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Rice starch granule characterization by flow cytometry scattering techniques hyphenated with sedimentation field–flow fractionation

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Abstract

Sedimentation field–flow fractionation (SdFFF) elution mode of micron sized particle is described generically as "Hyperlayer" and involves particle size, density, shape and rigidity. It requires the use of specific detectors of mass, size, surface, or of other characteristics of the eluted particles. Correlation of FFF retention data with such signals gives hyphenated information about particle properties. Flow cytometry (FC) is a multi dimensional particle counter, which permits specific particle property characterization using light scattering and fluorescence principles. It appears therefore as a powerful technique for micron sized species description. FC is mostly known for cell analyses, while its potential is much broader once proper calibration performed. In this report, forward angle signal (FS) is calibrated in size by using standard latex beads and produces, for a given particle sample, a number versus size histogram, describing particle size distribution. These histograms can be an alternative to Coulter counting. That methodology is tested with rice starch population (RSP) fractions obtained from FFF separation.

Keywords: Flow cytometry; Sedimentation field–flow fractionation; Particle size distribution; Size calibration; Rice starch

1. Introduction

In recent papers, our group has shown that substantial information can be deduced using association of flow cytometry (FC) with sedimentation field–flow fractionation (SdFFF) in the case of cell sorting [1,2]. It is therefore suggested that flow cytometry can be extended to other micron-sized species like, in this report, starch particles.

FC has been widely used for qualitative analyses of individual cells [3] as well as particle suspensions of other origins [4]. It has also been used coupled with FFF techniques for cell characterization by Gascoyne and coworkers [5] and Zborowski et al. [6]. FC versatility allowed particle counting as well as measurements of particle characteristics using light scattering principles and/or fluorescence [7,8]. When a single particle passes in front of a laser beam, the scattered (or the emitted fluorescence) light generates a signal dependent on the studied parameter of that particle. If the diffused light is collected at low angles [7–9] (10° forward scattering (FS)), diffraction predominates and the Mie Law [10] gives the dependence of scattered intensity on particle refractive index and geometrical cross-section, which is size and shape dependent. It is therefore possible to calibrate FC operated in the FS mode to obtain number versus size particle distribution histograms. Because of the complexity of this signal function and in order to limit or avoid bias, calibrating particles should have analogous refractive indices to the suspension under study. A complete calibration procedure must consider some specific features of the FC operated in the FS mode, such as different electronic gain [7,9]. Size calibration must be performed using numerous series of standard latex beads of reduced dispersities [11]. The ability of generating particle size distribution (PSD) histogram, also requires calibrating FC–FS histograms in terms of polydispersities. It is therefore possible and recommended, a posteriori, to control

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its accuracy using different sets of calibrated latex particles before or during experimental series.

Sedimentation field–flow fractionation is a chromatographic like technique known for its remarkable separation power in the case of micron-sized particles [12–16]. The field is generated by the rotation of the channel positioned at the periphery of a rotor basket. The elution mode of micron-sized species in such system is described as "Hyperlayer" [14–17]. It predicts that suspension separation is, at least, particle size, density and shape dependent [16]. Qualitatively, it can be stated from experimental data that particles of the same density and shape are eluted according to their volume (or size) while particles of identical shape and volume are eluted according to their density. Hyphenation of information provided by FFF in terms of retention with that provided by FC provides a rather sophisticated description of the sample population in terms of size, density, surface and composition characteristics, with the help of complex multi dimensional data analysis procedures or strategies [1,2]. Moreover, the separation power of SdFFF permits purification of fractions characterized by their multi dimensional FC pattern (population fingerprint) using FS in combination with side scattering (SS) and/or fluorescence.

Starch granules are of major use in the food, pharmaceutical and cosmetic industries [18,19]. Fraud and quality control require the development of sophisticated methods [20,21] including granulometry [22]. The average size of rice starch granules is 4.2–4.3 μm with a 3–8 μm size distribution [23,24]. They are irregularly shaped and polygonal. Starch size and size distribution plays a critical role in food fermentation processes as well as in the use of starch in pharmaceutical preparations [25]. There are therefore important requirements for complete rice starch population (RSP) descriptions and FFF hyphenated with FC (mainly in the FS and SS mode) corresponds to these needs. Numerous FFF studies using different techniques have been reported with starch materials [26–31]. Considerable information can be provided if associated with systematic and multivariate granulometric analyses. In this report, we demonstrate the usefulness of coupling FFF separations with calibrated FC–FS measurements for a rather accurate determination of RSP characteristics, especially size.

2. Experimental

2.1. Starch material

Rice starch (Fluka Biochemica, France) solutions were prepared at a concentration of 10 g/l in distilled water. Suspensions were ultrasonicated during 10 min, care was taken to prevent heating, and consequently, to prevent starch hydrolysis. These solutions were kept at 4 °C during a maximum of 5 days. Before every analysis, the solution was ultrasonicated during 1 min.

2.2. Field–flow fractionation system

The SdFFF system used in this work consists of a separation channel connected to a UV detector and to a classical HPLC pump, with a sample chromatographic-like injection device [1,2]. The FFF channel was made of two polystyrene plates, one described as the depletion wall and the other called the accumulation wall, separated by a Mylar spacer in which the channel was manually carved. The thickness of the Mylar band defines the channel's height. The dimensions of the channel were 795 mm long, 12 mm wide and 0.08 mm thick, with two V-shaped ends of 50 mm. The polystyrene plates and Mylar spacer were sealed in a centrifuge basket. The channel–rotor axis distance was measured as r = 14.8 cm. Sample inlet and outlet 0.254 mm i.d. Peek® tubings (Upchurch Scientific, Oak Harbourg, USA) were connected to the channel via the accumulation wall. The measured total void volume was 781 ± 3 μl (n = 15, σ). Void volume was calculated from the elution time obtained after injection of a non-retained compound (1 g/l of sodium benzoate, with UV detection at 254 nm). Sedimentation fields are expressed in units of gravity (g = 980 cm/s^2), and were calculated from the measured rotational speed (rpm: rotation per minute) and the channel radius, r (cm), according to the following equation:

$$G = \left(\frac{\text{rpm} \cdot 2\pi}{60}\right)^2 r \quad (1)$$

A Gilson 307 (Gilson Inc., Middelton, WI, USA) chromatographic pump was used to deliver mobile phase (distilled water). Sample injections were performed via a 7525 Rheodyne (Cotati, CA) valve with a loop of 25 μl. In each experiment, flow rates were measured. The elution signal was recorded at 254 nm by means of a Spectroflow 757 UV–vis spectrophotometer and a 14-byte (100 mV input) acquisition device (Keithley Metrabyte, Tauton, MA, USA) operating at 2 Hz and connected to a Macintosh computer. Size characterization was performed by flow cytometry on collected fractions all along the elution time. Fractions were collected and designated as follows: (1) the total peak fraction: TP, from 1 min 35 s to 5 min 30 s; (2) peak fractions F1–F4: F1, 1 min 45 s/2 min 40 s; F2, 2 min 50 s/3 min 25 s; F3, 3 min 30 s/4 min 20 s; and F4, 4 min 25 s/5 min 30 s. Elution procedures already described for cell sorting were systematically applied in order to limit or avoid channel poisoning [1,2].

2.3. Flow cytometry calibration

An EPICS-XL (Beckman Coulter France, Villepinte, France) with 1024 channels, equipped with an argon ion laser tuned to 488 nm at a beam power of 15 mW, was used for measurements. Flow-Check™ fluorospheres were used to check the stability of the optical and fluid systems. Polymer microsphere size standards (Duke Scientific, Palo Alto, CA, USA) of certified diameters (Table 1) were used for FS size

Table 1
Flow cytometry forward scattering calibration procedure

Electronic amplification	Characteristics of standard polymer microspheres					
	Measured mean channel			Calibration equation		
	3.063 ± 0.027 μm (0.03 μm) 1.0% CV	4.996 ± 0.035 μm (0.05 μm) 1.0% CV	6.992 ± 0.050 μm (0.07 μm) 1.0% CV	*A*	*B*	r^2
Gain 10.1	221 ± 7 (3.16%)	388 ± 11 (2.06%)	648 ± 12 (1.85%)	3.716	−17.088	0.9995
Gain 12.5	272 ± 8 (2.94%)	480 ± 11 (2.29%)	806 ± 16 (1.98%)	3.680	−17.657	0.9993
Gain 13.5	300 ± 10 (3.00%)	530 ± 13 (2.45%)	888 ± 20 (2.25%)	3.683	−18.038	0.9993
Gain 15	325 ± 10 (3.08%)	573 ± 12 (2.09%)	960 ± 20 (2.08%)	3.680	−18.302	0.9993

Standard polymer microsphere data are provided by the manufacturer and NIST compliant: mean diameter ± standard deviation, NIST compliant population standard deviation expressed as size and coefficient of variation expressed as the ratio of standard deviation vs. mean diameter. Results are displayed according to the same method: mean channel ± standard deviation (CV in percentage) for $n = 3$ (n corresponds to the number of independent experiments). Values of A and B parameters of Eq. (2); $n = 3$, r^2 is determination coefficient.

calibration. Duke polymer microspheres, with a refraction index of 1.59 at 589 nm at 23 °C (Duke Scientific Corporation) and nominal diameters of 3, 5 and 7 μm (1.0% CV), have a refractive index value very close to that of starch granules, 1.54 at 25 °C. These standards were chosen to cover a size range including rice starch granule diameters [23]. The forward signal (FS) histograms were recorded at different gains, depending on sample characteristics. Samples were vortexed for 10 s to prevent settling just before acquiring data. They were diluted at a concentration of 25 μl of stock suspension in 20 ml of deionized water. All samples were analyzed three times and data acquisition was stopped after 20,000 events were counted (monodisperse populations) or 200,000 (if populations appeared polydisperse). Flow cytometer software created Listmode files, which recorded cytometric data. WinMDI software version 2.8 (©Joseph Trotter) was used to transform data files in Excel format, in order to determine median channel value and associated data.

2.4. Coulter Counter

The determination of rice starch granule diameters was performed with a Coulter Multisizer II (Beckman Coulter France, Villepinte, France). Calibration was done with Beckman latex microspheres of 3.24 and 5.80 μm nominal median diameters. Glass tubes had an aperture diameter of 100 μm. The starch suspension was diluted in Isoton® to a final volume of 15 ml. The counting conditions were: 500 μl sample volumes, cumulating three successive assays.

2.5. Calculations

All data treatment and calculation were performed using Mathematica 4.1 software (Wolfram Research, Inc., Champaign, IL, USA) using included toolboxes as well as specifically programmed sequences (statistical moment calculations, filtering and deconvolution algorithms).

3. Results and discussion

In order to calibrate the flow cytometer, for rice starch population analyses of both the crude sample and the fractions collected from SdFFF elution, a series of appropriate standard populations was selected. The two critical parameters, which must be respected were the refractive index known at 1.54 for rice (25 °C) and the size range (3–8 μm). Standard latex beads described in Section 2 have a refractive index of 1.59 (23 °C) and nominal certified average diameters of 3, 5 and 7 μm are available. These two physical criteria made the use of FC–FS possible for starch analyses once calibration performed. They permit the construction of the RSP/PSD histogram as well as experimental determination of SdFFF selectivity and size performance of the flow cytometer operated in the FS mode.

3.1. Flow cytometry FS size calibration

In order to encompass RSP size distribution, three latex standards of average diameters 3, 5 and 7 μm were chosen. They were used pure or in some cases mixed for calibration. The flow cytometer was set at different amplification gains corresponding to a different range of histogram channels. The signal generated by the forward scattering after 200,000 particle counting gave an histogram with particle number as a function of FS signal intensity as shown in Fig. 1A obtained at gain 12.5, an amplification parameter perfectly set up for starch analyses.

This histogram used the overall FS signal intensity (1024 channels). From such a signal it was possible to calibrate the *x*-axis by means of certified average values of the latex standards. Mean diameter of every standard latex particle was plotted as a function of the corresponding FS median channel thereby establishing a FS calibration function. Calibrated size was a function of the logarithm of the flow cytometry FS channel number:

$$\text{Diameter} = A \ln(\text{FS channel}) + B \quad (2)$$

A and B values are given for different amplification gains in Table 1. Standards chosen must encompass not only sample

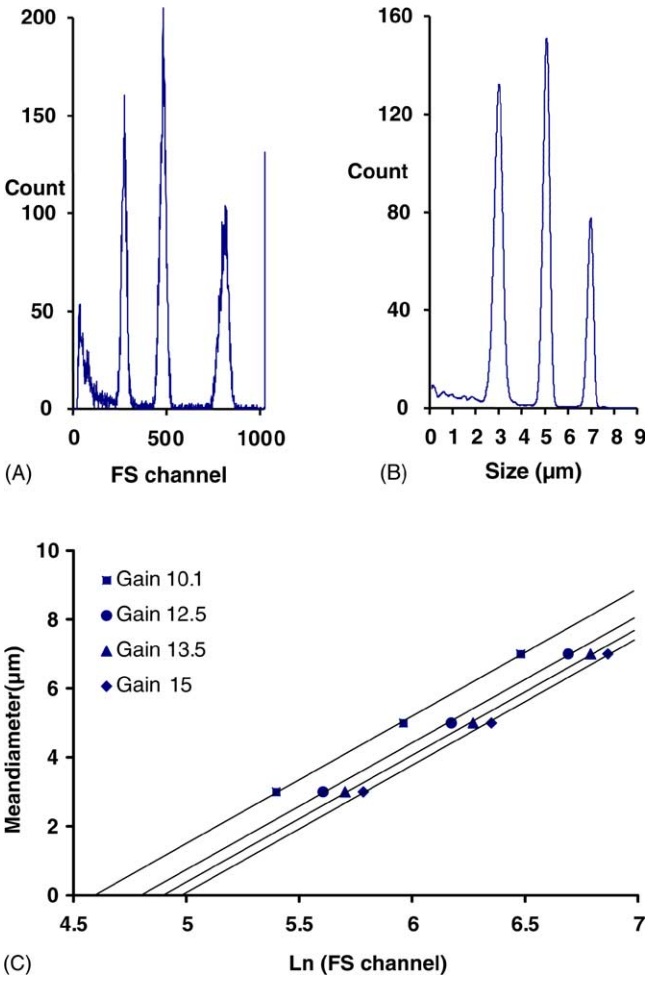

Fig. 1. Forward scattering signal from standard latex beads (NIST certified). (A) Count vs. signal intensity channel FS gain = 12.5. Channel range [1–1024] (20,000 particles counted). (B) Particle size distribution calibrated in size using Table 1 function at gain 12.5 after signal treatment (noise deconvolution, filtering). Latex particle (3, 5 and 7 μm) characteristics given in Table 1. (C) Calibration curves obtained from Table 1 data, used for flow cytometry FS-dependent size determination.

size distribution but also the 1024 channels of the FS signal, in order to avoid accuracy bias as shown in Fig. 1A. Data in Table 1 show that size/signal resolution was lowest at gain 10.1, while gain 15 led to a too wide abscissa range. Gain 12.5 or 13.5 could be used interchangeably for experiments, in the former case the FS channel minimum and maximum size resolutions were, respectively, 0.037 and 0.0036 μm. The signal profile of Fig. 1A resembles chromatographic ones authorizing analogous signal processing. Noise filtration can be performed as well as peak profile parameter calculations [32] leading to measured polydispersities (from peak spread) as shown in Fig. 1B and Table 2. Complete moment parameters were established from Fig. 1B profile analysis giving a complete PSD of each standard that can be therefore correlated with the manufacturer's data. Resulting informations are plotted in Fig. 1C for practical calibration procedures. Data from Table 2 show that the effective measured polydispersities from FC–FS were larger, but in the same range (two- to five-fold).

3.2. Crude rice starch granulometric distribution

Two different methods were performed. The first one exploited the FS signal of FC while the second used the more classical Coulter Counter principle. Coulter measurements were used to test the accuracy or to determine inter-techniques measurement biases.

3.2.1. Flow cytometry using FS mode

Rice starch suspensions were analyzed using FC–FS at different amplification gains. At amplification gain 13.5, the average size was 3.9 ± 0.2 μm (n = 3, σ) with a standard deviation calculated with the second moment method at 1.4 ± 0.2 μm (n = 3, σ). At amplification gain of 12.5 slightly different values were observed using identical calculation algorithms with an average value of 4.2 ± 0.2 μm (n = 3, σ) and a standard deviation of 1.5 ± 0.2 (n = 3, σ). The gain value intensity was of low impact in determining sample standard deviation while a light shift in the average size was observed. This can be explained by the logarithmic size scaling which weights the particle size distribution histogram differently. After calibration, the use of different amplification gain may induce a bias in rice starch PSD profile as shown in Fig. 2.

3.2.2. Coulter Counter measurements

FC results, obtained at optimal gain (12.5), were then compared to approved size measurement method, i.e. Coulter Counter. Average value was 4.30 ± 0.15 μm (n = 5, σ) and standard deviation was recalculated at 1.45 ± 0.05 (n = 5, σ). Such results are in agreement with those obtained at a 12.5 amplification gain of the flow cytometer.

Table 2
Calibrated flow cytometry standard latex particle data

Standard	3 μm	5 μm	7 μm
Calibrated average value (μm)	2.98 (moment), 3.02 (mode), 3.03 (parabolic)	5.06 (moment), 5.09 (mode), 5.08 (parabolic)	6.99 (moment), 7.00 (mode), 7.00 (parabolic)
Standard deviation (μm)	0.192 (moment), min = 0.187, max = 0.192	0.137 (moment), min = 0.137, max = 0.147	0.120 (moment), min = 0.120, max = 0.129
Measured polydispersity from FC (%)	6	2.6	1.7

Classical chromatographic like peak profile analyses were performed on Fig. 1B data: average values were calculated by means of the moment theory, mode and a parabolic regression at peak summit. Standard deviation was calculated from the second moment: min and max correspond to the standard deviation obtained after measurement at different peak heights.

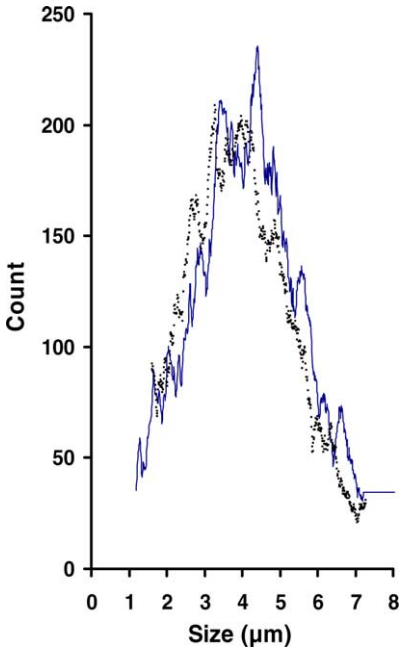

Fig. 2. Calibrated forward scattering signal of crude rice starch particles (50,000 particles counted). Full line at gain 12.5 and dotted line at gain 13.5. Crude rice suspension was analyzed and particle size distribution calibrated on the FS axis by means of Table 1 functions.

3.3. SdFFF of rice starch

3.3.1. Fractogram

In SdFFF, retention and peak profile of starch granules depended on the channel characteristics, operating conditions (field and flow) and sample characteristics (size, density, shape). From the previous granulometric measurements, rice starch particles were in the 3–8 μm range, with a size polydispersity varying from 33 to 35% depending on the measurement technique (FC Gain and Coulter). These micron-sized particles can be eluted in SdFFF according to the "Hyperlayer" mode [16]. The high polydispersity found by granulometric measurements led us to expect a wide distribution in retention time of the sample, which would allow fraction collection.

The separation development procedures in SdFFF combine field intensity and flow rate. First, series of elutions were performed at high flow rates to select a field range leading to an appropriate retention of the starch population. The selected field range must also assume a "Hyperlayer" elution mode. Then flow rate was reduced, in order to reduce lift force and enhance retention (compatibly with the above Hyperlayer requirements). Table 3 presents results obtained from different operating conditions leading to the establishment of optimized conditions (0.8 ml/min, 15*g*) whose fractogram is shown in Fig. 3. In all elution conditions described in Table 3, the "Hyperlayer" elution mode was respected. At a constant field (15.4 ± 0.1)*g*, the increase in flow rate induced an increase in retention ratio R_{obs}: R_{obs} = 0.250 ± 0.002 at 0.6 ml/min and R_{obs} = 0.351 ± 0.002 at 1.4 ml/min

Table 3
Separation development fractogram data

	R_{obs}	σ (R)	s
Mobile phase flow rate (ml/min)/constant external field = (15.4 ± 0.1)*g*			
0.6	0.250 ± 0.002	0.049	6.09–2.32
0.8	0.293 ± 0.004	0.054	6.81–2.51
1.0	0.317 ± 0.012	0.052	6.99–2.74
1.2	0.343 ± 0.016	0.049	5.81–3.12
1.4	0.351 ± 0.002	0.055	7.26–3.09
External field (*g*)/constant mobile phase flow rate = 0.8 ml/min			
10.0	0.338 ± 0.010	0.051	6.88–3.18
12.6	0.318 ± 0.002	0.054	7.27–2.78
15.4	0.293 ± 0.004	0.054	6.81–2.51
17.2	0.282 ± 0.004	0.052	6.92–2.42
19.9	0.271 ± 0.016	0.049	6.23–2.42

Retention ratio R_{obs} and standard deviation σ expressed in term of R for rice starch elution. Starch granule elevation value s, in μm: first value was calculated at the front of the fractogram, second value at the end of the fractogram. Elution conditions: flow injection of 5 μl starch suspension (10 mg/ml in mobile phase distilled water), flow rate: from 0.6 to 1.4 ml/min (distilled water); external multi-gravitational field: from (10.0 ± 0.1)*g* to (19.9 ± 0.1)*g*, spectrophotometric detection at λ = 254 nm. Results were expressed as mean ± S.D. for n = 3.

(mean ± S.D. for n = 3). An increase of field at a constant flow rate (0.8 ml/min) decreased R_{obs} with R_{obs} = 0.338 ± 0.010 at 10.0*g* and R_{obs} = 0.271 ± 0.016 at 19.9*g* (mean ± S.D. for n = 3). The second order choice is directed by a retention ratio minor to 0.3 (retained enough) and large peak dispersity.

The working hypotheses and definitions were the following. First, we assumed a single characteristic elution mode, according to particle size. Second, peak start and end retention ratio parameters are associated with PSD range, here 8 and 3 μm, respectively, leading to the calculation of their average position in the channel thickness. The consequence of the "Hyperlayer" elution mode was that every particle

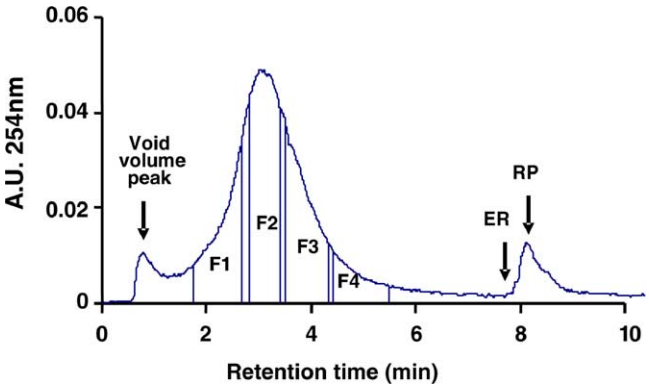

Fig. 3. Rice starch suspension fractogram. Experimental conditions 15.4*g*, 0.8 ml/min flow rate, channel (795 mm × 12 mm × 0.08 mm) made of polystyrene walls, flow injection of 25 μl of 10 mg/ml starch particles suspended in the carrier phase (distilled water). UV detection at 254 nm. FFF system void volume position signaled as well as the field stopped—end of rotation (ER) and the signal obtained provoked by release of reversibly trapped starch particles (RP). Collected fractions F1–F4 are shown.

subpopulation was restrained in a focused zone in the channel thickness.

From Eq. (3) [14–16],

$$R = \frac{6s}{\omega} \tag{3}$$

where R is the retention ratio, ω the channel thickness (80 μm), and we calculated s, the distance between the center of the focused zone to the channel wall [33], using the observed R values (Table 3).

Under optimal elution conditions, R = 0.293 ± 0.004 (n = 4, σ), the approximate average particle elevation was 3.91 μm. With an average rice starch granule diameter of either 4.20 or 4.34 μm depending on the granulometric method (FC or Coulter Counter), the approximate average starch granule elevation value s was greater than the mean rice starch granule radius (2.17 μm). The use of a channel with reduced thickness (80 μm) will emphasize size selectivity, leading to a broad elution profile, which facilitates time dependent fraction collections. However, a systematic size analysis procedure of collected fractions has been performed in order to determine the PSD.

3.3.2. Fraction collection and FC analyses

SdFFF fractograms have been calibrated in terms of size distribution in numerous cases [16]. However, this calibration procedure (associating, under given experimental conditions, a size to a retention time) requires the verification of numerous working hypotheses such as sphere-like shape and surface homogeneity as well as density function over the PSD. These hypothesis could be verified after sub-population separation and purification processes. In order to describe and characterize the separation process underlying the fractogram, fraction collections were performed as described in Fig. 3. PSD of the fractions were determined by means of their average values (first moment) and PSD standard deviation (square root of the second centered moment) as shown in Table 4. From granulometric measurements of any origin (FC or Coulter), fractions of different average values can be obtained, with modified polydispersities compared to the crude population. When Coulter data are compared with FC ones, average mean diameter values as well as population standard deviation (dispersity) were very close. The very slight differences could be attributed to the different measurement

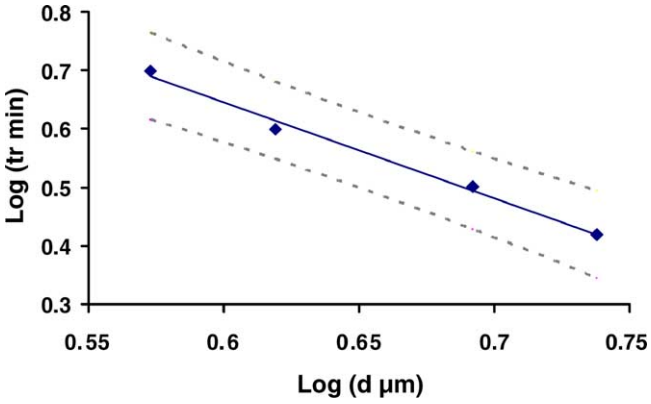

Fig. 4. Regression plot and associated data of FFF size related selectivity curve. Data used were extracted from PSD fraction results (Table 4). Statistics are given in Table 5. Experimental data are shown with linear regression curve. Dotted lines represent the confidence interval hyperbola (±2σ).

principles. We observed that the larger particles were eluted first and the smaller ones last. This elution order is compatible with the "Hyperlayer" one driving to retention versus size selectivity plot corresponding to both the sample and the operating conditions.

3.3.3. Size selectivity curve

The logarithm of the fraction retention times was plotted versus the logarithm of the particle average diameter d. It has been previously reported that, in the case of the elution of populations containing particles of different size but of analogous densities, the graph presented a straight line expressed as follows [34]:

$$\log t_R = -S_d \log d + \log t_{R1} \tag{4}$$

where $-S_d$ is the slope of the curve, represents the selectivity coefficient, and t_{R1} a constant value, equal to the retention time of a 1 μm diameter particle. The selectivity curve obtained plotting fractions average size versus fraction elution time is shown in Fig. 4. It was possible using four fractions to determine size/retention linearity using regression procedures, the results of which are shown in Table 5. With an intense S_d value of 1.63, high size selectivity was obtained. With a linear regression coefficient of 0.99, the selectivity curves demonstrate that SdFFF elution can produce size defined RS subpopulations, a conclusion which is supported by

Table 4
Measured mean diameters of rice starch granules eluted in the total peak and in the four collected fractions (n = 3)

Collected fractions	Flow cytometry, mean diameter (μm) and standard deviation (μm) (CV%)		Coulter Counter, mean diameter (μm) and standard deviation (μm) (CV%)	
Crude	4.20 ± 0.2	1.56 (34.59%)	4.34 ± 0.2	1.36 (31.41%)
F1	5.47 ± 0.2	1.63 (29.79%)	5.41 ± 0.03	1.41 (26.80%)
F2	4.92 ± 0.3	1.50 (30.49%)	5.13 ± 0.3	1.18 (26.22%)
F3	4.16 ± 0.2	1.35 (32.45%)	4.09 ± 0.2	1.19 (28.20%)
F4	3.74 ± 0.2	1.12 (29.94%)	3.71 ± 0.2	1.33 (31.67%)

Left column: results from flow cytometric data according to calibration equations for gain 12.5. Right column: measurement with Counter Coulter. Mean diameters were calculated from flow cytometry first moment given with precision scale (±) and standard deviation from profile centered second moment.

Table 5
Size selectivity regression data

Size selectivity data				*Regression parameter*	Estimate	S.E.	*T*-stat	*P*-value		Confidence interval
d (μm)	t_R (min)	log d	log t_R							
5.47	2.62	0.74	0.42	1	1.6302	0.0691	23.58	0.00179	R^2 = 0.9919	0.3444–0.4933
4.92	3.17	0.69	0.50	x	−1.641	0.105	−15.636	0.004065	Adjusted R^2 = 0.988	0.4279–0.5609
4.16	3.97	0.62	0.60	*Estimated variance*	0.0001794					0.5475–0.6805
3.74	5.00	0.57	0.70	*ANOVA table*						0.6154–0.7643
					d.f.	Sum of squares	Mean squared	*F*-ratio	*P*-value	
				Model	1	0.0439	0.04388	244.478	0.004065	
				Error	2	0.000359	0.0001795			
				Total	3	0.0442				

Linear regression on the decimal logarithm of the data was performed using Mathematica 4.1 software. A list of commonly required diagnostics such as the coefficient of determination R^2 and the Adjusted R^2 are given, the analysis of variance table (ANOVA table) and the mean squared error described as estimated variance. Confidence interval (95%) data were used to draw Fig. 4—a curve envelope. Ellipsoid of confidence region is given as well as S.E. and *P* values used for error estimation. Ellipsoid of confidence region: (1.63019, −1.644141); (0.774087, 003452); (0.549299, −0835626); (−0.835626, −0.549299).

the literature [35]. The linearity of the selectivity curves obtained from time dependent collected fractions suggest that RSP density function is constant or correlated to size.

4. Conclusions

Flow cytometry operated in the forward scattering mode appeared as a versatile tool to accurately determine particle size distribution of organic particles such as starch. The size resolution obtained using proper calibration procedures taking account not only the FS function of size but also the dimension added by the gain amplification resulted in a unique versatility. Hyphenation with SdFFF can define new particle characteristic in particular when subpopulations are considered. The high size selectivity observed as well as the potential choice of fraction collection, position and bandwidth, results in purified fractions of almost predictable properties (average size and polydispersity) at the expense of recovery percentage.

Such strategies already developed in the qualitative mode for cell separations [2,36] can be optimized quantitatively for organic particles, for example SS signal calibration attempts linked to particle composition or surface properties. In that regard, the versatility of fraction collections of predicted properties (size, polydispersity) opens new possibilities. It appears so far, that hyphenation of FFF with powerful light scattering techniques allows sophisticated description of both the crude sample and every collected fraction. For every eluted fraction, information provided by SdFFF retention associated with granulometric measurements may lead to fraction population density assessments.

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