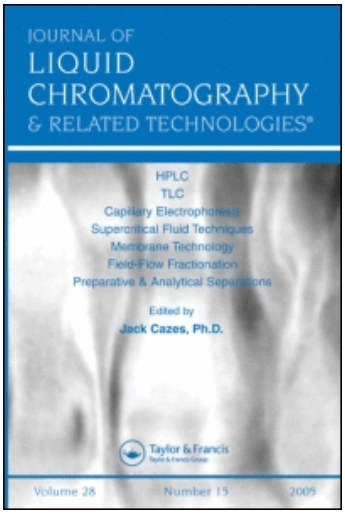

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PROGRESS IN FIELD-FLOW FRACTIONATION: THEORY, METHODOLOGY AND APPLICATIONS

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INTRODUCTION

The history of Field-Flow Fractionation (FFF) began in 1966 when Giddings (1) described a new separation concept based on a coupling of solute concentration and fluid flow nonuniformities inside a narrow channel, which cause the differential migration of the solute species and thus the separation. Physical or chemical lateral field acting across the channel composed usually of two planparallel walls (e.g., temperature gradient, electrical, magnetic or gravitational forces, chemical potential gradient etc.), interacts with molecules or particles of the solute and compresses them to one of the channel walls in the direction of x-axis, perpendicular to this wall. This concentration gradient induces a diffusion flux in the reverse direction. After certain time a steady state is reached and the distribution of the solute across the channel can be characterized by a mean layer thickness l. The velocity of the flow of the fluid inside the

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channel also varies in the direction across the channel. This velocity profile is caused by viscosity effects, accompanying the flow processes. At a laminar isothermal flow of a Newtonian fluid along a narrow channel, usually a parabolic velocity profile is formed inside the channel. The molecules or the particles of the solute are transported in the direction of the longitudinal axis of the channel at varying velocities, depending on the distance from the channel walls, in which they occur.

FFF is similar to chromatography in many aspects. However, all of the processes associated with the separation take place in the fluid phase and there is no stationary phase which would play an active role. This simpleness is, however, characteristic of classical FFF only and not of its combination with the chromatographic technique, e.g., with the use of a channel packed with a chromatographic bed. This is why FFF is sometimes classified as a one-phase chromatography (2-6) or polarization chromatography (7). The absence of the stationary phase that has a large surface area can be of fundamental significance for fractionations of the materials of biological origin. These materials often are very sensitive to a type and intensity of interactions with active surfaces of the packings of chromatographic columns and on contact with large surface areas of these packings they can denaturate in an undesirable way. The total surface area of the FFF channel plays no active part in separation process and is lower by several orders of magnitude than active surface area of the chromatographic column with a comparable separation capacity. Moreover, it can be deactivated so that undesirable interactions with the substances under separation are suppressed to the minimum. The strength of the

physical field applied can be varied within a very wide range continuously, starting from very fine up to very strong fields that induce intensive transversal migration of substances under separation. Consequently, the range of retentions is fairly variable, theoretically from zero retention, determined by a mere passage of the solute through the channel, to a total retention when the solute is completely compressed to the channel wall. The retention in the range from zero to the retention determined by a ten up to approximately fifty-fold of the void volume of the channel comes into account in practice. A simple geometry of the fractionation channel, permitting a lucid mathematical description of the separation process, also belongs to the advantages of FFF.

Giddings (1) assumed the possibilities of programming the field strength, programming the flow velocity, forming the shape of the velocity profile by varying the geometric characteristics of the channel, using casually a channel packing and thus combining the separation based on the above concept with the chromatographic technique, and applying the gases and liquids as the fluids, etc. So far only some of these ideas have been verified experimentally in practice and associated problems have been solved to rather a limited extent even from the theoretical viewpoint.

FFF is important methodology for fractionation and separation of macromolecules (8) within a wide range of molecular weights, starting from several thousands of daltons up to 10^{12} and even perhaps to 10^{15} of daltons (9) even for particles in submicron and micron range and for organized structures, such as cells and microorganisms etc. (4,10-13).

None of the chromatographic techniques nowadays posesses such a flexibility.

Numerous reviews (13-19) appeared in the last years. Each latest one brings up, together with a historical overview, some reports concerning the newest findings and developments. This also demonstrates a revolutionary growth of the FFF methodology and technology.

THEORY

Retention

Nonequilibrium theory of FFF (20) was elaborated on the principles parallel to the nonequilibrium theory of chromatography (21). The solute is displaced in a moving fluid by a combined action of the flow and the field applied. Velocity vector v can be decomposed into two perpendicular vectors: U, induced by the applied field and flow induced component v

$$v = U + v \tag{1}$$

The flux of the solute j can also be written as a sum of the axial component along the longitudinal axis of the channel z and the lateral component in the direction of the x-axis

$$j = j_x + j_z \tag{2}$$

$$j_x = -D_x (dc/dx) + Uc \tag{3}$$

$$j_z = -D_z (dc/dz) + vc \tag{4}$$

where $D_{x,z}$ are the diffusion coefficients in the

direction of the corresponding axes, c is the concentration. It follows that

$$dc/dt = - \nabla j = - (\nabla j_x + \nabla j_z) \qquad (5)$$

where ∇ is a gradient operator. As long as no axial flow occurs in the system, the concentration gradient induced by the field will be balanced by the diffusion, which will lead to a steady state or to a quasiequilibrium concentration, c'. As soon as the flow starts its action, the quasiequilibrium will permanently be disturbed. The deviation from the equilibrium can be characterized by the coefficient ε

$$c = c' (1 + \varepsilon) \qquad (6)$$

It holds for the lateral flux at equilibrium

$$D_x (dc/dx) - Uc' = 0 \qquad (7)$$

By solving this differential equation we obtain the distribution of solute concentration across the channel

$$c' (x) = c'_o \exp (xU/D) \qquad (8)$$

In the present coordinate system, x designates the distance from the wall at which the solute accumulates, U is then a negative magnitude. Equation (8) can thus be rewritten

$$c'(x) = c'_o \exp (-x|U|/D) \qquad (9)$$

Constant c'_o is the solute concentration at the

coordinate x = 0. On defining $l = D/|U|$, Equation (9) can be written as

$$c'(x) = c'_o \exp(-x/l) \tag{10}$$

where l is the mean layer thickness defined above. The average velocity of the zone in the axial direction z is given by

$$V = \langle c'(x) \cdot v(x) \rangle / \langle c'(x) \rangle \tag{11}$$

where v(x) is the actual velocity of the streamline at the coordinate x, brackets $\langle\ \rangle$ designate the average values. Retention ratio R is then defined by

$$R = V/\langle v(x) \rangle = \langle c'(x) \cdot v(x) \rangle / \langle c'(x) \rangle \langle v(x) \rangle \tag{12}$$

For the isothermal, isoviscous flow of a Newtonian liquid between the two parallel infinite planes that is not affected by any outer field, it applies that

$$v(x) = \Delta P x(w-x)/2L\mu \tag{13}$$

where ΔP is the pressure drop along the channel of the length L, the thickness w is the distance between the walls of the channel, and μ is the viscosity of the fluid. For the average velocity it holds

$$\langle v(x) \rangle = \Delta P w^2/12\ L\mu \tag{14}$$

The solution of the Equation (12) gives (22)

$$R = 6\lambda[\coth(2\lambda)^{-1} - 2\lambda] \tag{15}$$

where $\lambda = l/w$. This equation is a basic theoretical relationship that describes quantitatively the

retention in FFF. In the limit when λ approaches to zero it holds that

$$\lim_{\lambda \longrightarrow 0} R = 6\lambda \qquad (16)$$

In a number of practical applications of FFF the approximation given by Equation (16) is fully justified. Hence it can be seen that the relationship between R and λ is very simple. For even a better illustration of the physical meaning of λ , it can be written (4)

$$U = F/f \qquad \text{and} \qquad D = R^{\circ} T/f \qquad (17)$$

and hence

$$\lambda = R^{\circ}T/Fw \qquad (18)$$

where F is the effective field force acting on a mole of the solute, f is the friction coefficient, R° is the gas constant, and T is the absolute temperature. Equation (18) demonstrates that λ is the ratio of the thermal energy and the energy related to the effect of the field.

Zone spreading

Zone spreading is characterized quantitatively by the height equivalent to a theoretical plate (22)

$$H = \zeta^2/L = 2D/R\langle v(x)\rangle + \chi w^2\langle v(x)\rangle /D + \sum H_i \qquad (19)$$

where ζ is the standard deviation of the concentration zone at the end of the channel and χ is a dimensionless parameter. The first term in Equation (19) describes the longitudinal diffusion, the second one the nonequilibrium effects and the third one the sum of different contributions following from

relaxation processes, finite width of the injection etc. Equation (19) is analogous to the description of chromatographic processes. However, with respect to the character of FFF a term corresponding to eddy diffusion in the classical packed chromatographic column is not included in the above equation. The term that describes nonequilibrium processes (23), derived theoretically (20) is the most significant contribution to the total value of H in Equation (19). As follows from the nonequilibrium theory (20,22,23), the dimensionless parameter χ is expressed

$$\chi = 2D \langle c'(x).\varepsilon.v(x)\rangle / \left[\langle c'(x).v(x)\rangle w^2.\langle v(x)\rangle.(d\ln c'/dz)\right] \quad (20)$$

The complex relationship for χ was discussed in detail (23). In the limit when λ tends to zero, it holds simply (22)

$$\lim_{\lambda \longrightarrow 0} \chi = 24\,\lambda^3 \quad (21)$$

if λ tends to infinity, then it holds (23)

$$\lim_{\lambda \longrightarrow \infty} \chi = 1/105 \quad (22)$$

Relaxation

Immediately after the injection of the sample into the channel, the solute is distributed across the channel homogeneously. Only due to the action of the field does the concentration gradient start its formation until the steady state is reached. The time period till the establishment of the quasi-equilibrium is called the relaxation time. Relaxation

processes contribute to zone spreading according to the relationship (12,22)

$$H_r = (17\ n/140\ L)\ (\lambda w^2 \langle v(x)\rangle / D)^2 \qquad (23)$$

where n is an effective number of relaxation processes along the channel, usually equal to one. While increasing the total zone spreading undesirably, this contribution (often negligible) can be eliminated by stopping the flow through the channel after the injection for the time period that makes it possible to obtain the quasiequilibrium.

Relaxation processes occurring after the injection of the solute obviously affect also the retention. If the relaxation time of the solute, t_r, is defined as the time necessary for overcoming the distance between the centre of the channel and the centre of gravity of the quasiequilibrium zone, then the relationship between the apparent retention ratio, R', and theoretical R, i.e., not including the relaxation processes, is given by (22)

$$1/R' = 1/R - n\langle v(x)\rangle t_r(1-R)/RL \qquad (24)$$

and the relaxation time is given by (22)

$$t_r = \frac{w^2\lambda}{D}\left[\frac{1}{2} - \lambda + (\exp(1/\lambda) - 1)^{-1}\right] \qquad (25)$$

The choice of the above relaxation distance is rather arbitrary. In practice, it depends also on the arrangement of the given FFF experiment. Actually, in another paper by Giddings and co-workers (24), this distance is defined as the whole thickness of the channel w, and consequently t_r is expressed as

$$t_r = \frac{w^2\lambda}{D} \qquad (26)$$

by neglecting the second and the third terms in rectangular brackets of the right hand side of Equation (25). In fact, each of the above simple equations is only an approximation of the real situations after the injection of the sample into the channel. Kirkland and co-workers (25) investigated the time necessary to obtain the retentions not influenced by relaxation when using the stop-flow technique. Their experimental results indicated much longer times than expected on the basis of simple equations.

Quite obviously, an additional study, both theoretical and experimental, is needed to explain these differences.

Resolution and peak capacity

The resolution R_s of two solutes 1 and 2 has been defined by the known equation

$$R_s = \frac{|2(t_{R1} - t_{R2})|}{t_{W1} + t_{W2}} \qquad (27)$$

where t_{Ri} are the retention times of solutes 1 and 2 and t_{Wi} are the widths of the elution curves of solutes 1 and 2 expressed in time units. Alternatively, an expression in retention volume units instead of time t, can be used in Equation (27). An almost complete separation is obtained when $R_s = 1$ (ca. 95 % separation for Gaussian elution curves). It also holds for Gaussian elution curves that $W = 4\zeta$ (W being the width of the elution curve expressed consistently in the same units as ζ). By using the definition Equation (27) and the definition equations for retention and zone spreading (e.g. Equations (12) and (19)) Martin and Jaulmes (26) expressed the retention R_s as

$$R_s = \frac{\left| \frac{1}{R_1} - \frac{1}{R_2} \right|}{2\left(\frac{1}{R_1\sqrt{N}_1} + \frac{1}{R_2\sqrt{N}_2} \right)} \qquad (28)$$

where N_i is the number of theoretical plates for solutes 1 and 2 calculated simply from

$$N_i = L/H_i \qquad (29)$$

The peak capacity is defined as the maximum number of components which can be resolved, usually at unit resolution. It depends on a large number of experimental variables and solute characteristics. In order to generalize the conclusions concerning the peak capacity of various FFF techniques, Martin and Jaulmes (26) used several dimensionless parameters such as the reduced plate height h, the reduced velocity υ, and the reduced channel length ξ

$$h = H/w, \qquad \upsilon = \langle v(x) \rangle w/D, \qquad \xi = L/w \qquad (30)$$

The use of the reduced parameters leads to the following relationships

$$N = \xi/h \qquad (31)$$

and

$$h = \frac{2}{R\upsilon} + \chi\upsilon \qquad (32)$$

In Equation (32), which follows from Equation (19), the contribution due to relaxation and extra-channel zone spreading is neglected.

Martin and Jaulmes (26) used the above relationships and a set of typical values characterizing the parameters of actual FFF experiments and analyzed the

peak capacity of some of the FFF techniques with respect to the maximum accessible retention volume, the channel length, eluant velocity, detectability, and analysis time. The maximum accessible retention volume comes from the fact that at a certain point the solute cannot approach closer to the wall and the normal order of elution is disturbed by this steric effect. The details will be discussed below. The theoretical analysis (26) indicated that while the maximum peak capacity increases with increasing field strength, the size of solute which is retained before the occurence of the steric effect, decreases. Consequently, the maximum retention volume under typical conditions is about 24-100 times the channel void volume. The variation of the peak capacity with retention volume for a constant plate number is faster in FFF than in chromatography (26).

The peak capacity as well as resolution is proportional to the square root of the reduced channel length ξ, and decreases with increasing reduced eluant velocity in the practical retentions range.

Under the typical experimental conditions the peak capacity is not limited by detectability because the concentration of the solute at the maximum of the elution curve either increases or remains approximately constant with increasing retention volume (26). Providing the time allowed for the fractionation is limited, the highest peak capacity is obtained at the maximum retention volume.

All of the results of the theoretical analysis by Martin and Jaulmes (26) are, however, limited by the validity of Equations (15) and (19). It follows from the practical experience (26) as well as from the theoretical analysis (27) that some deviations have to be taken into consideration.

Optimization of FFF

By analyzing Equation (19) it was found (28) that the minimal obtainable value of H, i.e., the maximal efficiency with regard to the flow-rate, is given by an approximative relationship

$$H_{min} \doteq R\ w \tag{33}$$

and the optimal flow-rate corresponding to this efficiency is

$$\langle v(x) \rangle_{opt} = \sqrt{18}\ .\ D/R^2 w \tag{34}$$

The above relationships show that H_{min} decreases as the retention increases, i.e., with a decreasing retention ratio R, and hence the best resolution will be obtained for solutes that are retained most. For these solutes the optimal flow-rates will be the highest ones. It follows from the above that flow programming, i.e., a gradual increase of the flow rate, would make it possible on the one hand to work in the range of the optimal parameters permanently and to decrease the time of the analysis on the other. The speed of the FFF analysis can be expressed as the maximal number of theoretical plates generated per unit of time (28)

$$\dot{N}_{max} = D/4w^2\lambda^2 \tag{35}$$

It is obviously desirable to minimalize the values of w and λ, i.e., to increase the field strength and to bring up D to the maximum.

Deviations from idealized FFF model

All the theoretical relationships described above were derived considering some assumptions and

simplifications. Firstly, it was expected that the concentration profile is fully developed during elution. Secondly, the channel was considered to be formed between two infinite parallel planes (i.e., its width being infinite) but in reality it has finite all three dimensions.

Krishnamurthy and Subramanian published an exact theoretical analysis of FFF (29), based on their generalized dispersion theory. They formulated a model of FFF for a two-dimensional system with transverse flow of solute between parallel planes. It means that the effect of the side walls was neglected. Their results can be used to describe the zone spreading as well as the retention for all values of time since the injection of the solute into the channel. For the large values of time the results asymptotically approach those described by previous equations of the nonequilibrium theory by Giddings. The solution of Krishnamurthy and Subramanian (29) makes it possible to explain some experimental artefacts in detail. These artefacts could not be explained by means of the nonequilibrium theory. Perhaps the most important discrepancy between the nonequilibrium theory and the experimental data is that the zone spreading that is observed is considerably larger than the predicted one. Similar results were obtained by Doshi and Gill (30).

Jayaraj and Subramanian (31) further extended the original analysis (29) by a detailed theoretical study of relaxation phenomena in FFF. Using a numerical solution, they were able to model the processes occurring in the FFF channel in various phases of the development. According to this model the zones of the high concentration are situated in the vicinity of the channel centre, however, also in

the vicinity of its walls. In the vicinity of the channel centre the velocity gradient is low and thus axial dispersion is minimal. Although the velocity gradient is high in the vicinity of the wall, the actual velocity is very low and for this reason the axial dispersion is also relatively low. As a result, the concentration distributions in various cross-sections of the channel are rather complex functions. The results of this work (31) demonstrated a complex dependence of the concentration profiles across the channel on the axial coordinate during the relaxation.

Later on, Takahashi and Gill (32) analyzed quantitatively the problem of retention and dispersion in rectangular channel of finite dimensions. They found out that both the retention and dispersion are the functions of the aspect ratio a, that is, of the ratio of the width to the thickness of the channel. The higher is a, the closer is R to the asymptotic value obtained by neglecting side walls effect. On the other hand, the dispersion is allways higher in a real rectangular channel than in hypothetical one without wall effect. The comparison of the theoretical analysis with experiments confirmed the good agreement.

Janča (33) discussed the influence of various factors, like some experimental variables and the channel design, influencing the retention R, the dispersion characterized by χ, and the resolution R_s. The ratio R_{true}/R_∞ (where R_{true} and R_∞ are the retention ratios in real and hypothetical channel, respectively) approaches to unity with increasing a value. From the practical point of view when $a \geq 20$ there is no substantial difference between R_{true} and R_∞. The ratio $(\chi_{true}/\chi_\infty)_{opt}$ derived for optimal flow velocity $\langle v(x) \rangle_{opt}$ varies within

1.7 to 71 for the practically exploited region of the λ values (λ = 0.1 to 0.01). For the velocities higher than $\langle v(x) \rangle_{opt}$, the ratio $(\chi_{true}/\chi_{\infty})$ is higher. Also the influence of extra channel dispersion was discussed (33) with respect to the minimum attainable total zone width under the real conditions of a rectangular channel of finite dimensions. It is advantageous to operate in the region of higher retention values as in this case relatively larger volumes of solute solutions can be injected (33). The relative percentual distinguishable difference $(\Delta\lambda/\lambda)$x100 calculated for R_s=1 is lower in the case of a real rectangular channel than it is in the hypothetical case. However, relative resolution increases with the decreasing value of λ. Consequently, the required resolution can be achieved in higher retentions region.

Rigorous theory undoubtly brings a valuable contribution to the exact mathematical description of FFF. On the other hand, it is necessary to understand that in actual experimental conditions a number of nonidealities doubtlessly exist, such as imperfect smoothness of the surface of the FFF channel walls and some others, which can cause fundamental deviations of the experimental data from the theory. The above and a number of other possible nonidealities are treated by none of these theories. It is this simpleness and an easy telling physical conception of the derived relationships that are, in spite of some simplifying asymptotic assumptions, an advantage of the nonequilibrium theory.

FFF TECHNIQUES

While the principal arrangement of the contemporary experimental equipment for FFF is, except the

FFF channel proper, identical with the arrangement for liquid chromatography, various FFF techniques differ from each other by the character of the field applied. Their survey, including the main applications in the analysis of macromolecules and particles is given in the following paragraphs.

Thermal FFF

Thermal field-flow fractionation (TFFF) belongs to the historically oldest techniques of FFF. A channel for TFFF is relatively simple. It is usually composed of two metallic blocks with high-polished surfaces, which clamp a spacer. The upper of the two blocks is heated electrically, the lower one is cooled with water. The channel shape proper is cut into the spacer. A temperature gradient between the walls or rather the thermal energy flux causes the non-selective thermal diffusion of the solute species as a consequence of entropy production leading to the accumulation of solute usually near the cold wall.

In early works (34,35) a basic experimental arrangement was described and a successful fractionation of polystyrene (PS) standards with narrow distribution of molecular weights was demonstrated. Some fundamental theoretical and experimental aspects of TFFF were studied in papers by Giddings and co-workers (22,36). The value of λ is expressed by (22)

$$\lambda = \left[w(\alpha_T/T) \cdot dT/dx \right]^{-1} \qquad (36)$$

where α_T is dimensionless thermal diffusion factor, associated with the thermal diffusion coefficient, D_T

$$\alpha_T = D_T \cdot T/D \qquad (37)$$

From the theoretical viewpoint TFFF is the most complex technique. Owing to the temperature gradient across the channel, the flow is not isoviscous and, consequently, the velocity profile is not parabolic (36). An exact analysis of the shape of the velocity profile in TFFF with regard to nonisothermal flow was presented by Westermann-Clark (37).

Several applications of TFFF were oriented toward the separation of synthetic polymers. TFFF measurement of thermal diffusion factors (38) was demonstrated. Theoretical comparison of TFFF and size exclusion chromatography (39) for the separation of polymers pointed out a number of advantages of TFFF. It was further used to study thermal diffusion of PS in various solvents differing in their thermodynamic quality (40). The use of the pressurized system operating at elevated temperatures provided an effective fractionation of low-molecular weight PS (41). Potentialities of this technique were shown even for the fractionation of polymers with extremely high molecular weights (9), up to 10^{12} daltons. Temperature gradient programming made it possible to fractionate PS standards in a wide range of molecular weights from 4000 up to 7 000 000 daltons in a single experiment (42).

Miniaturization of the channel for TFFF and some other design modifications made it possible to reduce the time of analysis to several tens of seconds up to several minutes (43), and to increase the resolution (44).

The subsequent studies were oriented at the explanation of the factors that cause and affect zone spreading in TFFF (45) and at the determination of the precise polydispersity of the polymer samples

(46) by measuring at various solvent velocities and by extrapolating to zero velocity. An improved separation in TFFF can be obtained by using thermogravitational effect, i.e., by using thermal convection in a nonhorizontal channel. The resulting velocity profile formed under such conditions has a more complicated nonparabolic shape (47). All of the experimental results mentioned above were obtained with the use of PS samples. Only recently TFFF was used for the fractionation of other polymers (48) including polyethylene and polypropylene (49) that represent an experimental problem due to the solubility of these polymers at high temperatures. Martin (50) demonstrated an advantage of a coupling of the TFFF channel with the photogoniodiffusometer for low-angle laser light scattering for the analysis of polymers. Janča and Klepárník (51) showed potentialities of TFFF for the determination of molecular weight distribution of polymers and a procedure for an exact interpretation of experimental results of TFFF by using a calibration method.

The conditions of the effective separation by TFFF were discussed in relation to the matter of Soret effect (thermal diffusion) (52) and to the possibility to fractionate polyelectrolytes (53). Theory of retention in TFFF of macromolecules was progressed (54). In a critical review on polymer analysis by TFFF, Martin and Reynaud (55) specified the requirements for successful separations and demonstrated that samples of polymethylmethacrylate accumulate at the cold wall in given conditions.

Sedimentation FFF

Sedimentation field-flow fractionation (SFFF) belongs, besides TFFF, to the oldest techniques of

FFF. It was predicted conceptually already in Giddings' work (1), Berg and co-workers (56-58) published their results independently. Either natural gravitational or centrifugal forces in the centrifuge serve here as an effective field.

For the value of λ of spherical particles it holds (59)

$$\lambda = 6kT / \pi d_p^3 g w \Delta\varrho \qquad (38)$$

g is centrifugal acceleration, k is Boltzmann constant, d_p is particle diameter, and $\Delta\varrho$ is the difference in the densities of the particles and the solvent used. Berg and Purcell (56) presented the elementary theoretical analysis of fractionation of particles by using gravitational or centrifugal forces in the centrifuge. In their first experimental paper (57) they described the fractionation of PS latex with the particle size of 0.796 /um and 1.305/um. Their experimental arrangement was quite simple, but the time of the analysis was very long, 76-125 hours. In their subsequent paper (58) they described the separation of R 17 E. Coli bacteriophage, having the molecular weight of 4×10^6 daltons, in the centrifuge. The time of the analysis was substantially shorter in this instance, approximately 4 - 12 hours.

Giddings and co-workers (59) described the device in which the channel was coiled along the internal wall of the centrifuge basket. The basic theoretical and experimental aspects of SFFF were discussed and the fractionation of a series of monodisperse spherical PS latex was demonstrated (59). In the following paper (60) a theory of programmed SFFF using the programming of the intensity of centrifugal force and the programing of the density of the solvent was elaborated. Utilization of

programming expands considerably the range of molecular weights which can be fractionated in a single run. The programming of the field strength in SFFF was effectuated by decreasing gradually the number of revolutions of the centrifuge in the course of the separation of PS latex samples and the programming of the solvent density by increasing gradually concentration of saccharose in water which was used as a solvent.

Another type of programming, the flow-rate, was elaborated theoretically and verified experimentally (61). Extension and narrowing of the channel, slowing down the flow, field programming and an increase in the field strength applied (62) provided a high resolution of particle separation by SFFF in a thousand-fold range of masses in a single measurement.

Yau and Kirkland (25,63-65) also dealt with the programming of SFFF in the analysis of the particle size distribution. They also used the time-delayed exponential-decay programming of the intensity of the centrifugal forces which allowed to linearize the dependence of retention time on a logarithm of dimensions of fractionated solutes. Moreover, the total analysis time was shortened without sacrificing the resolution.

A new design of the equipment for SFFF (25) allowed to work with very high intensities of the centrifugal field, up to 15 000 G at 12 000 r.p.m. Innovations in technology of a rotor for SFFF permit further increase in rotational speed up to 32 000 r.p.m. corresponding to 100 000 G (66). The absolute dimensions or molecular weights of the particles under analysis can be calculated from the retention data (67). In such a way molecular weight

of T 2 bacteriophage was determined. According to the Equation (38), the retention is a function of the product $d_p^3\Delta\varrho$, among other parameters. Thus it is possible to determine the size and the density of the solute particles independently, providing the fractionation is made in various solvents differing in densities, e.g. in aqueous sucrose solutions (68). When the dilute sample of solute is injected during rotation it concentrates at the begining of the channel due to the fact that the average volume flow-rate of the retained solute is lower than the average one of the injected solution. This effect was actively used to concentrate the dilute colloidal samples by SFFF (24). One can even operate in such a way that the injection is run at higher field forces and after the whole quantity of the solute solution is injected, the field force is decreased to the required value. Of course, this procedure could be used in principal not only for SFFF but also for another FFF techniques. Above a certain limit of retention, determined by the size of the solute macromolecules or particles, there is no effective separation because the steric effect comes to be operative, as mentioned in theoretical part. This limit was found theoretically for SFFF (69). Accordingly, chain molecules larger than about 10^{10} in mass would difficult to separate (69).

The character of SFFF makes this technique very attractive, particularly for biological applications, but several other uses were described (70). SFFF was used for the characterization of liposomes (71), BSA microspheres (72), various viruses (73,74), colloidal particles in river water (75) and nuclear energy related materials (76,77). The consistent methodology of colloid characterization by SFFF was

developed, concerning the analysis of monodisperse populations (78), particles having size distributions (79), and emulsions (80). An experimental verification of theory indicated good agreement (81). SFFF was compared with several competitive chromatographic methods and conventional non-chromatographic methods, like exclusion chromatography, hydrodynamic chromatography and disc centrifugation. The comparison was made on the basis of resolution, accuracy, resolving power, peak capacity and dynamic range. SFFF provides superior or at least the same above parameters over the compared methods (82).

Sedimentation-Flotation Focusing FFF

The principle of a newly proposed (83) sedimentation-flotation focusing field-flow fractionation (SFFFFF) is substantialy different. Solute particles or macromolecules sediment or float in the density gradient of a liquid phase according to the differences between the local density of the liquid and that of the particle or macromolecule. At quasiequilibrium, the solute species are focused in a thin layer at the position where the density of the environment is the same as the solute density. The distribution of the solute can thus be characterized by a Gaussian function (83)

$$c(x) = c_{max}\exp\left[-\vartheta\omega^2 x_{max}(d\rho/dx)(x_{max}-x)^2/2R^oT\right] \quad (39)$$

where ϑ is the molar volume of the solute, ω is the rotational velocity of the rotor, and x_{max} is the coordinate of the maximum solute concentration c_{max} where the densities of solute and environment are identical. Originaly it was proposed to use a

rectangular cross-section of the fractionation channel so that the formed fluid velocity profile is parabolic (83). In a recent paper, Janča and Jahnová (84) proposed the new-shaped cross-section of the channel which is either trapezoidal or parabolic. In such a case the channel has a modulated permeability resulting in the variation of the fluid flow velocity across the channel width. A theoretical analysis of such parameters like the efficiency, the resolution and the retention was given (83,84). SFFFFF is anticipated for the separation of particles or macromolecules differing in their densities. For example, polymers could be analyzed from the point of view of tacticity because of differences in densities for chains having the same molecular weight but different structures. In a similar way, biological macromolecules or particles could be effectively fractionated. These substances are very often monodisperse with respect to molecular weight, but exhibit differences in structure which decide on density. The principle of the channel with modulated cross-sectional permeability can also be applied to other techniques of FFF (84).

Electrical FFF

Electrical field-flow fractionation (EFFF) belongs to experimentally most sophisticated techniques of FFF. This may also be the reason why a relatively less number of papers has been published, in spite of the first work (85) having been published as early as in 1972. The altogether homogeneous field is induced by electrical current across the channel. The walls of the channel for EFFF are composed of two semipermeable membranes permitting the passage

of small ions and separating the channel space from the electrode compartment. Dimensionless quantity λ is determined by the electrophoretic mobility, $/u_e$, electrical field strength, E, diffusion coefficient and channel thickness according to (86)

$$\lambda = D/_{/}u_e E\, w \qquad (40)$$

In the first paper (85), the principle of EFFF was described qualitatively and the method was applied to the analysis of some proteins - albumin, lysozym, haemoglobin and γ-globulin. In a subsequent paper (86) the theory of EFFF was elaborated in more detail, the experimental arrangement using regenerated cellulose as semipermeable flexible membrane that composed the channel walls was described and, again, some proteins were fractionated.

An explanation of the deviations, observed during the fractionation of the above proteins was presented by Subramanian and co-workers (87) as a result of the electrical field gradient in the vicinity of the membrane interface.

In order to eliminate the disadvantages following from the use of flexible membranes, Giddings et al. (88) designed a new channel in which both of the semipermeable membranes were carried by polyethylene frits. This gave rise to a better reproducibility and a better agreement between the theory and experiments for the separation of native proteins. The separations of denaturated proteins and PS latex, however, were not successful.

Reis and Lightfoot (7) also treated the separation of proteins by using a method from the EFFF class. The channel was composed of a circular

semipermeable tube and the electrical field was applied perpendicularly to the central axis of the channel. Recent studies of electroretention of proteins (89,90) have resulted in more detailed understanding of previously observed retention anomalies in EFFF. Homogeneous proteins were used to characterize the improvements of the separation system and a detailed critical review of both theoretical and experimental aspects of EFFF was presented (91). The solutes that show only minor differences in the mobilities, but differ substantially in D, can be separated by EFFF in spite that their electrophoretic resolution is poor. Hence EFFF and direct electrophoretic methods complement each other. The high voltage gradients per unit of length are obtained at low absolute values of the voltage across the EFFF channel. The heat, generated due to high voltage values impairs separation characteristics of direct electrophoretic methods.

Flow FFF

Flow field-flow fractionation (FFFF) is the most universal technique of FFF. The flow of the solvent, perpendicular to the flow of the basic medium in the channel, creates an external field. The earliest experimental works belonging to this class of FFF were published by Lee and co-workers (92,93), but were called one-phase chromatography (92) or ultrafiltration-induced polarization chromatography (93). They elaborated a basic theoretical model of the separation in circular semipermeable tubes and fractionated blue-dextran and human plasma (92), bovine serum albumin and some polydextrans (93).

Giddings and co-workers (94,95) designed the FFFF channel in a classical manner, i.e., of two

planparallel semipermeable membranes. They developed theoretical bases of FFFF and fractionated successfully a series of monodisperse spherical PS latex and a number of proteins. The separation in FFFF is determined only by the differences in the values of the diffusion coefficient, D, or the friction coefficient, f, because the perpendicular flow having the velocity U acts on all of the solutes uniformly. The retention parameter, λ, is then determined by (10)

$$\lambda = R^{o}TV^{o} \; / \; 3\pi N_{A}\mu V_{c}w^{2}d^{o} \qquad (41)$$

where V_c is the volumetric perpendicular flow, μ is the viscosity of the medium, V^o is the dead volume of the channel, d^o is the effective Stokes' diameter, N_A is Avogadro's number.

The effect of relaxation on the retention and resolution in FFFF was studied (96). A substantial improvement of the fractionation of f2 virus was achieved by using the stop-flow technique (96). FFFF can be applied as a dialysation or ultrafiltration cell (97) to a continuous separation. The operation of one such unit was demonstrated for the isolation of low-molecular weight ethylene blue from bovine serum albumin (97). Various viruses (98) and a number of proteins (99) were separated, purified and characterized as well as colloid silica gel samples (100). FFFF of water-soluble polyelectrolytes, sulphonated polystyrene and sodium salt of polyacrylic acid proved its applicability to the separation of macromolecules (101). FFFF complements to advantage SFFF as far as size distribution analysis is concerned (11).

Magnetic FFF

Magnetic field-flow fractionation (MFFF) has been studied in the only work (102), dealing with theoretical principles of the separation and demonstrating retentions of bovine serum albumin in the presence of nickel(II) ions in a magnetic field of 400 G. A coiled Teflon capillary was used as a channel. In the absence of nickel(II) ions no retention was observed.

For the value of λ of spherical particles the relationship was derived

$$\lambda = (8r/w)(R^{o}T/N_{A}\,/u_{p}\,\Delta H)^{2} \qquad (42)$$

where r is the particle radius, ΔH is the gradient of the magnetic field strength and $/u_{p}$ is the magnetic moment of the particle. Equation (42) obviously represents an approximation valid for λ approaching to zero. Moreover, the comparison of experimental retentions with the theoretical model indicates that besides the described effect of magnetic field on solute molecules other phenomena also play an important role. This conclusion results from the fact that the observed retention at 400 G is higher than the calculated one by using the simplified model (102).

Concentration FFF

Concentration field-flow fractionation (CFFF) is the only technique of FFF that makes use of a concentration gradient of a mixed solvent across the channel in order to induce effective chemical forces or chemical field (103). When chemical

potential gradient is $d/u^o/dx$, it follows from the theory (103) that the value of λ is

$$\lambda = R^oT/\Delta/u_c^o \qquad (43)$$

where $\Delta/u_c^o = (d/u^o/dx).w$ is the total increment of the chemical potential across the channel. If the ratio of the concentrations near the both walls is $\alpha_c = c_o/c_w$, then it holds for the retention ratio, R,

$$R = (6/\ln\alpha_c)\left(\frac{\alpha_c + 1}{\alpha_c - 1} - \frac{2}{\ln\alpha_c}\right) \qquad (44)$$

It was found by analyzing Equation (44) that for an effective fractionation α_c must be at least 10 to 100.

CFFF represents the most difficultly realizable technique in classical arrangement, but there exists a prospect that, owing to its unique retention mechanism, the effort required for its practical realization and application will be made.

Steric FFF

Steric field-flow fractionation (steric FFF) occupies among other techniques of FFF an exceptional position. It represents the upper limit of the field strength applied. The particles are compressed closer to the wall as the field strength increases. When the mean distance of Brownian motion is less than the particle radius, r, steric FFF takes place. The mean layer thickness is thus controlled by steric exclusion. Hence larger particles migrate into the streamlines of higher velocities than smaller particles do and are eluated more rapidly.

Giddings (104) treated theoretical aspects of steric FFF and its comparison with the mechanism of hydrodynamic chromatography. He derived limit relationship for the value of R, and for $\alpha \longrightarrow 0$

$$R = 6\alpha(1 - \alpha) \tag{45}$$

where $\alpha = r/w$. Of course, α in brackets can be neglected for $\alpha \longrightarrow 0$ and consequently

$$R = 6\alpha \tag{46}$$

When taking into consideration both normal and steric FFF, R was defined (105) as

$$R = 6\gamma\alpha + 6\lambda \tag{47}$$

where $\gamma \doteq 1$ is a dimensionless factor allowing for some nonidealities. λ is related to the solute diameter, d, for TFFF, SFFF, and FFFF by

$$\lambda = \lambda_o/d^n \tag{48}$$

where the exponent n has different typical values for various FFF techniques. It is obvious from Equation (47) that R will increase with increasing d in the region of normal TFFF, SFFF, and FFFF, and will decrease with increasing d in steric FFF. Hence it exists the inversion value R_{inv} for some of the FFF techniques for which $dR/dd = 0$. This was derived (105) as

$$R_{inv} = 3(n+1)(\gamma/nw)^{n/(n+1)}(2\lambda_o)^{1/(n+1)} \tag{49}$$

Theoretically, any effective field may be applied to the steric FFF mode. However, gravitational field represents the most practical means of the

utilization of the principle of steric FFF for fractionations of 1 - 100 /um particles. Experimental evidence for the applicability of steric FFF was presented by Giddings and Myers (106), by an example of the fractionation of glass beads having 10 - 32 /um in diameter. The column was composed of a spacer clamped between two glass plates. The channel proper was cut into this spacer. Various types of the chromatographic spherical packings were fractionated and characterized from the viewpoint of dimensions in the subsequent work (107). In this case, a dependence of the retention ratio, R, on the flow-rate, which was not predicted by the theory, was observed. Caldwell and co-workers (108) explained the dependence of R on the flow-rate observed previously (107) by the existence of lift forces.

By inclining the transversal axis of the channel and by injecting the sample into the upper part of the channel, it was provided that particles under separation were carried and slid towards the lower part of the channel where collection "pockets" were placed along the channel (109). The particles that were carried along the channel slid at the same time to lower parts of the channel and were trapped in the "pockets". Larger particles were trapped in a shorter distance from the injection port, smaller particles were trapped further from the injection port. Continuous fractionation of particles could be obtained in this manner by selecting the channel design properly. The distance from the injection point Z at which the particles are trapped into the "pocket" can be calculated from

$$Z = \frac{27\, Q \mu}{w^2\, r\, \Delta\rho G \cos\varphi} \qquad (50)$$

where Q is the fluid flow-rate, G is the gravity force, and φ is the inclination angle. Hence, both steric and sedimentation effect decide on separation.

Steric FFF represents a further principal progress in the methodology of FFF, and permits a simultaneous extension of applications into the range of the large-diameter particles.

PROSPECTS OF FFF

Several recent papers have treated both theoretically and practically further possibilities than can be provided by FFF.

An increase in the retention and capacity of the FFF channel, and an increase in the selectivity can be obtained by modifying surface of the channel accumulation wall with the aid of transversal barriers (110). These barriers form the grooves in which the solvent does not move and where the solute can penetrate both in and out by diffusion only. The grooves could be used to trap the second phase and to combine the action of the field and the partition between the phases. Preliminary results were obtained in experiments with the fractionation of PS standards by TFFF method using the channel with transversal grooves (110).

Subramanian (111) proposed that a perpendicular field across a short part of the channel establishes the concentration distribution without the flow and, later on, separation proceeds with the aid of the flow without the field action. By selecting properly the experimental conditions, i.e., the intensity and the time of the field action, the channel length and the solvent flow-rate, a high efficiency of fractionations can be reached within a relatively short time (111).

Except TFFF and SFFFFF all of the other techniques of FFF involved until now the establishment of a parabolic velocity profile. Theoretical analysis of the retention and the zone spreading when the velocity profile is asymmetric with regard to the longitudinal central axis of the channel and can be described by a general function of a polynomial type, was performed by Martin and Giddings (112). Janča and Giddings (113) showed a prospective possibility of utilizing non-Newtonian behavior of some liquids. They used flexible Ellis' three-parameter equation, describing non-Newtonian phenomena, to derive the dependence of R on λ for different conditions of the non-Newtonian flow. This phenomenon could be utilized to increase the selectivity of strongly retained solutes. The variation in the shape of the velocity profile in a single separation run was also suggested (113), i.e., programming of the properties that are decisive for non-Newtonian behavior of the liquid applied.

An extension of FFF to the separation of non-spherical particles and the influence of the wall effect were studied both theoretically and experimentally by Gajdos and Brenner (114).

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