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Short Communication

Improved accuracy in the determination of field-flow fractionation elution volumes

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ABSTRACT

Conventional operation of field-flow fractionation (FFF) systems involves carrying out the analysis at a constant flow of carrier; the flow is temporarily interrupted after injection of a sample in order to permit its equilibration under the applied field. Retention is calculated as the ratio of elution times for a non-retained species and the sample of interest, respectively. Such time-based retentions are only valid if the flow-rate is precisely known at all times during the run. The peristaltic pumps often used with FFF equipment are shown to have an output which varies unpredictably in time. Furthermore, initiation of flow after relaxation is shown to result in significant periods of transient behaviour while the system adjusts to the operating pressure. These and other variations in flow-rate can be eliminated as sources of error by basing the retention measurement on effluent weight, rather than on time. For this purpose, an electronic balance is interfaced with the system's computer, so that detector response/effluent weight data pairs are continuously monitored during the course of the FFF analysis.

INTRODUCTION

Elution volumes in field-flow fractionation (FFF), as in ordinary column chromatography, are generally determined from observed elution times at what is assumed to be constant rates of delivery of carrier, or mobile phase. These times are measured either directly, from the run-time associated with the peak maximum for an eluting component, or indirectly, from measurements of the distance on a chart recording between points representing the start of the separation process and the location of the peak maximum, respectively. Since the fundamental parameter of thermodynamic significance in these quasi-equilibrium processes is the elution volume, which directly relates to elution time only at a constant or well-controlled flow-rate, it is evident that significant errors may be introduced into the measurements if the mobile phase is delivered at an unstable or uncontrolled rate [1].

Characteristic for the FFF columns is their open geometry which provides little resistance to liquid flow; in fact, under normal operating conditions, the column

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pressure rarely exceeds 15 p.s.i. The carrier is, therefore, often successfully delivered to these columns by means of relatively inexpensive, low-pressure peristaltic pumps. Despite the many conveniences offered by these pumps, such as a wide range of flow-rates, the potential for electronic control of the pump speed, and the low cost, they have a clear disadvantage in that their output tends to vary with time. Factors such as temperature fluctuations in the laboratory, slight obstacles in the flow path, and ageing of the pump tubing, all have a significant influence on this output. Even the more elaborate pumping systems normally used in high-performance liquid chromatography are known to occasionally generate flow-rates which vary in time [1–3]. Retention data from FFF experiments using peristaltic or other pumps with non-constant output should, therefore, ideally be based directly on recorded elution volumes, rather than on elution times as is commonly done.

A parameter of utmost importance to any FFF analysis is the void volume, V^0, which is the reference point in any determination of retention ratio R [4,5]. Often, V^0 is determined from the elution volume of a non-retained compound mixed in with the injected sample. Since the void volume is small compared to most practical elution volumes of retained compounds for which analytical information is sought, any error in this number is of particularly large consequence for the accuracy of such retention-derived characteristics as the particle diameter or molecular weight.

Very often, the field-induced relaxation of a sample into its equilibrium distribution is allowed to occur in the absence of carrier flow [6]. As pumping is resumed, there is likely to be a certain lag period during which the system's pressure builds up to the steady state level associated with a desired flow rate. If the flow is assumed to be constant during this period, and elution volumes are calculated from elution times, serious errors could result. In particular, if the lag period is comparable in length to the time required to sweep out one column volume, such errors would strongly affect the measured void volume and, by implication, the accuracy of any analytical information gained from the FFF experiment.

In the present work, we have chosen to replace the time-based measurements of elution volume with a direct read-out of effluent weight via an inexpensive electronic balance interfaced to the system's computer. The advantages of this approach are illustrated in runs of both short (void volume) and long duration.

Upon injection into the FFF channel a sample is exposed to the applied field, which in the present context is a sedimentation field. Several other fields have been used successfully to accomplish separation in the FFF mode [5], and the experimental approach described here is generally applicable to any FFF system. Under the influence of the field, the different sample components migrate to that channel wall where their potential is minimized. After a certain relaxation time [6] the field-induced migration to the wall is exactly balanced by diffusion; at this point, each component has established a unique equilibrium distribution in the channel the thickness of which is reflected in the observed retention. From measurements of the volume V_e needed to sweep out the sample, and the void volume, V^0, one can therefore determine this thickness which, in turn, leads to information about the appropriate sample property, *e.g.* molecular mass or particle size in the case of sedimentation FFF [4].

During their migration through the channel, the sample zones will broaden due to a variety of causes. For a monodisperse material, the most important factor is generally the velocity-dependent non-equilibrium effect [7], which results from the fact

that the laminar flow of carrier will move different layers of the sample cloud at different velocities, thus disturbing its equilibrium distribution. Diffusive corrections to these disturbances are not instantaneous, and the non-equilibrium zone broadening (H_{neq}) will therefore increase with increasing carrier velocity. In the limit of high retention,

$$H_{neq} = 24\lambda^3[w^2 < v > /D] \quad (1)$$

where parameter λ [approximately equal to $(V^0/V_e)/6$] represents the dimensionless thickness of the sample layer [4]. Here, w is the channel thickness and D is the sample's diffusion coefficient. From this expression it is easily seen that the reduced efficiency resulting from fast channel flow $< v >$, can well be offset by operation at higher retention (smaller λ values). Early on, this condition suggested that rapid, high-resolution separations could be obtained at constant field, using a carrier flow which is programmed to increase during the course of the run [8]. The use of this promising approach will require precise knowledge of the magnitude of the flow-rate throughout the entire separation process.

METHODS

The overall experimental arrangement is shown in Fig. 1. Here, three items are of particular importance, namely the valve A, which permits by-passing the column

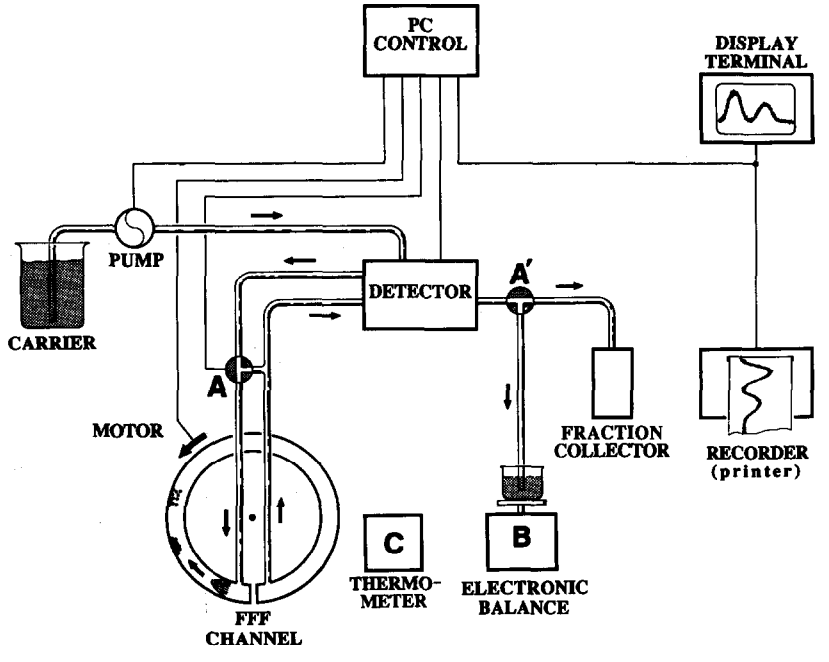

Fig. 1. Experimental arrangement involving the use of an electronic balance to measure flow-rates. A and A' are two magnetic valves under computer control, which determine the directions of flow before the inlet to the channel and after the detector, respectively. A read-out of the temperature from the thermometer C is fed into the computer before the program begins. Aside from controlling the pump, the system's computer also controls the centrifuge motor, and thus the channel's spin rate, via a feed-back mechanism. See also text.

during relaxation, the balance B which monitors the weight of effluent from the system, and the digital thermometer C. The first two items are interfaced with the system's computer, as indicated in the figure, and the third provides a separate record of the ambient temperature to enable a correct assessment of the carrier density. At no time was the temperature fluctuation larger than ± 0.25°C during the course of a 2-h run.

The sedimentation FFF system was built in house, essentially according to descriptions published previously [9]; the length, breadth, and thickness dimensions of the separation channel are 94.0 cm, 2.0 cm, and 0.0254 cm, respectively, and both ends are tapered with an angle of 60°. The void volume V^0 is 4.78 ml, as determined from the elution volume for non-retained acetone. Carrier (0.1% aqueous FL-70 from Fisher Scientific) is fed to the system via a peristaltic pump of type Minipuls 3 from Gilson, which is controlled by the computer. The optical density of the effluent is monitored at 254 nm by means of a Linear Model 106 UV detector, the signal of which is fed to the system's IBM-compatible AT personal computer (PC), which stores the output as amplitude/time pairs of data. The effluent is collected in a vented polythylene receptacle which is placed on top of an OHAUS electronic balance Model C501 with an interface port, designed to weigh with an accuracy of 0.1 g in the 0–500 g range. The output port from this balance is connected to the computer's RS-232 port, which stores its signal as weight/time pairs of data. By-pass valve A is likewise connected to the computer. A digital thermometer of type Sigma, Model HH22 is reporting the temperature of the experiment with an accuracy of 0.1°C. The samples used to establish Table I are polystyrene latex standards with a density of 1.057 g/ml, obtained from the BASF Corporation.

The following is an outline of the course of the analysis: The pump is instructed by the computer to deliver carrier at a low rate (0.5 ml/min) in preparation for the injection (commonly 1–25 μl), which is made directly at the head of the channel. In order to ensure that the sample fully enters the separation chamber, the pump is allowed to deliver carrier for an additional 15 s before being turned off. At this point valve A is automatically closed, and the rotor accelerated to a specified spin rate; as this

TABLE I

REPRODUCIBILITY OF PARTICLE SIZE ASSIGNMENTS UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Sample: polystyrene latex spheres with a density of 1.057 g/ml. Carrier: 0.1% (v/v) aqueous FL-70 with a density at 25°C of 0.9973 g/ml. Average flow-rate was 3.10 ml/min.

No.	Diameter (nm)						Average diameter (nm)
	RPM						
	1000	1100	1200	1300	1400	1500	
1	257	248	254	251	253	253	253 ± 3
2	308	300	303	301	301	300	302 ± 3
3	357	349	352	347	344	342	349 ± 5
4	425	421	421	409	—[a]	—[a]	420 ± 5

[a] Data excluded due to the strong wall effects seen at high retention.

rate is reached, the computer starts the count-down towards the end of the relaxation period; 2 min prior to reaching this end, while the channel is still being by-passed, the pump is automatically adjusted and starts delivering carrier at the faster flows desired for elution. This pressurizes all parts of the system with the exception of the channel itself. At the end of the relaxation period the computer opens valve A, now shunting all flow through the channel whose effluent is being collected in the receptacle on the balance. The detector signal, recorded by the computer as a function of effluent weight, is plotted versus the computed elution volume V_e on the computer screen, which also displays the instantaneous rate of flow through the channel. Data analysis is performed at the end of the run. The valve labelled A′ in the figure permits a temporary interruption of the flow-rate measurements to allow the collecting of fractions.

RESULTS AND DISCUSSION

Peristaltic pumps are inexpensive tools for delivering mobile phase to low-pressure chromatographic systems, and their output is, in principle, easily controlled by an external voltage regulator. In practice, however, the output is not fully predictable, but shows fluctuations both of high [3] and low frequency. The high-frequency pulsing is inherent in the operation of these pumps; however, its time constant is such that it has no effect on flow-rates measured during a typical FFF run, which lasts several tens of minutes. By contrast, slow changes over long periods of time, such as those recorded in Fig. 2, tend to introduce errors in retention ratios R based on measured elution times rather than on elution volumes. The figure displays the weight-based flow-rate at time t_n, $V(t_n)$, computed as the arithmetic mean of five consecutive weight/time data pairs:

$$V(t_n) = \frac{1}{5} \sum_{i=n-4}^{n} \frac{(w_i - w_{i-1})}{(t_i - t_{i-1})} \tag{2}$$

Here, w_i is the weight of the collected effluent at time t_i. Whether the variations in flow-rate are undesired, as those displayed in Fig. 2, or intended, as in the case of operation under programmed flow [8], the continuous measurement of effluent weight

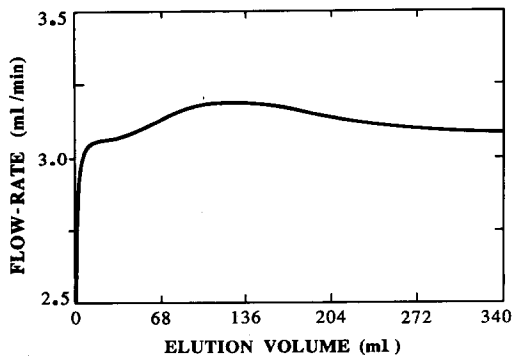

Fig. 2. An illustration of the flow-rate variations observed during a typical 2-h run.

offers clear analytical advantages, due to its direct proportionality to V_e at constant temperature.

Short-term fluctuations in flow-rate have a particularly severe impact on the determination of the small void volumes V^0 (typically less than 5 ml), which relate linearly to the retention ratio R from which particle diameters, d, or molecular weights are determined [4,5]. In the limit of high retention, R becomes a linear function of λ. Under these conditions, the sedimentation FFF technique generates retentions proportional to d^{-3}, while the flow FFF analogue retains particles in proportion to d^{-1}; thus, an error of *e.g.* 20% in V^0 translates into an error of 6% in d for a sedimentation FFF analysis, while diameters determined by flow FFF, with its lowersize selectivity, will have an error similar to that of V^0.

The measurement of effluent weights, in lieu of elution times, immediately highlighted a source of error that had previously been neglected. Traditionally, resumption of carrier flow at the end of the relaxation period is made by letting the start of pumping coincide with the beginning of separation. From the flow-rate–time relationship shown in Fig. 3 it became obvious that a significant number of seconds elapse between the onset of flow and the establishment of a steady-state of carrier delivery. By assuming that the flow had remained constant throughout, at the steady-state level, the observed elution time for the void peak of 1.3 min translated into a value for V^0 of 3.70 ml. This value is 23% below the weight-based value, determined to be 4.78 ml in good agreement with the 4.76 ml known from the channel geometry. In case one determines the void volume from the elution time of a non-retained compound admixed with the sample, as is commonly done, the sedimentation FFF analysis may therefore yield diameters in error of more than 6% as discussed above. It should be noted that, although R is the ratio of two elution volumes which are both affected by the transient behavior at the beginning of separation, the sample elution volumes, which ideally should be in excess of five column volumes [10], are much less in error than is V^0.

The effect illustrated in Fig. 3 led to the installation of by-pass valve A, as described above under Methods. By starting the pump and pressurizing the system before allowing flow into the channel it is now possible to significantly reduce the time required to reach a steady flow, as shown in Fig. 4. The oscillating behavior seen in these graphs is an artifact introduced by the low precision of the balance (0.1 g) in combination with the high sampling frequency, and persists despite use of the five-point smoothing routine described in eqn. 2.

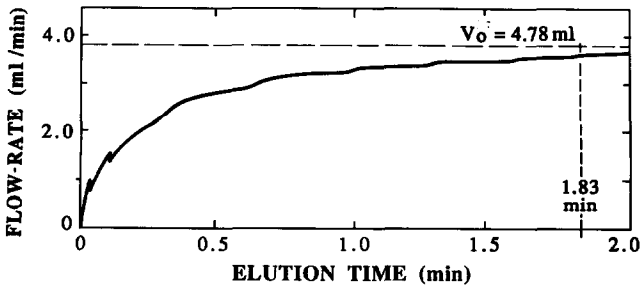

Fig. 3. The time course of effluent weight shows the slow approach to a steady flow during the early stage of a run in the absence of by-pass valve A.

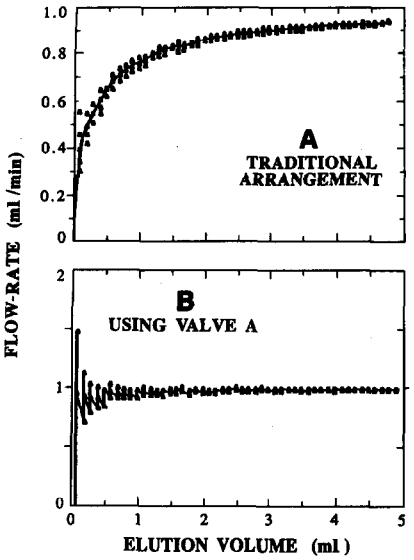

Fig. 4. (A) In the absence of by-pass valve A, the flow is slowly approaching the set level of 1 ml/min. (B) Operation of the by-pass valve as described in the text ensures a rapid establishment of steady flow.

By introducing the operational procedures described above, *i.e.* by weighing the effluent, and pressurizing the system prior to supplying carrier to the separation channel, it is now possible to determine particle diameters under widely varying field strengths, *i.e.* retention times, with a standard deviation of just above 1%, as seen in Table I.

While illustrated by samples from FFF analysis, the use of electronic balances to measure flow-rates is generally applicable to all liquid chromatography techniques, particularly where the system operates under computer control. By incorporating a high-precision balance into the system, one would thus be able to correct such discrepancies as those discussed in ref. 1.

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