HIGH CAPACITY COUNTERCURRENT CHROMATOGRAPHY FOR FAST ISOLATION OF NATURAL PRODUCTS

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1 INTRODUCTION

Countercurrent chromatography (CCC) is a technique that was first invented in the mid 1960's.[1] After a brief period of interest by many scientists, the technique declined in popularity, being effectively out-competed by rival techniques that were faster and more reliable. To many, CCC was known as a slow technique with separations measured in hours or days, with poor capacity allowing injections of only tens of milligrams, poor reliability and difficult scale-up because of a poor understanding of the factors required to scale up or instruments on which to do so. However, recent engineering improvements and advances in the understanding of the process have transformed the technique to one which provides separations in minutes, injection loadings in the scale of grams to hundreds-of-grams, equipment that is considerably more robust and a scale up from analytical to pilot level that is quick and easy.[2]

These developments have led to a resurgence of interest in CCC, especially for natural products, with the technique offering numerous advantages over both solid-phase chromatography and traditional liquid-liquid extraction. The current range of CCC instruments is a worthy inclusion in the arsenal of techniques necessary for the purification of challenging natural product molecules. This article describes the technique and its advantages over other purification methods and demonstrates the purification potential with a number of natural product examples.

2 COUNTERCURRENT CHROMATOGRAPHY

2.1 Mechanism of Action

CCC is a liquid-liquid purification technique comprising a liquid stationary phase and a liquid mobile phase. Separation of the components of a mixture is based on differential distribution between the two liquid phases. The immobilisation of the liquid stationary phase and the

multiple mixing and separation of phases are achieved by centrifugal forces. The instruments are consequently termed CCC-centrifuges.

A CCC centrifuge consists of a drum on which tubing is wound. When any coil is filled with liquid and rotated, every object within, whether it is lighter (e.g. an air bubble) or heavier (e.g. a glass bead), tends to move towards one end of the coil as a consequence of trying to maintain its original position. The end towards which the bubble and bead move is called the head of the coil, and the other end is called the tail.

If the air bubble represents the upper solvent phase and the glass bead the lower solvent phase, it can be seen that both phases "screw" themselves towards the head of the coil. Since they cannot both occupy the same space, one phase dominates and thus displaces the other towards the tail. So if the dominant phase is selected as the stationary phase of the system, then it will be retained in the coil by this screwing action, making the displaced phase the mobile one being pushed by a pump through the coil in the direction it is tending to go. In countercurrent chromatography terms, the mobile phase is being pumped from head to tail.

It can be seen that, if the coil were rotated in the opposite direction, the other phase is selected as the stationary one which is retained in the coil. In countercurrent chromatography terms, the mobile phase is being pumped from tail to head. In practical terms, the coil is always rotated in the same direction but the pump is switched to the other lead from the coil. The coil thus contains a liquid stationary phase, held in place purely by the "screwing" motion of the coil and a liquid mobile phase being pumped through the coil in the direction in which it is naturally being displaced. This gives a hydrodynamic countercurrent flow.

However, if simply spun on its own axis, the two phases inside the coil would not mix due to the high $r\omega^2$ rotational force (the "centrifugal" force). There would be laminar flow of the mobile phase over the stationary one and extremely poor partitioning of solutes. Therefore, the coil is spun in planetary motion, revolving around the central axis of the sun gear while simultaneously rotating about its own axis at the same angular velocity (Figure 1).

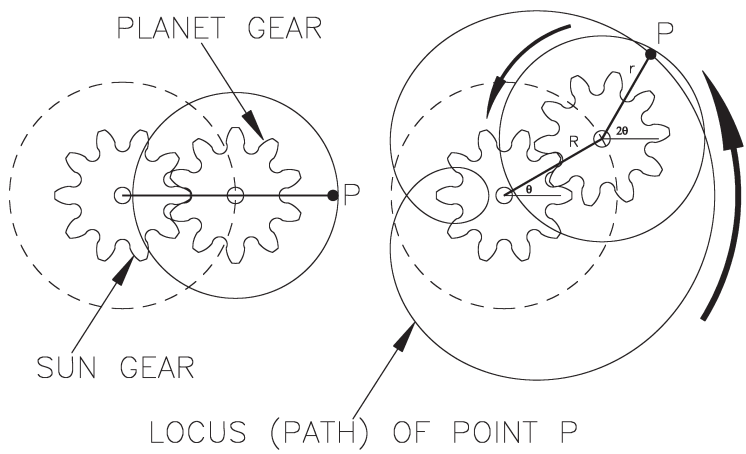


Figure 1 *Motion of the bobbin in the CCC centrifuge.*

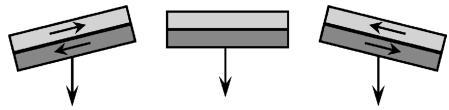


Figure 2 *Tilting tube effect that produces the wave mixing in the planetary motion centrifuge.*

This creates a mixing effect akin to the "swish-swosh" motion that occurs when a tube of two liquids is tilted from side to side (Figure 2). The net result of this motion is the formation of waves and a wave mixing effect (Figure 3).

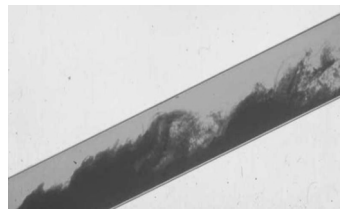


Figure 3 *A model of the wave mixing that occurs in the CCC centrifuge.*

When a sample is introduced into the centrifuge, it will experience a series of mixing and settling steps according to the low or high acceleration vectors (Figure 4). The components of the mixture introduced into the coil will separate according to their distribution ratio between the two solvent phases (KD = concentration of solute in stationary phase / concentration of solute in mobile phase).

It will be noticed in the diagram that there is one mixing and one settling zone per coil loop. Furthermore, the coil in Figure 4 is simply wound in an axial direction, producing a helical coil. In practice, a typical CCC coil will be multilayer wound and may have 30 loops spinning at 1200 rpm. It will therefore undergo 2.16 million partitioning steps per hour (30 x 1200 x 60). This provides for high resolution separations within the instrument.

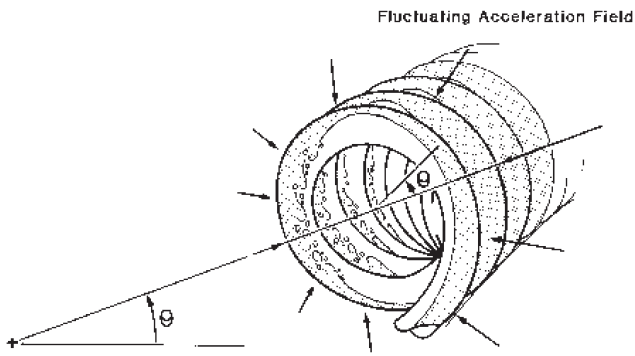


Figure 4 *The mixing and settling that takes place in a J-type centrifuge.*

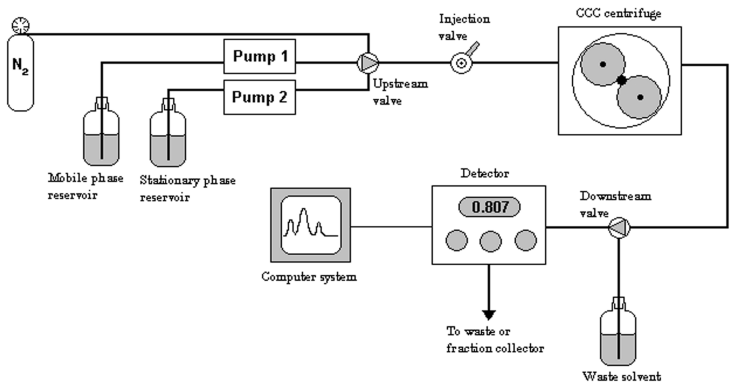


Figure 5 *Schematic layout of a typical preparative CCC setup.*

The synchronous planetary motion of the CCC coil has a special feature significant in the design of instruments: there is no twisting of the flow tubes linking the coil to the pump and detector. This is because the rotation about its own axis unwinds the twist produced by its motion around the sun gear. Thus, there are no rotating seals to wear out, leak or provide the risk of cross-contamination.

A schematic layout for a typical CCC setup is shown in Figure 5. In effect, the CCC coil is equivalent to a preparative HPLC column that does not wear out or need replacing. All the ancillary equipment is the same - pumps, injection valves, detector, fraction collector and chromatography software. Two pumps allow the easy switching of the liquid stationary to the liquid mobile phase, while a nitrogen cylinder allows the final extraction of the stationary phase from the coil if that is desired. The injection valve can be a standard Rheodyne type, but when the sample is particularly crude, viscous or may precipitate, direct injection from a syringe may be necessary. In the diagram a UV detector has been shown since this form of detection is non-destructive. However, in natural product purifications, CCC is commonly used with a bleed to a light scattering detector (ELSD) and has even been used with mass spectrometry detection.[3]

Since both phases are liquid, CCC can be operated in either normal or reverse phase mode, depending on which solvent phase is selected to be stationary. It is even possible to switch over mid-run to elute slow moving compounds. Alternatively, components that elute very slowly can be recovered by pushing out the stationary phase without any compound losses whilst maintaining resolution.[4]

At the Brunel Advanced Bioprocessing Centre, we have a range of CCC centrifuges running from analytical to pilot scale, the latter capable of processing several kilograms of crude material per day (Table 1). Known as Mini, Midi, Maxi and Super-Maxi, the Mini centrifuge is the size of a microwave oven, the Midi fits into a normal chemistry fume cupboard, and the Maxi instruments are pilot machines about 2 metres cubed (Figure 6). Commercially available machines are now produced by Dynamic Extractions Ltd (www.dynamicextractions.com). Importantly they have now developed a composite machine, the Spectrum, which includes both analytical and preparative coils in one machine, providing a versatile instrument.

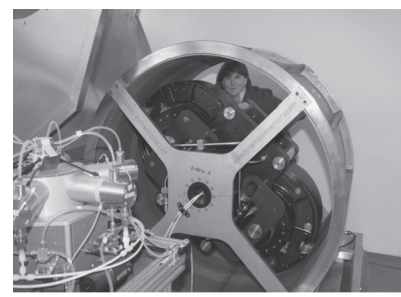
Instrument	MINI	MIDI	MAXI	"SUPER" MAXI
Scale of application	Analytical	Preparative	Pilot	Pilot
Processing rate	mg/hr	g/hr	kg/day	kg/hr
Rotor radius (mm)	50	110	300	300
Volume of coils (ml)	17	930	4600	18000
Coil bore (mm)	0.8	3.6	10.0	10.0
Max rotor speed (rpm)	2100	1400	850	850
Typical flow rate (ml/min)	0.25-2	5-80	100-1000	400-3000
Typical elution time (min) for K_D=1 peak	20	20	20	20
Typical loading (g)	0.005-0.050	1-30	50-300	250-1000

Table 1 *The range of CCC centrifuges at the Brunel Advanced Bioprocessing Centre with their specifications.*

Mini (analytical scale)

Midi (preparative scale)

Maxi (pilot scale)



"Super" Maxi (pilot scale)

Figure 6 *The Mini, Midi, Maxi and "Super" Maxi range of CCC centrifuges.*

2.2 Operational Advantages of CCC

The key advantages of countercurrent chromatography in the purification of natural products:

1. Far lower solvent usage compared with solid phase chromatography systems operating at the same scale. Furthermore, a simple analysis of solvent composition allows the recycling of the solvents, reducing the usage still further.[5]
2. 100% recovery of the sample components. Since there is no solid phase, there is no possibility of losses arising from irreversible adsorption onto the solid matrix. The target compound will be in one liquid phase or the other so it can always be recovered.
3. Particulates, such as cell debris are tolerated, so filtering a crude sample is not necessary and precipitation of the sample on meeting the solvent system does not adversely affect the purification process. A direct extraction of compounds from a crude natural mixture is possible.
4. High loading capacity with short processing times. The smallest Mini-CCC centrifuge, with a 17 ml coil and running at 1 ml/min flow rate, can routinely accept injections of 5-20 mg and is capable of loadings of up to 50 mg crude material.
5. The scale up from milligram to kilogram level is quick and predictable. Optimisation runs performed on the Mini CCC are immediately transferred to the Maxi machines (1000 times scale up) and show almost identical chromatograms.
6. The technique can be operated in normal batch injection, chromatography mode or as a continuous extraction process for better throughput.[6]
7. A wide range of polarities can be processed due to the range of solvents that may be used, from highly polar molecules such as peptide antibiotics[7] to highly non-polar molecules such as lycopene.[8] A recent review of over 200 papers on the purification of Chinese herbal medicines by CCC showed an ACD logP polarity range from -4 to +12 with the majority falling in the -2 to +8 range (Figure 7).[9] In this review, 354 natural product compounds were isolated in total from 108 different plant species and 56 plant families.

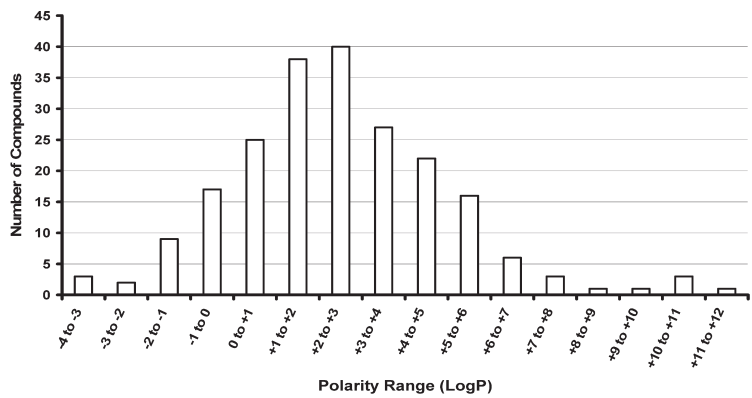


Figure 7 *The natural product compounds purified by CCC sorted by LogP polarity (taken from a review of 200 papers).*[9]

8. The coils are tough and the machinery robust. A set of coils would be expected to last the lifetime of the centrifuge and so maintenance and running costs are low.
9. Unlike solid phase chromatography, there is no change to component retention over time (no column aging effects) as a freshly-filled coil of solvents is used for each run. This makes it easier consistently to satisfy current regulatory requirements when performing purifications under a GLP or GMP environment.

2.3 Natural Product Application Examples

Approximately 30 natural product compounds have been purified using the Brunel CCC-centrifuges in a number of collaborations with academic groups active in natural products. A major focus has been on developing scaled up separations, currently up to kilogramme scale.

A typical CCC natural product purification performed at Brunel Advanced Bioprocessing Centre involved the separation of the isomers, honokiol (ACD logP 4.199) and magnolol (ACD logP 3.938) from the traditional Chinese medicine, Houpu, an extract of *Magnoliae Officinalis*.[10] This was done in collaboration with the State Key Lab of Biotherapy in Chengdu, China. Honokiol has potent anti-angiogenesis activity and material was required for pre-clinical trials and further chemical modification. A suitable two phase solvent system (hexane/ethyl acetate/methanol/water 1/0.4/1/0.4 v/v) was identified using the selection table described in reference[11] operated on a liquid handling robot. The upper, organic phase was chosen as the mobile phase (normal phase mode) in order to make the subsequent drying down of fractions more rapid. Loading studies were then performed on the small scale Mini CCC centrifuge (17.2 ml coil, 0.8 mm bore, 1800 rpm spin speed) to determine both the optimum injection concentration and volume. The chosen conditions were then directly scaled-up to the 4.6 litre Maxi centrifuge and the same run performed on a preparative scale (75 g injection loading in 230 ml phase i.e. 5% of column volume, spin speed 600 rpm, 600 ml/min flow rate, 30 minute run time) to yield 30 g of honokiol per injection with a purity greater than 99%.

This material was then used by the Chengdu group in a series of *in vivo* studies to improve its therapeutic effectiveness by formulation in liposomes[12-14] or in hydrogels.[15,16] In a programme to synthesise a series of novel derivatives of honokiol as potential analogues for anti-proliferative and anti-tumour treatment, they have produced three C-formyl derivatives of honokiol (3'-formyl, 5-formyl and 3',5-diformyl).[17] Not only was CCC used to provide the honokiol starting material, but it was also used to isolate these derivatives, including the 3'- and 5'-isomers.

In another example, the highly polar glucosinolate, glucoraphanin (ACD logP 0.53) has been isolated from extracts of broccoli seeds, using a phase system of 1-propanol/acetonitrile/saturated ammonium sulphate/water (1/0.5/1.2/1 v/v) to separate it from glucoiberin (ACD logP -0.81). Using a Midi centrifuge, 50 g was obtained in three days with *ca.* 30 runs of 30 min.[18] In partnership with Dynamic Extractions, this was scaled to 1 kg using a Maxi CCC centrifuge, again with a series of 30 min. runs over three days.

The flavonoids baicalein (ACD log P 0.314) and its 7-O glucoside, diglucoside and glucuronide, chrysin (ACD logP 2.879) and its 7-O-glucuronide and diglucoside, biochanin A (ACD logP 3.139), have all been separated by CCC from extracts of the seeds of *Oroxylum indicum*; analytically with a Mini CCC centrifuge with inline MS monitoring,[19] preparatively with a Midi CCC centrifuge[20] and also from the leaves.[21] Chloroform/methanol/water (9.5/10/5 v/v) was the solvent system used.

The terpenoid triptolidelode (ACD logP 1.268) and the alkaloids peritassine A, wilforgine and wilforine (ACD logP 6.244) have been isolated from extracts of *Tripterygium wilfordii* Hook f using pre-fractionation by medium pressure liquid chromatography followed by preparative CCC on a Midi-CCC centrifuge.[22] In a novel way of using a conventional twin column CCC centrifuge, intermittent CCC extraction has been used to enrich triptolide from 2% in a crude extract to 98% by retaining the material in the column while washing away all other components, either as lower or higher partitioning material.[23]

Another novel approach developed by the Brunel team, has been to optimise the resolution of an extract from *Millettia pachycarpa*[24] using model compounds, thereby preserving valuable starting material. In this way, tephrosin, pyranoisoflavone, dehydrodeguelin and deguelin have been purified using a hexane/ethyl acetate/methanol/ water (1/0.8/1/0.8v/v) system.[25]

Two recent examples of scaling up from the analytical scale Mini CCC centrifuge to the preparative scale Midi centrifuge are provided by the isolation of the biflavonoids amentoflavone, robustoflavone, bilobetin, hinkoflavone, isocryptomerin and an apigen-diglucide from *Selanginella tamarisca*[26] using heptane/ethyl acetate/methanol/water (2/3/2/3/v/v) and salvianolic acid B (ACD log P 2.14) from *Salvia mitiorrhiza* Bunge[27] using hexane/ethyl acetate/methanol/acetic acid/water (1/5/1.5/0.006/5 v/v).

Finally, in a very recent purification study, two new minor coumarin compounds, alsaticol and alsaticocoumarin A have been isolated from extracts of *Peucedanum alsaticum* L fruits using a heptane/ethyl acetate/methanol/water (1/1/1/1/v/v) phase system.[28]

3 CONCLUSION

On its invention back in the mid 1960's, CCC was a technique that showed great promise. Although it has been slow to deliver that promise, both engineering improvements and developments in the understanding of the process over the last ten years have developed the technique into a powerful purification application. The complexity of natural product purifications is such that a range of suitable purification techniques is required and CCC is now a technique that is wonderfully suited to large-scale preparative separations, with advantages over solid-phase techniques such as the ability to accept particulates, no columns to replace, and high speed and high resolution relative to current liquid-liquid techniques.

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