

How to Develop a Counter Current Chromatography Separation

Introduction

Counter current 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 depends solely upon the phase volume ratio and the partition coefficient 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 almost limitless. This means it should be possible to always find a solvent system that can perform the separation required.

The Criteria for a CCC Solvent System

Listed below are the criteria for selecting a CCC solvent system. The first four criteria are essential in order to successfully operate the J-type high-speed counter current chromatograph. The last two criteria are desirable, especially if the separation is being done on a larger scale and fractions are collected.

1. The solvent system must consist of two immiscible phases.
2. The selectivity of the phases must be sufficient for good resolution of the components to be separated in the sample.
3. The density difference between the phases must be at least 0.1 g/ml in order to retain the stationary phase in the column.
4. The settling time should be less than approximately 60 seconds in order for the mixing and settling steps inside the CCC column to operate efficiently. Note that the settling time will vary with the size of the vessel used.
5. The mobile phase should be volatile so it can easily be removed from collected fractions.
6. The phase volume ratio of the two phases should be acceptable in order to avoid excessive wastage of solvents.

Steps Involved in developing the separation method

1. Perform partition study experiments.
2. Analyse the samples from the experiments.
3. Select the preferred solvent system.
4. Make up the solvent system.

It is assumed that an HPLC machine is available for the protocol and that a suitable analytical method has been developed for identifying and quantifying the compound of interest on HPLC. The partition study experiment is an adaptation of the "shake flask method" used to measure distribution ratios.

Step 1: Partition Study Experiment

- a. Label a set of vials (capable of taking a 1.6ml volume) 1, 6, 12, 17, 22 and 28.
- b. Weigh out 5 – 10 mg of the test mixture into each vial.
- c. 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.

- d. Using a 1 ml adjustable pipette for accurate metering of the liquids, add 1.6ml ml total of the solvent systems highlighted in red in table 1, to the appropriate vials. Table 1 shows the solvent proportions for information. When making the solvent systems, be sure to minimise evaporation by capping each vial immediately after any addition of solvent.
- e. Cap the vials securely and allow them to equilibrate to the desired temperature (ideally 20° C) before shaking them vigorously for several minutes. A vortex or inverting mixer may be used.
- f. Allow 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

- a. Pipette 0.4 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.
- b. Analyse all the HPLC vials using the accepted HPLC method for the components to be separated.
- c. From the chromatograms, measure the peak areas of each component and calculate the Distribution Ratio D for each solvent system tested using the following equation. Note that the units for peak areas are not important so long as they remain consistent within each calculation:

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

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

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

Step 3: Select Solvent System

- i. The method described above is an initial screening. What you are looking for, for any given compound is a D value between 0.2 and 5.0 and a difference of at least 0.5, ideally 1.0 between different compounds to enable their separation.
- ii. Once you have identified which of the highlighted system(s) is closest to these requirements then you can repeat the screening process with adjacent solvent systems, for example if solvent system 17 gives the best values, then repeat with systems 15, 16, 18 and 19.
- iii. 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 is better than one with the values 2.8 and 5.0 respectively.

- iv. If a more polar system than No1 is required, add a volatile salt e.g. triethylammonium sulphate, to the aqueous layer and retest the distribution ratio as described above.
- v. For very polar compounds which contain charged acidic groups such as carboxylic acids (with pKa's below 6.0), you will often find these compounds stay only in the aqueous phase. One solution to this is to add 0.05% trifluoroacetic acid (TFA) to the solvent systems, as a charge suppressant. This will make the compounds more non-polar changing their distribution dramatically.
- vi. If a less polar system than No28 is required, substitute acetonitrile for some or all of the methanol e.g. heptane-acetonitrile-methanol (3:1:3) or heptane-acetonitrile (1:1).
- vii. 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).
- viii. Note that, as mentioned above, this procedure details the testing of an unknown compound and is ideally suited to an automated liquid handling system.

Step 4: Making up the Phase System

1. From the component ratio of the selected solvent system, calculate the volumes of each solvent required. For example, if the system ethyl acetate-water (1:1) is chosen, and 2 litres of solvent is estimated for the run, then 1 litre of each solvent is required.
2. Ensure the solvents to be used are at room temperature e.g. not just collected from a cold storage area, and that they have been filtered free from particulate matter.
3. In a fume cupboard, and wearing the necessary protective gear, measure out the volumes of solvent required in a measuring cylinder. Carefully transfer the solvents to a suitably sized flask using a glass or stainless steel funnel. Avoid any spillages. A separating funnel is ideal, since the two layers will be separated later.
4. Stopper the flask and carefully shake to mix the solvents. Periodically release the stopper when shaking in case any vapour pressure has built up inside the vessel.
5. Leave the flask until both layers are completely clear and full equilibrium has been achieved. This may take an hour or more.
6. Decant the lower layer from the upper and store the two phases in separate containers, clearly labelled.
7. The above procedure assumes the solvents will be used at room temperature. If a different temperature is required, then it is important that the solvents are equilibrated to this temperature before mixing as well as after mixing and before separating the layers.

No		Heptane	EtOAc	MeOH	Butanol	Water
1		0.00	0.00	0.00	2.00	2.00
2		0.00	0.40	0.00	1.60	2.00
3		0.00	0.80	0.00	1.20	2.00
4		0.00	1.20	0.00	0.80	2.00
5		0.00	1.60	0.00	0.40	2.00
6		0.00	2.00	0.00	0.00	2.00
7	More	0.10	1.90	0.10	0.00	1.90
8	Polar	0.20	1.80	0.20	0.00	1.80
9		0.29	1.71	0.29	0.00	1.71
10	↑	0.33	1.67	0.33	0.00	1.67
11		0.40	1.60	0.40	0.00	1.60
12		0.50	1.50	0.50	0.00	1.50
13		0.57	1.43	0.57	0.00	1.43
14		0.67	1.33	0.67	0.00	1.33
15		0.80	1.20	0.80	0.00	1.20
16		0.91	1.09	0.91	0.00	1.09
17	Entry	1.00	1.00	1.00	0.00	1.00
18	point:	1.09	0.91	1.09	0.00	0.91
19		1.20	0.80	1.20	0.00	0.80
20		1.33	0.67	1.33	0.00	0.67
21		1.43	0.57	1.43	0.00	0.57
22		1.50	0.50	1.50	0.00	0.50
23	↓	1.60	0.40	1.60	0.00	0.40
24		1.67	0.33	1.67	0.00	0.33
25	Less	1.71	0.29	1.71	0.00	0.29
26	Polar	1.80	0.20	1.80	0.00	0.20
27		1.90	0.10	1.90	0.00	0.10
28		2.00	0.00	2.00	0.00	0.00

Table 1: Selecting a CCC Solvent System – Proportions of Test Solvents

Running the CCC Apparatus

The exact procedure for running the CCC apparatus should be obtained from the instruction manual of the particular model employed. The following is a general procedure suitable for application to a typical analytical size J-type HSCCC instrument, such as the DE Mini Counter current Centrifuge supplied by the Dynamic Extractions.

The schematic layout of a typical CCC set-up is shown in Figures 1 and 2, below, using a DE Mini (18 ml coil) or DE Midi (34 and 948 ml coils) as examples. It is assumed for the purposes of this document that the instrument has already been set up and that the operator is familiar with the workings of the equipment.

Figure 1: Installation outline of DE Mini Centrifuge

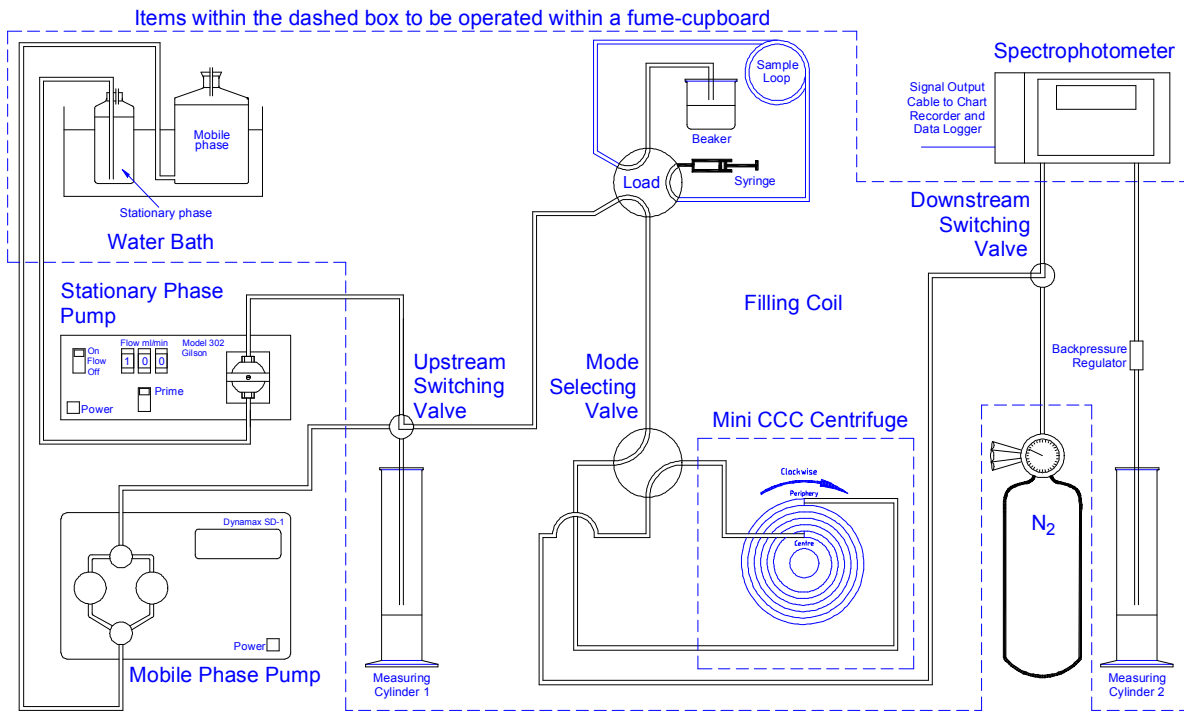
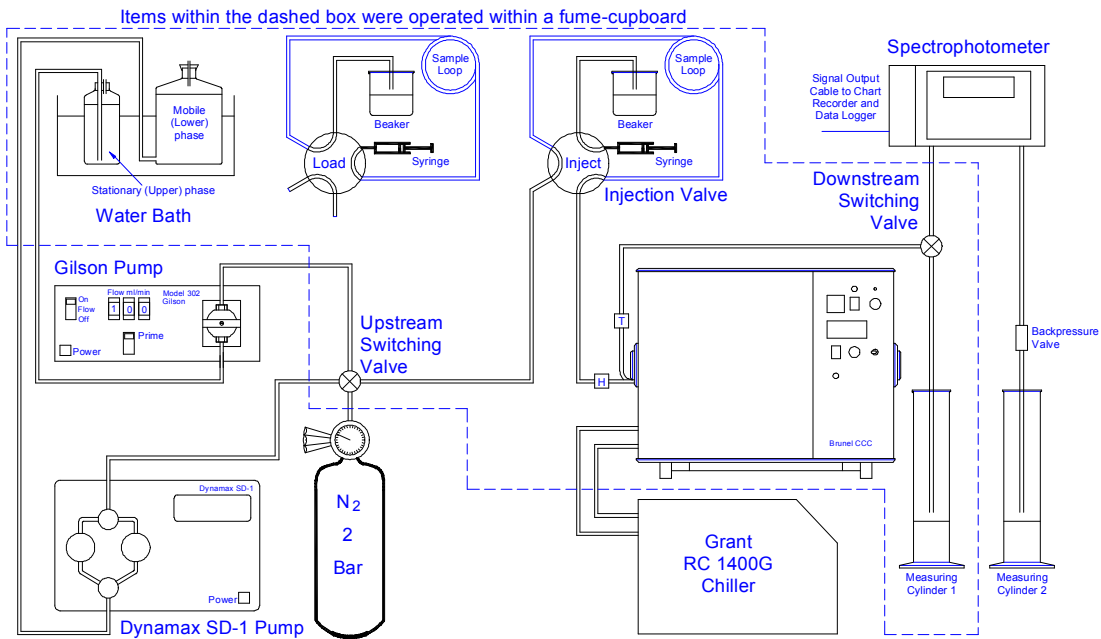


Figure 2: Installation outline of DE Midi Centrifuge



Step 5: Choosing the mode of operation

1. There are multiple modes for carrying out CCC such as Normal Phase (NP) mode, Reverse Phase (RP) mode, Dual mode, and Co-current mode. These are described in more detail in the

CONFIDENTIAL

literature, for simplicity we are going to describe only NP and RP mode which are most frequently used and always the starting point.

2. In NP mode the organic phase is the mobile phase and the aqueous the stationary phase while in RP mode the opposite applies with the aqueous phase the mobile phase. This is important because it affects the mode of operation described below.
3. Generally it is always best to choose the NP mode because this means the eluted compounds will be in a volatile phase which is easier to dry down. The other consideration is speed versus separation. Obviously if you choose the phase which your compound prefers as the mobile phase, then the run will be faster, however separation is best if the stationary phase is preferred.
4. In practice we suggest you start of with NP mode and change the solvent system to optimise the separation, this will give the best processing times.

Step 6: Preparing the CCC Column

1. Decide upon the mode of operation of the CCC. With the upper phase mobile, pump from the tail periphery to the head centre. With the lower phase mobile, pump from the head centre to the tail periphery. If collecting fractions, then it is generally convenient to have the more volatile, organic phase as the mobile one. Therefore, for these guidance notes, it will be assumed that the upper, organic phase is mobile and thus the apparatus is being used in normal phase chromatographic mode (as opposed to reverse phase mode).
2. For NP mode connect the tail end of the coil to the injection valve and the head of the coil to the detector. If in doubt as to which end of the coil is which, fill up the coil then operate the CCC machine with both ends of the coil open. Fluid will come out of the head end of the coil.
3. Depending upon the last use of the CCC column, it is good practice to wash the column with methanol and then rinse with water. This process should be considered each time a new solvent system is used, since droplets of the previous solvent system or sample may remain on the internal PTFE surfaces of the coil, affecting the properties of the new solvent system.
4. The coil may now be filled with stationary phase at a flow rate of approximately 1 ml/min for coils up to 10ml volume, or 10 ml/min for larger coils. (It is assumed that the lines to the pumps are primed with their respective solvents up to the upstream switching valve). Note that the rotor must be stationary during this process and the downstream switching valve is set to waste to avoid passing stationary phase through the detector.
5. The CCC is ready for operation when a steady stream of stationary phase can be seen coming from the outlet with no bubbles present.

Step 7: Performing the Separation

1. Start the rotor in the normal direction of spin and initially select a slow speed e.g. 500 rpm to allow the oil in the gearbox to disperse evenly. Then increase until the desired rotational speed for equilibration is reached
2. Start pumping the mobile phase at your desired flow rate.
3. Measure the volume of stationary phase eluted and note the reading. This volume equals the volume of mobile phase in the coil, plus the volume of the inlet and outlet tubes.
4. This information is useful in calculating how quickly the component peaks will appear and how much sample can be loaded onto the column.
5. Turn the downstream valve so the mobile phase flows through the detector and wait for a steady baseline to appear.
6. Introduce the sample through the injection valve. The sample can be dissolved in either the mobile or the stationary phase, or a mixture of both.

Step 8: Optimising the separation

1. Solvent system screening gives a very good idea of where to start in the development of the separation method but does not always give the final answer. To do this you need to optimise the separation to improve separation or to reduce run time.
2. Separation is always improved by one of two ways, either by decreasing flow rate or by using a more non-polar solvent system. This means in practice increasing by one level, for example from solvent system 17 to 18.
3. This will increase run time but improve separation. To counter this increase in run time you can try increasing flow rate to get a balance between these two parameters.
4. If separation is good but run time too long just decrease solvent system or increase flow rate.
5. To optimise throughput there are three methods, increasing flow rate, increasing loading concentration or increasing loading volume.
6. We suggest you try increasing flow rate first then increase the loading concentration. If stationary phase retention is good but separation reduces with loading then change solvent system to a more non-polar one. This will increase run time but overall the increase in throughput will more than compensate.