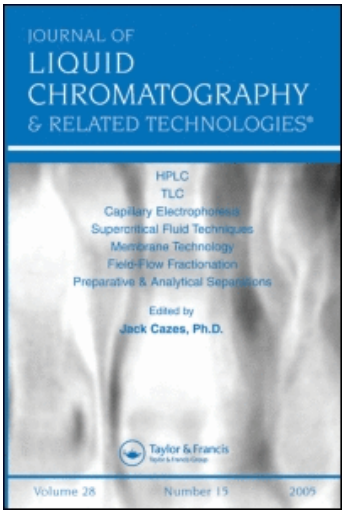

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D. Melucci[a]; A. Zattoni[a]; S. Casolari[a]; M. Reggiani[a]; R. Sanz[b]; P. Reschiglian[a]
[a] Department of Chemistry "G. Ciamician", University of Bologna, Bologna, Italy [b] Department of Analytical Chemistry, University of Barcelona, Barcelona, Spain

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WORKING WITHOUT ACCUMULATION MEMBRANE IN FLOW FFF. EFFECT OF SAMPLE LOADING ON RECOVERY

D. Melucci,[1,*] A. Zattoni,[1] S. Casolari,[1] M. Reggiani,[1] R. Sanz,[2] and P. Reschiglian[1]

[1]Department of Chemistry "*G. Ciamician*", University of Bologna, via F. Selmi 2, I-40126 Bologna, Italy
[2]Department of Analytical Chemistry, University of Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain

ABSTRACT

Steric/hyperlayer flow field-flow fractionation (St/Hyp/FlFFF) is suitable for the separation and characterization of micrometer-sized particles. In this technique, an ultrafiltration membrane is commonly used as the surface of the accumulation wall. St/Hyp/FlFFF has been recently tested in membraneless mode and an improvement in performance was found. Recovery was also improved and second-order effects were reduced.

In the framework of St/Hyp/FlFFF optimization, the effect of sample loading is a problem of a certain importance. For quantitative purposes, the conversion of peaks into mass particle size distributions is of prime importance and, therefore, the conditions in which there is no effect of sample loading on recovery should be investigated.

*Corresponding author. E-mail: melucci@ciam.unibo.it

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In this paper, systematic work was performed in order to study the effect of sample loading on recovery. We have found the conditions in which recovery is independent of sample loading. For these conditions, the limit of detection for various micrometer-size standard polystyrene particles was calculated. The absolute sample recovery was calculated by applying a quantitative method for single-run analysis in FFF with UV/Vis detectors.

Key Words: Flow field-flow fractionation; Recovery; Sample loading; Micrometer-sized particles

INTRODUCTION

One appealing characteristic of flow field-flow fractionation (FlFFF) is the possibility of rapidly fractionating and accurately characterizing samples from macromolecules to micrometer-sized particles. The fractionation of micrometer-sized particles can be achieved by the steric/hyperlayer subtechnique of FlFFF (St/Hyp/FlFFF). In this case, the conversion of retention times into diameters is usually performed via calibration by spherical standard particles. The use of standards, whose density may be different from unknown samples, is possible because retention is not significantly dependent on sample density.[1] When the samples are not spherical, the size information obtained is the hydrodynamic diameter. The possibility to perform calibration independently of density and shape is a great advantage, since for real samples shape and density are often unknown or inhomogeneous, and standard samples are rarely on hand. Moreover, the separation of supermicron particles in St/Hyp mode has the advantage of rapidity, due to the high elution flow rates employed; each run takes only few minutes. These characteristics of the St/Hyp/FlFFF technique make its use for separation and characterization of real samples of micrometer size very appealing.

In St/Hyp/FlFFF, an ultrafiltration membrane usually covers the porous accumulation frit to prevent particle penetration. However, the membrane is responsible for many problems. One of the most serious drawbacks of the use of membranes as accumulation wall is the possibility of sample adhesion, which can affect recovery and give rise to memory effects. Frequent membrane replacement is necessary. Additional costs and low reproducibility of void volume and related retention data are the consequence. For this reason, some authors have recently proposed[2] to work in St/Hyp/FlFFF without membrane.

The special prototype studied in this work was the same used in the previous work.[2] This system has already shown a general performance

improvement. No massive sample immobilization on the microporous accumulation frit was observed. Very good reproducibility and selectivity as high as with membrane were observed. The logic development of the previous work on membraneless St/Hyp/FlFFF is the optimization of both peak quality and sample quantitation. In this work, the quantitative aspects are dealt with. It has already been demonstrated that recovery is improved once the membrane was removed.[2] However, the effect of sample loading on recovery should also be investigated. Sample overloading must be avoided because it can affect quantitative results. In fact, when recovery is shown independent of sample loading, signal is linearly dependent on injected mass and peak area is proportional to the injected amount. Hence, the signal can be accurately converted into injected mass and a linear calibration plot of area vs. injected mass is obtained. Data may be used for quantitative applications, such as the evaluation of the limit of detection (LOD) from area vs. injected amount data. The conversion of fractograms into mass size distributions (Particle Size and Amount Distribution, PSAD)[3,4,5] could also be performed.

The first systematic study on recovery in FFF was proposed by Ratanathanawongs et al.,[6] who defined the fundamental categories of recovery. *Absolute recovery* is the percentage ratio between eluted and injected sample mass. Proportionate recovery is the relative recovery of one sample with respect to the others. *Linear recovery* corresponds to recovered amounts linearly dependent on injected mass.

In this work, we focus the attention on absolute recovery. The aim is to find out experimental conditions in which absolute recovery is high and uneffected by sample loading (case of *linear recovery* and proportionate recovery independent of sample loading). The maximization of recovery is a prime goal of every analytical technique. The importance to have recovery independent of sample loading has already been explained above. To study the effect of sample loading on recovery, we used standard monodispersed particles with calibrated diameter. We validated the single run quantitative method[5,7] and applied it to calculate absolute recovery. We collected a wide set of peak area vs. concentration data for the evaluation of the effect of the injected amount of sample on recovery. The conditions for which recovery is independent of sample loading were found. In case of no overloading, the limit of detection was also evaluated. By this approach, it was also shown that membraneless St/Hyp/FlFFF has, with respect to the standard membrane mode, the great advantage that recovery is much higher and in some cases equal to 100%. The experimental conditions to assess total recovery are particularly useful when the absence of memory effects is sought (e.g., with live biological samples in preparative experiments).

Data Handling

FlFFF Basic Principles

The theory of FFF and the principles of FlFFF were described elsewhere.[1,6,8] For the readers' convenience the basic principles are briefly summarized.

In FlFFF, separation takes place within a ribbon-like tapered channel where a laminar flux is maintained. A cross flow is set up perpendicularly to the longitudinal flow. During elution the cross-flow pushes analytes towards one wall (accumulation wall), while the longitudinal flow at the same time transports the analytes and gives rise to lift forces. For micrometer-sized particles, which are the analytes of interest in the present paper, the balance between the combined viscous and lift forces keep the particles at different distances from the accumulation wall depending on their hydrodynamic diameter. Larger particles experiment faster velocities and are eluted earlier than smaller ones.

This elution mechanism that operates in our experiments is then called steric/hyperlayer (St/Hyp). This is the mechanism when the intensity of the hydrodynamic lift forces is relatively high with respect to the viscous field.

System Calibrations

The calibration of instrumental parameters (spectrophotometric cell path length, injector loop volume) is accurately performed here using a spectroscopic standard of known molar absorptivity. The equations employed are the Beer-Lambert law and the Beer-Lambert-like law modified for flow-through UV/Vis spectrophotometers.[5,9]

Recovery Evaluation

In this work, is applied the single-run quantitative method for FFF-UV/Vis that has been developed and already tested by some of the authors.[5,7] This method allows for the direct calculation of eluted sample mass m (g) from a single peak area measurement:

$$\hat{A}\dot{V} = \kappa\, b\, m \tag{1}$$

where $\hat{A}$ (min) is the peak area, $\dot{V}$ ($cm^3\ min^{-1}$) the longitudinal flow rate, κ ($cm^2\ g^{-1}$) the extinction coefficient of particles, and b (cm) is the detector path

length. The proportionality coefficient between turbidity and concentration (κ) can be directly calculated from sample specifications by the following equation:

$$\kappa = \frac{3}{4 \ln 10 \ \rho \ a} Q \tag{2}$$

where ρ (g cm^{-3}) is the particle density, a (cm) is particle radius and Q is the extinction efficiency. For the instrumental and experimental conditions of the present work, Q is assumed equal to 1.[7]

EXPERIMENTAL

Instrumental Setup

The fractionator was the same as in the previous work.[2] It is a prototype version derived from the commercial model F-1000 Universal Fractionator (FFFractionation LLC, Salt Lake City, UT). The standard ceramic accumulation frit of 5 μm porosity is substituted with a new frit made of sintered alumina. This frit has a porosity low enough to prevent penetration of particles with diameter higher than 1 μm, and it is machined to obtain a perfectly flat and smooth surface. Because of its low porosity, this frit can be used as accumulation wall with micrometer-sized dispersed samples, with no need of a membrane. The fractionator was disposed vertically to prevent the influence of gravity on the perpendicular field. Channel nominal dimensions were: 29.4 cm in length from tip to tip, 2.0 cm in breadth, 0.0254 cm in thickness. Nominal channel volume was 1.41 cm^3.

Sample injection was made through a Rheodyne valve, model 7125 (Rheodyne, Cotati, CA), whose loop was 17.83 ± 0.06 μL (N = 30). The loop volume was calibrated by applying the Beer-Lambert-like law for flow through systems to measured peak areas from flow injection of K_2CrO_4 in Na_2HPO_4 0.05 M at 373 nm, for which the molar absorptivity ε is known (4820 L mol^{-1} cm^{-1}).[10] The loop volume can be obtained from the slope of the linear regression analysis on peak area $\hat{A}$ (min) vs. $10^3 \ \varepsilon \ b \ c / \ \dot{V}$[9] (six values of c, five repeated measurements).

The nominal longitudinal flow rate ($\dot{V}$) was always 6.0 cm^3 min^{-1}, and it was generated by a SSI pump series II (SSI, State College, PA). For each injection, the actual value of $\dot{V}$ was measured on line with a burette. The difference between nominal and experimental values was never higher than 0.2 cm^3 min^{-1}. In the case of analysis with a field applied, the cross-flow rate was always 2.3 cm^3 min^{-1}, and it was generated by a Varian pump, model 2510 (Varian, Walnut Creek, CA). Two four-way valves were employed: the first to switch between the two cross-flow modes (recirculating, non recirculating), the

second to switch between the two longitudinal flow modes (direct and back-flushing). One six-way valve Valco model E60-230 (VICI, Onsala, SE) was employed to switch between stop-flow mode and run mode.

The UV/Vis detector, used as a turbidimeter, was a Dynamax model UV-1 (Varian) operating at 330 nm. The acceptance angle was measured as reported elsewhere.[5] The result was 8.3°. The path length was 0.813 ± 0.009 cm ($N = 15$). It was calibrated using the same spectroscopic standard used to calibrate the loop. Various solutions of known concentration c were continuously flushed through the detector cell. The path-length b was obtained as the slope of the linear regression analysis on absorbance A vs. (ε c) (five values of c, three repeated measurements).

The signal obtained from the Dynamax detector was captured through a 12-bit I/O DAQ board model Lab PC+ (National Instruments, Austin, TX), plugged into a PC Pentium III 350 MHz driven by LabView based software.

Samples, Mobile Phase, and Injection Procedure

For all measurements, standard NIST/Traceable monodisperse polystyrene microspheres (PS) (Duke Scientific Corp., Palo Alto, CA) were employed. Diameters and other specifications of the employed PS are reported in Table 1.

When the cross flow was applied, injection was performed at a flow-rate lower than the elution flow rate, and calculated to correspond to an injection time of 3 s. The injection time is the time required to sweep the sample down to the tapered inlet of the channel. Experiments were performed in stop-flow mode. Stop-flow time was calculated as the time to allow the cross-flow to fill one void volume. Elution was performed with the cross-flow line switched to recirculating mode. Back-flushing at 10 $cm^3\,min^{-1}$ with the cross flow switched off, was applied after each elution for cleaning purposes. When no field was applied, injection was performed in stop-less mode.

Table 1. Specifications for the PS Samples

Standard Sample	d (μm)	St. Dev. for d (μm)	ρ (g cm^{-3})	Batch Concentration (% w/w)
PS 4 μm	4.000	0.04	1.05	0.35
PS 7 μm	6.992	0.07	1.05	0.30
PS 10 μm	9.975	0.09	1.05	0.22
PS 10 μm	10.15	0.10	1.05	0.20
PS 15 μm	15.02	0.15	1.05	0.30
PS 20 μm	20.00	0.20	1.05	0.31

Mobile phase was pure Milli-Q grade water produced by Simplicity 185 (Millipore, Bedford, MA) with sodium dodecyl sulfate (SDS) 0.01% w/v (Sigma-Aldrich, Steinheim, Germany) and Tris (tris-hydroxymethylamino-methane) 5 mM at pH = 9.6 (Sigma-Aldrich) added.

RESULTS AND DISCUSSION

All the obtained data are calculated with 95% probability. Each measurement was repeated at least three times. Errors bars reported in Figures correspond to the error on the mean value. In some cases, the error bars are not shown, since the results were smaller than the displayed symbols.

Validation of the Quantitative Single-Run Method

Absolute recovery is defined as the ratio between eluted and injected sample mass. To evaluate absolute recovery of the sample, a common practice is to determine the ratio between sample peak areas obtained for on-channel and off-channel runs.[6] In this method, two different experiments are required to evaluate absolute recovery. The sample is first injected through the channel at given values of longitudinal and cross flow rate. Successively, the channel is excluded from the system and the same amount of sample is directly injected in the detector cell, at the same longitudinal flow rate. The main drawback of this method is that peak areas are measured by independent runs. Consequently, the evaluation of absolute recovery is affected by inter-run uncertainty. However, if the area obtained by the second experiment (off-channel peak area) is predictable, this problem can be bypassed. Prediction of off-channel peak area can be done by the single-run quantitative method (Eq. 1). In fact, all the instrumental parameters have been accurately calibrated, the flow rate $\dot{V}$ was measured during each injection, and the extinction coefficient κ can be calculated through Eq. 2. However, Eqs. 1 and 2 should be validated in the membraneless St/Hyp/FlFFF-UV/Vis system used here.

In order to perform the validation of the single-run quantitative method for the prediction of off-channel peak areas, three PS with different d were employed, with diameters 10, 7, and 4 μm. For each diameter, various PS samples were prepared at different concentrations. Each sample was run off-channel, and each run was repeated three times. For each flow injection, the percentage ratio between the observed peak area and the area predicted by Eqs. 1 and 2 was calculated. This ratio was called $\hat{A}_{obs}/\hat{A}_{calc}$. The model is validated if $\hat{A}_{obs}/\hat{A}_{calc}$ is not significantly different from 100%. Figure 1 reports the obtained results. It can be seen, that the average values of $\hat{A}_{obs}/\hat{A}_{calc}$ never differs from 100% more than

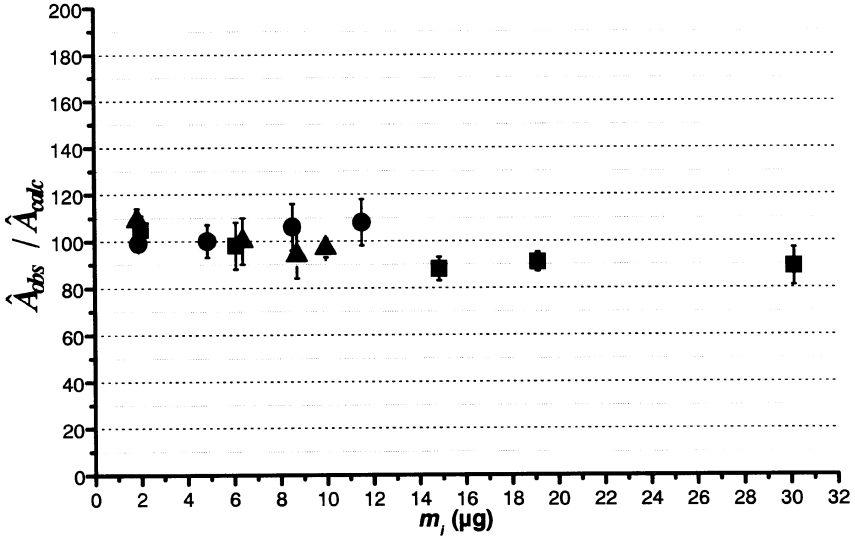

Figure 1. Prediction of off-channel area by the single-run quantitative method (Eq. 1). ●: PS 4 μm. ■: PS 7 μm. ▲: 10 μm.

7%. Such an error is not significant if one considers that the discussed equations contain numerous parameters determined by calibration and, hence, affected by experimental errors. Moreover, the batch concentration of samples is given by the manufacturer as "approximate". The sum of all these sources of errors may likely be as high as 10%. The wider error bars observed for low sample loading values can be due to the low signal to noise ratio, which makes the choice of baseline for peak integration critical. The results reported in Fig. 1 allow calculation of absolute recovery, as the ratio between the experimental peak areas and the off-channel peak areas calculated by Eq. 1, where m is the injected mass.

In previous literature, the area measured by injecting the sample through the channel without any applied field was, in fact, employed in order to determine the absolute recovery, instead of the off-channel peak area. However, it is not at all evident that this practice is correct for FlFFF operations. In fact, in the absence of an applied field, sample particles experience the whole channel volume and they could be lost by diffusion through the depletion frit, that in our case has a standard porosity of about 5 μm. In order to test whether this practice can be applied to the system employed here, experiments and calculations analogous to those performed for data reported in Fig. 1 were performed. In this case, the only difference is that the channel was on line and the cross flow was switched off. Runs were then performed by using the same samples and longitudinal flow rate,

reported above. Results are reported in Fig. 2. It can be seen that $\hat{A}_{obs}/\hat{A}_{calc}$ values are not significantly different from 100%, with differences between average values and 100% ascribable to the experimental uncertainty in sample concentration and instrumental calibration. Hence, the experimental evaluation of off-channel peak areas as the peak areas obtained on-channel without field, works correctly in the present system. However, it is noteworthy, that this practice involves two separate injections. The method based on area calculated through Eq. 1 is, therefore, preferred here.

Effect of Sample Loading on Recovery

Figure 3 reports the absolute recovery values determined as the ratio between measured peak areas and area values calculated by Eq. 1. The same three samples used above, each one eluted alone in a separate run, are considered for different injected amounts. For all the sample diameters, the observed recovery is very high with respect to previous results obtained with membrane in similar conditions.[2] For each diameter, no effect of sample loading is found. This implies; that absolute recovery is independent of the injected mass here. As a consequence, quantitative applications, such as the calculation of the limit of

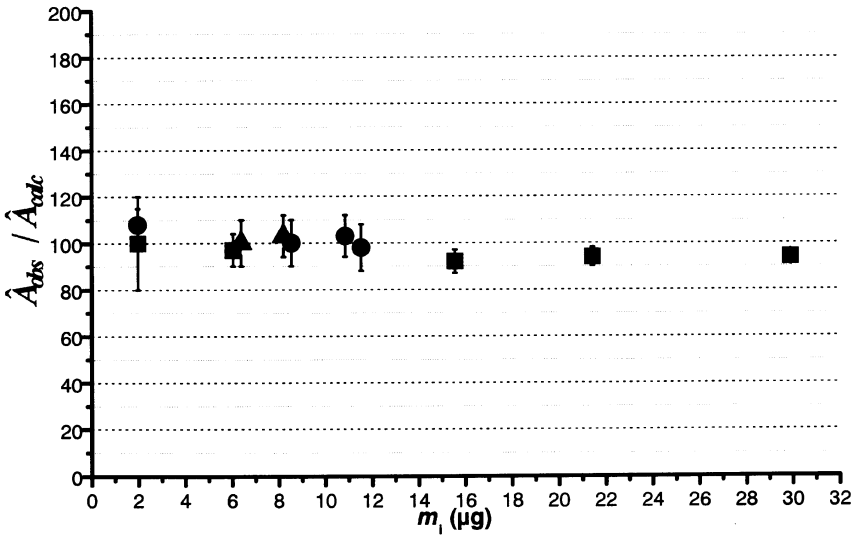

Figure 2. Prediction of off-channel area by areas measured without applied field. ●: PS 4 µm. ■: PS 7 µm. ▲: 10 µm.

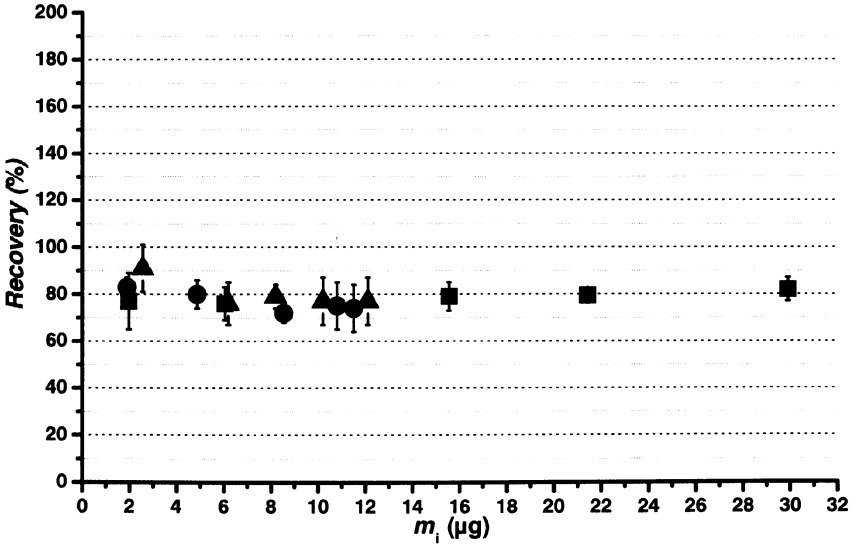

Figure 3. Percentage recovery with respect to area predicted by Eq. 1. Separate elutions. ●: PS 4 µm. ■: PS 7 µm. ▲: 10 µm.

detection (LOD) from $\hat{A}$ vs. c ($c =$ concentration in g cm^{-3}), and the accurate conversion from signal to mass in PSAD practice are possible.[3,5]

Some interesting features on recovery can be noticed when samples constituted of mixtures of particles of different diameters are examined. Figure 4 reports recovery values as a function of total injected amounts in the case of mixtures of all the PS particles, which were each one in equal quantity. Recovery is evaluated only for PS 4 µm and 7 µm, because for PS 10, 15, and 20 µm, peak resolution resulted to be lower than unity. In this case, the evaluation of retention times is obtained from peak maxima, while accurate peak area measurements are not possible. Otherwise, peaks for PS 4 and 7 µm were baseline resolved. Although it was possible to change $\dot{V}$ and $\dot{V}_C$ in order to obtain baseline resolution for all the PS samples, it was preferred to maintain $\dot{V} = 6\,cm^3\,min^{-1}$ and $\dot{V}_C = 2.3\,cm^3\,min^{-1}$ in order to compare data obtained in previous works under the same conditions.[2] Figure 4 shows a decreasing trend in the absolute recovery with increasing total sample loads. Moreover, when PS 4 µm is injected in mixture, its recovery is significantly lower than when it is injected alone (see Fig. 3). The data point in Fig. 3 that corresponds to the range of 5 µg of injected PS 4 µm can be compared, for instance, to data points in Fig. 4 that correspond to the range 20–30 µg of the injected PS mixture amount, in which PS 4 µm was added at 4–6 µg. It can be observed, in fact, that in the case of single elution, the

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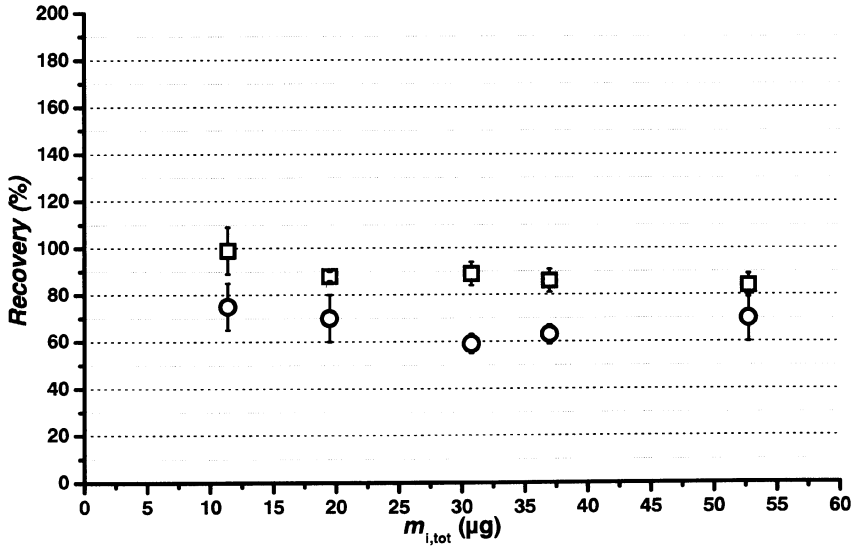

Figure 4. Percentage recovery with respect to area predicted by Eq. 1. Elution of mixtures of 4, 7, 10, 15, 20 µm in equal quantity. ○: PS 4 µm. □: PS 7 µm.

average absolute recovery for PS 4 µm is 80%, while in the case of PS mixture the average absolute recovery is 60–70%. For PS 7 µm, the opposite effect is, indeed, observed. Recovery in mixture is higher than for the separate injection of PS 7 µm. The recovery of PS 4 µm, thus, resulted to be significantly lower with respect to PS 7 µm at any sample load if the two PS were injected in mixture. This is equivalent to saying, that the proportionate recovery of PS 4 µm with respect to PS 7 µm is lower than 100%. This finding may be related to the particle elevation values during elution, which can be influenced by the total particle mass. Further investigation about this issue is in progress and may be discussed in a future paper.

A different effect of sample loading on absolute and *proportionate recovery* when different analytes are simultaneously injected, was not expected. In fact, it seems difficult to explain this result through theoretical models. However, an empirical interpretation of this effect may be carried out. Figure 3 shows that even at a sample load of 30 µg for a single analyte, when this analyte is injected alone no effect on absolute recovery is evident. A sample load of 30 µg for a single analyte would indeed correspond to a total injected mass of 150 µg, if a mixture of all five PS is considered. However, a strong effect of the injected amount on absolute recovery is observed with mixtures (Fig. 4), even at total injected amounts of 10 µg. Such a result could be related to effects that take place during

relaxation processes. Further experimental work is needed to explore relaxation when particles of different size are simultaneously present in the sample. Results in this paper suggest that particular care to total sample loading is necessary when mixtures are injected. Figure 4, in fact, shows that for the PS 7 μm injected in mixture at low sample loads, the relevant recovery is not significantly different from total recovery. This is the first case in which a recovery of 100% was obtained in our FlFFF experiments. It is not easy, indeed, to find many cases of total recovery in the relevant literature. It must be recalled, that in the case of total recovery, the conversion from signal to eluted mass is straightforward. As a consequence, no verification of sample loading effect on quantitative results is needed for the application of the PSAD method. Total recovery can also escape sample carry over effects. This has been found a fundamental feature for FFF applications to real samples.

Evaluation of the Limit of Detection

When absolute recovery is independent of sample loading, the calculation of the limit of detection (LOD) from area vs. injected mass data is immediate. The same data reported in Fig. 3 was then re-elaborated to calculate relevant LOD values. Points were re-plotted as $\hat{A}$ vs. c. Each c data point corresponds to three repeated measurements of $\hat{A}$. Hence, the degree of freedom for the statistic determination of the LOD is 13. The LOD was determined by interpolation, as the abscissa of the point of the lower 95% confidence hyperbole, whose ordinate is equal to the intercept of the upper 95% confidence hyperbole.[11] Linear regression results for the determination of LOD are reported in Table 2. In all cases, the linear correlation is good and the intercept is not significantly different from zero, as requested. As expected, sensitivity is higher for smaller particles. In fact, sensitivity is determined by the extinction coefficient, which is inversely proportional to the diameter (see Eq. 2). However, the LODs obtained for the three diameters turned out to be comparable. This should be ascribed to the worse

Table 2. Linear Regression Parameters for the Determination of the Limit of Detection

Diameter (μm)	Correlation Coefficient	Intercept (min)	Slope (min μg^{-1})	LOD (μg)
4	0.995	$(6 \pm 8)\ 10^{-5}$	$(1.49 \pm 0.09)\ 10^{-4}$	2.0
7	0.999	$(-5 \pm 5)\ 10^{-5}$	$(9.8 \pm 0.3)\ 10^{-5}$	2.3
10	0.992	$(3 \pm 4)\ 10^{-5}$	$(6.1 \pm 0.5)\ 10^{-5}$	2.4

signal to noise ratio for smaller particle peaks. In fact, for the same area value, the peaks that elute at higher retention times have lower height and, hence, the choice of the baseline position affects the peak area evaluation more critically.

CONCLUSIONS AND PERSPECTIVES

A very good absolute recovery (at least 80%) has been always observed in membraneless St/Hyp/FlFFF on PS samples eluted in separate runs. Limits of detection of about 2 μg were determined. The conditions at which the absolute recovery is independent of sample loading have been found. At low sample loading, total recovery has been measured. At high sample loads of particles in mixture, an effect on absolute and proportionate recovery appears.

These results show that membraneless St/Hyp/FlFFF can be used for quantitative applications in the fractionation and characterization of supermicron real samples. Applications to samples such as cells, bacteria, and yeasts are in progress.

A natural development of the present work is the investigation of the effect of sample loading on retention parameters. The choice of conditions for which retention is independent of sample loading effects would allow for the accurate conversion of retention times into size. This will be the subject of a future paper.

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REFERENCES

1. Ratanathanawongs-Williams, S.K. Flow Field-Flow Fractionation. In *FFF Handbook*; Schimpf, M., Caldwell, K.D., Giddings, J.C., Eds.; John Wiley & Sons Inc.: New York, 2000; 257–278.
2. Reschiglian, P.; Melucci, D.; Zattoni, A.; Mallò, L.; Hansen, M.; Kummerov, A.; Miller, M. Anal. Chem. **2000**, *72*, 5945–5954.
3. Reschiglian, P.; Melucci, D.; Zattoni, A.; Torsi, G. J. Microcolumn Sep. **1997**, *9*, 545–556.
4. Reschiglian, P.; Melucci, D.; Torsi, G.; Zattoni, A. Chromatographia **2000**, *51*, 87–94.
5. Reschiglian, P.; Zattoni, A.; Torsi, G.; Melucci, D. Rev. Anal. Chem. **2001**, *20*, 239–269.

6. Ratanathanawongs-Williams, S.K.; Giddings, J.C. Sample Recovery. In *FFF Handbook*; Schimpf, M., Caldwell, K.D., Giddings, J.C., Eds.; John Wiley & Sons Inc.: New York, 2000; 325–344.
7. Zattoni, A.; Melucci, D.; Torsi, G.; Reschiglian, P. J. Chromatogr. Sci. **2000**, *38*, 122–128.
8. Giddings, J.C. Science **1993**, *260*, 1456–1465.
9. Reschiglian, P.; Zattoni, A.; Melucci, D.; Locatelli, C.; Torsi, G. Recent Res. Devel. Appl. Spectros. **2000**, *3*, 61–80.
10. Burke, R.W.; Deardoff, E.R.; Menis, O. J. Res. Natl. Bur. Stand. **1972**, *76* (A), 469.
11. Miller, J.C.; Miller, J.N. *Statistics for Analytical Chemistry*; Ellis Horwood: Chichester, UK, 1993; 101–168.

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