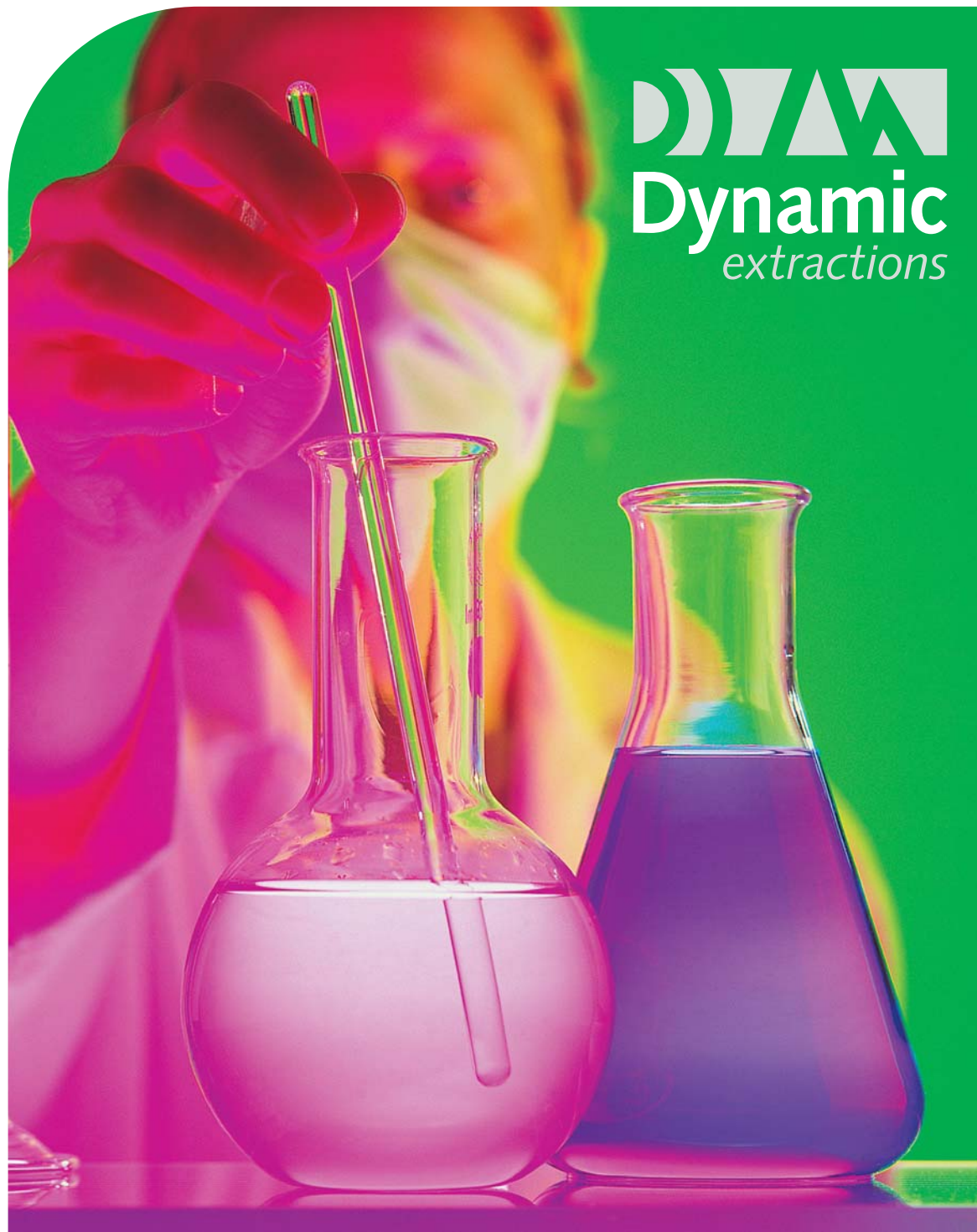


An introduction
to high
performance CCC
for sample
purification



When should you consider using High Performance CCC in your laboratory?

HPCCC is a unique technique that allows you to achieve high quality chromatographic purifications at low pressure, with high sample injection loadings and low solvent usages.

There are many challenges for purification techniques in chemistry; however HPCCC is particularly suited to the following types of application:

- Where solubility of your sample is problematic to your existing purification techniques
- Where you want to purify target compounds from crude samples, which are early in their chemistry development and can not be handled by other techniques without significant sample preparation
- Product development where you do not want to redevelop your purification processes at each differing scale



Key benefits of using HPCCC:

- No sample loss - Since both phases are liquid the sample can be totally recovered
- High throughput technology - Although primarily the liquid/liquid nature of the technique helps with sample solubility issues, it also enables high sample loadings, allowing high throughput to be achieved
- Low solvent consumption - The technique uses 10% of the volume of solvents to process equivalent masses of crude samples.
- Simple scale-up - Since column packing is very reproducible, at any and all scales of operation, the duplication of separation conditions is easy.

Now read on to gain a basic understanding of the theory behind the technology and how you operate a HPCCC in your laboratory. In fact everything you need to know to start using this exciting technology today. By the end of this booklet we hope you will understand enough for you to see how this technique will expand the capabilities of the purification toolbox in your laboratory.



Welcome to Dynamic Extractions

Dynamic Extractions is a company focused on the development of novel purification technology for use by the international pharmaceutical industry by providing improved separation solutions to their existing and future, development and manufacturing needs

Contents

A short history of the development of HPCCC	4
Developing a better understanding of HPCCC	6
The advantages over other partitioning chromatography techniques	8
Operating strategies	8
Developing a purification method for your separation	10
Using a HPCCC in your laboratory	12
Performance in application	13
Minimising sample preparation	13
Further reading	14

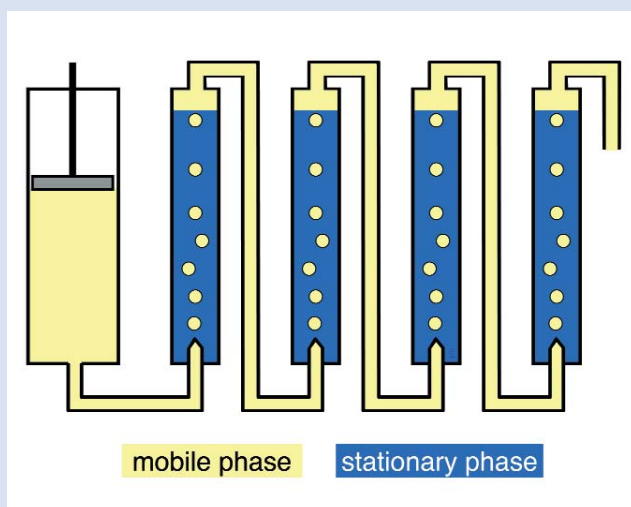
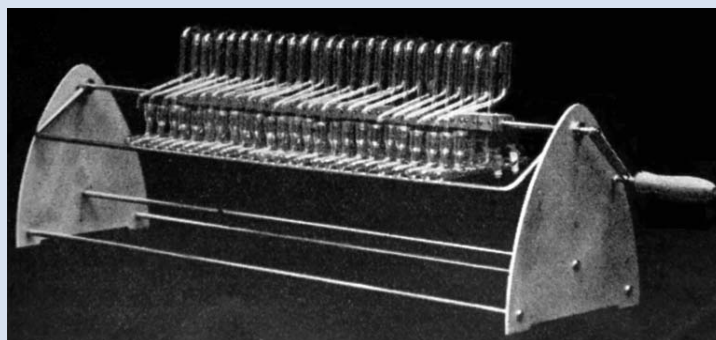
A short history of the development of HPCCC

The roots of HPCCC lay in the well known process, to chemists, of liquid-liquid extraction.

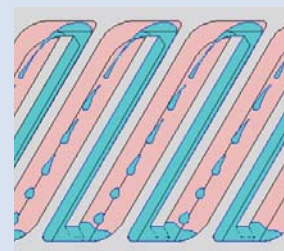


A sample is dissolved into a two-phase solvent system, normally in a separatory funnel, vigorously shaken, left to settle to allow the two phases to separate. The compound of interest will partition between the two phases, where the solvents have been selected so that the compound is more soluble in one than the other. This process can be repeated by first removing the heavier phase from the separatory funnel and mixing another fresh quantity of lighter phase or adding a further quantity of fresh of heavier phase to the separatory funnel and performing the procedure again. The compound of interest will partition again and become more purified.

Obviously although effective this is a time consuming and laborious process, therefore attempts were made to improve upon the separating funnel. The next generation of equipment was the Craig Counter-Current Distribution Apparatus, which mechanised the above process with a series of interconnected separating funnels.

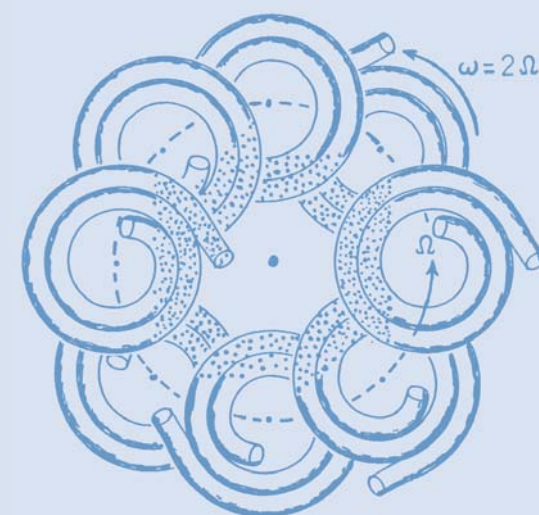


The next progression was the advent of Droplet Counter-Current Chromatography (DCCC), where a series of vertical tubes are connected by capillaries. The design enables one phase, designated the stationary phase (SP) to be retained in the tubes while the other phase, mobile phase (MP) is then either pumped to the top of the apparatus, if the phase is denser, and to the bottom, if lighter. The MP then passes through the SP, and where compounds are more soluble in the MP will pass through the apparatus more quickly and a separation will take place due to partitioning.

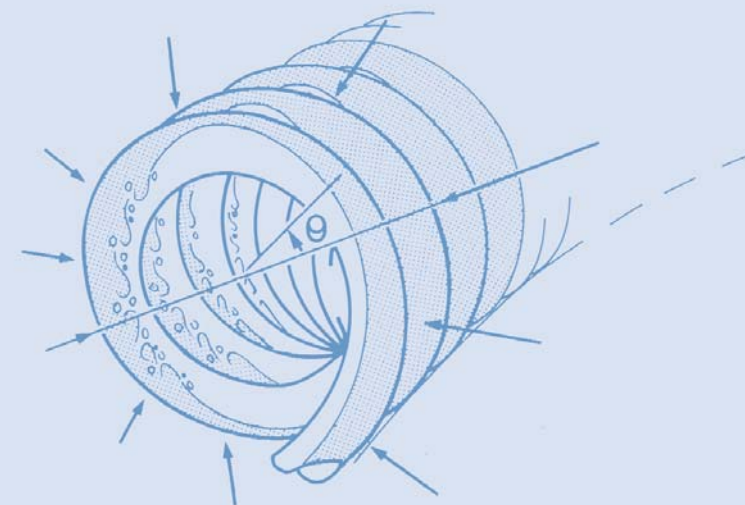


Obviously DCCC only uses gravity to retain the SP and so the next enhancement was centrifugal DCCC, where small tubes and capillaries are rotated in a centrifugal Partition chromatograph (CPC). The performance of this CPC equipment far exceeds DCCC due to using centrifugal force rather than just gravity, but as the illustration shows the construction mimics DCCC.

CPC machines rotate around only one axis; the next development was to rotate the coil on two axes. This has commonly become known as the "J" configuration and the instruments became known as High Speed Counter current Chromatograms (HSCCC). These machines have a continuous length of tubing, the column, helically wound on a bobbin that rotates on its own axis, which itself rotates around a central axis to achieve a planetary motion. This motion sets up an oscillating hydrodynamic force field, which causes a mixing and settling step to occur with each revolution of the bobbin. This hydrodynamic force field also causes phases of differing density to travel to opposite ends of the coil; it is through this phenomenon alone that retains the SP.



However, despite the complex centrifugal force field the SP retention of these early machines was often poor when compared to CPC machines. This was due to a limited understanding of the hydrodynamics present. The relationship between SP and operational parameters such as: mobile phase flow rate [1, 2], direction of pumping mobile phase through the coil [3], coil radius [3], tubing bore [4] and rotational speed [4] is now understood. This has finally led to the development of true high speed machines that has now enabled High Performance Counter Current Chromatography (HPCCC) to evolve. It is these latest machines that are now allowing chemists for the first time to consider the use of CCC technology to reliably and speedily solve their purification problems.



Developing a better understanding of HPCCC

Having discovered the development path that has driven this technology, now we must develop a better basic understanding of the technique and some of the key parameters, as explained below.

Distribution Constant (D):

All separations in HPCCC occur because of the differing solubility that components have in the biphasic solvent system. This effect is measured by the distribution constant D (also known as Kd):

$$D = C_S / C_M$$

C_S is the concentration of the sample component in the SP and C_M is the concentration of the component in the MP. A component of the sample with a high distribution constant will have a higher concentration in the SP than in the MP and will elute late from the column. Another component with a low distribution constant will have a higher concentration in the MP, than in the SP, and will elute earlier. If a compound is equally distributed in the two phases ($D=1$) the compound will elute after one column volume no matter which phase has been chosen as the MP.

Stationary Phase Retention (Sf):

This is the ratio between the SP volume and the total column (system) volume. The larger Sf, the larger the column (system) capacity available for the separation, hence, the better the resolution.

$$SF(\%) = \frac{V_{\text{column}} - V_{\text{mp}}}{V_{\text{column}}} \times 100$$

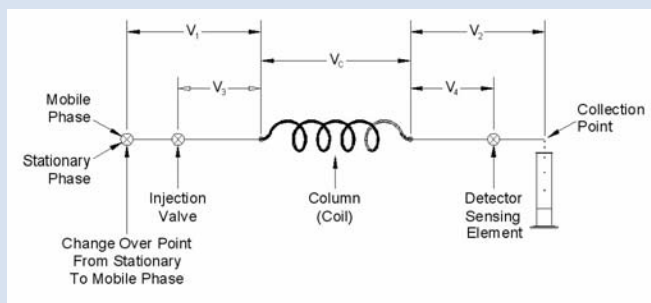
V_{mp} is equal to the volume of SP eluted during the equilibrium (this is discussed later in operation of HPCCC) and V_c is the column volume of the HPCCC, indicated on the next diagram:

Elution Time (t):

Once you have determined the distribution constant for your target compound and determined the SP retention for the biphasic solvent system you can then calculate the peak elution time for any of your target compounds from the following equation:

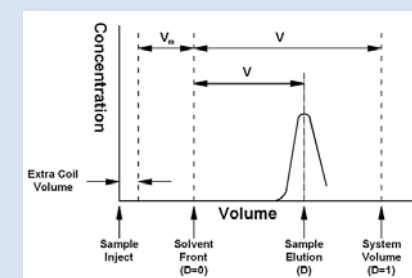
$$t_D = \frac{V_c}{F} [S_f (D-1) + 1]$$

where V_c is the column (coil) volume, F the MP flow rate, S_f the SP retention (expressed as a fraction) and D the distribution ratio. Your target peak will always elute after the same time, regardless of loading, from that size of column. Once your system is developed, prediction of elution times is easy.

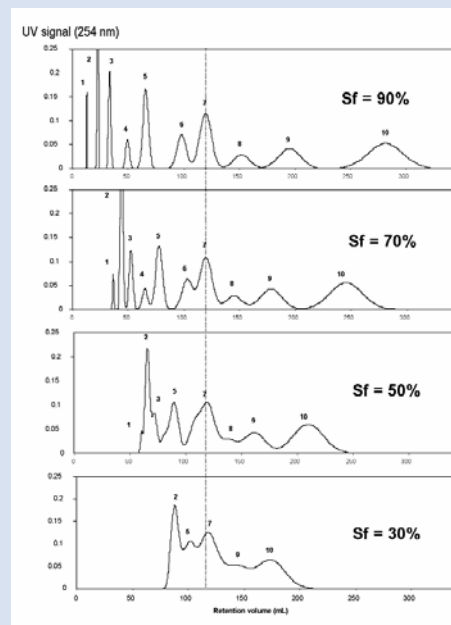


Peak elution:

This diagram, on the right, shows the theoretical elution of a compound with a distribution coefficient D. A component with a low distribution constant (i.e. <1) will have a higher concentration in the MP than in the SP and elutes quickly. A component of the sample with a high distribution constant (i.e. >1) will have a higher concentration in the SP than in the MP and elutes slowly. In the section that describes separation development, we shall use this property to show how elution time can be accurately predicted for any scale of separation.



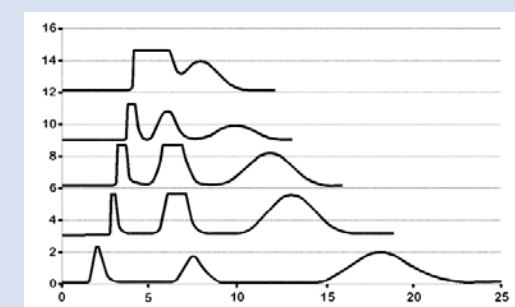
Resolution:



Key to the high performance of this technology is high SP retention. What impact does this have on separation performance? As shown in the diagram, to the left, the better the SP retention the better the resolution achieved. As we can see the higher, the SP retention the better resolution that is achieved. Previous HSCCC machines have struggled to provide high SP retention (i.e. better than 70%); now that HPCCC equipment is available SP retentions of better than 85% are the norm.

This has two major benefits. The first is that across the range of biphasic systems used high SP retentions can be achieved. It is well understood that as you move from non-polar biphasic solvent systems to polar biphasic systems SP retention reduces (i.e. becoming more polar). Therefore, in the past because of poor SP retention it has not been possible to use the technique reliably across the range of purification challenges.

Since industry has been well served by reverse phase HPLC for the non-polar range, this technology has not been adopted. The second benefit relates to throughput and speed of separation. In the past, although the technology has been called high speed, this has been a misnomer because separation times are typically measured in hours rather than minutes. High SP retention allows high MP flow rates to be used without loss of resolution. This is demonstrated by the figure on the right. Now that HPCCC machines are available, high throughput separations can be achieved across the range of polarity.



Effect of column length:

Resolution of any separation is improved by increasing the length of the column; this is possible because unlike chromatography techniques that use solid SP, HPCCC is a low-pressure technique and that does not limit the length of the column, within reason. The resolution is improved by the square root of the ratio of the lengths of the column (e.g. doubling the column length improves resolution by the square root of 2, which is 1.4). Obviously increasing the length of the column increases the time of separation, however throughput is unaffected because the sample load is a % of the column volume (i.e. in this example the sample load doubles, but the separation takes twice as long therefore throughput remains the same.). Hence separations can be developed on small scale machines to show the suitability of the technique, allowing manufacturing columns to be custom made to maximise the most important process characteristic (throughput, yield or purity).

The advantages of HPLC

HPLC has a number of advantages over other types of partitioning chromatography techniques, such as HSCCC or CPC and FCPC. Firstly, because the SP retention is so much better than HSCCC equipment, MP flow rates can be far higher and therefore speed of separation and throughput are dramatically reduced and increased respectively.

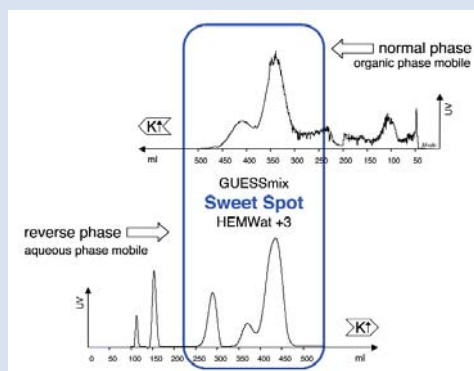
HPLC is a low-pressure technique, operating in the 50-200psi range, at all scales. This allows column length to be increased improving resolution when required. More importantly because operating pressure is not a limiting factor, scale-up is easy to predict.

Finally there is now a range of HPLC equipment available that allows the technique to be used through from analytical scale (mgs per injection) through to pilot plant scale (kilo per injection) and shortly even larger scales of equipment will be available.

Operating strategies

The advantage of a liquid SP is that it allows a number of differing operating strategies to be used, that are not possible when you are working with a solid SP. You now have access to the volume of the liquid SP and it's contents.

Another major difference when using HPLC is that unlike solid phase chromatography techniques running a normal phase separation is just the reverse process to running reverse phase. The compounds will now elute with the fast running compounds eluting slowly and the slow running compounds eluting early. This is because the partitioning coefficient of each compound becomes its reciprocal value to the one it had in the reverse phase. The figure to the right demonstrates this property [5].



Achieving a high definition separation

Firstly, let us deal with defining the conditions that lead to high-resolution separations being achieved on HPLC. The conditions should be selected such that the compound of interest has a D value between 0.5 and 2.0. The average D value of the analytes contained in a more complex sample should also be "set" to this range when doing an experiment on a mixture of unknowns. The best way to adjust the D value is to modify the two-phase solvent system; this is dealt with, later on, in the method development section. As you will read below one may also change the mode of operation (i.e. switch from normal phase to reverse phase, or vice versa during a single separation) to also achieve this purpose.

Single-mode CCC

Single-mode CCC is the most basic type of separation. This is a one direction separation, and it can be either normal or reversed phase depending on the selection of the MP.

- In "normal phase" mode, solutes elute in the order of increasing polarity (normal phase isocratic mode). Upper organic phase mobile, lower aqueous phase stationary.
- In "reverse phase" mode, solutes elute in the order of decreasing polarity (reverse phase isocratic mode). Lower aqueous phase mobile, upper organic phase stationary.

Elution Extrusion

Elution extrusion makes use of the fact that compounds may be fully separated inside the column before eluting from it. Due to the fact we are using a liquid SP, we are able to recover the separated compounds without completing the full elution cycle.

In elution extrusion, the separation is started in the same manner as in single-mode CCC. However, when the run reaches a certain point (e.g. $D=1.0$), MP will be stopped and stationary phase pumped in to extrude the column contents (i.e. the phase initially used as SP will be introduced in the instrument as a new MP) [6].

The advantages of this method are:

- The peak width of compounds with higher D will be kept narrow, so that the resolution can be improved for otherwise strongly retaining compounds
- The overall run time will be shortened since only one column volume of initial SP (i.e. the new MP) is needed to extrude the entire sample
- Another advantage of the elution extrusion is that at the end of the experiment the instrument is loaded with stationary phase and ready for another injection

Dual-mode CCC

In Dual-mode CCC, the MP is first pumped in the normal phase mode, in the middle of the run, the flow is stopped and then the MP is pumped as in reverse phase mode. Dual-mode can also be run vice versa if required [7, 8, 9].

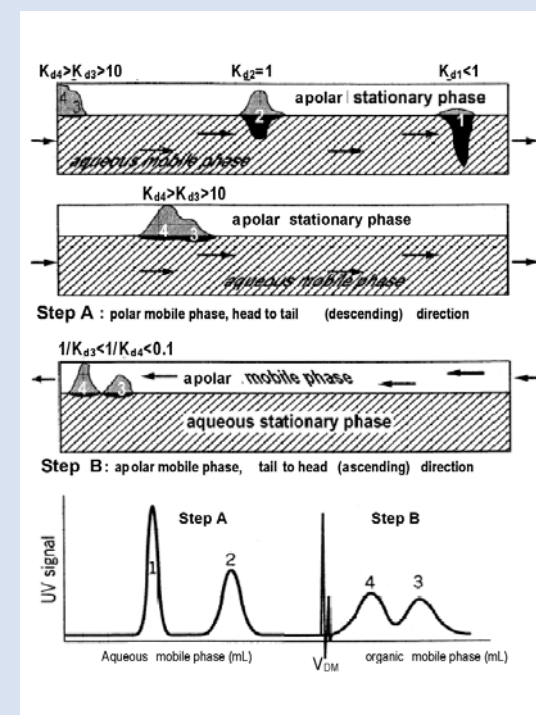
The advantage of this method is that compounds having strong affinity for the original SP can also be separated quickly rather than waiting a long time for them to elute in the MP.

pH Zone Refining

This method relies on the phenomena that charged entities (ions) prefer the aqueous phases and uncharged molecules prefer organic phases and the strategy employs basic organic phases and acidic aqueous phases (or vice versa) [10, 11]. The analytes dissolved in the SP (aka the "retainer acid" in pH zone refining, e.g. HCl) are eluted by MP (aka the "eluter base", e.g. TEA) according to their pKa values and solubility. The two major advantages of this strategy are for those types of molecules it enables very large loading capacity and high resolution separations.

Co-current CCC

Sometimes misleadingly called Dual CCC. The MP and the SP are pumped simultaneously. Depending on the retained volume of each phase in the column, the residence time of each will be different, eluting the sample in a defined band whose volume or width is determined by the respective flows [12].



Developing a purification method for your separation

MP and SP Selection

The key to successful HPLCC purifications is the selection of the most appropriate pair of mobile and stationary liquid phases [5, 13]. This is achieved by performing a partitioning study, where small quantities of the crude to be purified is added to one of the solvent combinations from the screen and the distribution coefficient of the component in the two phases is then measured. This is a systematic and predictable process.

The basic requirement for a HPLCC solvent system is that it consists of two immiscible phases (a biphasic solvent system). A particular compound will have a different relative solubility in each of the phases. Therefore, the compound is effectively distributed between the two phases. The distribution can be quantified by taking the concentration of the compound in the upper phase and dividing it by the concentration of the same compound in the lower phase. The distribution ratio determines how the compound will behave during a HPLCC separation. In general, the more soluble a compound is in the phase that has been chosen as the MP, the faster (lower volume) it will elute.

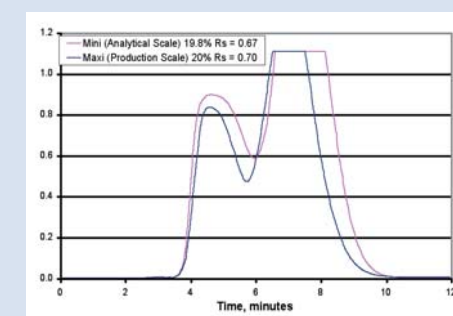
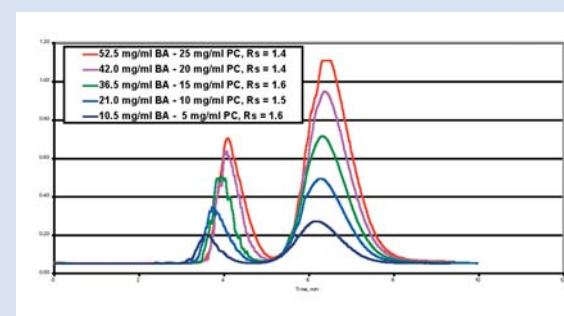
No.	Heptane	EtOAc	MeOH	Butanol	Water
1		0	0	2	2
2	0	0.4	0	1.6	2
3	0	0.8	0	1.2	2
4	0	1.2	0	0.8	2
5	0	1.6	0	0.4	2
6	0	2	0	0	2
7	0.1	1.9	0.1	0	1.9
8	0.2	1.8	0.2	0	1.8
9	0.29	1.71	0.29	0	1.71
10	0.33	1.67	0.33	0	1.67
11	0.4	1.6	0.4	0	1.6
12	0.5	1.5	0.5	0	1.5
13	0.57	1.43	0.57	0	1.43
14	0.67	1.33	0.67	0	1.33
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

This is normally done by initially using every 5th system in the table, selecting the two most appropriate systems and performing the tests again using the solvent combinations between the initial combinations selected. Alternatively, you can move up or down the table, depending upon the HPLC results obtained, testing one solvent system at a time. To do this, first, make up the solvent system at the mid-point of the table, and calculate the distribution coefficient. If the compound of interest appears mainly in the lower, aqueous layer (i.e. D value too small) then move up the table and try a more polar solvent system. On the other hand, if the compound of interest appears mainly in the upper, organic layer (i.e. D value too large) then move down the table and try a less polar solvent system. This procedure is repeated until a suitable system is found.

The solvent selection can be performed either manually or automatically, if the necessary equipment is available. Solvent screen for moderate and polar compounds is shown opposite. The Heptane/Ethyl Acetate/Methanol/Water solvent combinations shown are applicable to a wide range of polarity and Butanol is added when a more polar system is required. Currently there are screens for moderate, polar and nonpolar compounds, with aqueous-aqueous polymer tables being developed for use on protein and large molecules.

Loading and Scale-up

Having selected the optimum solvent system we then perform initial runs on a DE Mini (analytical scale) to demonstrate that the separation run gives the initial purity and reasonable run time required. Once these conditions have been determined then we can look to increase the separation to the appropriate scale of machine. Loading studies are then performed on the Mini; firstly, we increase concentration of sample at a fixed column volume of 2.5% until we can no longer maintain the necessary stationary phase retention. Secondly, we fix at this concentration and increase the percentage column volume of the sample, increasing normally in 2.5% volume steps, until we lose the required resolution. We then simply multiply up by the product of coil volumes to provide us with our scale-up conditions. Below you will find a scale-up example between a DE Mini and a DE Maxi. The chromatograms of the runs are shown below on the right.



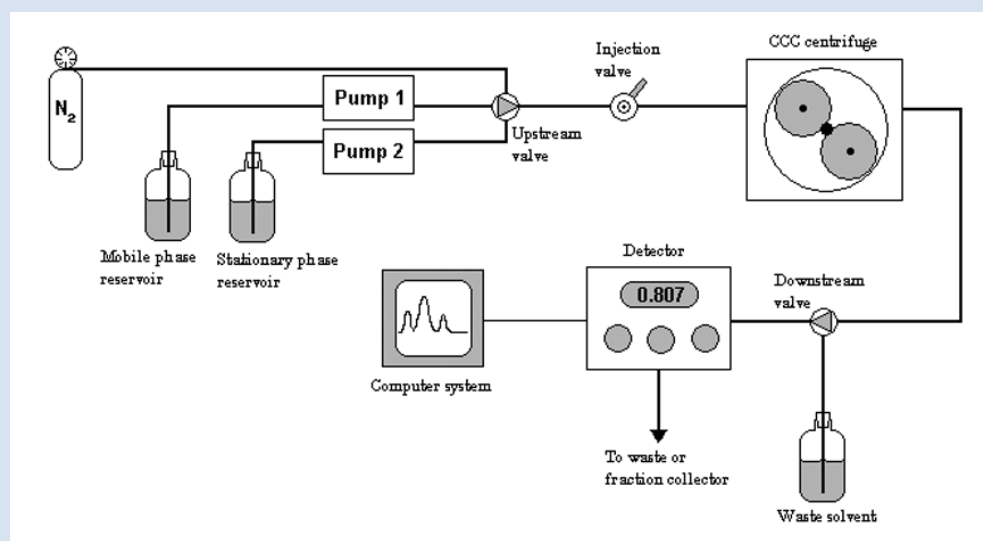
Actual scale-up example

Parameter	Mini HPLCC	Maxi HPLCC
Coil volume	5.4ml	4600ml
Rotational speed	2100rpm	600rpm
Flow rate	1ml/min	850ml/min
Volume of Stationary phase	3.47ml	2180ml
Phase System	Heptane-Ethyl Acetate-Methanol-Water (by vol. 1.4:0.1:0.5:1.0)	Heptane-Ethyl Acetate-Methanol-Water (by vol. 1.4:0.1:0.5:1.0)
Injection Mode	In aqueous mobile phase in Reverse Phase	In aqueous mobile phase in Reverse Phase
Injection Volume	20% of coil volume	20% of coil volume
Sample Description	Benzyl Alcohol and p-Cresol	Benzyl Alcohol and p-Cresol
Sample Concentration	42mg/ml of Benzyl Alcohol and 20mg/ml of p-Cresol	42mg/ml of Benzyl Alcohol and 20mg/ml of p-Cresol
Run Time	10 minutes	10 minutes
Pressure During Run	58psi (4bar)	80psi (5.5bar)

Using HPCCC in your laboratory

The schematic installation diagram shows how simply a HPCCC unit can be incorporated within your laboratory. Typically when people are first introduced to using a HPCCC unit they are told to treat it just as a different type of column and simply install it in an existing LC system (HPLC, Flash), replacing the solid SP column. The primary alteration required by the system will be that a restrictor requires fitting on the pumps, if they operate at high pressure, because HPCCC works at significantly lower pressures than HPLC.

Once installed the simple steps of working with HPCCC are as follows: select the preferred solvent system [14], make up the solvent system, prepare the HPCCC instrument and perform the separation. (Detailed operating instructions are available.) However, the following needs to be noted regarding sample preparation and injection. The sample for injection can be made-up in either the MP or SP, as long as the equilibrium of the solvent system is not disturbed. It is recommended to dissolve sample in a mixture of both phases. In any case, all compounds in the sample must be brought in solution by use of one or both phases.



The typical size of the sample loop is 5% of the column volume. If at this loading you are achieving good separation then you may increase loading further, but it is always better to achieve this increase by further increasing the volume of sample injected. It is possible to dissolve samples in larger volumes where solubility problems are encountered; however, this will cause a loss in resolution, but this can be compensated for by increasing the length of column used.

The pressure of the instrument should be monitored while the sample is injected and throughout the run. This is especially important on the analytical scale machine where a rapid, but small change in pressure can cause a loss of SP and a reduction in the resolution of the separation.

Performance in application

Below are presented two examples of HPCCC equipment in action on purifications of material that was used in a pharmaceutical development project:

Type of separation: Hydrophobic (Non-polar)

Processing regime: Single pass

DE Centrifuge: Midi

Phase system: Heptane/Ethyl Acetate/Methanol/Water

Loading per injection: 25 grams of crude

Target compound isolated per injection: 6 grams (average)

Purity: >92%

Recovery: >95%

Cycle time: 40 minutes

Total quantity of crude processed: 9.0 kg

Total solvent necessary: 468 litres

Type of separation: Hydrophobic (polar)

Processing regime: Single pass

DE Centrifuge: Maxi

Phase system:

Loading per injection: 160 grams of crude

Target compound isolated per injection: 23.6 grams (average)

Purity: >95%

Recovery: >90%

Cycle time: 40 minutes

Total quantity of crude processed: 6.7 kg

Total solvent necessary: 480 litres

Minimising sample preparation

To illustrate the nature of the crude samples that can be directly injected and purified by HPCCC, we have shown photographs of typical samples that have been processed. Note the high particulate contamination.



Further reading

Our intention with this booklet has been to introduce the possibilities that HPCCC offers and how a chromatographic technique without solid phase media is easy and practical to use in the laboratory. On all aspects of the HPCCC that we have discussed Dynamic Extractions can provide information that is far more comprehensive. For further reading consult the bibliography that follows.

Bibliography

A Short History of the Development of HPCCC

- 1 Du, Q., Wu, C., Qian, G., Wu, P., Ito, Y., Relationship between the flow-rate of the mobile phase and retention of the stationary phase in countercurrent chromatography, *J. of Chromatography A*, 835, 231-235, 1999
- 2 Wood, P., Janaway, L., Hawes, D., and Sutherland, I., A., Determination of J-type centrifuge Extra-coil volume using stationary phase retentions at differing flow rates, *J. Liq. Chrom. & Rel. Technol.*, 26 (9-10), 1417-1430, 2003
- 3 Wood, P., Jaber, B., Janaway, L., Sutherland, I., A., and Terlinden, N., Effect of beta-value on the Head and Tail Distribution of the Upper and Lower phases in Helical Coils, *J. Liq. Chrom. & Rel. Technol.*, 28 (12-13), 1819-1837, 2005
- 4 Wood, P., Janaway, L., Hawes, D., and Sutherland, I., A., Stationary phase retention in Countercurrent Chromatography: Modelling the J-type centrifuge as a constant pressure drop pump, *J. Liq. Chrom. & Rel. Technol.*, 26 (9-10), 1373-1396, 2003

Operating Strategies

- 5 Friesen, J. B., Pauli, G. F., G.U.E.S.S.-A Generally Useful Estimate of Solvent Systems for CCC, *J. Liq. Chrom. & Rel. Technol.*, 28, 2777- 2806, 2005
- 6 Berthod, A., Hassoun, M., Harris, G., Using the Liquid Nature of the Stationary Phase: The Elution-Extrusion Method, *J. of Liq Chrom. & Rel. Tech.*, 28 (12-13), 1851-1866, 2005
- 7 Alvi, K., Screening natural products: bioassay-directed isolation of active components by Dual-Mode CCC, *J. Liq. Chrom. & Rel. Technol.*, 24 (11-12), 1765-1773, 2001
- 8 Agnely, M., Thiebaut, D., Dual-mode high-speed countercurrent chromatograph: retention, resolution and examples, *Journal of Chromatography A*, (790), 17-30, 1997
- 9 Lee, Y. W., Cook, C. E., Ito, Y., Dual Countercurrent Chromatography, *J. Liq. Chrom.*, 11 (1), 37-53, 1988
- 10 Pennanec, R., Viron, C., Blanchard, S., Lafosse, M., Original uses of the pH-Zone-Refining principle: Adaption to synthesis imperatives and to ionic compounds, *J. Liq. Chrom. & Rel. Technol.*, 24 (11-12), 1575-1591, 2001
- 11 Ito, Y., Shinomiya, K., Fales, H. M., Weisz, A., Scher, A. L., pH- Zone-Refining Countercurrent Chromatography. A New Technique for Preparative Separation, American Chemical Society Symposium No. 593 Modern Countercurrent Chromatography, 156-183, 1995
- 12 Berthod, A., Hassoun, M., Using the liquid nature of the stationary phase in countercurrent chromatography IV. The cocurrent CCC method, *Journal of Chromatography A*, (1116), 143-148, 2006

Developing a purification method for your separation

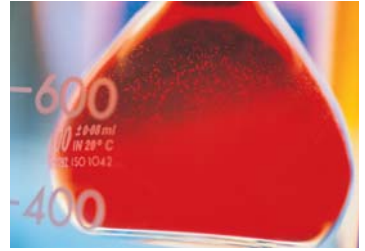
- 13 Garrad, I. J., Simple Approach to the Development of a CCC Solvent Selection Protocol Suitable for Automation, *J. Liq. Chrom. & Rel. Technol.*, 28 (12-13), 1923-1935, 2005

Using a HPCCC in your Laboratory

- 14 Ito, Y., Golden rules and pitfalls in selecting optimum conditions for high-speed countercurrent chromatography, *Journal of Chromatography A*, (1065), 145-168, 2005



Dynamic
extractions



further information

For further information contact Dynamic Extractions on:

+44 (0)1753 696979

or visit our website:

www.dynamicextractions.com

Dynamic Extractions Ltd

890 Plymouth Road

Slough

Berkshire

SL1 4LP

t: +44 (0)1753 696979

f: +44 (0)1753 696976

e: enquiries@dynamicextractions.com