focus on
Chromatography

Chromatography…but not as we know it.

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The use of countercurrent chromatography (CCC) as a preparative technique is seen to occupy a niche area of separation science and is largely used to isolate natural products. However, the technique has considerable untapped potential both at the laboratory preparative scale and also at larger scale.

Introduction

This article gives a chromatographer's perspective of the potential of countercurrent chromatography (CCC) in the pharmaceutical industry, specifically relating to high-performance countercurrent (HPCCC) instruments. The work described has been performed by a consortium consisting of GlaxoSmithKline, Pfizer, Dynamic Extractions and Brunel University and is part of a three-year project sponsored by the UK Government's Technology Strategy Board as part of its high value manufacturing programme.

Although CCC has always had a relatively low profile in separation science we believe that it has considerable unrealised potential to improve both laboratory and manufacturing efficiency. In the laboratory for example the opportunity is to enhance overall separation capability. This will require the integration of CCC technology to a similar extent to that achieved in HPLC giving the same degree of instrumental control and automated method development. This will allow the instrumentation to be integrated into a broader strategy for preparative separations. As a manufacturing tool, CCC promises lower costs compared to other large scale chromatographic separation technology with potential applications such as reclamation of waste streams for high value products.

CCC is applicable to preparative separations covering a range of scales from a few milligrams through to kilograms and can be operated in both batch and semi-continuous modes. Relatively large-scale chromatographic separations can be achieved using CCC and in batch mode for example throughputs of the order of 10kg/day have been projected [1]. Potential loading in a semi-continuous mode has not been established but initial research indicates that it will be about 5 to 6 times higher than for batch mode.

An overview of countercurrent chromatography

CCC was first introduced by Yoichiro Ito in 1966 [2] – the basic principle involves subjecting two immiscible liquids to an external acceleration field generated by centripetal motion. Therefore unlike solid phase chromatography both stationary and mobile phase are liquids. The technique has been variously described as a multi-stage liquid-liquid extraction and a continuous countercurrent chromatography process. The column in CCC is open tubing, which is initially filled with the liquid phase that becomes the stationary phase and the sample is injected with the mobile phase. Separation is based on the distribution of the sample between two immiscible liquid phases and is characterised by the distribution ratio (Kd) defined as the concentration in the stationary phase divided by the concentration in the mobile phase. This is also known as liquid-liquid partition chromatography.

The liquid nature of the stationary phase leads to many unique features – high injected sample loading, high yields of purified compounds, high reliability of retention and a number of different processing methods that can be used for this liquid–liquid extraction/chromatography process. Method transfer from one instrument to another, or one scale to another, is simple and predictable. From a quality angle, the reliability of retention that is available with CCC is seen to offer particular advantage, especially at manufacturing scale.

Stationary phase is retained in the column as a result of complex centripetal acceleration. Either liquid phase can be the mobile phase as illustrated in *Figure 1*. The advantage of having two liquid phases is that other operating modes rather than just standard elution are available. These can either save time or solvent usage and can also ultimately allow semi-continuous liquid chromatography to be performed.

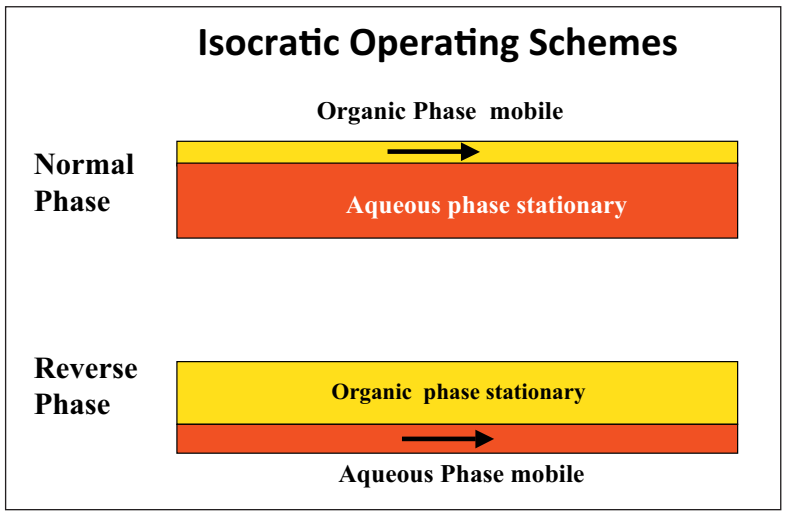

Figure 1. Isocratic operating schemes for CCC.

The latest high-performance countercurrent chromatography (HPCCC) instruments run at a higher rotational speed (typically 'g' fields up to 240g) compared to conventional high speed countercurrent chromatography (HSCCC) units (~70g) significantly reducing separation time by a factor of 10 to between 20 to 40 minutes, while maintaining resolution.

HPCCC instruments have two bobbins that enable additional processing versatility where semi-continuous processing can be achieved by continuously injecting sample in between the two bobbins and intermittently switching the direction of flow between the aqueous mobile phase from one end of the column to the organic mobile phase from the other end of the column. In this processing configuration, the more hydrophilic compounds elute with the aqueous phase and the more hydrophobic compounds elute with the organic phase while a chosen target compound can be concentrated inside the column and harvested at regular intervals.

Why has CCC had such a low profile?

CCC has had a relatively low profile in the separation science community with applications being largely confined to the preparative isolation of natural products [3] where its appeal is two fold; 1) the absence of a solid stationary phase to cause problems with irreversible adsorption and decomposition of sensitive compounds and 2) the provision of an alternative separation tool for difficult separation problems such as those arising from closely related structural isomers.

It is only with the advent of HPCCC instruments relatively recently that commercial instrumentation capable of producing separations on a similar time scale to HPLC has been available. The introduction of high g-level instruments also enabled small bore columns requiring only milligrams of sample to be used for scouting purposes. Finally, previous generations of CCC instruments have traditionally not been marketed as integrated separation units, in contrast to the situation with HPLC, where over the last 20 years instrument integration under software control has revolutionised practice [4,5]. This gap is now being addressed by integration of HPCCC instrumentation with conventional computer controlled HPLC systems, which can then be programmed to perform the analysis automatically, including the automated proportioning and delivery of the mobile and stationary phases [6]. A photograph of an integrated instrument is presented in *Figure 2*.

Where does CCC fit?

There are two clear areas where CCC can add value. The first is as a complementary preparative technique to HPLC and other techniques at the lab scale where CCC acts to enhance overall preparative capability. The objective here is to generate a first pass approach for preparative chromatography that will provide a separation solution in the fastest timescale possible with near 100% success rate. The aim is to maximise laboratory operating efficiency by removing the requirement to 'hand craft' that small proportion of separations that will not yield to existing approaches.

The second is to provide a large scale, cost competitive, preparative capability (of the order of 10kg/day) which can be used in a range of applications from reclamation of high value materials from recrystallisation liquors for example to applications involving continuous processing activities. Here the cost advantages of not having an expensive stationary phase are very attractive. Add to this the ability to process materials containing particulates, the retention time reliability that arises because of the predictability of liquid phase partition and the possibilities to run semi-continuously and we have a very exciting capability.

A photograph of a prototype large scale HPCCC instrument is presented in *Figure 5* to allow the reader to get an appreciation of the size of the equipment, which is capable of producing material at a rate of approximately 10kg per day.

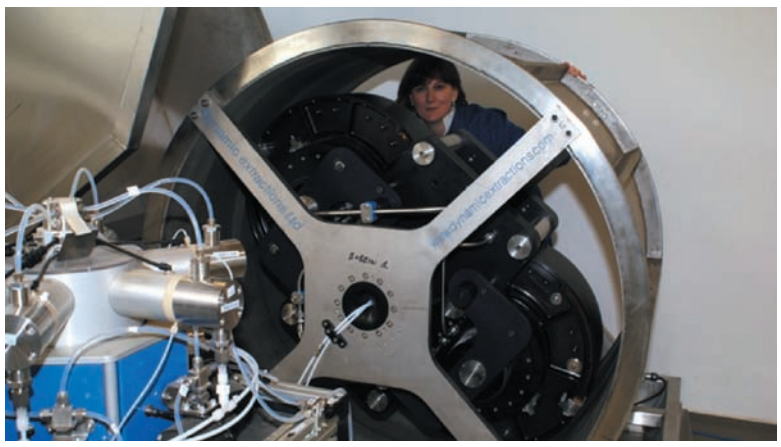

Figure 5. Dynamic Extractions Maxi 18L prototype HPCCC instrument at Brunel University's Advanced Bioprocessing Centre.

Alternate operating strategies

An interesting enhancement to CCC already mentioned in the introductory overview is the use of intermittent counter-current extraction (ICcE) mode, which takes advantage of having two liquid phases – either of which can be used as the mobile phase [11]. With ICcE the two phases (mobile/stationary) are continuously alternated with the sample being continuously injected into the middle of the column or between columns if a standard two bobbin CCC instrument is used – this allows either the separation of binary mixtures or the concentration of a selected compound from a complex mixture while impurities are washed away. *Figure 6* illustrates this process diagrammatically. Sample is continuously loaded between the two columns and flow switched regularly between reverse and normal phase modes. Under optimised conditions the target peak is held inside the instrument and gradually increases in concentration while the impurities are washed away in either the upper or lower phase. This process is illustrated by the column fraction photos, which have been obtained for the preparative isolation of the target in the complex mixture application discussed earlier. These photos nicely illustrate the fractionation of this very crude material. This is confirmed by the analytical HPLC *(Figure 7)*. With ICcE column loading and yield is substantially enhanced compared to isocratic elution while solvent consumption is reduced making it the mechanism of choice for large scale and continuous operations.

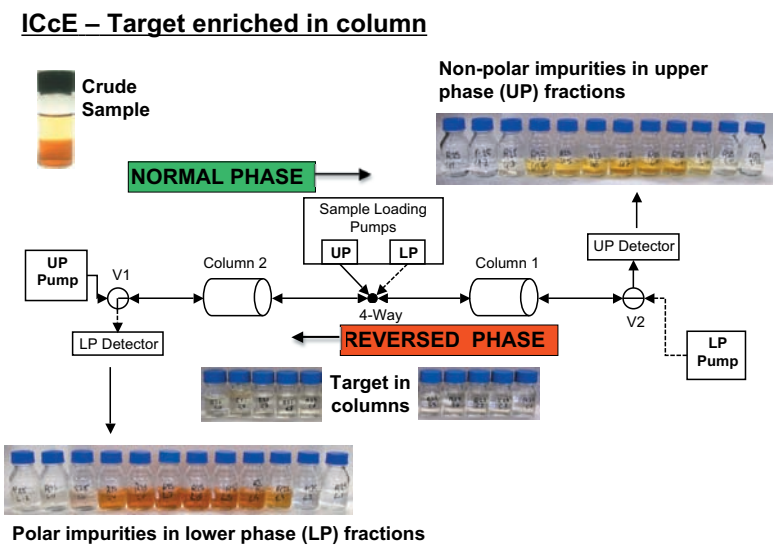

Figure 6. Diagrammatic representation of Intermittent Counter Current Extraction (ICcE) applied to the separation of a target material from a complex mixture. The photos give a pictorial indication of the separation of this crude mixture with the pure (clear) target fractions remaining in the columns while the impurities are washed away – the polar materials in one direction and the non polar materials in the other.

This alternative elution method is expected to offer greatly enhanced loading and substantial reduction in solvent use compared to conventional CCC and is the subject of intensive investigation by our consortium. It is clearly very well suited to larger scale separations and continuous operation.

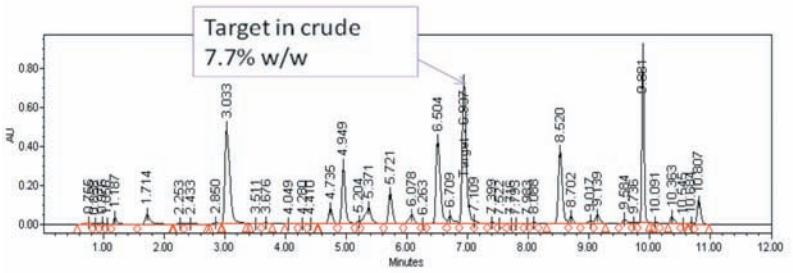

Figure 7a. ICcE separation of a target peak from a crude mixture - HPLC analytical separation of input material.

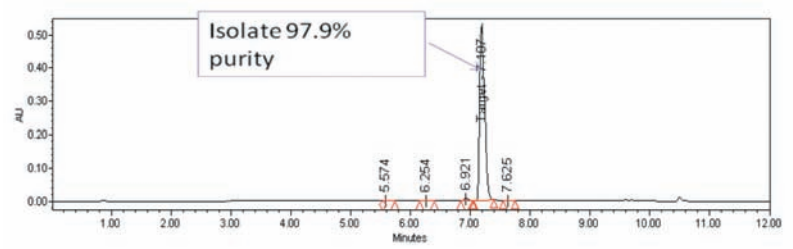

Figure 7b. ICcE separation of a target peak from a crude mixture - HPLC analytical separation of pooled fractions isolated by ICcE.

The future of the project

The project is just starting its second year and, having established a wide-ranging applications portfolio for HPCCC, the next year of the project will focus on further simplifying the method development protocols to make them even quicker and easier for a chromatographer to use; extending the range of solvent systems available for use to enhance solubility/loading and enable the use of greener solvents; and further developing, understanding and demonstrating the ICcE operating method.

All of these objectives will further enhance the capability of HPCCC instrumentation to easily integrate into existing workflows. Updates throughout the year and until the end of the project will be available at **www.dynamicextractions.com/TSB**.

Conclusion

CCC holds considerable promise as a preparative technique to enhance current laboratory capability to rapidly react to separation problems. The challenge here is to integrate the instrumentation and control systems to allow CCC to take its place alongside existing preparative separation capability. This will give a greater overall chance of finding generic solutions to preparative separation problems quickly and efficiently. For larger scale separations CCC again offers the potential to lower overall costs, opening up the possibility of using preparative chromatography in new areas.

References

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Figure 2. An integrated HPCCC system (Shimadzu (UK)/ Dynamic Extractions Spectrum).

The key benefits that HPCCC instruments offer the chromatographer are:
- higher sample loading per injection;
- easy scale-up;
- very high recoveries of injected samples.

This is due to the high stationary phase volumes in the column, a single primary mechanism of separation and the lack of expensive solid stationary phase packing materials, which also clearly add considerable expense as the scale of the separation is increased. In addition, CCC is capable of handling relatively 'dirty' samples containing particulates as shown in *Figure 3* [7,8] which would normally require considerable sample preparation.

Figure 3. Two views of a sample loading tube reported in [8] showing particulate matter, which was subsequently successfully processed.

Separation capability

At the outset of the project the consortium has focussed on generating a set of applications, which illustrate the selectivity and versatility of CCC. The aim here was to allow an understanding to be gained of the capability of CCC and to use the resulting separations portfolio to influence the separations community. A variety of purification challenges have been successfully overcome including the separation of isomers, purification of crude reaction products and recovery of product from mother liquors. Compounds spanned a range of polarity and structural types. The data that has been obtained to date [9] illustrate that the technique has excellent applicability. Thirteen out of the fifteen mixtures studied were separated at loadings suitable for preparative use and of these nine were achieved with a simple heptane, ethyl acetate, methanol, water system, known as the HEMWat solvent system. This work has also shown that CCC has the potential to provide an alternative to solid phase chromatography and produce the quantities required to support the development of drug candidates.

Table 1. Relationship between selectivity (α), plate count, N and resolution, R.

N required to give R_S for given α values		
	R_S = 1.0	R_S = 1.5
α	N	N
1.000	-	-
1.005	650000	1450000
1.010	163000	367000
1.020	42000	94000
1.050	71000	16000
1.100	1900	4400
1.250	400	900
1.500	140	320
2.000	65	145

All CCC, including HPCCC instruments have relatively low efficiencies with plate counts per column amounting to only several hundred compared with the thousands or tens of thousands of plates (N) per column which are typically available when using HPLC. However, as shown in *Table 1*, baseline resolution ($R \geq 1.5$) of two components in CCC is achievable with extremely modest efficiency values if selectivity (α) can be sufficiently enhanced. Although the efficiency of CCC systems is modest, the options for enhancing selectivity are extensive, virtually any combination of solvents can be used as long as it can produce two (or more), readily separable, immiscible phases. This indicates that high-resolution purification is possible, but other factors also need to be taken into consideration. HPCCC instrumentation offers an alternative orthogonal approach to preparative chromatography.

To the chromatographer, used to HPLC, although this appears counter intuitive, by focussing on selecting the optimum partitioning conditions, using automated methods, it is possible to achieve some very challenging separation objectives. This is illustrated by the example presented below.

Separation of a target from a complex mixture – purification of a waste stream

The isolation of a target material from a complex mixture is a very good illustration of the capability of CCC. A crude mother liquor sample from a crystallisation has been reprocessed by CCC to yield a purified fraction *(Figure 4a, b & c)* [10]. This material has subsequently been processed by further crystallisation to give pass quality product.

Instrument	Dynamic Extractions MIDI HPCCC Preparative 945mL column
Solvent system	Heptane : Ethyl acetate : Methanol : Water (12:11:12:11) Stationary phase retention : 77%
Operation mode	Normal phase mode, upper phase as mobile
Method	41mL/min ; 1400rpm ; 30°C; isocratic ; elution-extrusion
Sample loading	8.2g in 41mL UP (200mg/mL)

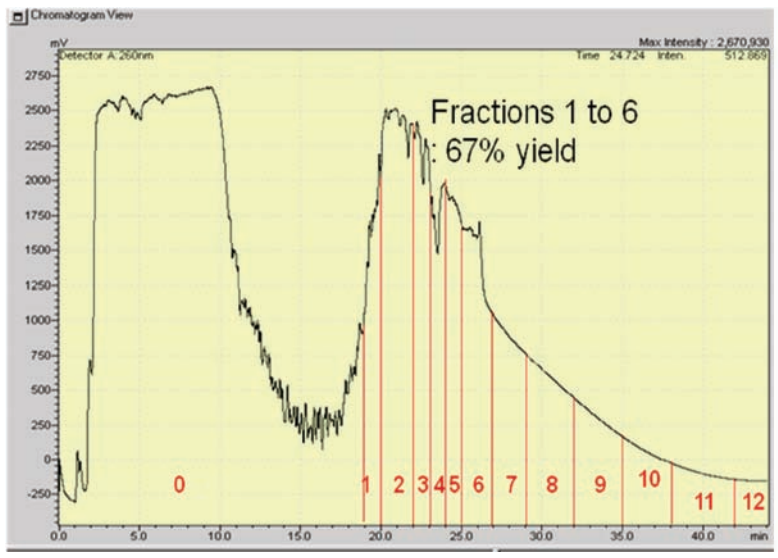

Figure 4a. Isocratic CCC separation of a target peak from a crude mixture.

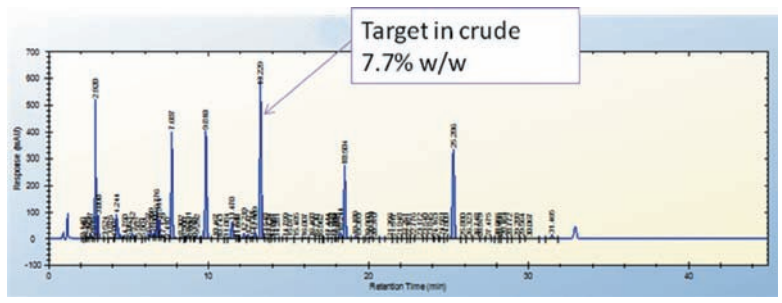

Figure 4b. Separation of a target peak from a crude mixture -HPLC analytical separation of input materials.

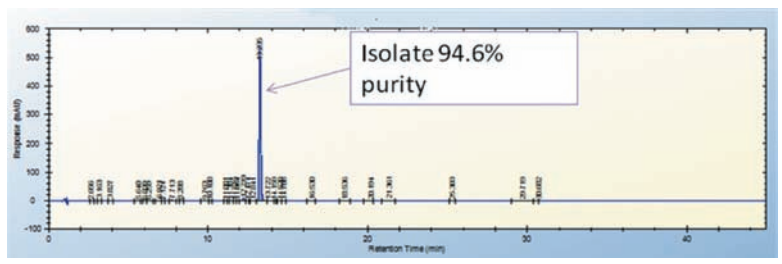

Figure 4c. Separation of a target peak from a crude mixture - HPLC analytical separation of pooled fractions isolated by CCC.