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Validation procedures of sedimentation field-flow fractionation techniques for biological applications

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Abstract

Sedimentation field-flow fractionation (SdFFF) offers great potential for the separation of submicrometer and micrometer-sized species. The availability of commercial instrumentation and the versatility of this method originated its success. At this stage of development, SdFFF techniques are mature enough for use in analytical research, development and even routine work. However, prior to their use, these techniques like any other methodologies, have to be validated. As the application of SdFFF techniques to cell separation is being constantly developed, we have investigated separation performance according to validation rules classically defined for separation methods (chromatography) in the case of cellular materials.

Keywords: Sedimentation field-flow fractionation; Validation; Cells

1. Introduction

Field-flow fractionation (FFF) is a family of separation techniques conceptualised in 1966 by J.C. Giddings [1]. The techniques are based on the coupling of a laminar flow in a ribbon-like channel and an external field perpendicular to the flow direction. The applied field can be of very different natures [2], either electrical [3], magnetic [4], hydrodynamically generated [5] or thermal [6]. Sedimentation FFF can either use the simple earth gravity (gravitational field-flow fractionation: GrFFF) or a centrifuge force (multigravitational field-flow fractionation: SdFFF) as the external field. This latter subtechnique is largely successful in the separation and analyses of inorganic [7,8] and organic species [9,10] or biological ones including cells [11–13], viruses [14,15] and bacteria [16]. However, the success of separation sciences relies on validation procedures which were developed over 10 years ago and are now described by many organisations such as FDA (Food and Drug Administration) [17], ISO (International Standard Organization) [18], ICH (International Conference on Harmonization) [19] and the EEC (European Economic Community) [20]. These procedures include statistical, as well as functional criteria. The statistical criteria usually

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involved are: detection and quantification limits, precision, accuracy and linearity. The functional criteria are selectivity–specificity and sensitivity [21,22]. The purpose of this report is to describe and analyse cellular material elution with SdFFF in the light of the above procedures.

2. Theory

An extensive description of the separation principle has already been published [23,24]. To summarise, the ribbon-like channel with tapered ends is cut in a mylar sheet of standardised thickness (250 μm) and sandwiched between two polycarbonate plates. The channel is then set in a basket and it encircles a centrifuge axis like a belt, as schematically described in Fig. 1A. Two rotating seals made of polished inox and of graphitized carbon enable the carrier phase to flow through the channel by means of inlet and outlet tubing.

2.1. Spinning and external field

The spinning of the channel generates differential acceleration forces whose direction is perpendicular to the flow direction. The sedimentation field G (unit of gravity 1 $G=980$ cm/s^2) can be calculated from the rotational speed (rpm) and the rotor radius r:

$$G = k \cdot \mathrm{rpm}^2 \times r \quad (1)$$

$$k = 4\pi^2/(60^2 \times 980) \quad (2)$$

It is obvious from Eq. (1) that the effective field, applied to a given particle, is related to its position in the channel thickness. For each given channel thickness (ω), a given field gradient is generated. This gradient depends only on the spinning rate. It can be calculated according to the following equation:

$$\partial G/\partial x = k \cdot \mathrm{rpm}^2 \quad (3)$$

with $r-\omega<x<r$.

The maximum calculated intensity of this gradient is around 0.1% of the applied field strength.

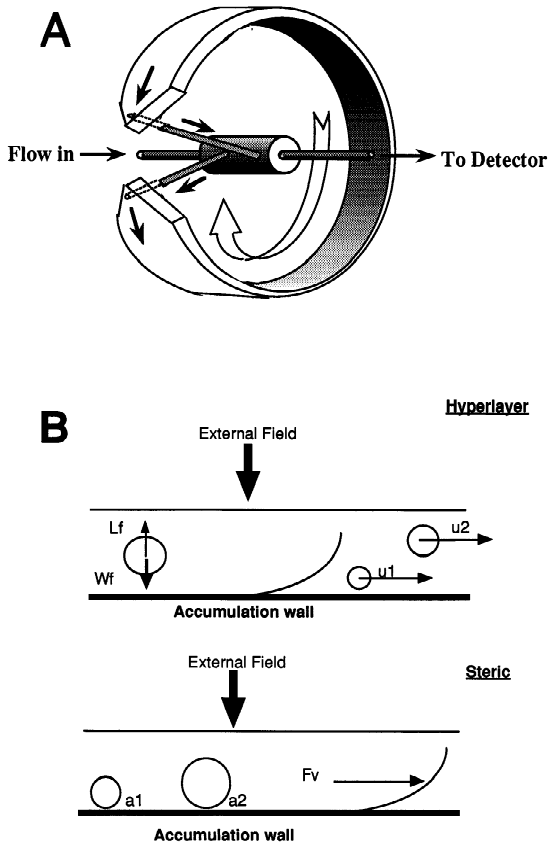

Fig. 1. (A) Schematic representation of the sedimentation field-flow fractionation apparatus. (B) Schematic representation of the hyperlayer (upper part) and steric (lower part) elution mechanism. W_f is the field-induced force acting on the particle, L_f the lifting force; $u1$ and $u2$ are particles' 1 and 2 velocities, respectively; $a1$ and $a2$ are particles' 1 and 2 respective mean diameters.

2.2. Steric hyperlayer red blood cells elution mechanism

The elution mechanism of red blood cells and nucleated cells is now qualitatively well established and described as 'steric hyperlayer' [23,24], as illustrated in Fig. 1B (upper part). In such elution mode, injected species are focused by the external field towards the accumulation wall [23–25]. Because of the channel geometry and the carrier-phase characteristics, the flow velocity is parabolic [24]. Therefore, the particles closer to the accumulation wall moved more slowly than those less affected by the external field [23–25]. Previous experiments in

sedimentation FFF with red blood cells (RBC) have supported this elution mode [24,26]. The most important assumption made in this elution mechanism is that species are not only affected by the external field (W_f) but also by opposite hydrodynamic lift forces (L_f) which drive away the species from the wall [23] till an equilibrium position in the channel thickness is found. In that case, field effects are exactly balanced by lift forces, the nature of which is not totally assessed to date. However, their influence and qualitative description have been investigated intensively [23–26]. When flow velocity is sufficiently low (inducing therefore lift forces of low intensity), and/or when the external field is high enough to overcome the lift ones, particles are focused on the surface of the accumulation wall. They hinge along the wall as described in Fig. 1B (lower part). This limit condition is now described as steric elution mode [23–25]. In that case, large particles whose centre of gravity is situated in high velocity stream lines, elute ahead.

3. Experimental

3.1. Multigravitational field-flow fractionation separation device

The design of the apparatus set up in our laboratory has already been described [27]. The final channel used in this report was 0.025 cm thick, 1 cm wide and 78.5 cm tip-to-tip long. The associated theoretical void volume including connection and detection tubing was calculated to be 2.17 ml. The experimental void volume including connection tubing was measured at 2.38±0.05 ml using 0.1% (w/w) sodium benzoate solution (Darrasse Frères, Paris, France). Rotor axis to accumulation wall distance was measured at 14.0 cm. A Carpanelli engine M71B4 (Carpanelli, Bologna, Italy) associated to a pilot unit Mininvert 370 (Richard System, Les Ulis, France) allowed control of the rotor rotation whose precision was measured at 0.01%. A Gilson pump model 302 (Gilson Medical Electronics, Middleton, WI, USA) associated with a pressure damper allowed controlled flow-rates from 0.05 to 5 ml/min. The carrier phase was made by mixing phosphate buffered saline solution pH 7.2; 300 mOsm (PBS 75511, Biomérieux, Marcy-l'Etoile, France) with 0.1% (w/w) of bovine albumin (No A-4503, Sigma Chemical, St Louis, MO, USA). Flow-rates were systematically controlled by weighing. Samples were flown into the channel by means of a classical Rheodyne valve model 7125i (Rheodyne, Cotati, CA, USA). Sample injections were performed with a 2.5-μl loop directly in the established flow through the accumulation wall (flow injection). At the separator outlet, a Waters model 440 photometer (Waters Corporation, Milford, MA, USA) preset to 350 nm was connected. Data were recorded on a Daewoo computer (Daewoo Europe, Roissy Charles de Gaulle, France) using a Sof 30160 acquisition card with laboratory-made software allowing 16 bytes precision operated at a 3-Hz frequency.

3.2. Human red blood cells

Samples were drawn from a healthy donor, after his informed consent, with the help of a Vacutainer system (Vacutainer, Maylan, France) and mixed with a potassium salt of EDTA, and stored at 4°C. Before FFF elution, extemporaneous dilution of the blood sample with the carrier phase (v/v) was performed. Final concentrations were measured with a Coulter counter TAII (Coulter Electronics, Luton, UK).

3.3. Fractogram measurement procedures

For the determination of validation parameters, calculations were performed using a spreadsheet programmed in Excel 5 (Microsoft, Redmond, WA, USA) on a Commodore 486 DX2 computer (Commodore Business Machines, West Chester, PA, USA). The peak area, when needed, was taken as the response signal for a given sample. It was evaluated using Foley 's method [28]. As FFF is a chromatographic-like technique [1], classical retention and dispersion parameters from that method (retention ratio, asymmetry factor and HETP) [29] were used for the analysis of sample stability, repeatability and intermediate precision. Retention ratio is commonly defined in FFF as the ratio of t_0/t_r, where t_0 is the dead-time and t_r the elution time of retained particles. As described by Bildlinmeyer and Warren [29], the asymmetry factor is computed as follows:

$A = b/a$, where a and b are the distances, measured at 10% of the total peak, respectively before and after the peak maximum.

3.4. Validation procedures

As blood is a particular living medium, continuous biological processes are expected to occur [30]. It is therefore necessary, before all validation procedure attempts, to check if the sample elution characteristics are affected or not by blood sample storage. This is why an additional criterion must be preliminarily tested, i.e. sample stability.

3.4.1. Precision and accuracy

Precision and related definitions have been extensively described [31–34]. It was proposed to assess precision using at least six to ten replicates [34]. We have chosen to analyse the system precision (repeatability) using six replicates for 10-, 100- and 200-fold dilutions. Assessment of the intermediate precision, which is the reproducibility by the same operator using the same material, was performed for five days. Each day, runs were carried out in five replicates (triplicates recommended [17–20]). For biological samples, an R.S.D. of ±15% is appropriate except for the quantification limit where ±20% is acceptable [35].

The accuracy of the method was evaluated by calculating the bias, i.e. the percentage difference between the measured mean concentration (μ) and the corresponding nominal concentration (c) as follows [36]:

$$\text{Bias\%} = 100.(\mu/c) \quad (4)$$

3.4.2. Linearity

Linearity determines the ability of the procedure to obtain test results which are proportional to the concentration of the analyte in the sample with a given range either directly [31,34] or via a well defined mathematical transformation [32–34,37]. Eighteen concentrations from 24 875 to $5.0 \cdot 10^6$ cells per μl were used in the calibration study; triplicates were used for each experiment.

3.4.3. Limits of detection (LOD) and quantification (LOQ)

Limit of detection (LOD) is the minimal amount of an analyte in the sample which can be detected but not necessarily quantified [32,34], whereas limit of quantification (LOQ) is defined as the lowest analyte concentration at which the accuracy and precision are less than 20% [32,33]. LOQ is determined by analysing successively diluted samples until the requisite levels of accuracy and precision [38].

3.4.4. Sensitivity

Sensitivity is determined as the smallest concentration difference that can be detected [39].

3.4.5. Statistical procedures: ANOVA tests

The ANOVA test principle is based on the hypothesis that the total variability of experiments is due to sampling fluctuations. The null-alternative hypothesis method was therefore used [40] considering the total variability defined as the sum of within-run variability and between-run variability.

4. Results and discussion

4.1. Sample stability

Blood stability was studied for five days. The initial sample with EDTA was divided into five fractions and stored at 4°C. Every day, a fraction was taken for elution studies. Before SdFFF analysis, the stored fraction was equilibrated at room temperature for 2 h. Five-fold dilution samples were injected into the SdFFF device. Experiments were monitored at three fields (5, 10, 15 G). For each field, three flow velocities (0.47, 0.67 and 1.00 cm/s) were established. Each experimental condition was run in triplicate. Examples of typical fractograms obtained under different elution conditions are shown in Fig. 2. These RBC elution profiles are specific of RBC elution in SdFFF as already described in numerous reports [6–8] and because of their chromatographic-like general shape, chromatographic peak description methods can be applied. Therefore stability analyses were performed by means of cell elution profile descriptions. For this purpose, three parameters were

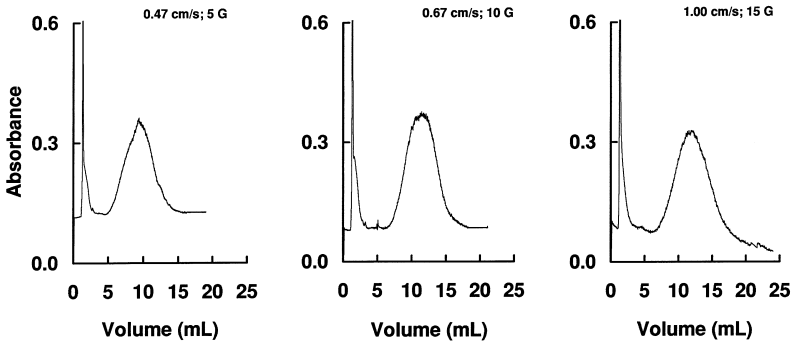

Fig. 2. Fractograms of red blood cells in sedimentation field-flow fractionation. Injection volume: 2.5 μl, flow injection, carrier phase: isotonic phosphate buffer saline solution with 0.2% (w/w) bovine albumin.

chosen: retention ratio (R), asymmetry factor (A) and the classical dispersion parameter described as height equivalent to a theoretical plate (HETP). Special attention was paid every day to elute the same quantity of particles during cell stability experiments by means of a sample Coulter counter numeration.

Results of the stability study are given in Table 1. For identical elution conditions, i.e. for an external field of 10G and a linear velocity of 1.0 cm/s, the mean retention ratio value varied from 0.13±0.01 to 0.14±0.01 over five days. For the same period, the asymmetry factor and HETP ranged respectively from 0.92±0.05 to 0.99±0.03 and from 1.75±0.12 to 1.94±0.05 cm. R.S.D. values were lower than 7% for all the parameters under study. It can be noticed that the highest R.S.D. was found for the dispersion parameter. Fisher coefficient (F) calculations at 5% risk led to the conclusion that the parameters under

Table 1
Summary of stability study

Field strength: (G)		5		10		15	
Flow velocity: (cm/s)		0.67	1.00	0.67	1.00	0.67	1.00
Retention ratio							
Days	1	0.14±0.01	0.20±0.01	0.10±0.01	0.13±0.01	0.09±0.01	1.07±0.01
	2	0.16±0.01	0.21±0.02	0.10±0.01	0.13±0.00	0.09±0.01	1.03±0.01
	3	0.17±0.01	0.21±0.01	0.11±0.01	0.14±0.01	0.09±0.00	0.10±0.01
	4	0.16±0.02	0.23±0.00	0.11±0.00	0.14±0.02	0.09±0.01	0.10±0.01
	5	0.16±0.01	0.23±0.01	0.10±0.02	0.13±0.01	0.08±0.01	0.10±0.00
Asymmetry factor							
Days	1	0.79±0.01	0.93±0.00	0.99±0.00	0.92±0.05	1.24±0.01	1.26±0.00
	2	0.68±0.01	0.80±0.01	1.20±0.01	0.94±0.01	1.41±0.02	1.46±0.01
	3	0.95±0.00	1.02±0.02	1.10±0.00	0.95±0.01	1.46±0.01	1.35±0.00
	4	1.11±0.01	1.15±0.01	1.16±0.01	0.99±0.01	1.43±0.01	1.24±0.01
	5	1.04±0.01	1.13±0.01	1.28±0.01	0.99±0.03	1.36±0.01	1.30±0.00
HETP (cm)							
Days	1	1.99±0.04	2.75±0.01	1.90±0.01	1.75±0.12	1.39±0.01	1.92±0.02
	2	1.91±0.01	2.63±0.02	1.79±0.01	1.81±0.02	1.34±0.01	1.96±0.02
	3	2.18±0.00	2.52±0.01	1.67±0.01	1.93±0.02	1.52±0.01	1.86±0.01
	4	2.47±0.01	2.86±0.01	1.98±0.01	1.94±0.05	1.47±0.01	1.78±0.01
	5	2.65±0.02	2.97±0.00	2.05±0.01	1.83±0.05	1.63±0.00	2.07±0.02

Flow-rates: 0.67 and 1.00 cm/s, 5 days, $n=3$; three field strengths: 5, 10, 15 G.

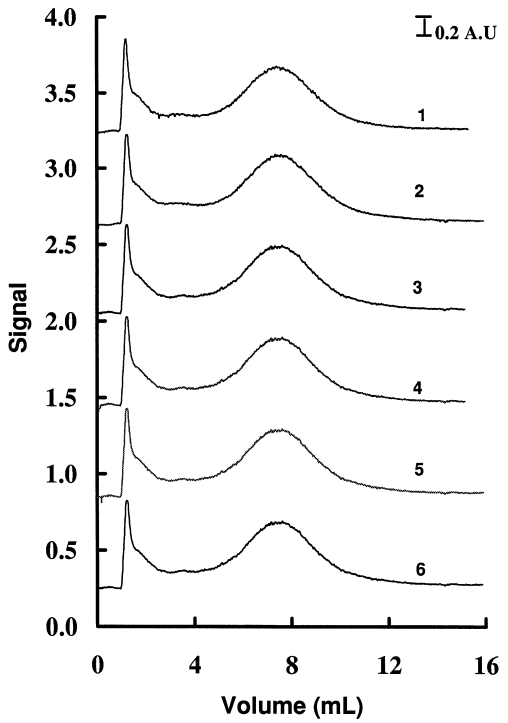

Fig. 3. Fractograms of red blood cell samples eluted in the same conditions. Flow-rate: 1.0 cm/s; field intensity: 10 *G*, 2.5-μl loop; dilution factor: 100; carrier phase described in the legend to Fig. 2.

study are significantly independent of red blood cell (RBC) modifications for a five-day period. It can be concluded that RBC samples, stored for a 5-day period at 4°C, can be used without a significant bias as a 'standard' sample to evaluate cell elution performance of any SdFFF system.

4.2. Precision and accuracy

Precision was assessed from repeatability experiments. These experiments were performed at three different RBC concentrations (dilution factor of 10, 100 and 200), as recommended [17]. The data of RBC elution, shown in Fig. 3, corresponds to the most diluted sample. It can be observed that each fractogram shows two peaks whose origins have been already described [12]. The first one corresponds to the elution of unretained species and is used to determine either the real flow-rate or the system void volume. The second peak corresponds to the elution of RBC. Qualitatively it is observed in Fig. 3 that almost all fractograms are strictly superposed. For each fractogram, peak characteristics were calculated and the results are displayed in Table 2. Repeatability experiments consisted of 6 runs of the same RBC sample; the R.S.D. for retention ratios was lower than 6%. However, in all cases, calculated R.S.D. values were lower than 9%. According to classical validation rules, these results of RBC elution in SdFFF are highly repeatable. It must be noticed that quantities eluted may play a role in the

Table 3
Intermediate precision

	Days					
	1	2	3	4	5	Between-day
Retention ratio						
Mean	0.16	0.15	0.16	0.15	0.15	0.15
S.D.	0.01	0.00	0.01	0.00	0.01	0.01
R.S.D.	6.39	2.09	4.99	2.40	8.46	5.90
Asymmetry factor						
Mean	1.22	1.30	1.23	1.25	1.34	1.27
S.D.	0.07	0.06	0.05	0.10	0.04	0.08
R.S.D.	5.50	4.63	4.14	8.32	3.23	5.97
HETP						
Mean	1.69	1.70	1.83	1.84	1.86	1.78
S.D.	0.14	0.15	0.18	0.10	0.07	0.14
R.S.D.	8.20	9.05	9.60	5.46	3.56	9.44

Flow-rate: 1.0 cm/s; dilution factor: 80; field intensity: 10*G*, 5 days, $n=5$.

Table 2
Precision study

Dilution factor	10			100			200		
	Mean	S.D.	R.S.D.	Mean	S.D.	R.S.D.	Mean	S.D.	R.S.D.
Retention ratio	0.17	0.01	3.83	0.15	0.01	5.00	0.15	0.01	5.17
Asymmetry factor	1.15	0.08	6.63	1.22	0.09	7.23	1.34	0.08	5.87
HETP	1.68	0.15	8.94	1.71	0.16	9.34	1.78	0.15	8.42

Experimental conditions are described in Fig. 3.

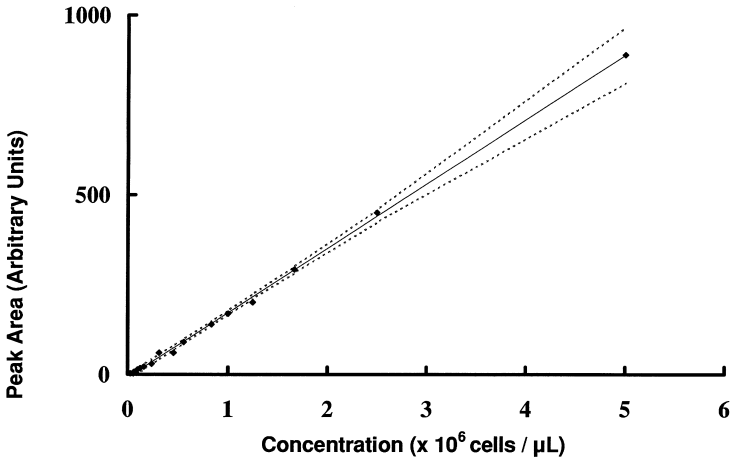

Fig. 4. Effect of red blood cell concentration on peak area. Red blood cell concentrations ranging from 25 000 to 5 000 000 cells per μl; field strength: 10 *G*; flow velocity: 1.0 cm/s, $n=3$; solid line: linear-model fitting line; dashed line: confidence interval curve.

RBC peak characteristics. Comparison of mean values and variance analyses showed a significant difference between both series of experiments. An intermediate precision series of elution were performed. Peak characteristics were calculated and are displayed in Table 3. Statistical analyses showed that between-day R.S.D. values were lower than 9.5%, and that within-day R.S.D. values were lower than 9.6%. The calculated bias varied from −8% to 3% thus meeting the Washington criteria [41]. It can therefore be stated that SdFFF elution of RBC within 5 days is highly reproducible at a 10% risk.

4.3. Linearity

Linearity was assessed using samples whose dilution factor varied from 0 to 400. A standard deviation of 5% ($\pm 2\sigma$, $n=12$) with Coulter counter TA II was observed for concentration control. The quantities of eluted cells were estimated by means of area measurement of the RBC elution peak on each fractogram, and the results are plotted in Fig. 4. As a result of a first-order unweighed least-square fitting procedure, a calculated slope of $179 \cdot 10^6 \pm 1 \cdot 10^6$ A.U. μl per cell and an intercept of -7.83 ± 2.02 cells per μl were found. The validity of that linear model was assessed using the classical *F*-test comparison whose characteristics are summarised in Table 4. As a result, it can be stated with a 5% risk that the detector response is concentration-dependent with a first order regression coefficient estimated at 0.9989, in the concentration range under study.

4.4. Limits of detection and quantification

Limits of detection (LOD) and quantification (LOQ) are critical stages in the validation process [34]. The LOD and LOQ determination procedure

Table 4
Goodness of fit and lack of fit for red blood cells

ANOVA table of goodness of fit and lack of fit				
	Sum of squares	Degrees of freedom	Mean squares	$F_{calculated}$
Due to regression	$2.54 \cdot 10^6$	1	$2.54 \cdot 10^6$	17487.20
Lack of fit	$2.44 \cdot 10^3$	16	$1.53 \cdot 10^2$	1.05
Residuals	$5.23 \cdot 10^3$	36	$1.45 \cdot 10^2$	
Total	$2.54 \cdot 10^6$	53		

Sample concentration varying from 25 000 to 5 000 000 cells per μl; field intensity: 10*G*; flow-rate: 1.0 cm/s, $n=3$.
$1.84 < F_{tabulated}\ (\alpha = 5\%) < 4.17$.

was the following: with a detector sensitivity set at 0.01 AUFS, the injected concentration was decreased after each series of three to four runs. A signal corresponding to three times the blank signal was taken as the LOD response, whereas 10 times the blank signal was taken as the LOQ [37]. When LOD and LOQ values were reached, statistical confirmation was assessed six and ten times with respective results of 12 500±3 500 and 25 000±4000 cells per μl. However, other LOD and LOQ methods were proposed [40] and led to 24 493±1531 and 183 577±2723 cells per μl. A third method of LOD determination [40], is related to the confidence interval curve of the regression model described in Fig. 4; its calculated value is in the 500-cells-per-μl range. Discrepancies between the LOD determination methods may be related to poor correlation between the experimental data and the straight line, below 25 000 cells per μl, as shown in Fig. 4.

4.5. Sensitivity

Sensitivity experiments were monitored using an initial red blood cell sample of 500 000 cells per μl. Variable cell quantities were successively added to the initial quantity. Response areas were calculated and plotted against the concentration as shown in Fig. 5. Two regions can be observed, the first one ranges from 500 000 to 516 667 cells per μl where peak area seemed to be identical for all concentrations. The second region at a concentration higher than 520 000 cells per μl, corresponds to a domain where the relation between concentration and area is linear. Then, if we assume that sensitivity is the smallest concentration difference that can be detected, the inspection of Fig. 5 shows that with the sedimentation FFF system used in this report, the sensitivity is 16 667 cells per μl. ANOVA tests were used to verify the existence of both regions. A global ANOVA test taking into account all the seven concentrations studied in the sensitivity experiment was not necessary because Fig. 5 shows evidence that the curve is not linear. Suppose the seven concentrations *A*, *B*, *C*, *D*, *E*, *F* and *G* with *A*= 500 000 cells per μl, *B*=505 000 cells per μl, *C*= 510 000 cells per μl, *D*=512 500 cells per μl, *E*= 516 667 cells per μl, *F*=525 000 cells per μl and *G*=550 000 cells per μl. Two ANOVA tests were performed on two series of data. The first one comprised concentrations *A*, *B*, *C* and *D* and the second one was made up of concentrations *B*, *C*, *D* and *E*. The mean peak areas of the first series of data were found to be identical ($\alpha = 5\%$), whereas those of the second series appeared to be significantly different ($\alpha = 5\%$). It is therefore possible to state that the concentration difference between *A* and *E* concentrations is the sensitivity of the system. Both ANOVA tests confirm the result obtained from Fig. 5.

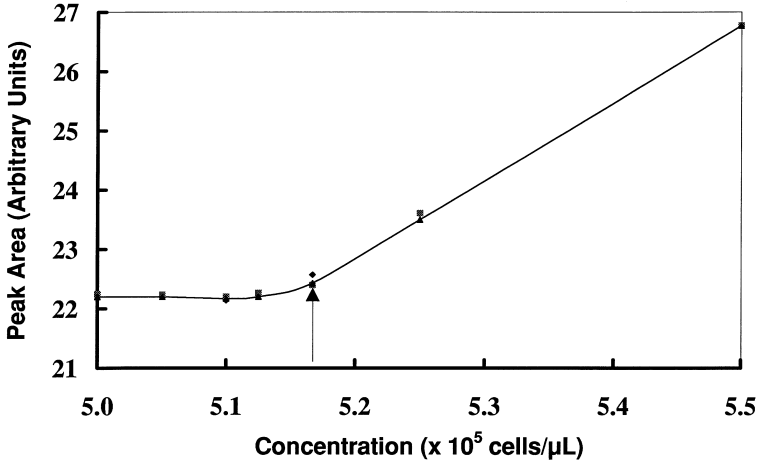

Fig. 5. Effect of small variations of red blood cell concentration on detector response. Red blood cell concentrations ranging from 500 000 to 550 000 cells per μl (500 000; 505 000; 510 000; 512 500; 516 667; 525 000; 550 000); field strength: 10 *G*; flow velocity: 1.0 cm/s, $n=3$. The arrow indicates the red blood cells concentration from which the detector detects a variation in red blood cells concentration.

4.6. Recovery

In FFF, many studies pointed out the existence of particle–particle or/and particle–wall interactions [7,24,25] which may lead to irreversible cell sorption or destruction, whose relative intensity must be evaluated. Wall modifications were sometimes used to eliminate interaction effects [25,26]. Even in this case, a flushing procedure was used by Hoffstetter-Kuhn et al. to flush out stuck particles [11]. To evaluate the recovery, a sample is divided into two equal fractions, 20 μl of one is considered as the reference. The second fraction is injected into the FFF system and the red blood cells are collected at the outlet of the separator. Cells were monitored by means of a Coulter counter. Five observations were done. The mean recovery found was 94.0%±3.0% with a low R.S.D. of 3.15%.

4.7. Application to the steric hyperlayer elution mode of RBC

To assess RBC elution mechanism, a series of experiments was therefore performed with RBC considered as standards. The elution parameters plotted in Fig. 6 show that the RBC retention ratio at a given external field is flow-rate dependent as predicted by the steric-hyperlayer elution mode. The more intense the external field, the more retained the RBC. These results are in total accordance with those described under analogous conditions in the early eighties by Caldwell et al. [42] and a decade later by Metreau et al. [27] who systematically studied retention properties of living RBC in SdFFF. The concomitant increase in the retention ratio when the flow-rate increases and/or when the field intensity decreases was also characterised in SdFFF for nonbiological micrometer-sized species [1]. However, in order to obtain a biologically compatible recovery (over 60%), one has to avoid experimental conditions leading to an elution mode as ‘steric’ as possible to limit particle–wall interactions [11]. Using a channel of equivalent thickness, retention ratio data of Fig. 6A are in the same range as those observed by Caldwell et al. [42] for the living RBC, regardless of the species concentration, injection mode and channel-wall nature differences. These two series of results can be compared to those described by Metreau et al. [27] for a thinner channel. When

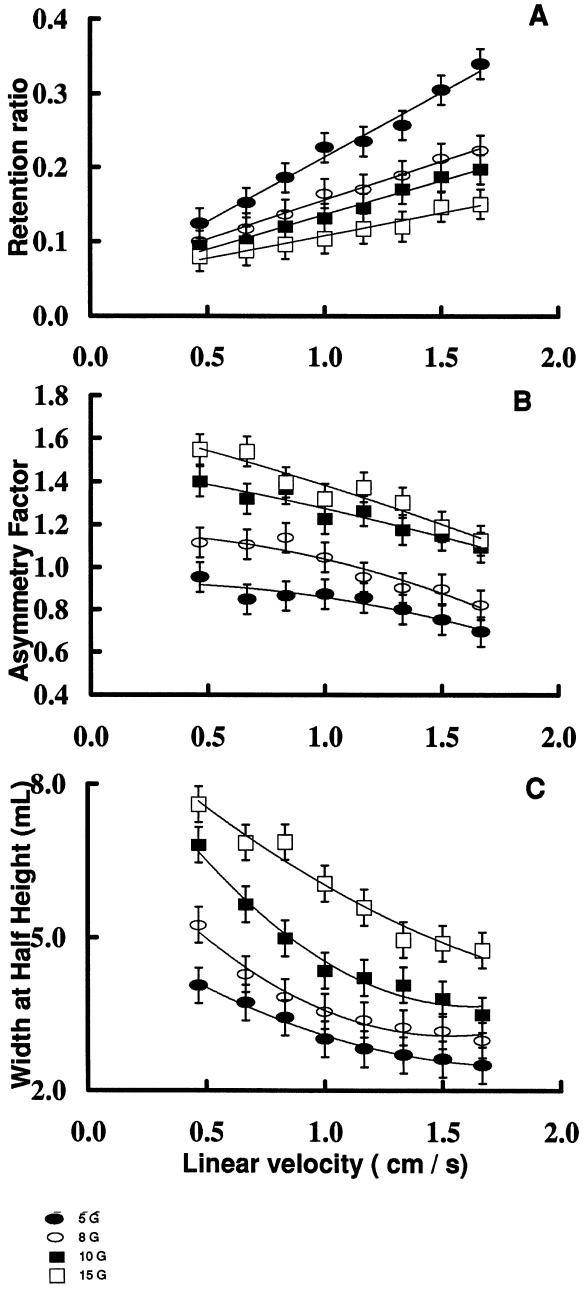

Fig. 6. Effect of flow velocity and field intensity on fractogram characteristics. Flow-rate ranging from 0.47 to 1.67 cm/s (0.47, 0.67, 0.83, 1.00, 1.17, 1.33, 1.50, 1.67); four field intensities: 5, 8, 10 and 15 G, $n=4$; 2.5-μl loop; carrier phase described in Fig. 2 legend. A: Retention ratio; B: asymmetry factor; C: peak width at half height.

retention ratio data of this report and of Caldwell et al. [42] are compared to those described by Metreau et al. [27], it can be observed that, at equivalent external field and linear flow velocities, the data of Metreau et al. [27] presents systematically increasing retention ratio values. This is in accordance with the steric–hyperlayer elution mode which predicts that for a given particle eluted under equivalent conditions (field, linear velocity), the hydrodynamically generated lifting force is higher in a thinner channel. RBC peak shape is also modified, as shown in Fig. 6B with a systematic asymmetry ratio decrease with increasing flow-rate and field intensity. The most interesting feature of the systematic study shown in Fig. 6C is that peak dispersion decreased systematically when flow-rate increased and external field decreased. Bibliographic analysis of equivalent results described by Caldwell et al. and Metreau et al. [27,42] showed slight discrepancies. HETP curves described by Metreau et al. differed from those obtained by Caldwell et al. both in intensity and in shape. Fig. 7 shows the values calculated from the data of this report. It is observed that for these three series of data (Metreau et al., Caldwell et al., Assidjo et al.) the HETP values were in the same range, although curve shapes differed. These differences may be due to two factors: the nature of the cells (fixed versus living) and the channel characteristics (geometry, wall material). In the present report, channel thickness is analogous to the one used by Caldwell et al., cells and SdFFF are of the same type as the ones described by Metreau et al. [27]. Calculated HETP values obtained by us were close to the ones already described by Metreau et al. [27]. These results point out an important fact: a living RBC population appeared to be more polydispersed in size, density and shape than the fixed ones. A balance between external field and flow-rate is to be found for maximal separation within the RBC population.

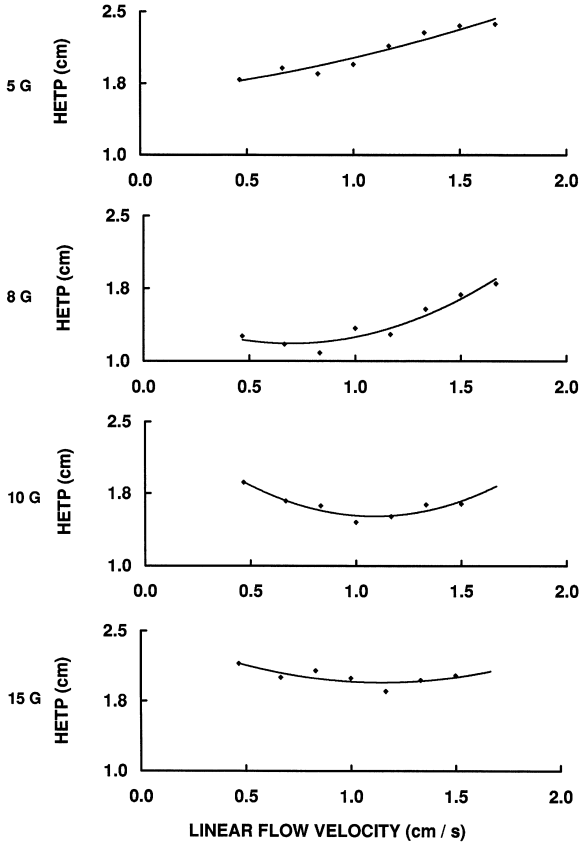

Fig. 7. Effect of flow velocity and field intensity on peak dispersion parameter (HETP). Experimental conditions described in Fig. 6.

5. Conclusion

As selectivity is extensively studied in SdFFF, mainly in the case of nonliving particles, the functional and statistical criteria studied in this report showed that FFF and SdFFF are mature enough to be validated even in the case of biological materials. The main result of this investigation is that SdFFF can be considered accurate and precise when validated under conditions close to those classically used for other elution methods, like chromatography and electrophoresis. In terms of elution conditions, data shown in this report closely match those described in the bibliography for living RBC, regardless of small differences in peak dispersion characteristics, so far difficult to interpret. In that case some bias can be pointed out: fixed RBC are known to behave differently from living ones as described by Parsons et al. [43]. Apparatus design is also different as well as some methodological approaches which can be at the origin of these differences.

If the polydispersity effects of colloidal (brownian) particles and their impact on SdFFF elution characteristics have been extensively studied [44], this is not the case for micrometer-sized particles,

and particularly in biology where no standard particles could be found. Systematic studies using biochemical probes may help to establish guidelines for elution performances and therefore optimisation rules in FFF as preliminarily described by Cardot et al. [45] in the case of RBC-selective elution according to age.

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