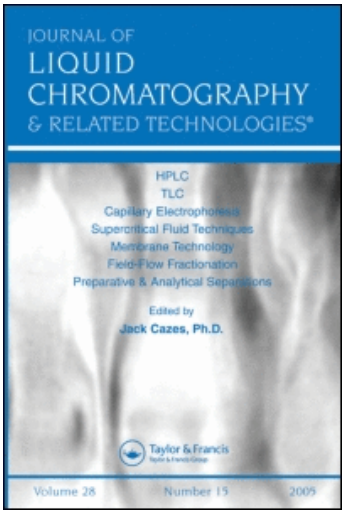

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Assidjo, Emmanuel and Cardot, Philippe J. P.(1997) 'Sedimentation Field-Flow Fractionation at Gravitational Field of Red Blood Cells: Systematic Studies of Injection Conditions', Journal of Liquid Chromatography & Related Technologies, 20: 16, 2579 — 2597
To link to this Article: DOI: 10.1080/10826079708005581
URL: http://dx.doi.org/10.1080/10826079708005581

J. LIQ. CHROM. & REL. TECHNOL., 20(16 & 17), 2579–2597 (1997)

SEDIMENTATION FIELD-FLOW FRACTIONATION AT GRAVITATIONAL FIELD OF RED BLOOD CELLS: SYSTEMATIC STUDIES OF INJECTION CONDITIONS

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ABSTRACT

Field Flow Fractionation (FFF), which uses the gravitational field for retention, elution, and separation of cellular materials, appeared to obtain a constant success. Blood cells, as well as parasites, were separated and isolated using this very simple method. A need emerged, therefore, to evaluate its separation performance. Towards that objective, and because of numerous common features of FFF with chromatography, the injection conditions appeared to be critical. Therefore, we have analysed the effect of injected concentration, of injected volume, and of the specific injection procedure widely used in FFF the "stop flow time relaxation process."

In the light of numerous reports, red blood cells (RBC) appeared to be considered as a "model" for cell analysis in FFF, and were eluted according to a mechanism described as "Steric

Hyperlayer," retention properties of RBC were therefore analysed versus carrier flow rate intensities. Guidelines are given to elute RBC in gravitational FFF depending on the separation objectives i.e., analytical or semi preparative.

INTRODUCTION

Field Flow Fractionation is a family of dynamic separation methods, based on the elution of different sample constituents, in a ribbon-like channel, flowed by a carrier phase of various velocities.[1-3] Sample components are retained according to their sensitivity to a field applied in a direction perpendicular to the flow. Separations are induced and controlled by that field, and the flow.[4-6] Gravitational Field Flow Fractionation[7-10] belongs to the family of FFF sedimentation techniques,[11] the external field is simply the gravitational one.

One of the advantages of this technique in the elution of micron sized material is that the separation occurs in one phase (the carrier medium), which can be very simple. Because of the well established "Steric Hyperlayer" elution mode of micronic material in GFFF[12,13] one can expect that particle-separator interactions would be limited. Such a feature is particularly interesting in the case of biological materials, whose characteristics may be modified because of an interaction process.

For more than ten years, the use of FFF techniques in biological analysis has been in constant development. The feasibility of cell sorting with FFF was demonstrated, for the first time, in 1983, using the sedimentation (multigravitational) technique.[14,15] Flow FFF methods also were employed.[13,16,17]

In the early nineties, the simplest technique which uses a gravitational field regained interest because of its design and operating simplicity.[13,18] Retention and purification of different species of health and biological interest were obtained with this method: filariae,[19] trichomonas,[20] and Pneumocistis.[21] In the case of red blood cell analysis, normal and pathological material were analysed and sub population purifications were obtained.[20-24]

Because of the complex polydispersity of cellular material in terms of shape, of size, of density, it is obvious that it would be difficult to analyse retention data of RBC for the purpose of defining an "elution model." However, some empirical rules will emerge and will be of help for future developement, for separation enhancement, or optimization. Questions which

are studied in this report can be summarized follows: How do we inject the RBC into the FFF Channel? In what volume? At what concentration and flow rate?

MATERIALS AND METHODS

Human Red Blood Cells

Samples are drawn from the same healthy donor, prior to experiments, with the help of a Vacutainer® system (Vacutainer, Maylan, France) and mixed with a sodium salt of ethylene diamine tetraacetic acid (EDTA 1.5% w/w). Samples were stored at 0°C for a maximum duration of 24 hours. Extemporaneous dilutions were performed before FFF analysis by simple mixing of the sample with the carrier phase (v/v). Final concentrations were measured with a Coulter Counter TAII (Coulter Electronics LTD, Luton, UK). Flow system: A Gilson Model 302 (Gilson Medical Electronics, Inc., Middelton, WI, USA) HPLC pump connected to a pulse damper system allowed to obtain 0.1 to 5 mL/min flow rates. It was connected to the FFF separation device by means of a sample injection device.

The carrier phase used in this study is a classical buffer system pH=7.2, 0.15 Mol/L from BioMerieux (PBS 75511, BioMerieux Sa, Marcy l'Etoile, France) added with 0.1%(w/w) of bovine albumin (No A-4503, Sigma Chemical Company, St Louis, Mo, USA); the final carrier phase density was 1.002 g/mL, its viscosity 1.016 cp. Flow rate intensities were systematically controlled by weighing.

The injection device, a Rheodyne valve model 7125i (Rheodyne, Incorporated, Cotati, CA, USA), was used to flow the sample into the channel. Injected volume was set up using Upchurch Peek tubes of different lengths and internal diameters (from 0.508 mm id and 99 mm long for a 20μL loop to 1.016 mm id and 432 mm long for a 350 μL loop). This injection device was connected to the FFF channel inlet by means of a connecting tubing of known length and internal diameter (0.254 mm id, 20 cm long).

When stop-flow injection procedures were needed, the samples were injected into the channel inlet at low flow rate (0.3 mL/min) for a time corresponding to a volume slightly greater than the one corresponding to the injection volume measured by the connection tube.

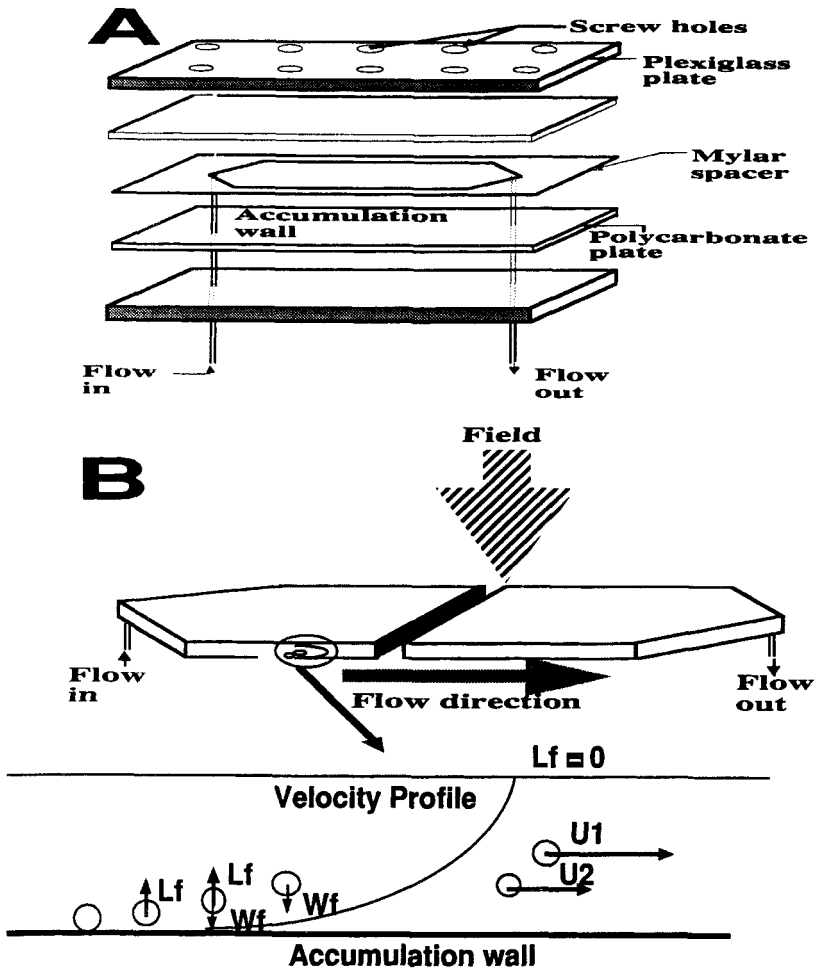

Figure 1. A: Design of the FFF channel, dimensions are: 49.5 ,2, 0.001 cm, inlet and oulet angle of 60°. B: Upper part channel geometry, field and flow directions. b lower part: schematic representation of the "hyperlayer" elution mechanism, Wf is the field-induced force acting on the particle, Lf the lifting force.

Ahead of the injection device, a switching valve model V.100L (Upchurch Scientific, Oak Harbour, WA, USA) was placed to divert the flow away from the channel. Systematic studies were performed by means of ink samples to visualise the sample entrance into the channel for the purpose of defining the experimental value of this volume.

Sedimentation Field Flow Fractionation Device

The gravitational FFF apparatus used in this report was set up in our laboratory; it as been modified from the one previously published by J. C. Giddings[1] to be compatible with biological conditions, and has been completely described by A. Bernard et al.[7] The paralellepipedic dimensions are 49.5 cm in length, 2 cm breadth, 0.01 cm thick; its volume was calculated at 0.99 mL. Including connection tubings and detection cell volume, the total void volume of the system was experimentally measured at 1.132 ±0.06 mL (n = 10) using 25 % (w/w) Dextran solutions (Dextran D-4626, Sigma Chemical Company) in the carrier phase. Planarity in length and breadth was set up and controlled over all experiments. A schematic representation of the system is shown in Figure 1A.

Detection device: A Waters Model 440 Photometer, set at 350 nm (Waters Corporation, Milford, MA, USA), operated in the turbidimetric mode, is connected to the outlet of the FFF channel by means of a Peek tube (0.254 mm id , 20 cm long). Data were recorded with an Atari ST 520 Computer (Atari Corp. , Sunnyvale, CA, USA) using a 14 bytes Keithley M1111 acquisition kit (Keithley Metrabyte Corporation, Taunton, MA, USA) at a 1Hz frequency.

Peak profile analysis: As the peak parameters of the gaussian-like fractogram, which signified the elution of RBC, will be analysed by the method described by B. A. Bidlingmeyer & F. V. Warren Jr.[25] For practicability, retention will be defined with the help of the peak summit elution time; as no statistical differences in the void volume measurements were found when plasma dilutions and Dextran solutions were compared, the characteristic sample plasma protein peak found on each fractogram was used as a probe for void volume measurement. The ratio, summit of the protein peak versus RBC signal summit is defined in this report as the RBC retention ratio. Peak dispersion parameters also will be calculated using analogous procedures, using peak width at different percentage (%) of the peak height, peak profile asymmetry, and equivalent plate height (HETP).

RESULTS AND DISCUSSION

Elution Mode of RBC in GFFF

Elution modes in FFF are now well-defined,[3,13,14,26,27] even in the case of biological material.[14] The separation process is based on the parallelepipedic geometry of a channel, which controls the interaction of particle velocity in the

carrier flow profile with an external field perpendicular to the great surfaces of the channel as schematized in Figure 1A. A pulse of mixed species is flowed into the ribbon-like channel, and species-external field interactions force the former toward one wall. As, usually, the carrier flow profile is parabolic in the channel thickness (laminar flow conditions), the species closer to the wall moves more slowly, being therefore, more "retained" compared to those not or less affected by the field. Therefore, band species are eluted at different speeds. It was established by K. Caldwell[14] and other research groups[13,16,19,20] that RBC and nucleated cells are eluted according to the so called "steric-hyperlayer" elution mode whose specificities are briefly exemplified in Figure 1B, upper part, and have been described by J. C. Giddings.[25] In the representation of Figure 1B, lower part, cells are shown scaled to the channel thickness. In such a model, the cells are focused by the external field (gravitational or multigravitational) into stream lines of the parabolic flow profile of the mobile phase. This focalisation process, whose kinetics and mechanism are, so far, not totally assessed, is one of the most important features in the analysis of cellular material by FFF techniques. The original position of the cell in the channel thickness (at the inlet), and the dynamics of the equilibrium process, as well as the cell characteristics, play a role in the cell travel along and across the channel. Because of the symmetry of the parabolic flow profile, it is of interest to place all the material under study in the accumulation half part of the channel as shown in Figure 1A; this is made possible by driving the cell into the channel directly on the accumulation wall, as set on our device. Moreover, it as been demonstrated that, during elution, even at high flow rates, particle-wall interactions could occur.[15,21] The channel wall, made of polycarbonate material (Lexan®), has been chosen[7] to minimize these interactions; such demonstration is made by simply flushing the channel after elution. Contrary to what has been observed in SdFFF with cellular material,[15] the flushing procedure did not remove any material from the channel, and fractions collected during this procedure were found to be free of any material, as shown on the elution volume dependent fractogram. The flushing procedure, made by simply flowing the channel at high flow rate (3 mL/min) was associated with a single step modification of the baseline intensity, probably due to the modification of the pressure drop in the detection cell as shown in Figure 2A; even at very low flow rates, no sorption of the samples were diagnosed by means of signal intensity and fraction collection.

Because of the linked elution characteristics of species with their position in the channel thickness, the use of retention ratio in FFF is rather practical. It is, therefore, relatively simple to plot fractograms using retention ratio units, as shown, for different flow velocities with the fractograms of Figure 2B. One can observe that such fractogram design showed, clearly, the focalisation process

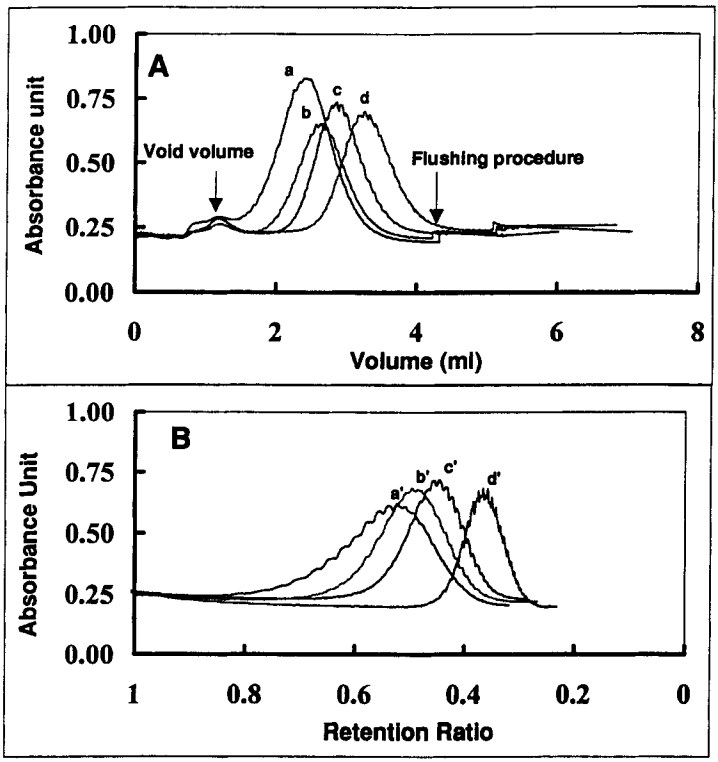

Figure 2. Fractograms of red blood cells in gravitational field flow fractionation, flow injection. 50 μL loop, dilution factor 300, flushing procedure :3ml/min. A: volume scaled signal (a = 0.33 cm/s, b = 0.28 cm/s, c = 0.23 cm/s,d = 0.17cm/s). B: retention ratio scaled signal (a' = 0.30 cm/s, b' = 0.25 cm/s, c' = 0.20 cm/s, d' = 0.13 cm/s).

obtained for micronic species. In particular, the fractogram obtained at 0.13 cm/s appeared much more retained and sharper than at higher average flow velocities. These retention ratio scaled fractograms demonstrated, clearly, the "focused" effect of lifting forces.

Sample Concentration Effect on RBC Peak Profiles

Total blood samples were diluted in a range from 4 (i.e 1 volume of blood added to 3 volumes of carrier phase) to 2000. The RBC injected concentration, thus, ranged from 1.2×10^6 to 2.5×10^3 cells/μL. The injection volume was

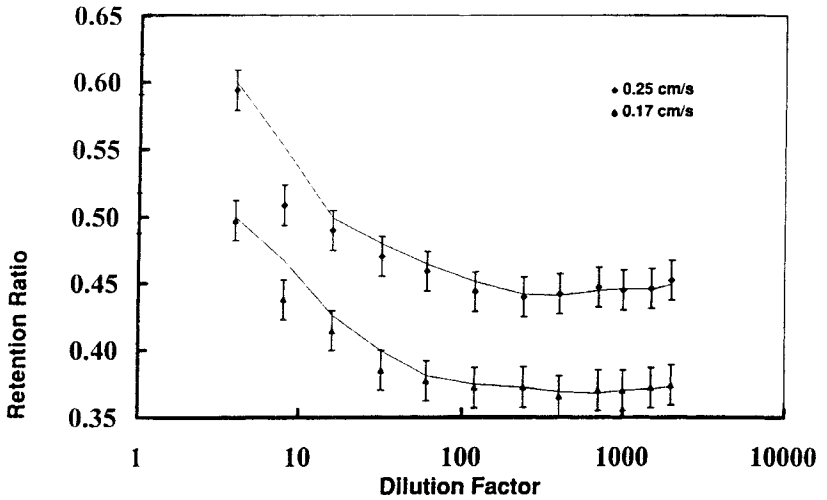

Figure 3. Effect of RBC dilution factor on retention ratio. Flow injection, 50 μL loop, dilution factor varying from 4 to 2000. Statistical data: n = 4, standard deviation = 1%.

chosen at 50μL and flow injections were performed. As the blood's main population is the RBC, these simple dilutions can be assumed to represent a rather pure RBC suspension. Elutions were obtained at two different linear velocities of the carrier phase: 0.17 and 0.25 cm/s. In Figure 3, retention ratio data at different concentrations and statistical estimation were plotted on a semi logarithmic scale, each experiment being performed four times.

Most of the described retention mechanism of micronic species in FFF assumes that, when the sample flows into the channel, the species undergo a field effect which leads to an equilibrium profile in the channel thickness. In the case of a Newtonian suspension, at a sufficient dilution (4% w/w ratio) this equilibrium can be shaped by a decreasing exponential profile. But, there is little information about the modification of the equilibrium profile in the case of concentrated suspensions.

It is observed that retention ratio decreased as dilution factor increased. This can be explained by some unmastered comportement of concentrated samples and/or by some specific features of living RBC suspensions. Rheological studies[28] have shown that a suspension of RBC will behave in a Newtonian manner when its concentration is lower than 10^5 cells/μL. Therefore, one can expect that the elution process is strongly modified, as the

sample band may behave differently at high concentration. It is obvious, in the light of data plotted in Figure 3 that, at high dilution, samples seemed to behave in an analogous way. However, chemometric methods will be of help to define the maximum analytical concentration of RBC.

The chemometric method employed in this report uses a linearity test (ANOVA), for the data. A linear model fitting test is performed over data and we determine the total variation due to the linear model and the variation due to the departure from this model. For that purpose, a total variability which is the sum of the residual variability, the linear model variability, and the variability due to a non linear model, is estimated. Fisher coefficients from the linear model and from the non linear one are calculated and compared, respectively. These Fisher coefficients enable to determine if the data fit a linear model or a non linear one. Such methodology was proposed by M. Feinberg.[29] That procedure is repeated using a mobile averaging technique of 5 successive data points.

For data plotted in Figure 3, it is observed that, at a constant flow rate, no modification of the retention ratio occurred when a dilution factor higher than 240 was used (the linearity test and regression gave a slope value of 0.0±0.02). This dilution value can be considered, for this channel geometry, as the upper usuable one for analytical purposes. Retention ratios were systematically higher when a higher carrier phase velocity was employed, an effect characteristic of the "hyperlayer mechanism."

However, the retention ratio measurement technique, using the summit of the RBC peak profile, may be biased if this profile is strongly asymmetrical, which is observed experimentally in some cases. Therefore, using the same chemometric approach, we have analysed the asymmetry ratio, whose data are plotted in Figure 4. Again linearity test and moving average methods showed that asymmetry ratio was constant as long as the dilution factor is higher than 240, whatever the flow rate.

It is surprising that, in both cases (retention and asymmetry ratios) the "limit" high value was the same. Therefore, as the dilution factor increased, peak profile behaved more symmetrical and more retained; such a combined effect may have an impact on what is considered in chromatography as an "efficiency" measurement, the HETP, whose data are plotted in Figure 5. Again, the chemometric approach of these data showed two domains, whose boundary was a dilution factor of 240. These results showed that the peak efficiency was increased at low flow rate and, when Figures 3 and 5 are compared, such increase depends on the retention of the sample.

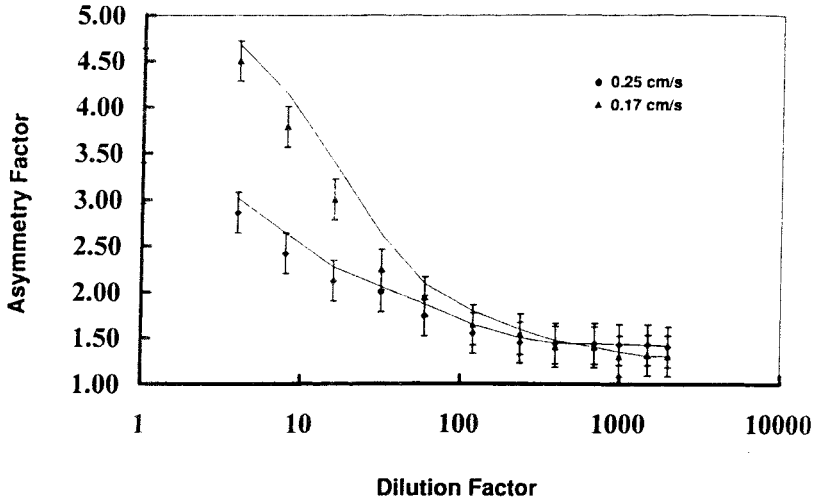

Figure 4. Effect of RBC dilution factor on asymmetry ratio: injection conditions identical to those of Figure 3. Standard deviation 2%.

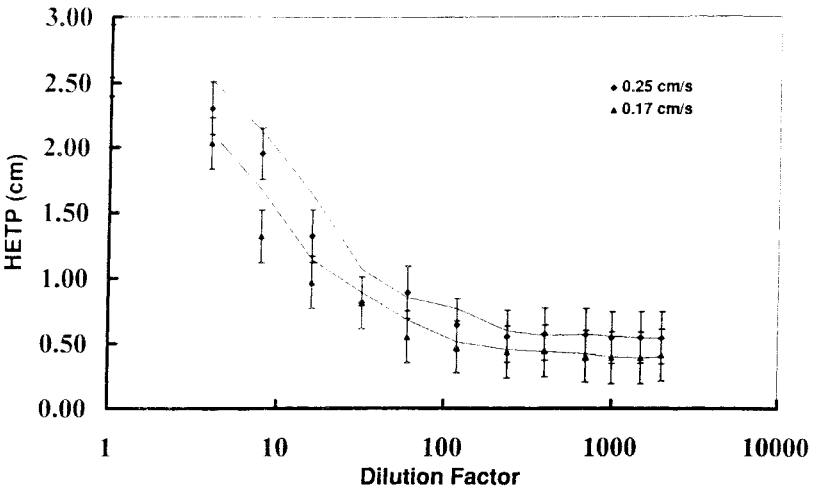

Figure 5. Effect of RBC dilution factor on HETP. Conditions identical to Figure 3, n = 4, standard deviation = 3%.

Because of the complex polydispersity of RBC samples, it is not obvious that the elution conditions of highest efficiency are those of highest separation power. However, HETP can be considered here as a probe for elution conditions comparison. Even if compared to chromatographic techniques, the HETP values are low (0.5 cm), one must have in mind that the species under study are much bigger than any molecule, meso or macromolecules. If we compare the ratio of the length of a theoretical plate (HETP) to the one of a cell (5μm), only 1000 queueing species can be found in a "plate;" this allowed to state that, even with a low plate number per column, such separation system is highly efficient.

Sample Volume Effect on the RBC Elution Characteristics

In the previous developement, the standard injection volume was 50μL, that is to say, 4.4% of the channel void volume. Because of the channel geometry, 4.4% of the channel length or surface is occupied by the injected volume. However, to avoid cell interactions, destruction, or viability decrease, it is necessary to use the greatest dilution of the cells.

Somehow, such procedure may lead to a larger injection volume, which may occupy a larger channel portion. As this injection volume increases, it becomes evident that the available and effective separation channel volume (length) will decrease. There is, therefore, a compromise to be found between dilution factor, injection volume and detection. For that purpose, a constant quantity of cells is injected into the channel at different concentrations, that is, with different injection volume, varying from 20 to 350 μL. The flow rate was chosen to obtain a rather low retention ratio.

As retention ratio values are obtained using the "protein peak method," modifications in the injection volume may modify retention factor calculations. The thicker is the channel, the more important is that effect. To take account of the channel length modification provoked by the injection volume, the protein peak elution volume was decreased by half of the injection volume to simulate the effect of a WISP injection; however, data obtained with both retention ratio methods were not significanlty different. Therefore, the most simple method was used whose results are shown in Figure 6A. Two domains can be found, up to 200μL the retention ratio is constant; this is confirmed again using the chemometric approach described above.[29] However, asymmetry description was also analysed; results are shown in Figure 6B. It is observed that asymmetry ratio was constant up to 200 μL injected into the channel, that is, a volume equivalent to 16% of the system total volume. When efficiency measurements were made, as shown in Figure 6C, major differences arose.

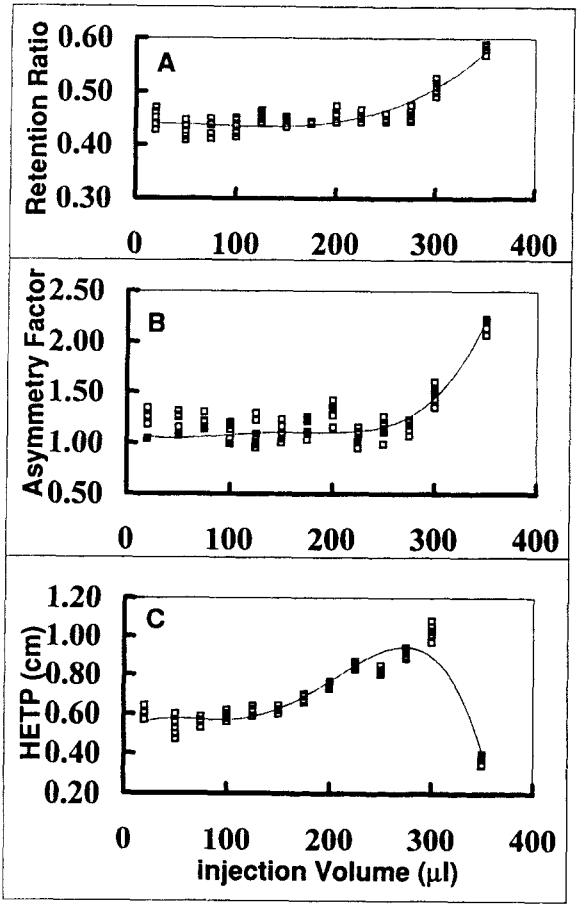

Figure 6. Injection volume effects on RBC fractogram characteristics. RBC dilution factor = 300. Flow linear velocity = 0.25 cm/s. Injection volume varying from 20 to 350 µL (20, 50, 75, 100, 125, 150, 175, 200, 225, 250,275, 300,350). n = 5, maximum standard deviation = 5%. A: Retention ratio, B: Asymmetry factor, C: HETP.

Constant HETP values were only obtained up to 100 µL of injected sample; at 300 µL, that is, at 25% of the void volume, a 100% efficiency loss was observed. Therefore, it can be concluded that, for this channel geometry, a maximum injection volume of 100 µL is possible, which is around 10% of the FFF void volume.

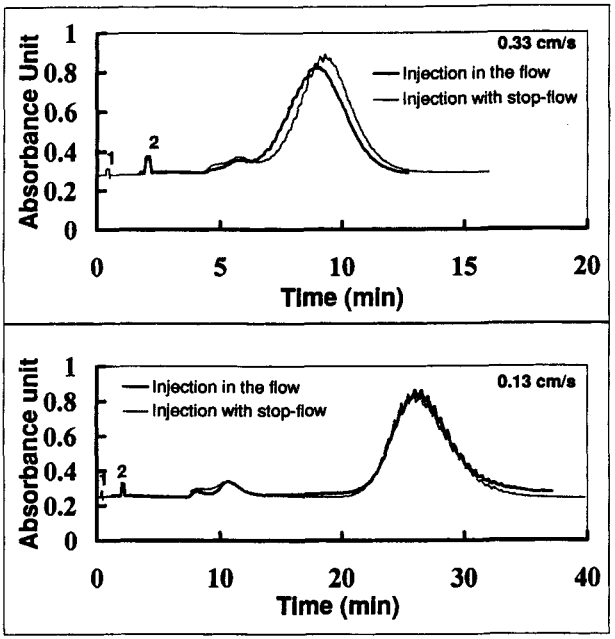

Figure 7. Effect of injection procedure on RBC fractograms. 50 μL loop, dilution factor 300. Stop flow time (when performed) = 2 min. 1: injection with stop flow, 2: injection into the flow. Upper: fractogram differences at high flow rate. Lower: fractogram differences at low flow rate.

This series of results showed clearly that, for a channel of 1 mL, up to 500,000 cells can be injected in a volume lower than 100 μL. As we know now how many cells in a given volume have to be injected in GFFF, the final step of this study is to analyse or define how to place this sample into the separation channel.

Flow and Stop Flow Injection Procedures

Since the early developemental stage of FFF techniques, a relatively original injection mode (compared to chromatography) was used and is now called: Stop Flow Injection or Primary Relaxation Process. If we consider particles or species injected into the channel without flow migration profile, the external field undergoes the distribution of the particles in the channel thickness. After a given time, the field-induced concentration effect, thwarted

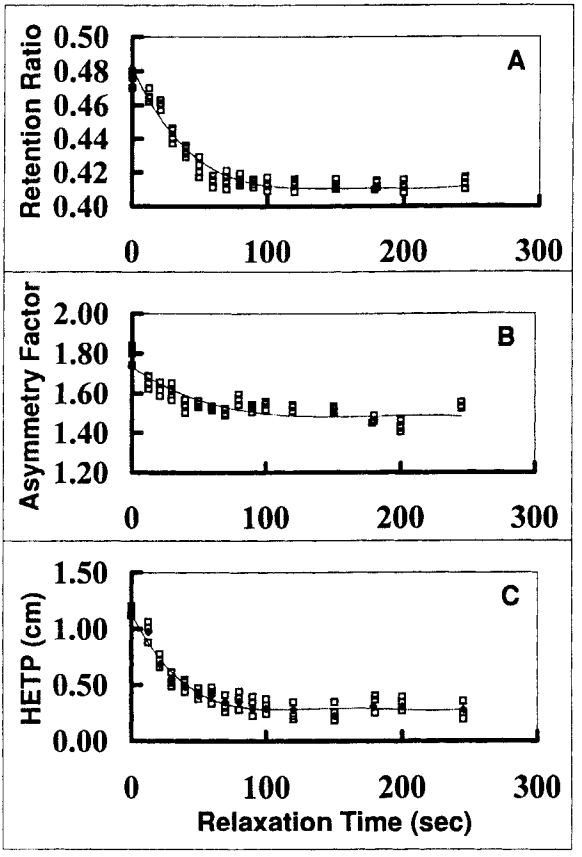

Figure 8. Effect of stop flow time on fractogram characteristics. RBC sample characteristics identical to Figure 7. Carrier phase linear velocity = 0.25 cm/s. Stop flow time varying from 0 to 300 s (0, 10, 20, 30, 40, 50, 60, 70, 85, 100, 120, 150, 180, 210, 250, 300). n = 4, maximum standard deviation = 4%.

by the particle diffusion effect, leads to an equilibrium state. It is obvious that, when particles are injected into the channel when the flow profile is established, a time is needed for the particles to reach this equilibrium state. The stop flow injection procedure, consisting of stopping the flow when all the sample is at the channel inlet for a time long enough where particles can reach this equilibrium state. At high flow rate, the retention factor is decreased when the stop flow procedure is used, as shown in Figure 7, upper part. At low flow rates, fractograms did not show any differences, as demonstrated by fractograms of Figure 7, lower part. Such differences are light because the set up of our device was designed to minimize them.

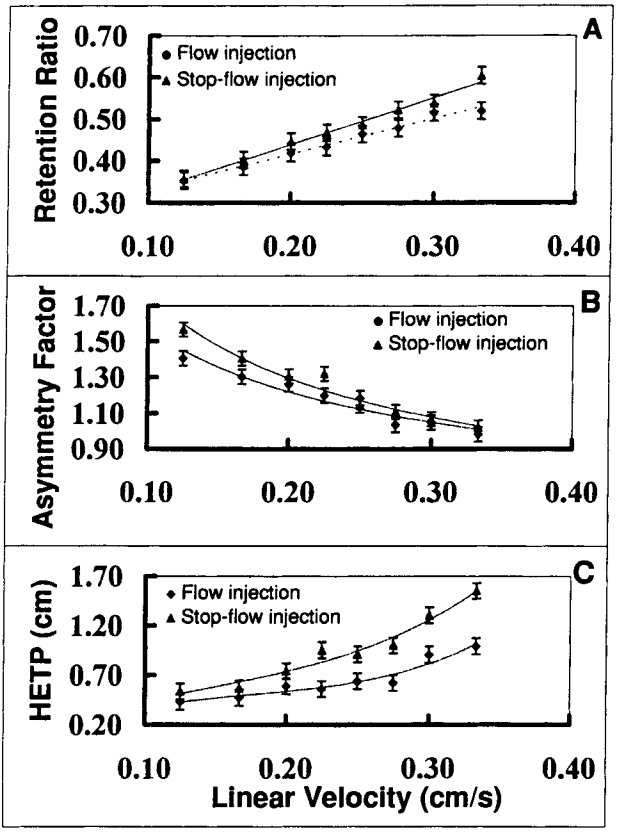

Figure 9. Flow rate and injection procedure effects on fractogram characteristics. RBC samples identical to Figure 7. Linear velocities varying from 0.13 to 0.25 cm/s (0.13, 0.17, 0.20, 0.23, 0.25, 0.28, 0.30, 0.33). A: Retention ratio, B: Asymmetry factor, C: HETP.

However, a closer investigation of peak characteristics allowed to describe the effect of both of these procedures. In some elution cases, even when the inlet tube is connected to the accumulation wall, retention, asymmetry factor, and HETP modifications are important, as shown in Figures 8 A, B, and C. In this study, injection volume and concentration of the samples are kept constant, and stop flow time will vary.

Strong modifications of the parameters under study are observed in the first minute; however, the chemometric approach using the three peak desciption parameters define a minimum stop flow time of 100 sec. There is

an empirical reference method to evaluate this relaxation time. This reference time is considered as the time required for a particle to fall through all the channel thickness (w), for example, with a spherical particle of diameter dp and density rp, falling under the effect of the gravity (981 cm/s^2) in a medium of viscosity h and density rm this time (sed_t) has been established for sedimentation FFF:[30]

$$sed\_t = 18\ h\ w / [\ dp^2(\ rp\text{-}rm)\ G]$$

If we consider the RBC as a sphere whose diameter and density are dp = 5 μm and rp = 1.051, respectively, a channel thickness of 100 μm, a liquid density of rm = 1.002, whose viscosity is 1.016 cp, a sed_t time is calculated at 137 sec. Therefore, this sed_t time is a probe for the "relaxation time" which slightly overestimated the relaxation time. However, if stop flow procedure and relaxation time can be of interest and estimated for monodisperse populations, in the case of highly polydisperse population like RBC, this estimation may be biased for RBC subpopulations. Flow injection of these latter materials may be of help to increase the selective elution of some species subpopulations according to their different sed_t times. As this time is a linear function of the channel thickness, at low flow rates, the channel length covered by the sample is low compared to the channel length.

If the flow increases, that covered length increases also. In the case of a flow injection, the displacement of the sample is a complex function of sed_t and of the "hyperlayer" equilibrium position. There is, therefore, a need to evaluate, experimentally, the balanced effects of "relaxation" and of flow rates.

Data are shown in Figures 9 A, B, and C. Whatever the injection procedure, the retention ratio increase with flow rate increase is characteristic of the presence of "lifting forces" which are at the origin of the "hyperlayer" elution mode of RBC. In all cases, systematic differences are found when the two injection procedures are compared. To point out that injection with "stop flow" procedure or not, generated higher differences at high flow rates and that, if flow rates are low, differences are reduced, driving to non statisitically significant differences.

It can be assumed then, that introducing the samples into the channel at low flow rates may simulate the stop flow procedure. This point is important in the case of biological samples, where species-species interactions will occur if the "local" concentration of the sample is too high or when species channel material interactions appear.

CONCLUSION

Of the specific features of gravitationnal field flow fractionation, in terms of simplicity in the separator design, in terms of simplicity of operation, the results presented in this report may be of practical use in most of the FFF techniques dealing with cellular species. Modifications, easily evaluated, can be set up if channel geometry is modified; however, extrapolation of these results with different wall materials are possible only if proofs are given of the absence of particles sticking at the channel accumulation wall surface. It appeared, in the light of these and, according to other previous work,[31] that it is practical to simply replace the HPLC column of a chromatographic system by an FFF separator to set up an FFF system. Both injection procedures are, therefore, possible, with some slight modifications. For biological elutions in FFF, instead of stop flow procedures, we recommend low-flow injections as already described in other studies.[15,31] When inlet tubing is positionned through the accumulation wall, it could be of interest to use connections of relatively large diameter to avoid complex hydrodynamic effects during injection.

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Received January 2, 1997
Accepted April 10, 1997
Manuscript 4443