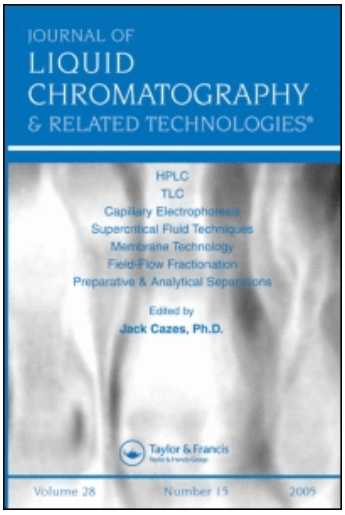

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Simple Approach to the Development of a CCC Solvent Selection Protocol Suitable for Automation

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Simple Approach to the Development of a CCC Solvent Selection Protocol Suitable for Automation

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Abstract: One of the biggest problems with countercurrent chromatography, when it comes to its practical application in an industrial environment, is developing a protocol and corresponding solvent system for its operation. This paper critically reviews previous proposals for solvent selection and suggests a scheme that is suitable for use by an inexperienced user and for automation using a modern liquid handling robot.

Keywords: Countercurrent chromatography, CCC, Solvent selection

INTRODUCTION

Countercurrent chromatography (CCC) is a form of liquid-liquid chromatography in which either a centrifugal or gravitational force is used to retain one liquid phase in a coil or train of chambers, while a second, immiscible phase is passed through as a stream making contact with the other phase. There is no adsorption, because there is no solid matrix, so retention of a sample depends solely upon the phase volume ratio and the distribution ratio, or differential solubility, of the solute.

The two phases used in CCC may be obtained from two, three, four, or even five or more liquids. This makes the possibilities for solvent selection

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almost limitless. On the plus side, this means it should be possible (in theory at least) to find a solvent system that can perform the separation required. The down side is that, unless some form of systematic and logical approach is used to identify a suitable system in a reasonable time frame, the search for a solvent system becomes a random, trial-and-error hunt with an almost infinite number of possibilities. It is the required systematic approach that this paper is addressing.

SOLVENT SELECTION APPROACHES

In the literature, a number of different approaches can be found for selecting a CCC solvent system. Many articles on CCC list classes of compounds and suggested solvent systems to try.[1–3] Table 1 gives an example. This is of limited use, for it is hard to know how to adjust each system if you are trying to purify a compound that is not listed. It is also common to find general tables giving suggested solvents for broad classes of compounds.[4,5] Table 2 gives an example.

Another approach, described by Foucault & Chevolot[6] and shown in Table 3, involves finding a "best solvent" for the compound of interest, and then partitioning this solvent between two others as suggested in the table. Unfortunately, a certain degree of skill with solvents is required to successfully operate the system; otherwise it becomes another trial-and-error approach. To be industrially useful, a protocol must be simple and robust enough to be followed the first time by an inexperienced operator or a robot.

The approach most likely to be universally acceptable is that of a selection table of solvents. A number of examples exist in the literature.[6–9]. The selection table used in this protocol (Table 4) is a combination of that produced by Margraff[9] and Oka, and Oka & Ito[7] with heptane substituted for the less industrially-acceptable hexane. The result is a list of 28 two-phase solvent systems running from polar (butanol-water) to nonpolar (heptane-methanol). Table 4 shows the volumes in millilitres to make up 4 mL of each solvent system. There are at least three ways of using such a selection table.

The approach most commonly found in the literature is to have an entry point in the middle of the table, with a corresponding solvent system. For example, system No 17 is highlighted in dark grey (heptane-ethyl acetate-methanol-water, 1:1:1:1). The compound of interest is partitioned in this solvent system (details of a simple test are given below) and the table traversed according to the results. The process is repeated until a suitable system is found.

An alternative is to scan across the table making up, say, every 6th solvent system to find the approximate area of interest, and then focus in on that area to find the precise system required. For example, solvent system

Table 1. Example solvent systems, from reference[2]

Substance separated	Instrument	Solvent system	Ratio
Polyene antibiotics	Synchronous coil planet centrifuge	$CHCl_3$-MeOH-H_2O	4:4:3
Glycoside antibiotics	Shmadzu CCC	n-BuOH-ET_2O-H_2O	10:4:2
Trichoverrins	CCC-1000	n-C_6H_{14}-$CHCl_3$-MeOH-H_2O	1:1:1:1
Benarthin	Sanki NMF	n-BuOH-HOAc-H_2O	15:1:15
Pristinamycins	SFCC CPHV 2000	$CHCl_3$-EtOAc-MeOH-H_2O-HCOOH	12:8:15:10:2
Siderochelin A	HSCCC	$CHCl_3$-MeOH-H_2O	7:13:8
Efrotomycin	HSCCC	CCl_4-$CHCl_3$-MeOH-H_2O	5:5:6:4
Pentalenolactone	HSCCC	$CHCl_3$-MeOH-H_2O	1:1:1
Tirandamycin	HSCCC	n-C_6H_{14}-EtOAc-MeOH-H_2O	70:30:15:6
Dunaimycins	HSCCC	n-C_6H_{14}-EtOAc-MeOH-H_2O	8:2:5:5
Actinomycins	HSCCC	n-C_6H_{14}-EtOAc-MeOH-H_2O	1:5:4:5
Valinomycin	HSCCC	n-C_6H_{14}-MeOH-H_2O	10:9:1
Pipericidin A1	HSCCC	n-C_6H_{14}-Et_2O-MeOH-H_2O	4:1:4:1
Concanamycin	HSCCC	n-C_6H_{14}-EtOAc-MeOH-H_2O	1:1:1:1
Tomaymycin	HSCCC	n-C_6H_{14}-EtOAc-MeOH-H_2O	1:3:1:3
Acetoxycycloheximide E73	HSCCC	$CHCl_3$-C_6H_5Me-MeOH-H_2O	5:4:5:4
Tiacumicin	HSCCC	CCl_4-$CHCl_3$-MeOH-H_2O	7:3:7:3
Benzanthrins	HSCCC	CCl_4-$CHCl_3$-MeOH-H_2O	4:1:4:1
Coloradocin	HSCCC	$CHCl_3$-MeOH-H_2O	1:1:1

Table 2. Example general table giving suggested solvents for broad classes of compound. From reference[4]

Polarity of solutes	Nonpolar	Medium polarity	Polar
System of solvent: Ito's classification	Hydrophobic	Intermediate	Hydrophilic
Basic solvents	Hydrocarbons-acetonitrile or methanol	Chloroform-methanol-water	Butanol-ethanol-water
Modifiers	Methyl acetate Ethyl acetate Dichloromethane Ethanol	Ethyl acetate Propanol Acetic acid	Methanol Acetone Acetic acid

Table 3. The "best solvent" approach as described in reference[6]

Less-polar solvent	"Best solvent"	More-polar solvent
Heptane, toluene, MiBK, $CHCl_3$, EtOAc, Et_2O, cyclohexane, hexane	*Acetone*	Water, EG
Hexane, heptane, cyclohexane	*Benzene, toluene*	DMF, sulfolane, DMSO, MeCN
Heptane, hexane, toluene, $CHCl_3$, EtOAc, CCl_4, benzene	*1-BuOH, 2-BuOH*	Water, MeCN, EG
Cyclohexane	*$CHCl_3$*	MeCN
EtOAc	*Dimethyl acetamide*	Water
Toluene, $CHCl_3$	*DMF*	Water
THF	*DMSO*	Water
Cyclohexane	*Et_2O*	MeOH
Heptane	*EtOAc*	MeOH, MeCN
Heptane, hexane, $CHCl_3$, EtOAc, Et_2O	*EtOH*	Water, MeCN
$CHCl_3$, benzene, dichloroethane	*HCOOH*	Water
Perfluorohexane	*Hexane*	Benzene
Heptane, toluene, $CHCl_3$, MiBK, EtOAc, 1-BuOH, cyclohexane, Et_2O	*HOAc*	Water
Toluene, MtBE, MiBK, EtOAc, chlorobenzene	*MeCN*	Water
Heptane, cyclohexane, hexane	*MEK*	Water
Heptane, hexane, benzene, toluene, $CHCl_3$, EtOAc, 1-BuOH, CH_2CH_2	*MeOH*	Water, MeCN
Heptane, toluene, benzene, $CHCl_3$, EtOAc, isopropyl ether, 1-BuOH	*1-PrOH, 2-PrOH*	Water
Toluene	*Pyridine*	Water
Heptane, $CHCl_3$, cyclohexane, CCl_4, DCM. dichloroethane	*THF*	Water, MeCN

Table 4. Selection table for determining a CCC solvent system: volumes in mL to make 4 mL. The table entry point is highlighted in dark grey and selected systems to allow rapid screening of the table in light grey. Adapted from references[7] and [9]

No	Heptane	EtOAc	MeOH	Butanol	Water
1	0	0	0	2	2
2	0	0.4	0	1.6	2
3	0	0.8	0	1.2	2
4	0	1.2	0	0.8	2
5	0	1.6	0	0.4	2
6	0	2	0	0	2
7	0.1	1.9	0.1	0	1.9
8	0.2	1.8	0.2	0	1.8
9	0.29	1.71	0.29	0	1.71
10	0.33	1.67	0.33	0	1.67
11	0.4	1.6	0.4	0	1.6
12	0.5	1.5	0.5	0	1.5
13	0.57	1.43	0.57	0	1.43
14	0.67	1.33	0.67	0	1.33
15	0.8	1.2	0.8	0	1.2
16	0.91	1.09	0.91	0	1.09
17	1	1	1	0	1
18	1.09	0.91	1.09	0	0.91
19	1.2	0.8	1.2	0	0.8
20	1.33	0.67	1.33	0	0.67
21	1.43	0.57	1.43	0	0.57
22	1.5	0.5	1.5	0	0.5
23	1.6	0.4	1.6	0	0.4
24	1.67	0.33	1.67	0	0.33
25	1.71	0.29	1.71	0	0.29
26	1.8	0.2	1.8	0	0.2
27	1.9	0.1	1.9	0	0.1
28	2	0	2	0	0

numbers 1, 6, 12, 17, 22 and 28 (highlighted in grey) are particularly easy to create, all being multiples of 0.5 mL when 4 mL of solvent system is made up. If the results of testing these systems show that No 17 is too polar and No 22 too nonpolar, then the systems between 17 and 22 are tested to fine-tune the selection.

The third and favoured approach is to use a modern liquid handling system or robot to make all the systems suggested in the table and test them automatically. Thereafter, it is simply a case of studying the results and selecting the appropriate system for the CCC separation. If such equipment is available, this is the approach that requires the least time, effort, and skill on the part of the operator.

STEPS INVOLVED IN DEVELOPING THE PROTOCOL

The steps involved in selecting a CCC solvent system and performing a separation can be listed as follows:

A. Perform distribution ratio experiments.
B. Analyse the samples from the experiments.
C. Select the preferred solvent system.
D. Make up the solvent system.
E. Prepare the CCC instrument and column.
F. Perform the separation.
G. Shut-down procedure for the instrument.

This paper deals only with the first three of these steps.

For the purposes of this protocol, it is assumed that an HPLC instrument is available and that a suitable analytical method has been developed for identifying the target compound. It is not necessary to obtain HPLC baseline resolution of the target compound from other peaks in the chromatogram, since peak heights can be used for the distribution ratio calculations. However, the results will be better if peak areas are used. The distribution ratio experiment itself is an adaptation of the "shake flask method."[10]

Step 1: Distribution Ratio Experiment

Make a small amount (1/2 to 1 mL) of a highly concentrated solution (50 mg/mL or greater) of the test mixture to be separated using a suitable solvent, preferably one of the phase component solvents, e.g., methanol. It is, of course, assumed that the compound of interest is stable in the solvents used, at least for the duration of the experiment.

Label a set of vials (capable of taking 4 mL volume) from 1 to 28.

Ensure the solvents to be used are equilibrated to room temperature e.g., not just brought in from a cold storage area. Make a note of the room temperature. If possible, it is better to equilibrate the solvents to a fixed temperature, ideally 20°C.

Using a pipette for accurate metering of the liquids, or a liquid handling robot, make up 4 mL of each solvent system detailed in Table 4. When making the solvent systems, be sure to minimise evaporation by capping each vial immediately after any addition of solvent.

Accurately pipette a very small amount (e.g., 30 μL or less) of the concentrated test solution into each solvent system vial, 1 to 28. The exact volume chosen will depend upon the concentration and the response factors of the components on HPLC, but should be as little as possible in order to avoid affecting the solvent ratios in the vial.

Cap the vials securely and shake them vigorously for several minutes. A vortex or inverting mixer may be used.

Allow the liquids in the vials to settle at the selected temperature (e.g., 20°C) until two distinct layers have formed and any heat generated from the mixing process has been allowed to disperse. This may take an hour or more.

Step 2: Analyse Samples

Pipette 1 mL of each layer from every vial into separate HPLC vials, labelled "1 Upper Layer", "1 Lower Layer," etc. When doing this, avoid drawing up any of the opposing layer for that may affect the final results.

Dry all the vials in a centrifugal concentrator, redissolve in an HPLC-friendly solvent, e.g., methanol, and analyse using the standard method for the compound of interest. It should be noted that, depending upon the HPLC method and the solvents used in the solvent systems, it may be possible to skip the drying step and analyse the samples directly. This can only be judged on a sample-by-sample basis.

From the chromatograms, measure the peak areas of each component, taking care that the integration is consistent, and calculate the Distribution Ratio D for each solvent system (1 to 28) using the following equation. Note that the units for peak areas are not important so long as they remain consistent within each calculation:

$$D = \frac{\text{peak area in stationary phase}}{\text{peak area in mobile phase}}$$

$$D = \frac{\text{peak area in lower layer}}{\text{peak area in upper layer}} \quad \text{if normal phase operation is chosen}$$

$$D = \frac{\text{peak area in upper layer}}{\text{peak area in lower layer}} \quad \text{if reverse phase operation is chosen}$$

Step 3: Select Solvent System

For the component of interest, identify a suitable solvent system, i.e., one in which the component has a D value between approximately 0.2 and 5. This will be the system of preference for the purification of the component on CCC.

If there is a choice of solvent systems, then select the system which has a D value around unity for the main component of interest, plus as great a difference as possible between this component and the contaminants present in the mixture. For example, a system where the component of interest has a D value of 0.78 and the main contaminant a value of 2.4 ($\alpha = 3.07$) is better than one with the values 2.8 and 5.0 respectively ($\alpha = 1.78$). The α factor D_2/D_1 gives a clue as to the best separation system, but note, also, that higher D's take longer to elute.

If the optimum D value falls between two adjacent solvent systems, then create a new system with proportions half-way between the two. For example,

if system No 4 ethyl acetate-butanol-water (3:2:5) is too polar and system No 5 ethyl acetate-butanol-water (4:1:5) is not polar enough, try testing a system between the two, such as ethyl acetate-butanol-water (3.5:1.5:5).

A CASE STUDY

In the following example, the target compound (arrowed in each figure) is a pharmaceutical product eluting at 12.9 minutes in the HPLC chromatograms shown. The following HPLC conditions were used: Waters 2695 Alliance with 2995 detector, Waters Symmetry C_{18} column, 100 × 4.6 mm, 3.5 um, 40°C, 1 mL/min, PDA detection 210–400 nm, water/acetonitrile gradient 30–95% acetonitrile over 17 minutes, returning to initial conditions at 17.5 minutes. Chromatograms shown are a MaxPlot of the wavelength range quoted.

Each solvent system in Table 4 was made as described, i.e., 4 mL total volume with the crude sample added as 30 uL of a 50 mg/mL solution in methanol. After equilibration, 1 mL of each layer was removed, dried in a centrifugal concentrator at 40°C and redissolved in 1 mL methanol before analysis on the HPLC. In solvent system No 1 (butanol-water, 1:1), it can be seen that all the compound are in the upper layer (Figure 1). Note the AU axis of the chromatograms is autoscaled, so the full scale deflection is 0.7 AU for the upper layer but only 0.035 AU for the lower layer.

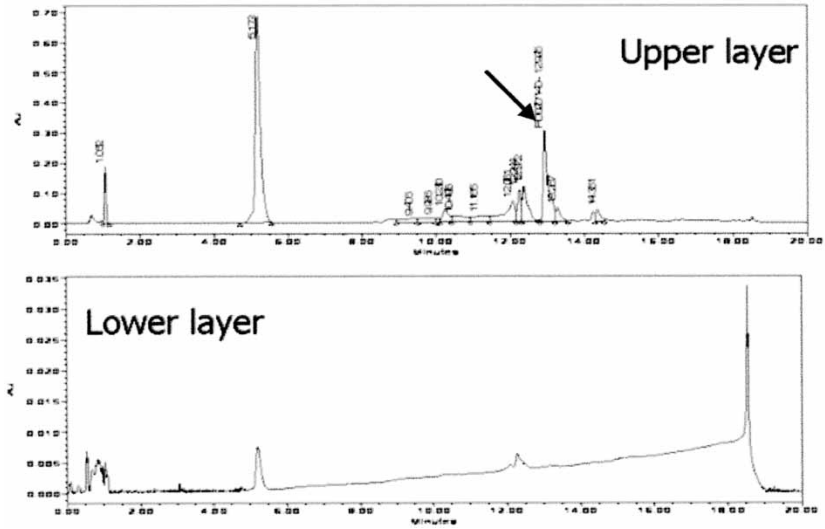

Figure 1. HPLC analysis of the upper and lower layers of solvent system No 1 (butanol-water, 1:1). The target compound is in the upper layer only. (HPLC conditions as given in the text.)

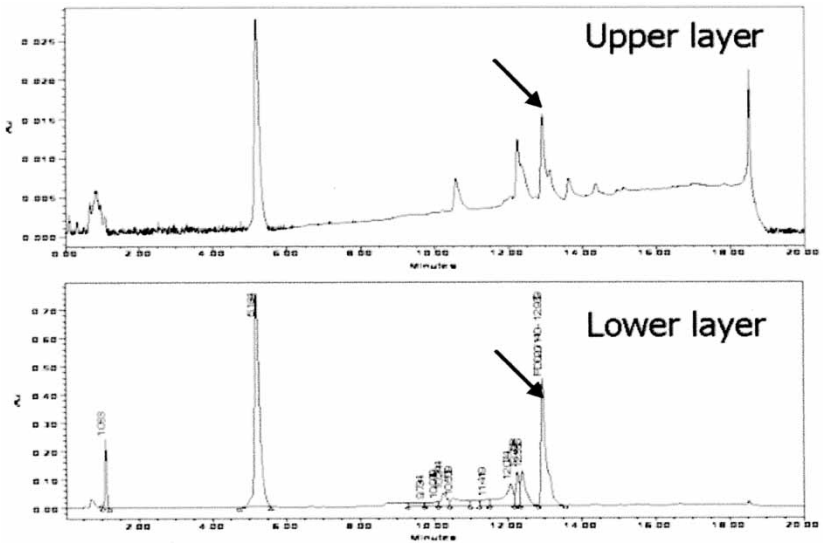

Figure 2. HPLC analysis of the upper and lower layers of solvent system No 28 (heptane-methanol, 1:1). The target compound is almost entirely in the lower layer.

In solvent system No 28 (heptane-methanol, 1:1), the compound is almost entirely in the lower layer (Figure 2). However, with solvent system No 22 (heptane-ethyl acetate-methanol-water, 3:1:3:1) the target compound is fairly evenly distributed across the two layers (Figure 3). Measurement of

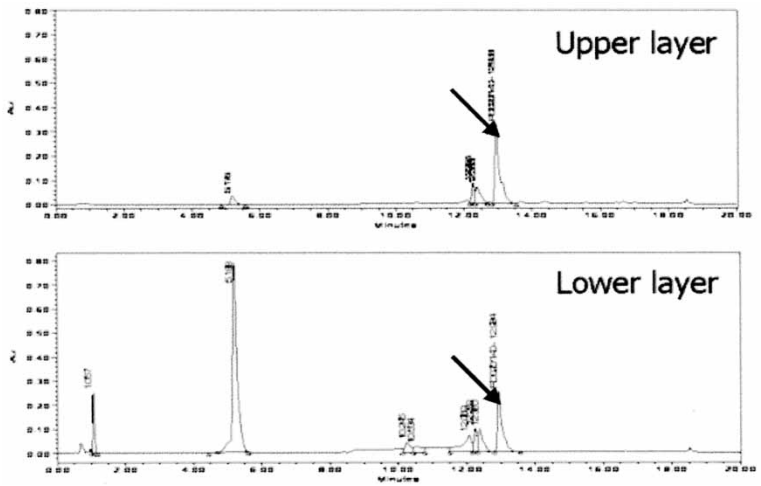

Figure 3. HPLC analysis of the upper and lower layers of solvent system No 22 (heptane-ethyl acetate-methanol-water, 3:1:3:1). The target compound is evenly distributed across the two layers.

Table 5. The distribution ratios of the target compound and its main contaminants in solvent system No22

Component HPLC retention time (min)	HPLC peak area upper layer (uV.sec)	HPLC peak area lower layer (uV.sec)	Distribution ratio D = lower/upper
Target compound 12.9 minutes	31,23,832	23,50,793	0.75
12.4	6,97,508	9,319,58	1.34
12.3	4,86,917	6,253,30	1.28
5.2	3,42,592	7,614,358	22.2

the peak areas, as shown in Table 5, gave a calculated Distribution Ratio (D = peak area in lower layer/peak area in upper layer) of the target compound of 0.75, with the large 5.2 minutes contaminant having a D value of 22.2. Values this far apart mean separation of these two components should be easy on a typical CCC machine. Furthermore, the D values of the two closely eluting peaks at 12.3 and 12.4 minutes are 1.28 and 1.34, respectively. Knowing this information, a CCC coil length can be selected that will achieve the desired separation.

If the D values of the target compound and/or contaminants is plotted against the solvent system number (which of course runs from polar No 1 to nonpolar No 28) then a trend is seen as expected (Figure 4). This may help in the selection of a suitable system.

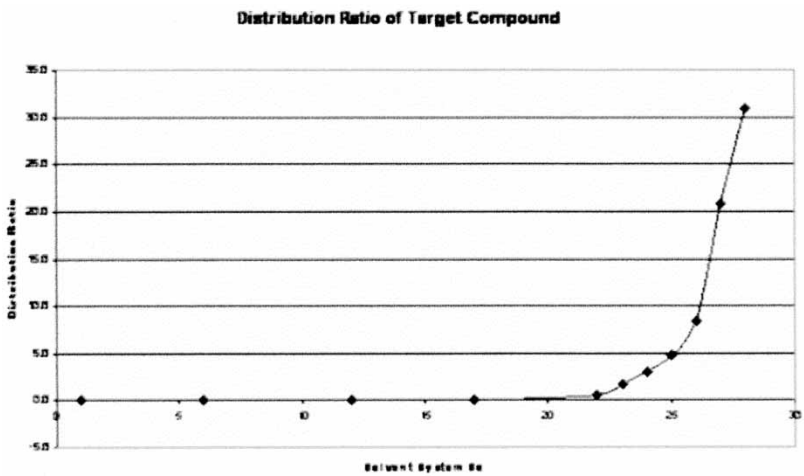

Figure 4. A plot of the distribution ratio against the solvent system number for the target compound.

The crude mixture was, therefore, run on a CCC centrifuge using solvent system No 22 (heptane-ethyl acetate-methanol-water, 3:1:3:1) and the following conditions: Brunel Mini CCC, 17.2 mL coil, 1.0 mL/min flow rate pumping from tail to head, upper organic phase mobile, 2100 rpm spin speed, 30°C, UV detection at 277 nm, 50 uL sample loop, crude sample 32 mg/mL in mobile phase. The CCC chromatogram shown in Figure 5 was obtained. One minute fractions were collected across the chromatogram. These were dried in a centrifugal concentrator at 40°C, redissolved in methanol, and analysed on HPLC using the conditions previously given. The target compound was detected in fractions 24–30 but the majority was found in fractions 25–29. These were pooled, dried as before, and analysed again on HPLC at 1 mg/mL concentration in methanol to give a purity by peak areas of 98%.

FUTURE WORK

The selection table shown in this article covers an intermediate range of polarities. If a more polar solvent system than No 1 is required, then there are three likely options:

1. pH control, especially for ionisable compounds. The addition of acids or bases, e.g., 1% ammonia solution in place of the water.
2. Salt solutions, e.g., the addition of ammonium sulphate to the water.
3. Polymer phase systems, e.g., PEG/phosphate.

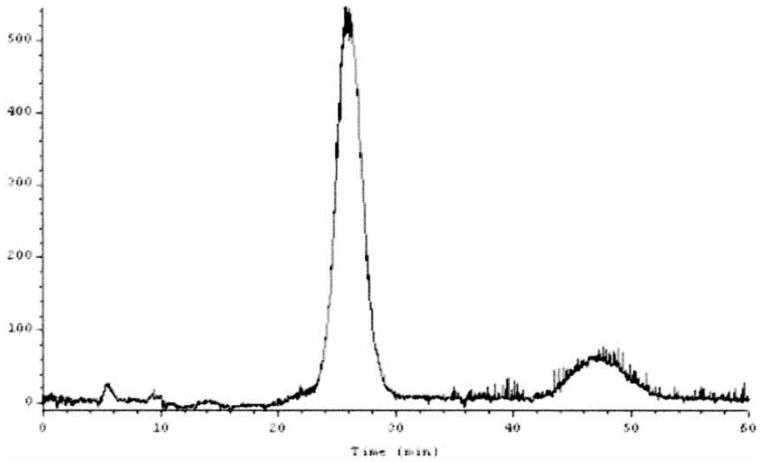

Figure 5. CCC chromatogram of the example mixture using solvent system No 22 (heptane-ethyl acetate-methanol-water, 3:1:3:1). Other conditions as given in the text.

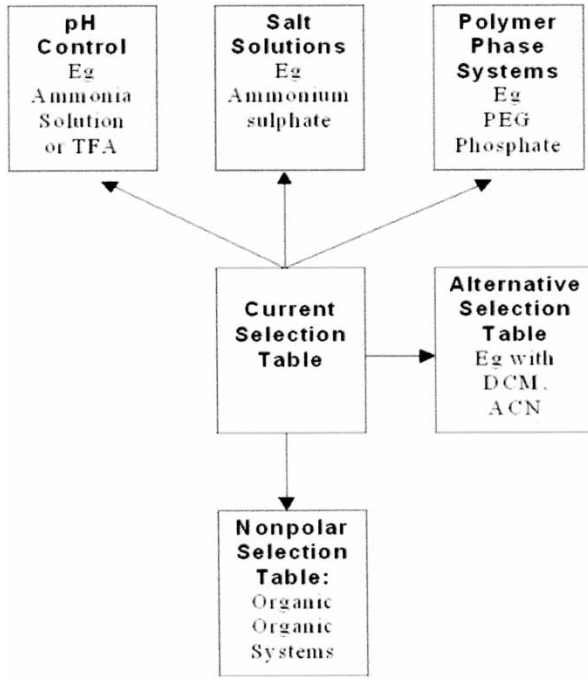

Figure 6. Schematic diagram of possible future solvent selection tables for CCC solvent systems. TFA = triflouroacetic acid, PEG = polyethylene glycol, DCM = dichloromethane, ACN = acetonitrile.

Each of these will require a selection table for the automated robot to make up as required.

On the other hand, if a more non-polar system than No 28 is required, then another selection table using highly non-polar organic/organic systems is required.

Finally, an alternative selection table is proposed, covering the same polarity range as the current table but with different solvents. This is because, whilst it may be possible to find a solvent system which has the correct distribution ratio for the target compound, there may be contaminants with very similar D values and which, therefore, co-elute on CCC. An alternative table with different solvents is an option to try to solve this problem, since these solvents may show a different selectivity to the first. These proposals are shown in diagrammatic form in Figure 6.

CONCLUSION

With the complete set of 6 solvent selection tables, the ideal CCC solvent system can be determined semi-automatically overnight using a

liquid-handling robot and an HPLC. Furthermore, the extent of separation of the target compound from its contaminants can be estimated by the relative D values and, thus, an assessment made concerning the likely extent of purification by CCC. This makes CCC a particularly powerful and predictable preparative separation technique.

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