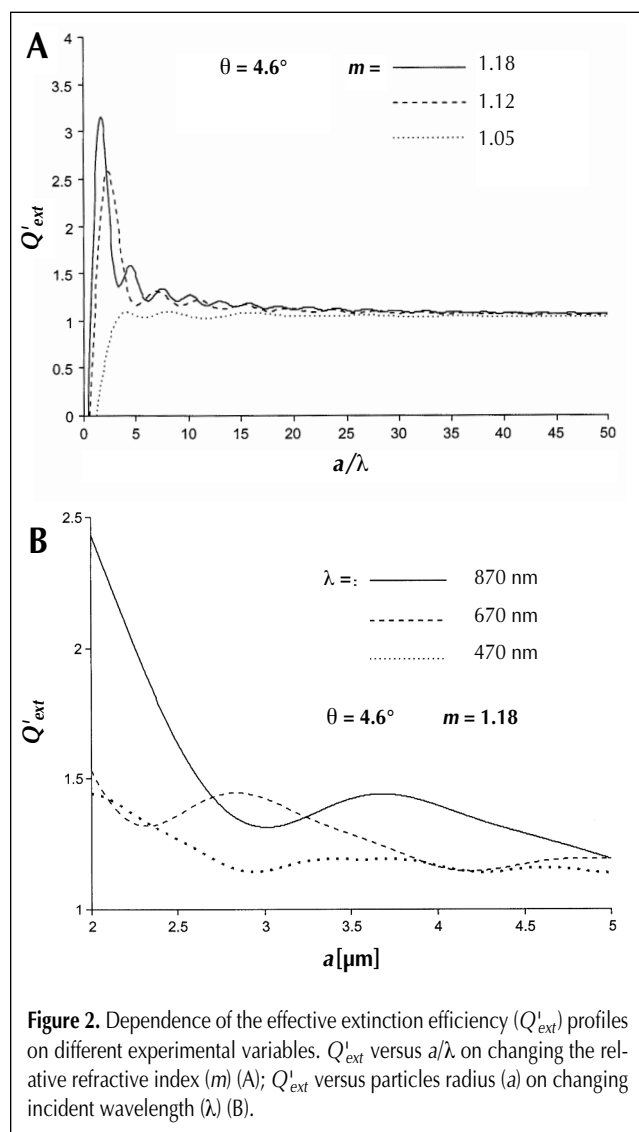
An example of linear regression carried out in order to experimentally determine  $Q'_{ext}$  confirmed the linear dependence (intercept =  $0.001 \pm 0.005 \text{ cm}^{-1}$ ; standard deviation = 0.00326; correlation coefficient  $r = 0.9997$ ) of turbidity on particulate sample concentration over a broad concentration range (up to  $15 \times 10^{-5} \text{ g cm}^{-3}$ ), as in the case already reported for supermicron silica particles (2,4,9).

It is known that constant, experimentally determined  $K$  values are actually average values with respect to the dimensional distribution of the samples. Therefore, it is important to

bear in mind that experimental, effective extinction efficiency values ( $Q'_{ext}$ ) determined by Equation 2 must be also considered as average values. It was reported (4,17) that for polydisperse particles, Equation 2 still holds true if half of the volume-surface average diameter ( $D_{3,2}$ ) is assumed for particle radius ( $a$ ). As a consequence, the average  $Q'_{ext}$  values obtained by means of Equation 2 from average experimental  $K$  values can be considered valid for particle diameters corresponding to their  $D_{3,2}$  mean values.

From the analysis of Figure 3, a very good agreement between experimental and predicted values of  $Q'_{ext}$  can be observed in a range where they tend to be constant and close to unity. However, deviation from the predicted profile is evident with increasing  $a/\lambda$ ; that is, when light absorption of PS particles begins to occur below a selected value (in air) of incident wavelength  $\lambda \cong 370 \text{ nm}$ . From this finding, it is evident that a constant, effective extinction efficiency approximately equal to 1 can be predicted for absolute analysis purposes only when particles do not absorb light.

In Figure 3, the arrow indicates the point above which the experimental conditions that fulfill necessary requirements for the application of the absolute method can be obtained.



**Figure 2.** Dependence of the effective extinction efficiency ( $Q'_{ext}$ ) profiles on different experimental variables.  $Q'_{ext}$  versus  $a/\lambda$  on changing the relative refractive index ( $m$ ) (A);  $Q'_{ext}$  versus particles radius ( $a$ ) on changing incident wavelength ( $\lambda$ ) (B).

#### Limitation: restriction of the experimental domain

One may remark that if the entire procedure described above is to be followed whenever absolute analysis is to be made, it would be rather time consuming and, therefore, may not be competitive with common calibration methods. We have, therefore, determined guidelines to choose the experimental conditions and indicate the limiting cases in which absolute analysis is possible. To this end, the van de Hulst-Walstra equation was solved as a function of two independent variables: the phase coefficient  $\rho$ , which includes particle size, incident wavelength, and relative refractive index; and the acceptance angle of the detector. The solution as a 3-dimensional plot is reported in Figure 4. In this surface plot, the region where the effective extinction efficiency values ( $Q'_{ext}$ ) do not differ more than  $\pm 5\%$  from the unit value is highlighted. Although Figure 4 generally allows for the prediction of extinction efficiency within the entire range of experimental and instrumental conditions, such a flat region can be considered as the preferable domain for obtaining absolute analysis. In fact, an accurate knowledge of the values for all the experimental and instrumental parameters is not strictly necessary where the extinction efficiency shows constant. Nevertheless, to predict the extinction coefficient, the value of which is inversely proportional to particle size (Equation 2), the exact knowledge of the relationship existing between retention and particle size is strictly required. However, the accurate conversion of retention to size belongs to FFF fundamentals, and it cannot be thought of as a limiting factor of the quantitative method presented here.

#### Application

##### Experimental test of the absolute analysis model

The experimental conditions indicated in Figure 3 have been used to experimentally verify the model of absolute analysis based on Equations 1 and 2. A set of fractograms of known amounts of PS sample ( $3.05 \mu\text{m}$ ) was collected. The mobile

phase composition (SDS  $3.08 \times 10^{-3}\text{M}$ ) and flow rate ( $3 \text{ cm}^3/\text{min}$ ) were chosen in order to minimize the effects on recovery played by electrostatic and second-order phenomena (10). SDS at the concentration used was known not to influence effective extinction efficiency values (2,4). Agreement with the model was performed by relating recovered quantities determined by applying Equations 1 and 2 to known injected quantities ( $N_i$ ). Weighted, linear regression analysis based on seven values of  $N_i$ , for which three repeated measurements were performed, shows a very good agreement with the model. The intercept does not significantly differ from zero, and the slope does not significantly differ from unity both at the 95% ( $0.1 \pm 2$  and  $1.00 \pm 0.02$ , respectively) and at the 99% confidence level ( $0.1 \pm 3$  and  $1.00 \pm 0.03$ , respectively). The linearity test by analysis of variance (ANOVA,  $F$  test) (18) accepts the null hypothesis (no significant difference between the regression function and the straight line  $y = x$ ) both at 95% and 99% confidence level ( $F_{\text{exp}} = 1.21$ ,  $F_{p=0.01} = 9.77$ ,  $F_{p=0.05} = 4.64$ ).

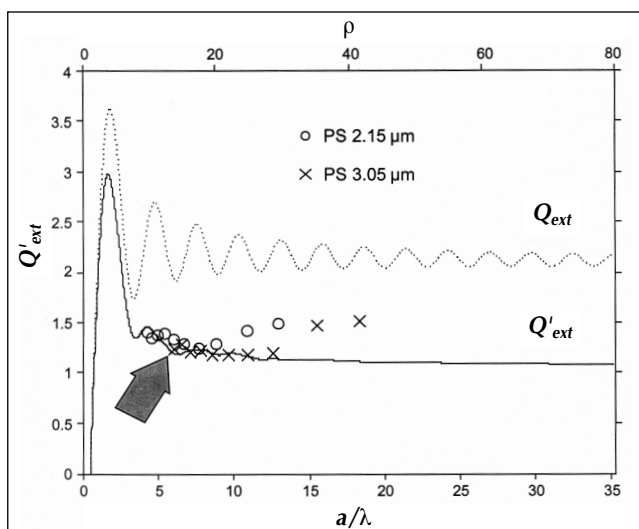
Once the application of the method for absolute analysis has been verified, an FFF fractogram of particulate matter can be directly recorded in concentration units, and sample recovery can be rapidly obtained from a single run without previous calibration, as discussed in the following paragraph.

#### Sample recovery by absolute analysis

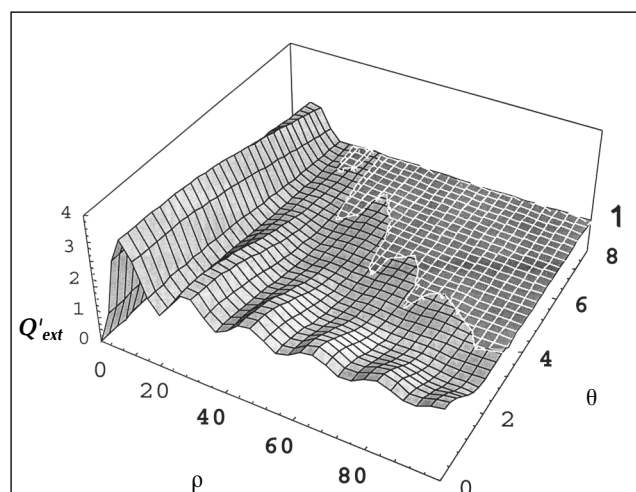
Sample recovery determination in FFF practice has been often performed by measuring peak areas of a set of samples and by comparing the fractionated sample amounts to those determined by loading identical quantities directly into the UV-vis detector (i.e., with the FFF channel excluded from the mobile phase stream). On the contrary, the described method based on the derived BL law requires neither this comparison nor the use of uncorrelated experiments. In fact, it is clear from the previous section that when recovery is 100%, the eluted quantities evaluated by the absolute method always correspond to the injected quantities in a broad range of sample

loads. In our previous work (2), it was shown that by plotting the recovered quantities ( $N_0 = \bar{A}F/Kb$ ) against known quantities ( $N_i$ ) of the sample directly injected into the FFF system, a linear regression plot whose slope directly gives the sample recovery is obtained. For those studies, however, an experimentally determined (rather than predicted)  $K$  value was employed and, therefore, analysis with a set of different sample loads and a calibration procedure to obtain  $K$  values was required. However, because it has been demonstrated here that  $K$  values can be theoretically calculated, the recovery from just a single run can be obtained with no previous calibration.

In the case of silica particles, it was shown in all cases that recovery is independent of the injected sample amount and generally increases by either reducing the ionic strength of the mobile phase or adding a surfactant (2). Moreover, in GrFFF, it is known that because of the longer residence times in the channel and reduced particles elevations from the accumulation wall, with decreasing flow rate, sample recovery also decreases. Therefore, different runs were performed under different conditions for the same PS sample ( $3.05 \mu\text{m}$ ) used for the validation of the model reported in the previous paragraph, either by changing the mobile phase composition or flow rate. Based on a predicted extinction coefficient  $K$  value, all of the recovery measurements have been made from single area measurements. Figure 5 shows a series of fractograms obtained for the same sample (PS,  $3.05 \mu\text{m}$ ) by changing flow rates. In Figure 5A, recovery is determined directly by means of area measurements of fractograms. Recoveries were evaluated by means of Equations 1 and 2, with the same value of calculated extinction coefficient ( $K = 1240 \text{ cm}^2/\text{g}$ ) used in the model validation step. The mobile phase was still SDS ( $3.08 \times 10^{-3}\text{M}$ ). Because peak area values depend on the flow rate, for the sake of easier comparison, the turbidity values are multiplied by the flow rate. Peaks are therefore plotted as the detector signal ( $\tau$ ,  $\text{cm}^{-1}$ ) multiplied by the mobile phase flow rate ( $F$ ,  $\text{cm}^3/\text{min}$ ) versus the retention time. As previously observed, it is con-



**Figure 3.** Comparison between predicted (—,  $Q'_{\text{ext}}$ ; ---,  $Q_{\text{ext}}$ ) and experimental extinction efficiency values (○, PS =  $2.15 \mu\text{m}$ ; ×, PS =  $3.05 \mu\text{m}$ ). Acceptance angle  $\theta = 4.6^\circ$ ,  $\lambda$  (in water) =  $165\text{--}500 \text{ nm}$ .



**Figure 4.** Three-dimensional plot of the effective extinction efficiency ( $Q'_{\text{ext}}$ ) versus  $\rho$  and  $\theta$ .

firmed here that recovery decreases with decreasing flow rate. At 0.5 cm<sup>3</sup>/min, recovery is in fact 69% of the total recovery obtained at 2.0 cm<sup>3</sup>/min. In Figure 5B, an analogous study with a different mobile phase is reported. The ionic strength was kept constant in both the experiments (SDS was there substituted by NaN<sub>3</sub> at the same value of ionic strength). It is again confirmed that, without surfactant, recovery strongly decreases with decreasing flow rate, although the decrease is more pronounced than in the previous case (with SDS), particularly at low flow rates. In fact, with SDS, recovery decreases from 85% at 2.0 cm<sup>3</sup>/min to 59% at 0.5 cm<sup>3</sup>/min, whereas with NaN<sub>3</sub>, it is reduced from 66% at 2.0 cm<sup>3</sup>/min to 36% at 0.5 cm<sup>3</sup>/min. The role of the surfactant with respect to the modulation of GrFFF retention as a consequence of its effect on particle-wall interaction has already been discussed both for PS (19) and silica particles (10,20). A systematic study of the effects that different experimental conditions have on sample recovery is beyond the aims of the present paper.

## Conclusion

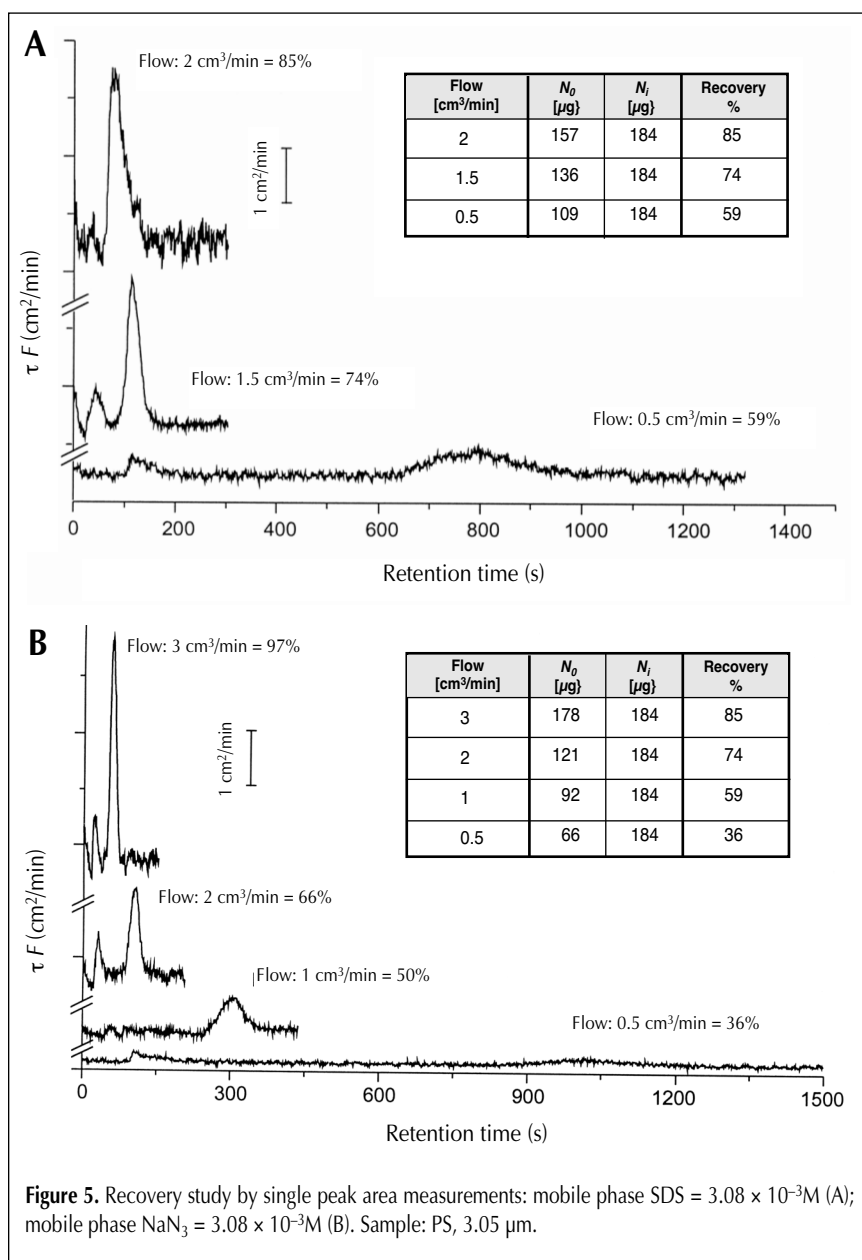
The approach presented here shows that quantitative analysis in FFF can be performed through standard UV-vis detectors in absolute mode. Because of the predictability and constancy of particle extinction efficiency within broad experimental boundaries, it is shown that one of the main limitations in the use of UV-vis detectors for quantitative analysis in FFF (i.e., the lack of information on particles' optical properties) can also be overcome. The proposed approach has been based on the ideal case of spherical particles of well known optical properties. Further work on the predictability of the UV-vis extinction properties of dispersed samples, either for particles of irregular shape or more complex optical properties dispersed in different media, is currently in progress.

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