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Practical approach to high-speed counter-current chromatography

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ABSTRACT

Counter-current chromatography (CCC) is mainly used in the preparative separation, extraction and purification of samples. CCC does not operate with a solid stationary phase. Two immiscible liquid phases are used. One is the liquid mobile phase and the other is the liquid stationary phase. Centrifugal fields are used to retain the liquid stationary phase while the mobile phase is pushed through it. The CCC "column" is often a continuous open tube, coiled on a spool which is rotated in a centrifuge. Most high-speed counter-current chromatographs are scheme IV Ito coil planet centrifuges. A practical CCC approach is reported in this paper. The user-adjustable parameters are divided into (i) configuration parameters and (ii) operating parameters. The configuration parameters are the tube internal diameter, the spool radius, the number of turns and the total internal apparatus volume. The operating parameters are three active parameters (mobile phase flow-rate, spool rotation speed and temperature) and two passive parameters (the stationary phase retention percentage and the driving pressure). The choice of solvents to be used as the biphasic liquid systems is critical. Tie lines in ternary phase diagrams are used to optimize the solvent choice with hexane–methanol–water and the chloroform–methanol–water systems. The effects of interdependent parameters on the CCC chromatograms are discussed and illustrated with examples. It is shown that a highly efficient counter-current chromatograph can have a poor resolving power if it cannot retain a sufficient amount of liquid stationary phase.

INTRODUCTION

More than 20 years ago, Ito and co-workers [1,2] initiated the development of modern counter-current chromatography (CCC). Today, this technique is mainly used in the preparative separation, extraction and purification of samples. CCC is a separation method which does not employ any solid stationary phase. Two immiscible liquid phases are used. The first is the mobile phase and the second is the stationary phase. The CCC "column" is usually a continuous open tube coiled on a spool which is rotated in a centrifuge. The centrifugal field holds the liquid stationary phase tightly so that the mobile phase can be pushed through it.

Ito and co-workers [3–6] developed numerous variations of the CCC technique, including locular, horizontal flow-through, droplet, helix, and coil planet centrifuge CCC. The publications of Ito and co-workers [3–6] and Conway [7] have described

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extensively the principle of hydrodynamic equilibrium in CCC, the various flow-through schemes, the distribution of centrifugal force vectors and the twist-free mechanism avoiding the use of a rotary seal.

From the various possible designs, Ito and co-workers [4–6] showed that scheme IV planetary motion was the arrangement which best held the liquid stationary phase and allowed the fastest and most efficient separation. Consequently, much of the commercially available high-speed CCC equipment derives from scheme IV coil planet centrifuge CCC. Such CCC devices are becoming very efficient and reliable, so that the use of CCC for preparative extraction and purification is rapidly spreading in academic and industrial laboratories. The growing interest for CCC is due to the following advantages of the technique: (a) a solid support is not required, which precludes any irreversible solute adsorption responsible for sample loss and damaged columns; (b) the direct introduction of crude liquid extracts can always be performed; and (c) a wide range of solvent systems can be used.

The aim of this paper is to present a practical approach to high-speed CCC. This paper discusses which parameters of the CCC system are important, how the adjustable parameters are related and the effects on the chromatogram. Special attention is given to the last cited advantage. The choice of suitable CCC solvent systems is so wide that it may be difficult to determine which system should be used in a given situation. Some physico-chemical properties of liquid systems will be used to facilitate the choice of the best liquid system required to obtain an acceptable separation.

BASIC COUNTER-CURRENT CHROMATOGRAPHY EQUATIONS

The equations used in CCC are very similar to the classical chromatographic equations. The basis retention equation is:

$$V_R = V_0 + (V_T - V_0)P \tag{1}$$

or

$$V_R = V_T + (P - 1)V_S$$

in which V_R is the retention volume, V_T is the internal volume of the apparatus, V_0 is the elution volume of an unretained solute of the volume of the mobile phase inside the apparatus and V_S is the stationary phase volume inside the apparatus; $V_T = V_0 + V_S$. P is the solute partition coefficient, *i.e.*, the ratio of the solute concentration in the stationary phase to the solute concentration in the mobile phase. For example, with an apparatus of 120 ml internal volume, equilibrated with a liquid system in such a way that 70 ml of stationary phase were trapped inside, with a 50-ml mobile phase moving through, the retention volume of a compound with a partition coefficient $P = 2$ is 190 ml. The retention time is 3 h 10 min at a 1 ml/min mobile phase flow-rate, or 45 min at 4 ml/min. When the partition coefficient is as high as 100, the retention volume is 7 l and the retention time is 24 h at 5 ml/min. The partition coefficient range which can actually be used is limited, as discussed later.

The solute capacity factor, k', is related to the partition coefficient, P, and to the phase ratio by:

$$k' = \frac{V_R - V_0}{V_0} = \frac{V_S}{V_0} P \tag{2}$$

The solute partition coefficient is directly proportional to the capacity factor, k'.

USER-ADJUSTABLE CHROMATOGRAPHIC PARAMETERS

The adjustable parameters vary according to the CCC apparatus used. They can be divided in two kinds of parameters. The configuration parameters are, in the case of the coil planet centrifuge CCC devices (P.C., Potomac, MD, USA; S.F.C.C., Neuilly-Plaisance, France; Pharma-Tech, Baltimore, MD, USA; Kromatron SEAB, Villejuif, France [8]), the internal diameter of the coiled tube, the spool radius, the number of turns (number of cartridges in the case of the Sanki device described recently [9], Sanki Lab., Sharon Hill, PA, USA), and the total internal volume of the apparatus. The operating parameters can be divided in active parameters, *i.e.*, the mobile phase flow-rate, the rotation speed and the operating temperature, and passive parameters, *i.e.*, the stationary phase retention percentage and the driving pressure. As these parameters are interdependent, it is important to have an idea of the effect of one change on the separation obtained.

Configuration parameters

The Ito coil planet centrifuge chromatographs contain one or several coiled plastic tube spools. Defining d_t (the internal diameter of a tube of length L), r (the spool radius), n (the number of turns) and V (the spool volume), the relationships between these parameters are:

$$n = \frac{L}{2\pi r}; \; V = \frac{\pi d_t^2 L}{4} \tag{3}$$

$$n = \frac{2V}{\pi^2 d_t^2 r} \tag{4}$$

Introducing R, the spool gyration radius, Ito defined the β ratio as:

$$\beta = \frac{r}{R} \tag{5}$$

Eqn. 4 clearly shows that all configuration parameters are interdependent. The internal volume, V_T, depends on the square of the tube internal diameter (eqn. 3). The number of turns, n, is directly proportional to the tube length and inversely proportional to the spool radius.

Operating parameters

The operator can tune the active parameters and measure the passive parameters. The operating parameters, mobile phase flow-rate, rotating speed and percentage of stationary phase retention are intimately related. Fig. 1 illustrates the phase retention evolution *versus* flow-rate and rotation speed. The percentage of retention increases with rotation speed and decreases when the flow-rate increases. If the centrifugal field is higher, the stationary phase is held more tightly. A high flow-rate displaces more stationary phase. The amount of stationary phase retained is also depending on (a) the tube internal diameter, (b) the spool radius and (c) the liquid system used and the way it is used (upper or lower liquid phase mobile). The terminal of the rotating coil where all the objects tend to move is defined as the head. The other terminal is defined as the tail. For scheme IV coil planet centrifuge CCC, Ito [10] showed that with a sufficient rotation speed (>300 rpm), the maximum stationary phase retention was obtained under the following conditions: the heavy (lower) mobile phase must be pushed from the head to the tail through the light (upper) stationary phase. The light (upper) mobile phase must be pushed from the tail to the head if the stationary phase is the denser (lower) liquid. Unfortunately this useful rule is not valid in any instance. The rule is inverted when a very hydrophilic liquid system, such as *sec.*-butanol–water or *n*-butanol–acetic acid–water, is used [5,7,10].

The percentage of stationary phase retention also depends on the coiled tube internal diameter, d_t [11] and the injection conditions [4]. It is difficult to retain the stationary phase with a narrow-bore tube ($d_t < 0.9$ mm) [11]. Owing to solvent–wall interactions, the organic phase displaces the aqueous phase. The injection of concentrated solutions may also disturb the phase equilibrium inducing stationary phase loss. Ito [4] recommended the injection of large volumes (*e.g.*, 0.2 V_T) of concentrated solution dissolved in the stationary phase. These two effects show that the percentage of stationary phase retention depends largely on the liquid system used.

Temperature changes affect mainly the liquid system. A rise in temperature induces an increase in the mutual solubility of the phases, a decrease in liquid viscosity and a change in solute partition coefficients.

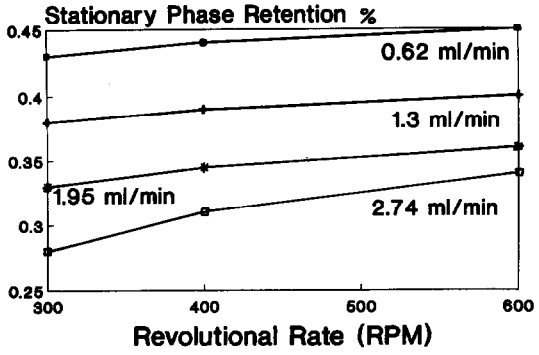

Fig. 1. Percentage retention of stationary phase *versus* the revolutional rate (rpm) for different flow-rates. Conditions: multilayer coil planet centrifuge CCC apparatus; liquid system, hexane–methanol–water; mobile phase, upper organic phase. Data from ref. 5.

BIPHASIC LIQUID SYSTEMS

In limited CCC applications two immiscible liquids are used. For example, octanol and water are used in the determination of the octanol–water partition coefficient [12]. In most other situations, the great advantage of CCC is the ability to use any liquid mixture that forms two phases. Water is a widely used liquid for its price, non-toxicity, ease of purification, availability and polar solvent properties. An apolar solvent forms the apolar phase and a third solvent, with an intermediate polarity, is used to adjust the solute partition coefficients. This looks easy; however, the possible compositions are boundless. The choice of solvents depends on the solutes to be separated. Once the solvents have been chosen, a knowledge of the ternary system is obtained using the ternary phase diagram.

Choice of solvent system

The range of liquid systems forming two immiscible layer is so wide that choice may be a problem. Furthermore, it is very difficult to find the partition coefficient of

TABLE I

BIPHASIC LIQUID SYSTEM USED IN CCC

The polarity index is the chromatographic elution strength on alumina, from ref. 15. (N) normal, lower phase mobile → input head, output tail; upper phase mobile → input tail, output head. (R) Reversed, lower phase mobile → input tail; upper phase mobile → input head.

Liquid	Polarity index	Liquid	Polarity index	Liquid	Polarity index	CCC mode
Hydrophobic						
n-Heptane	0.01	Chloroform	0.40	Water	>2	N
n-Heptane	0.01	Ethyl acetate	0.58	Methanol	0.95	N
				Water	>2	
n-Heptane	0.01	Chloroform	0.40	Acetonitrile	0.65	N
n-Hexane	0.01	Methanol	0.95	Water	>2	N
n-Hexane	0.01	Pentanol	0.61	Water	>2	N
n-Heptane	0.01	Acetic acid	1.00	Methanol	0.95	N
Toluene	0.29	Chloroform	0.40	Water	>2	N
Intermediate						
Dichloromethane	0.42	Methanol	0.95	Water	>2	N
Chloroform	0.40	Methanol	0.95	Water	>2	N
Chloroform	0.40	Acetic acid	1.00	Water	>2	N
Ethyl acetate	0.58	*n*-Butanol	0.72	Water	>2	N
		Acetonitrile	0.65			
Ethyl acetate	0.58	*n*-Propanol	0.82	Water	>2	N
Hydrophilic						
n-Butanol	0.72	Water	>2			N
n-Butanol	0.72	Methanol	0.95	Water	>2	R
n-Butanol	0.72	Acetic acid	1.00	Water	>2	R
n-Butanol	0.72	Pyridine	0.71	Water	>2	N
n-Butanol	0.72	*n*-Propanol	0.82	Water	>2	R
n-Pentanol	0.61	Methanol	0.95	Water	>2	R

a compound in the literature when more than two solvents are used. The data compiled by Wisniak and Tamir [13] is very helpful. Ito [4] classified some liquids systems as hydrophobic, intermediate and hydrophilic. The physico-chemical properties of the pure liquids are important. A low viscosity, high interfacial tension and high density difference are desirable. Ito and Conway [14] have shown that the settling time of the two solvent phases, *i.e.*, the time required for the hand-shake solvent mixture to be completely separated into two layers, provides a reliable numerical index for the hydrodynamic behaviour of the system in CCC. Table I lists some systems commonly used in CCC, along with a polarity index for the pure liquids and the CCC mode that should be used with the system.

A quick method of selecting a solvent system consists in checking the sample by thin-layer chromatography on silica or cellulose using the organic layer as the eluent [16,17]. As described by Hostettmann *et al.* [17], if the R_F values of the compounds to be separated are higher than 0.4, the less polar phase can be used as the mobile phase. For more polar substrates ($R_F < 0.4$), the more polar phase should be used as the mobile phase.

Ternary phase diagram

The ternary phase diagram of a three-liquid system fully characterizes the system at a given temperature. Unfortunately, few ternary phase diagram can be found in the

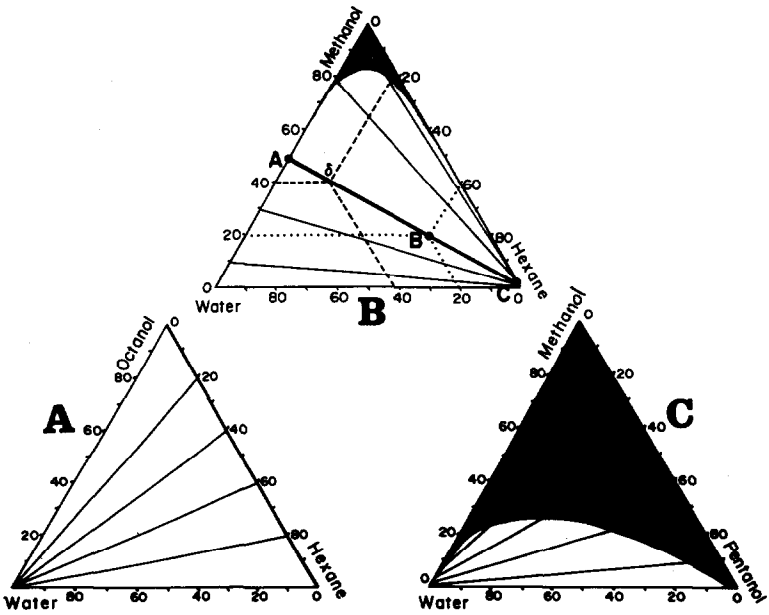

Fig. 2. (A) Hexane–octanol–water system; the organic upper layer is a mixture of hexane and octanol and the aqueous lower layer is essentially water. (B) Hexane–methanol–water system; the organic upper layer is mainly hexane; the aqueous lower layer is a methanol–water mixture. For points A, B, C and δ, see text. (C) Pentanol–methanol–water system, the pentanol–methanol upper layer can dissolve large amounts of water. The lower aqueous phase contains methanol and pentanol. This diagram is considerably modified by temperature changes. Dark areas, monophasic; open areas, biphasic with tie-lines. Temperature, 22°C.

literature [13,18,19]. To delineate a ternary phase diagram is easy but tedious work. Fig. 2 shows three recently obtained ternary phase diagrams [20]. The hexane–octanol–water system (Fig. 2A) has a very low mutual solubility between the aqueous and organic phase. With the hexane–methanol–water system (Fig. 2B), hexane is the main constituent of the organic phase. The aqueous phase is practically a water–methanol solution. A small monophasic area exists near the methanol apex. For the pentanol–methanol–water system (Fig. 2C), a large monophasic area exists. Water partitions between the two phases in the biphasic area. Small additions (<0.5%, w/w) of a fourth component do not significantly alter the phase diagram. For example, the addition of 0.1 *M* hydrochloric acid in water does not change the phase diagrams in Fig. 2. If four liquids are used to obtain the biphasic liquid system, it is possible to map a pseudo-ternary phase diagram by keeping the ratio of two liquids constant. One of the three diagram apexes will be, for example, methanol–water (50:50, w/w), and the two other apexes will represent the two other liquids. Temperature rises affect the mutual solubility of the liquids; usually, the monophasic area increases with temperature.

The tie-lines, which are indicated in the biphasic areas of Fig. 2, are extremely useful in CCC. They allow the quantity and composition of the two phases obtained when three liquids are mixed to be calculated. This is illustrated using the diagram for hexane–methanol–water (Fig. 2B). Point B in this diagram corresponds to the mass composition 60:20:20. For a 100-g preparation, 60 g of hexane are 91 ml (d_{hexane} = 0.660 g/cm^3), 20 g of methanol are 25.3 ml (d_{methanol} = 0.791 g/cm^3) and 20 g of water are 20 ml. The volume percentage ratio is: 66.8:18.5:14.7. This mixture is given in the CCC literature as hexane–methanol–water (18:5:4, v/v/v). The corresponding tie-line is drawn in bold. It gives the composition of the two phases that separate when mixture B is prepared. The aqueous phase, point A, has the mass composition methanol–water (49:51) (hexane, traces). The organic phase mass composition, point C, is hexane–methanol–water (99:0.9:0.1) [20]. Once the composition of A and C are known, the relative masses of A and C can be calculated using the lever rule:

$$\text{distance AB } (AB) \times \text{mass of A} = \text{distance BC } (BC) \times \text{mass of C} \quad (6)$$

and

$$\text{mass of A} + \text{mass of C} = \text{mass of B} \quad (7)$$

On Fig. 2B, $AB = 0.61AC$ and $BC = 0.39AC$. From eqns. 6 and 7 this becomes:

$$\frac{0.61\ \overline{AC}}{0.39\ \overline{AC}} = 1.56 = \frac{\text{mass of C}}{\text{mass of A}} = \frac{100 - \text{mass of A}}{\text{mass of A}}$$

which gives: mass of A = 39 g and mass of C = 61 g (or 61% of the initial mass mixture). The densities of the upper organic phase and the lower aqueous phase were 0.65 and 0.90 g/ml, respectively. This allows the calculation of the two volumes 93.8 and 43.3 ml for the upper and the lower phases, respectively.

Point δ (mass composition hexane–methanol–water, 18:40:42) belongs to the

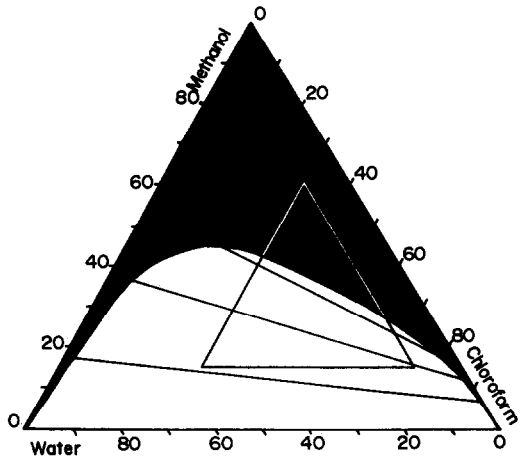

Fig. 3. Chloroform–methanol–water system with tie-lines. Methanol partitions between the lower organic phase and the upper aqueous phase. The inset triangle corresponds to Fig. 4. Temperature, 22°C.

same tie-line as point B (Fig. 2B). The mixture separates into two phases of composition A and C. Applying the lever rule for composition δ, the upper and lower phase volumes can be calculated as 27.2 and 91.4 ml, respectively (for a 100-g δ mixture). As the two phases separated from points B and δ have exactly the same composition, the optimum use of solvents is when composition B is prepared to use the lower aqueous phase as the stationary phase and the upper hexane phase as the mobile phase. Composition δ is prepared when the lower aqueous phase is the mobile phase.

Another important property of the tie-lines is that the longer the line, the greater the difference between the two liquid phases. The interfacial tension, density, viscosity and polarity difference increase with length of tie-line. The polarity difference between the upper and the lower phases explains the partition of a solute between the two phases. The tie-lines of Fig. 2A and B show little variations. The tie-lines of Fig. 2C have very different lengths. The popular CCC system chloroform–methanol–water (Fig. 3) also has tie-lines with variable lengths [21]. The solute partition coefficients depend on the length of the tie-line.

Liquid systems and partition coefficients

The separation power of CCC is based on small solute partition coefficient differences (eqn. 2). A change in the liquid composition induces various changes in the solute partition coefficients. Table II lists the partition coefficient for four compounds in various compositions of the Fig. 2B and Fig. 3 liquid systems. Compositions a to k are given in Fig. 4, which is an enlargement of the chloroform–methanol–water phase diagram (Fig. 3) with the tie-lines. The compositions listed in Table II do not indicate the relative polarity of the two phases obtained. Fig. 4 shows that the tie-lines delineate the polarity of the biphasic system. Composition a has the greatest polarity difference between the two phases (longest tie-line). The partition coefficients of the polar test solutes were 10 and 17. Composition k belongs to the shortest tie-line close to the biphasic area boundary (binodial line). It has the lowest polarity difference between

TABLE II

LIQUID SYSTEMS AND PARTITION COEFFICIENTS

System	No.	Volume composition			Mass percentage			Partition coefficient ($P_{org/aq}$)	
		$CHCl_3$	CH_3OH	H_2O	$CHCl_3$	CH_3OH	H_2O	DNP glucosaminc	PNP-α-D-glucopyr.
Chloroform–methanol–water[a]	a	13	7	8	58.3	16.9	24.8	10	17
	b	4	4	3	48.6	25.9	25.5	3.4	7.1
	c	5	9	7	34.7	33.2	32.1	3.1	6.3
	d	13	7	4	66.7	19.1	14.2	2.9	5.6
	e	5	6	4	44.5	29.4	25.1	2.6	4.4
	f	5	5	3	52.2	27.9	19.9	2.3	4.0
	g	43	37	20	56.4	25.9	17.7	2.1	3.5
	h	10	12	7	46.7	30.1	23.2	2.0	3.1
	i	7	13	8	36.2	35.5	28.3	1.9	2.9
	j	36	42	22	49.1	30.6	20.3	1.8	2.8
	k	5	10	6	35	37.4	27.6	1.7	2.7
		C_6H_6	CH_3OH	H_2O	C_6H_6	CH_3OH	H_2O	Promazine	Simazine
Hexane–methanol–water[b]	l	2	2	0	45.5	54.5	0	2.6	3.6
	m	2	2	1	33.8	40.6	25.6	2.0	5.0
	n	2	1	1	42.4	25.4	32.2	0.9	6.2
	o	2	1	2	32.1	19.2	48.7	0.3	1.6
	p	2	0	2	39.8	0	60.2	0.07	0.5

[a] Data from ref. 28.
[b] Data from ref. 27.

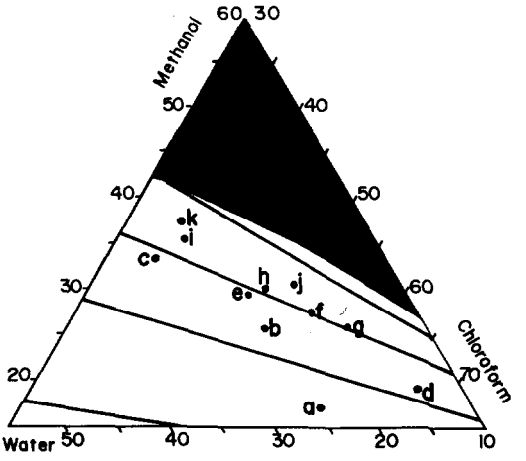

Fig. 4. Enlargement of the diagram chloroform–methanol–water with the tie-lines. Points a–k correspond to the compositions listed in Table II from ref. 28.

the phases. The partition coefficients of the two polar solutes were 1.7 and 2.7. Compositions f, g and h almost belong to the same tie-line. For these compositions, the partition coefficients are almost identical, *e.g.*, 2.3, 2.0 and 1.9, respectively, for dinitrophenylglycosamine, because all three separate in the same biphasic system. Only the relative phase volumes are different. Two points must be emphasized: (a) in different compositions belonging to the same tie-line, the solute partition coefficients do not change because the compositions of the separated phases do not change; and (b) the shorter the tie-line, the closer to unity the partition coefficient of all solutes. A tie-line of length zero is the critical limit of the monophasic area.

If the addition of electrolytes in the aqueous phase, such as acids, salts or buffers, does not significantly affect the ternary phase diagram, it can induce remarkable changes in the solute partition coefficients. The ionizable solutes are particularly sensitive to aqueous phase pH changes. Changes of several orders of magnitude with pH are commonly observed for the partition coefficients [4,5,7].

The partition coefficient of a given solute may be close to unity when that of another solute is very different, as illustrated by Table II, compositions l–p. The shortest tie-line of the diagram for hexane–methanol–water is the hexane–methanol binary mixture, with no water. Composition l belongs to that line. The partition coefficient for simazine was 3.6. It increased up to 6.2 when water was added up to 30%, v/v (composition n) and then decreased. This means that simazine has a greater affinity for the methanol–water solution than for the hexane-rich phase. However, this trend is reversed when the methanol content is low. Promazine has a high affinity for methanol. It is "pushed" into the hexane phase by increasing amounts of water (Table II). Note that with composition n, promazine partitions equally between the two phases ($P = 0.9$) and simazine has a high preference for the methanol–water phase ($P = 6.2$). Such a difference in partition coefficients will give a good CCC separation.

INFLUENCE OF VARIOUS PARAMETERS ON CHROMATOGRAMS

Configuration parameters, operating parameters and the liquid system used all have an important influence on the CCC separation. Obviously, if no stationary phase is retained, there is no chromatographic separation. Four different chromatographic merits are considered. These are: selectivity, efficiency, duration of analysis and loading capacity. Selectivity and efficiency both contribute to the chromatographic resolution. Duration of analysis and loading capacity both contribute to the chromatographic throughput.

Resolution

Selectivity is the ability of a chromatographic system to retain the components of a mixture differently. When the selectivity ratio $\alpha = k'_2/k'_1$ is higher than unity, compound 2 is separated from compound 1. The chromatographic efficiency is linked to the peak sharpness and is measured by the number of theoretical plates (N). A commonly used equation is: $N = (V_R/W_{0.6H})^2$, in which V_R is the retention volume and $W_{0.6H}$ is the peak width, expressed in volume, at 60% of the peak height. CCC efficiency depends on the number of turns of the coil [5], the way in which the solute injection was performed [22], the flow-rate and rotational speed [23], the temperature and the physico-chemical properties of the liquid system used (density, mutual solubility, interfacial tension and viscosity). These parameters act on the mechanical mixing quality which drives the kinetics of the solute exchange between the two liquid phases. The efficiency is directly related to the kinetics of the solute exchange between phases.

The resolution equation combines selectivity and efficiency:

$$R_s = \left(\frac{N^{\frac{1}{2}}}{2}\right)\left(\frac{k'_2 - k'_1}{k'_2 + k'_1 + 2}\right)$$

Using eqn. 2, R_s can be expressed as:

$$R_s = \frac{N^{\frac{1}{2}}}{2} \frac{P_2 - P_1}{P_2 + P_1 + 2\dfrac{V_0}{V_S}} \tag{8}$$

The baseline resolution is obtained when R_s is higher than 1.5. Fig. 5 shows the partition coefficient difference, $P_2 - P_1$, necessary to obtain a baseline resolution ($R_s = 1.5$) between compounds 1 and 2 plotted *versus* the CCC efficiency, N. A maximum separation power is obtained when $P_1 - P_2$ is at a minimum. This is obtained with a high efficiency and a maximum stationary phase retention (Fig. 5) [24,25]. In practice, an increase in efficiency up to *ca.* 800 plates produces an important gain in resolution. Also, a percentage of stationary phase retention of 65% (80 ml with the 125-ml volume apparatus used to draw Fig. 5) is satisfactory. Further improvements in efficiency or stationary phase retention above 800 plates and 65%, respectively, do not produce a dramatic gain in resolution.

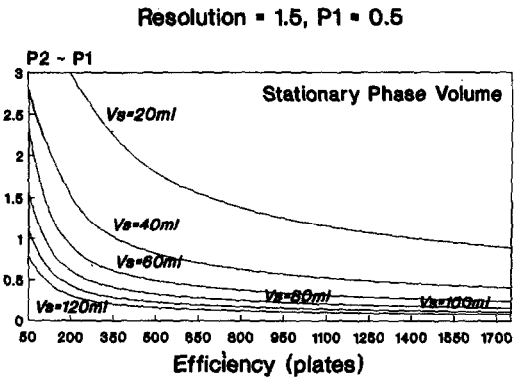

Fig. 5. Partition coefficient difference, $P_2 - P_1$, needed to obtain a baseline peak resolution ($R_s = 1.5$) *versus* the apparatus efficiency for different stationary phase retention volume. $V_T = 125$ ml; $V_0 = V_T - V_S$; $P_1 = 0.5$; eqn. 8.

Throughput

A high throughput is obtained when the loading capacity is high and the duration of separation is low. The loading capacity depends on the volume of stationary phase retained in the apparatus. The higher the internal volume, V_T, the higher the stationary phase volume that can be retained. An apparatus with a high loading capacity should be used with a long (L), large bore (d_t) tube to increase the spool volume (V, eqn. 3) and the internal volume (V_T). It should also have a small spool radius (r) to obtain a high turn number (n, eqn. 4) and a small gyration radius (R) to maximize the β ratio (eqn. 5) and to have an acceptable resolution capability [5,26].

The duration of separation depends on the mobile phase flow-rate and the highest partition coefficient (eqn. 1). The mobile phase flow-rate cannot be increased indefinitely for two reasons. (a) The stationary phase is washed off by a high flow-rate (Fig. 1) which significantly decreases the resolution power (Fig. 5). (b) The driving pressure, ΔP, increases with flow-rate and cannot pass a pressure limit of about 30 bar above which PTFE tubes rupture and leaks occur. The driving pressure is the sum of a hydrodynamic term (Darcy law) and a hydrostatic term [29].

$$\Delta P = \frac{64\eta LF}{\pi d_t^4} + n\Delta\rho\omega^2\Phi \tag{9}$$

ΔP is expressed in Pascals (N/m^2, 10^5 Pa = 1 bar). In the hydrodynamic terms of eqn. 9, η is the mobile phase viscosity [cP, $\times 0.001$ to take account of the units (cm^3) of the flow-rate] and F is the flow-rate (cm^3/s). In the hydrostatic term, $\Delta\rho$ is the density difference between the two liquid phases (g/cm^3), ω is the rotational speed (radian/s), Φ is a geometrical parameter (m^2) function of the β term, the rotor radius R and the spool radius r.

The separation duration depends on the solute partition coefficients and the stationary phase volume retained. Decreasing the V_S term in eqns. 1 or 2 decreases the solute retention volume V_R at the cost of the resolution which vanishes (Fig. 5 and eqn. 8). Once again, CCC parameters are intimately related. Fig. 6 recapitulates the

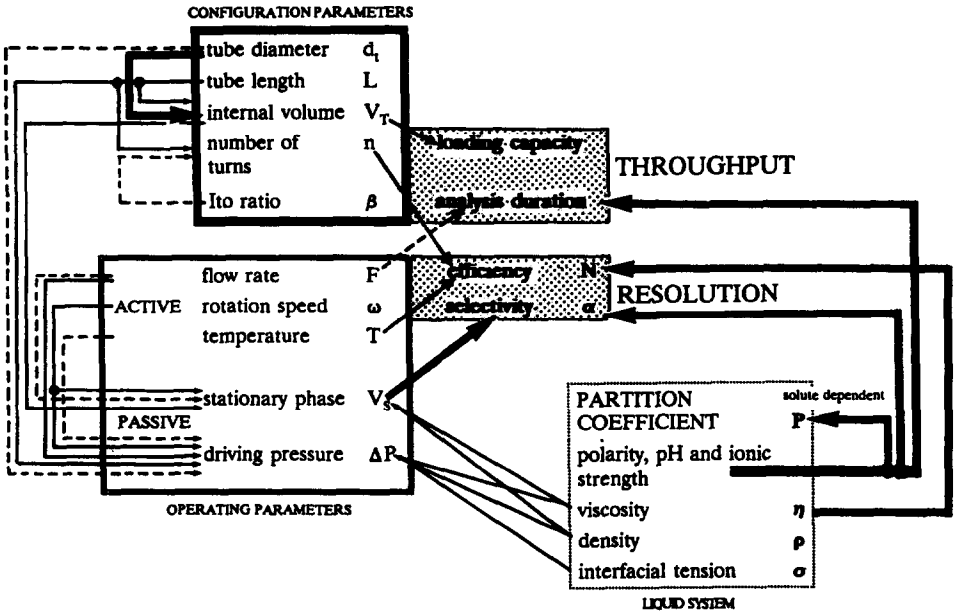

Fig. 6. Interconnections of the CCC parameters. Solid line indicates that an increase in the starting parameter increases the parameter to which it is connected. Broken line indicates that an increase in the starting parameter decreases the parameter to which it is connected. Thick line represents an important effect. P, the solute partition coefficient, depends on the way the system is used, $P_{org/aq} = 1/P_{aq/org}$. For symbols, see text.

relationships between the numerous parameters of CCC. The arrows join one parameter to another. The arrows with solid lines mean that an increase in the parameter at the start of the arrow induces an increase in the parameter to which it is connected. Arrows with broken lines mean the opposite: the connected parameter decreases. A thick line corresponds to a critical control. The liquid system is the most important in CCC optimization. The two key parameters are P, the solute partition coefficient, and V_S, the amount of stationary phase retained. P depends only on the liquid system and the solute. V_S depends on the configuration parameters, mainly V_T, on the operating parameters and on the liquid system. The optimization of a CCC separation may often need two different sets of apparatus. The first, with a low internal volume and a high efficiency, will sacrifice throughput for resolution. The liquid system can be rapidly optimized to adjust the solute partition coefficients and the resolution of separation. A large-volume CCC apparatus can then be used to obtain a high throughput.

EXAMPLES

To conclude this paper, some literature examples are discussed. Fig. 7 is an interesting example of the versatility of high-speed CCC. Fig. 7A–C shows three chromatograms of an extract of *Guttiferae* root bark obtained with the mobile phase

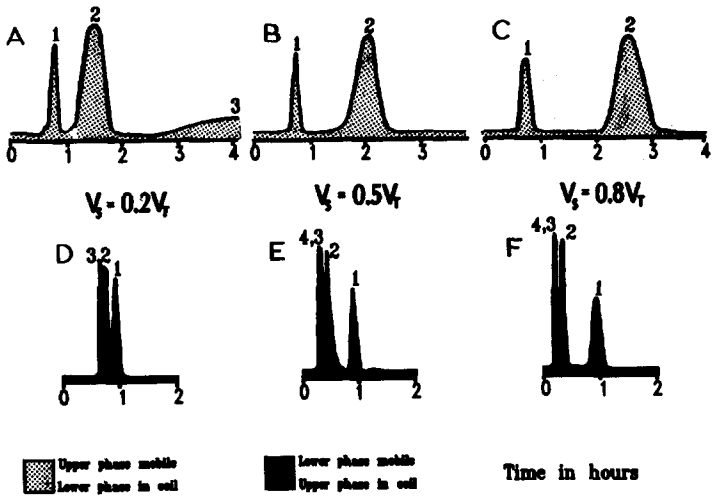

Fig. 7. Effect of V_S, the stationary phase volume, on the CCC chromatogram. The CCC apparatus retained 20% (A and D), 50% (B and E) and 80% (C and F) of the total apparatus volume in the coil. Apparatus: multilayer coil planet centrifuge CCC; compounds 1–4, anthranoid pigments; solvent system, hexane–acetonitrile–methanol (40:25:10, v/v/v); flow-rate, 4 ml/min; rotational speed, 700 rpm. From ref. 30.

consisting of the apolar upper phase of the waterless mixture hexane–acetonitrile–methanol (8:5:2, v/v/v) [30]. The stationary phase volume, V_S, was adjusted using two pumps running together. Fig. 7D–F shows the chromatograms obtained with the same sample and liquid system, but with the mobile phase as the lower polar phase. Table III lists the retention volumes of compounds 1 and 2 corresponding to the six chromatograms. The total volume of the apparatus, V_T, is 360 ml [30]. The partition coefficient of the solute can be calculated because the percentage of stationary phase is known and is indicated in Fig. 7 as a V_T percentage. Eqn. 1 allows the P value listed in

TABLE III

PARTITION COEFFICIENT DATA FOR FIG. 7

Abbreviations: lo. = lower phase in coil, upper phase mobile; up. = upper phase in coil, lower phase mobile. The aqueous/organic partition coefficients were calculated using: (1) for chromatograms A, B and C, $P = [(V_R - V_T)/V_S] + 1$; (2) for chromatograms D, E and F, $P = 1/\{[(V_R - V_T)/V_S] + 1\}$.

Fig. 7 chromatogram	Stationary phase	Retention volume (ml)		Partition coefficient with V_T = 360 ml		Partition coefficient with V_T = 195 ml	
		1	2	1	2	1	2
A	0.2 lo.	188	347	<0	0.82	0.82	4.8
B	0.5 lo.	186	480	0.03	1.65	0.90	3.9
C	0.8 lo.	173	613	0.35	1.88	0.86	3.7
D	0.2 up.	214	180	<0	<0	0.67	1.6
E	0.5 up.	216	110	5	<0	0.82	7.8
F	0.8 up.	221	77	1.9	58	0.86	4.1

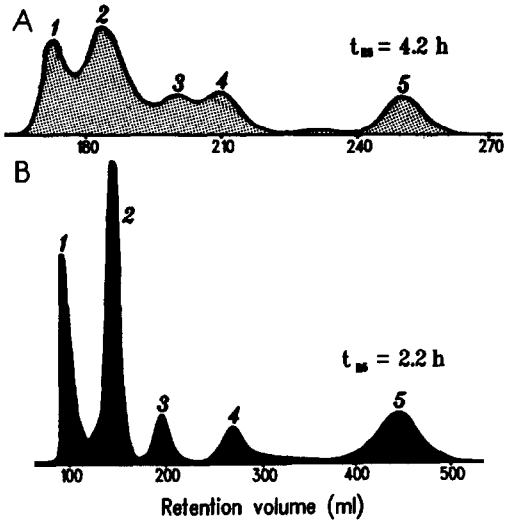

Fig. 8. CCC separation of the same sample (flavonoids) with the same liquid system [chloroform–methanol–water (4:3:2, v/v/v); mobile phase, lower organic phase] with two different apparatuses. Apparatus A: twelve-column horizontal flow-through coil planet centrifuge; V_T = 220 ml; V_S = 55 ml (25%); flow-rate: 1 ml/min; 300 rpm. Apparatus B: multilayer coil planet centrifuge; V_T = 300 ml; V_S = 255 ml (75%); flow-rate: 3.3 ml/min; 800 rpm. From ref. 24.

Table III as polar phase/hexane phase partition coefficients to be calculated. The P values for a compound are not constant. In some instances, the computation produced a negative P value which has no meaning. It was noted [30] that "the elution time of 1 was hardly affected by changing the stationary phase percentage in either the two modes". This behaviour is typical of a solute distributing equally in both phases (P close to unity). With this in mind, it was assumed that the apparatus volume V_T was inaccurate and it was estimated as V_T = 195 ml. Table III lists the corresponding P values of 0.85 ± 0.05, 4.2 ± 0.5 and *ca.* 20 for compounds 1, 2 and 3, respectively.

Fig. 8 shows two chromatograms obtained with the same sample and liquid

TABLE IV

EFFICIENCY AND RESOLUTION DATA FOR FIG. 8

For apparatus A: V_T = 200 ml; V_S = 0.25 V_T = 55 ml. For apparatus B: V_T = 300 ml; V_S = 0.75 V_T = 255 ml.

Fig. 8 peak No.	Apparatus A				Apparatus B			
	V_R (ml)	$P_{aq/org}$	N plates	R_S	V_R (ml)	$P_{aq/org}$	N plates	R_S
1	174	0.16	1100	–	100	0.21	500	–
2	186	0.38	1100	0.55	142	0.38	560	2.05
3	200	0.63	1200	0.63	196	0.59	530	1.84
4	213	0.87	1800	0.67	270	0.88	530	1.83
5	252	1.58	2400	2.05	450	1.59	500	2.80

system, but with two different counter-current chromatographs [24]. The CCC apparatus A that produced the Fig. 8A chromatogram was a horizontal coil planet centrifuge device. Fig. 8B was obtained with a multilayer coil planet centrifuge CCC apparatus B [24]. Table IV lists the retention volumes, partition coefficients, peak efficiencies and resolutions obtained for the five peaks separated from the vegetable extract sample. The first observation is that the throughput of the two sets of apparatus is very different. If A was able to separate the last solute (5) with 252 ml, it took 4.2 h because the flow-rate could not exceed 1 ml/min due to a poor 25% retention of the stationary phase. Apparatus B used 450 ml of aqueous mobile phase to elute compound 5, but it did so in only 2.2 h because the stationary phase was held tightly. A 3.3 ml/min flow-rate was possible with a 85% retention of the stationary phase. The partition coefficients of the five solutes are identical, within experimental error, whatever apparatus is used. They depend only on the liquid system (Fig. 6). Apparatus A has a higher efficiency, in the 1200 plate range, than apparatus B, which is two times less efficient. Apparatus A contains sixteen coiled columns connected serially, which was about 1850 turns, probably many more turns than apparatus B (not given) [24]. The two times higher efficiency of apparatus A is not obvious on Fig. 8. The B peaks look sharper because the volume scale is different and the origins are out of the figure. The resolution power of apparatus A is much lower than that of apparatus B. All the compounds separated using B were baseline-resolved ($R_s > 1.5$). The high stationary phase retention in B is responsible for its high resolution capability compared to A. In eqn. 8, the ratio $2V_0/V_S$ was 0.35 for B, and 6 for A. The two times better efficiency advantage of A could produce a 40% increase in resolution power at a constant percentage retention of the stationary phase. Unfortunately, the low retention of the stationary phase by A causes a very low selectivity (Fig. 6) and a low resolution power.

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