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Use of Secondary Equilibria for the Separation of Small Solutes by Field-Flow Fractionation

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The dynamic range and selectivity of field-flow fractionation (FFF) can be increased by using secondary chemical equilibria (SCE). SCE are established by adding a macromolecular additive or aggregate, which strongly interacts with the field, to the carrier solution. In this study an oil-in-water (O/W) microemulsion was used as the carrier solution in a sedimentation FFF apparatus. The microemulsion droplets (referred to as the "support") interact with the field and are retained relative to the bulk water. Small solutes that partition or bind to the microemulsion droplets are also retained relative to solutes that do not interact with the support. In this way it is possible to separate somewhat polar compounds, such as ascorbic acid and sodium benzoate, which prefer bulk water, from apolar solutes, such as toluene, which prefers the support. In addition, the study of retention times in this system allows one to calculate the average microemulsion droplet radius. It appears that SCE-FFF could be a useful way to obtain important information on the physicochemical properties of a variety of colloidal supports.

In a recent communication, the theoretical study on the use of secondary chemical equilibria (SCE) for the separation of small solutes by field-flow fractionation (FFF) was reported (*1*). The FFF technique uses an external field to drive macromolecules or particles, carried by a solution in a long ribbonlike channel, toward one wall of the channel. Because of the parabolic flow profile, the molecules or particles, forced against the wall, travel more slowly and are retained. Particles that interact more strongly with the field show the greatest retention (*2*). This technique is very useful for analyzing solutes of high molecular weight, such as synthetic polymers, proteins, or latexes. However, it cannot be used with low molecular weight solutes.

In order to retain small molecules that do not interact with the field, a secondary chemical equilibrium (SCE) can be used. The principle, outlined and derived in a recent paper (*1*), is illustrated in Figure 1. In SCE-FFF, the liquid carrier solution contains particles, for example micelles, oil droplets, or macromolecules, that interact with the external field and are retained (open circles in Figure 1). These particles are referred to as "support" (*1*). If a sample is introduced that contains a mixture of solutes that interact weakly with the field, but that have a certain affinity for the "support", then a separation of the solutes may be possible. Solutes having a strong affinity for the "support" will be retained because they will be carried at a low speed by the "support" (hexagons, Figure 1B). Solutes with little or no affinity for the "support" will be carried at a higher speed by the continuous phase (small dark circles, Figure 1B).

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A practical study of the feasibility of the SCE-FFF technique is given here. Using the sedimentation FFF method, micellar solutions and microemulsions were used as mobile carrier solutions. Micellar solutions contain surfactant molecules aggregated in micelles. A micelle of polyoxyethylene-6-cetyl ether, $C_{16}H_{33}(OCH_2CH_2)_6OH$, written for convenience C16E6, molecular weight 506, can consist of 10000 surfactant molecules. The micellar aggregational weight is as high as 5×10^6 g/mol (*3*). Microemulsions are clear systems that contain a polar liquid (generally water or saline), a hydrophobic liquid (oil), and one or a mixture of surface-active agents (*4*). The classical structural model for microemulsions is that of a monodisperse population of dynamic and interacting microglobules. In oil in water (O/W) microemulsions, the oil phase consists of submicroscopic oil droplets whose diameters range from about 6 to 80 nm, depending on the composition. The use of ordered systems in chemical separations has been described recently (*5*).

Sedimentation FFF theory was developed by Giddings, Yang, and Myers (*6*, *7*). In this technique, the field is induced by a centrifuge. The basic retention parameter, R, is defined as the ratio of the average velocity of the solute to the mean velocity of the carrier. The basic retention equation for FFF shows the retention ratio, R, to be uniquely related to the reduced layer thickness of the zone, λ, in the following manner:

$$R = 6\lambda[\coth(1/2\lambda) - 2\lambda] \quad (1)$$

The reduced layer thickness, λ, is defined as the ratio of the mean layer thickness, l, of the solute to the channel thickness, w

$$\lambda = l/w \quad (2)$$

When secondary chemical equilibria are used, the solute reduced layer thickness is (*1*)

$$\lambda = \frac{3 + C_{sm}\bar{V}(P-1)}{6 + (w/d)C_{sm}\bar{V}(P-1)} \quad (3)$$

in which C_{sm} is the "support" concentration in the carrier solution, $\bar{V}$ is the molar volume of the "support", and the $C_{sm}\bar{V}$ product corresponds to the volume percentage of "support" in the carrier. P is a dimensionless partition coefficient representing the solute affinity for the "support" phase and d is the mean layer thickness of the "support" (*1*).

EXPERIMENTAL SECTION

Carriers. The nonionic surfactants used to make the micellar and microemulsion solutions were polyoxyethylene 23 dodecyl ether (C12E23, or Brij 35), polyoxyethylene 10 dodecyl ether (C12E10), and polyoxyethylene 20 stearyl ether (C18E20, or Brij 78). They were obtained from Sigma Chemical (St. Louis, MO). The physicochemical properties related to FFF are listed in Table I with the microemulsion compositions used. Sodium dodecyl sulfate (SDS), pentanol, and hexane were obtained through Sigma. Water was distilled and filtered on a Barnstead Nanopure system.

Sedimentation FFF Apparatus. Detailed characteristics of the sedimentation FFF device used have been described elsewhere

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Table I. Physicochemical Parameters and Composition of the Ordered Media Used (25 °C)

Micellar Solutions (4% w/v)[a]

surfactant	mol wt, g/mol: monomer	mol wt, g/mol: micelle	CMS, mol/L	$\bar{V}$, L/mol	$C_{sm}\bar{V}$, %(v/v)	ρ_m, g/cm^3	ρ_s
C12E23	1200	6×10^4	6×10^{-4}	1.064	3.55	1.000	1.128
C12E10	630	2×10^5	10^{-4}	0.595	3.80	0.998	1.058
C18E20	1150	7×10^5	4×10^{-7}	1.208	4.20	0.994	0.952

Microemulsion Composition[b]

no.	water, %(w/w)	SDS, %(w/w)	pentanol, %(w/w)	hexane, %(w/w)	$C_{sm}\bar{V}$, %(v/v)	ρ_{uE}, g/cm^3	ρ_s
1	78.8	3.5	7.0	10.7	25.6	0.9436	0.780
2	83.3	3.9	7.8	5.0	19.5	0.9661	0.827

[a] $\bar{V}$, molar volume; $C_{sm}\bar{V}$, volume fraction of the organic phase; ρ_m, density of the 4% (w/v) micellar solution; ρ_s, calculated density of the micellar phase. [b] ρ_{uE}, density of the microemulsion. Density calculation were made assuming that the aqueous phase has the pure water density value (0.9970 at 25 °C).

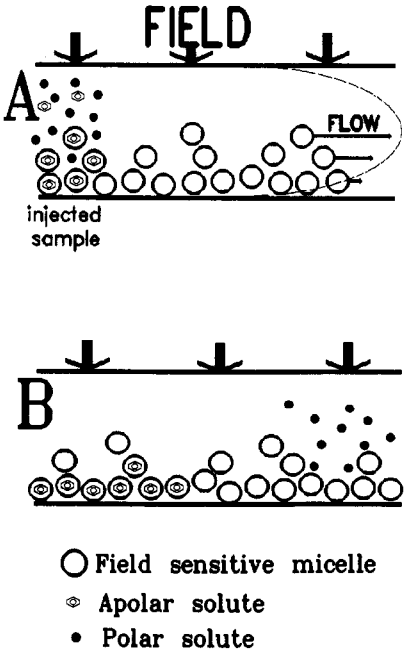

Figure 1. Principle of SCE-FFF. The "support" particles or droplets (open circles) are field sensitive and move more slowly than the overall carrier. A: Simple injection. B: The polar solute (dark circles) moves with the carrier, the apolar solute (hexagons) moves with the "support".

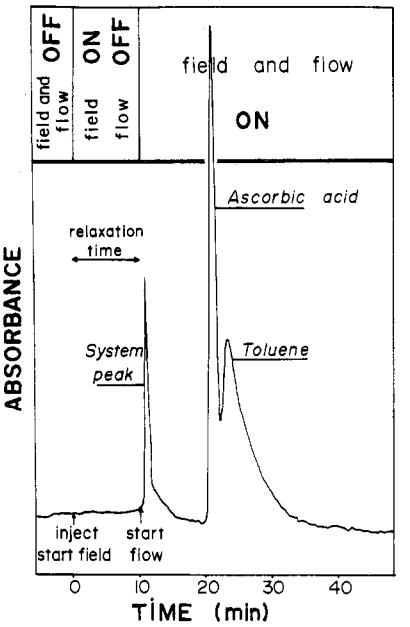

Figure 2. Separation of ascorbic acid (57 nmol) and toluene (220 nmol) by SCE-FFF using microemulsion 1 as carrier, 1400 rpm, 337g, relaxation time 10 min, then 0.22 mL/min, absorbance 1.28. Note that the "system peak" in this system is simply the detector response that occurs when the pump is turned on and flow begins.

(*6*, *8*). Basically, it consists of a 16 cm radius centrifuge basket in which the inside wall is fitted with a FFF channel fabricated from two pieces of 762 μm thick 304 stainless steel sheets with a 127-μm Mylar spacer from which the desired channel shape had been cut and removed. The three pieces were bolt-welded together on the outside edges while bent to fit inside the centrifuge basket. The channel dimensions were as follows: radius r, 15.8 cm; thickness w, 127 μm; length L_c, 93.0 cm; breadth b, 2.0 cm; and volume v_c, 2.34 mL. Two special rotary seals, designed and built in the Utah laboratory, allowed continuous flow to and from the channel. Each seal consisted of a hard tungsten carbide stationary sleeve with an internal flow channel and a moving body of stainless steel. The flow stream was isolated from the stabilizing bearings and directed into the rotating FFF channel by means of double O-ring seals. An injection port was placed between the seal and the FFF channel inlet enabling sample injections by microsyringe when the centrifuge was stopped.

The rotation rate range of the device was between 50 and 1400 rpm, controlled by a laboratory-built microprocessor-driven unit. The carrier fluid was pumped by a Minipuls Model 2 metering peristaltic pump from Gilson Medical Electronics (Middleton, WI). The effluent was monitored by a 254-nm Altex Analytical UV detector, Model 153, from Beckman Instruments (Berkeley, CA).

Procedure. As indicated in the theoretical treatment, the channel must be equilibrated before any sample injection (*1*). As the "support" (micelles or microemulsion droplets) is field sensitive, it is retained in the channel. After channel equilibration, the internal concentration of the "support", C_s, is higher than the analytical concentration, C_{sm}, in the carrier outside the channel. Using eq 1 for the retention parameter, we have (*1*)

$$C_s = C_{sm}/R \tag{4}$$

As this apparatus utilized a stop-field stop-flow injection, the following procedure was used. After a 10-min equilibration time, with both field and flow on, the centrifuge rotation and the flow were stopped simultaneously. The outlet tubing was clogged with a needle to avoid any concentration change inside the FFF channel. Then, the sample was injected (2 μL) and the injection was followed by a 10-min stop-flow period with the field on, to allow for relaxation (Figure 2). The theoretical relaxation time, t_r, was defined by Giddings et al. as (*6*, *9*)

$$t_r = \frac{w^2\lambda_s}{D_s}\left[\frac{1}{2} - \lambda_s + \frac{1}{\exp(1/\lambda_s) - 1}\right] \tag{5}$$

in which D_s is the diffusion coefficient of the "support" and λ_s is the "support" reduced layer thickness. With this system and

Table II. Peak Characteristics Corresponding to Figures 2 and 3[a]

microemulsion	solute	V_R, mL	λ	N, plates	resolution	R, nm
1	ascorbic acid	2.48	no	500		
		(0.09)	ret	(100)		
	toluene	2.98	0.25	60	0.55	35
		(0.15)	(0.04)	(20)	(0.06)	(2)
2	ascorbic acid	2.60	no	300		
		(0.20)	ret	(100)		
	toluene	3.20	0.20	12	0.38	41
		(0.30)	(0.04)	(4)	(0.08)	(3)
3	sodium benzoate	2.40	no	100		
		(0.20)	ret	(50)		
	toluene	3.20	0.20	13	0.33	41
		(0.30)	(0.04)	(4)	(0.07)	(3)

[a] V_R, retention volume; λ, reduced layer thickness of the solute; N, plate count; R, estimated radius of a microemulsion droplet. Numbers in parentheses correspond to the experimental error margin.

an estimated value of 0.3 for λ_s and 6×10^{-8} cm^2/s for D_s (corresponding to 40 nm radius spheres) the theoretical relaxation time, t_r, is 5 min. The use of a 10-min stop-flow relaxation time ensured a complete reequilibration of the system. However, on restarting flow, this equilibrium is destroyed at the beginning of the channel because the incoming eluent cannot equilibrate instantaneously. A steady state ensues, with the volume fraction of the "support" particles over the cross section of the channel increasing with the distance from the channel entrance (where the "support" concentration is C_{sm}) to a steady-state "support" concentration of C_s (eq. 4) at a relaxation distance, h_0, down the channel. For any distance higher than h_0, the "support" concentration is constant and equal to C_s. The relaxation distance is the product of the mean velocity of the carrier and the relaxation time, t_r (eq 5). By use of low flow rates, it is possible to minimize the distance h_0. If h_0 is small compared to the channel length, then the assumption that a state of equilibrium exists throughout the run is reasonable and produces minimal error. However, if h_0 is significant, the equilibration problem can be avoided by injecting the sample into the channel at some distance ($\geq h_0$) from the inlet.

RESULTS AND DISCUSSION

To demonstrate the feasibility of SCE-FFF, the separation of two different polarity solutes was attempted. Ascorbic acid (vitamin C) and sodium benzoate were chosen as relatively polar compounds that would be located in the aqueous phase of the systems used. Toluene was chosen as an apolar compound that would be located in the oil phase. Both are easily detected. To avoid any oxidation, fresh solutions of ascorbic acid were made every day.

By use of micellar carriers, it was not possible to separate ascorbic acid from toluene. Some runs were done at 2000 rpm to obtain a centrifugal field as high as 690g. Because of damage to the seals caused by organic components, all experiments using microemulsions as a carrier were limited to a rotation speed of 1400 rpm.

Figure 2 shows the separation of ascorbic acid and toluene with the microemulsion system 1 (Table I). In this system and all other analogous experiments, the ascorbic acid eluted at the dead volume of the system, indicating that it is completely unretained. Table II presents the FFF characteristics of each peak and Figure 3 presents the same separation with the microemulsion system 2. Sodium benzoate was also separated from toluene by using identical microemulsion supports. These results are also given in Table II. Figure 3 shows the reproducibility of two successive runs obtained in the same conditions. Quantitative changes in the elution profile, such as those indicated in Figure 3, may be due to the injection process or to sample overloading. Table II also presents the retention parameters and plate counts with the corresponding experimental error margins.

Retention. The retention of a small molecule lies between two limits: (i) when $P = 0$, the solute is excluded from the

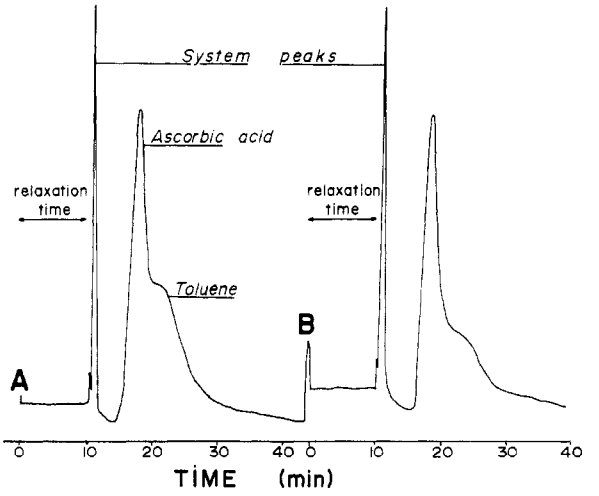

Figure 3. Reproducibility in two successive runs with microemulsion 2: ascorbic acid, 10 nmols; toluene, 200 nmol; flow rate, A = 0.361 mL/min, B = 0.356 mL/min; absorbance 0.32. Note that the "system peaks" in this system is simply the detector response that occurs when the pump is turned on and flow begins.

"support" and moves with the continuous phase; (ii) when P is very large, the solute has a very high affinity for the "support" and moves at the "support" speed (Figure 1). To have an idea of the microemulsion droplet distribution in the channel, we assume that toluene has a high affinity for the hexane droplets, so we can obtain from eq 3

$$\lambda_{toluene} = d/w = \lambda_{support} \quad (6)$$

where λ is the basic retention parameter in FFF. In the case of sedimentation FFF, λ can be expressed as (6)

$$\lambda = kT/[mGw(\Delta\rho/\rho_s)] \quad (7)$$

in which m is the mass of one "support" particle, $G = \omega^2 r$ is the centrifugal acceleration term, w is the channel thickness, ρ_s is the "support" density, and $\Delta\rho$ is the density difference between the continuous phase and the "support" phase. In the present case, eq 7, the experimental values of Table II and the density values of Table I lead to an estimate of m, the mean mass of a microemulsion droplet. By use of the density ρ_s, it also is possible to obtain the mean radius of a microemulsion droplet. Given the assumptions that the aqueous phase density is that of pure water and the droplets are monodisperse, the values 35 (±2) and 41 (±3) nm can be calculated for microemulsion 1 and 2, respectively. Alternative methods for determining the radii of dynamic microemulsion droplets are not simple. However, the results obtained in this study correlate in droplet size and behavior (i.e., droplet size increases upon dilution) with those of an analogous system

characterized by small angle neutron scattering (*10*).

The corresponding droplet masses are 1.4×10^{-16} and 2.4×10^{-16} g for microemulsion 1 and 2, respectively. The mass of the heaviest micelle (C18E20, Table I) is only 1.2×10^{-18} g, 2 orders of magnitude lower than that of a microemulsion droplet. With micellar solutions as carriers, eq 7 shows that it is necessary to have a field, G, 2 orders of magnitude higher than with microemulsions, in order to obtain comparable retention parameters. Since G increases as the square of the spin rate, a rotation speed in the 15 000 rpm range, 1 order of magnitude higher than the one we used with microemulsion carriers (i.e. 1400 rpm) would be necessary to obtain solute separations with micellar carriers.

Efficiency. From Figures 2 and 3 and Table II, it is apparent that the efficiency (plate count) of the toluene peak is lower than the one of the ascorbic acid peak. In FFF, the factors influencing the plate height, H, can be summarized in a relationship similar to the equation governing chromatography in capillary tubes (*11*, *12*)

$$H = \frac{2D}{R\langle v \rangle} + \chi \frac{w^2\langle 2 \rangle}{D} + \sum H_i \qquad (8)$$

where D is the average diffusion coefficient of the solute in the carrier fluid, $\langle v \rangle$ is the mean carrier linear velocity, χ is the λ-dependent nonequilibrium coefficient, w is the channel thickness, and the H_i terms are plate height contributions due to any disturbances such as the relaxation process, end effects, extracolumn void volumes, imperfect channel design, and so forth.

The first term on the right-hand side of eq 8 describes the zone spreading due to longitudinal diffusion, the second accounts for nonequilibrium and mass transfer effects, and the last term is a composite term that includes mainly the extrachannel effects.

In the case of micellar systems, we have shown that the diffusion coefficient of a solute having a strong affinity for the micellar phase is very close to the diffusion coefficient of the micelle itself (*13*). Similar results were obtained in microemulsion systems (*14*, *15*). Although the R value of toluene (eq 8) is lower than the corresponding value of ascorbic acid, the diffusion coefficient of toluene (when associated with the microemulsion) is *2 orders of magnitude lower* than that of the unassociated ascorbic acid. Consequently the first term of eq 8 ($2D/R\langle v \rangle$) is much lower for toluene than for ascorbic acid. Therefore, the fact that the toluene plate height is greater than that of the unretained ascorbic acid cannot be attributed to the longitudinal diffusion term. The third term (eq 8) should differ very little for the two solutes. Clearly, any band-broadening effects occurring in the h_0 zone at the beginning of the channel would be greater for toluene (which is associated with the microemulsion) than for an unretained solute. Also, a substantial part of the toluene band broadening may originate in the second term of eq 8, which corresponds to nonequilibrium and mass transfer effects. This conclusion is supported by evidence that the toluene exchange between microemulsion droplets is slow. The loss of efficiency in micellar chromatography, compared to classical mobile phase chromatography, is a well-known phenomenon attributed to poor mass transfer (*16*, *17*). It is very likely that an analogous process occurs in this system.

In conclusion, it should be pointed out that the use of secondary chemical equilibria to separate small solutes by FFF currently cannot compete with normal- or reversed-phase HPLC in analytical scale separations. However, SCE-FFF can be a very useful tool to obtain insights and data concerning structure, size, and other physicochemical properties of ordered media and to obtain some information on the solute partitioning between the two phases of such systems. As more is learned about SCE-FFF and the technique and instrumentation improves, its usefulness undoubtedly will expand.

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