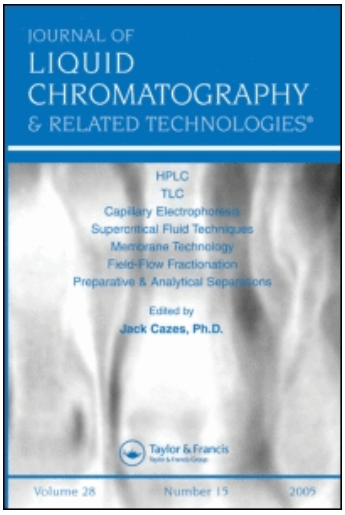

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Countercurrent Chromatography (CCC) and its Versatile Application as an Industrial Purification & Production Process

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COUNTERCURRENT CHROMATOGRAPHY (CCC) AND ITS VERSATILE APPLICATION AS AN INDUSTRIAL PURIFICATION & PRODUCTION PROCESS

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ABSTRACT

This paper describes the versatile operation of CCC, its potential for scale up and compares its operational performance with HPLC as a generic preparative purification process. In the words of the UK Biology & Biotechnology Science Research Council (BBSRC), there is a need for a "generation of new, robust, and usable techniques for bioprocess intensification and simplification" and, in particular, technology that can be scaled from laboratory to process scale easily and cheaply without any fundamental change to the principle of separation. CCC offers the potential to do this.

INTRODUCTION

Liquid/Solid Chromatography

There are limitations to currently available liquid/solid phase chromatography when scaling up from analytical to preparative or process scale. The majority of analytical separations use reverse phase, but this process can become too expensive for preparative LC, resulting in the need for new methodology using normal silica when changing from laboratory (1-5mg) to preparative (5mg-kg+) scale. The chromatography options, therefore, become more limited at scale-up unless the product retail price is extremely high and reverse phase and chiral supports can be considered. Target compounds may be adsorbed, denatured, hydrolysed or change configuration on solid supports. Such media have a limited life and are more prone to contamination and potential blockage from particulates. Furthermore, the capital cost of auxiliary equipment, consumables, and column replacement/refurbishment becomes orders of magnitude more expensive, as columns become larger, to cope with target compound capacities required in laboratory scale preparation (5-100mg), pilot preparation (100mg - 10g) and process scale (10g - tonnes). For example. solvent pump systems alone may increase from £1000s to £100,000s plus.

Countercurrent Chromatography (CCC)

CCC[1-3] is a process that avoids these difficulties. It is a form of liquid-liquid chromatography, without a solid support, which separates soluble substances such as synthetic and natural biochemical products on their partition, or differential solubility, in two immiscible solvents. The principle of

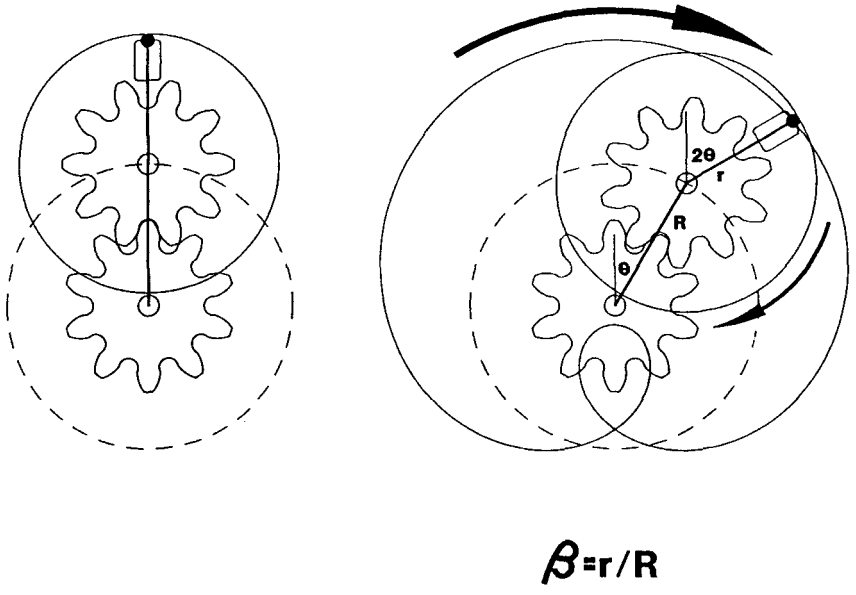

Figure 1. Locus of coil planet centrifuge motion with the central gear fixed. A bobbin/coil system would be mounted on the planetary gear.

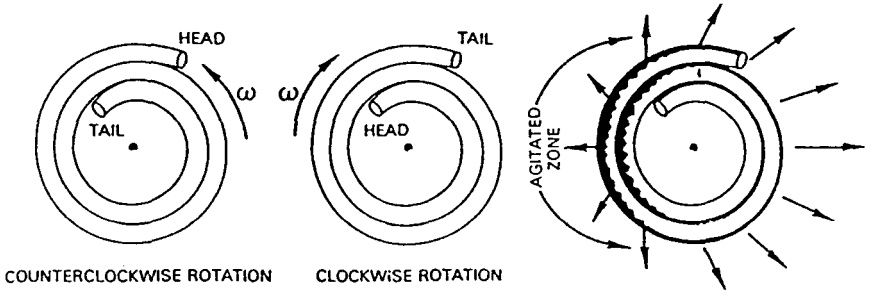

Figure 2. The notation and relationship between the variable force field and the mixing and settling zones (from (6) ibid).

Figure 3. The Quattro CCC instrument with the rotor compartment and control panel doors open showing the twin rotor and controls.

separation (partition) is the same in both the laboratory and the process plant. It is also universal in that it can be applied to an extremely broad range of purification problems, both organic and inorganic, in many industries. Furthermore, because there is no solid support, there is 100% sample recovery and no need for any pre-purification.

The operational process is extremely simple. The system consists of a sample, a length of tubing and two immiscible solvent phases. The tubing is initially filled with the solvent phase intended to be the stationary phase and the sample is injected with the mobile phase. After an appropriate period of time, fractions of the injected sample emerge from the downstream end of the tubing in the order of their partition coefficient.

The tubing (usually PTFE) is wound on a drum which is centrifugally rotated in planetary motion (Figure 1). This sets up alternating zones of mixing and settling[4] which travel along the length of the tube synchronous with the high and low "g" sides of the coil (Figure 2).

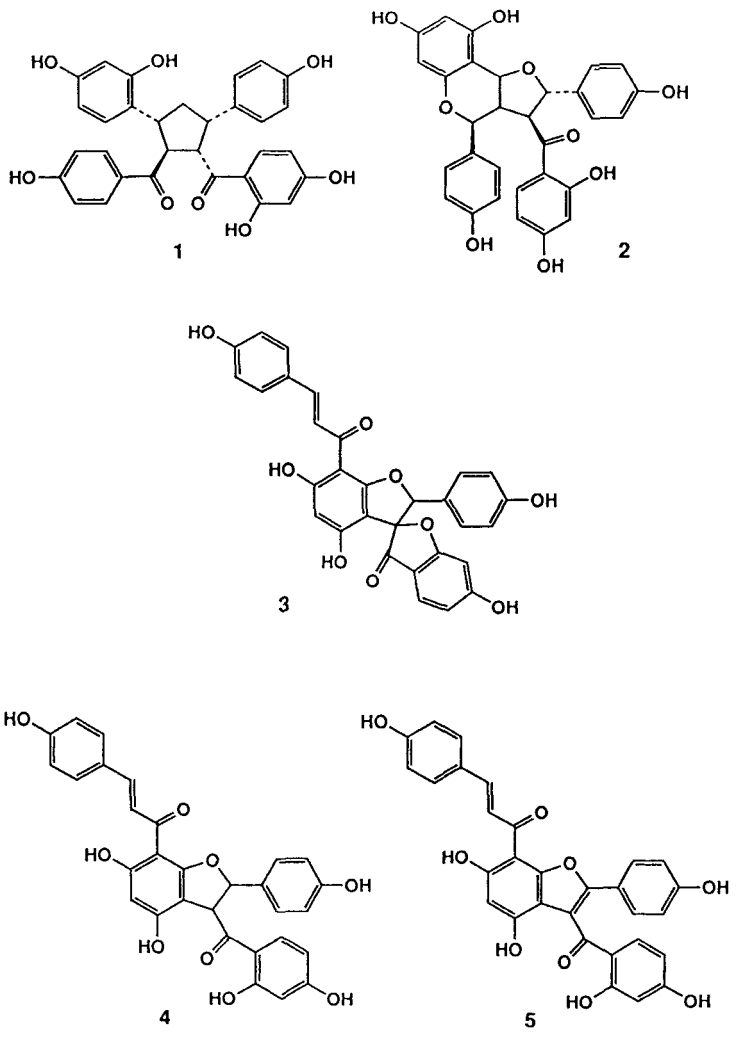

Figure 4. The purification of polyphenols from the stem bark of *Brackenridgea zanguebarica* (Ochnaceae) for A) a sample loading of 50mg on a 64 mL coil and B) a sample loading of 200 mg on a 630 mL coil.

Samples injected with the mobile phase undergo as many as 50,000 partitioning steps per hour with the retained stationary phase, resulting in high resolution separations with no sample adsorption onto solid supports. The mixing efficiency is excellent and the process is not limited by hydrostatic pressure.

A breakthrough was made in CCC technology with the development of the Type J coil planet centrifuge[5] which formed the basis of the first commercial CCC device.[6] The market is now demanding a new generation of reliable, safe, well engineered, temperature controlled CCC Instruments. This paper describes the operation and application of such an instrument (Figure 3), capable of convincing industry that CCC is the new emerging technology for bioprocessing and generic sample purification in the future.

CCC Instrumentation

The CCC instrumentation used in all of these applications was the Quattro CCC (supplied by AECS, PO Box 80, Bridgend,CK31 4XZ, UK) with twin bobbins, each with twin coils of 95 mL and 260 mL volume. The tubing was 3.2 mm o/d x 1.6 mm i/d PTFE. It is designed around the twin coil planet centrifuge first proposed by Ito[7] but has a novel flying lead arrangement that reduces wear and has no central shaft, allowing higher β values.

Comparing CCC with HPLC

There are three key problems when scaling up from analytical (<0.1 to 5mg) to prep-scale (5mg-1kg+) HPLC:

1. Most analytical separations use microparticulate bonded phase HPLC packings (both reverse and normal phase) while many laboratory and pilot preparations still use cheaper normal silicas in order to keep costs down. This change of packing material during scale-up, therefore, requires further chromatographic method development work to be carried out.
2. As the required target compound capacity increases, the quantity of solid phase needed and the solvent flow rates increase considerably as shown in Table 1. This range of flows not only requires several ranges of progressively more expensive pumps and other hardware, but also leads to major solvent acquisition and disposal problems.
3. Target components may irreversibly adsorb, hydrolyse, change steric/chiral confirmation or denature on solid stationary phases.

The following applications demonstrate **how** ***one CCC laboratory instrument*** can 1) operate over the traditional analytical, laboratory preparative and pilot preparative ranges of HPLC; 2) be operated in both normal and reverse phase mode in one fractionation; 3) be used as a rapid method development, and 4) as an extraction process.

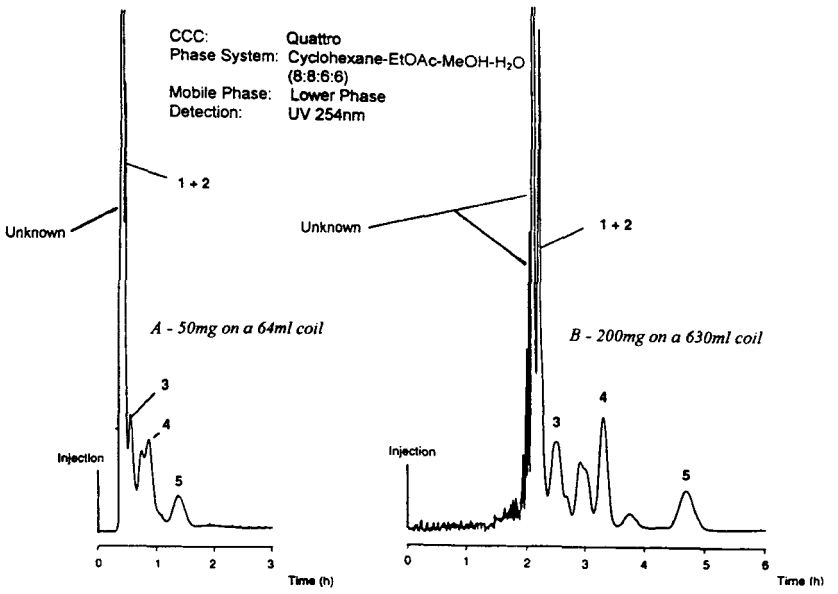

Figure 5. The structures of CCC chromatographed polyphenols identified in Figure 4.

Table 1

Typical Solvent Flows and Yields for HPLC Columns Compared to Laboratory Scale CCC

	Column o/d (mm)	Flow (mL/min)	Yield (g)	Hardware Cost (£K)
Analytical HPLC	1-5	1-10	<0.005	5-20
Lab Prep HPLC	5-20	4-50	0.005-0.1	20-100
Pilot Prep HPLC	20-150	50-800	0.1-10	100-1,000
Lab CCC	3	1-10	0-10	25
Proc Prep HPLC	150-1,000+	800-7,000+	10-100,000	1,000+

Purification of Polyphenols from the Stem Bark of *Brackenridgea zanguebarica*-Ochnaceae[9]

This application demonstrates scale-up from 50 mg to 5 g by firstly increasing coil length to improve resolution and peak separation and, secondly, increasing sample loading to the point where the resolved peaks start to run into one another again.

Figure 4(a) & 4(b) show the resolution of polyphenols from the stem bark of *Brackenridgea zanguebarica* (Ochnaceae) for various sample loadings. These polyphenols have fungicidal activity. Temperature was maintained at 30°C throughout. Figure 4(a) shows the fractionation obtained with a sample loading of 50 mg on the short 64 mL coil. Six major peaks are eluted in less than 1.5 hours, containing five enumerated target polyphenols (Figure 5). There is a bifurcation between the first eluted peak (co-extractives) and the next peak (target components 1 & 2) and a similar bifurcation containing target component (4).

The same fractionation was then repeated (Figure 4(b)) using the full coil capacity (630 mL) with an increased sample loading of 200 mg. The six peaks evident in Figure 3(a) are now resolved into seven eluted within 5 hours. The bifurcated peak, containing target component (4), is completely resolved. The fractions containing target components 1 & 2 are virtually resolved from the first peak (co-extractives) and a barely perceptible tailing shoulder on target component (4) is resolved into a new peak between target components (4) and (5).

It was possible to increase sample loading by 25x on the 630 mL coil without significant loss of resolution. The 5 g fractionation is not shown, as the absorbance at this concentration saturated the fixed wavelength detector used. Fractions were collected and analysed separately by TLC confirming the required resolution.

Purification of Flavonoid Glycosides from *Tephrosia vogelii* (Leguminosae) Leaves[10]

This fractionation demonstrates the versatile option of using either the upper phase (normal) or the lower phase (reverse) as the mobile phase and that the elution order reverses when changing from one to the other. It also demonstrates that the mobile phase can be changed at any time to elute the retained peaks in reverse order.

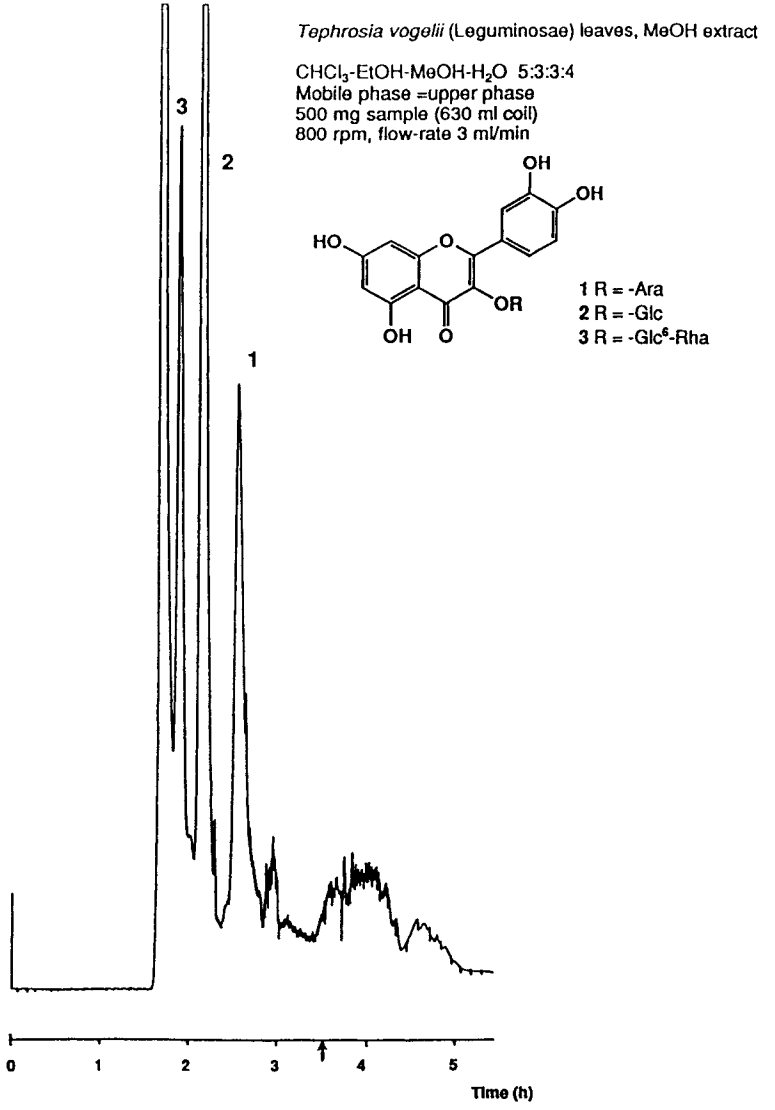

Figure 6. The fractionation of flavonoid glycosides from a 500 mg sample of *Tephrosia vogelii* (Leguminosae) leaves, MeOH extract on the 630 mL coil with the upper phase mobile.

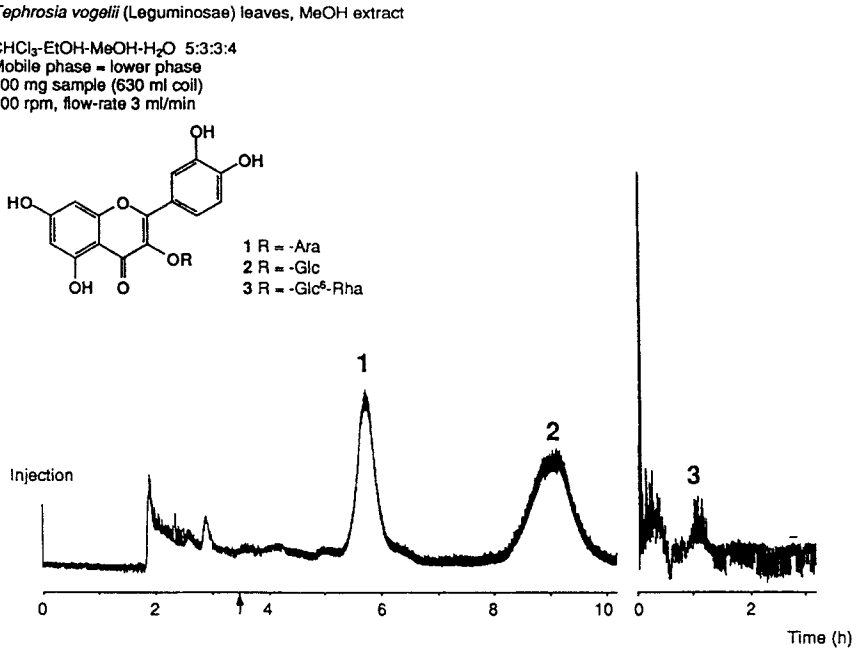

Figure 7. The fractionation of flavonoid glycosides from a 500 mg sample of *Tephrosia vogelii* (Leguminosae) leaves, MeOH extract on the 630 mL coil with the lower phase mobile.

Flavonoid glycosides may have several interesting biological properties, including antioxidant activity, and use in receptor inhibition assays. Figures 6 and 7 shows the fractionation of flavonoid glycosides from 500 mg sample of *Tephrosia vogelii* (Leguminosae) leaves, MeOH extract on the 630 mL coil with (Fig. 6) the upper phase mobile and (Fig. 7) the lower phase mobile. Again, temperature was maintained at 30°C throughout. With the upper phase mobile, the target components are eluted early and close together within 3.5 hours. Note that the k=1 point (arrowed) is at 3.5 hours so that all the eluted peaks have a partition coefficient greater than unity, favouring the mobile phase. The partition coefficient (k) is defined here as the concentration in the upper phase over the concentration in the lower phase. When k=1 the sample is equally partitioning in each phase as if it is a single phase. By repeating the same separation with the upper phase stationary and the lower phase mobile, the partition coefficients are all less than unity and will be retained longer, giving better resolution.

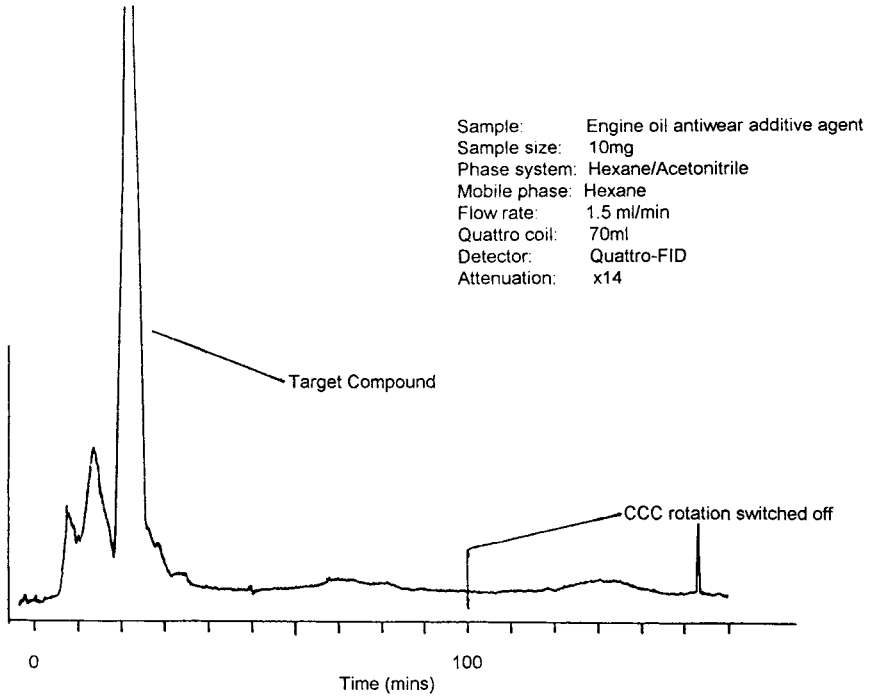

Figure 8. The fractionation of an anti-wear agent using an acetonitrile/hexane phase system with a 10 mg sample and the acetonitrile as the stationary phase.

Note, also, that the target components identified in Figure 6 now elute in reverse order. Components (1) and (2), eluting after 5.5 and 9 hours respectively, are so well separated that the sample loading capacity could be increased by a further 5x without losing resolution.

Another operating feature of CCC is demonstrated in Figure 7. After 10 hours with some components still retained in the stationary phase, the pumped phase is "reversed" again with the upper phase now becoming the mobile phase once more and the remaining components are eluted in reverse order.

Thus, the component eluting first is the one that was most retained in the upper phase (the first peak to elute in Figure 6) and the second is the target component (3). An alternative approach would have been to stop the coil rotation and pump out the retained components in the order in which they had been retained.

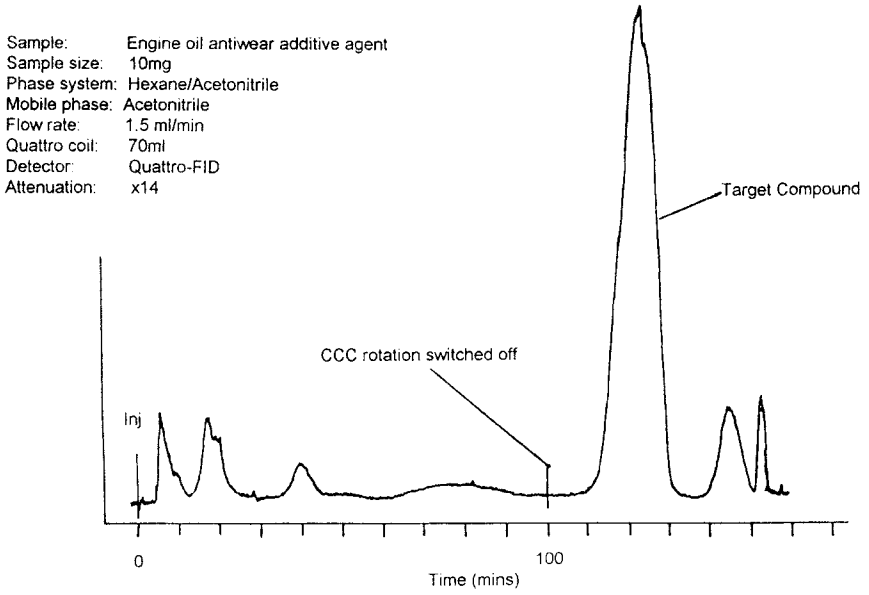

Figure 9. The fractionation of an anti wear agent using an acetonitrile/hexane phase system with a 10 mg sample and the hexane phase as the stationary phase.

Fractionation of Engine Oil Anti-wear Agent[11]

This application is an example of how a CCC method can be quickly developed. It also demonstrates how pumping out the coils' contents while they are stationary preserves the elution order and resolution.

The use of a non-aqueous hexane/acetonitrile phase system gives a convenient polarity screen. With the hexane phase retained as the stationary phase, the polar compounds elute early while non-polar compounds are retained. Alternative phase systems can use heptane as a hexane substitute or ethyl acetate as an acetonitrile substitute.

The procedure is as follows: 1) choose the phase in which the target component(s) are most soluble in the stationary phase. Based on the retention characteristics, either normal or reverse phase can be chosen, and the percentage retention of stationary phase optimised to allow elution of target components approximately at the switch off point; 2) fill the coil with this chosen stationary phase while the coil is stationary; 3) rotate the coil at 800

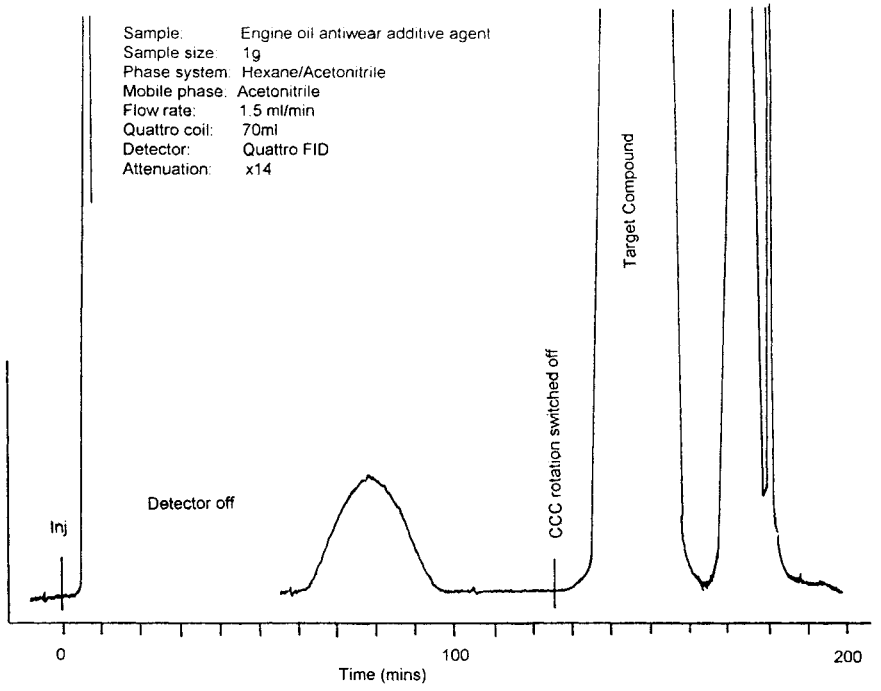

Figure 10.The fractionation of an anti wear agent using an acetonitrile/hexane phase system with increasing the sample loading by a factor 100x to 1,000 mg with the hexane phase still as the stationary phase.

rpm and flow the mobile phase, noting the volume of the coil used and the volume of the stationary phase eluted prior to mobile phase breakthrough (the percentage stationary phase retention is typically 65 to 80+% given appropriate tail/head orientation. For example, with a 70 mL coil, 60 mL of stationary and 10 mL mobile phase would be typical.); 4) work out the volume of mobile phase (V_m) in the coil (Volume of mobile phase = Coil volume less eluted volume of stationary phase less volume of inlet/outlet leads); 5) split the eluent stream between the fraction collector and the FID transport detector; 6) flow the mobile phase until 4 to 10+ times V_m (depending on difficulty of resolution) has eluted; 7) switch off coil rotation; 8) continue flowing the mobile phase to displace the remaining stationary and mobile phase from the coil. This will maintain the spatial separation of components already chromatographed, as the Archimedian forces retaining the stationary phase no longer apply when the coil is stationary.

The components which had been previously retained are all now eluted with 'infinitely' retained components eluting in the final drops of the eluting contents of the coil.

Figures 8-13 show normal and reverse phase chromatography of a commercial engine oil anti-wear agent. By choosing a low partition coefficient (approximately k=0) target components can be extracted or focused onto the front upstream end of the coils when using large injection volumes while higher partition coefficient impurities are eluted. The target component is then eluted after switch off has occurred. Using this technique, scale up from 10 mg to 1000 mg, using only the smallest CCC column of 70 mL total volume, has been achieved. This procedure can also be the foundation of an 'on-line' extraction/purification process.

Figure 8 shows a 10 mg fractionation with hexane as the mobile phase. The target compound elutes as the third peak. Figure 9 illustrates the same fractionation with the acetonitrile phase mobile. The peaks elute in reverse order and the target peak is retained longer, giving better resolution, and is arranged to elute just after the switch off point. Figure 10 shows the result of increasing the sample loading by a factor 100x to 1000 mg. Note the target component is still resolved from those components near it. Figures 11 and 12 give the HPLC-UV traces of trade supplied anti wear agent and of the same target component purified by CCC (Figure 10). It should be noted that the baseline perturbation in the region where the target component elutes is due to the mobile phase gradient employed for the separation. Figure 13 confirms the purity of the target compound after fractionation in the CCC by mass spectrometry.

Should a simple binary system not give adequate resolution, tertiary/quaternary modifiers such as ethyl acetate, MTBE, chloroform, dichloromethane, THF, dioxane, volatile organic acid, or volatile organic bases may be added as required. To test miscibility take 1 mL each of hexane and acetonitile in a 5 mL test tube. Add a tertiary phase progressively until the phase system becomes miscible. Repeat, but this time reduce the percentage addition until a two phase system is achieved which will settle in less than 30s after shaking.

TLC may be used as a valuable pre-screening method for CCC. Take appropriate volumes of each equilibrated phase and independently chromatograph the sample using silica TLC plates. The inspection of the two plates will give a good indication of the number of components and the range of the partition coefficients. If available, repeat the procedure using known standards. Change solvent choice and relative percentages to optimise

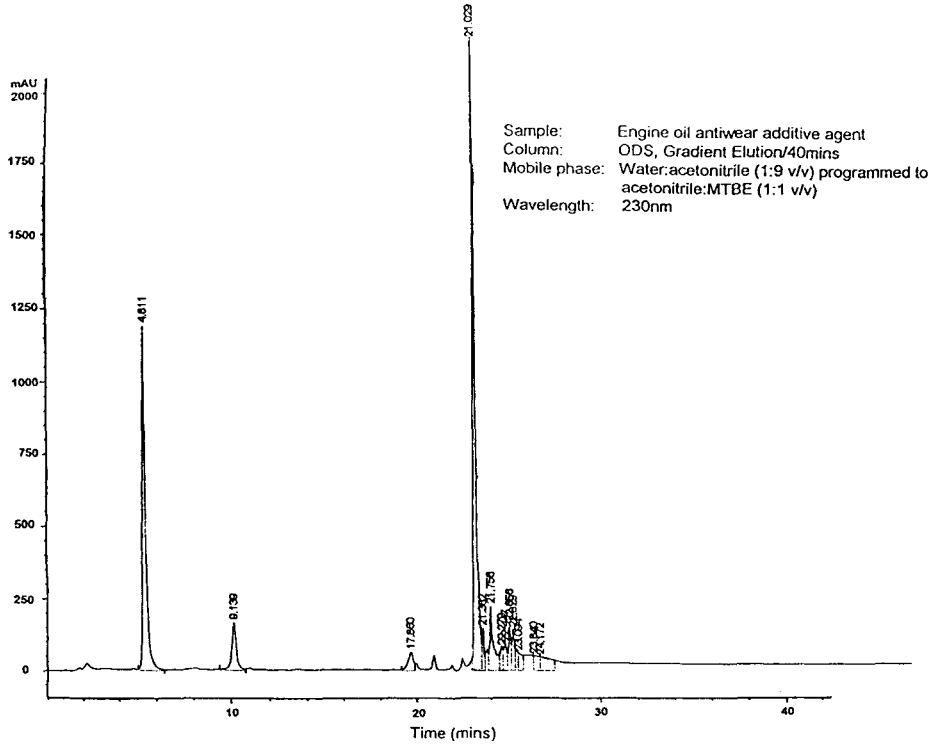

Figure 11. The fractionation of an anti wear agent using an acetonitrile/hexane phase system with the HPLC-UV trace of trade supplied anti wear agent.

separation of target components. In many cases, optimisation of phase solvents and composition by TLC may be adequate to transfer the chosen solvent system in either normal or reverse phase directly to CCC. If not, further optimisation by CCC will be required.

CONCLUSIONS

Purity & Stability

A broad spectrum of separations has been performed. There was no loss or degradation of sample by irreversible adsorption or hydrolysis. There were

also no cross contamination problems from one run to another as the coils were emptied, solvents re-distilled or thrown away, and the coils refilled for each new separation.

Applications

The versatility of CCC in rapid method development, polarity screening and as an extraction process has been demonstrated. It also has the potential for many more applications. The fact that CCC can cope with crude extracts containing particulates means that it has the potential for the separation of cells, organelles and miscelles. Modifier enzymes or catalysts can be immobilised in the stationary phase and the coils used as "on column" or coil reactors.[13]

Similarly, chiral and affinity ligands can be used for highly selective chromatography. Dissolution of a modifier in one or other of a user-chosen pair of suitable immiscible phases is a much easier and less costly alternative to the involved chemical manipulations required to bond modifiers onto solid chromatography media. It also has the potential for cost effective chiral scale-up.

For example CCC only requires dissolution of a chiral selector in one of a pair of user chosen immiscible phases rather than expensive chemical manipulation to irreversibly bind the chiral entity to a solid phase support.[14] Finally, it can be used for inorganic separations given appropriate choice of chelating agents. Thus it can be seen that CCC is a very versatile technique capable of both organic and inorganic separations.[15]

Operations

The versatility of CCC in moving from one application to another in a completely different field has been demonstrated. There are a near infinite number of options of immiscible phase systems that can be used for the mobile and stationary phases of CCC. The option of using either reverse or normal phase has been demonstrated and it is an advantage that switching from one to the other can take place at any time.

This wide range of phase systems is facilitated by the use of the FID transport detector coupled to the CCC; the FID is a universal detector and is essentially unaffected by the solvents used for CCC.

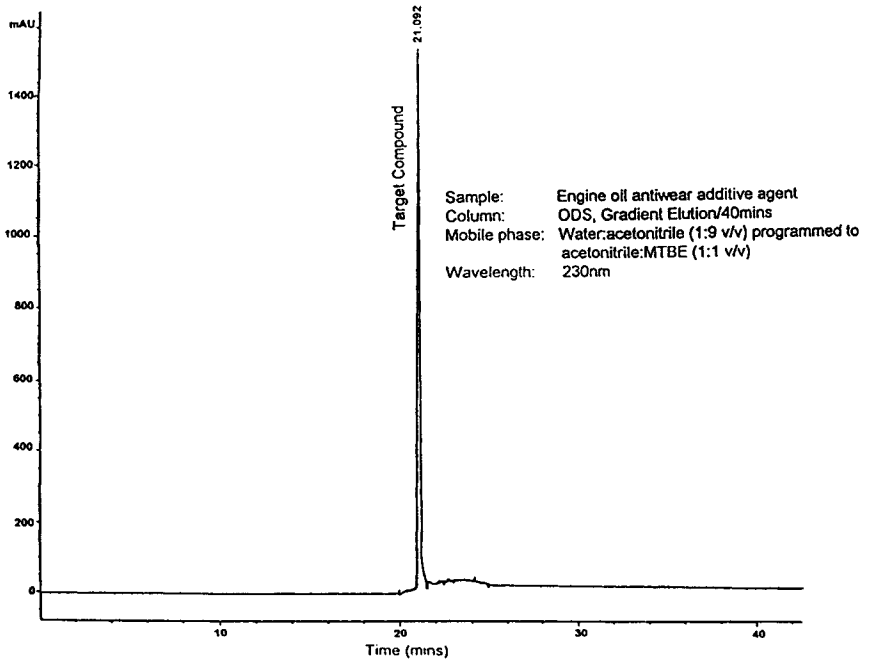

Figure 12. The fractionation of an anti wear agent using an acetonitrile/hexane phase system with the same target component after 1 g purification by CCC.

While the versatility of CCC in scale up was demonstrated (moving from the smallest to the largest coil configurations), it should be noted that, with CCC, it is possible to have special bobbins manufactured with four identical 175 mL coils. In this way, four identical chromatographic separations may be accomplished at a time, using only one CCC instrument, thereby providing an extremely cost-effective method for screening large numbers of samples for bioactivity.[16]

Further Scale-up Potential

Existing laboratory scale CCC is capable of 1 mg to 10 g separations in one instrument with flow rates of 1 to 10 mL/min of the mobile phase. This would require three different instruments in the case of HPLC - analytical (1-5 mg); lab prep (5-100 mg) and pilot prep (0.1-10 g).

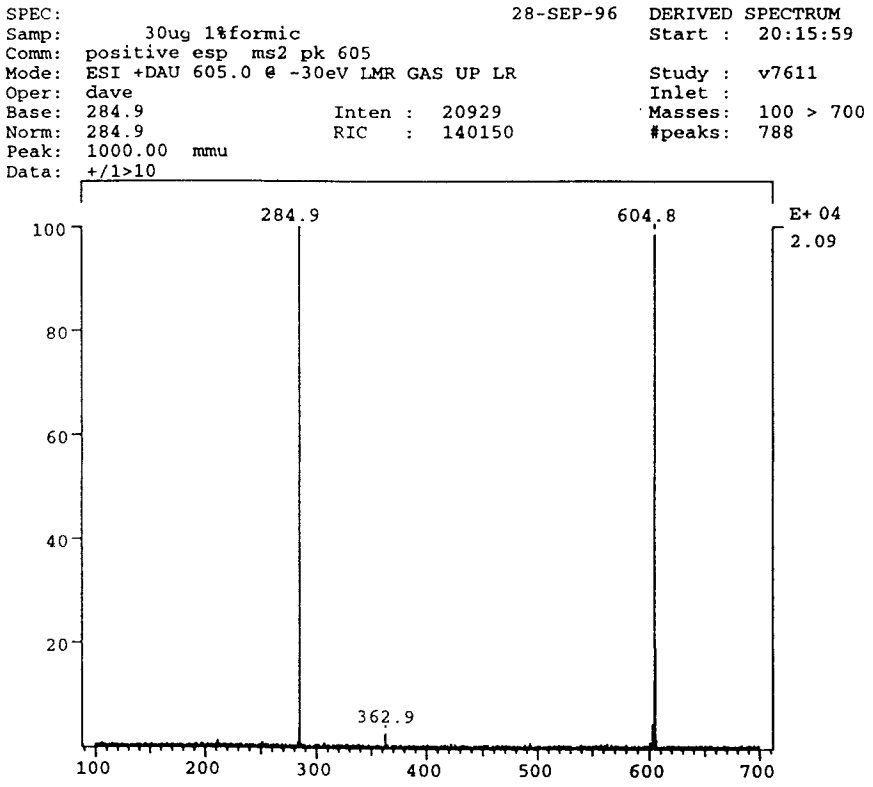

Figure 13. The fractionation of an anti wear agent using an acetonitrile/hexane phase system with the purity of the target compound after fractionation in the CCC demonstrated by mass spectrometry.

Preparative-HPLC uses 5x to 80x more solvents than CCC for the equivalent separation. 1-10 mL/min for a 1200 mL (10g) capacity CCC compared to 50-800 mL/min for 0.1-10 g capacity HPLC with separations in an equivalent time-scale. Potential kg to tonne CCC might only use flows of 10-100 mL/min as opposed to 1000-7000 mL/min - with a massive difference in solvent cost and cost of auxiliary hardware such as pumps.

If the current machine, without optimisation, can manage at least 10 g per run and, with optimisation, 50 g per run, then 50 kg per run would become possible with a 1000x scale-up (i.e., building a machine at least 10x the size). With between 2-4 runs a day and 250 operating days a year, then a single machine could process 25-50 tonnes a year in standard chromatography mode

and 250-500 tonnes per year in extraction mode. Furthermore, as the machines get larger, the rotational speed for a given "g" would become slower giving a longer mixing/settling time and the choice of a broader range of solvent systems or an increase in flow to reduce throughput time and increase turnover still further. However, there will be a trade-off against a reduced number of mixing/settling cycles per hour resulting in slightly reduced resolution.

More research will be needed on the effects of scale up on retention and resolution but, if such instruments become commercially available in the future, then industry would, for the first time, have a cost effective preparative technology that could be scaled up linearly from mg to tonne quantities.

ADDENDUM

The companies participating in the applications reported in this paper do not imply their commercial endorsement of the instrument used.

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