Intermittent Counter-current Extraction

A new continuous dynamic liquid-liquid extraction methodology

A thesis submitted for the degree of Doctor of Philosophy

By

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Abstract

For the pharmaceutical industry, the manufacture of high value pharmaceuticals from natural products, chemical synthetic routes or fermentation processes all require intensive downstream processing steps to produce a pure final product. A small footprint liquid-liquid processing method would help to reduce the capital cost and process development time of this downstream processing.

In this thesis, it is hypothesised that continuous liquid-liquid extraction can be achieved using a standard hydrodynamic counter-current chromatography (CCC) instrument by switching the flow of the liquid phases between normal phase and reversed phase intermittently, so separating a feed stream into two eluant flows.

A model of the process was derived and tested on three scales of instrument, from the semi-preparative to the pilot scale. The method developed, Intermittent Counter-current Extraction (ICcE) was compared to dual-flow counter-current chromatography (DFCCC), the classical method of applying continuous extraction using a counter-current chromatograph. ICcE was found to be advantaged due to the more stable phase volume ratio achievable in the columns and the ability to operate the procedure on standard commercial twin-column CCC instruments which operate at high g-field.

The robustness of the ICcE method was successfully demonstrated across a range of phase system polarities and at high throughput (1kg/day on a preparative instrument) with model mixtures of pharmaceutical compounds.

The effectiveness of this new processing method was confirmed on three industrially relevant case studies. Firstly a polar extract from natural senna pods to extract important sennosides, secondly an intermediate polarity highly complex active pharmaceutical ingredient waste stream to recover the main active component and thirdly a non-polar natural product extract to recover macrocarpal compounds.

In summary, the ICcE method now offers another tool in the range of liquid-liquid separation methods available to the pharmaceutical and other high value industries.

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Acknowledgements

To my grandmother for lettin' me to play down by the river in the summer

Abbreviations

ACN - Acetonitrile

API – Active pharmaceutical ingredient

AU – Absorbance units

BPR – Back pressure regulator

BSA – Bovine serum albumin

CCC – Counter-current chromatography

CCCE – Continuous counter-current extraction

CCD – Counter-current distribution

CDCD – Counter double current distribution

CHM – Chinese herbal medicine

CLR – Closed loop recycling

CLRPIPI – Closed loop recycling periodic intro profile injection

CPC – Centrifugal partition chromatography

DAD – Diode array detector

DFCCC – Dual flow counter-current chromatography

DF – Dual flow

DMCCC – Dual mode counter-current chromatography

DuCCC – Dual counter-current chromatography

g-field – Gravitational field strength

GUESS – Generally Useful Estimate of Solvent Systems for CCC (Friesen and Pauli 2005)

GUESSmix – A range of naturally occurring bioactive small molecules as described in Friesen and Pauli 2005

HPCCC – High performance counter-current chromatography

HPLC – High performance liquid chromatography

HSCCC – High speed counter-current chromatography

HEMWat – Heptane/Ethyl Acetate/Methanol/Water or Hexane/Ethyl Acetate/Methanol/Water phase system

ICcE – Intermittent counter-current extraction

I.D. – Inside diameter

Kel-F - Polychlorotrifluoroethylene

LC – Liquid Chromatography

LLE – Liquid–liquid extraction

LP – Lower phase

MDM – multiple dual mode

MS – Mass spectroscopy

NP – Normal phase

O.D. – outside diameter

PEEK – polyetheretherketone

PFA – perfluoroalkoxy

PTFE – Polytetrafluoroethylene

RP – Reversed phase

RT – Retention time

SB – Switching on the bench

SC – Switching on the column

sCPC – Sequential centrifugal partition chromatography

SMB – Simulated moving bed

SSR – Steady state recycling

TCM – Traditional Chinese medicine

TFA – Trifluoroacetic acid

TMB – True moving bed

TSB – Technology Strategy Board

UP – Upper phase

UV – Ultraviolet

Nomenclature

c_L – Concentration of the solute in the lower phase

c_m – Concentration of the solute in the mobile phase

c_s – Concentration of the solute in the stationary phase

c_U – Concentration of the solute in the upper phase

d – Drop diameter

d_c – Column diameter

D – Molecular diffusivity

F_p – Flow rate of peak progressing through a column

F_L – Eluant flow rate when the lower phase is the mobile phase

F_m – Mobile phase flow rate

F_{SL} – Sample solution flow rate when the lower phase is the mobile phase

F_{SU} – Sample solution flow rate when the upper phase is the mobile phase

F_U – Eluant flow rate when the upper phase is the mobile phase

H – Henry constant

k’ – Retention factor

k_c – Mass transfer coefficient in the continuous phase

K_d – Partition coefficient

L – Characteristic length over which mass transfer occurs

m_m – Mass of a compound in the mobile phase

m_s – Mass of a compound in the stationary phase

μ - Viscosity

R – Ratio of the superficial velocities of the dispersed to continuous phases

R_e – Reynolds number

ρ - Density

S_f – Stationary phase retention in a column

Sh – Sherwood number

τ – Ratio of upper phase step time to lower phase step time

t – Time

t_L – Time for which the lower phase flows as the mobile phase

t_m – Time for which the mobile phase flows

t_p – Time for which a peak progresses in the column

t_U – Time for which the upper phase flows as the mobile phase

U_c – Superficial velocity of the continuous phase

$U_{c,f}$ – Flooding velocity of the continuous phase in a liquid-liquid extraction column

U_d – Superficial velocity of the dispersed phase

$U_{d,f}$ – Flooding velocity of the dispersed phase in a liquid-liquid extraction column

U_o – Characteristic velocity

U_s – Slip velocity

V_c – Column volume

V_d – Dead volume

V_m – Volume of the mobile phase in the column

V_p – Volume of the column progressed by a solute peak

V_s – Volume of the stationary phase in the column

x – Ratio of eluant to sample flow rate in upper phase

x_f – holdup at flooding, the fraction of the column occupied by the dispersed phase

y – Ratio of eluant to sample flow rate in lower phase

Chapter 1: Introduction

Counter-current chromatography (CCC) is a liquid-liquid chromatographic system developed in the late 1960's by Yoichiro Ito (Ito et al. 1966). A coil-planet centrifuge is used to maintain one liquid, of a two phase system, quasi-stationary in a column while the other liquid is flowed past. Ito produced numerous prototype instruments developing the technology throughout the 1970's (Ito and Bowman 1977) culminating in the hydrodynamic J-type CCC centrifuge design (Ito 1980). This design became the standard for CCC separations, however instruments were largely lab built bespoke units lacking the robustness of commercial instruments and only able to achieve relatively modest *g*-fields (up to 80*g*). In the late 1990's the robustness of these instruments was improved (Sutherland et al. 2005) to provide scalable twin column CCC instruments, which have latterly been manufactured commercially by Dynamic Extractions[1]. In parallel similar developments occurred with hydrostatic centrifugal partition chromatography (CPC) instruments which were first developed in the early 1980's by Sanki Engineering (Murayama et al. 1982) before further commercial exploitation by Armen Instruments and Kromatron.

Both these types of liquid-liquid chromatography instrument allow batch chromatography to be performed in a similar way to solid-phase chromatography except the liquid stationary phase is retained by a centrifugal *g*-field rather than being immobilised on a solid support. The lack of solid support with liquid-liquid instruments gives much higher proportion of liquid stationary phase therefore allowing much higher sample loading per injection. The design of the columns in both CCC and CPC instruments is important to ensure good retention of the liquid stationary phase. Because of the liquid nature of the phases, the instruments are also amenable to liquid-liquid extraction (LLE) methods, giving improved efficiency though with lower productivity over standard LLE processes.

Both CCC and CPC have been used almost exclusively as batch systems to achieve chromatographic separations, in a similar way to high performance liquid chromatography (HPLC) and preparative batch liquid chromatography (LC). However continuous processes have been developed using solid stationary phases, most notably, simulated moving bed (SMB) which was first developed at a very large scale for the petrochemicals industry (Broughton and Gerhold 1961). While LLE methods include processes such as fractional

[1] Dynamic Extractions Ltd, Slough UK; founded in 2002 as a spin-out company from Brunel University, London, UK

extraction (Treybal 1980) which operates in a similar fashion. A number of approaches to continuous processing using CCC instruments exist, including dual flow (DFCCC) in hydrodynamic instruments (Ito 1985) and multiple dual mode (MDM) in hydrostatic instruments (Delannay et al. 2006). On hydrostatic CPC instruments an initial attempt to achieve the equivalent of SMB was developed in France (Couillard et al. 2003). The literature review will assess these methods and what improvements are required to achieve continuous down-stream processing in liquid flows.

1.1) Scope of the thesis

At the present time, particularly in the pharmaceuticals industry there is a move to design continuous processes to remove batch variability and reduce relatively large batch reactor footprints. Although much work has been done to achieve flow reaction there is still a bottleneck in the down-stream processing and purification of reaction mixtures from reactors, whether they are batch or continuous processes. Up to 70% of the cost of production can be in the downstream processing stages. Therefore an important field of research is the possibilities for achieving down-stream purifications in continuous liquid-flow streams.

In this thesis, it is hypothesised that the approach, described as intermittent counter current extraction (ICcE), will work on a standard twin bobbin hydrodynamic CCC instrument as a continuous processing method to separate a sample into two streams. If this can be achieved, then it could be envisaged that a CCC instrument with modifications to ancillary equipment could be attached to the outlet of a continuous reactor to give a fully continuous process for the production of active pharmaceutical ingredients. The feasibility of the ICcE method is to be examined and compared with DFCCC and the potential for scale-up of these methods will then be investigated for use as continuous down-stream processing units.

As the methods being investigated use standard CCC instruments with chromatographic and extraction methods the resultant continuous processing methods developed will effectively build upon the standard chromatographic theory, two phase systems, phase properties, partitioning behaviour and batch CCC and CPC theory and practice (Ito 2005).

Therefore the aim of this thesis is to focus on the following research questions:

1) Can the liquid-liquid SMB type method be transferred from CPC to CCC instruments?

2) If so can ICcE be competitive with the DFCCC continuous process already developed on CCC instruments?
3) If ICcE is competitive can it be scaled for preparative and pilot production on industrial applications?

Further, based on the literature review given in Chapter 2, two supplementary questions need to be addressed:

4) How does the back pressure applied to the outlets of a DFCCC column affect the phase ratio within the column?
5) How robust is the ICcE method to changes in phase system polarity?

From the literature review it is clear that although DFCCC has been used for continuous processing there is very little understanding of what controls the retention of the phases within the DFCCC column, while the ICcE method is practically untested.

The thesis is structured such that, Chapter 2 will review recent literature covering continuous processes both in liquid-liquid systems and liquid-solid phase systems including LLE, SMB and DFCCC; Chapter 3 covers the methods used; Chapter 4 develops a model for the continuous liquid-liquid process using CCC; Chapter 5 discusses in detail the research questions and finally Chapter 6 addresses the industrial uses of the new ICcE liquid-liquid methodology.

Chapter 2: Literature review of continuous processing

Continuous processing, extraction and chromatographic techniques are used across the chemical industry for separations ranging from the fractionation of crude oils in the petrochemicals industry to the purification of active pharmaceutical ingredients (APIs) in the pharmaceuticals industry. The three sections of this literature review cover liquid-liquid extraction, solid phase liquid chromatography and counter-current chromatography processes.

Section 2.1 details liquid-liquid extraction or solvent extraction, where a wide range of devices for continuous extraction were developed, including mixer-settler units and agitated columns, both of which are unit gravity systems.

Section 2.2 covers the methods of continuous processing used in solid phase liquid chromatography, focusing on steady state recycling and simulated moving bed chromatography.

Finally, Section 2.3 covers continuous counter-current chromatography (CCC) processes including dual-flow CCC, sequential centrifugal partition chromatography (CPC) and intermittent counter-current extraction which are continuous processes based on liquid-liquid chromatographic techniques using a variety of centrifuge designs to retain one of the liquids as a stationary phase under the influence of a high *g*-field.

For all these continuous processes discussed, the systems can be envisaged as a black box with eluant feeds and a sample feed, which are continuously fed into the box and two liquid streams leaving the box. For solvent extraction the feed is often in one of the eluant flows, whereas for fractional extraction and the continuous CCC processes the feed is separate.

2.1) Liquid-Liquid Extractors (LLE)

Liquid-liquid extraction, or solvent extraction allows the separation of compounds based on their distribution between two immiscible liquids. As a separation technique it is second only to distillation in importance to the chemical industry. Initially used by the petrochemical industry it is now common place in the pharmaceutical, hydrometallurgy, nuclear and environmental sectors (Mohanty 2000).

With liquid-liquid extraction, a degree of separation will occur if the distribution of substances (solutes) in a feed mixture are different when contacted with an immiscible solvent. This separation can be enhanced by multiple contacts either in a batch wise or continuous mode. The various types of extractor are designed to maximise the rate of mass transfer of the solute between the immiscible liquids.

For these processes the solution containing the solute to be extracted is called the *feed*, the immiscible liquid the feed is contacted with is the *solvent*, the solvent rich product is the *extract* and the liquid from which the solute is removed is the *raffinate* (Figure 2.1).

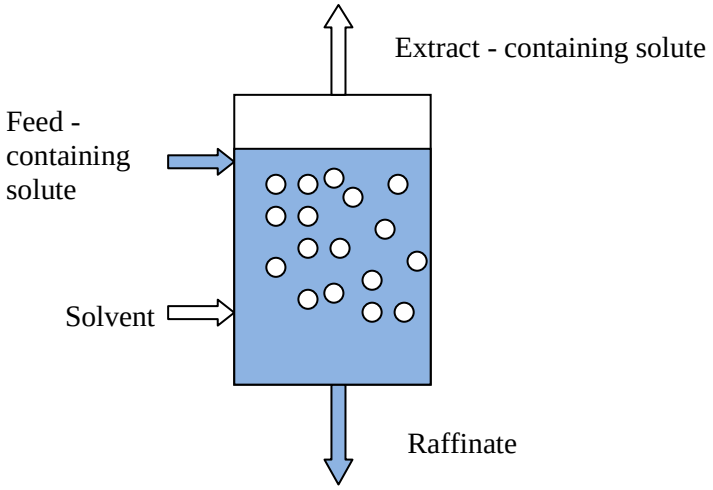


Figure 2.1: Solvent extraction terminology

The process may be complicated slightly by the use of two solvents such that two solutes in the feed distribute differently between the immiscible solvents and leave from opposite ends of the extraction unit, *fractional extraction* (Figure 2.2). This type of operation is analogous to dual-flow CCC discussed in Section 2.3.

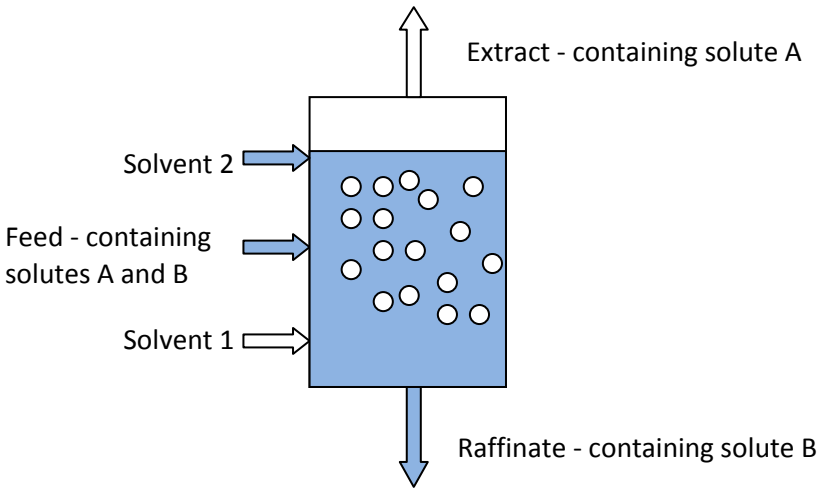


Figure 2.2: Fractional Extraction, Solute A prefers Solvent 1 and solute B prefers Solute 2

2.1.1) Theoretical considerations of liquid-liquid extraction

In a liquid-liquid extraction column or settler one of the liquids will be a continuous phase with the other immiscible liquid dispersed within it. Maximum separation efficiency, in terms of mass transfer and throughput is dependent on a number of key terms. Firstly, *holdup, x*, is the fraction of the column volume occupied by the dispersed phase and should be maximized. However, when the holdup increases such that the dispersed phase coalesces the column becomes *flooded*. Once flooding occurs in a column separation efficiency drops dramatically as the smallest droplets of the dispersed phase cannot overcome the opposing flow of the continuous phase and either one and possibly both liquids will fail to pass through the column and instead leave from the same end they entered (Treybal 1980; Cusack and Fremeaux 1991).

Practical column design therefore focuses on achieving the highest phase flow velocities without flooding occurring. The greater the *flooding velocities* for both the continuous and dispersed phase the higher the column capacity, though flows are limited to, at most, 75% of flooding velocity to maintain stable operating conditions. Flooding velocities of the continuous phase, $U_{c,f}$ (Equation 2.1) and dispersed phase, $U_{d,f}$ (Equation 2.2) are calculated from the holdup at flooding, x_f (Equations 2.3), where R is the ratio of the dispersed to continuous phase *superficial velocities* $(R = U_d/U_c)$. The *superficial velocity* is the hypothetical velocity of the phase passing through the column, rather than the measured velocity at a given point which can be variable across a column and from point to point

within the column. U_o, the characteristic velocity, is a measure of the maximum velocity that the dispersed droplets can achieve dependant on the internal restrictions of a column and the constant, *k*, which is dependant on the specific type of column extractor being used. (Cusack and Fremeaux 1991).

$$U_{c,f} = \frac{U_0 x_f (1 - x_f)^{k-1}}{x_f + (1 - x_f)R} \quad \text{... (Eq. 2.1)}$$

$$U_{d,f} = R U_{c,f} \quad \text{... (Eq. 2.2)}$$

$$x_{f=} \frac{R(k+2) - [R^2(k+2) - 4R(R-1)(k+1)]^{0.5}}{2(R-1)(k+1)} \quad \text{... (Eq. 2.3)}$$

The characteristic velocity is calculated from the *slip velocity*, U_s, which is a calculated number related to the difference in velocity of the immiscible phases and their holdup (Equation 2.4).

$$U_s = \frac{U_c}{(1-x)} + \frac{U_d}{x} \quad \text{... (Eq. 2.4)}$$

The slip velocity is independent of the column design, however it is related to the characteristic velocity, U_o, through the constant, *k*, related to the specific type of column extractor being used (Equation 2.5).

$$U_s = U_o (1 - x)^k \quad \text{... (Eq. 2.5)}$$

Mass transfer between a drop and a continuous phase is fundamentally driven by the molecular diffusivity, *D*, and the drop diameter, d, which was first described theoretically by Langmuir (Langmuir 1918). From this work a lower limit for the mass transfer coefficient in the continuous phase, k_c, can be assigned (Equation 2.6).

$$k_c = 2\frac{D_c}{d} \quad \text{... (Eq. 2.6)}$$

If the drop is in motion then the continuous phase mass transfer coefficient will increase. Therefore, a dimensionless Sherwood number can be defined which is the ratio of the convective mass transfer coefficient and the diffusive mass transfer coefficient, where the characteristic length over which mass transfer occurs is *L* (Equation 2.7).

$$Sh = \frac{kL}{D} = \frac{Convective\ mass\ transfer\ coefficient}{Diffusive\ mass\ transfer\ coefficient} \quad \text{... (Eq. 2.7)}$$

For extraction columns a dimensionless number, the Reynolds number (Reynolds 1883) can be defined which relates the inertial to viscous forces within the flow (Equation 2.8).

$$R_e = \frac{d_{32} U_s \rho_c}{\mu_c} \quad \text{... (Eq. 2.8)}$$

where d_{32} is the mean drop diameter, ρ_c and μ_c are the density and viscosity of the continuous phase respectively and U_s is the velocity of the continuous flow.

The mass transfer coefficients for extraction columns are obtained from this Reynolds number, measured concentration profiles and by applying simulation models.

For example with Kühni columns Kumar (Kumar and Hartland 1988) empirically fits Equation 2.9 for solute extraction from the continuous to dispersed phase and Equation 2.10 for solute extraction from the dispersed to continuous phase.

$$Sh_{oc} = -40 + 23Re^{1/3} \quad \text{... (Eq. 2.9)}$$

$$Sh_{oc} = 200 + 0.047Re^{1.44} \quad \text{... (Eq. 2.10)}$$

Similarly empirical solutions have been derived for pulsed, spray, Karr and rotating disc columns (Kumar and Hartland 1999), which leads to the following section in which the specific design of liquid-liquid extractions is discussed.

2.1.2) Equipment for liquid-liquid extraction

In the chemistry lab liquid-liquid extraction is done on a small scale batch-wise using a separating funnel, but this technology has been scaled up to allow large industrial scale separations in both batch and continuous modes.

Industrial scale processes can be divided into two distinct sections. *Stage wise extractors*, where each stage has the facility for first mixing the insoluble solutions then separating the product streams (Section 2.1.2.1) and *continuous contact extractors* where the insoluble liquids flow continuously counter-current to each other (Section 2.1.2.2).

2.1.2.1) Stage-wise extractors

Stage-wise extraction either occurs using a *single stage mixer-settler* (Section 2.1.2.1.1) consisting of a mixing unit for contacting the immiscible liquids followed by a setter unit for their physical separation, which can be linked to form a continuous chain of mixer-settler units or using *sieve-tray towers* (Section 2.1.2.1.2) where one immiscible liquid is dispersed by a sieve then allowed to collect on a tray to coalesce, this being repeated on numerous occasions in an extraction tower.

2.1.2.1.1) Mixer – settlers

The mixer-settler usually has a mixing unit and separate settling unit, though for a batch process the mixing unit could act as the settler after agitation has finished (Figure 2.3).

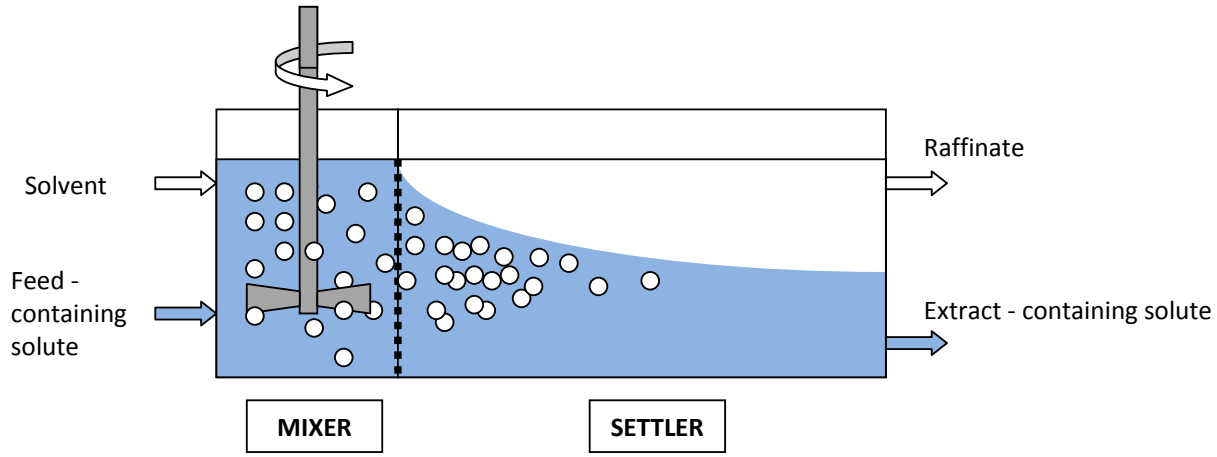


Figure 2.3: Mixer-settler unit

The mixing unit is normally a tank with an impeller to disperse droplets of one liquid in another immiscible liquid, the maximum volume fraction of the dispersed phase being in the

order of 0.7. Above this value flooding occurs, the phases invert and the continuous phase will become dispersed and vice versa. The aim is to form droplets with diameters in the order of 0.1 to 1.0 mm to allow good mass transfer rates. Smaller droplets, in the order of 1 to 2 µm are likely to form stable emulsions, which take too long to separate efficiently while larger droplets, greater than 1.0mm in diameter settle too rapidly reducing the rate of mass transfer (Treybal 1980).

Once agitation ceases, assuming a stable emulsion is not formed, the mixture will rapidly coalesce into two phases, with a defined interface, known as the *primary break*. Often though, one of the phases, usually the one in excess, will remain cloudy due to very small droplets of the other phase still dispersed within it. This phase will clear with time, but this *secondary break* takes much longer. Therefore, with multistage units, the liquids will be passed to the next stage after the primary break has occurred.

The settling unit receives the dispersion from the mixer and separation occurs. To minimise disturbance of the liquid in the settler the dispersion entering is passed through a baffle. The velocity of the solution in the settler must be slow enough to stop any turbulent flow which would create further mixing. Improved settling can be achieved by replacing the baffle with a coalescer, which increases the droplet diameter hence reducing the settling time. Coalescers are simply fine mesh materials such as fibre glass, steel wool, glass wool or polypropylene mesh.

Mixer-settler units are cascaded (Figure 2.4) to achieve the required level of extraction with the liquids being pumped between the stages. Each mixer-settler providing 0.8 to 0.9 theoretical stages[2] (Cusack and Fremeaux 1991).

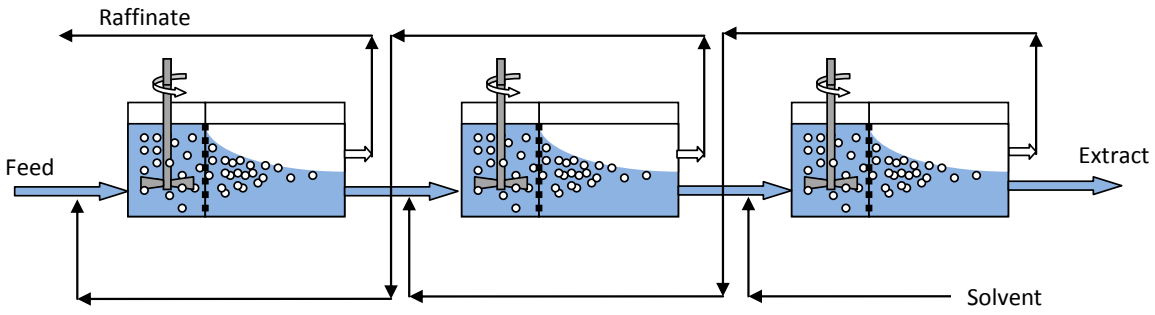


Figure 2.4: Three cascaded mixer-settler units

[2] The hypothetical space in which the two phases will reach equlibrium

Integrated mixer-settlers have been developed where the mixing vessel is inside a larger settling vessel to minimise the complexity of the interconnecting pipe work. The more dense liquid is flowed by gravity, while the lighter liquid can be raised by an airlift pump so the overall system has minimal moving parts.

2.1.2.1.2) Sieve-tray multistage towers

Agitation from a mixer is important if the immiscible liquids have high interfacial tension as this mixing improves drop formation dramatically. However, if the liquids have a relatively low interfacial tension then sieve-tray multistage towers become practical as droplets will form without mechanical mixing. The trays in the tower contain perforated plates which the dispersed phase passes through forming droplets. For the tower shown in Figure 2.5 the light phase is dispersed and the droplets formed rise through the heavy continuous phase and coalesce on the underside of the next tray, before passing through the perforations in that tray to form more droplets.

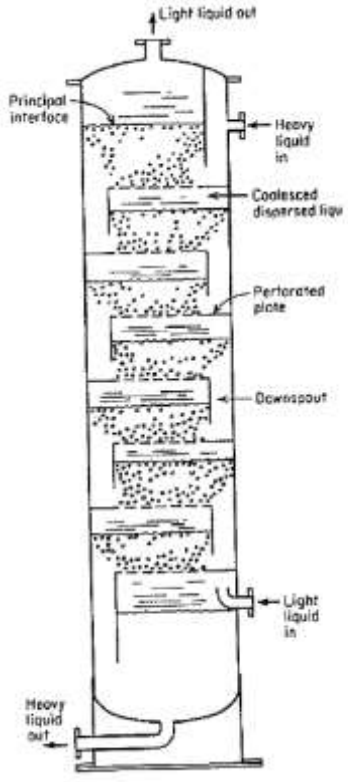


Figure 2.5: Sieve-tray extraction column with the light phase dispersed (Treybal 1980)

The heavy phase travels across each tray and then down the column via downspouts in each tray. The formation and coalescence of droplets at each tray stops any concentration gradient within the droplets persisting throughout the column. Ideally the dispersed and continuous

phases should exhibit plug flow behaviour within the column, to maximize the concentration gradient, however non plug flow, axial mixing can occur. The trays constrain axial mixing to the volume between adjacent trays rather than allowing the axial mixing to spread throughout the column. Both plug flow and reduced axial mixing improve the mass transfer of the column.

2.1.2.2) Continuous-contact extractors (Differential extractors)

With continuous-contact extractors the immiscible liquids flow counter-current to each other through the unit. Due to the difference in density of the liquids, under the force of gravity counter-current flow will occur, therefore the extractors are usually designed as large vertical towers, the heavy phase entering at the top of the tower or column, while the light phase enters at the bottom. The columns are operated either without energy input, *spray* and *packed towers* (Section 2.1.2.2.1) or more usually with energy input, either *mechanically agitated* or *pulsed columns* (Section 2.1.2.2.2). Instead of using gravity as the driving force for flow, *centrifugal extractors* (Section 2.1.2.3) have been designed which rotate at high speed giving a high centrifugal force to drive the radial counter-current flow of the immiscible liquids.

2.1.2.2.1) Columns without energy input: spray and packed towers

Spray columns are based on the types of columns used for gas liquid contact, where the density difference is high (800 kg/m^3) and therefore good dispersion can be achieved. However, spray and packed towers are only effective for LLE with liquids of low interfacial tension where dispersion is relatively easy. A spray tower is the most basic type of column being a simple shell with no internal baffles or trays where the heavier liquid enters at the top and the lighter liquid enters at the bottom. In practice the freedom of the liquids to move throughout the column allows excessive axial mixing reducing mass transfer to the extent that any column is not much greater than a single extraction stage (Treybal 1980).

To reduce axial mixing the column can be packed with a material which allows more controlled movement of the dispersed liquid through the column. The packing should not be wetted by the dispersed liquid droplets otherwise the droplets will coalesce on the packing forming rivulets reducing the interfacial area and hence the mass transfer. Therefore, the packing is chosen to be wetted by the continuous phase, often ceramic materials for aqueous phases and plastics for organic phases.

Both spray and packed towers have limited ability to mix immiscible fluids, especially those liquids with high interfacial tension, therefore further mechanical aid in the form of internal rotating impellers or equivalent are required to create dispersions.

2.1.2.2.2) Mechanically agitated columns

A range of columns has been developed since the early 1950s with different internal mechanical agitation of the column contents, achieved by either rotating or reciprocating internal elements or by pulsation of the liquids entering the column, Table 2.1. By these methods good dispersions are formed and therefore good mass transfer rates occur.

Table 2.1: Mechanically agitated column types

Column Type	Agitation Method
Rotating Disc contactor (RDC)	Spinning discs
Scheibel extractor	Drive impeller with baffles
Kühni column	Drive impeller with baffles and perforated plates
Karr column	Reciprocating plates
Pulsed columns	Liquids pulsed through fixed perforated trays

Rotating disc contactors are columns where agitation of the immiscible liquids is achieved using simple rotating discs attached to a rotating shaft and separated by baffles. Its relatively simple design has meant it initially found widespread use in the petrochemical industry for the separation of lubricating oils. The spinning discs break up the dispersed droplets increasing the interfacial area so improving mass transfer, while the baffles fixed to the shell of the tower help to limit axial mixing (Strand et al. 1962; Morís et al. 1997).

Scheibel extractors were developed for the pharmaceutical industry where a larger number of theoretical stages are required (Figure 2.6). The column consists of a tower with a central mixer to drive turbine impellers separated from each other by inner baffles to control the mixing pattern and direct the flow to the outside of the column where further baffles promote settling and inhibit flow on the internal tower walls. Initial designs separated the impellers with packed wire mesh (Scheibel and Karr 1950).

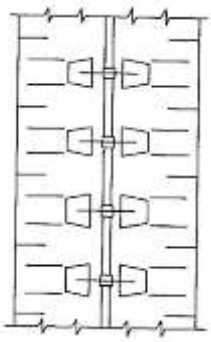


Figure 2.6: Scheibel extractor, inner baffles are supported by tie rods to direct the flow towards the outer baffles where settling occurs (Treybal 1980)

Kühni columns (Figure 2.7), widely used today, are similar to the Scheibel extractor, except baffled impellers are mounted between perforated plates, creating torus and radial shaped mixing zones between the plates. The perforated plates between the agitated compartments confine axial mixing, in a similar way to sieve-tray columns, further improving mass transfer. A 1.26 m high, 0.15 m diameter column can produce up to 7.5 theoretical stages per meter (Kumar and Hartland 1988) while columns are up to three meters in diameter and can achieve up to 30 theoretical stages in a single column.

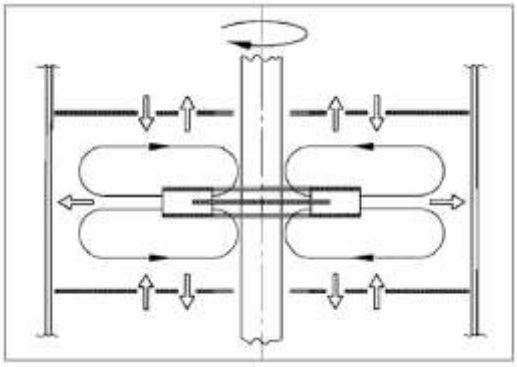


Figure 2.7: Kühni column, vortex mixing between perforated plates

Karr columns create the dispersion using reciprocating plates, rather than rotating impellers or disks. They are based on an original design by van Dijck with plates joined by chains (van Dijek 1935). Karr improved this design with open perforated plates (55-60% void space) fitted to a central shaft reciprocated vertically. This design gives very uniform shear mixing, therefore gives a narrow droplet size range, which is good for systems which tend to emulsify, so allowing high throughput (Karr 1959; Cusack and Fremeaux 1991).

The agitated columns described above use rotating or reciprocating parts to improve mixing and dispersion, whereas pulsed columns impart energy to disperse the liquids by providing

pulses to the liquid external to the column (Mar and Babb 1959). Development of these types of columns has been dominated by the nuclear industry as it is possible to design a column with no moving parts so that it can be behind radiation shielding and therefore minimise the need for maintenance. The Purex process was developed in the 1950s for the reprocessing of irradiated uranium. Tributyl phosphate will extract uranyl nitrate from an aqueous feed solution in the presence of nitric acid and also allow the separation of uranium and plutonium from each other (Irish and Reas 1957). The basic column design is similar to the sieve-tray multistage towers, where the immiscible liquids are dispersed and coalesced by perforated trays, with up or down spouts to allow flow of the continuous phase. A pulsed column dispenses with the up or down spouts and the trays (or plates) have such small perforations that the immiscible liquids can only flow through the holes when pulsed. The light and heavy phases are alternately pulsed through the perforated plates to form dispersions, which are then allowed to coalesce at the trays between pulses (Cusack and Fremeaux 1991).

Pulsation can potentially be applied to any type of column to improve mass transfer, though this is at the expense of increased energy costs and reduced flow rates and therefore throughput.

2.1.2.3) Centrifugal contactors and extractors

Initially for the pharmaceuticals industry, centrifugal contactors or extractors were designed to have short residence times to allow the separation of relatively unstable compounds and therefore also find use in the nuclear industry (Modolo et al. 2007), where counter-current gas centrifuges have been used since the early 1950s (Whitley 1984). Centrifuges generates a high g-field therefore are ideal for immiscible liquids with very small density differences (less than 0.05 g/cm^3).

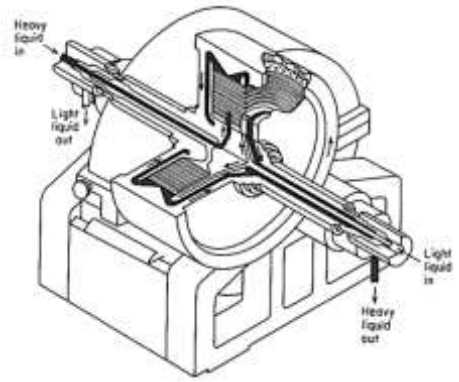


Figure 2.8: Podbielniak extractor with internal concentric cylinders (Treybal 1980)

The classic Podbielniak extractor centrifuge (Figure 2.8) is formed from a cylindrical drum containing perforated concentric cylinders (Podbielniak 1949). The more dense liquid is flowed in from the centre of the centrifuge while the lighter liquid flows from the periphery of the centrifuge to the centre. The acceleration generated by the rotation drives the immiscible liquids through the perforated cylinders and the interface and dispersion of the phases is controlled by the pressure difference between the inlet pressures of the phases and the outlet pressure of the lighter phase.

Annular centrifugal contactors (Figure 2.9) have been developed where mixing occurs in the annulus between the rotor and the fixed housing of the rotor and separation is driven by the enhanced g-field due to rotation. A single unit will give just under one theoretical stage (Meikrantz et al. 2001)

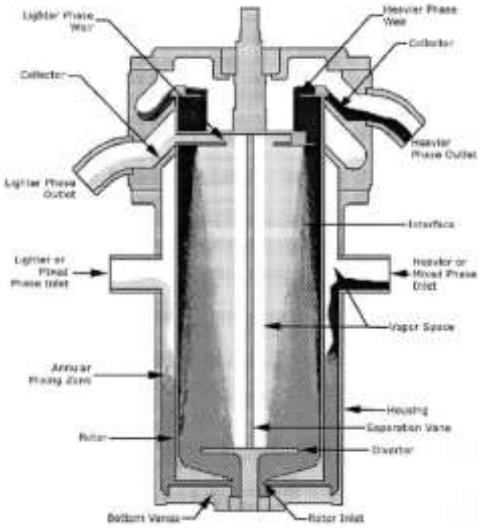


Figure 2.9: Annular centrifugal contactor (Meikrantz et al. 2002)

Recently multi-stage centrifugal contactor separators have been applied to the full enantioselective separation of one enantiomer of 3,5-dinitrobenzoyl-leucine using a cinchona alkaloid as a chiral selector (Schuur et al. 2009). This though is highly unusual, most chiral separations are done using high performance liquid chromatography, where the stationary phase is bound to a solid silica support and packed in a column. Continuous extraction or separation has to be achieved by the use of valving to simulate the movement of the packed stationary phase, the subject of the following section, as mobilisation of the solid phase is impractical.

2.2) Continuous processing in solid phase liquid chromatography

In the late 1930s and early 1940s Martin and Synge used the early techniques of liquid-liquid extraction combined with those of chromatography to develop partition chromatography (Martin 1952; Synge 1952) and so open up the field of first liquid chromatography and then high performance liquid chromatography (HPLC), where a packed solid acts as the stationary phase.

Continuous processing using this solid phase liquid chromatography is predominately achieved using simulated moving bed (SMB) chromatography where a sample or feed is continuously injected between a series of columns and two fractions are continuously collected (Section 2.2.1). Separation occurs due to the movement of the liquid phase and the solid phase counter-current to each other. The movement of the solid phase is simulated by periodic switching of the inlet and outlet ports of the columns. This technology allows binary separations where a sample is separated into two product streams such as for chiral separations where the sample is a racemic mixture consisting of two enantiomers. Chiral separations are of particular importance to the pharmaceutical industry where the two enantiomers may have significantly different biological modes of operation therefore many active pharmaceutical compounds are sold as single enantiomers, having been separated from their chiral partner. SMB is complex to achieve in practice due the valving needed to simulate the movement of the solid phase, but can be cost effective at large-scale. Therefore, further refinements have been developed to ensure the efficient use of the stationary phase including Varicol (Section 2.2.2).

Steady state recycling (SSR) chromatography (Section 2.2.3) was developed as a less complex alternative to SMB chromatography. A circulating chromatographic profile is setup by passing the eluted mobile phase back through the column (closed loop recycling) and periodically injecting a sample onto this profile and periodically collecting fractions containing the separated components. However, in reality the system is repetitive rather than continuous as both the sample and fraction collection must be periodic.

Finally, Section 2.2.4 and Section 2.2.5 discuss attempts to realize true-moving bed (TMB) chromatography where the phases are physically moved counter to each other with an apparatus which transports the solid phase media in a rotary (Annular Chromatography) or a "step-wise" manner (true moving bed belt chromatography).

2.2.1) Simulated Moving Bed (SMB) Chromatography

2.2.1.1) SMB methodology

SMB consists of a number of solid phase chromatographic columns connected by a valving system which allows the simulated movement of the solid phase support counter to the mobile phase flow (Figure 2.10). The columns in a typical SMB unit are split into four sections. Though two columns per section are common, the number of columns in each section can be varied dependant on the requirements of the separation. Five pumps are required, one to continuously recycle the fluid flow around the loop (clockwise in this example), one each for the feed and desorbant inlet flows and one each for the extract and raffinate outlet flows. The feed, containing two components **A** and **B**, enters the SMB unit between Section 2 and 3. Component **B** which is less retained flows preferentially with the mobile liquid phase and is collected between Section 3 and 4 as the raffinate. The system is run until component **A** is almost eluting with the raffinate, the end position of the first cycle shown in Figure 2.10.

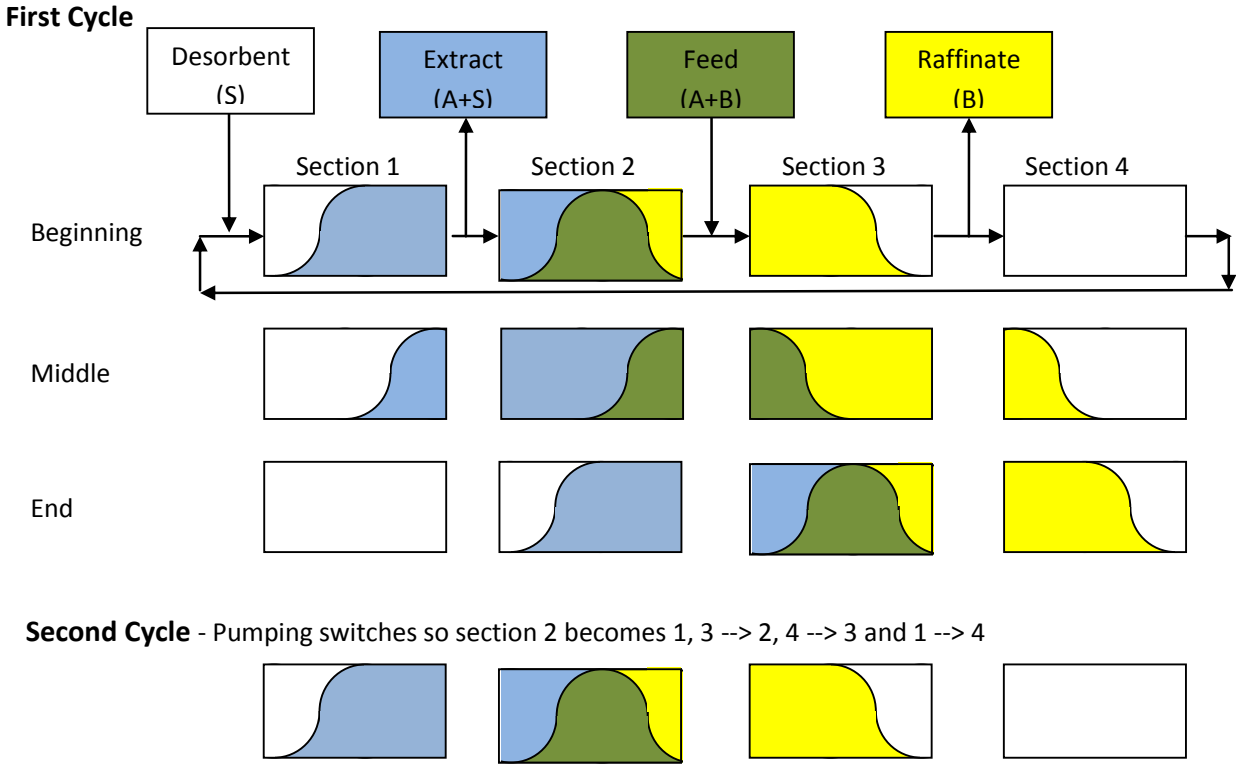


Figure 2.10: SMB operation at steady state, from the beginning position liquid is flowed around the system until the profile resembles the end position, then the valving is switched such that the inlet and outlet points move in the direction of the liquid flow by one section, so simulating the counter-current movement of the stationary phase

The movement of the solid phase is then simulated by the switching of the inlet and outlet ports in the same direction as the fluid flow. Section 4 becomes Section 3, Section 3 becomes Section 2, Section 2 becomes Section 1 and Section 1 becomes Section 4. Section 1 now contains pure **B**, which is extracted by the desorbant S and collected between Section 1 and 2 as the extract **A+S**. As the liquid flow continues all the less well retained compound **B** is washed from section 2 to section 3, as more feed stock enters between Section 2 and 3 before the next simulated movement of solid phase, at which point the cycle will continue.

An effective working design and process to operate SMB chromatography is achieved through the use of computer simulations, based on modelling of non-linear chromatography. These simulations allow the evaluation of feed concentrations, number of columns per section, column length and diameter, particle size and the flow rates of the recycling pump and the inlet and outlet pumps (Charton and Nicoud 1995).

The "triangle theory" of SMB operation, Figure 2.11, was developed in the late '90s (Mazzotti et al. 1997). This methodology allows an operating zone to be defined for both linear and non-linear isotherms, though as the theory is based on equilibrium theory mass transfer effects are ignored.

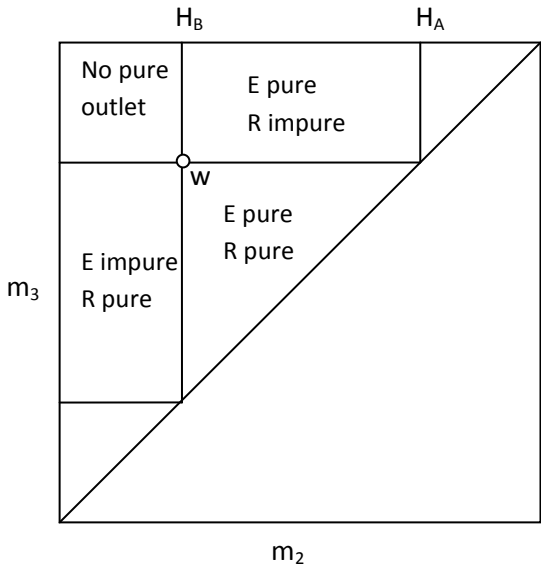


Figure 2.11: Triangle theory of SMB operation, regions of extract and raffinate purity are shown dependant on the liquid to solid flow ratio and the Henry constants of the compounds to be separated, w is the optimal separation point

Graphically the flow rate ratios, m_2 and m_3, in Section 2 and Section 3 of the SMB unit, respectively, are compared to the linear adsorption isotherms for the two components to be separated.

The flow rate ratios are defined by:

$$m = \frac{net\ liquid\ flow\ rate}{adsorbed\ phase\ flow\ rate} \quad \text{... (Eq. 2.11)}$$

While the linear adsorption isotherm is defined by:

$$n_i = H_i c_i, (i = A, B) \quad \text{... (Eq. 2.12)}$$

Where n_i is the mass concentration in the absorbed phase, c_i is the mass concentration in the liquid phase and H is the Henry constant.

From this analysis the triangular region for pure raffinate and extract streams can be determined. The optimal point for separation, *w*, at the apex of the pure extract and raffinate triangle is also the least robust point as a shift in parameters can move the operating point directly into a region of incomplete separation. The theory was further developed for non-linear isotherms and a topographically similar diagram can be produced, however the boundaries between the regions will be dependent on the feed composition and the flow rate ratios become coupled.

This type of topographical graph of the optimal operating region for a continuous process is clearly very useful to the SMB community and a method of visualising the separations using liquid-liquid systems is developed in the modelling chapter of this thesis (Chapter 4).

2.2.1.2) Development of SMB

The Universal Oil Products Company first patented a "Continuous sorption process employing fixed bed of sorbent and moving inlets and outlets" (Broughton and Gerhold 1961). A plurality of fixed bed columns were connected such that the movement of the stationary phase was simulated by a multi-way valve which simultaneously switched all the inlet and outlets of the columns in the same direction as the liquid flows.

The technology was further developed in the early 1960s by the petrochemical industry and given the generic name of the "Sorbex" process as a method for the separation of multiple thousands of tons per annum of hydrocarbons. About half of the Sorbex systems produced

were used for the separation of p-xylene from alkyl-aromatic C_8 fractions using Y-zoelites as an adsorbant and toluene or p-diethylbenzene as the deabsorbant, the "Parex" process (Juza et al. 2000). The Sorbex system was also applied for the large scale separation of mono- and oligosaccharides (Ruthven and Ching 1989) by the sugars industry.

The use of SMB at the process scale, by the petrochemicals and sugars industries allowed relatively quick development of SMB for use in the fine chemicals industries and then in pharmaceuticals separations, using HPLC chromatographic equipment, though at over an order of magnitude smaller scale. For clarity the operation of a standard SMB unit is described below. Improved modelling (Mazzotti et al. 1997), the demonstration of successful scale-down and new chiral stationary phases through the 1990's meant pharmaceutical companies developed SMB for enantiomer separations (Schulte and Strube 2001). Parameters measured by analytical scale HPLC (solubility, retention times and selectivity) can be used to evaluate the scale-up of these separations. The critical parameters of an SMB process, product concentration, purity, productivity and eluate consumption are often adjusted to optimise one parameter, critical to a specific separation, at the expense of the other parameters. (Bae and Lee 2006; Bae et al. 2008).

SMB has been shown to provide significant improvements in production rate and solvent usage over batch preparative HPLC without loss of purity or yield (Grill et al. 2004). 247 kg of a racemic mixture were separated at the process scale using a six-column SMB unit with solvent usage of only 0.11 l/g racemate compared to 0.71 l/g for a two step batch process. Although it was noted that at lower lab-scale the extra optimisation required for SMB meant a steady state recycling system was competitive (Section 2.2.3).

Applications are often limited to high value products due to the complexity of both the valving system and the number of columns required for an SMB process, although attempts to produce less complex systems have been made. Three zone systems, where Section 4 is removed, can reduce the number of columns, valves and pumps required, though at the expense of increased desorbant usage and increased dilution of the raffinate (Chin and Wang 2004). At the limit a SMB process has been demonstrated using only two columns (Rodrigues et al. 2008). This configuration minimises the use of stationary phase though the paper makes it clear that with more columns it would be possible to implement a more effective SMB system using more zones with a similar quantity of pumps and valves.

2.2.2) Varicol

Varicol is another multi-column continuous process which may be considered as a refinement of SMB, whereby the inlet and outlet lines are shifted non-synchronously causing the number of columns in each zone to vary periodically, such that, over time on average there are a non integer number of columns, giving more efficient use of stationary phase and therefore higher productivity compared with standard SMB (Adam et al. 2000). 6, 5 and 4-column Varicol units have been compared to a 6-column SMB unit for the separation of isomers of SB-553261 racemate. The 6-column Varicol unit gave a 10% increase in productivity and 3.7% drop in eluant usage over the 6-column SMB unit. However the 4-column Varicol unit gives a 50% increase in productivity, though with a matched 50% increase in eluant usage. Therefore the economics of the process are dependent on whether increased productivity or minimising the cost of the separation is most important (Ludemann-Hombourger et al. 2002).

2.2.3) Steady State Recycling (SSR)

Steady state recycling was developed as a slightly less efficient method than SMB, for separating enantiomers, with the restriction that the feed is loaded repetitively rather than continuously but has the advantage of using only one column. Initially described as Closed Loop Recycling with Periodic-Intra Profile Injection (CLRPIPI) (Grill 1998), the process was originally developed from closed loop recycling for a gas chromatograph system (Porter and Johnson 1959), where the mobile phase eluted from a column is passed back onto the column through the mobile phase pump, that is recycled, so simulating a longer column and therefore increasing the resolution. Mixing in the pump reduces some of the separation achieved, therefore the column volume needs to be significantly greater than the pump volume (Grill 1998). Sample is injected onto the column and the pure leading edge is collected, the unresolved part of the chromatogram is re-injected onto the column with a further injection of sample, the resolved trailing edge of the chromatogram is then collected, followed by the next leading edge. This sequence can be repeated and a steady state will be reached with time (Grill and Miller 1998). The system has been modelled successfully (Quiñones et al. 2000) using a competitive Langmuir model and equilibrium dispersive model. To correctly model the system the extra dispersion caused by recycling through the column needed to be accounted for as this contributed significantly to band broadening. The method was shown to be useful at a lab-prep scale where throughput of 2.05 kg racemate per kg chiral stationary phase per day (kkd) could be achieved, compared to 1.45 kkd for lab scale SMB and only

0.44 kkd for batch chromatography though solvent usage, at 0.21 l/g for SSR, is higher than for SMB, though still much better than for batch chromatography (Grill et al. 2004).

2.2.4) Annular Chromatography

In annular chromatography (Figure 2.12) movement of the stationary phase is achieved by packing in an annular ring which is rotated very slowly (180°/h) cross-current with respect to the eluant flow (Fox 1969, Hilbrig and Freitag 2003). The feed is introduced at the top of the annular ring and flows through the column as it slowly rotates. The rotation drives separation of the compounds in the feed dependant on their retention to the packed solid phase. For example sugars, glucose and fructose are separated with a feed concentration of 200 g/L, feed flow rate of 2.8 L/h, eluant flow rate of 9.2 L/h and rotation rate of 600°/h giving a 90% yield of fructose at 90% purity. Although the separation performance of annular chromatography is inferior to SMB type processes, it does allow multi-component separations (Howard et al. 1988).

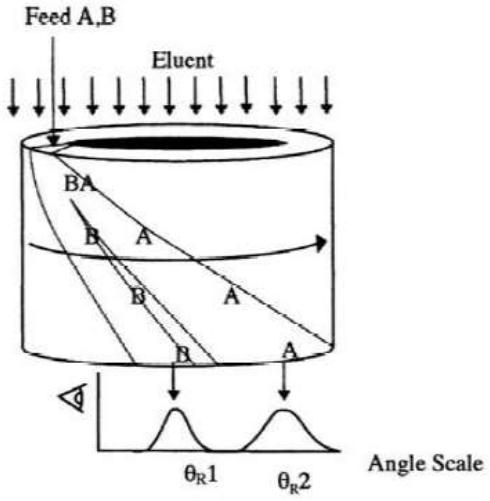


Figure 2.12: Schematic of continuous annular chromatography column (Hilbrig and Freitag 2003)

2.2.5) True moving bed (TMB) chromatography using solid phase media

SMB, where the movement of the solid phase is simulated by the use of valving, is the main method of achieving continuous separation with solid phase media due to the extreme difficulties in mobilising the solid phase media to allow it to move counter-current to the liquid phase. However two approaches have been used to attempt to truly move solid phase, so creating true moving bed chromatography. A belt coated in chiral stationary phase, cellulose tris(3,5-dimethylphenylcarbamate), is used to separate racemic oxprenolol, a β-blocker. The belt is passed through four solutions, an enantioselective-absorbant solvent, an enantioselective-desorption solvent, a desorption receiving solvent and finally a rinse solvent, though in this example the solvents are not moved counter-current (Yashima et al. 1995).

Secondly, a rotary holder with 12 individual cells was used to give stepwise movement of the solid phase counter-current to the liquid phase. Each cell contains a piston filled with solid phase which can be reciprocated in the cell to equilibrate with the liquid before all the pistons transfer to the next available cell. Liquid is removed as extract and raffinate from the first and ninth cells and replaced by solvent and feed. The cycle can be repeated continuously to separate the feed stock. Difficulties were noted due to the solid phase not wetting with the liquid phase as the liquid is not pressurised, which would restrict the solvents that can be used with the device (Nishizawa et al. 1999).

Neither TMB device is, in reality, particularly practical and neither fully realises the continuous movement of the solid and liquid phases counter-current to each other. So TMB is still a theoretical concept. The final section of this review therefore focuses on the development and use of liquid-liquid chromatographic techniques using a variety of continuous processes.

2.3) Continuous processing in counter-current chromatography

Within the CCC field, although the term counter-current is used extensively, implying the phases move in opposite directions to each other most methods used on instruments have one liquid phase held quasi-stationary, by the applied gravitational field, while the other phase is flowed past. The composition of the phases is not changed with time and this is known as the classical isocratic elution mode.

Unlike CPC, if the flow is stopped in CCC the upper phase moves to the head end of the column and the lower phase moves to the tail end of the column – true counter-current flow (hence the name). This therefore allows the option of continuous running where the phases are flowed truly counter-current to each other. Section 2.3.1 reviews this mode of operation which has historically been described as dual CCC (DuCCC), dual flow CCC (DFCCC) and more recently as continuous counter-current extraction (CCCE). In all these modes the eluant phases are flowed counter-current through a modified single column, with the sample to be separated being loaded continuously through an additional T-piece close to the mid-point of the column. These modes are a realisation of true-moving bed chromatography. The sample is split into too streams with compounds eluting dependant on their distribution ratios within the two-phase system used. More polar compounds will elute with the more polar phase and less polar compounds will elute with the less polar phase.

Section 2.3.2 reviews a number of recently developed “semi-” or “quasi-continuous” running scenarios using conventional CCC and CPC instruments. With these modes the flows of the two phases are alternated such that the stationary phase also alternates. The sample can be loaded either as repeated batch injections from one end of the column or continuously between two columns on a twin bobbin instrument, while the more and less polar eluants flow intermittently from alternate ends of the system. Section 2.3.2.1 reviews multiple dual mode (MDM), while Section 2.3.2.2 discusses the continuous sample loading between two CPC columns and finally Section 2.3.2.3 reviews intermittent counter-current extraction (ICcE), the study of which is the main focus of this thesis.

2.3.1) Dual flow counter-current chromatography (DFCCC)

Many of the fundamental concepts and theories used in counter-current chromatography were developed from Craig counter-current distribution instruments. These devices have long chains of glass tubes, each containing a two-phase system. To operate the device, a sample is introduced into the first tube and all the tubes are shaken and allowed to settle. The mobile phases, either upper or lower, in each tube are transferred to the next tube in the chain and the shake and settle steps are repeated. This is continued until separation of the sample is achieved. From this original work, the overarching concept of separating a sample by "the introduction of the mixture at the centre of a perfectly operating continuous column (of test tubes)", with the separated products eluting from opposite ends of the column, is first introduced (Craig and Craig 1956). Post and Craig describe the system as counter double current distribution (CDCD) (Post and Craig 1963). 10 g of alpha and beta protein chains of haemoglobin were fed into the middle of a line of 58 test-tubes and were separated after 200 mixing and settling transfers using a 2-butanol, 0.5M acetic acid, 10% dichloroacetic acid (9:10:1) phase system. Though the paper describes in detail the possibility of continuously feeding the sample in at the centre of the chain of tubes the results only describe a batch separation.

From the initial development of a coil planet centrifuge (Ito et al. 1966), Ito went on to develop a flow through centrifuge, later known as the I-type centrifuge (Ito and Bowman 1971), an instrument which could potentially be used for continuous separations. The I-type instrument provided a system with many discrete loops, each containing about 50% of the two phases, which go through a mixing and settling step with each rotation on the centrifuge. The design did not require rotating seals for the two leads to flow the mobile phase into and out of the column. Therefore, it was relatively simple to attach the pairs of inlet and outlet leads to allow both fluid phases to be flowed truly counter-current to each other.

The earliest example of CCC instruments to provide counter-current flow for removal and concentration of a target compound were using foam CCC separations (Ito and Bowman 1976) of dyes Rhodamine B and Evans Blue, where lauryl sulphate was used as the collector for the Rhodamine B. The columns were formed by winding 30 m of 2.5 mm I.D. PTFE tubing on to 13 mm O.D. pipes to give approximately 1000 individual mixer/settler loops. Flow tubes were attached for nitrogen gas feed and liquid collection on the head side and liquid feed and foam collection on the tail side (Figure 2.13).

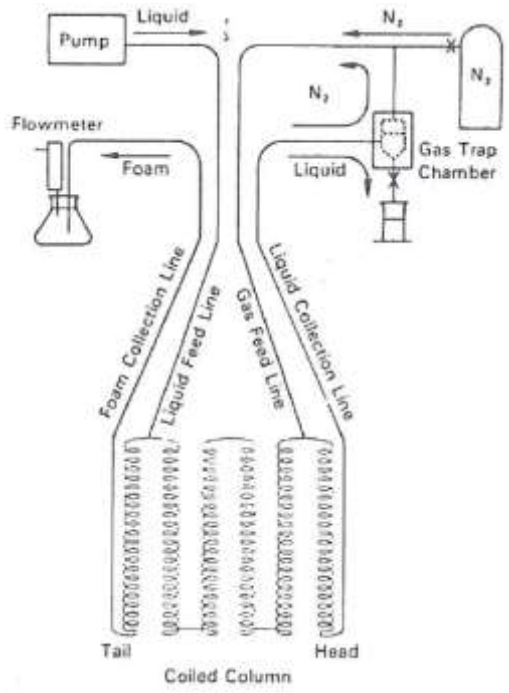


Figure 2.13: Foam CCC: The first example of true counter-current flow in a CCC instrument(Ito and Bowman 1976)

The term dual CCC was first used by Ito in 1985. He had developed the J-type centrifuge which gives better retention of stationary phases than an I-type instrument (Ito 1985). To achieve counter-current flow, a novel design of multilayer CCC bobbin was produced with end fittings containing both an inlet and an outlet at each end of the column. An inlet was also added at the mid-point of the column for sample injection (Figure 2.14). Solutes in the sample fed into the column through the mid-point inlet distribute between the two continuously flowing phases and are eluted from the outlet of the phase into which they preferentially partition. The column used was made from a 10 m x 2.6 mm I.D. section of PTFE tubing, wound on a 12.5 cm diameter holder, with 3-way Kel-F (polytrifluoromonochloroethylene) T-pieces to connect the inlet, outlet and feed tubing. The inlet tubes for the eluants were extended into the column by one complete turn (about 50 cm) to prevent back flow of the eluant phases from the local outlet. The apparatus was used for a foam separation as previously demonstrated, nitrogen gas (600 mL/min, 5.5 bar) being fed into the head inlet and a 10^{-3} M sodium dodecyl sulphate surfactant solution (3.6 mL/min) being feed into the tail inlet. A sample solution containing 5 x 10^{-4} M each of rhodamine B and Evans blue was fed into the midpoint of the column at a continuous flow rate of 0.36

mL/min resulting in each compound being successfully and separately continuously eluted from opposite ends of the column.

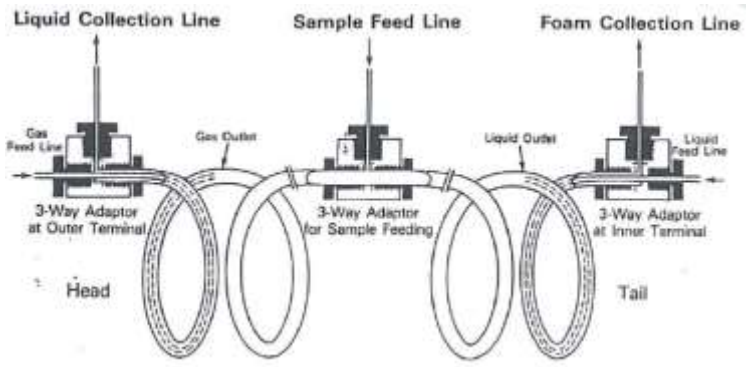


Figure 2.14: Ito's novel coil design with inlet and outlets at each end of the column and a mid-point inlet for sample injection (Ito 1985)

Ito (1985) also applied the method to the separation of proteins. Bovine serum albumin (BSA) and sheep haemoglobin were separated using similar conditions to those for the dye separation, except the liquid phase used was 0.2 M sodium phosphate solution (pH 8.9). Though it is acknowledged this separation is unusual as many proteins lack any active foam producing capability.

The same apparatus was used to demonstrate the concentration of small quantities of natural products contained in large volumes of aqueous solution (Oka et al. 1989; Oka et al. 1991). Using a dual flow foam separation the hydrophobic components of bacitracin were concentrated by producing thick foam with nitrogen gas and using distilled water as the liquid.

Though the dual-flow instrument had only been used for foam separations, it was clearly amenable to true counter-current liquid-liquid separations and this was for the separation of various indole and steroid mixtures (Lee et al. 1988). A 400 mL coil wound from 1.6 mm I.D. PTFE tubing was used with similar designs for the inlet, outlet and feed flow tubes as described previously. A mixture of indole-3-acetic acid, indole and biphenyl was separated with a pentane, ethanol and water (5:4:1) phase system, with both phases flowing at 1.5 mL/min. The biphenyl eluted with the upper phase, while the other two components eluted with the lower phase. Further, the separation and purification of a synthetic target from a crude chemical reaction mixture, containing unreacted starting materials and by-products was achieved using a hexane, ethanol and water phase system (6:5:4). The flow rate was 2.0 mL/min for both phases and the target eluted with the lower phase while all other impurities

were removed with the upper phase. Finally, the separation of a mixture of related steroids was achieved with a hexane, ethyl acetate, methanol and water phase system (6:5:5:5). For all these separations the sample was loaded as a bolus rather than a continuous injection. Therefore, for the more polar compounds eluting with the lower phase and the less polar compounds eluting with the upper phase, there was some resolution seen between the various steroids. Polar prednisone (Δ^1cortison) eluting first with the more polar lower phase, followed by II α-OH-progesterone, while the non-polar compounds eluted as a partially resolved band in their expected polarity order, the least polar first, a reduced estrone-3-methyl ether derivative followed by estrone-3-methyl ether and finally progesterone. The paper shows for the first time the potential to achieve continuous extraction based on the partitioning of compounds between two liquid phases for a CCC instrument.

All these papers (Ito 1985; Lee et al. 1988; Oka et al. 1989; Oka et al. 1991) use a needle valve on the head, upper phase, outlet to control the flow leaving the column. Oka specifically describes how the setting of the needle valve (and therefore by implication the back pressure) can change the outlet from which a peak elutes. Between 4 and 6% fully open a peak elutes with the foam outlet, yet once the valve is set between 6 and 9% of fully open the same peak elutes with the liquid outlet stream (Oka et al. 1989). This implies back pressure is required to control the phase ratio and the outlets from which the upper and lower phases exit the column although neither back pressure or the column phase ratio are discussed in any of these papers.

Dual flow CCC was seen as a method for achieving normal and reversed phase elutions at the same time so providing a highly efficient separation method for natural products. The bioactive lignin schisanhenol was separated from a crude ethanol extract of *Schisandrra rubriflora* Rhed at Wils containing schisanenol acetate, using a hexane, ethyl acetate, methanol and water (10:5:5:1) phase system (Lee 1991). A second example is given for the isolation of the topoisomerase inhibitor, boswellic acid acetate from its triterpenoic mixture using a hexane, ethanol and water (6:5:1) solvent system with a 400 mL column volume dual flow unit. Both examples used a flow rate of 2.0 mL/min for both phases. Importantly Lee (1991) discusses for the first time the possibility of selecting a solvent system to "*... allow stripping the crude extract with DuCCC* (DF-CCC) *to remove the impurities or inactive compounds. Consequently, the bioactive component will be concentrated inside the column for subsequent collection.*" This concept is expanded on and demonstrated in the later part of this thesis using intermittent counter-current extraction (ICcE), when applied to the

purification of an industrial waste stream. Lee goes on to demonstrate the use of DFCCC with highly polar phase systems and compounds for the isolation of a polypeptide from a synthetic mixture (Lee 1996). [2-D-penicillamine, 5-D-penacillamine] enkephalin (DPDPE), synthesised by solid phase means, was purified using DFCCC with a 1-butanol containing 0.1% trifluoroacetic acid (TFA) and 0.1% TFA solution (1:1) phase system, both at a 2.0 mL/min flow rate. Though the DFCCC instruments and applications demonstrated had the potential to be run continuously, all the examples to this time were for bolus injections and details of injection times and volumes were not given.

In 2006, analytical DFCCC is described for rapid sample preparation for pesticide analysis (Goto et al. 2006) and the first theory and model (Ito et al. 2006) for predicting the retention time and position of solutes, either in the upper or lower phase streams of a DFCCC run is derived. The retention time is derived from the following equation,

$$t_R = \frac{KV_U + V_L}{F_L - KF_U} \qquad \text{... (Eq. 2.13)}$$

Where K, the partition coefficient, is the ratio of the concentration of the solute in the upper phase divided by the concentration in the lower phase and where, V_U, F_U, V_L and F_L are the volumes and flows in the upper and lower phases respectively.

From this equation three cases are described;

1. $KF_U<F_L$, then t_R is positive so the solute flows with the lower phase from head to tail
2. $KF_U>F_L$, then t_R is negative so the solute flows with the lower phase from tail to head
3. $KF_U=F_L$, then t_R is infinite implying the solute remains in the column

Ito's very limited analysis of this theory, for $KF_U<F_L$ using four dyes (acridine orange, sudan black, sudan red and sudan blue) with a n-hexane, acetonitrile and 0.1% formic acid (45:45:10) phase system, showed that although the compounds flowed with the lower phase as expected the elution times varied significantly, with differences of up to 35% change in the expected elution time (Table 2.2). The column used was 10 m long, 2.6 mm I.D. PTFE tubing with a column volume of 50 mL.

However, the paper did demonstrate the effectiveness of dual-flow as an analytical technique for carbamate pesticide analysis, by using the fact that the hydrophilic pesticides could be separated from the hydrophobic oils and fats in cereal and beans which are washed from the

column in the upper phase, so repetitive injections are possible without risk of contamination from retained components in the column and the samples could be analysed by LC/MS as the interfering aliphatic compounds had been removed.

Table 2.2: Theoretical and experimental retention times for four dyes in a 50 mL, 10 m, 2.6 mm I.D. PTFE DFCCC column (Ito et al. 2006)

Compound	K	Theoretical t_R (min)	Experimental t_R (min)	% Difference
Acridine orange	0.00	1.30	1.30	0
Sudan black	0.16	3.11	2.58	-21
Sudan red	0.31	5.66	8.08	30
Sudan blue	0.80	40.9	30.3	-35

Further, the need for back pressure on the upper phase, head outlet of a dual flow is again mentioned, as a 70 mm length of 0.25mm I.D. Polyetheretherketone (PEEK) tubing was "*needed for establishing the hydrodynamic equilibrium to restrain the natural pumping force*". However the effect of this tubing or lack of it on the phase ratio within the column is not commented on any further.

Following on from this work, a bespoke coil was constructed to optimise the analytical DFCCC to allow it to be directly linked to MS/MS (Ito et al. 2008). The coil was manufactured by forming a spiral groove in a disk of Kel-F plastic (polymonochloro-trifluoroethylene) then sealing the channel with a PTFE sheet.

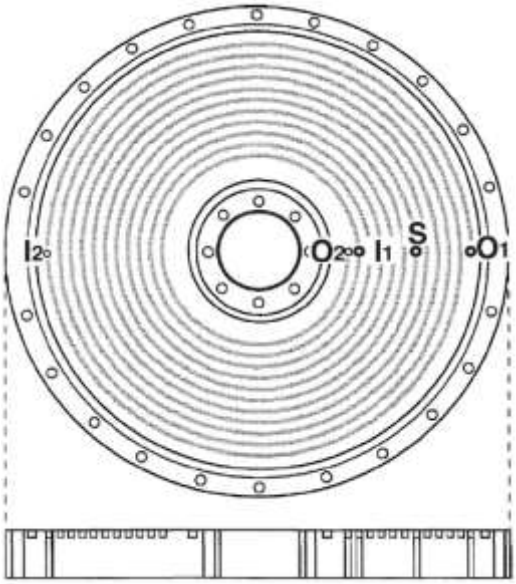


Figure 2.15: DFCCC coil machined in Kel-F polymer, 6 mL coil volume (Ito et al. 2008)

Using the same n-hexane, acetonitrile and 0.1% formic acid (45:45:10) phase system the eluted lower phase (flow rate 1 mL/min), containing the separated pesticides, was split with a 1:4 ratio before direct injection into the MS/MS. The optimised column had a 1 mm x 2 mm profile, to give a total coil volume of 6 mL, resulting in a coil only 3 m long. The optimised coil volume and design shortened the time to reach dynamic equilibrium. The design also eliminates the need for feed tubes inserted into the column to stop back flow, though the position of the outlets must be chosen at the design stage as once fitted the outlet position cannot be adjusted.

The model described by Ito is a starting point for determining where a compound might elute from a DFCCC. However, Kostanian et al. compared the standard eluting counter-current distribution (CCD) model with a longitudinal mixing cell model for CCC more generally and defined a specific set of equations for DFCCC (Kostanian and Voshkin 2007; Kostanyan et al. 2007). Using the longitudinal mixing cell model the distribution of the solute between the upper and lower phases is shown to be determined by three dimensionless parameters, the extraction factor, defined as the distribution ratio of the solvent multiplied by the ratio of the phase system flow rates; the total number of equilibrium stages and the sample inlet position. While for the eluting CCD model a formula was obtained to evaluate the solute eluting from each end of the chain of cells, when the sample is fed exactly into the middle of the chain and the phase transfers take place simultaneously.

The phases in a DFCCC column, wound with transparent 5 mm I. D. PFA tubing, were visualised (van den Heuvel and Sutherland 2007) to understand the hydrodynamic behaviour of the phases and assess the potential for scale-up of DFCCC to a large bore instrument. This paper shows that the phases do not distribute evenly along the length of the column and that a transition area occurs between the normal and reversed phase ends of the column.

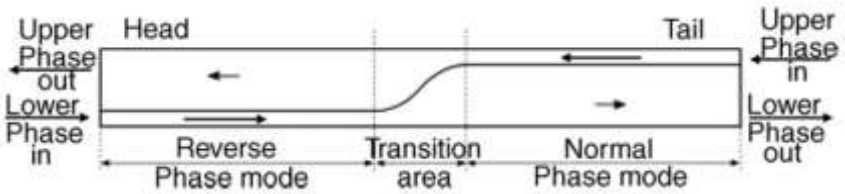


Figure 2.16: Distribution of phases in a DFCCC column and the transition area (van den Heuvel and Sutherland 2007)

A 2.2 m coil was wound as a spiral of five and a half loops on a cantilevered centrifuge. The inlet tubes were extended into the column by one turn (35 cm) at the centre, lower phase

inlet, and by half a turn (25 cm) at the periphery, upper phase inlet. The intermediate polarity heptane, ethyl acetate, methanol, water (1.4:0.6:1.0:1.0) phase system used for visualization was dyed with Sudan blue which partitions into the upper phase completely and Procion brilliant yellow, which partitions into the lower phase completely. Stroboscopic photographic images of the column were taken, triggered by the rotor so all images were taken at the same position in the rotation cycle. These images were analysed to determine the phase distribution within the column at various flow rates. In this idealised, short coil, for the phase system used, with equal flow rates the transition area was located close to the head of the coil, so the column is largely full of lower phase. To get equal volumes of each phase in the column the upper phase flow rate had to be higher than the lower phase flow rate.

A method for predicting the position of this transition area was derived (van den Heuvel and Sutherland 2009).

$$\%L_U = \frac{\%V_U + B_U\sqrt{Q_U}}{B_L\sqrt{Q_L} + B_U\sqrt{Q_U} + 1} \qquad \text{... (Eq. 2.14)}$$

Where $\%L_U$ is the percentage length of the column where reversed phase mode dominates and B_U and B_L are the gradients of the normal phase and reversed phase Du plots repectively, $\%V_U$ is the percentage volume of the upper phase in the column and Q_U and Q_L are the flow rates of the upper phase and lower phase. By definition the percentage of the column where normal phase dominates is $100-\%L_U$. The same short column is used to test this equation, with the non polar heptane, ethyl acetate, methanol and water (1.4:0.1:0.5:1.0) phase system, and the term continuous counter-current extraction (CCCE) is used to describe the technology for the first time. Interestingly, the effect of the initial condition of the column on the final equilibrium position was tested. Filling the coil initially with only upper phase, only lower phase or a 50%/50% mix of the two phases had no effect on the final equilibrium distribution of the phases in the column. Therefore it was summarised that, in continuous running, any disruption of the column equilibrium by the sample introduction should be corrected by the continued flow of the phases and any stripping seen in isocratic CCC runs can be minimised as the phases are continuously replaced.

More recently, DFCCC was demonstrated at the preparative scale, when it was again described as CCCE, using a specially constructed 625 mL DFCCC column (van den Heuvel

and Sutherland 2009). A proprietary synthetic pharmaceutical liquor, from Pfizer Ltd., containing seven components in isopropanol/water solution, was successfully split into two streams at a throughput of 30g/hour. The column used had six layers of 5 mm I.D. PFA tubing giving a total coil length of about 32 m, and the inlet leads were extended one metre into each end of the column to prevent backflow of the phases. At the midpoint of the column, between layers three and four, a T-piece was fitted to allow sample injection. The sample mixture was screened across eight heptane, ethyl acetate, methanol and water phase systems from a polar (3:1:3:1) phase system to a non polar (1:3:1:3) phase system. Using the phase system (3:1:2:2) the compounds split the mixture into two groups of three and four compounds around a K_d value of one, as predicted by their K_d values, for equal flow rates of 30 mL/min. Successful continuous loading was demonstrated as 20 g of reaction liquor (118 mg/mL) was loaded over a 40 minute period. Though, it must be noted, none of these papers discuss the back pressure applied to the head-centre outlet of the column and what effect this might have on the phase volume ratio retained in the column.

A numerical model was developed using computational fluid dynamics (König and Sutherland 2007). A mesh model was constructed to model the design of the short visualisation column described previously (van den Heuvel and Sutherland 2007). The model showed how the phases behave as they enter a DFCCC column through the inlet leads which extend into the column by one metre at each end. Upper phase entering at the periphery was observed to move towards the centre outlet as long as the coil was rotated to put the head at the centre of the column. If the rotation was reversed, such that the head was at the periphery, the upper phase was seen to exit the coil directly by the periphery outlet. Even when the head was at the centre of the column, some upper phase was still seen to exit from the periphery outlet. It was hypothesised that this might be caused by lack of pressure at the outlet points. The model was setup with the outlets as pressure boundaries, but with no pressure reference values set and as the outlet tubes are very short they provide very little resistance to flow, unlike in the experimental setup.

This model and the previously described literature clearly show the need for a more detailed understanding of how the pressure at the outlets to the DFCCC column changes the hydrodynamic equilibrium within the column. The effect of the pressure applied to the outlets is therefore investigated as the first part of this thesis in an attempt to discover a route to a robust continuous liquid-liquid extraction technology.

2.3.2) Quasi-continuous CCC

The most recent developments in continuous processing with counter-current chromatography have been around the quasi-continuous operating modes where the flow of the eluant is switched between normal and reversed phase modes at specified time intervals. This type of operation has been described as true-moving bed (TMB), Section 2.3.2.2, when used with hydrostatic instruments and intermittent counter-current extraction (ICcE), Section 2.3.2.3, when used with hydrodynamic instruments. The concept of switching the eluant flow direction was developed in the late 1980's as dual mode CCC and then later as multiple dual mode (MDM) where multiple switches occur, Section 2.3.2.1. These two methodologies are reviewed in detail before moving on to the quasi-continuous modes.

2.3.2.1) Dual mode and multi dual mode CCC

The benefit of a liquid stationary phase is that the elution mode can be switched part way through a separation. This was first used to reduce the time of a separation and increase the solute concentration in the latter fractions for the separation of five hydroxyanthraquinone derivatives, extracted from rhizome of *Rheum palmatum* L. (Zhang et al. 1988A; Zhang et al. 1988B). The separation used a n-hexane, ethyl acetate, methanol and water (9:1:5:5) phase system on a Pharma-Tech model CCC-2000 HSCCC instrument fitted with a 70m x 0.85 mm I.D. PTFE column, total volume 40 mL, rotating at 1800 rpm. The first three components, physcion, aloe-emodin and rhien, were eluted in reversed phase at 1 mL/min within 40 minutes. Without interrupting the run, the flow was switched to normal phase mode and the final two peaks, chrysophanol and emodin, were eluted. In their second paper, a similar method was used for the separation of a methanol extract from *Stephania Tetrandra* S. Moore of the alkaloids, tetrandrine, fangchinoline and cyclanoline. On the same instrument as in the first paper, an n-hexane, ethyl acetate, methanol and water phase system (3:7:5:5) was initially flowed in reversed phase to elute cyclanoline followed by fangchinoline within 60 minutes. The flow was then switched to normal phase mode to elute tetrandrine within another 30 minutes. This approach of swapping the mobile phase flow part way through the separation was seen of particular importance for the separation of natural products as the crude extracts from plant materials usually contain a large number of components spread over a relatively wide polarity range.

A similar result can be achieved by this swapping of the flow direction, phase reversal, when using a hydrostatic CPC instrument (Marston et al. 1988). An ascending mode isocratic run is compared with a phase reversal run for the separation of flavanoids; hesperetin, kaempferol and quercetin using a Sanki Model LLN CPC instrument fitted with six cartridges of total volume 125 mL, rotating at 600 rpm. The separation in ascending isocratic mode with a chloroform, methanol and water phase system (33:40:27) took 6.5 hours at a flow rate of 1 mL/min. Using phase reversal, quercetin was eluted first with the upper phase in 55 minutes at 1.6 mL/min, then the mode was switched to descending mode to elute the hesperetin and Keampferol in a further 35 minutes, also at 1.6 mL/min. This paper shows the significant improvements in throughput that can be achieved by using phase reversal.

Slacanin, Marston and Hostettmann went on to repeat the separation using a hydrodynamic CCC instrument (Slacanin et al. 1989). Using a P.C. Inc. instrument fitted with a 360 mL coil, 2.6 mm I.D tubing, rotating at 700 rpm separation by reversed phase with the same chloroform, methanol and water phase system (33:40:27) took 3 hours at a flow rate of 3 mL/min, whereas in normal phase the separation took 8 hours at 3 mL/min. Using phase reversal, quercetin was eluted first with the upper phase in one hour, then the mode was switched to reversed phase to elute the hesperetin and Keampferol in the second hour, also at 3 mL/min. Because the column is run in both normal and reversed phase within a single run, rather than equilibrating the column in the normal manner, the column was filled with a 50%/50% mixture of the upper and lower phase by running two pumps simultaneously at equal flows, such that for each part of the run the stationary phase retention would be 50%. Interestingly, this paper also demonstrates the use of a phase ratio gradient within the column, whereby the upper and lower phases were pumped into the column co-currently at rates of 4 mL/min and 1 mL/min respectively to reduce the length of a normal phase separation from eight to three hours.

In later papers (Gluck and Martin 1990; Menges et al. 1990; Duret et al. 2000; Alvi 2001; Berthod et al. 2003), the concept of phase reversal, switching the mobile phase flow part way through the run was described as dual-mode.

Equations for calculating retention time using dual mode CCC and for the increased resolution when dual mode is compared to a standard HSCCC run have been derived from the basic equations of chromatography (Agnely and Thiébaut 1997). Theoretically, improved resolution will be achieved if the difference in retention times between two components can

be increased. The paper also analyses the phase distribution within the column before and after phase reversal. Using a Pharma-Tech model CCC2000 instrument with a 1.6 mm I.D. PTFE column of volume 130 mL, rotated at 800 rpm, with a water-heptane (1:1) phase system the distribution of stationary phase in the column was measured by equilibrating the column in reversed phase at 6 mL/min then stopping the column and gently emptying and fractionating the column contents with compressed air. In this reversed phase mode, uniform distribution of phase was seen throughout the column with 85% stationary (upper) phase at all points along the its length. However, phase reversal had a significant effect on the distribution of phases along the column. The column was again initially equilibrated in reversed phase, giving a stationary phase retention of about 85% (upper phase), however after phase reversal the volume of stationary phase, now the lower phase, is therefore only about 15% of the coil volume. As the upper mobile phase is flowed, the relatively small volume of lower stationary phase was seen to collect towards the tail of the column. After 17 minutes, the stationary phase was starting to collect at the tail of the column and only distributed across about 60% of the coil length, while after 50 minutes the stationary phase was almost entirely collected at the tail of the column, the first 16% of the column volume, the remainder of the column being flooded with mobile upper phase. This is the first evidence that the phases do not distribute evenly along the length of the column, predating the later visualisation work on phase distribution in DFCCC columns discussed in Section 2.3.1 (van den Heuvel and Sutherland 2007)

A further development by repeating the dual mode cycle on a CPC instrument, moved dual mode from a simple application to shorten run time and potentially improve resolution to a semi-continuous method for separating two components. This methodology is termed multiple dual-mode (MDM) CPC (Delannay et al. 2006). Using a hydrostatic CPC, a bolus of sample was loaded then run though an ascending and descending cycle before loading another bolus and repeating the cycle. This cycle can be repeated many times each time eluting a slug of the separated component from the column before the next switch, one component flowing with the upper phase preferentially, while the other component flows with the lower phase preferentially. The separation of Acenaphthylene and naphthalene was done using a Kromaton Technologies CPC, with 20 partition disks (1320 individual cells in total), and a column capacity of 200 mL, rotating at 1800 rpm, using a heptane and acetonitrile phase system. A 1 g 50:50 mixture of the two compounds was injected onto the CPC column and subjected to a dual mode cycle at flow rates of 8 mL/min for both phases,

then a further 0.5 g injection was made and the dual mode cycle repeated. This was repeated for three more injections, with a loading of 3 g in total. Due to the hydrostatic nature of CPC, a significant back pressure of 25 bar was recorded for the run. Timing for the switching between ascending and descending mode were evaluated from two preliminary runs: firstly, an ascending mode run where acenaphthylene elutes ahead of naphthalene, the critical time for switching is 23 minutes, just before naphthalene elutes; secondly, a single dual mode cycle using the 23 minute switching time, giving an ascending mode cycle, followed by a descending mode cycle where the naphthalene starts eluting before the acenaphthylene with a critical switching time of 16 minutes. Therefore, for the MDM run, switching should occur at 23 and 16 minute intervals, with reinjection every 39 minutes, though inspection of the chromatogram shows that times were adjusted while the MDM run was in progress. Ascending phase timings reduce from 23 minutes to 10 minutes and descending mode timings reduce from 16 minutes to 9 minutes (Figure 2.17).

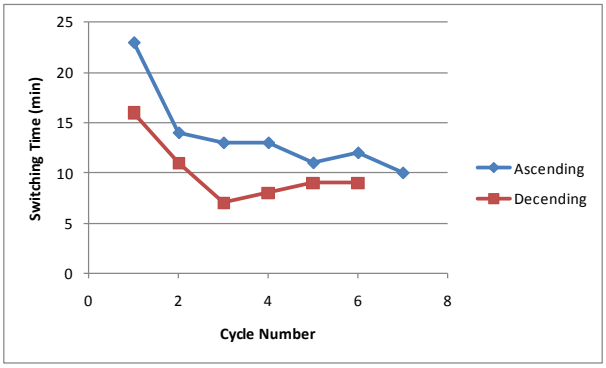


Figure 2.17: Change in switching time for MDM semi-continuous separation (Delannay et al. 2006)

Although not discussed in the paper, this reduction in the switching time is required as for all subsequent injections after the first injection, the leaded edge of the next peak to elute is inside the column and therefore does not have to travel a full column length before it starts to elute. The described run has empirically determined the repeat switching times of the ascending and descending modes to achieve steady state operation of the MDM for these compounds and this phase system.

2.3.2.2) Sequential Centrifugal Partition Chromatography (sCPC)

In a patent, Couillard et al. describe a method for separating a crude sample into two component groups using a CPC device (Couillard et al. 2003). The patent specifically claims "*...a device including at least one centrifugal chromatographic column constituted by the serial interconnection of at least one set of separation cells, characterised in that it comprises: injecting the feed at an intermediate point of said set of cells; and carrying out alternate cycles of the two phases...*" The figures and detailed description are of a CPC instrument where the feed is loaded continuously between two columns and the upper and lower phase eluants were flowed alternately in ascending (less dense phase mobile) and then descending (more dense phase mobile) modes so splitting the feed into two groups which eluted from opposite ends of the system. The patent also claims the method could be used for separating optical isomers with the addition of a chiral selector. Sutherland (2007) mentions the testing of the Armen 12.5 L CPC, which has two 6.25 L rotors, each containing 528 cells, where a mixture of two dyes was separated continuously with an efficiency between 3000 and 3800 theoretical plates, which was shown as a video at the CCC2006 conference (Sutherland 2007). The product literature for this technology[3] brands the concept as TMB "True moving bed" chromatography although this term should correctly only apply if both phases are moving continuously counter-current to each other as in DFCCC discussed in Section 2.3.1. Another concept introduced by the patent is the cascading of multiple instruments to allow separation of more complex mixtures, though this has previously been discussed for SMB systems (Wankat 2001).

A recent paper by Hopmann, shows the separation of analytical levels of pyrocatechol hydroquinone (5 mg/mL of each in the feed) using an Armen Instrument TMB-250 CPC with two columns of 125 mL, each rotor having 10 discs with 93 cells per disc (Hopmann et al. 2012). The paper defines a set of operating parameters, for running sequential CPC and graphically maps out a region of complete separation similar to the model discussed in Chapter 4 of this thesis. The graphical representations presented in Chapter 4 were independently derived and shown by the author in a presentation at CCC2010 in Lyon, France (Hewitson et al. 2010). The model presented in Chapter 4, also shows the regions of partial separation where only one of the two flow streams is pure.

[3] http://www.armen-instrument.com

2.3.2.3) Intermittent counter-current extraction (ICcE)

Based on the previous success of both dual mode and dual flow methods, the potential of the sequential method as described for a CPC instrument, containing a series of cells, might be transferable to a CCC instrument, with its long coiled tube, where one phase is held quasi-stationary by the J-type centrifugal rotation. A study of the practical difficulties and scalability of the method using CCC instruments is the focus of the second half of this thesis, but some preliminary work using this technique, both by myself and other groups, and relevant modelling are discussed as part of this literature review for completeness in this section.

A similar principle to ICcE, using a pair of CCC columns with a bolus injection between the columns but elution from one end was described as a "simulated dual-flow" approach by Dubant in a presentation at CCC2006 (Dubant et al. 2006). This work is a stepping stone between dual mode and the full realisation of ICcE. Using a twin column CCC with a heptane, ethyl acetate, methanol and water (7:13:10:10) phase system, Dubant injected a 250 mL sample between the bobbins and then alternated the flow between normal and reversed phase for an undefined number of times, before eluting the compounds from one end of the system with the target 96% pure at 82% recovery. With a classical isocratic CCC run the resolution between the peaks was only 0.25, whereas with this method the resolution between the peaks increased to 0.85.

Kostanian and Voshkin's (2007) modelling work describes DFCCC, where the phases flow continuously counter-current to each other, or as he terms it, "simultaneous transfers of the phases" occur. They also briefly discussed the concept of alternately transferring phases, as seen with dual mode, sequential CPC and ICcE. They state that, theoretically, alternating the phase transfer gives improved separation efficiency although this is not confirmed experimentally. Kostanyan (2008) also shows the theoretical benefits which might accrue from a pulsed mobile phase flow, controlled-cycle counter-current chromatography, where a delay period in the flow allows full equilibration to occur. This technique had previously been used effectively in both distillation and liquid-liquid extraction fields (Mar and Babb 1959; Belter and Speaker 1967).

The first results for sample separations with ICcE, where a sample was injected between a pair of CCC columns and the phases were alternately switched between normal and reversed phase were published by the author (Hewitson et al. 2009). The aim of this work was to

make an initial assessment of ICcE as a high throughput continuous process either for separating compounds into two groups or for isolating and enrichment of a target compound within the columns while washing away the rest of the sample, as Lee had previously discussed for DFCCC (Lee 1996). Using a model mixture sample, based on the GUESSmix containing caffeine, vanillin, naringenin, and carvone (Friesen and Pauli 2005), the feasibility of ICcE to successfully separate compounds from a sample into two eluant streams was demonstrated. Using a conventional twin column DE-Midi preparative instrument (4mm I.D. PFA columns, column volume 912.5 mL) with a hexane, ethyl acetate, methanol and water (4:5:4:5) phase system the sample loading was increased by 56 times (from 200 mg to 11400 mg), the runtime was reduced by a factor of three (from 280 minutes to 90 minutes) and the timings were optimised to split the model mixture into two pairs, whereby caffeine and vanillin eluted with the lower phase and naringenin and carvone eluted with the upper phase. Peak broadening, and therefore lower resolution between the eluting peaks, was observed when compared to the expected resolution for these GUESSmix compounds with high separation factor. Where a target is present at a low concentration in the original crude, which is typical of bioactive compounds found in many herbal extracts, the approach where the target is retained in the column while impurities are washed away from it is useful for enrich and concentration. A representative Chinese herbal medicine (CHM), the herb *Tripterygium wilfordii* Hook. f. (TWHF) was used to study this concentration method. This plant has recently attracted attention due to its anti-cancer activity (Yang et al. 2003; Phillips et al. 2007). However, the main compound responsible for bioactivity, triptolide is present at only 2% in the crude and is difficult to isolate from its neighbour, peritassines A, with sufficient purity, recovery and throughput. Using the same preparative Midi instrument with a hexane, ethyl acetate, methanol and water (2:3:2:3) phase system triptolide was successfully isolated from peritassines A and enriched by retaining the target within the column while washing away all other components of the crude material. The throughput of the ICcE concentrating method was 3100 mg crude extract /hour which was 4.8 times greater than the throughput of a conventional reversed phase isocratic CCC run at 650 mg of crude extract /hour. A total of 188 mg of greater than 98% pure triptolide was extracted in a three hour ICcE run, compared with 8 mg of triptolide from a two hour isocratic CCC run (Ye et al. 2008). For the initial optimisation runs with the GUESSmix, the sample was loaded as a bolus over a slightly extended period of 16 minutes, whereas for the CHM extract the sample was loaded continuously over a two hour period, showing that continuous operation of ICcE was feasible.

2.3.2.4) ICcE scale-up and practical applications

The availability of a 4.6 L twin column CCC unit at Brunel allowed for the scale up of ICcE from prep to pilot scale to be tested (Sutherland et al. 2009). A similar GUESSmix containing carvone, naringenin, caffeine and vanillin was loaded for a total of 20 minutes. The same phase system was used and flow rates had been increased by a factor of five using volumetric scale-up (Wood et al. 2007). The control software for the Maxi instrument did not allow any changes to the flow rates after injection, unlike for the Midi extraction where eluant flow rates were increased after sample injection had stopped to keep the overall flow rate constant. The resultant chromatograms from the Midi and Maxi extractions compared well, with carvone and naringenin in the upper phase eluant and caffeine and vanillin in the lower phase eluant, though vanillin was retained for longer in the Maxi run. A second run, using a slightly more polar hexane, ethyl acetate, methanol and water phase (2:3:2:3) system demonstrated the potential to extract only caffeine with the lower phase from the tail of the column, while eluting the other components with the upper phase from the head outlet.

These preliminary results showed the potential of ICcE as a new scalable extraction method, but a more detailed study is required to understand the robustness of the method across both a range of instrument sizes, at different flow rates and using a broad range of phase system polarities and samples to understand the limitations of the method. This approach is the focus of the later chapters of this thesis.

Yang et al. formulated a model for the prediction of peak elution with ICcE, or intermittent dual CCC, as they describe it (Yang et al. 2009). For a solute fed into the middle of a column the linear velocity in the upper and lower phase steps is calculated. From this the average linear velocity can be determined and knowing the length of the column the retention time of any injected solute and the end from which that solute elutes can be determined. Using single hydrostatic and hydrodynamic columns operated in multiple dual mode, the effect of switching cycles, between normal and reversed phase, on the resolution between two solutes is analysed. The hydrostatic column was mounted on a Pharma-Tech instrument (2.4 m x 0.46 mm I.D. PTFE tubing, column volume 15 mL wound on a 4mm core to give 1200 turns). Using a hexane, ethyl acetate, methanol and 0.1 M HCl phase system (1:1:1:1) to separate DNP-amino acids, DNP-β-ala and DNP-glu, no improvement in resolution was seen between classical isocratic and the MDM separation for similar run times. It is speculated that the lack of improved resolution is due to the time taken to re-establish equilibrium in the

column due to the high (near 50%) dead volume in each loop, which has to be displaced with each switching cycle (Figure 2.18). When switching from descending mode (heavier phase mobile) to ascending mode (lighter phase mobile) the phase in the left had side of each loop in Figure 2.18 has to be displaced into the right had side of the loop before mixing can be re-established. While this displacement is occurring the column is in effect being extruded, so no separation is taking place, reducing the period for which separation occurs in each cycle.

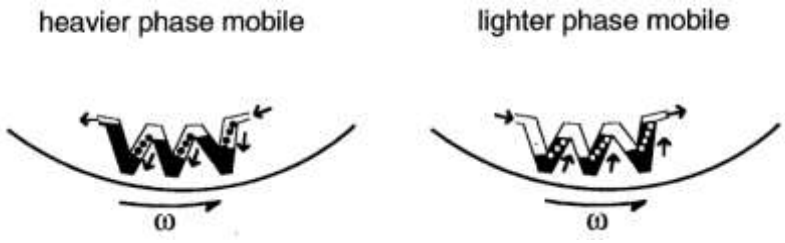


Figure 2.18: Descending and ascending flow in a toroidally wound coil. In both modes the left had side of the loop contains mobile phase while the right hand side of the loop contains the mixing zone (Ito et al. 1998)

Whereas with the hydrodynamic column, mounted on a PC Inc. instrument (50 m x 1.6 mm I.D. PTFE tubing, column volume 130 mL), an improvement in resolution was observed. A mixture of DNP-ser and DNP-asp was separated with a hexane, ethyl acetate, methanol and 0.1M HCl (4:5:4:5) in similar times by classical elution and MDM elution. The MDM elution improved the resolution between the peaks to 1.37 from 0.94 for a classical elution separation. Finally, the experimental retention times are compared with the theoretical retention times derived from the K_d values of the solutes. The results for the hydrostatic and hydrodynamic columns show similar variations of -5% to +6% and -6% to +6% respectively. These variations are in part explained by the effect temperature can have on the static K_d values measured for the solutes. Temperature control on the machines used was limited therefore; small variations in K_d value amplified the variations in retention time.

These results demonstrate the need for hydrostatic systems with minimised dead volume, such as a CPC instrument specifically designed to reduce the inter-chamber volume or the use of hydrodynamic instruments where this dead volume does not arise and the need for good temperature control of the instruments used. As only a single column is used further studies are required to understand the effects of using ICcE with a pair of bobbins connected in series.

Comparison between ICcE and batch CCC has been shown recently for the separation of honokiol and magnolol, the main bioactive isomers in the traditional Chinese medicine

"Houpu", an extract from Magnolia officinalis Rehd. Et Wils. (Peng et al. 2010). A hexane, ethyl acetate, methanol and water (2:1:2:1) phase system was chosen, which according to Yang's theory, gave a negative average linear velocity for magnolol (K_d=1.31), so it eluted with the lower phase and a positive average linear velocity for honokiol (K_d=0.67) so it elutes with the upper phase when the flow rates and switching times are equal. A DE-Midi CCC instrument equipped with twin bobbins, containing both analytical and preparative columns was used firstly for method development on the analytical columns (coil volume 33.0 mL, 0.8 mm I.D. stainless steel) and then scale-up on the preparative columns (coil volume 909 mL, 4.0 mm I.D. PFA tubing). For the analytical separation a standard eluant flow rate of 1 mL/min was used, and a range of sample concentrations was tested and a concentration just below the solubility limit was used (50 mg/mL). The sample flow rate was optimised to 0.2 mL/min giving over 98% purity of both compounds. Scale up to preparative scale was achieved by linear volumetric scale-up of the flow rates using a factor of 25X (Ignatova et al. 2007). With a sample flow rate of 5 mL/min and eluant flow rates of 25 mL/min for both upper and lower phases, a total of 30g of the sample mixture was purified in a two hour period. This is a 3.75X increase in loading over the previously presented classical isocratic results (Chen et al. 2007). The purity of the honokiol fractions was maintained at 98.6%, though the purity of the magnolol fraction was reduced from 99.9% isocratically to 93.7% by the ICcE extraction.

2.4) Conclusions from the literature review

In conclusion, this literature review has highlighted that although DFCCC has been used for continuous processing there is very little understanding of what controls the retention of the phases within the DFCCC column, while the ICcE method is practically untested. Specifically:

- For dual flow counter-current chromatography (DFCCC) the effect of the pressure applied to the outlets will be investigated in Chapter 5, Section 5.4 in an attempt to discover a route to a robust continuous liquid-liquid extraction technology.
- For intermittent counter-current extraction (ICcE) preliminary results have shown the potential of ICcE as a new quasi-continuous scalable extraction method. A more detailed study is now required to understand the robustness of the method across both a range of instrument sizes, at different flow rates and using a broad range of phase system polarities and samples to understand the limitations of the method. This will be the focus of Chapter 5, Sections 5.5. Further in Chapter 6, the application of ICcE to novel applications including industrial waste streams and plant extracts will demonstrate the practical use of the method to industry.

Therefore, two supplementary research questions were formulated:

4) How does the back pressure applied to the outlets of a DFCCC column affect the phase ratio within the column?
5) How robust is the ICcE method to changes in phase system polarity?

These will be investigated in Section 5.

Chapter 3: Methods and materials

Chapter 3 gives the methods used for the experiments discussed in Chapters 5 and 6. The reagents, solvent systems and analysis methods are given in Section 3.1; Section 3.2 describes the stationary phase retention test procedures on all scales of CCC instrument; Section 3.3 describes the DFCCC instrument and standard procedures for operation and Section 3.4 describes the ICcE setups.

3.1) General methodology

Section 3.1 gives the reagents used (Section 3.1.1), solvent system preparation (Section 3.1.2), test tube partitioning studies (Section 3.1.3) and the HPLC analysis of the fractions (Section 3.1.4).

3.1.1) Reagents

The solvents used were purchased from Fisher Chemicals (Loughborough, UK). The methanol and ethyl acetate used for all retention tests were of analytical grade, while hexane was specified grade from a fraction of petroleum. All solvents for HPLC analysis were HPLC grade also supplied by Fisher Chemicals (Loughborough, UK). Deionised water was purified by reverse osmosis from a Purite Select Fusion pure water system (Thame, UK).

A group of compounds which spanned the polarity range was required to test ICcE with the phase systems of different polarities. Therefore, compounds from the GUESSmix (Figure 3.1), a range of naturally occurring bioactive small molecules (Friesen and Pauli 2005) which satisfied this criterion were selected. Biphenyl was added as an alternate non-polar compound due to its ready availability for large scale trials.

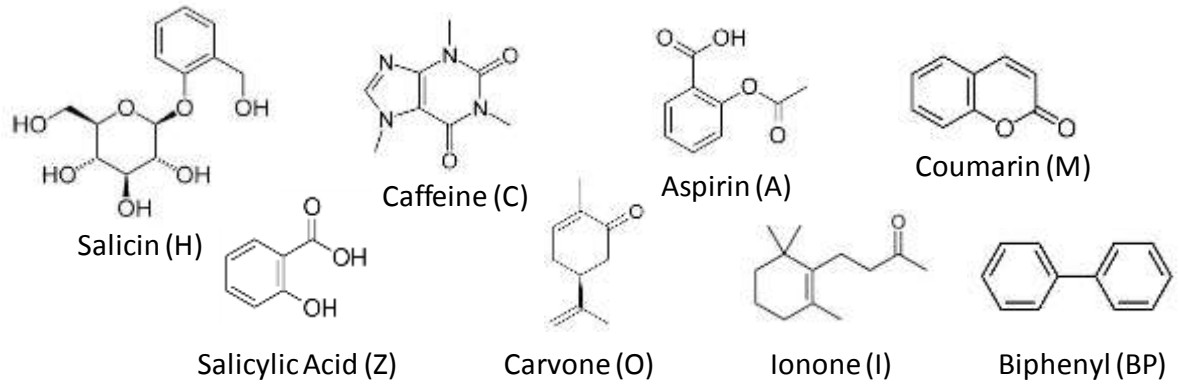


Figure 3.1: Compounds from the GUESS mixture (Friesen and Pauli 2005)

Carvone, salicylic acid, coumarin and caffeine were purchased from Fisher Chemicals (Loughborough, UK) and biphenyl, ionone, aspirin and salicin were purchased from Sigma–Aldrich (Gillingham, UK).

3.1.2) Preparation of phase systems

3.1.2.1) Apparatus for preparation of phase systems

- Measuring cylinders to solvent volumes
- Separating funnel for phase separation

3.1.2.2) Procedure for preparation of phase systems

For all stationary phase retention tests the phase systems were made up classically at room temperature by mixing the solvents required in the given ratio in a separating funnel, shaking vigorously and then allowing them to equilibrate. Five solvent systems were used consisting of hexane, ethyl acetate, methanol and water with volume ratios given in Table 3.1.

Table 3.1: Phase system composition and solvent ratios

Phase System Number	Arizona Letter	Composition	Solvent Ratio
23	U	Hexane:Ethyl Acetate:Methanol:Water	4:1:4:1
19	Q	Hexane:Ethyl Acetate:Methanol:Water	3:2:3:2 (1.5:1:1.5:1)
17	N	Hexane:Ethyl Acetate:Methanol:Water	1:1:1:1
15	L	Hexane:Ethyl Acetate:Methanol:Water	2:3:2:3 (1:1.5:1:1.5)
11	G	Hexane:Ethyl Acetate:Methanol:Water	1:4:1:4

From the solvent ratios given in Table 3.1 the required volume of each solvent was calculated. For example, for HEMWat#17 with a volume ratio for Hexane:Ethyl Acetate:Methanol:Water of 1:1:1:1, to make one litre of phase system, 250 mL of each component was required.

Each component was measured using a clean measuring cylinder and poured into the separating funnel. The order of solvent addition was hexane, ethyl acetate, methanol and finally water to aid initial mixing of the phases.

The separating funnel was shaken vigorously and the phases were allowed to equilibrate for one hour. To ensure the phase system was fully equilibrated, the phase ratio at equilibrium was marked on the separating funnel. The phase system was shaken again and allowed to re-equilibrate for one hour. If the phase ratio had not changed the phase system was confirmed as fully equilibrated.

Further batches of phase system could then be made up with a shorter equilibration time of 15 minutes and without the need for a secondary equilibration as the phase ratio could be checked each time in the separating funnel.

The phases were then separated and stored in Winchesters for use in the various retention tests. As no samples were injected for the retention tests, the phase systems were reused as required. The upper and lower phases were collected at the end of each retention test and separated for reuse in repeat experiments.

3.1.3) Test tube partitioning measurements

Distribution ratios were required for the GUESSmix compounds and various target compounds and impurities in the phase systems used. Static distribution ratios were determined by dispensed upper phase (0.5 mL) and lower phase (0.5 mL) into a HPLC vial. The model compound or crude mixture (0.5 mg) was added. The vial was shaken vigorously until equilibrium had been established between the phases. Equal volumes (0.1 mL) of upper and lower phases were pipetted into separate HPLC vials and evaporated to dryness under vacuum. Finally, the residues were dissolved with methanol (1 mL) and analysed by HPLC. The distribution ratio/partition coefficient (K_d) of a particular compound in reversed phase mode was calculated as the ratio of peak area in the upper (stationary) phase to the peak area in the lower (mobile) phase. In normal phase mode, the K_d value would be the reciprocal of these values.

3.1.4) HPLC analysis of fractions

For all ICcE runs the fractions produced were analysed by HPLC to confirm the position and purity of the eluted peaks. For example, the modified GUESSmix was used as the model sample system. This mixture contained caffeine, aspirin, coumarin, salicylic acid and carvone at 10 mg/mL each and salicin and biphenyl at 5 mg/mL made up in a mixture of upper and lower phases. 50 µl samples from the fractions collected in the ICcE run were diluted with 950 µl of methanol and were analysed on a reversed-phase Waters Symmetry

C18 column (75 mm × 4.6 mm I.D. 3.5 μm) thermostated at 40 °C. Mobile phase was a mixture of A (0.1% aqueous formic acid) and B (acetonitrile) in a gradient program with a flow rate of 1 mL/min: 0-6 min ramp up from 5 to 95% B, 6-8 min hold 95% B. Eluant was monitored using a DAD detector.

3.2) Isocratic CCC stationary phase retention methodology

A standard isocratic CCC system is used, as shown in Figure 3.2, for the measurement of stationary phase retention. On all three scales of equipment, the semi-prep Spectrum scale (143.5 mL), the Midi scale (912.5 mL) and the pilot Maxi scale (4.6 L), a similar methodology was adopted. Section 3.2.1 describes the apparatus, while Sections 3.2.2 and 3.2.3 detail the normal and reversed phase methodology for the Midi instrument. Section 3.2.4 briefly discusses the differences when using the Spectrum and Maxi instruments. The instrument parameters are given in Table 3.2. For all cases the stationary phase retention was measured on a single column, with the second column being filled with a 20% methanol water solution to keep the rotors mechanically balanced.

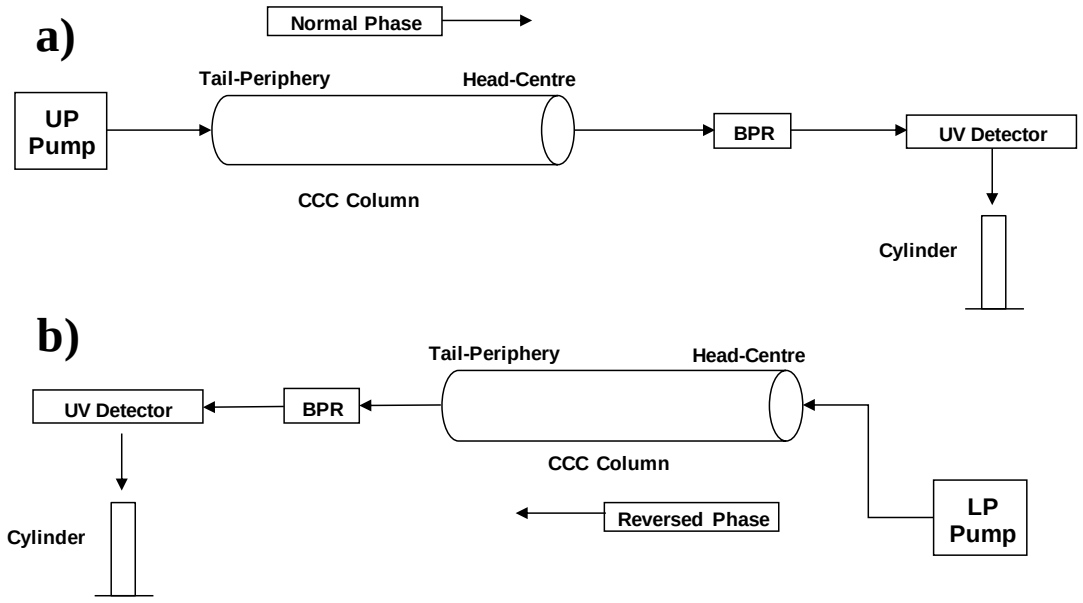


Figure 3.2: Isocratic CCC flow path: a) Normal phase elution, b) Reversed Phase Elution

Table 3.2: CCC Instrument parameters

Instrument	Spectrum	Midi	4.6L Maxi	18L Maxi
Column volume (mL)	143.5	912.5	4600	17150
Column material	PTFE	PFA	PFA	PFA
Column bore (mm)	1.6	4.0	10.0	10.0
Rotor radius (mm)	85	110	300	300
Rotor speed (rpm)	1600	1400	600	600
Max *g*-field	240	240	120	120

3.2.1) Apparatus for stationary phase retention measurements

- High performance J-type Midi CCC instrument (Dynamic Extractions, Slough, UK) fitted with 4 mm I.D. preparative columns made of polyfluoroalkoxy tubing (PFA) with column volumes of 459.5 mL and 453.0 mL.
- Chilled water system to cool the centrifuge
- Water bath to maintain the phase system temperatures
- Upper and lower phase solvent containers
- Mobile phase pump – Knauer K-1800 pump with 1000 mL heads (Berlin, Germany)
- Stationary phase pump – Armen preparative pump with 100 mL heads (Armen Instrument, Vannes, France)
- Measuring cylinders to collect displaced phases
- 4bar nitrogen supply to empty columns
- Stopwatch

3.2.2) Normal phase retention procedure on the Midi instrument

For normal phase elution and retention studies the upper organic phase is the mobile phase and the lower aqueous phase is the stationary phase as shown in Figure 3.2a. The HEMWat phase systems given in Table 3.2 were used.

The preparative columns on the DE-Midi were initially emptied with nitrogen gas, from the centre inlet to periphery outlet, while rotating the column at 200 rpm in reverse to place the head of the column at the periphery outlet. This mode of operation purges all the liquid from the column.

The upper and lower phase pumps were initially primed with their respective phases. Both pumps had 6.9 bar (100 psi) back pressure regulators fitted directly after the pump heads to ensure the action of the CCC centrifuge as a positive pressure pump did not allow siphoning or draw through of phases when the instrument was operating.

All liquid phases and CCC columns were thermostatically controlled at 30 °C. The empty columns were filled with the lower stationary phase at a flow rate of 200 mL/min from periphery to centre so displacing the air in the columns. Once filled, the columns were rotated forwards, at 1400rpm, placing the head at the centre of the column and the tail at the periphery, to displace any remaining air inside the columns. When a clear flow of phase was observed from the column, the stationary phase flow was stopped.

Once the instrument temperature was stable at 30 °C, the flow of the mobile phase and the stopwatch were started. The eluted phases were collected in measuring cylinders. The initial flow was 20 mL/min.

Only stationary phase was eluted until breakthrough, when the mobile phase starts to elute from the column. For a period of time both mobile and stationary phase elute from the column. Collection of phase system continued until no more stationary phase had been observed eluting from the column for at least two minutes. The total stationary phase eluted was calculated from the sum of the portions of stationary phase in each cylinder.

The mobile phase flow rate was then increased to 40 mL/min and the eluted phases were collected, as previously, until no more stationary phase was displaced for at least two minutes. The stationary phase eluted (V_c) at this new flow rate was determined and the total stationary phase remaining in the column (V_s) can therefore be calculated as follows:

$$V_s = V_c - V_d \qquad \text{... (Eq. 3.1)}$$

Where V_d is the dead volume of the system which is calculated from the known lengths and bores of the tubing in the system, and was confirmed by Wood plots (Wood et al. 2003B).

The flow rate was increased stepwise and the methodology was repeated until at least 60% of the stationary phase had been eluted from the column or the flow rate had reached 420 mL/min.

At each stage the flow rate was confirmed by checking the volume of phase eluted over one minute.

At the end of the run the pump was stopped first and valves were closed to seal off the column, then the rotation of the centrifuge was stopped.

To confirm the volume of stationary phase remaining the column the system was emptied using nitrogen as described initially. The eluted phases were collected into a measuring cylinder to measure the total stationary phase remaining in the system. This was checked against the final calculated stationary phase volume as an error check.

3.2.3) Reversed phase retention procedure on the Midi instrument

The reversed phase stationary phase elution and retention procedure is similar to the normal phase method, using the same apparatus given above and the setup shown in Figure 3.2b, except that:

- for reversed phase the lower aqueous phase is the mobile phase and the upper organic phase is the stationary phase
- to retain the upper stationary phase in the column the mobile phase flows from head-centre to tail-periphery

As for the normal phase retention procedure, the columns are initially emptied with nitrogen flowed from centre to periphery with the columns slowly rotating in reverse. The columns are then filled with the upper stationary phase from tail-periphery to head-centre, displacing the air in the columns, and the columns are rotated forwards to ensure all air is displaced. The column rotation was then stopped. The column was then reconnected such that the mobile lower phase flows from head-centre to tail-periphery. The columns were rotated forwards at 1400rpm and once the instrument had reached full speed the flow of mobile phase was started. As previously the eluted phases were collected in measuring cylinders to determine the total stationary phase eluted at each flow rate, to a maximum of 420 mL/min. Similarly, at the end of the run the column was emptied to confirm the total stationary phase remaining in the column.

3.2.4) Stationary phase retention tests on the Spectrum and Maxi CCC instruments

The procedures used for testing the stationary phase retention on the Spectrum and Maxi instruments were similar to that described for the Midi instrument, except that due to the differences in scale different pumps and flow rates were required.

For the Spectrum instrument an Agilent 1200 preparative pumping system was used controlled by Agilent Chemstation software. The system also had 6.9 bar (100 psi) back pressure regulators installed directly after the pumps to ensure stable flow from the HPLC pumps. Flow rates from 1 mL/min to a maximum of 72 mL/min where used.

For the Maxi instruments a bespoke Armen Glider pumping system as used. This system has four triple headed pumps to minimise flow pulsatility. Each pump can operate up to 3000 mL/min. A visual display allows the setting of the required parameters, flows, operating pressures, serial or parallel flow through the columns, UV detection wavelength and operating modes, isocratic normal, reversed phase or intermittent switching (Figure 3.3). Flow rates from 100 to 1200 mL/min were used.

Figure 3.3: Armen Glider pumping system visual display for control of pumps, UV detector and fraction collector when operating the Maxi CCC instruments

3.3) Dual Flow Counter Current Chromatography (DFCCC) methodology

The dual flow counter-current chromatography (DFCCC) instrument is a specially designed preparative (561 mL coil volume) bobbin (supplied by Dynamic Extractions, Slough, UK) mounted in a standard Midi-CCC case. The instrument is designed to allow the continuous flow of phase systems truly counter-current to each other with a sample being loaded continuously at the mid-point of the column. Therefore, the sample will be split into two streams with more polar compounds flowing and eluting with the more polar phase and less polar compounds eluting with the less polar phase.

The column itself is constructed from 5mm polyfluoroalkoxy (PFA) tubing. The end fittings on the column were designed to allow both inlet and outlet connections at each end of the column, such that the phase systems can flow continuously past each other, the upper phase flowing from the tail-periphery to the head centre of the column while the lower phase flows in the opposite direction. The inlet tubes to the column are extended into the column by one metre to ensure there is no back flow of phase system directly down the adjacent outlet port. The midpoint of the column has a tee-piece fitted to allow continuous sample injection.

Although the scale-up of DFCCC had been successfully demonstrated two significant complications exist. Firstly, the phase ratio in the column is important as it determines the columns loading capacity. If the sample concentration or sample flow is increased this may lead to solvent system overload leading to either precipitation of the compounds in the column or compounds may elute from both ends of the column. For complex sample mixtures the best chance of high initial loading will be with a 50%/50%, or in the range 40%/60% phase ratio. Secondly, it has been observed that to maintain the upper phase in the column either a needle valve (Ito 1985) or a length of capillary tubing (Goto, Ito et al. 2006) was attached to the head-centre of the column to provide back pressure. No systematic studies have been reported into the back pressure requirements for a DFCCC or its effect on the phase volume ratio in the column. Therefore methodology was developed to investigate these parameters further.

3.3.1) Apparatus for the dual flow experiments

Figure 3.4 shows a schematic of the equipment setup used for the dual flow experiments. The equipment used is listed below. Back pressure regulation was achieved using lengths of 0.8 mm I.D. PTFE tubing or Swagelok precision back pressure regulators.

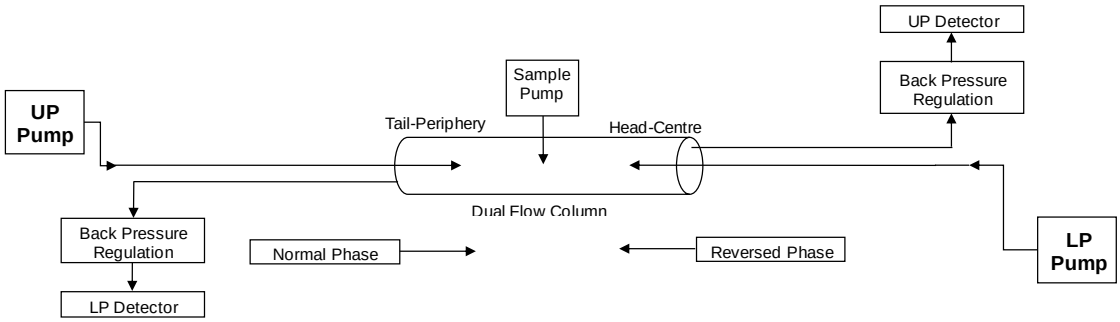


Figure 3.4: DFCCC flow path

- High performance J-type Midi CCC instrument case (Dynamic Extractions, Slough, UK) fitted with bespoke 5 mm I.D. preparative dual-flow column
- Chilled water system to cool the centrifuge
- Water bath to maintain the phase system temperatures
- Upper and lower phase solvent containers
- Upper phase pump – Knauer[4] K-1800 pump with 1000 mL heads
- Lower phase pump – Knauer K-1800 pump with 250 mL heads
- Two Knauer K-2501 Spectrophotometers
- Knauer K-501 Analytical pump to inject the sample
- Measuring cylinders to collect displaced phases
- 4bar nitrogen supply to empty columns
- Computer running Knauer Eurochrom HPLC software to collect UV spectrophotometry data

3.3.2) Methodology for maintaining the phase volume ratio for DFCCC

The effect of the back pressure applied to the centre outlet on the phase volume ratio within the DFCCC column was assessed. Section 3.3.2.1 details the filling and equilibration

[4] Knauer, Berlin, Germany

procedure, including pre-filling of the columns to achieve equilibrium quickly and therefore improve cycle times and Section 3.3.2.2 details the methodology for the measurement of back pressure generated by a length of capillary tubing.

3.3.2.1) Protocol for filling and equilibrating the DFCCC column

Firstly, a given length of capillary tubing was attached to the centre outlet of the DFCCC. The upper and lower phases and the DFCCC column were thermostatically controlled at 30 °C. The empty column was filled with upper phase at 100 mL/min from tail-periphery to head-centre. To purge any air, the column was rotated at 200 rpm and all outlets were opened in sequence. The instrument rotation was increased to 1000 rpm. The flows of the upper and lower phases were simultaneously started. The phases eluted from the centre and periphery outlets were collected at one minute intervals for the first ten minutes, then two minute intervals to 30 minutes and finally 5 minute intervals until the total volume of each phase leaving the column was equal to the volume entering the column for three consecutive fractions, showing the column was at equilibrium. The flows of the upper and lower phases were stopped and valves closed to seal the column. The rotation was stopped.

The column was emptied into a measuring cylinder to confirm the phase ratio. With the periphery outlet open, nitrogen gas was flowed into the centre of the coil, while rotating in reverse direction at 200 rpm to place the head at the periphery. This purged all the liquid phases from the column.

To pre-fill the DFCCC column with a given phase volume ratio the empty column was initially filled with upper phase at 100 mL/min and purged of air as described above. Once full of upper phase the rotation was stopped. Lower phase was then pumped into the column at 80 mL/min to displace a given volume of upper phase, which was measured in a cylinder. Once the required volume ratio had been reached the lower phase flow was stopped. The machine rotation was then set to 1000 rpm and the stability of the phase volume ratio set within the column was tested by flowing upper and lower phases and measuring the resultant eluant flows as described above.

For the separation runs with the DFCCC the back pressure on the outlets of the column was maintained with two adjustable 0-6.9 bar (0-100 psi) compact back pressure regulators (Swagelok, Kings Langley, UK, Serial no. KCB1F0A2D5P10000). The sample was loaded

through the mid-point inlet and the back pressure on the centre outlet was manually adjusted throughout the runs to keep the flow of eluant equal to the upper phase inlet flow.

3.3.2.2) Back pressure produced by lengths of capillary tubing

- Knauer K-1800 pump with 250 mL heads to pump phases
- Upchurch[5] 6.9 bar (100 psi) back pressure regulator P-787 (BPR)
- Pressure transducer 0-10 Bar, 0-5 V output (RS components[6] 348-8093)
- Computer with Picolog[7] ADC-11 analogue to digital convertor to collect pressure signal

Figure 3.5 shows the setup used to measure the back pressure generated by a flow of phase through a known length of capillary tubing. Knowing this, the actual back pressure required to maintain a stable phase ratio in the column could be calculated (See Section 5.4) and the capillary tubing could then be replaced by standard back pressure regulators.

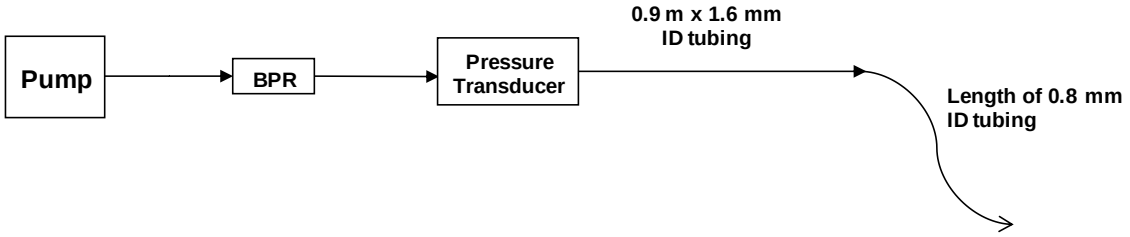


Figure 3.5: Back pressure measurement setup

Either 0.5, 1.0 or 2.0 m length of 0.8 mm ID PTFE capillary tubing was attached to the end of the flow path. Phase was flowed at 20, 40, 60, 80 and 100 mL/min using the Knauer HPLC pump and the back pressure was recorded for a 20 second period at each flow rate. The average pressure over this 20 second period was calculated from the recorded data. Blank measurements with no tubing connected were also recorded.

[5] IDEX Health & Science, Oak Harbor, WA, USA
[6] RS Components Ltd. Corby, UK
[7] Pico Technology, St. Neots, UK

3.4) ICcE methodology

For an intermittent counter-current extraction the sample solution is loaded as a bolus or continuous injection between the columns and the mobile phase was flowed alternately, first in normal phase (upper phase mobile, from tail-periphery to head-centre) and then in reversed phase (lower phase mobile, from head-centre to tail-periphery). Switching between normal and reversed phase was carried out at regular intervals. If the flow rates are equal and the switching intervals are equal, compounds with a distribution ratio below one (for reversed phase) should elute with the lower phase from one end of the column, and compounds with a distribution ratio above one should elute with the upper phase from the other end of the column. That is, more hydrophobic compounds tend to be carried towards the head of the columns with the organic upper phase, while more hydrophilic compounds tend to be carried by the aqueous lower phase towards the tail of the columns.

The methodology required to run ICcE on a Midi instrument is described, followed by the requirements to scale down to the Spectrum instrument and scale up to the Maxi instrument.

3.4.1) Apparatus

Figure 3.6 shows the setup used for ICcE with the flow paths used depending whether flow is a) normal phase or b) reversed phase depending on the positions of the two switching valves V1 and V2.

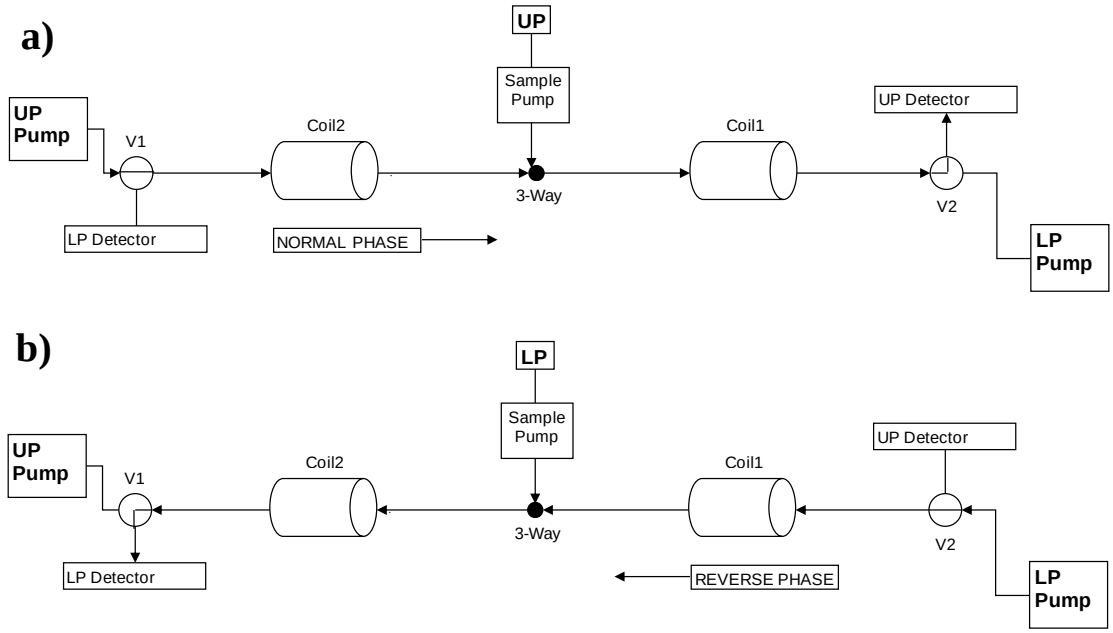


Figure 3.6: ICcE Setup and flow paths for normal and reversed phase switching operations

The apparatus required for ICcE is similar to that required for DFCCC except that a standard twin bobbin CCC instrument is used and additional valving and control software is required to switch the flow intermittently between normal and reversed phase operation.

- A high performance J-type Midi CCC instrument (Dynamic Extractions, Slough, UK) fitted with 4 mm I.D. preparative columns made of polyfluoroalkoxy tubing (PFA) with volumes of 459.5 mL and 453.0 mL.
- Two Knauer[8] K-6 (6-way/2-position) automatic valves to switch between normal and reversed phase
- Chilled water system to cool the centrifuge
- Water bath to maintain the phase system temperatures
- Upper and lower phase solvent containers
- Upper phase pump – Knauer K-1800 pump with 1000 mL heads

[8] Knauer, Berlin, Germany

- Lower phase pump – Knauer K-1800 pump with 250 mL heads
- Two Knauer K-2501 Spectrophotometers
- Knauer K-501 Analytical pump to inject the sample
- Measuring cylinders to collect displaced phases
- 4bar nitrogen supply to empty columns
- Computer running Knauer Eurochrom HPLC software to collect UV spectrophotometry data

3.4.2) Filling the coils and establishing hydrodynamic equilibrium

Using the water bath and the thermostatic control on the Midi instrument, all the liquid phases and the CCC columns were thermostatically controlled at 30 °C. For each ICcE run the initial phase ratio in the column was set to 50%/50% of upper to lower phase. This was achieved by equilibrating the columns in reversed phase at a constant flow rate of 80 mL/min while adjusting the rotational speed of the instrument based on the previously measured stationary phase retention data.

Firstly, the empty columns were filled with upper phase at a flow rate of 200 mL/min from tail-periphery to head-centre with the coils rotating at 200rpm to displace all the air inside the coils. The centrifuge rotation was increased to 1400rpm and the temperature was allowed to equilibrate at 30 °C. The rotational speed of the instrument was then reduced to the value given in Table 3.3 for the specific phase system being used. The lower mobile phase flow was started at 80 mL/min and the eluted phases were collected in a measuring cylinder to confirm the volume of stationary upper phase eluted. Once breakthrough had occurred the rotation speed was increased to 1400 rpm as required.

Table 3.3: Rotational speed required to achieve 50% phase retention in reversed phase for a DE-Midi centrifuge at 80 mL/min for given phase systems

HEMWat Phase system	Rotational Speed (rpm)	S_f (%)
23	580	53
19	575	53
17	650	55
15	1400	60
11	1400	47

3.4.3) ICcE running conditions

Compared to isocratic CCC, ICcE has additional operational variables that have to be optimised – the flow ratio, the flow difference and the respective time intervals between switching from normal to reversed phase. For the Midi setup, the flow rates of the pumps were controlled manually. While the switching times between normal and reversed phase cycles were controlled automatically from the Knauer Eurochrom software by setting up a digital output switching table to alternately switch the two Knauer 6-way/2-position valves.

3.4.3.1) Operating conditions for phase retention stability within columns

To test the stability of the phase retention within the columns ICcE was run without sample injection. After equilibration, switching cycles were run starting in normal phase. Twelve cycles were run each with four minutes of normal phase elution followed by four minutes of reversed phase elution. The four minute switching time (8 minute cycle time) was chosen to as a balance between ensuring a short enough time for separation to occur without target compounds eluting from both ends of the column, but long enough between cycle switches to ensure long term mechanical durability of the switching valves. The eluted phase was collected and volumes measured to check the volume of phase displaced with each switching cycle.

The mobile phase flow was stopped and then the rotation was stopped. The two columns were emptied separately by flowing nitrogen (4 bar) into the centre of the column while rotating at 200rpm in reverse, placing the head of the column at the periphery. The volume of each phase remaining in the coils was measured.

3.4.3.2) Separations using ICcE

With ICcE the sample is loaded between the columns with an HPLC pump, unlike for isocratic elution where the sample is normally loaded from a sample loop. At the midpoint between the columns a tee-piece is inserted into the system. The sample is made up in a mixture of upper and lower phase such that when the system is running in normal phase mode, the sample is loaded in upper phase, while when the system is running in reversed phase mode the sample is loaded in lower phase. Sample loading was achieved by drawing the sample solution from a measuring cylinder containing the sample in both upper and lower phase. The draw tube was manually moved between the upper and lower phases in the cylinder dependant on which phase was the mobile phase eluant.

For sample separations the columns were equilibrated as described previously. Then the system was run in normal phase with the upper phase mobile at 35 mL/min, for example. After four minutes the flow was switched to reversed phase mode with the lower phase mobile at 35 mL/min. This cycle was repeated. The sample, made up in equal volumes of upper and lower phase, was loaded through the sample pump, at the midpoint between the columns at 5.5 mL/min in the same phase as the eluant flow. The sample was loaded for the first eight complete cycles, 64 minutes in total. At the end of the sample loading stage the eluant flows were increased to 40 mL/min to maintain the same mean flow rate (previously it had been 35 + 5.5 mL/min) and continued for a further 14 cycles. For all runs the upper and lower phase eluants were monitored with a UV detector. Fractions were collected every four minutes for analysis by HPLC. From this data, fractograms were constructed showing the position of the eluted peaks with respect to time. At the end of each run the columns were emptied separately with nitrogen (4 bar), while rotating the coil in reverse at 200rpm and fractionated (100 mL fractions) for analysis by HPLC.

3.4.4) ICcE on the Spectrum and Maxi instruments

The procedure for ICcE on the smaller scale Spectrum instrument and larger sale Maxi instrument is similar to the used on the Midi instrument.

For the Spectrum instrument a pair of Agilent 1200 preparative pumping systems was used controlled by Agilent Chemstation software with valving to switch flows between normal and reversed phase and Agilent multiple wavelength detectors (MWD), with preparative flow cells (0.3mm cell path length) to record the UV absorbance of compounds in the eluant flow. Finally, fraction collectors were used to automate the fraction collection for later HPLC analysis. The Chemstation software was programmed with timetables to switch the flow

between normal and reversed phase as required and allowed the automatic adjustment of the eluant flow once the sample injection was completed.

For the Maxi instrument the bespoke Armen Glider pumping system was used (Figure 3.7). This allowed fully automated control of the flow of eluants and sample feeds. The triple headed pulse free pumps allow flow rates up to 3000 mL/min a preparative UV detector monitors the eluant flow. Switching of the flow paths is achieved with Swagelok ball valves controlled by the Armen Glider software. The switching intervals for the upper and lower phases can be set independently.

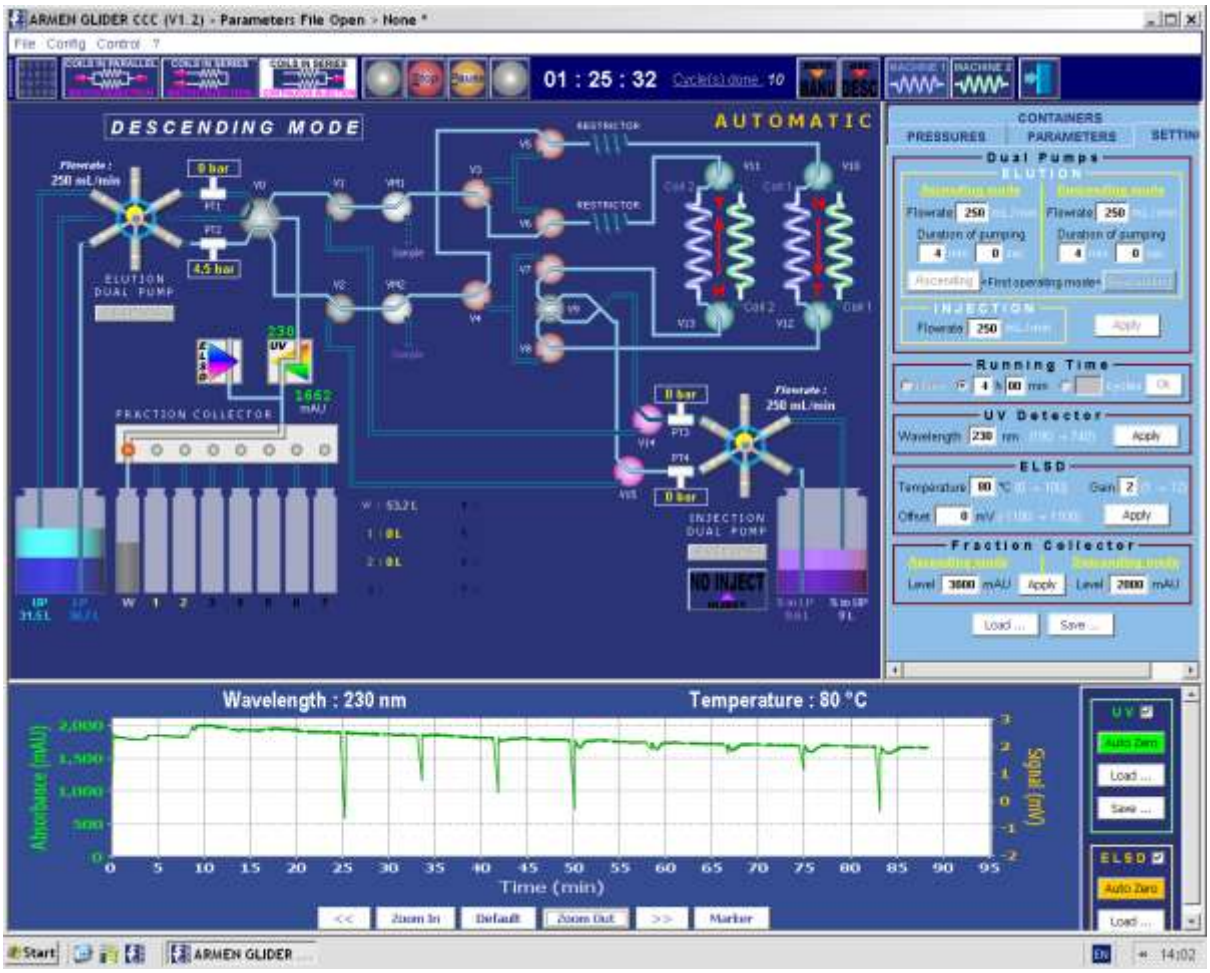


Figure 3.7: Armen Glider pumping system visual display for control of pumps, UV detector and fraction collector when operating the Maxi CCC instruments

Chapter 4: Theoretical Models of ICcE

A theory of chromatography "employing two liquid phases" was developed in the early 1940's (Martin and Synge 1941) based on the partition of compounds between two liquid phases. This theory was further developed into a theory of counter current distribution (CCD) based on the transfers between test-tubes with equal volumes of upper and lower phase (Williamson and Craig 1947) and also to determine the number of transfers required to give a significant difference between two peaks (Nichols Jr 1950). Chromatography being achieved with a column of test tubes where the mobile phase is transferred along the train of test tubes to the adjacent tubes, equilibrated and transferred to the next test tube in the train. This cycle is repeated to achieve separation of compounds based on their partition between the two liquid phases used. For continuous operation, a theory was described by Craig and Craig (1956), as discussed in Section 2.3.1, where a sample is introduced into the middle of the chain of tubes, known as counter double current distribution (CDCD). More recently, an ideally mixed cells model and a diffusion longitudinal mixing model were described by Kostanian (2002) and CCD was used as the basis for an eluting CCD model (Sutherland et al. 2003), both models being developed specifically for use with CCC instruments. An enhanced model based on eluting CCD has recently been described (de Folter and Sutherland 2009) to give predictions for isocratic, co-current and dual flow CCC operating modes.

In Section 4.1 an initial model for ICcE is developed to allow prediction of the compound elution order from each side of a pair of ICcE columns. The model allows adjustment of flow, stationary phase and timing to give the elution time for any given K_d value. This allows the setting of initial flow rate parameters to achieve separation in an ICcE system based on the range of flow rates and stationary phase retention values which can be used for an isocratic CCC run. These values are experimentally determined in Section 5.3.

This model is extended in Section 4.2 to describe the optimal and practical points for the separation of a pair of compounds. For a given time switching ratio (the time the upper phase flows compared to the time the lower phase flows for each cycle) the optimum flow rates and practical flow rates can be calculated.

In Section 4.3 the models described are compared to models developed independently by Yang and later Völkl and subsequently published (Yang et al. 2009; Völkl et al. 2013).

4.1) Separation of compounds from a mixture by ICcE with respect to time

In this section, a model is derived to relate the retention times in an ICcE method to the K_d values of compounds in a sample feed. The model allows the user to visualise how compounds with different distribution ratios, K_d (where K_d for this model is the concentration of solute in the stationary lower phase (C_L) divided by the concentration of solute in the upper phase (C_U), the normal phase distribution ratio) elute from either end of the column in terms of elution/retention time (t_p) for a given K_d value. Inputs to the model are the column volume (V_C), the stationary phase retention (S_f), the flow rates of the upper phase eluant (F_U) and lower phase eluant (F_L), the flow rate of the sample in upper phase (F_{SU}) or lower phase (F_{LU}) and the switching time of the upper phase step (t_U) or of the lower phase step (t_L). The initial model does not account for the effects of band broadening.

It also allows the user to determine which compounds will be retained in the columns dependant on the run time and which compounds will be seen to elute from both ends of the column.

The distribution ratios for the compounds to be separated can be determined from simple test tube partitioning measurements (Section 3.4.1). With these experimentally determined distribution ratios of the compounds to be separated it is possible to use the model to empirically set the flows and switching times to determine a starting point for the required separation.

For a single CCC column, the progression of a solute peak (F_P) of a compound of given distribution ratio (K_d) can be calculated, if the column volume (V_C), volumes of mobile (V_M) and stationary phase (V_S) and mobile phase flow rate (F_M) are known and from this flow rate the volume progressed by a solute peak (V_p) within the column can be calculated for each switching interval.

For the model it is assumed that both columns are identical in volume, that the volume of the flying leads is negligible, that switching between normal and reversed phase occurs instantly and that K_d values are not concentration dependant. Further, the maximum flow of the mobile phase cannot be higher than allowed by the stationary phase retention, otherwise then columns would strip stationary phase.

From first principles, the partition ratio (k') is defined, in Equation 4.1.1, as the ratio of the mass of a compound in the stationary phase (m_s) with respect to the mass of that compound in

the mobile phase (m_m). C_S and C_M are the concentrations of solute in the stationary and mobile phases respectively. Figure 4.1.1 shows the relationship of partition ratio to the column volume graphically.

$$k' = \frac{m_S}{m_M} = \frac{C_S V_S}{C_M V_M} = \frac{K_d V_S}{V_M} \qquad \text{... (Eq. 4.1.1)}$$

Equation 4.1.1 can be derived in terms of the distribution ratio (K_d) as above or the stationary phase retention (S_f) as follows. The stationary phase volume ratio (Sf) is defined as:

$$S_f = \frac{V_S}{V_C} \qquad \text{... (Eq. 4.1.2)}$$

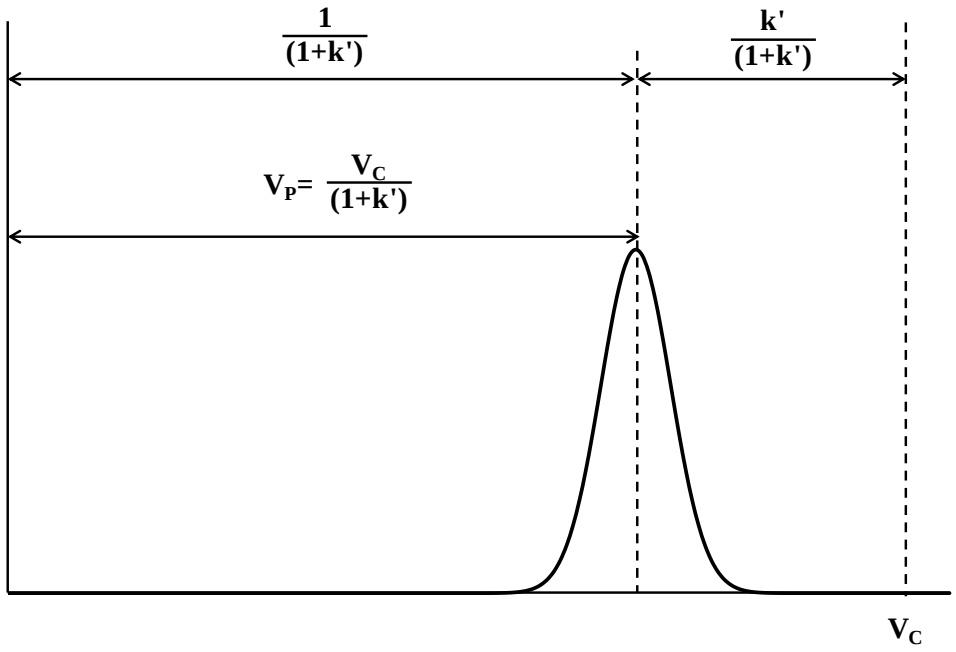


Figure 4.1.1: Distribution of a compound with partition ratio, k', in the column at the point of elution of solvent front

Rearranging equation 4.1.2,

$$V_S = S_f V_C \qquad \text{... (Eq. 4.1.3)}$$

The volume of the column is the sum of mobile and stationary phase volumes as follows:

$$V_C = V_M + V_S \qquad \text{... (Eq. 4.1.4)}$$

Combining equations 4.1.3 and 4.1.4 and making V_M the subject results in:

$$V_M = V_C - V_S = V_C - S_f V_C = (1-S_f)V_C \quad \text{... (Eq. 4.1.5)}$$

Substituting for V_M from equation 4.1.5 in equation 4.1.1 gives:

$$k' = K_d \frac{S_f V_C}{V_C - S_f V_C} = \frac{K_d S_f}{(1-S_f)} \quad \text{... (Eq. 4.1.6)}$$

The time (t_M) for the mobile phase to pass through the column volume (V_C) is the mobile phase volume (V_M) divided by the mobile phase flow rate (F_M).

$$t_M = \frac{V_M}{F_M} \quad \text{... (Eq. 4.1.7)}$$

In that time a peak of partition ratio (k') will have travelled $\frac{V_C}{1+k'}$ through the column in volume terms as shown in Figure 4.1.2. Therefore the time for this peak to elute (t_p) can be calculated.

$$t_P = t_M \frac{V_C}{\left[\frac{V_C}{(1+k')}\right]} = t_M(1+k') \quad \text{... (Eq. 4.1.8)}$$

Substituting for k' from Eq. 4.1.6 gives t_P in terms of K_d, S_f and t_M,

$$t_P = t_M\left(1 + \frac{K_d S_f}{(1-S_f)}\right) = \frac{t_M(1-S_f+K_d S_f)}{1-S_f} = \frac{t_M(1+S_f(K_d-1))}{1-S_f} \quad \text{... (Eq.4.1.9)}$$

Now the progression rate of the peak (F_P) can be calculated from the volume progressed by the solute peak (V_P) divided by the time for the mobile phase to pass through the column (t_M).

$$F_P = \frac{V_P}{t_M} \quad \text{... (Eq. 4.1.10)}$$

From Figure 4.1.1 it can been seen that,

$$V_P = \frac{V_C}{(1+k')} \quad \text{... (Eq. 4.1.11)}$$

Therefore combining Eq. 4.1.10 and 4.1.11 gives,

$$F_P = \frac{V_C}{(1+k')t_M} \quad \text{... (Eq. 4.1.12)}$$

The denominator of this equation is t_P (Equation 4.1.8), therefore,

$$F_P = \frac{V_C}{t_P} \quad \text{... (Eq. 4.1.13)}$$

Substituting for t_P using Equation 4.1.9 gives,

$$F_P = \frac{V_C(1-S_f)}{t_M(1+S_f(K_d-1))} \quad \text{... (Eq. 4.1.14)}$$

The numerator of this equation equals V_M (Eq. 4.1.5) and substituting for t_M (Eq. 4.1.7) gives,

$$F_P = \frac{F_M}{1+S_f(K_d-1)} \quad \text{... (Eq. 4.1.15)}$$

The coil volume progressed (V_P) in a given time (t) can therefore be calculated as the progression rate of the solute peak (F_P) multiplied by the time that solute peak progresses for,

$$V_P = F_P \cdot t \quad \text{… (Eq. 4.1.16)}$$

therefore,

$$V_P = \frac{F_M t}{1+S_f(K_d-1)} \quad \text{… (Eq. 4.1.17)}$$

With ICcE there are two bobbins, which are operated intermittently in normal and reversed phase and sample is flowed into the system between the bobbins in the mobile phase. There are therefore four potential progression rates for each solute peak depending on which bobbin the peak is in and whether the flow mode is reversed or normal phase. The flow rate in the second bobbin is increased by the volume of sample flow and the flow mode (reversed or normal phase) will change the flow direction as shown in Figure 4.1.2.

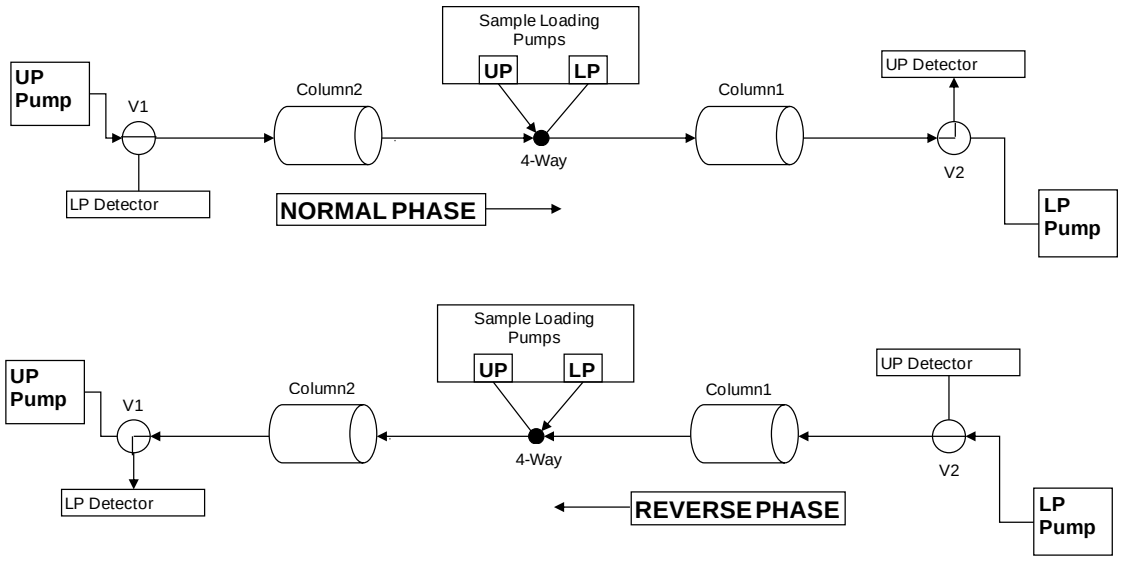


Figure 4.1.2: ICcE operating modes. Valves V1 and V2 are switched intermittently to allow operation in either normal or reversed phase modes

For normal phase flow with the two bobbins, Equation 4.1.17 can be rewritten as,

$$V_{PnC1} = \frac{(F_U + F_{SU})t_U}{1 + S_{f1}(K_d - 1)} \quad \text{… (Eq. 4.1.18)}$$

$$V_{PnC2} = \frac{F_U t_U}{1 + S_{f2}(K_d - 1)} \quad \text{… (Eq. 4.1.19)}$$

where t_U is the intermittent time for normal phase flow, S_{f1} and S_{f2} are the stationary phase retentions of the two bobbins in normal phase, V_{PnC1} is the volume in column 1 a solute peak travels in one step and V_{PnC2} is the volume a peak traverses in column 2.

For reversed phase flow, the normal phase K_d value is inverted and the stationary phase retention is $(1\text{-}S_f)$, therefore Equation 4.1.17 becomes,

$$V_{PrC1} = \frac{F_L t_L}{1 + (1 - S_{f1})(\frac{1}{K_d} - 1)} \quad \text{… (Eq. 4.1.20)}$$

$$V_{PrC2} = \frac{(F_L + F_{SL})t_L}{1 + (1 - S_{f2})(\frac{1}{K_d} - 1)} \quad \text{… (Eq. 4.1.21)}$$

where t_L is the intermittent time for reversed phase flow, V_{PrC1} is the volume in column 1 a solute peak travels in one step and V_{PrC2} is the volume a peak traverses in column 2. Figure 4.1.3 shows graphically these volumes plotted against K_d. Experimentally this graph is useful as it visually shows compounds with which K_d values will potentially elute from a column and which K_d values are retained within the column in each step.

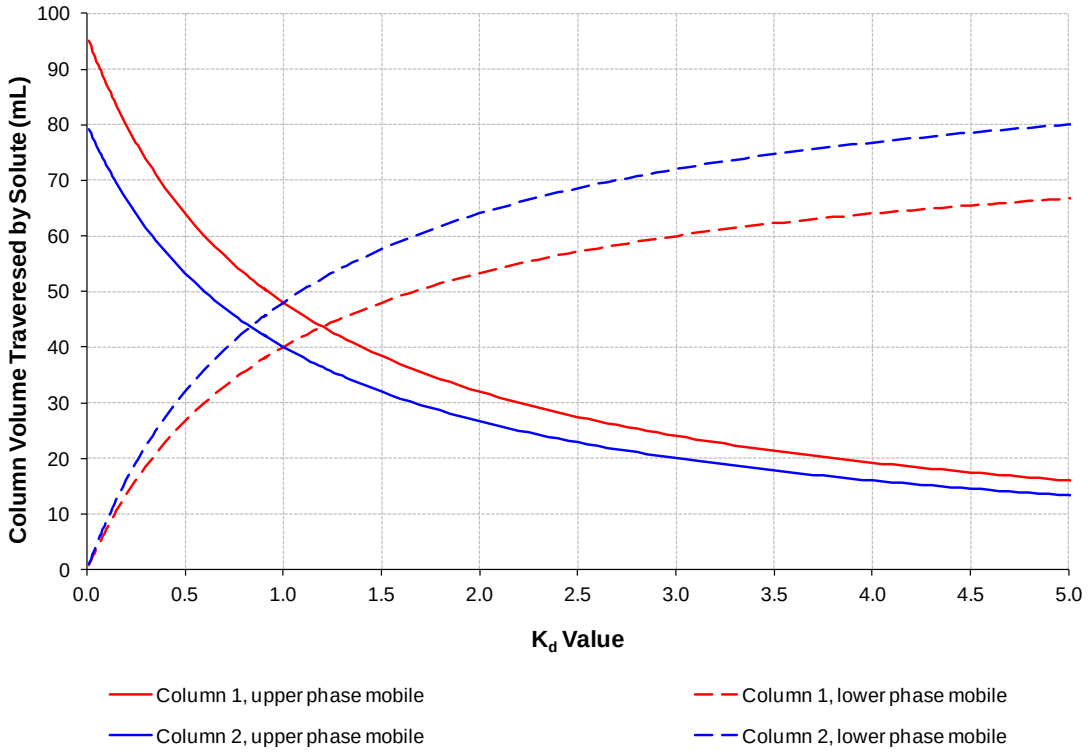


Figure 4.1.3: Volume traversed in by a solute in each column in each step, $F_U = F_L = 10$ mL/min, $F_{SU} = F_{SL} = 2$ mL/min, $S_{f1} = S_{f2} = 50\%$, $t_U = 4$ min and $t_L = 4$ min.

The volumes progressed by a solute peak in one cycle for each column are therefore given by Equations 4.1.22 and 4.1.23.

$$V_{PC1} = V_{PnC1} - V_{PrC1} \quad \text{… (Eq. 4.1.22)}$$

$$V_{PC2} = V_{PrC2} - V_{PnC2} \quad \text{… (Eq. 4.1.23)}$$

As the column volume is known, the time to elution of a compound of specific K_d value can be calculated from Equations 4.1.24 and 4.1.25.

$$t_{PC1} = \frac{V_C(t_U + t_L)}{V_{PC1}} \quad \text{… (Eq. 4.1.24)}$$

$$t_{PC2} = \frac{V_C(t_U + t_L)}{V_{PC2}} \qquad \text{… (Eq. 4.1.25)}$$

Where t_{PC1} are the retention times of compounds eluting from column 1 with the upper phase and t_{PC2} are the retention times of compounds eluting from column 2 with the lower phase. Figure 4.1.4 shows these times plotted against K_d. Experimentally this visualisation allows an understanding of which compounds will be eluted, how long they will take to elute, which compounds will be retained in the column and the minimum α-factor that can realistically be separated for a given set of operating conditions. The points were the vertical lines cross the curves gives the boundary condition for where a compound with a given K_d will either be retained in the column indefinitely or eluted from the column, as this is the K_d value at which it takes an infinite time for a compound to elute from that end of the column.

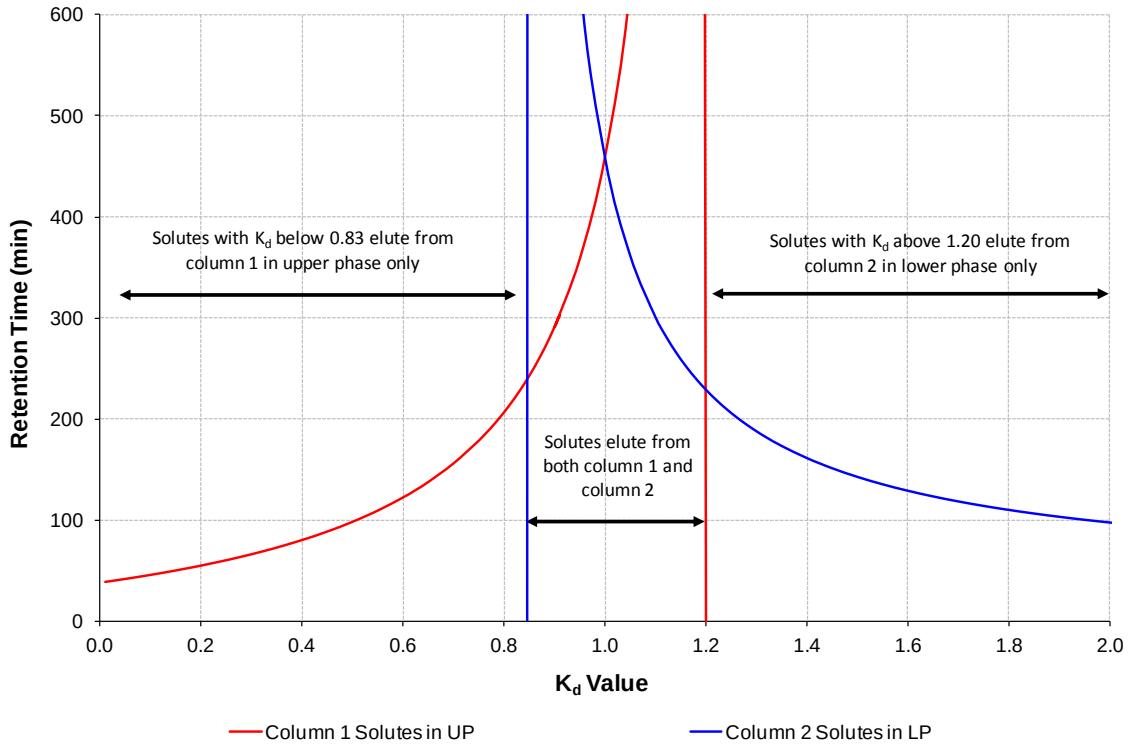


Figure 4.1.4: Retention time for components using an ICcE setup. $F_U = F_L = 10$ mL/min, $F_{SU} = F_{SL} = 2$ mL/min, $V_C = 460$ mL, $S_{f1} = S_{f2} = 50\%$, $t_U = 4$ min and $t_L = 4$ min.

The K_d value for the compound which would be retained in the column, K_{dR}, is calculated by equating Equations 4.1.24 and 4.1.25 and solving for K_d, as the point the two lines cross is the K_d value which while continuously move back and forth within the column (Figure 4.1.4).

Therefore, equating Equation 4.1.24 and 4.1.25 gives,

$$\frac{V_C(t_U+t_L)}{V_{PC1}} = \frac{V_C(t_U+t_L)}{V_{PC2}} \quad \text{… (Eq. 4.1.26)}$$

Substituting for equations 4.1.22 and 4.1.23 gives,

$$V_{PnC1} - V_{PrC1} = V_{PrC2} - V_{PnC2} \quad \text{… (Eq. 4.1.27)}$$

While substituting for equations 4.1.18-21 gives,

$$\frac{(F_U+F_{SU})t_U}{1+S_{f1}(K_d-1)} - \frac{F_L t_L}{1+(1-S_{f1})(\frac{1}{K_d}-1)} = \frac{(F_L+F_{SL})t_L}{1+(1-S_{f2})(\frac{1}{K_d}-1)} - \frac{F_U t_U}{1+S_{f2}(K_d-1)} \quad \text{… (Eq. 4.1.28)}$$

Assuming, S_{f1}=S_{f2}, which is normally the case, and rearranging gives,

$$\frac{(F_U+F_{SU})t_U+F_U t_U}{1+S_{f1}(K_d-1)} = \frac{(F_L+F_{SL})t_L+F_L t_L}{1+(1-S_{f1})(\frac{1}{K_d}-1)} \quad \text{… (Eq. 4.1.29)}$$

or,

$$\frac{1+S_{f1}(K_d-1)}{1+(1-S_{f1})(\frac{1}{K_d}-1)} = \frac{(2F_U+F_{SU})t_U}{(2F_L+F_{SL})t_L} \quad \text{… (Eq. 4.1.30)}$$

Which simplifies, for the distribution ratio always retained in the columns, K_{dR}, to:

$$K_{dR} = \tau\frac{(2F_U+F_{SU})}{(2F_L+F_{SL})} \quad \text{… (Eq. 4.1.31)}$$

Where τ is the ratio of the upper phase to lower phase step times.

Four ratios can be adjusted to change the separation observed in Figure 4.1.3 and Figure 4.1.4. These are:

- Ratio of upper phase to lower phase eluant flow rates
- Ratio of eluant to sample flow rates, in upper phase (x) and lower phase (y)
- Ratio of upper phase to lower phase step times (τ)
- Stationary phase retention (S_f)

Changes to either the ratio of the eluant flows or the ratio of the flow times of the eluants have similar effects as both parameters change the ratio of the total volumes of upper and lower phase flowed as mobile phase in each cycle. When the ratio of the volume of upper phase to lower phase equals 1 all compounds with a normal phase $K_d<1$ elute with the upper phase fractions and all compounds with $K_d>1$ elute with the lower phase fractions, while a compound with $K_d=1$ would be retained in the column (as shown in Figure 4.1.4 above).

If the ratio of the volume of upper mobile phase to lower mobile phase is greater than one then the split point moves to a higher K_d value, therefore more compounds elute with the upper phase (Figure 4.1.5a), whereas if the ratio is less than one the split point moves to the left and more compounds elute with the lower phase (Figure 4.1.5b)

The ratio between the eluant flow rate and the sample flow rate effects the range of K_d values which elute from both ends of the columns. Increasing the ratio such that the eluant flow rate is significantly greater than the sample flow rate minimises the range of K_d values which elute with both phases (Figure 4.1.5c), whereas decreasing the ratio so the sample flow rate is closer to the eluant flow rate increases the range of K_d values which elute from with both the upper and lower phase eluants (Figure 4.1.5d).

Changes to the stationary phase retention (that is the phase volume ratio) within the columns have no effect on the range of K_d values which elute from both ends of the column, rather the stationary phase retention effects the retention time of the compounds in the columns. An increased volume of lower phase in the columns, that is a higher normal phase stationary phase retention, causes the compounds with K_d values below the split point to elute with the upper phase fractions earlier, while compounds with a K_d value above the switch point elute in the lower phase fractions later (Figure 4.1.5e). With increased upper phase in the columns, i.e. lower normal phase stationary phase retention, compounds with K_d values below the split point elute later and compounds above the switch point elute earlier (Figure 4.1.5f).

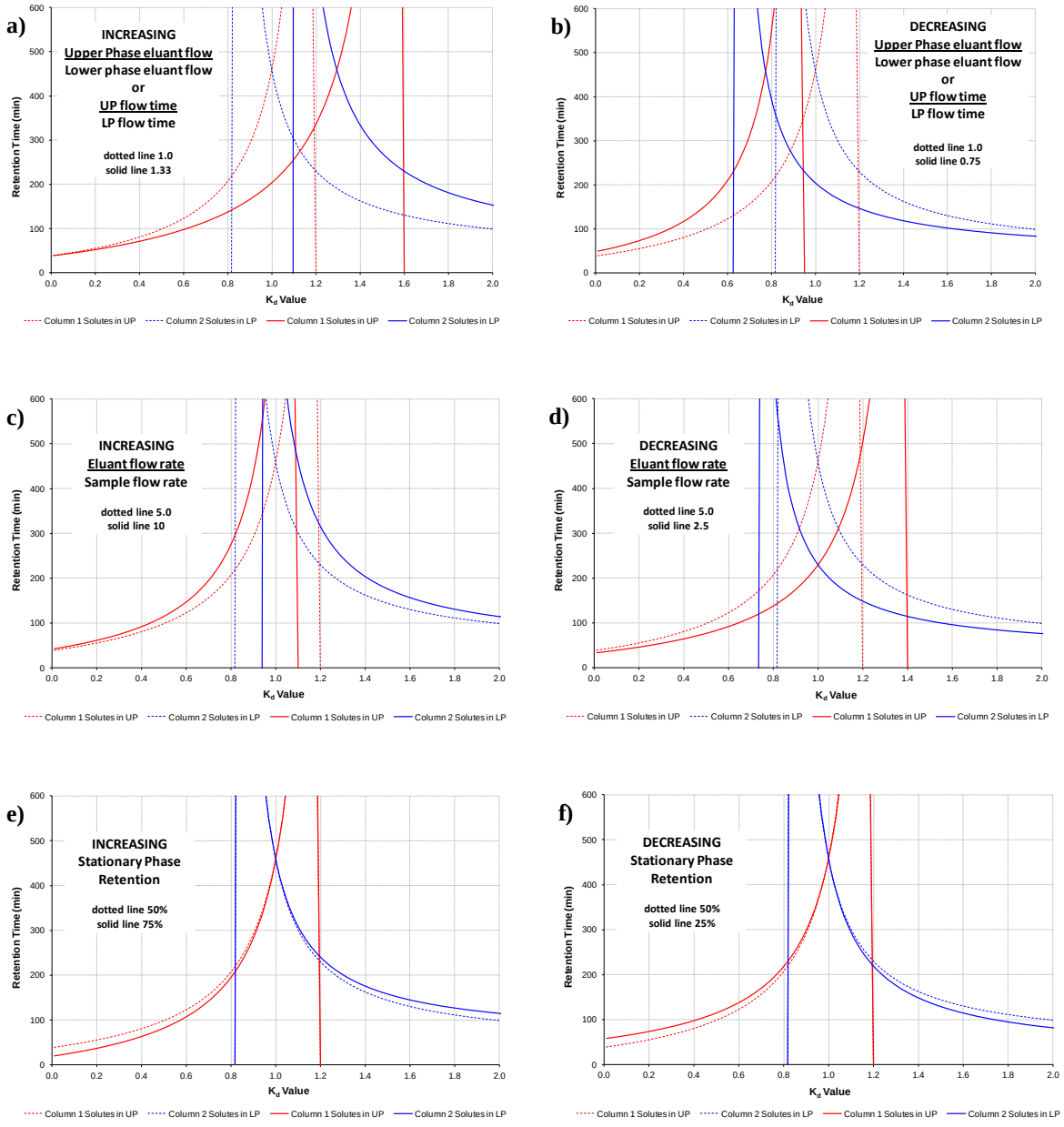


Figure 4.1.6: Retention time for components using an ICcE setup; V_C = 460 mL, t_U = 4 min and t_L = 4 min; dotted line a-f) $F_U = F_L$ = 10 mL/min, $F_{SU} = F_{SL}$ = 2.0 mL/min, $S_{f1} = S_{f2}$ = 50%, solid lines a) F_U = 10 mL/min, F_L = 7.5 mL/min, $F_{SU} = F_{SL}$ = 2.0 mL/min, $S_{f1} = S_{f2}$ = 50%; b) F_U = 7.5 mL/min, F_L = 10 mL/min, $F_{SU} = F_{SL}$ = 2.0 mL/min, $S_{f1} = S_{f2}$ = 50%; c) $F_U = F_L$ = 10 mL/min, $F_{SU} = F_{SL}$ = 1.0 mL/min, $S_{f1} = S_{f2}$ = 50%; d) $F_U = F_L$ = 10 mL/min, $F_{SU} = F_{SL}$ = 4.0 mL/min, $S_{f1} = S_{f2}$ = 50%; e) $F_U = F_L$ = 10 mL/min, $F_{SU} = F_{SL}$ = 2.0 mL/min, $S_{f1} = S_{f2}$ = 75% and f) $F_U = F_L$ = 10 mL/min, $F_{SU} = F_{SL}$ = 2.0 mL/min, $S_{f1} = S_{f2}$ = 25%

4.2) Visual representation of the flow ratios for the ICcE method

The graphical representations from the initial model described in Section 4.1 allowed for prediction of the distribution of compounds between the upper and lower phase fractions and the time compounds will remain in the columns. However, a clear graphical method to visualise the split point and how the closest compounds on each side of the split point would be separated was also required. Therefore a second set of equations were derived from the initial model to show the effect of the ratios ($x = F_U/F_{SU}$, $y = F_L/F_{SL}$, $\tau = t_U/t_L$) on the separation of the two compounds closest to the split point. This graphical representation will show the boundary conditions between where the two compounds have been fully separated, partially separated or contaminated by each other. Plotting x against y, for a given τ, can also be used to show the effect of changing the normal and reversed phase eluant flows (F_U and F_L). In this model the stationary phase retention was assumed to be 50% as S_f was shown to have a more limited effect on the separation in Section 4.1.

Assuming K_{dA} is less than K_{dB}, that is compound A is expected to prefer to elute with the more non-polar upper phase while compound B is expected to elute with the more polar lower phase then the following equations were empirically derived by interrogation of the initial model in Section 4.1. Equation 4.2.1 is the boundary condition between regions where both compounds elute with the lower phase or pure A starts to elute with the upper phase. Equation 4.2.2 is the boundary between the region where both compounds elute as pure fractions and the region where only the upper phase fractions are pure, while Equation 4.2.3 is the other boundary between the region where both compounds elute as pure fractions and the region where only compound B elutes pure with the lower phase fractions while A and B both elute with the upper phase fractions. Equation 4.2.4 is the boundary between the where nothing elutes in the lower phase or pure B elutes in the lower phase while both A and B elute with the upper phase.

The equations of the four linear lines in Figure 4.2.1 are:

$$y = \frac{K_{dA}}{\tau} x - 1 \qquad \text{… (Eq. 4.2.1)}$$

$$y = \frac{K_{dA}}{\tau} x + \frac{K_{dA}}{\tau} \qquad \text{… (Eq. 4.2.2)}$$

$$y = \frac{K_{dB}}{\tau} x - 1 \qquad \ldots \text{(Eq. 4.2.3)}$$

$$y = \frac{K_{dB}}{\tau} x + \frac{K_{dB}}{\tau} \qquad \ldots \text{(Eq. 4.2.4)}$$

Where, $\tau = \frac{t_U}{t_L}, x = \frac{F_U}{F_{SU}}, y = \frac{F_L}{F_{SL}}, K_{dA} < K_{dB}$

Therefore, Figure 4.2.1 compares the eluant flow to sample flow ratios for the upper (x) and lower (y) phases at a given cycle step time ratio (τ). This gives a clear visual representation of the region where pure compounds elute with the upper and lower phase fractions. The effect of any shift in the flow rates on the separation can be judged.

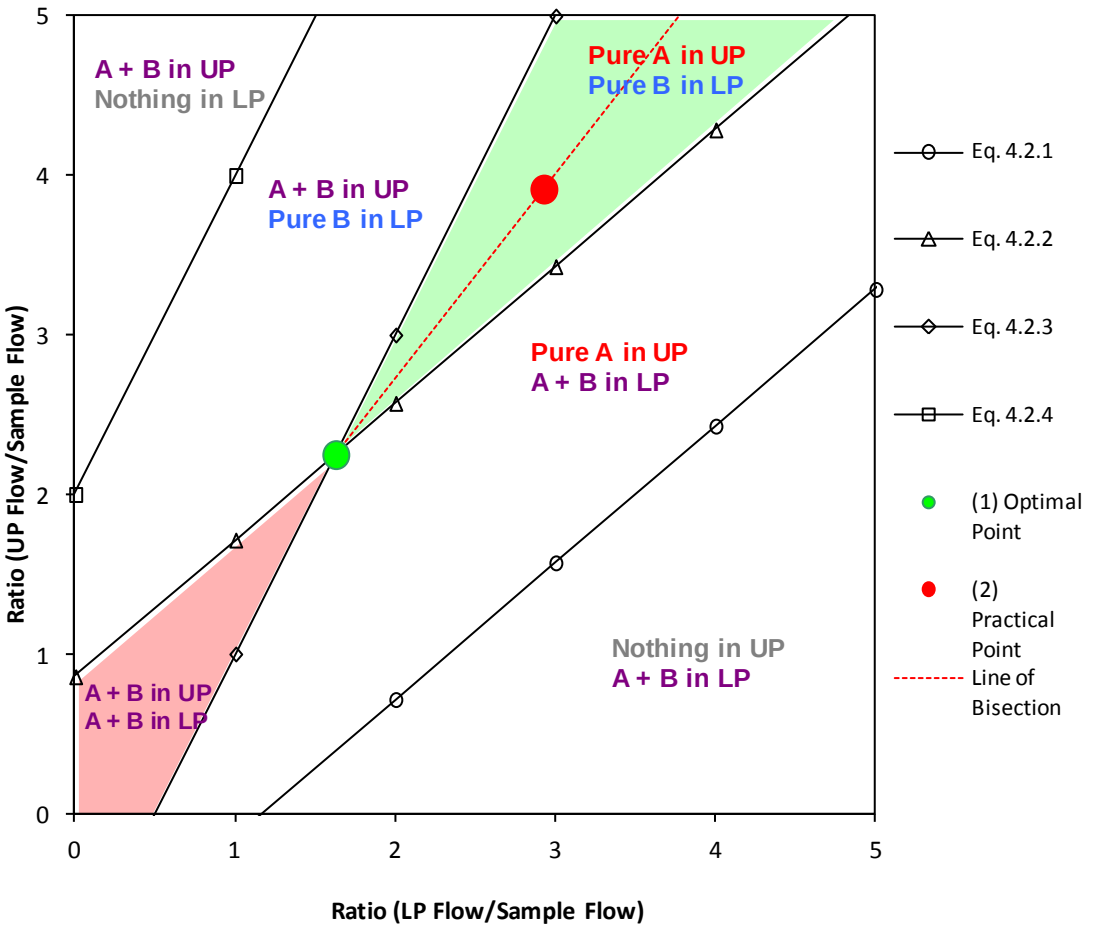


Figure 4.2.1: Graphic interpretation of the ICcE model showing the areas where separations occur between two compounds, A and B, of different K_d values (K_{dA} = 0.6 and K_{dB} = 1.4) at a range of flow ratios at fixed timing ratios (t_L = 4 minutes and t_U = 2.8 minutes, for this example)

The optimal point for the separation is where both ratios are minimised, yet both eluant streams continue to have pure compound in them (Point 1 - green, Figure 4.2.1). The smaller the eluant to sample ratio the less eluant required to separate the compounds and the less diluted the eluting fractions. However, in practice any shift from this point due to slight errors in the flow rates of the eluant or sample pumps would move the separation into an area of incomplete separation. Therefore a practical operating point is calculated on the bisecting line between the limits of the region of complete separation.

The optimal point (x_1, y_1, Point 1 in Figure 4.2.1) is the intersection of Equation 4.2.2 and Equation 4.2.3 and gives the point of highest throughput. x_1 (Equation 4.2.6) and y_1 (Equation 4.2.11) can be calculated by equating Equation 4.2.2 and 4.2.3 as follows:

$$\frac{K_{dA}}{\tau}x + \frac{K_{dA}}{\tau} = \frac{K_{dB}}{\tau}x - 1 \qquad \text{… (Eq. 4.2.5)}$$

Therefore rearranging Equation 4.2.5 gives,

$$x_1 = \left(\frac{K_{dA}}{\tau} + 1\right) / \left(\frac{K_{dB}}{\tau} - \frac{K_{dA}}{\tau}\right) \qquad \text{… (Eq. 4.2.6)}$$

From Equation 4.2.2,

$$x = \frac{y\tau}{K_{dA}} - 1 \qquad \text{… (Eq. 4.2.7)}$$

And from Equation 4.2.3,

$$x = \frac{\tau}{K_{dB}}(y + 1) \qquad \text{… (Eq. 4.2.8)}$$

Therefore equating Equations 4.2.7 and 4.2.8 gives,

$$\frac{y\tau}{K_{dA}} - 1 = \frac{\tau}{K_{dB}}(y + 1) \qquad \text{… (Eq. 4.2.9)}$$

So rearranging Equation 4.2.9 gives,

$$K_{dB}y\tau - K_{dA}K_{dB} = K_{dA}\tau(y + 1) \qquad \text{… (Eq. 4.2.10)}$$

Therefore,

$$y_1 = \frac{K_{dA}(K_{dB}+\tau)}{\tau(K_{dB}-K_{dA})} \qquad \text{… (Eq. 4.2.11)}$$

Although the most efficient separation, with respect to throughput and solvent usage, will be given at the optimal point (x_1, y_1) this gives no leeway for errors in either flow rates or timings as a shift in either will move the process into a region of incomplete separation. Therefore, a safer operating area is within the region between the lines described by Equations 4.2.2 and 4.2.3, given by the green shaded area in Figure 4.2.1, though this will reduce the throughput and increase solvent usage.

A good operating point will be along the bisector of the lines defined by these two equations, marked by the dotted red line in Figure 4.2.1, which gives the greatest degree of freedom for any shift in flow rates or timings.

The equation for the line of bisection can be calculated as follows. The inclination (angle between a line and the x-axis) of the lines described by Equations 4.2.2 and 4.2.3 is the arctangent of their slopes. The average of their inclinations is the inclination of the bisector and the slope of the bisector is the therefore the tangent of the bisector inclination. As the optimal point is known, using the point-slope form of the linear equation, the bisector equation can be derived.

The slopes of Equations 4.2.2 and 4.2.3 are given in Equations 4.2.12 and 4.2.13.

$$m_2 = \frac{K_{dA}}{\tau} \qquad \ldots \text{(Eq. 4.2.12)}$$

$$m_3 = \frac{K_{dB}}{\tau} \qquad \ldots \text{(Eq. 4.2.13)}$$

Using the above argument the slope of the bisector is therefore given by Equation 4.2.14.

$$m_b = \tan\left(\frac{\arctan m_2 + \arctan m_3}{2}\right) \qquad \ldots \text{(Eq. 4.2.14)}$$

A practical working point on the bisector (x_p, y_p - Point 2 in Figure 4.2.1), can be calculated using Equations 4.2.15 and 4.2.16, based on the point-slope linear equation, where n is an increase in flow above the optimum point flow values defined by Equations 4.2.6 and 4.2.11.

$$y_p = m_b x_p - m_b x_1 + y_1 \qquad \ldots \text{(Eq. 4.2.15)}$$

$$x_p = n x_1 \text{ where } n > 1 \qquad \ldots \text{(Eq. 4.2.16)}$$

This practical working point (Point 2, Figure 4.2.1) can then be used to produce a second graph (Figure 4.2.2) which visually shows the effect of any changes in the intermittent timing intervals at the practical flow rates set. Simply rearranging Equations 4.2.1-4 to plot t_U against t_L gives Equations 4.2.17-20 which are the boundaries that divide up the various areas of complete and incomplete separation. Equations 4.2.19 and 4.2.18 are the upper and lower boundaries between the region where both compounds elute as pure fractions and the region where either only the lower phase fractions or upper phase fractions are pure, respectively. While Equation 4.2.17 is the boundary between the region where either pure A or nothing elutes with the upper phase and the lower phase fractions are all impure and Equation 4.2.20 is the boundary between the where either pure B or nothing elutes in the lower phase while all the upper phase fractions are impure, containing both A and B.

$$t_U = K_{dA}\left(\frac{F_L}{F_U + F_{SU}}\right)\frac{F_{SU}}{F_{SL}}t_L \quad \ldots \text{(Eq. 4.2.17)}$$

$$t_U = K_{dA}\left(\frac{F_L + F_{SL}}{F_U}\right)\frac{F_{SU}}{F_{SL}}t_L \quad \ldots \text{(Eq. 4.2.18)}$$

$$t_U = K_{dB}\left(\frac{F_L}{F_U + F_{SU}}\right)\frac{F_{SU}}{F_{SL}}t_L \quad \ldots \text{(Eq. 4.2.19)}$$

$$t_U = K_{dB}\left(\frac{F_L + F_{SL}}{F_U}\right)\frac{F_{SU}}{F_{SL}}t_L \quad \ldots \text{(Eq. 4.2.20)}$$

The actual timings (t_U, t_L) used for Figure 4.2.1 are given by the model point (red) in Figure 4.2.2. While any point on the red dotted line has the same step time ratio, τ.

From Figure 4.2.2, it can be seen that the longer the timings used the more leeway there is for any changes in operating parameters which occur.

The line of equal τ, given by the red dotted line (Figure 4.2.2) is not the bisector of the lines of Equations 4.2.18 and 4.2.19 and therefore if the timings t_U and t_L are significantly different from each other then the practical operating point lies close to one of the lines of Equation 4.2.18 or 4.2.19.

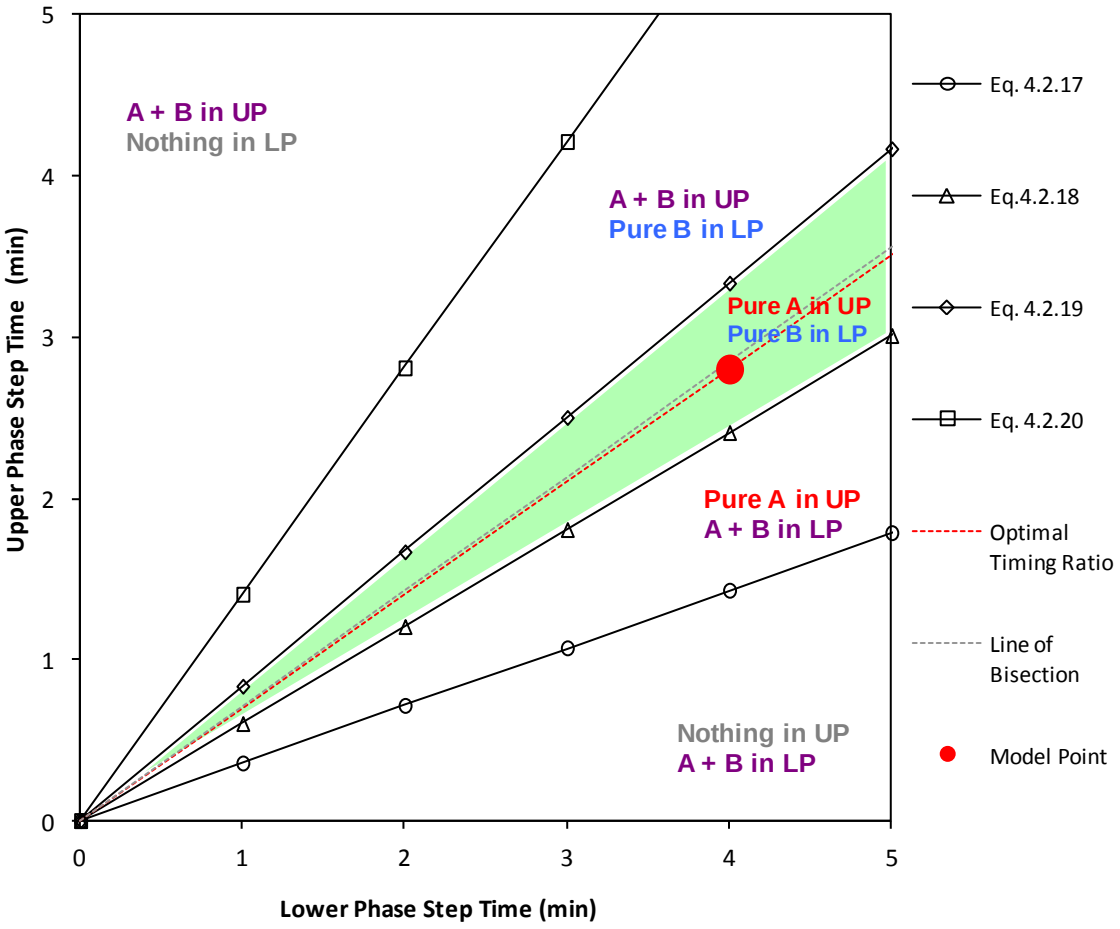


Figure 4.2.2: Graphic interpretation of model showing the areas where separations occur between two compounds, A and B, of different K_d values (K_{dA} = 0.6 and K_{dB} = 1.4) at a practical flow ratio for a range of intermittent timing intervals between normal and revered phase

Therefore, a point on the bisector would give more robust operating conditions. The equation for the line of bisection can be derived for this graph using the same methodology as used for Equation 4.2.14.

$$t_U = \tan\left(\left(\arctan\left(\frac{K_{dB}F_L F_{SU}}{(F_U + F_{SU})F_{SL}}\right) + \arctan\left(\frac{K_{dA}F_{SU}}{F_{SL}}\left(\frac{F_L + F_{SL}}{F_U}\right)\right)\right)\Big/ 2\right) t_L \quad \ldots \text{(Eq. 4.2.21)}$$

Figure 4.2.3, based on Figure 4.2.1, shows how changes to the flow ratios effect the separation of two compounds. An increase in the ratio of the eluant flow rate to the sample flow rate moves the separation point into a more robust region of the diagram, although with the penalty of increased solvent usage. Decreasing this ratio will improve throughput and solvent usage, however it risks the separation moving into a region of either no separation or only partial separation. While, an increase in the ratio of the upper phase eluant flow rate to lower phase eluant flow will cause the compounds to elute with the upper phase fractions to a greater extent, potentially contaminating the upper phase fractions compound B which should elute with the lower phase. A decrease in this ratio causes the compounds to elute more with the lower phase fractions which will therefore start to be contaminated with compound A which should elute with the upper phase.

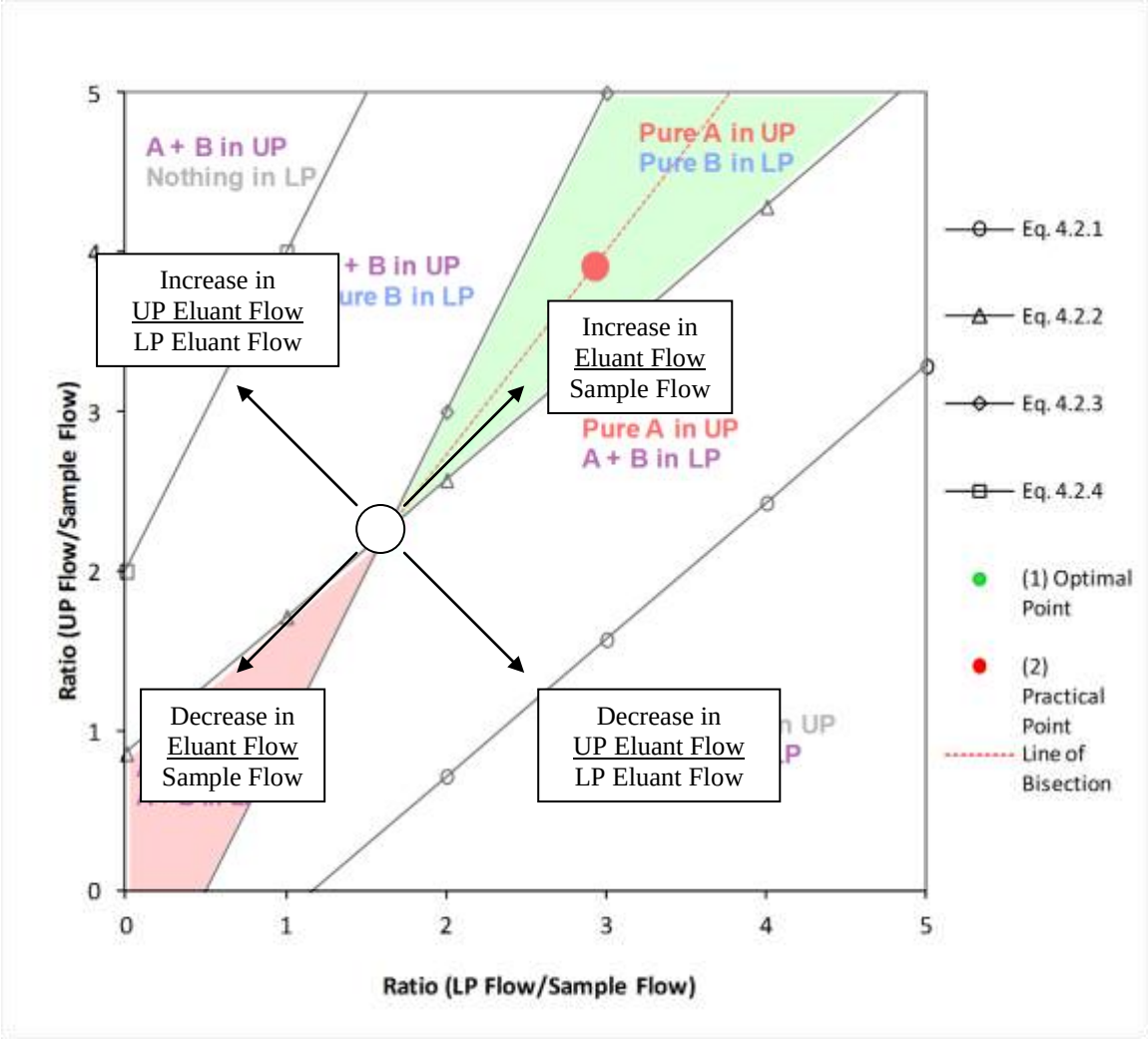


Figure 4.2.3: Graphic visualisation of the effect of flow rate changes on the separation of two compounds, A and B, of different K_d values (K_{dA} = 0.6 and K_{dB} = 1.4) based on Figure 4.2.1

Figure 4.2.4, based on Figure 4.2.2, shows how a change in the ratio of the upper phase flow rate time to the lower phase flow rate time will effect the separation of the two compounds. An increase causes the compounds to elute more with the upper phase, therefore potentially contaminating the upper phase fractions while a decrease in the ratio causes the compounds to elute with the lower phase so contaminating the lower phase fractions.

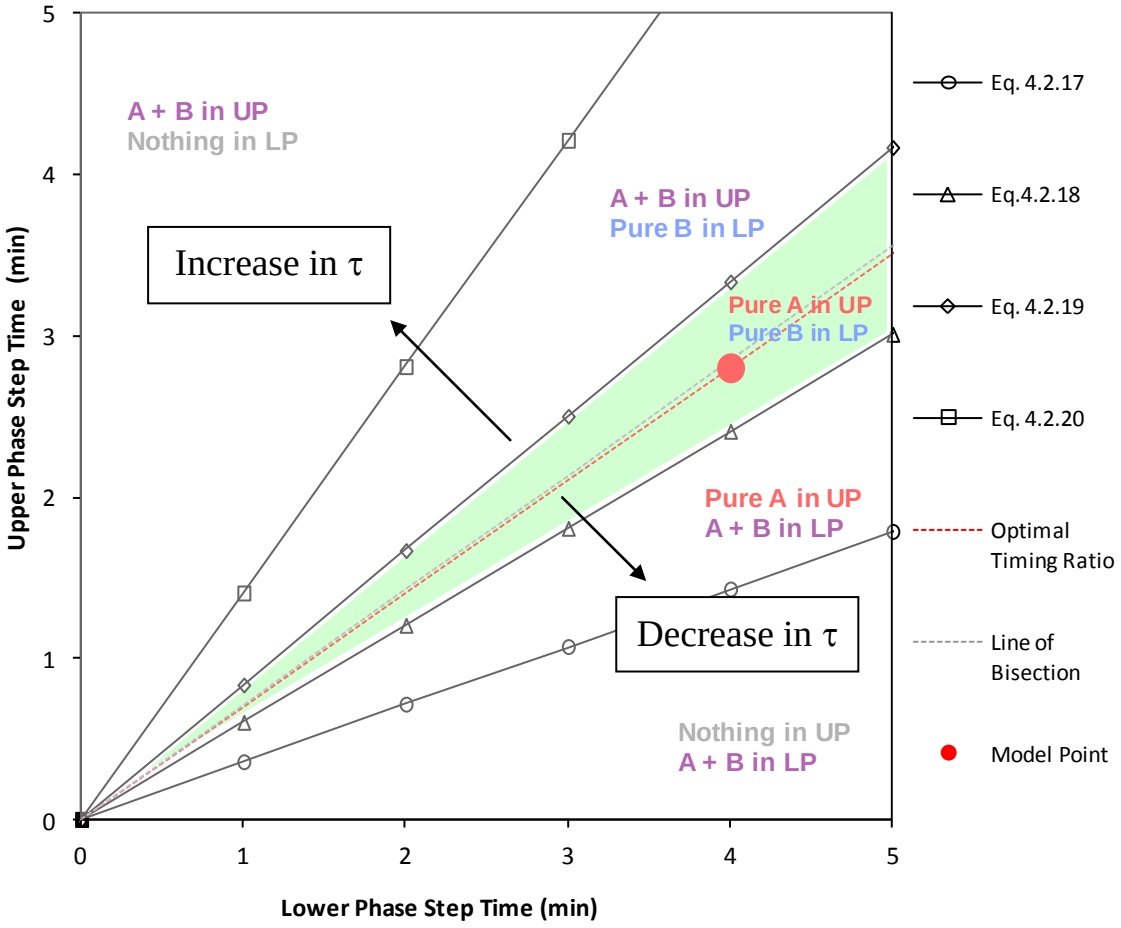


Figure 4.2.4: Graphic visualisation of the effect of changes in switching times on the separation of two compounds

4.3) Comparison to Yang and Volkl models for ICcE

Yang et al. recently published a model based on the similar logic to that presented above in Section 4.1, without the graphical representation, describing the separation process as intermittent dual countercurrent chromatography (Yang et al. 2009). An equation for the average linear velocity of the solvent peaks, $\mu_{X,i}$, is calculated, Equation 4.3.1.

$$\mu_{X,i} = \frac{(F_L / A_C)t_{i,L} - (\beta_L + 1)/(1 + K\beta_L) - (F_U / A_C)t_{i,U}K(1 + \beta_U)/(1 + K\beta_U)}{t_{i,L} + t_{i,U}} \quad \text{... (Eq. 4.3.1)}$$

Where A_C is the cross sectional area of the column, β_L is the volume ratio when the lower phase is mobile and β_U is the volume ratio when the upper phase is mobile.

While the retention time, t_R, of the peaks within the column is determined from Equation 4.3.2.

$$t_R = \frac{0.5V_C(t_{i,L} + t_{i,U})}{(F_L / A_C)t_{i,L} - (\beta_L + 1)/(1 + K\beta_L) - (F_U / A_C)t_{i,U}K(1 + \beta_U)/(1 + K\beta_U)} \quad \text{... (Eq. 4.3.2)}$$

As the cross sectional area of the column and the volume ratios are directly related to the stationary phase retention, Equation 4.3.2 is analogous to Equations 4.1.24 and 4.1.25 derived in the previous section, but Equation 4.3.2 does not take into account the sample flow rate. This sample flow rate causes the linear velocity of a peak to be different in each column, which in turn gives the range of compounds that will elute with both the upper and the lower phase fractions.

Völkl et al. also derives a model for the separation of two components where the process is named sequential centrifugal partition chromatography (sCPC) (Völkl et al. 2013). A similar set of equations is used to derive the linear distance a peak travels and therefore a velocity for the components within the column. Applying operating parameter constraints for the complete elution of two components with either the upper or lower phases allows the construction of equation 4.3.3 which defines the region of complete separation with respect to the upper and lower phase switching times.

$$\frac{F_{U,1} + F_{U,F}}{F_{L,2}} K_A < \frac{t_{Des}}{t_{Asc}} < \frac{F_{U,1}}{F_{L,2} + F_{L,F}} K_B \quad \text{... (Eq. 4.3.3)}$$

Where, K_{dA} and K_{dB} are the distribution ratios (where K_d is the concentration of a compound in the upper phase divided by the concentration of that compound in the lower phase), F_U and F_L are the upper and lower phase flow rates for a given column, F_F is the feed (sample) flow rate in either upper or lower phase and t_{Asc} and t_{Des} are the switching intervals for the upper and lower phase eluant flow steps.

Equation 4.3.3 can be related directly to Equations 4.2.18 and 4.2.19 of the earlier theory which describe the same region, as Völkl assumes the sample flow rates are equal, F_S, therefore assuming $F_S=F_{SU}=F_{SL}$, Equations 4.2.18 and 4.2.19 can be rearranged as,

$$\frac{t_U}{t_L} = \left[\frac{F_L+F_S}{F_U}\right] K_{dA} \qquad \text{... (Eq. 4.3.4)}$$

and,

$$\frac{t_U}{t_L} = \left[\frac{F_L}{F_U+F_S}\right] K_{db} \qquad \text{... (Eq. 4.3.5)}$$

which bounds the area,

$$\left[\frac{F_L+F_S}{F_U}\right] K_d < \frac{t_U}{t_L} < \left[\frac{F_L}{F_U+F_S}\right] K_d \qquad \text{... (Eq. 4.3.6)}$$

Equation 4.3.6 is equivalent to Equation 4.3.3 as the distribution ratio, K_d is inverted in the Völkl compared to the work in this thesis.

Völkl's work does not mention the outer regions of incomplete separation described by Equations 4.2.17 and 4.2.20 or show the graphical representation in Figure 4.2.1 which allows the optimal flow point to be visualised.

4.4) Conclusions on models developed

The models developed and discussed in this chapter independently reach a similar conclusion that the critical operating parameters are the four dimensionless ratios,

- Upper phase eluant flow/lower phase eluant flow
- Upper phase flow time/lower phase flow time
- Eluant flow/sample flow
- Upper phase volume/lower phase volume

Any model developed needs to effectively visualize the process it describes to allow the researcher to quickly setup initial conditions to perform a successful separation. The model developed in Section 4.1 gives a clear indication of how compounds of differing K_d values will be separated or retained in the column but does not take into consideration the important effects of band broadening.

Changing the ratio of eluant flow rates or the eluant flow times have similar effects as they both change the ratio of the total volume of upper or lower mobile phase flowed in each cycle. From the model it can be seen that when the ratio of these volumes is one then compounds with a normal phase $K_d<1$ elute with the upper phase fractions and all compounds with $K_d>1$ elute with the lower phase fractions, while a compound with $K_d=1$ is retained in the column.

When the ratio of the volume of upper mobile phase to lower mobile phase is greater than one then the split point moves to a higher K_d value however, if the ratio is less than one the split point moves to the left and more compounds elute with the lower phase.

The ratio between the eluant flow rate and the sample flow rate effects the range of K_d values which elute from both ends of the columns. A high ratio gives a very clean cut but uses large amounts of solvent, while a low ratio uses less solvent but gives a much less clean cut. This ratio is set with the aim of achieving acceptable separation for the closest running compounds and therefore minimising solvent usage for the process.

Changes to the stationary phase retention (that is the phase volume ratio) within the columns have no effect on the range of K_d values which elute from both ends of the column, rather the stationary phase retention effects the retention time of the compounds in the columns. Therefore the phase volume ratio in the column was set to one initially to ensure good

loading for the model mixtures used which contain compounds with a broad range of polarities.

A graph to show the regions of pure separation is derived, from which a point of optimum separation is derived as well as a practical operating point which should allow separation of a group of compounds.

The models developed in this section are validated in the following experimental chapter. The initial model developed in Section 4.1 is validated against experimental results for a sample mixture containing a range of naturally occurring bioactive compounds, the GUESSmix (Friesen and Pauli 2005), using a range of HEMWat phase systems in Section 5.5. While the model to calculate optimal points and practical operating points is used to setup initial operating conditions for the industrial applications given in Chapter 6.

How the models are further developed to allow useful visualization to the experimental chemist of the effect of changes to these main parameters will ultimately decide their usefulness.

Chapter 5: A route to continuous processing

5.1) Introduction to the continuous processing experimental work

As discussed in the literature review, Craig and Craig (1956) introduce the concept of continuously separating a mixture using liquid-liquid extraction techniques (Section 2.3.1). They describe the theoretical process of "the introduction of the mixture at the centre of a perfectly operating continuous column".

Post and Craig (1963) went on to describe a device in which transfers can be accomplished in this way to allow separation of mixtures.

With the development of both hydrodynamic and hydrostatic liquid-liquid chromatography through the following decades two possible routes to adapting this technology to continuous processing have been proposed.

Firstly **dual-flow CCC**, originally described by Ito and Bowman (1976) with foam CCC separations to separate a mixture of dyes, in which the phases flow truly counter-current to each other as occurs in a liquid-liquid extractor (Section 2.3.1). Secondly, **Intermittent counter-current extraction**, a modification of Post and Craig's original concept above in which the sample is loaded continuously in the centre of a column and the upper and lower phases are flowed alternately in normal and reversed phase (Section 2.3.2.3).

For successful commercial use of either or both of these technologies there is a requirement for robust technology.

5.2) Aims of the experimental work

The aims of the experimental work are to answer the five research questions detailed in Chapter 1:

1) Can the liquid-liquid SMB type method be transferred from CPC to CCC instruments?
2) If so can ICcE be competitive with the DFCCC continuous process already developed on CCC instruments?
3) If ICcE is competitive can it be scaled for preparative and pilot production on industrial applications?

4) How does the back pressure applied to the outlets of a DFCCC column affect the phase ratio within the column?
5) How robust is the ICcE method to changes in phase system polarity?

HEMWat phase systems are applicable to a wide range of liquid-liquid separations (Pauli et al. 2008). Therefore, in Section 5.3 the phase retention properties of this phase system will be studied to ensure continuous processing can be run robustly with a range of phase system polarities.

The alternate switching between normal and reversed phase had previously only been demonstrated on hydrostatic CPC instruments (Couillard et al. 2003), therefore to answer research question 1, the potential to transfer this technology to high performance hydrodynamic CCC instruments and the similar need to maintain a stable phase volume ratio within the columns will be discussed in Section 5.4.2.

In Section 5.4.3, to answer research question 2, DFCCC and ICcE will be compared and their relative advantages and disadvantages discussed.

In Section 5.5, to answer research questions 3 and 5, the applicability of ICcE at the semi-prep, prep and pilot scale will be examined, using a model system containing compounds with a range of polarities, across a range of phase system polarities. Section 5.6, will look briefly at the possibility of introducing a design change to the standard CCC instrument to allow on-column switching (instead of on-bench switching) of the phase systems so maximising the useful operational time of the instrument.

Dual flow has been tested at analytical (Goto et al. 2006) and preparative scale (van den Heuvel et al. 2009), however, a method to maintain a given phase volume ratio (the volume of upper phase in the column compared to volume of lower phase in the column) in the dual flow columns was not well understood.

Therefore, in Section 5.4.1, to answer research question 4, the effect of back pressure on phase volume ratio in the DFCCC column will be tested and try to improve the robustness of the dual-flow system.

5.3) Stationary phase retention of HEMWat phase systems

The HEMWat solvent system, or closely related systems, with a single substitution are the most widely used solvent systems (Pauli et al. 2008). 29% of the articles surveyed by Pauli use this solvent system and a further 18% use a closely related solvent system. The solvent system has been used for the separation of a wide range of both natural products and pharmaceutical compounds; including antibodies (Oka et al. 1996), flavanoids (Chen et al. 2003), alkaloids (Zhang et al. 1988A) and steroids (Du et al. 1995).

For isocratic CCC, stationary phase retentions above 70% are common. But for ICcE the mobile and stationary phases flow alternately, while for DFCCC, both phases are flowing continuously. As for both DFCCC and ICcE, where both phases are mobile, the phase volume ratio (the volume of upper phase in the column/volume of lower phase in the column) becomes important. Therefore, this raises the question; with what phase ratio should the columns be initially filled? As the phase volume ratio will determine the loading capacity of the column. So to maximise initial loading of the columns, especially for a complex mixture where the mass distribution of impurities may not be known, the initial phase volume ratio in the columns is set between 40 and 60%. Though, optimum will be dependent on the relative solubility of the respective compounds.

Therefore, it is important to understand the flow rates at which 50% stationary phase retention is achieved in isocratic CCC across the range of phase system polarities and machine sizes.

5.3.1) S_f of HEMWat phase systems across the polarity range

A linear relationship between the stationary phase retention and the square root of flow has been shown (Du et al. 1999). The aim of these experiments was to confirm this linear relationship exists for the range of HEMWat phase systems from polar HEMWat 11 phase system to non polar HEMWat 23 phase system and confirm the limiting flow rates at which 50% retention is reached for use of ICcE or DFCCC. Results were produced at both the Spectrum semi-prep scale and the Midi preparative scale at 240*g*, the standard operating *g*-field for these instruments. Further limited studies on the 4.6 L Maxi pilot scale used only the intermediate HEMWat 17 phase system at 120g which is the maximum operating g-field of this instrument.

At all scales the stationary phase retentions were determined in both normal phase and reversed phase. The experiments were performed as described in the protocols given in Chapter 3.1.

Figure 5.3.1 shows that in reversed phase the DE-Spectrum semi-preparative instrument gives linear Du-plots in the standard operating range, between 1 and 12 mL/min. In the extended, high flow operating range, between 24 and 72 mL/min some deviation from linearity is observed. For the more non-polar phase systems, HEMWat 19 and 23, the rate of stationary phase loss increases slightly, while for the more polar phase systems, HEMWat 11 and 15, the rate of loss of stationary phase decreases slightly.

While the DE-Midi preparative instrument in reversed phase (Figure 5.3.2) gives linear Du plots, both in its standard operating range of 10 to 80 mL/min and in the extended range from 120 mL/min up to 420 mL/min. For the more polar phase systems, HEMWat 11 and 15, a significant initial loss of stationary phase is observed at a flow rate of 10 mL/min, specifically, HEMWat 23 and 19 have similar stationary phase retentions of 91.3% and 91.7% respectively, HEMWat 17 gives very slightly lower stationary phase retention of 89.3%, but the HEMWat 15 and 11 give significantly reduced stationary phase retentions of 78.5% and 80.6% respectively. This excess 10% reduction in stationary phase is maintained as the flow rate is increased all the way to 420 mL/min. To confirm this behaviour, the stationary phase retention was measured for both HEMWat 15 and HEMWat 23 phase systems at different *g*-fields as discussed in the following section (Section 5.3.2).

When comparing the semi-preparative and preparative reversed phase results both show similar behaviour. Both give linear Du-plots for all phase systems, with the more polar phase systems having reduced stationary phase retention. For a given flow rate of the mobile phase the cross-sectional area the mobile phase occupies is independent of bore (Wood et al. 2003). Thus as expected, the preparative instrument gives slightly improved stationary phase retention when the results are compared scaled by the cross-sectional area of the column. From Figure 5.3.1 and Figure 5.3.3 respectively, the stationary phase retention is 52 – 72% for HEMWat 11 – 23 at 12 mL/min on the semi-preparative instrument in comparison to 57 – 77% for HEMWat 11 – 23 at 75 mL/min on the preparative instrument.

In reversed phase, for both instruments, acceptable linear Du plots are seen to the target limit of 50% stationary phase retention across the range of phase systems.

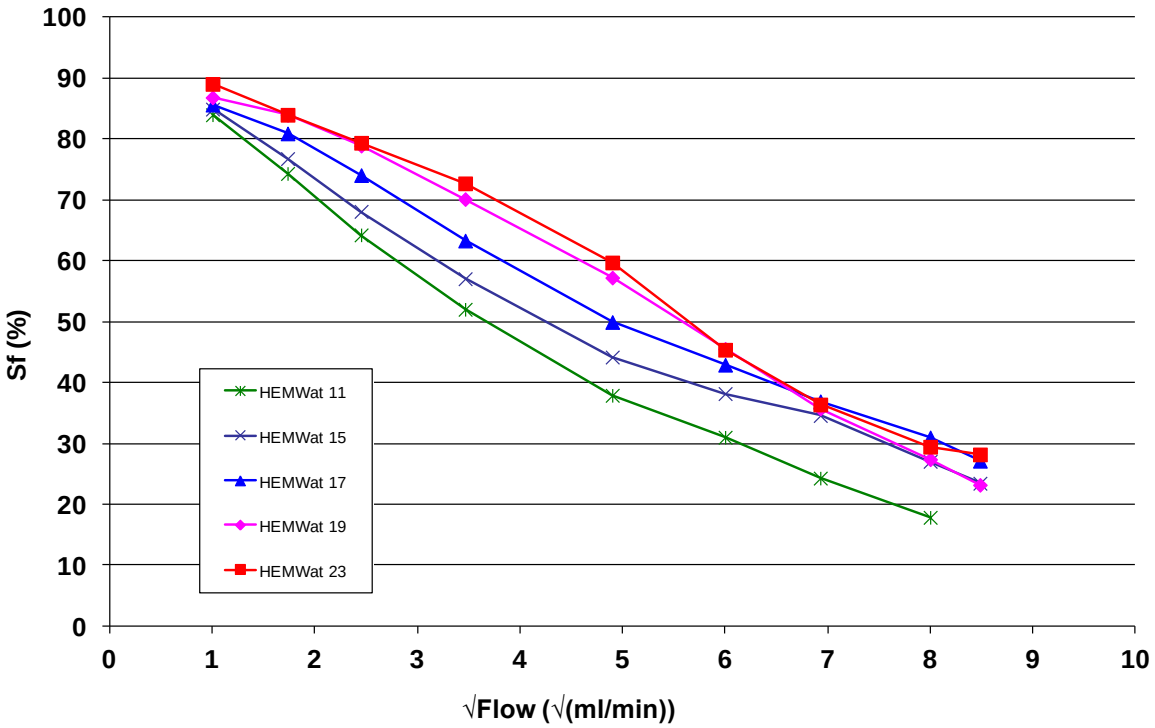


Figure 5.3.1 Reversed phase Du plots for HEMWat 11, 15, 17, 19 and 23 with DE-Spectrum semi-prep instrument, V_c = 73 mL, 1.6 mm bore PFA tubing, 240*g*, 30 °C

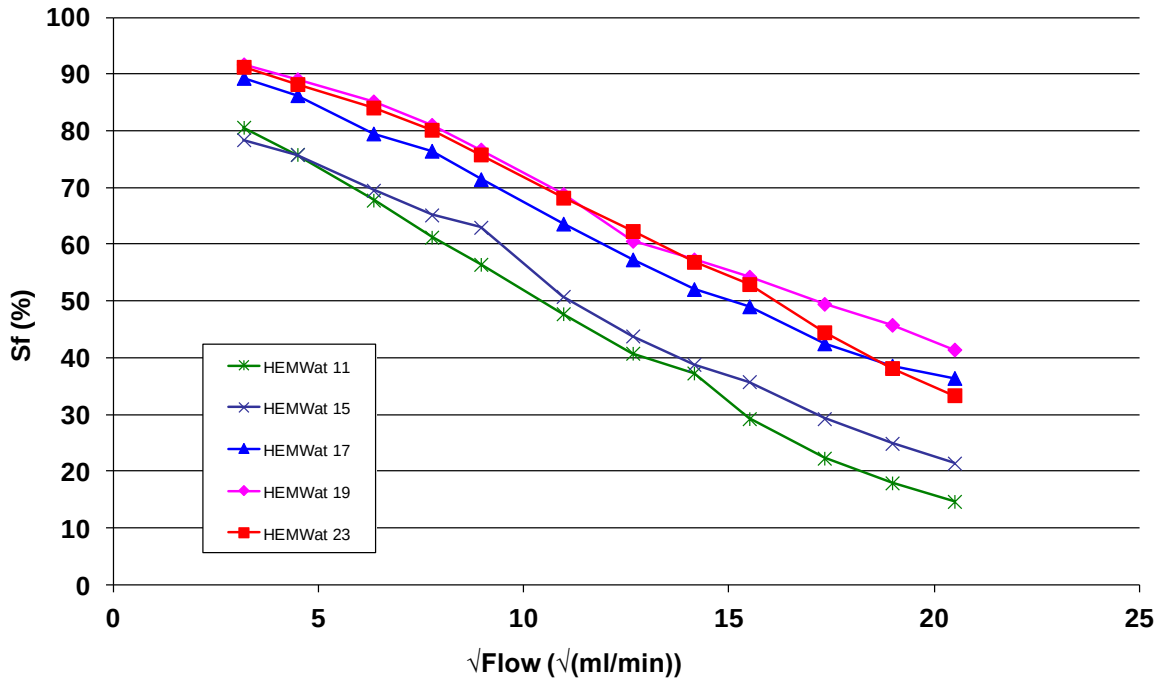


Figure 5.3.2: Reversed phase Du plots for HEMWat 11, 15, 17, 19 and 23 with DE-Midi preparative instrument, V_c = 459.5 mL, 4.0 mm bore PFA tubing, 240*g*, 30 °C

In normal phase on the Spectrum instrument (Figure 5.3.3), linear behaviour is observed for the stationary phase in the standard operating region, up to 12 mL/min. However, above this flow rate a significant reduction in stationary phase retention is seen across the range of HEMWat systems used. The rate of reduction in stationary phase increases as the phase systems become more polar and a distinct inflection point between two regions of stationary phase behaviour moves to a lower flow rate.

With the Midi instrument, similar non linear effects are observed for the stationary phase retention in normal phase (Figure 5.3.4). Initially linear behaviour is observed, up to flow rates of 40 mL/min however, above 40 mL/min there is an inflection point for the most polar phase systems. Less polar HEMWat phase systems 17, 19 and 23 all maintain linearity to 80 mL/min, but then show the same inflection point. Similarly to the Spectrum instrument, the point of inflection moves toward a slower flow rate as the polarity of the phase system is increased.

When comparing the semi-preparative and preparative normal phase results both show similar behaviour. Both have linear Du-plots until the inflection point and for both the inflection point occur at lower flow rates with more polar phase systems. As for reversed phase, the preparative instrument gives slightly improved stationary phase retention when the results are compared scaled by the cross-sectional area of the column. From Figure 5.3.2 and Figure 5.3.4 respectively, the stationary phase retention is 78 – 81% for HEMWat 11 – 23 at 12 mL/min on the semi-preparative instrument in comparison to 86 – 88% for HEMWat 11 – 23 at 75 mL/min on the preparative instrument.

In normal phase the change in behaviour, after the inflection point highlights a limit to Du's empirically derived equation. As the Du plots are not linear to the target 50% stationary phase retention, the inflection point will be used as the maximum flow for dual flow and ICcE runs in normal phase. This is clearly critical for the more polar phase systems where retention falls away very quickly after the inflection point, though with the more stable non polar phase systems, where the rate of decline in stationary phase is not so rapid, there is still the opportunity to work at higher flows.

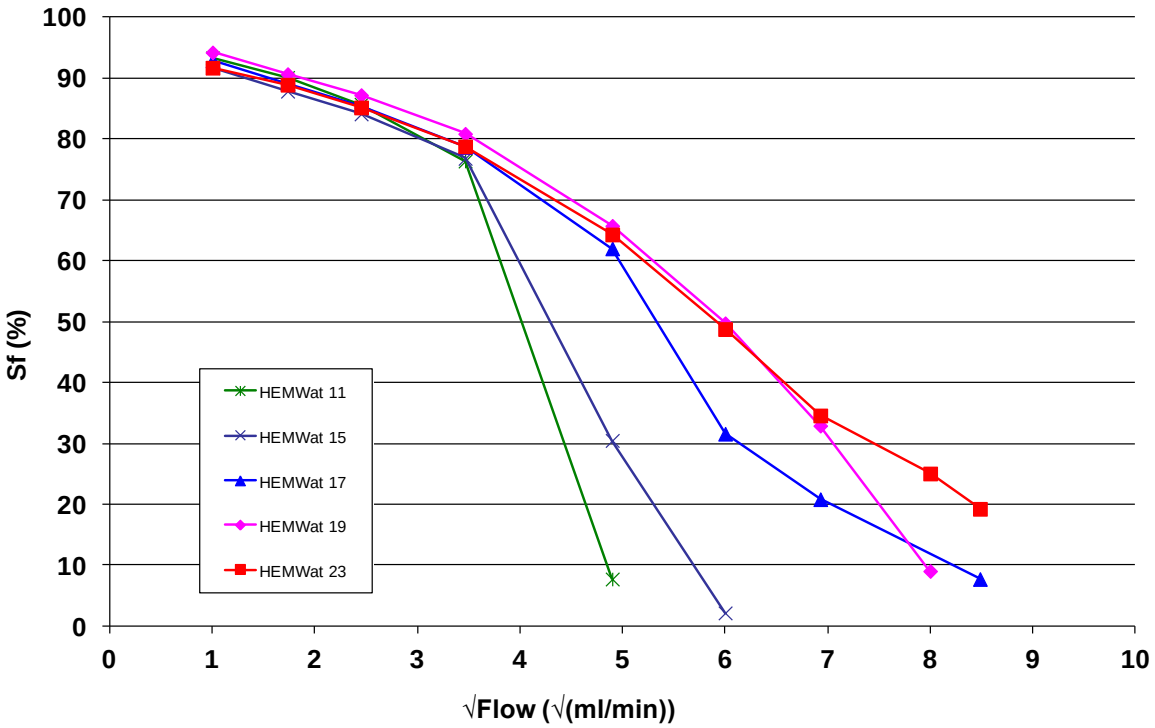


Figure 5.3.3: Normal phase Du plots for HEMWat 11, 15, 17, 19 and 23 with DE-Spectrum semi-prep instrument, V_c = 73 mL, 1.6 mm bore PFA tubing, 240*g*, 30 °C

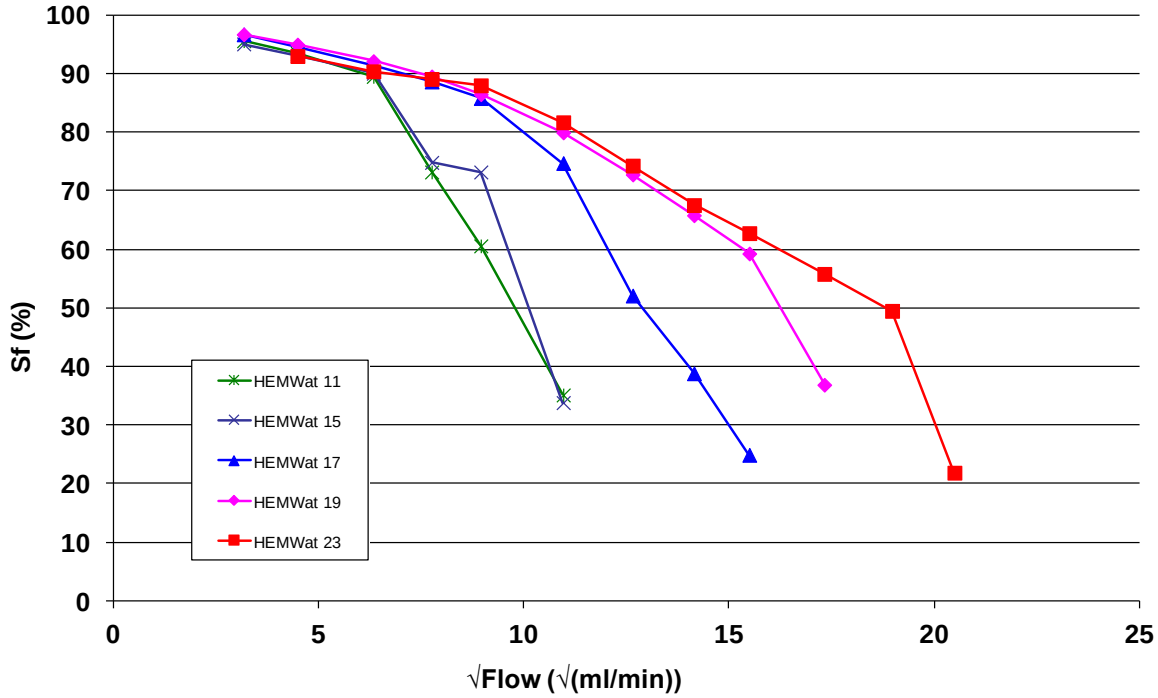


Figure 5.3.4: Normal phase Du plots for HEMWat 11, 15, 17, 19 and 23 with DE-Midi preparative instrument, V_c = 459.5 mL, 4.0 mm bore PFA tubing, 240*g*, 30 °C

5.3.2) S_f of HEMWat 15 and 23 phase systems for g-field changes

To confirm the inflection point seen in normal phase was not simply due to the high g-field (240g) used with the "high-performance" CCC instruments retention results for both the relatively unstable polar HEMWat 15 solvent system and the more stable HEMWat 23 solvent system at *g*-fields of 240*g*, 177*g*, 123*g* and 79*g* using the DE-Midi instrument in both normal and reversed phase were compared (Figure 5.3.5-5.3.8). HEMWat 15 and 23 were chosen for these experiments as practically they are closer to the most often used phase systems in real world industrial applications. Although HEMWat 11 seems slightly more unstable it is relatively unusual to use this phase system.

For HEMWat 15 (Figure 5.3.5) the initial reduction in reversed stationary phase retention is shown to be a real effect, with an initial reduction in stationary phase seen at all *g*-field levels tested. The retention curves are linear with respect to the square root of flow, as described by Du (1999), however there does seem to be a slight "S" shape to the plots. The 10% reduction in stationary phase retention seen in Figure 5.3.2, such that the extrapolated Du-plots do not pass through 100% retention at zero flow, was confirmed by these results. The gradient of the plots also becomes more negative with reducing *g*-field due to the reduced force to retain the stationary phase.

In normal phase with HEMWat 15, Figure 5.3.6, the point of inflection occurs at all *g*-levels, becoming more pronounced at slower rotational speeds. Although the loss of stationary phase at 79*g* (600 rpm) seems catastrophic it should be noted that the point reached at 50% retention with a flow rate of 60 mL/min is stable.

With HEMWat 23, in reversed phase (Figure 5.3.7) the gradients of the curves are less steep than for HEMWat15 implying the phase system is more hydrodynamically stable. Up to 12 mL/min the Du-plot is linear. As the *g*-field decreases and the flow increases the curve shape becomes "S" shaped rather than linear, as was the case with HEMWat15, making prediction of the stationary phase retention at lower rotational speeds less accurate.

The normal phase results for HEMWat23 confirm this solvent system is hydrodynamically more stable, than HEMWat15, with stable stationary phase retention to over 60 mL/minute at all g-levels. The inflection point is very clear, with two linear regions observed, firstly the classical region described by Du, followed by a second region with a steeper gradient for all rotational speeds.

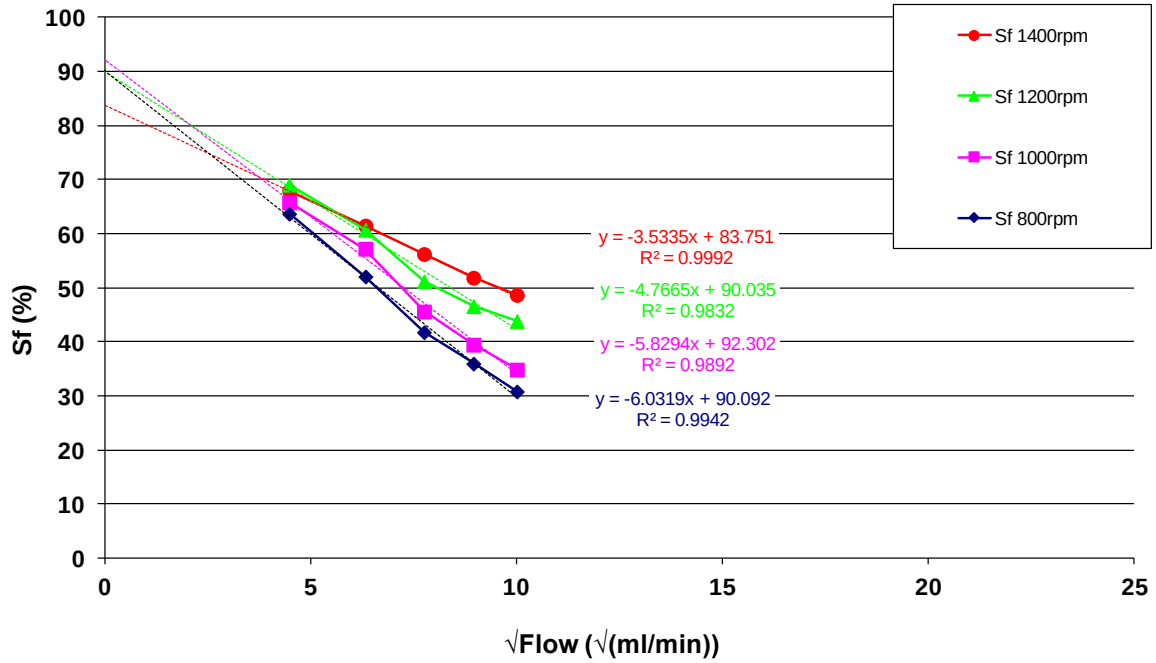


Figure 5.3.5: Reversed phase Du plots for HEMWat 15 at 240*g* (1400 rpm), 177*g* (1200 rpm), 123*g* (1000 rpm) and 77*g* (800 rpm) with the DE-Midi preparative instrument, V_c = 912 mL, 4.0 mm bore PFA tubing, 30 °C

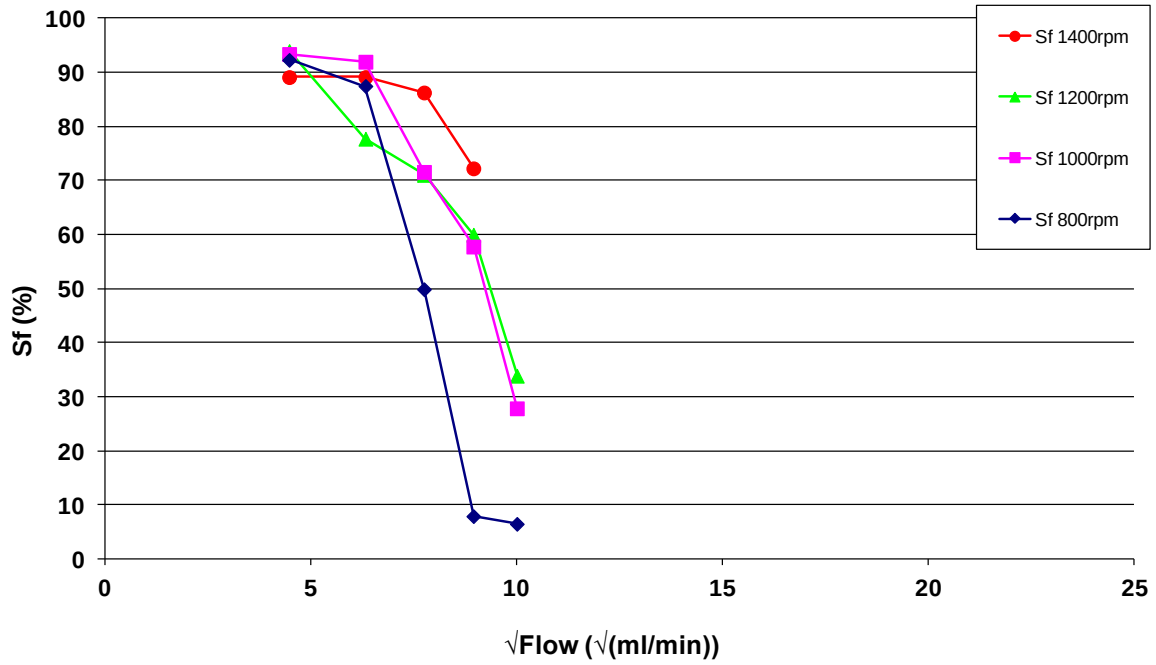


Figure 5.3.6: Normal phase Du plots for HEMWat 15 at 240*g* (1400 rpm), 177*g* (1200 rpm), 123*g* (1000 rpm) and 77*g* (800 rpm) with the DE-Midi preparative instrument, V_c = 912 mL, 4.0 mm bore PFA tubing, 30 °C

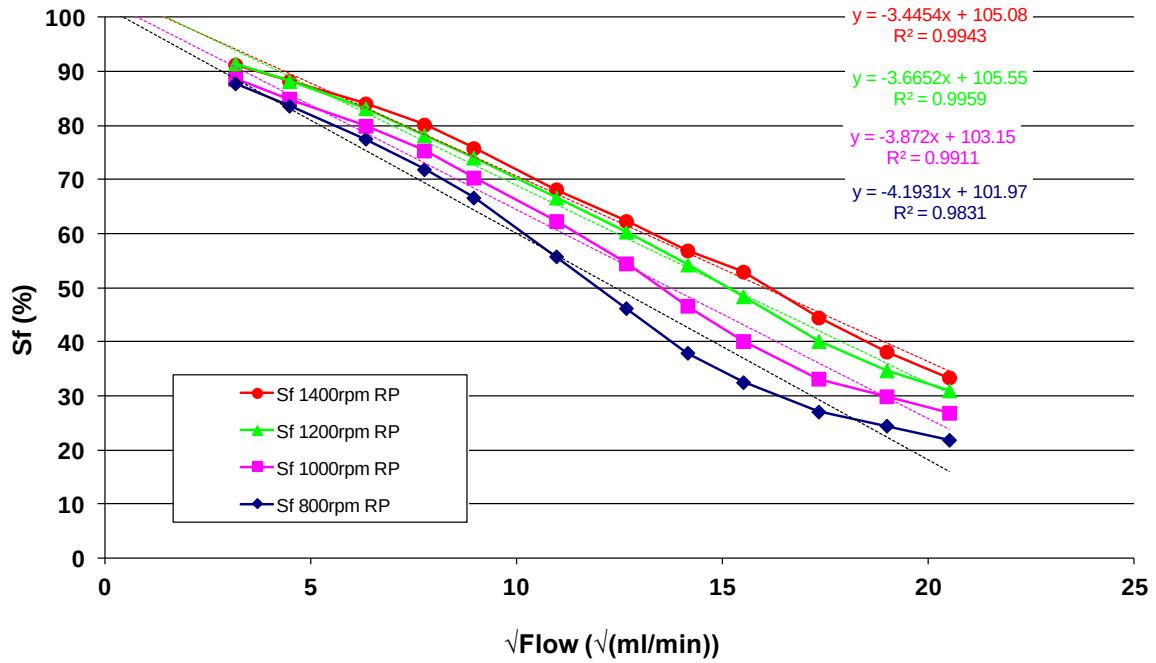


Figure 5.3.7: Reversed phase Du plots for HEMWat 23 at 240*g* (1400 rpm), 177*g* (1200 rpm), 123*g* (1000 rpm) and 77*g* (800 rpm) with the DE-Midi preparative instrument, V_c = 459.5 mL, 4.0 mm bore PFA tubing, 30 °C

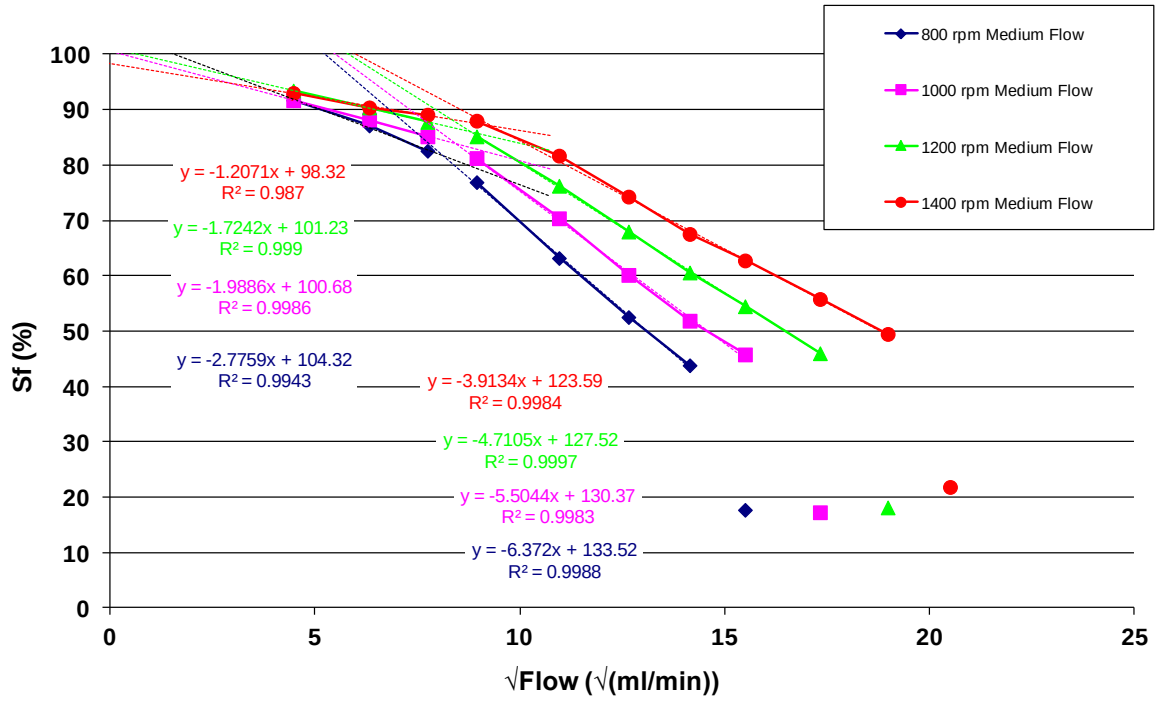


Figure 5.3.8: Normal phase Du plots for HEMWat 23 at 240*g* (1400 rpm), 177*g* (1200 rpm), 123*g* (1000 rpm) and 77*g* (800 rpm) with the DE-Midi preparative instrument, V_c = 459.5 mL, 4.0 mm bore PFA tubing, 30 °C

With increasing g-field the inflection point is at higher flow rates driven by improved retention in both these regions. Note that at 1400 rpm (240g, HPCCC) flow rates of 80 mL/min can be maintained with stationary phase retention of 88% whereas for HSCCC (800 rpm, 79g) only a flow rate of 60 mL/min with retention of 82% can be maintained.

5.3.3) Stationary Phase retention on the 4.6 L Maxi Instrument

With the 4.6L Maxi instrument, which operates at a maximum of 120*g*, the intermediate polarity HEMWat system 17 was tested in both reversed phase (Figure 5.3.9) and normal phase (Figure 5.3.10) to determine the operating limits for this pilot scale instrument. This intermediate polarity phase system is used as it is the starting point for solvent system searches when determining the best HEMWat solvent system to use for a separation (Garrard 2005). To compare this data with the semi-prep Spectrum and preparative Midi instruments, operating at the higher 240*g*, the flow rates were normalised by dividing the flow rate by the cross-sectional area of the tubing used.

Due to the reduced *g*-field used with the 4.6L Maxi the reversed phase stationary phase retention is reduced. The gradient of the reversed phase stationary phase retention curve is also steeper (-21 cf. -11) when compared with the instruments running at 240*g* (Figure 5.3.9).

In normal phase an inflection point is again observed for HEMWat system 17 between 400 and 600 mL/min (Figure 5.3.10). Therefore, although flow rates up to 500 mL/min could be used in reversed phase and the stationary phase retention would be maintained above 50%, due to the inflection point in the normal phase mode graph the flow rates will be have to be limited to 400 mL/min on the 4.6 L Maxi pilot instrument.

To confirm the extreme reduction in stationary phase seen at 120*g* on the 4.6 L Maxi instrument in normal phase, normal phase retention measurements were done on the other sizes of commercial instrument with the rotational speed set to give 120*g* (Figure 5.3.11). These results show very similar sudden loss of stationary phase retention when compared at normalised flow rates.

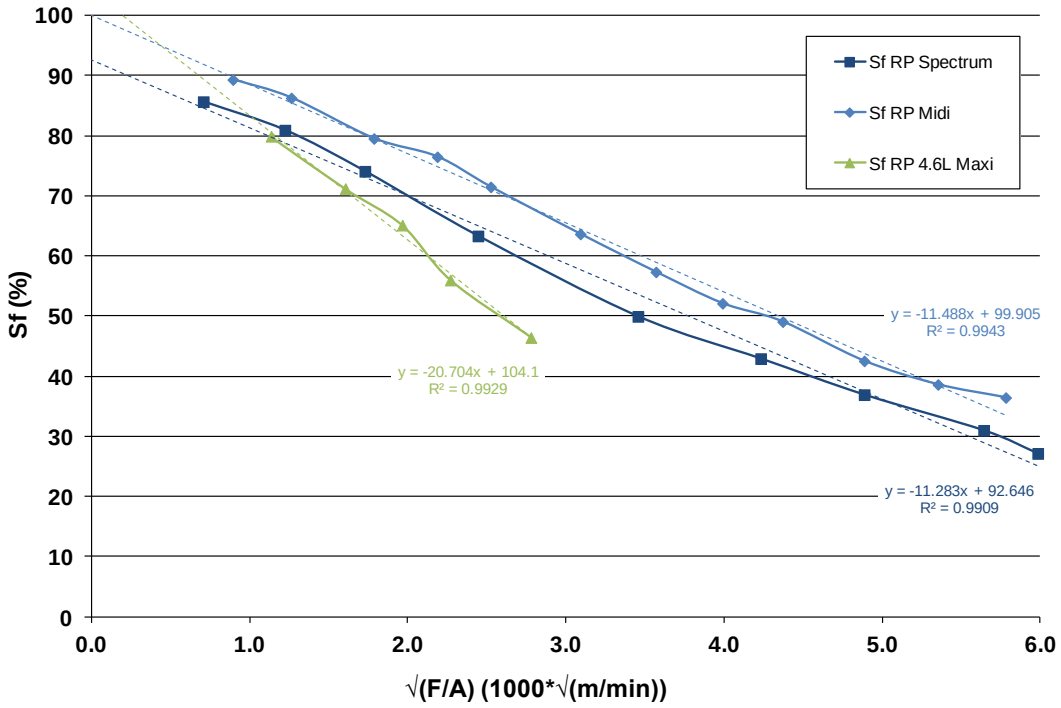


Figure 5.3.9: Reversed phase Du plots for HEMWat 17 at 120*g* (600 rpm) with the 4.6 L Maxi pilot instrument, V_c = 2300 mL, 10.0 mm bore PFA tubing, 30 °C compared to the DE-Spectrum and DE-Midi instruments at 240*g* (1400 rpm)

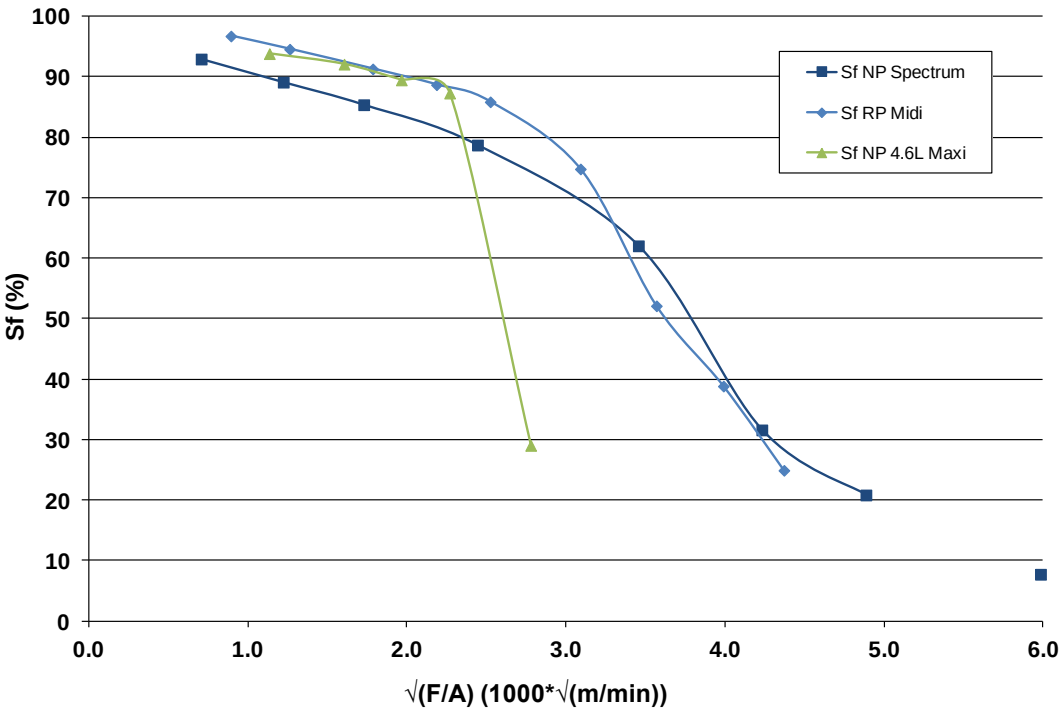


Figure 5.3.10: Normal phase Du plots for HEMWat 17 at 120*g* (600 rpm) with the 4.6 L Maxi pilot instrument, V_c = 2300 mL, 10.0 mm bore PFA tubing, 30 °C compared to the DE-Spectrum and DE-Midi instruments at 240*g* (1400 rpm)

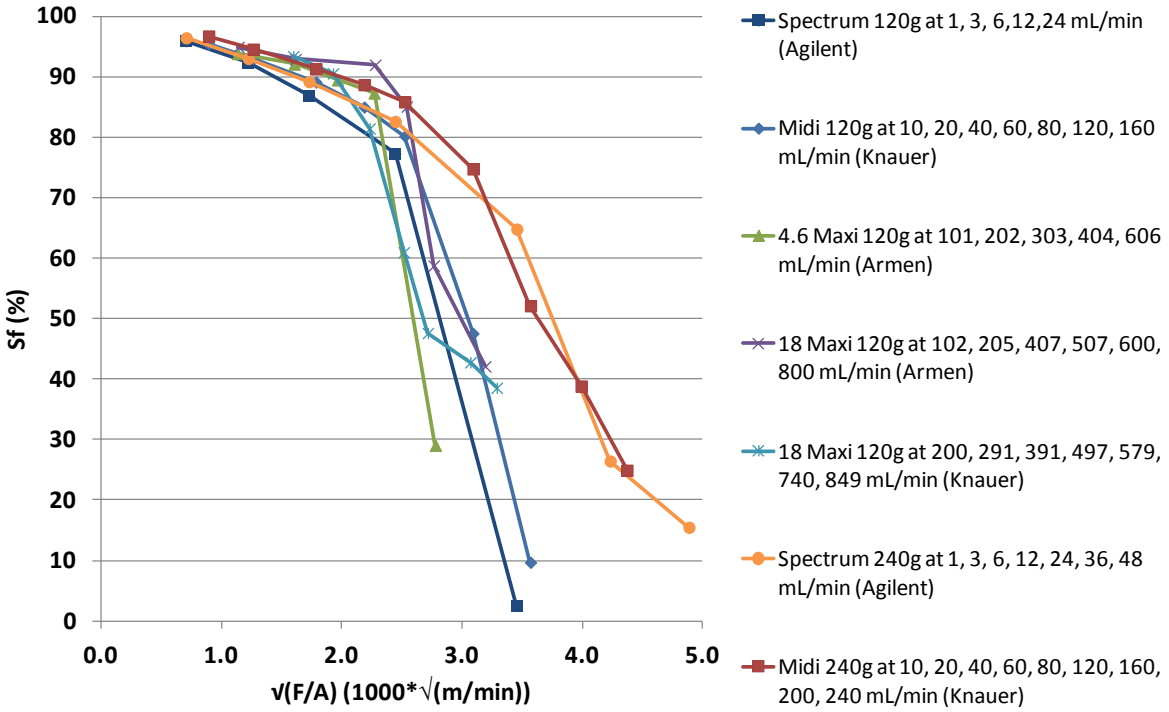


Figure 5.3.11: Normal phase Du plots for HEMWat 17 at 120*g* (600 rpm) with the DE-Spectrum V_c = 70 mL, 1.6 mm bore PTFE tubing, DE-Midi V_c = 453 mL, 4.0 mm bore PFA tubing, 4.6 L Maxi pilot instrument, V_c = 2300 mL, 10.0 mm bore PFA tubing, 18 L Maxi pilot instrument, V_c = 8510 mL 10.0 mm bore PFA tubing, 30 °C compared to the DE-Spectrum and DE-Midi instruments at 240*g* (1400 rpm)

Therefore, the volumetric scale-up used in CCC (Wood et al. 2007) will have to be used with caution when scaling-up to the pilot instrument, especially in normal phase where the inflection point and catastrophic loss of stationary phase occurs at lower relative flow rates as the bore increases.

Analysis of the Reynolds numbers is given in the following section (Section 5.3.4) to attempt to understand the cause of the inflection point seen in normal phase at all instrument scales.

5.3.4) Analysis of Reynolds numbers

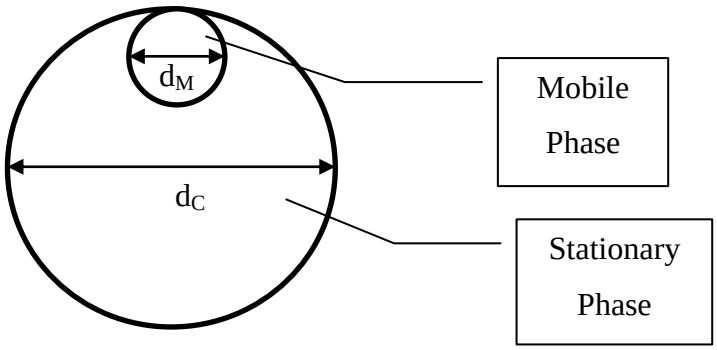
The Reynolds number for a fluid system is a dimensionless number relating the ratio of the inertial forces to the viscous forces. It potentially allows the characterisation of different flow regimes. Low Reynolds numbers (less than 2000), where viscous forces dominate, are regions of smooth laminar flow. High Reynolds numbers (greater than 3000), where inertial forces dominate, are regions of turbulent flow containing vortices and other instabilities. The transition between these two regions will occur between 2000 and 3000.

The Reynolds number for the mobile phase is calculated from Equation 5.3.1.

$$R_e = \frac{\rho_M d_M u_M}{\mu_M} \qquad \text{... (Eq. 5.3.1)}$$

Where, ρ_M is the density of the mobile phase (in kg/m^3), μ_M is the viscosity of the mobile phase (in Ns/m^2), u_M is the mean linear velocity of the mobile phase (in m/s) and d_M is the characteristic dimension of the mobile phase (in m).

The characteristic dimension of the mobile phase is the diameter of the cross sectional area the mobile phase would occupy assuming the area is circular.



From Eq. 4.1.5 described previously: $V_M = V_C(1 - S_f)$

And as, $V = \frac{\pi d^2 L}{4}$ and L is constant so, $V \propto d^2$

Therefore, $d_M = d_C\sqrt{1 - S_f}$ ... (Eq. 5.3.2)

While the mean linear flow velocity, $u_M = \frac{F_M}{A_M}$ ... (Eq. 5.3.3)

Where, F_M, is the flow rate of the mobile phase and A_M is the cross sectional area through which the mobile phase flows.

As, $$A_M = \frac{\pi d_M^2}{4} \quad \text{... (Eq. 5.3.4)}$$

Therefore, substituting for d_M from Equation 5.3.2,

$$A_M = \frac{\pi d_C^2 (1-S_f)}{4} \quad \text{... (Eq. 5.3.5)}$$

Therefore Equation 5.3.3 becomes, $$u_M = \frac{4F_M}{\pi d_C^2 (1-S_f)} \quad \text{... (Eq. 5.3.6)}$$

Therefore substitution Equation 5.3.2 and 5.3.6 into Equation 5.3.1 gives,

$$R_e = \frac{\rho_M 4F_M d_C \sqrt{1-S_f}}{\mu_M \pi d_C^2 (1-S_f)}$$

Which simplifies to,

$$R_e = \frac{\rho_M}{\mu_M} \cdot \frac{4F_M}{\pi d_C \sqrt{1-S_f}} \quad \text{... (Eq. 5.3.7)}$$

Using Equation 5.3.7, the Reynolds numbers were calculated for both normal and reversed phase across the HEMWat phase systems used. Figure 5.3.12 shows the Reynolds numbers for both normal and reversed phase elution using the Spectrum semi-prep instrument. For both modes of elution the Reynolds numbers increase with increased flow with the numbers for reversed phase being significantly less than those for normal phase. However, on this instrument even up to 70 mL/min flow rates the Reynolds numbers are always below 2000 implying continuous laminar flow.

Reynolds numbers for the Midi preparative instrument (Figure 5.3.13) show similar trends to the semi-preparative instrument, however, the Reynolds numbers achieved are on average two to three times as large at all flows. In reversed phase Reynolds numbers reach just over 2000, however with normal phase they reach 5000 with the relatively stable HEMWat 23. In normal phase all flows over 40 mL/min have the potential to be in a region of turbulent flow at which point excessive mixing of the phases may occur which could result in stripping a greater proportion of the stationary phase as the *g*-field will not be high enough to coalesce the two phases.

The Reynolds numbers for the pilot 4.6 L Maxi instrument (Figure 5.3.14) show even higher Reynolds numbers for both the normal and reversed phase results, the normal phase Reynolds numbers being over 3000 for flows of 200 mL/min and above.

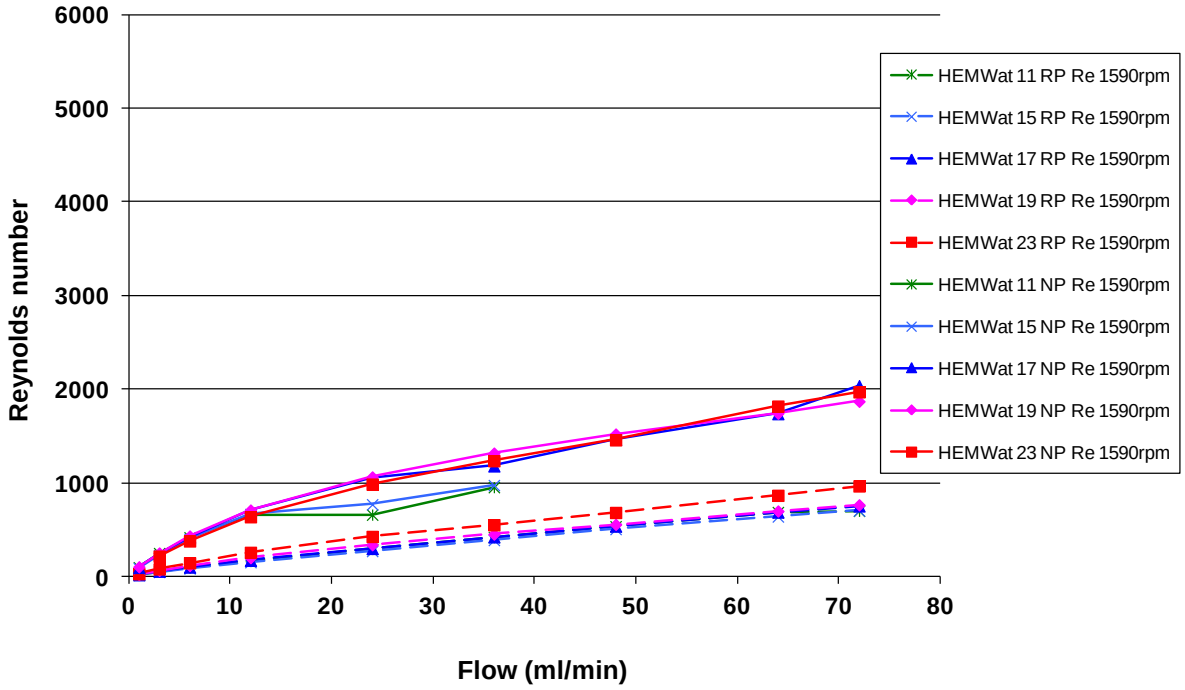


Figure 5.3.12: Reynolds numbers for HEMWat 11, 15, 17, 19 and 23 with DE-Spectrum instrument in normal (solid lines) and reversed (dashed lines) phase, V_c = 73 mL, 1.6 mm bore PFA tubing, 240*g*, 30 °C

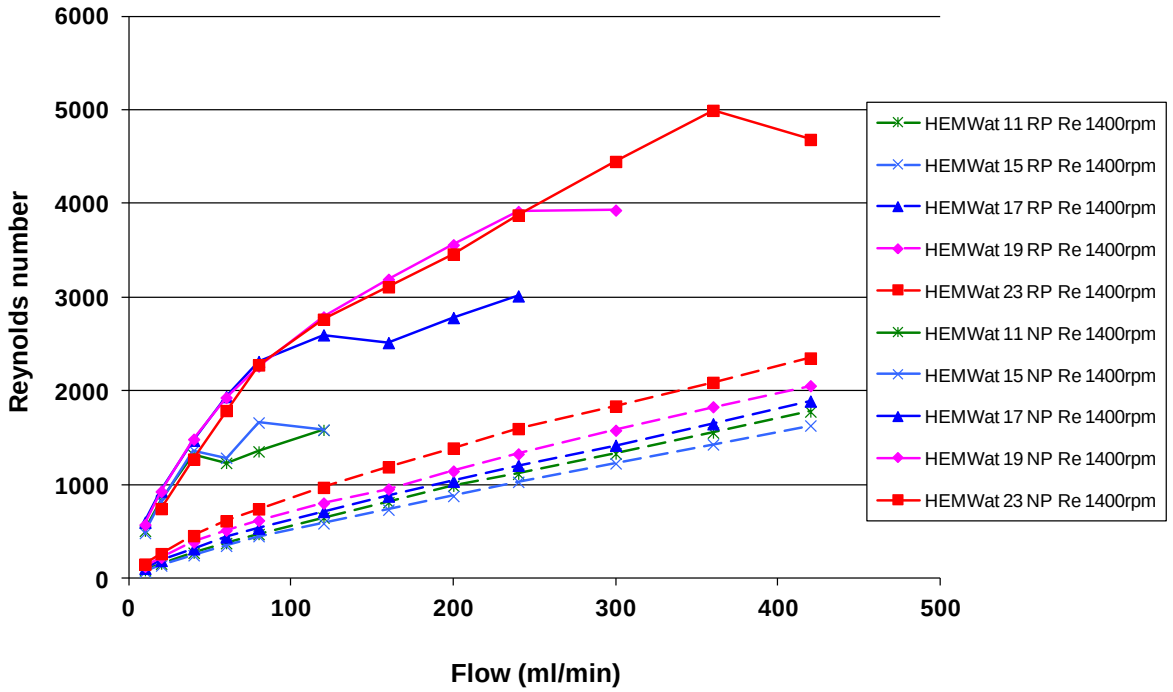


Figure 5.3.13: Reynolds numbers for HEMWat 11, 15, 17, 19 and 23 with DE-Midi instrument in normal (solid lines) and reversed (dashed lines) phase, V_c = 459 mL, 4.0 mm bore PFA tubing, 240*g*, 30 °C

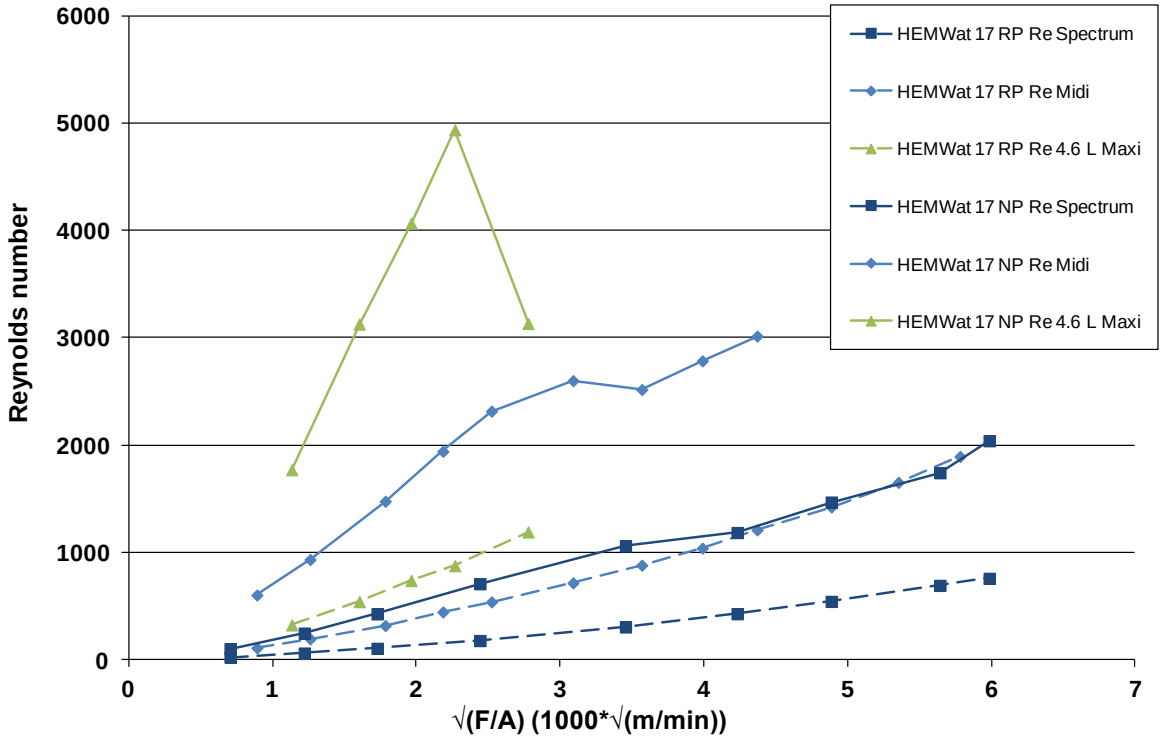


Figure 5.3.14: Reynolds numbers for HEMWat 17 (NP:solid lines, RP:dashed lines) at 120*g* (600 rpm) with the 4.6 L Maxi pilot instrument, V_c = 2300 mL, 10.0 mm bore PFA tubing, 30 °C compared to the DE-Spectrum and DE-Midi instruments at 240*g* (1400 rpm)

The Reynolds numbers calculated assume mean linear flow rates, however the distribution of phase in each loop of the CCC is unlikely to be linear (Wood et al. 2001) therefore there may be points of higher linear velocity in the column which would generate larger Reynolds numbers and therefore potentially a transition to turbulent flow which would change the column hydrodynamics.

5.4) Evaluation of DF and ICcE methods

The two CCC continuous processing methods, dual flow and ICcE, are compared in this section. Maintaining a stable phase volume ratio in the column is important for continuous operation of these instruments, therefore this was the focus of the study.

5.4.1) Maintaining stable phase volume ratio within the DFCCC column

Results from van den Heuvel (2009) show stable phase volume retention can be achieved within the dual flow bobbin. However, control of the ratio of upper phase to lower phase volume within the bobbin is difficult. His results state that both the upper and lower phase flows have a large effect on the phase volume ratio in the column. Therefore specific flows would be required to achieve a given ratio. However, if the phase flow ratio (the ratio of the upper phase flow divided by the lower phase flow) is fixed then the flow rates could not be changed to alter the distribution ratio at which compounds flow with the upper or lower phase. Even with phase volume ratios varying between 10 and 90% van den Heuvel achieved a group separation. Nevertheless, the phase volume ratio determines the column loading capacity as discussed in Section 5.3. Therefore the phase volume ratio needs to be at least in the range of 40 to 60% to give the opportunity for high loading with complex mixtures. Previous papers have used either needle valves (Ito 1985, Lee et al. 1988, Oka et al. 1989, Oka et al. 1991) or a length of capillary tubing attached to the centre outlet (Goto et al. 2006; Ito et al. 2006). The papers attribute this to helping control the upper phase outlet flow and establish equilibrium to counter the natural pumping action of the CCC column from tail periphery to head centre.

Therefore the effect of a tubing restriction attached to the upper phase outlet of the DFCCC column on its equilibration is investigated in this section. Based on these results, it is shown that these restrictions are a means of creating back pressure at the upper phase outlet which will control the phase volume distribution within the column.

5.4.1.1) Maintaining DFCCC phase volume ratio – experimental conditions

The detailed experimental methods used for dual flow equilibration are given in Section 3.3. Briefly, the DFCCC column (561 mL) was filled with lower phase and equilibrated at 20, 40 and 60 mL/min upper phase flow rates and 50, 30 and 10 mL/min lower phase flow rates

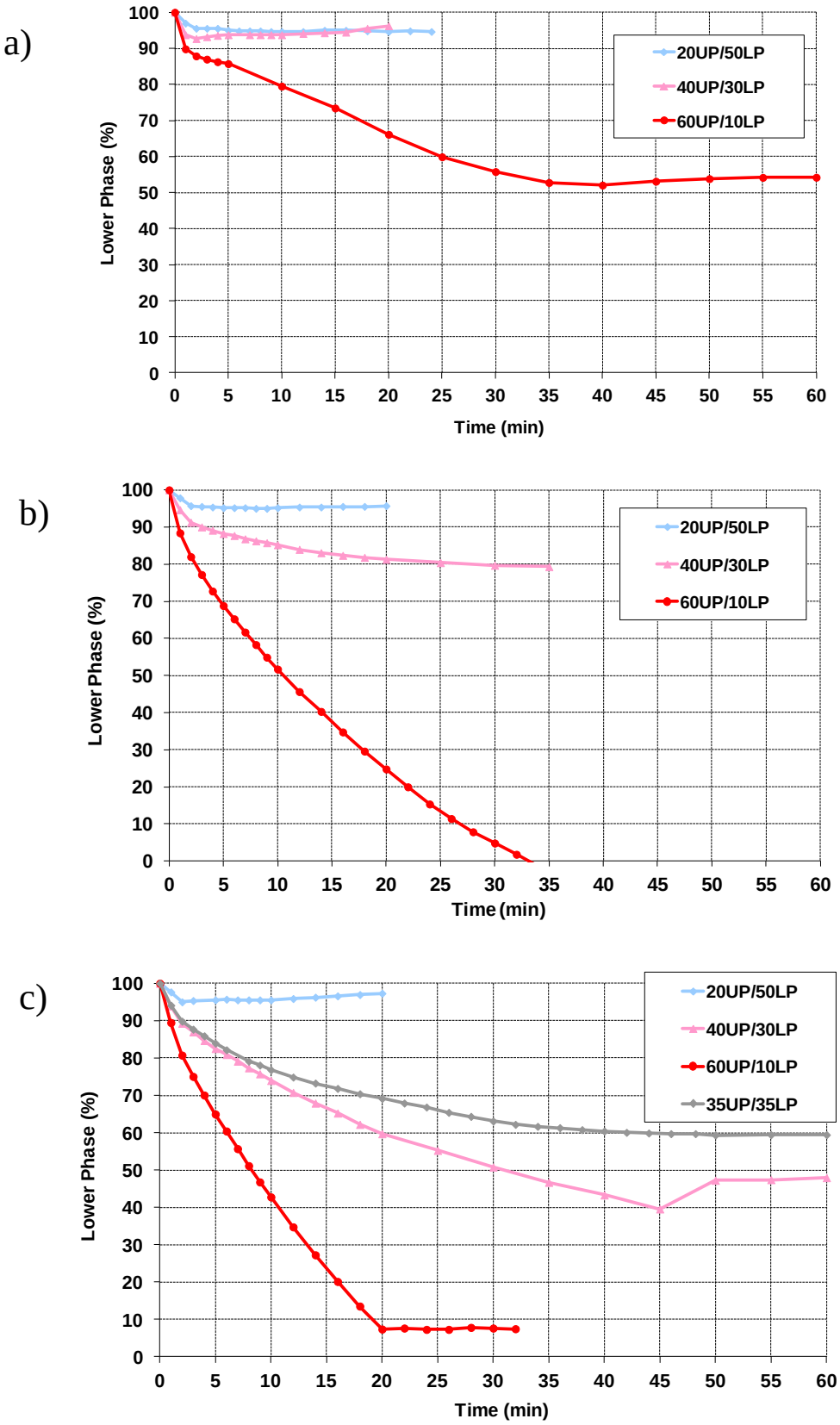


Figure 5.4.1: Effect of flow ratio on phase retention within a DFCCC column (561 mL), HEMWat 17 phase system, 1000 rpm, 5.0 mm I.D. PFA tubing, 30 °C for a) 0.0 m, b) 1.0 m, and c) 2.0 m 0.8 mm I.D. tubing attached to the centre (upper phase) outlet

respectively to give a relative flow rate of 70 mL/min in all cases. The centre (upper phase) outlet had either 2.0 m, 1.0 m or 0.0 m of 0.8 mm I.D. PTFE tubing attached to restrict the flow.

5.4.1.2) Maintaining DFCCC phase volume ratio – results and discussions

Figure 5.4.1 shows the lower phase in the column with respect to time, calculated from the displaced volumes of upper and lower phases from the upper and lower phase outlets respectively for the three restrictions on the centre upper phase outlet (Figure 5.4.1a – 0.0 m of tubing, Figure 5.4.1b – 1.0 m of tubing, Figure 5.4.1c – 2.0 m of tubing). With a low phase flow ratio of 0.4 (upper phase flow 20 mL/min, lower phase flow 50 mL/min, blue diamonds on Figure 5.4.1) the column equilibrates within two minutes with all restrictions, however the column is always over 90% full of lower phase. With a phase flow ratio of 1.3 (upper phase flow 40 mL/min, lower phase flow 30 mL/min, pink triangles) the greater the restriction the more upper phase is held in the column and the column can be equilibrated between 40 and 60% in 45 minutes (Figure 5.4.5c). While if the phase flow ratio is 6.0 (upper phase flow 60 mL/min, lower phase flow 10 mL/min, red circles) the column can be equilibrated in 35 minutes if no tubing restriction is used, but once the restriction is applied the column floods with upper phase within 20 minutes.

In all cases as the phase flow ratio increased the volume of upper phase in the column increased. Also, as the length of restrictive tubing used increased the volume of upper phase in the column increased, apart from at a flow ratio of 0.4. Both the increasing phase flow ratio and tube length increase the back pressure at the upper phase outlet.

Therefore, the back pressure generated with the given lengths of 0.8 mm I.D. PTFE tubing when HEMWat 17 upper phase was flowed, at 20 to 100 mL/min, was measured to allow calculation of the back pressure generated in the DF equilibration experiments using the experimental conditions given in Table 5.4.1. An empirical equation (Equation 5.4.1) of a response surface for the pressure at the upper phase outlet for any given length of tubing and flow rate (Figure 5.4.2) was generated based on a user defined three level factorial model Design of Experiment (Statgraphics Centurion).

$$P = 0.601 - 0.0259F - 0.494l + 0.00025F^2 + 0.0243Fl \quad \text{... (Eq. 5.4.1)}$$

Where pressure (P) is in bar, flow (F) is in mL/min and tube length (l) is in metres.

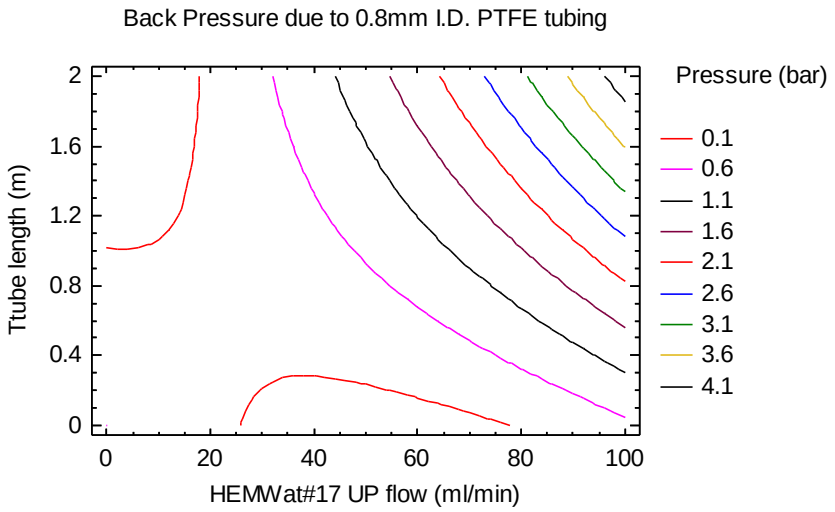


Figure 5.4.2: Back pressure due to flow of HEMWat 17 UP through 0.8mmm bore PTFE tubing for flows from 20 – 100 mL/min

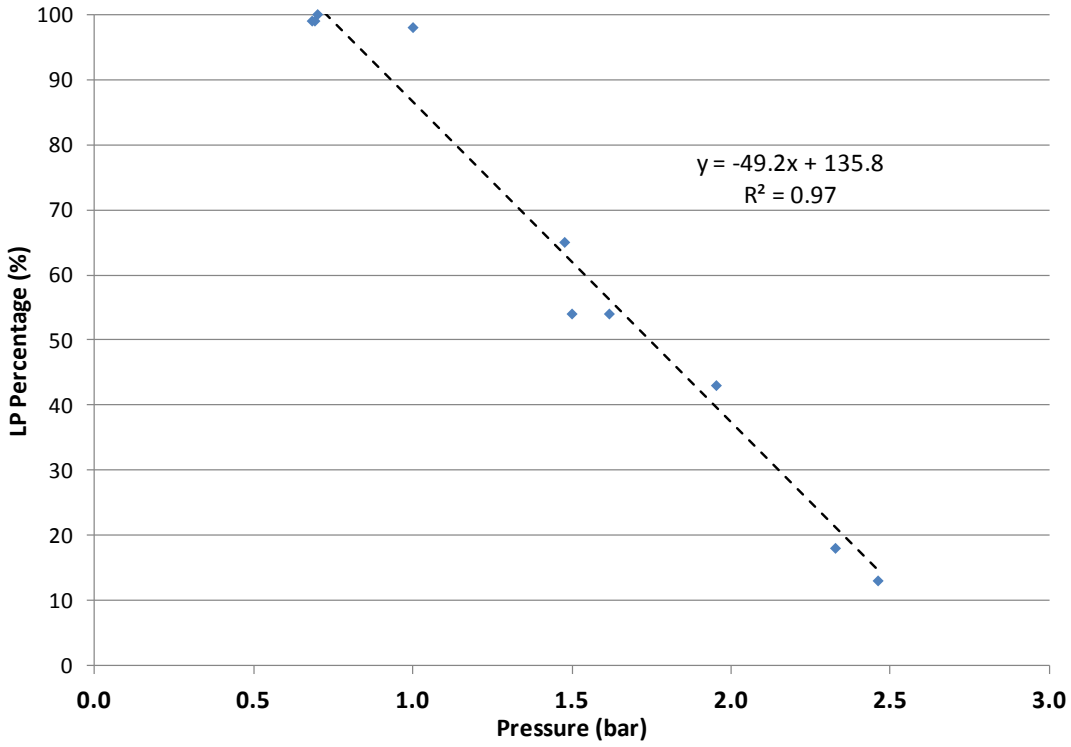


Figure 5.4.3: Retained lower phase in a DFCCC column (561 mL) due to changes in the back pressure applied to the centre outlet, DFCCC instrument, HEMWat 17, 1000rpm, 5.0 mm I.D. PFA, 30 °C

Table 5.4.1: Flow rates and tubing lengths used to determine back pressure on the outlet of the DFCCC instrument

Upper Phase Flow Rate (mL/min)	0.8 mm bore tubing length (m)	Pressure (bar)
20	0.0	0.019
40	0.0	0.061
60	0.0	0.129
80	0.0	0.208
100	0.0	0.349
20	1.0	0.166
40	1.0	0.421
60	1.0	0.909
80	1.0	1.549
100	1.0	2.456
20	2.0	0.343
40	2.0	0.835
60	2.0	1.706
80	2.0	2.970
100	2.0	4.532

Applying Equation 5.4.1 to calculate the back pressure being applied to the centre outlet for each experimental condition, then plotting these results against the final lower phase percentage in the column, measured by extrusion with compressed air of the column contents, gives a linear relationship (Figure 5.4.3).

Figure 5.4.3 includes runs at matched flow rates of 35 mL/min for upper and lower phase with 2.0 m and 4.0 m lengths of tubing further confirming the linear relationship between the back pressure on the centre outlet and the lower phase percentage in the DFCCC column.

Importantly, the system is very sensitive to applied back pressure. As can be seen from the slope of Figure 5.4.3, only a very small 100mbar change in back pressure will cause a 4.9% change in lower phase percentage in the column.

From the above study, equilibration to 50/50% phase ratio took nearly 40 minutes (Figure 5.4.4, filled circles and Figure 5.4.1a filled circles). Therefore, to try to reduced this time the column was initially filled with the target ratio of upper and lower phase (Figure 5.4.4, 52% triangles, 50% diamonds, 48% squares). When the initial phase ratio was in the target range of 40-60% the column was seen to displace 5-10% of the lower phase independent of the starting ratio then slowly drift over the next 40 minutes, to give final phase volume ratio in the range 35 to 49%. It was also observed that a small amount of lower phase stripped from the upper phase centre outlet (Figure 5.4.5, hatched blue area).

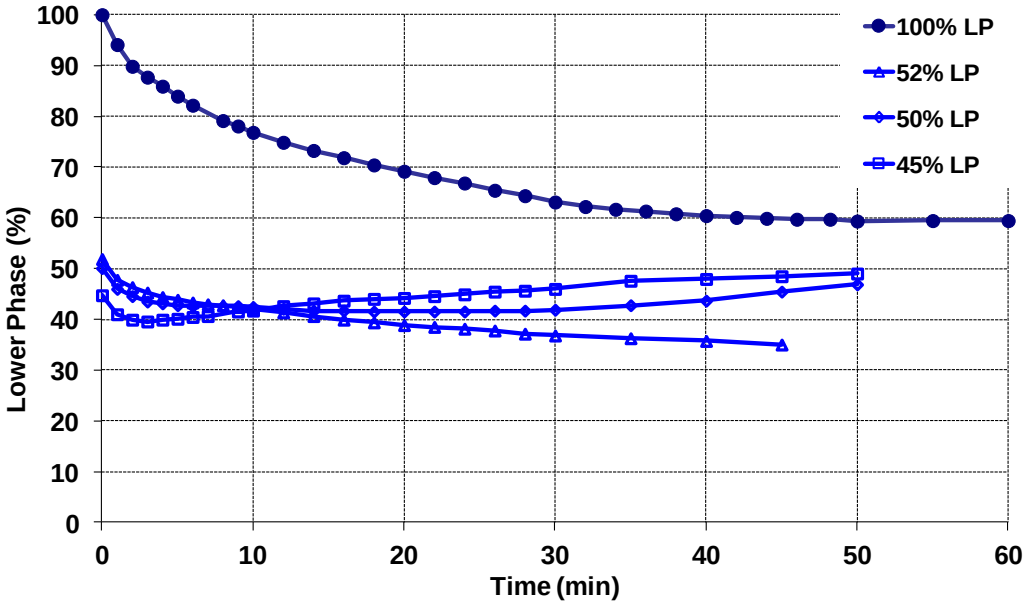


Figure 5.4.4: Comparing conventional filling with filling the DFCCC column with a phase ratio in the target range 40-60%, DFCCC bobbin (561 mL), HEMWat 17, 1000 rpm, 0.0 mm I.D. PFA tubing, 30 °C, Upper phase flow = lower phase flow = 35 mL/min, 2.0 m 0.8 mm I.D. tubing on the centre outlet

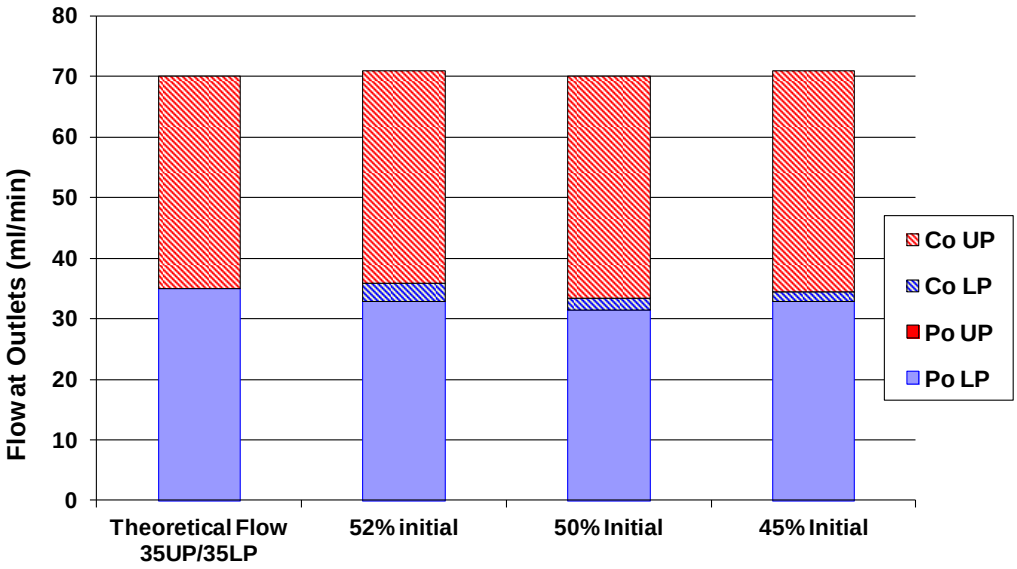


Figure 5.4.5: Equilibrium flow rates from centre upper phase outlet (Co) and periphery lower phase outlet (Po), DFCCC bobbin (561 mL), HEMWat 17, 1000 rpm, 0.0 mm I.D. PFA tubing, 30 °C, Upper phase flow = lower phase flow = 35 mL/min, 2.0 m 0.8 mm I.D. tubing on the centre outlet

Due to the limited space for attaching the outlet leads on the DFCCC bobbin, the design of the centre end fitting is such that the upper phase outlet is situated on the high *g* side of the bobbin where any lower phase collects. This striped lower phase has a higher viscosity and density than the upper phase so will cause increased back pressure at the centre outlet so may explain the variability of the final phase ratio within the column. Redesign of this end fitting is therefore required to reduce this observed stripping.

5.4.1.3) Maintaining DFCCC phase volume ratio – conclusions

For the DFCCC the back pressure applied to the centre outlet, when the periphery outlet is open to atmospheric pressure, controls the phase volume ratio in the column. A relatively small 0.1 bar change in back pressure gives a 4.9 % change in percentage lower phase in the column. Even when the column was filled with an initial 50/50% mix of upper and lower phase the phase volume ratio was seen to drift with time.

The difficulties in maintaining stable phase volume ratio with the DFCCC bobbin manually means it is likely continuous real-time flow and back pressure monitoring with automated feedback to pressure regulators will be required to maintain very stable phase retention.

Therefore, the next section (Section 5.4.2) tests phase retention with the ICcE method to determine if having only one phase flowing as the mobile phase at any one time helps to improve the stability of the phase volume ratio in the columns.

5.4.2) Maintaining column balance and phase volume ratio stability for ICcE

To allow continuous operation it is important that the phase volume ratio within the columns is maintained at a constant value and that within the two columns the phase volume ratio is similar so that the instrument does not become hydrodynamically and mechanically unbalanced. The previous results using the DFCCC have shown that when both phases flow at the same time, the phase volume ratio in the column was highly dependent on the back pressure applied to the centre outlet of the column. With ICcE the upper and lower phases are flowed alternately as mobile phase, therefore the original phase volume ratio could change with time and might lead to the bobbins becoming unbalanced. Further, the volume of the flying leads of a standard twin CCC instrument is likely to lead to the unbalance of the two columns when the phases are alternated, therefore this section describes modifications to the flying leads to eliminate this unbalance and further tests the stability of the phase retention across a range of HEMWat phase systems polarities from the polar system 11 through to non-polar system 23.

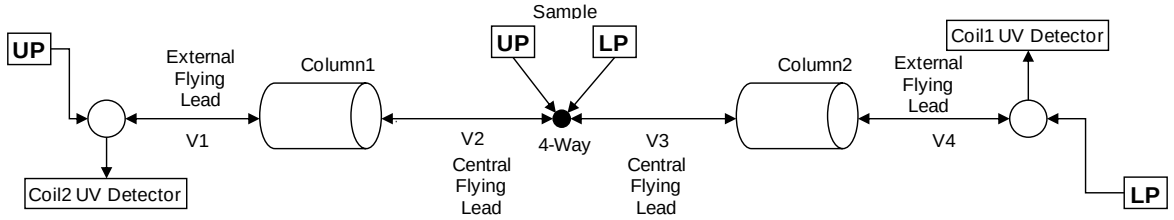


Figure 5.4.6: Flying lead positions on a standard twin bobbin CCC instrument

Theoretically the ICcE switching cycles will change the phase volume ratio of a standard twin bobbin CCC instrument where the flying leads (V1 to V4) are identical lengths of tubing (Figure 5.4.6). When running ICcE, after each switch of flow direction a dead volume of the previous mobile phase is pushed back onto the column from which it has just eluted. For example when switching from normal phase to reversed phase the upper phase which was mobile in the flying leads will be pushed back into the columns by the new flow of lower phase in reversed phase operation. The volume V4 of upper phase is pushed onto column 2 and the volume V2+V3 of upper phase is pushed onto column 1, while the volume V1 is eluted from the system. When the system is switched back to normal phase operation the opposite happens and a volume V1 of lower phase is pushed onto column 1 and a volume V2+V3 of lower phase is pushed onto column 2. As on a standard setup V2+V3 is double V1

or V4 with each switching cycle an excess of upper phase will build up in column 1, while an excess of lower phase will build up in column 2 until the bobbins become unbalanced. This imbalance will potentially put excessive mechanical load on the instrument making long term continuous running impractical.

However, column balance and true equilibrium can be maintained if V1=V2+V3=V4 as under this condition the dead volume of solution pushed onto each column when switching will be equal, therefore over time the bobbins should maintain their initial phase volume ratio.

5.4.2.1) Maintaining ICcE phase volume ratio – experimental conditions

The experimental methods used for ICcE are given in Section 3.4. Briefly, The HEMWat mobile phase was pumped alternately, first in normal phase (upper phase mobile, from tail-periphery to head-centre) and then in the opposite reversed phase direction (lower phase mobile, from head-centre to tail-periphery). Switching between normal and reversed phase was carried out at four minute time intervals for 12 cycles.

In practice on a preparative DE-Midi instrument, to achieve equal dead volumes for the polytetrafluoroethylene (PTFE) flying leads (3.2 mm O.D., 1.6 mm I.D., 2.5 m long), the external flying leads connecting the columns to the ancillary equipment (V1 and V4) were extended by 2.5 m each so that the total volume of V1 and V4 was 10 mL each, the same as the central flying lead section, connecting the two columns in series (V2+V3).

5.4.2.2) Maintaining ICcE phase volume ratio – results and discussions

The predicted lower phase within the columns with time and the actual ratio of phases observed after column pump out are given in Figure 5.4.7a for standard flying leads and Figure 5.4.7b for matched volume flying leads. With standard flying leads attached, the initial phase ratio changed from 46/54% (UP/LP) in each column to 73/27% (UP/LP) in column 1 and 36/64% in column 2 compared to predicted values of 61/39% in column 1 and 35/65% in column 2 after 96 minutes.

When the external flying leads were extended to match that of the internal flying leads, so that the dead volumes were equal (i.e. V1=V2+V3=V4), the final ratio of phases within the columns stayed close to the initial one. After 96 minutes, the phase ratio was 55/45%

(UP/LP) in column 1 and 48/52% in column 2 compared to the initial values of 50/50% (UP/LP) in each column.

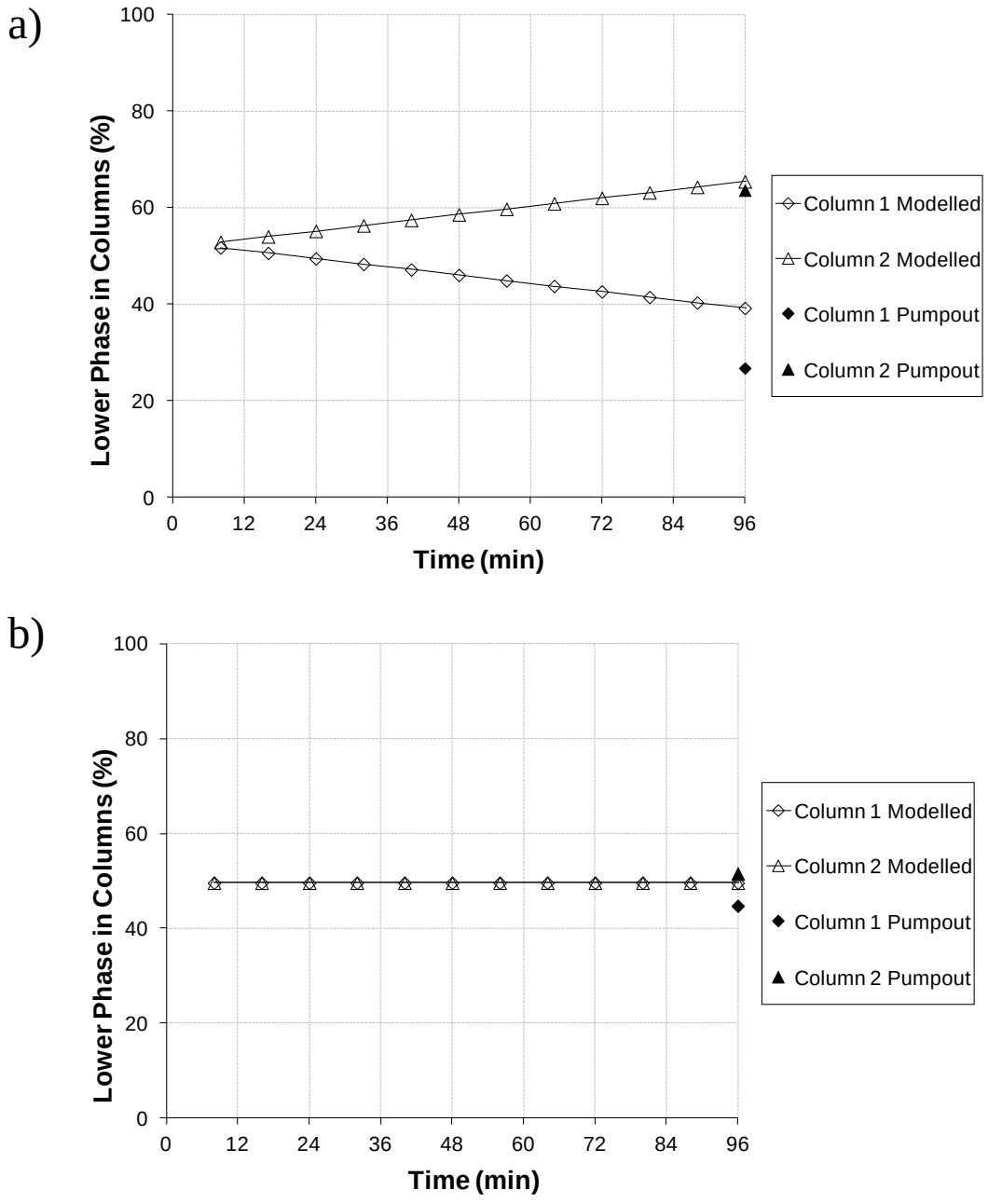


Figure 5.4.7: Predicted and actual lower phase in columns after 12 ICcE switching cycles for a) standard flying leads and b) matched volume flying leads; HEMWat 23, upper phase and lower phase flow rate 40 mL/min, flow switched every 4 min, rotational speed 1400 rpm, temperature 30 °C

As these results showed no obvious drift in the phase volume ratio over an extended 96 minute switching period, the study was extended to cover phase systems across the polarity range. Figure 5.4.8 shows that for HEMWat 11, 15, 17, 19 and 23 the columns maintained

their initial phase volume ratio, with no drift over time, in the range 40% to 60%, which would not cause imbalance between the columns. The results also confirmed that the protocol in Section 3.4.2 for setting up of the initial stationary phase retention to approximately 50% had been successful.

The lower phase in the columns was between 42%, for HEMWat 15, and 55% for HEMWat 11. Increasing the flow of each phase from 40 mL/min to 80mL/min for HEMWat 23 had practically no effect on the lower phase retention (from 48% to 45% respectively).

Figure 5.4.9 shows the percentage of upper and lower phases in the individual columns at the end of the ICcE runs were always in the range 40% to 60% when using the matched flying leads with flow rates of 40 mL/min for both phases. When the flow rate was increased to 80 mL/min for the HEMWat 23 system, although the overall phase volume ratio did not change significantly, column 1 where the upper phase entered ended the run with increased upper phase, whereas column 2 where lower phase enters had an increase in lower phase relative to the 40 mL/min run.

5.4.2.3) Maintaining ICcE phase volume ratio – conclusions

With a standard twin bobbin CCC instrument, to maintain similar phase volume ratio in both bobbins when operating the ICcE method it was shown that it is required to adjust the dead volumes of the flying leads such that the individual inlet and outlet flying lead dead volumes equal the total flying lead dead volume between the bobbins. Stable phase volume ratio was maintained for a 96 minute runtime, with 12 switching cycles across a range of polarity phase systems (HEMWat 11, 15, 17, 19 and 23) giving an operating mode that ought to be more robust than the phase volume ratio achieved with the DFCCC equilibration tests. Therefore the following Section 5.4.3 compares separations using the two modes to access which mode has the greater opportunities to achieve successful continuous processing.

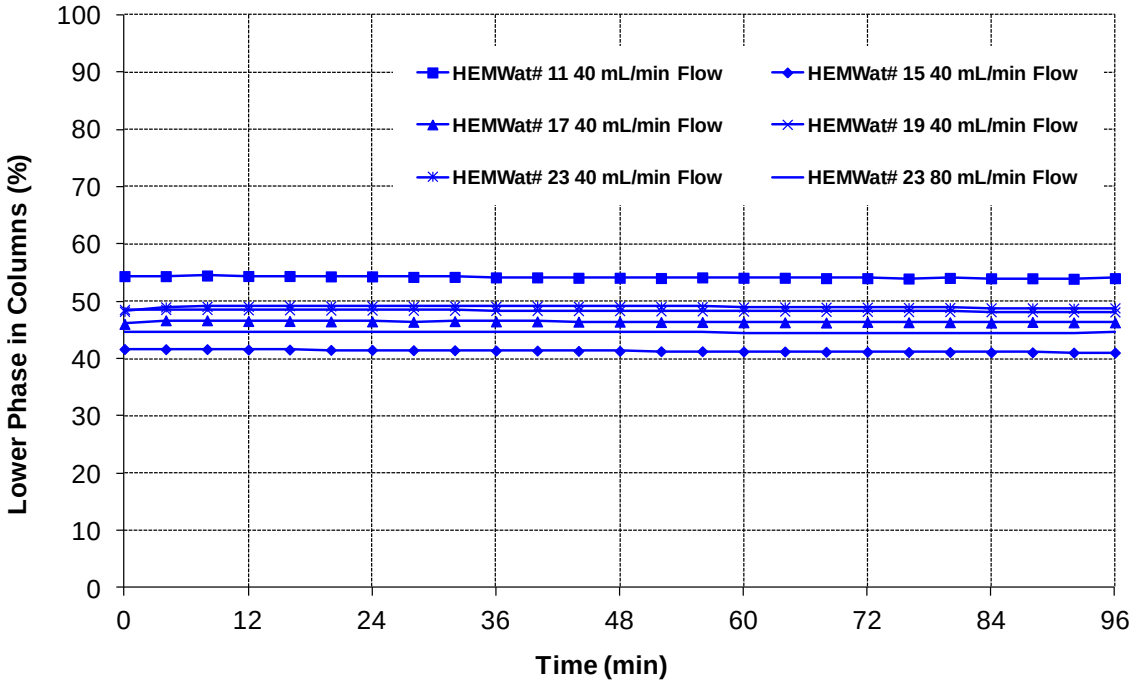


Figure 5.4.8: Lower phase in columns with respect to time calculated from displaced volume of stationary phase after each switching cycle, preparative DE-Midi (912 mL) 4.0 mm I.D.PFA, 1400 rpm, 30 °C

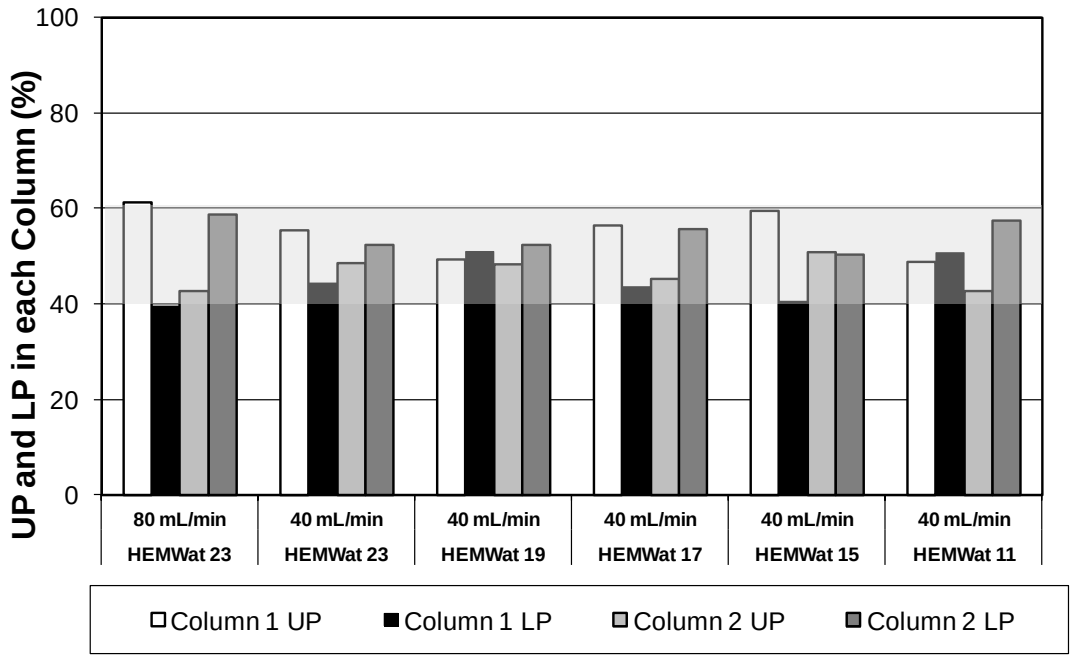


Figure 5.4.9: Percentage upper and lower phase in the columns after 96 min ICcE run with matched flying lead volumes, V1 = (V2 + V3) = V4), switching every 4 minutes, preparative DE-Midi (912 mL) 4.0 mm I.D.PFA, 1400rpm, 30 °C

5.4.3) Comparison of DFCCC and ICcE methods

Based on the retention results achieved, a short study to compare the two methodologies to evaluate their similarities, operating benefits and difficulties by using a model system containing a mixture of four compounds (caffeine, vanillin, naringenin and carvone) from the GUESSmix (Friesen and Pauli 2005) with hexane, ethyl acetate, methanol and water phase systems in continuous processing modes.

In Section 5.4.3.1 the feasibility of splitting a sample GUESSmix into two streams is discussed for ICcE and DFCCC. While in Section 5.4.3.3 target enrichment is qualitatively assessed by comparing, for ICcE, the concentration of a traditional Chinese medicine compound in the column with, for dual flow, the concentration of vanillin in the column from a GUESSmix. Section 5.4.4 gives the conclusions from this study comparing ICcE with DFCCC runs to split a sample into two streams or enrich a target inside the column.

5.4.3.1) Splitting a sample mixture into two streams – experimental conditions

The ICcE work was performed on a preparative Midi-HPCCC instrument using the HEMWat 16a (4:5:4:5) phase system. The methodology used is given in Section 3.4, and detailed run conditions and apparatus are described in Hewitson et al. (2009), Appendix 1.1.6. Briefly, an upper phase flow rate of 50 mL/min and lower phase flow rate of 60 mL/min were used switching every four minutes between upper and lower phase flow to split an 11.2g sample of carvone, naringenin, caffeine and vanillin (50 g/L, 224 mL) which was loaded for 16 minutes. These four compounds were chosen to broadly cover the polarity range, from highly non-polar carvone to polar caffeine, with naringenin and vanillin as two intermediate polarity compounds.

For DFCCC the same four compounds from the GUESSmix were used with HEMWat 15 (4:6:4:6). The methodology for the runs is given in Section 3.3 and detailed run conditions in Ignatova et al. (2011), Appendix 1.1.4. To split the four component sample into two streams the upper phase flow rate was set to 20 mL/min and the lower phase flow rate was set to 50 mL/min with manual pressure adjustment to maintain 50% phase volume ratio in the column. 7.4 g of sample in a solution of upper phase (50 g/L, 150 mL) was injected in the middle of the DFCCC column within 30 minutes.

For both runs the aim was to elute carvone and naringenin with the upper phase fractions and caffeine and vanillin with the lower phase fractions.

5.4.3.2) Splitting a sample mixture into two streams – results and discussion

For ICcE, the optimised conditions for splitting the sample mix into two streams gave 7.9 g/h throughput (Figure 5.4.10). Positive values of peak area corresponding to elution with the upper phase from the head and negative values to elution with the lower phase from the tail. Non polar carvone (K_d=7.4) and naringenin (K_d=1.25) eluted in the upper phase fractions with retention times of 10 and 31 minutes respectively, while more polar vanillin (K_d=0.55) and caffeine (K_d=0.09) eluted in the lower phase fractions with retention times of 6 and 30 minutes respectively, with complete separation of N and O from C and V between the upper and lower phase eluants from opposite ends of the columns.

For DFCCC, carvone (K_d=14.8) and naringenin (K_d=3.82) eluted with the upper phase and caffeine (K_d=0.14) with the lower phase according to their distribution ratios. Vanillin (K_d=1.21) eluted with the lower phase (though its K_d value in HEMWat 15 is above one) due to the higher flow rate of the lower phase compared to the upper phase (Figure 5.4.11). The separation cycle time was one hour, which gives 7.4 g/h throughput for sample processed. To maintain accurate flow throughout the run the back pressure on centre outlet was manually adjusted to achieve constant flow of 20 mL/min.

Both the ICcE and DFCCC methods worked to separate the component sample into two streams, with similar throughputs, however the need for manual control of flow from the upper phase outlet for the DFCCC run made this impractical to easily automate as a continuous process when compared to the ICcE method.

5.4.3.3) Target enrichment – experimental conditions

The ICcE method target enrichment demonstration was previously published by myself (Hewitson et al. 2009) as discussed in the literature review (Section 2.3.2.3). A preparative DE-Midi instrument was used to concentrate triptolide in the column from a Chinese herbal medicine extract. HEMWat 15 was flowed at 40 mL/min in normal phase and 35 mL/min in reversed phase, with the flow switched every 4 minutes.

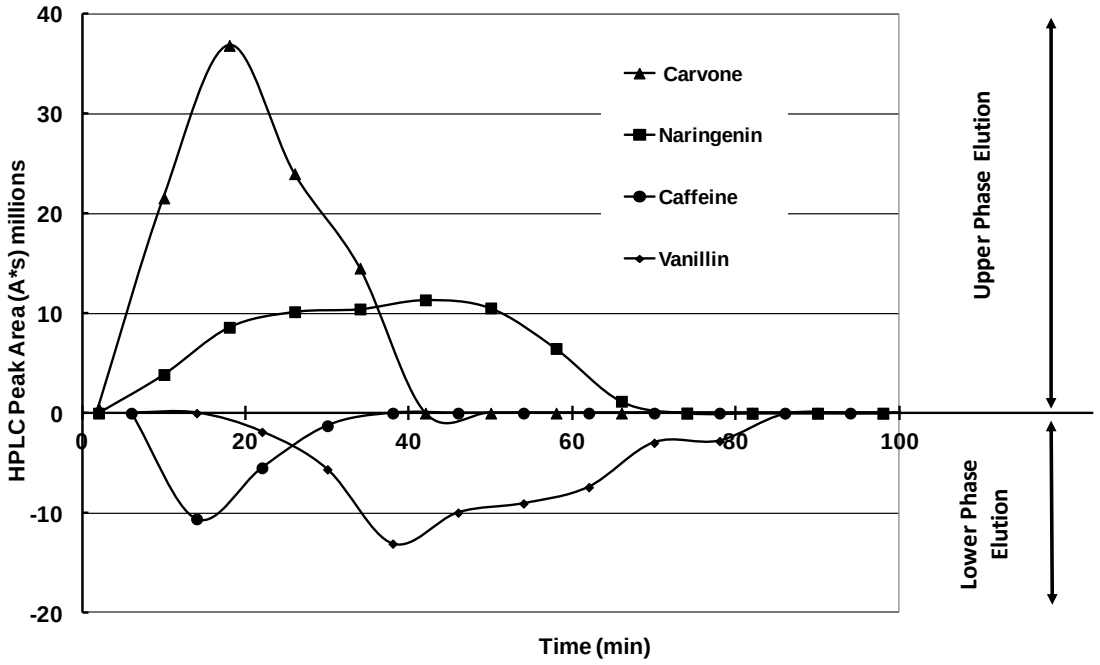


Figure 5.4.10: Fractogram constructed from HPLC fraction analysis after each ICcE cycle for the separation of GUESSmix compounds. HEMWat 16a, upper phase flow rate 50 mL/min, lower phase flow rate 60 mL/min, flow switched every 4 minutes, sample concentration 50.0 g/l, sample volume 224 mL, loading time 16 min, 1250 rpm, 30 °C

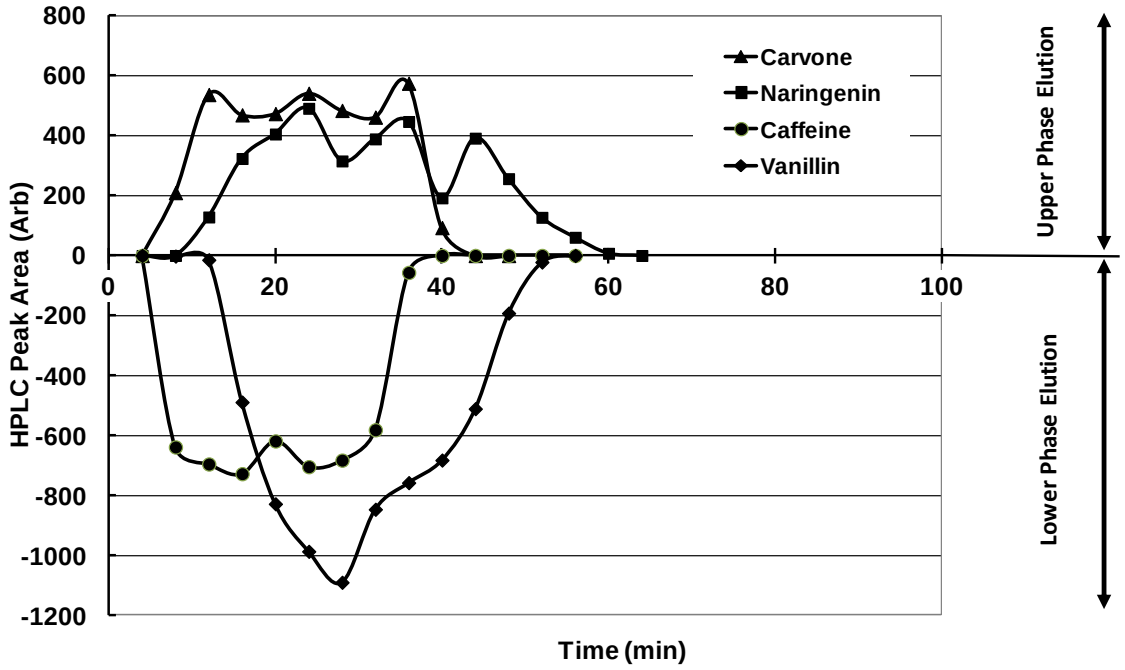


Figure 5.4.11: Fractogram constructed from HPLC fraction analysis after DFCCC separation of GUESSmix compounds. HEMWat 15, upper phase flow rate 20 mL/min, lower phase flow rate 50 mL/min, sample concentration 50.0 g/l, sample volume 150 mL, loading time 30 min, 1000 rpm, 30 °C

For the DFCCC method to demonstrate concentration in the column, the upper and lower phase flow rates were set equal to 35 mL/min, and a 7.4 g of sample solution containing carvone, naringenin, caffeine and vanillin in 150 mL of upper phase was injected in the middle of the DFCCC column at 5.0 mL/min. As vanillin has a distribution ratio near one, with equal flow rates, it would be expected to be largely retained in the column and slowly elute with the lower phase.

5.4.3.4) Target enrichment – results and discussion

For the ICcE method, 188 mg of 98% pure triptolide (C1) was collected inside the column while the rest of the compounds were washed away (Figure 5.4.12) with a throughput of 3 g/h of crude extract. The enrichment run, using the HEMWat 15 phase system, was stable enough to allow for the sample to be loaded continuously for 120 minutes, followed by a 56 minute wash cycle, which removed all the major remaining impurities (C2-4) from the target material in the column.

For the DFCCC method (Figure 5.4.13), carvone and naringenin eluted with the upper phase and caffeine with the lower phase. The main part of vanillin fraction was retained in the column, a small part eluted with lower phase and vanillin just started appearing in the upper phase when the separation run was stopped. The whole separation process was finished in just under one hour, since the column content could be pumped out at a very high flow rate (up to 200 mL/min for a preparative column), giving a 7.4 g/h throughput for the sample processed.

Both ICcE and DFCCC have been shown to be successful and practical methods for enriching a target in the column, washing that target and later collection by pumping out the column contents as had been theorised by Lee (1991) and previously discussed in Section 2.3.1.

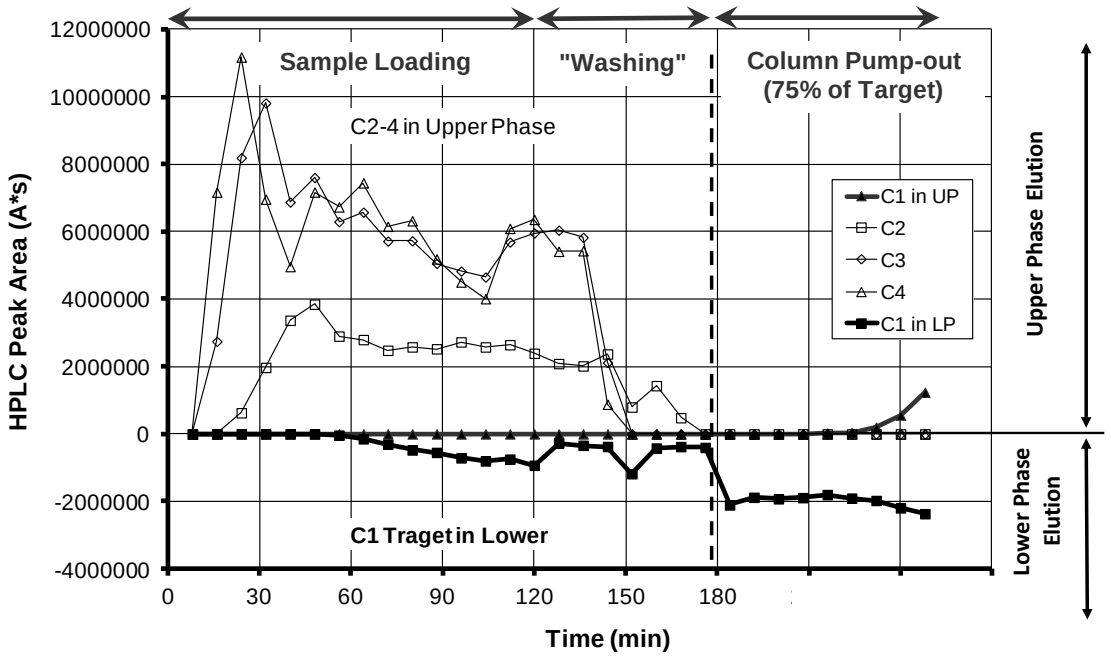


Figure 5.4.12: Fractogram constructed from HPLC fraction analysis after each ICcE cycle for the extraction of triptolide from a Chinese herbal medicine. HEMWat 15, upper phase flow rate 40 mL/min, lower phase flow rate 35 mL/min, flow switched every 4 minutes, sample concentration 12.0 g/l, sample volume 766 mL, 1250 rpm, 30 °C

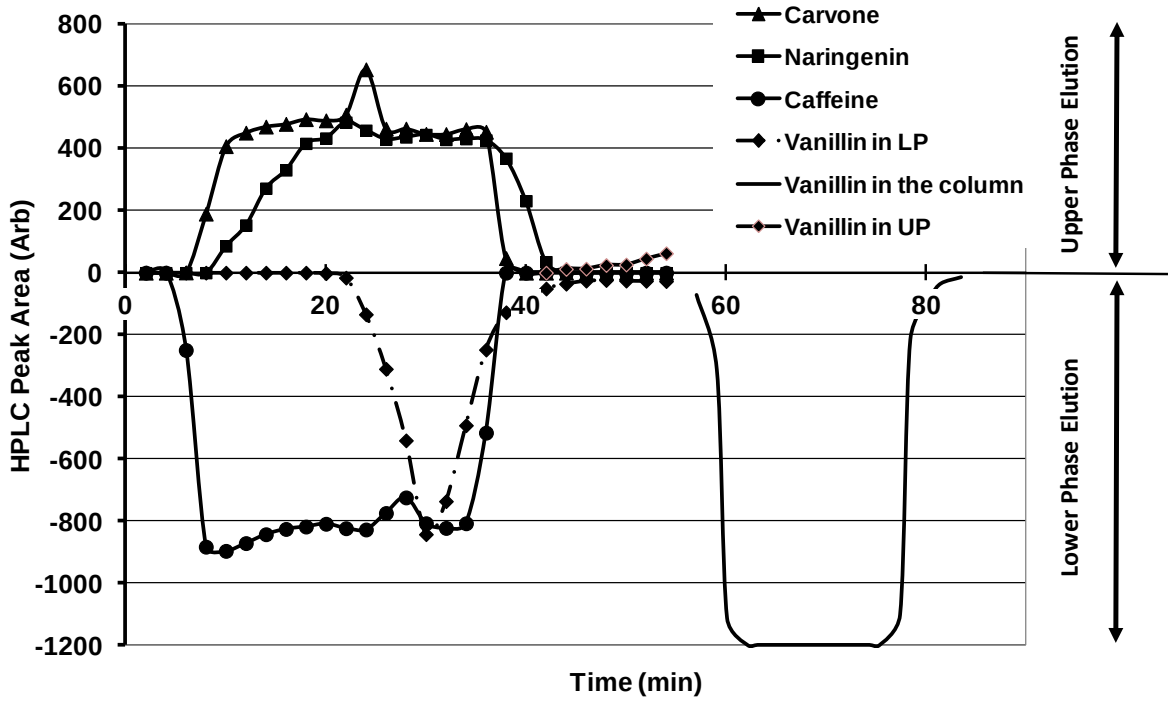


Figure 5.4.13: Fractogram constructed from HPLC fraction analysis after DFCCC separation of GUESSmix compounds. HEMWat 15, upper phase flow rate 35 mL/min, lower phase flow rate 35 mL/min, sample concentration 50.0 g/l, sample volume 150 mL, 1000 rpm, 30 °C

5.4.4) Comparison of the ICcE and DFCCC methods – conclusions

The evaluation study compared DFCCC and ICcE to show the advantages and disadvantages of the two methodologies. Based on the results presented above a comparison at preparative scale is summarised in Table 5.4.2. While the throughputs are similar, it can be seen that the set up for ICcE currently has three main advantages:

- ICcE can be set up using commercially available instrumentation with the simple addition of some valves, and a secondary pump and detector:

  The existence of a range of sizes of commercially available twin bobbin CCC instruments, from the analytical scale to the pilot scale, capable of operating at 240*g*, allow for the testing and potential adoption of ICcE as a continuous separation method relatively quickly. However for DFCCC the present design can only be operated at 120*g* due to the thin walled tubing used in its construction. This tubing limits the operating pressures which can be used within the bobbin. The DFCCC bobbin also exhibited some stripping of lower phase from the centre outlet, due to the design of the end fitting. All these design improvements need to be addressed before further experimentation to allow scaling of DFCCC both down, to produce an analytical instrument, and scaled up to produce a pilot instrument.

- For ICcE the phase volume ratio is easy to set up and maintain:

  As only one phase is flowing at a time with the ICcE method, as long as the flow rate is not increased to a point where the column starts stripping, the phase volume ratio in the column will be maintained, therefore once the column has been equilibrated to the initial phase volume ratio it has been shown that this ratio will be maintained throughout the run. On the DFCCC instrument, the need for back pressure control and the sensitivity of this back pressure control, a 100 mbar increase in back pressure reduced the lower phase in the column by 5%, make operating the instrument automatically extremely difficult. Whereas for ICcE, automated flow switching can be achieved with standard valving.

Table 5.4.2: Summary of comparison of ICcE and DFCCC

Criteria	ICcE	DFCCC
Instrument type	Any standard twin bobbin instrument at rotor speeds up to 240*g*	Requires a specialised column, the design of which at present is limited to 1000rpm (120*g*)
Required phase volume ratio	Required running conditions can be setup within 10 minutes	Column equilibration can take up to one hour
Stability of phase retention	Once set up the phase equilibrium is very stable	Maintaining the volumetric phase equilibrium is a challenge. Automated feedback control of the back pressures applied at the centre outlet, based on real-time flow and pressure data is required
Sample throughput	Similar throughput	Similar throughput
Flexibility of separation process	Split point of a mixture according to K_d can be altered by time cycles, flow rate or phase system	Only flow rate and phase system composition can be used to alter the split point

- For ICcE the split point can be controlled by both flow and timings:

  For DFCCC once the phase system has been chosen, only the flow rates can be used to adjust the split point therefore relatively wide variations in flow rates must be accommodated, whereas for ICcE the variation in flow rate can be limited by making variations to the switching intervals which will also determine the split point.

For the first time DFCCC and ICcE have both been practically demonstrated as effective methods for the enrichment of a target compound by trapping in the column as had been theorised by Lee (1991).

Overall, although DFCCC has the potential for continuous processing, further technical development of specialised bobbins and accurate pressure and flow control are required to allow further progress, therefore the focus of the next section of this thesis will be the scaling, modelling and application of ICcE with a range of varying polarity phase systems where opportunities for continuous processing can be realised with standard twin bobbin CCC instruments.

5.5) Scale up of ICcE using a model system based on the GUESSmix

Section 5.5.1 aims to establish the effectiveness of ICcE to successfully separate compounds of a sample into two eluant streams across a range of HEMWat phase system polarities at the semi preparative DE-Spectrum instrument (143 mL) and the preparative DE- Midi instrument (912 mL) scales. The compounds used for this study were a model sample mixture based on a modified GUESSmix (Friesen and Pauli 2005) with the aim of splitting this sample at different points dependant on the polarity of the compounds by polar (HEMWat 11), intermediate (HEMWat 17) and non-polar (HEMWat 23) phase systems.

In Section 5.5.2 the results across the range of phase systems are compared to the theoretical model given in the previous chapter.

In Section 5.5.3 the feasibility to achieve a throughput of over 1 kg of crude processed per day with a preparative scale Midi instrument was investigated by doubling the eluant and sample loading flows. The change of the split point, by adjusting the flow switching times of the phases, is also experimentally demonstrated with ICcE for the first time.

In Section 5.5.4 the further scale-up to the 4.6L pilot Maxi scale is investigated.

5.5.1) Effect of different polarity phase systems on the ICcE method

The following figures show reconstructed HPLC fractograms for three ICcE runs with the eight compounds from the modified GUESSmix (Friesen and Pauli 2005) containing salicin, caffeine, aspirin, coumarin, salicylic acid, carvone, ionone and biphenyl. The compounds eluting with the upper phase are shown in the positive domain while compounds eluting with the lower phase are shown in the negative domain. For both the semi-preparative (Figure 5.5.1a) and preparative (Figure 5.5.1b) instruments with the polar HEMWat system 11 the model mix is separated into two streams between the relatively polar caffeine and aspirin. Only salicin and caffeine eluted with the lower phase while the rest of the mixture eluted with the upper phase. With the intermediate polarity HEMWat system 17 (Figure 5.5.2) the model mix is split around coumarin, which is retained in the column, while the polar salicin, caffeine and aspirin elute with the lower phase and the non-polar compounds eluting with the upper phase. Whereas, with the non-polar HEMWat system 23 (Figure 5.5.3) the model mix is split around the relatively non-polar carvone, which is retained in the column. In this case, non-polar ionone and biphenyl elute with the upper phase. ICcE was shown to be stable across a range of polarities for over one hour of continuous sample injection and successfully split the model sample at various points according to the polarity of the solvent system used. The fractograms show the instrument reaches steady state with compounds eluting at a constant concentration in the fractions. Note also that as seen previously for the Chinese herbal medicine (Section 5.4.3.2) it is possible to concentrate up a target compound in the column if its K_d value is near unity as demonstrated for coumarin with HEMWat 17 and carvone for HEMWat 23. For all three HEMWat systems the number of cycles and times to maximum concentration give reasonable agreement at the semi-preparative and preparative scales. For all preparative results compounds elute slightly later and are retained in the column for slightly longer than with the semi-preparative instrument. These results where therefore compared to predicted results using the theory developed in Section 4.1.

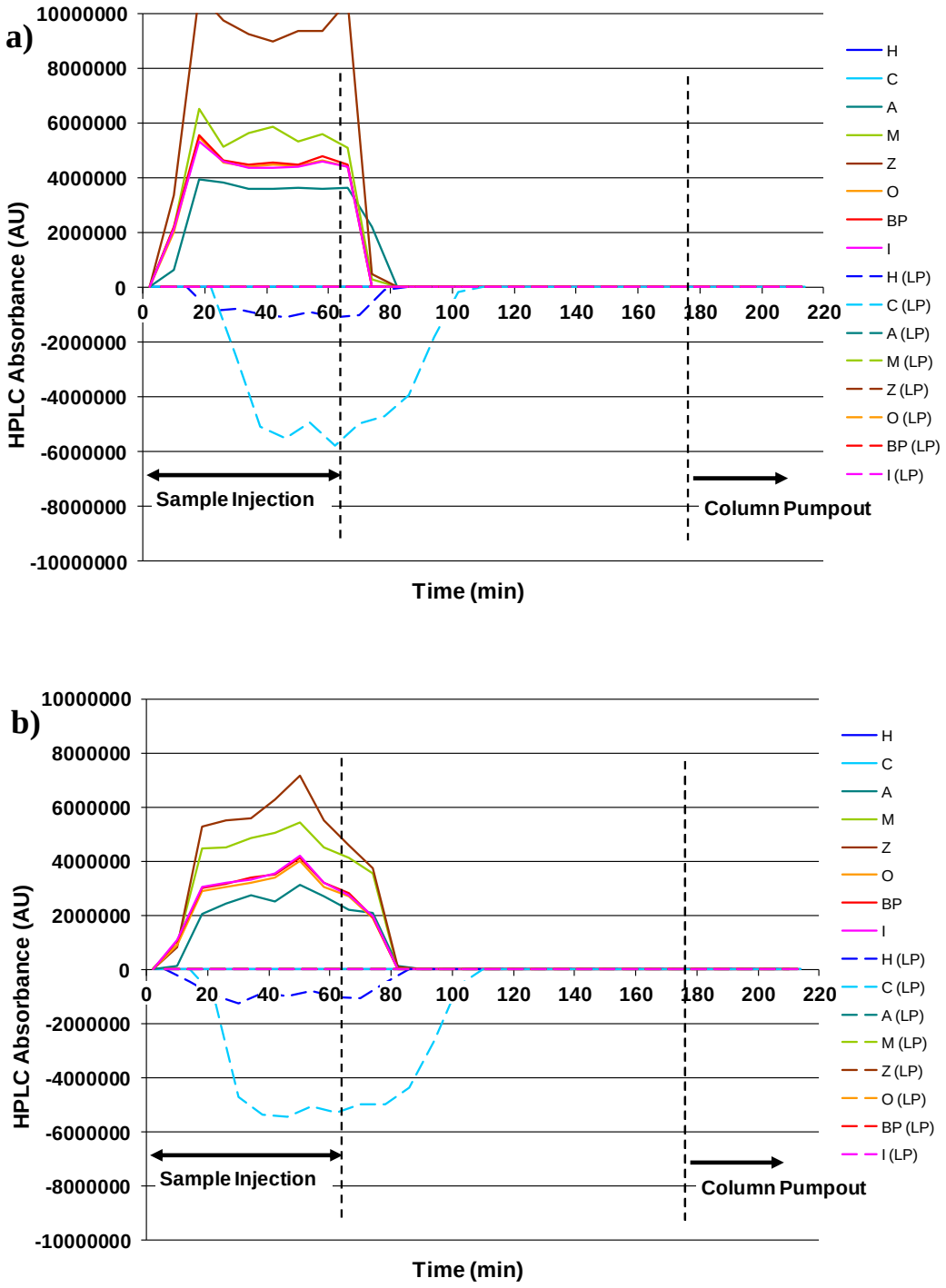


Figure 5.5.1: Fractogram constructed from HPLC peak areas of the solutes in each eluted fraction for the ICcE separation using Solvent system: HEMWat 11 at 30 °C for the separation of eight compounds from a modified GUESSmix on a) DE-Spectrum semi-preparative columns, coil volume 143.5 mL, upper and lower phase flow rate 5.5 mL/min switched every 4 minutes, sample flow rate 0.86 mL/min, sample volume 55 mL, rotational speed 1600 rpm (240*g*) and b) DE-Midi preparative columns, coil volume 912.5 mL, upper and lower phase flow rate 35 mL/min, flow switched every 4.0 minutes, sample flow rate 5.5 mL/min, sample volume 352 mL, rotational speed 1400 rpm (240*g*); upper phase elution (solid line) upper domain; lower phase elution (dotted lines) lower domain.

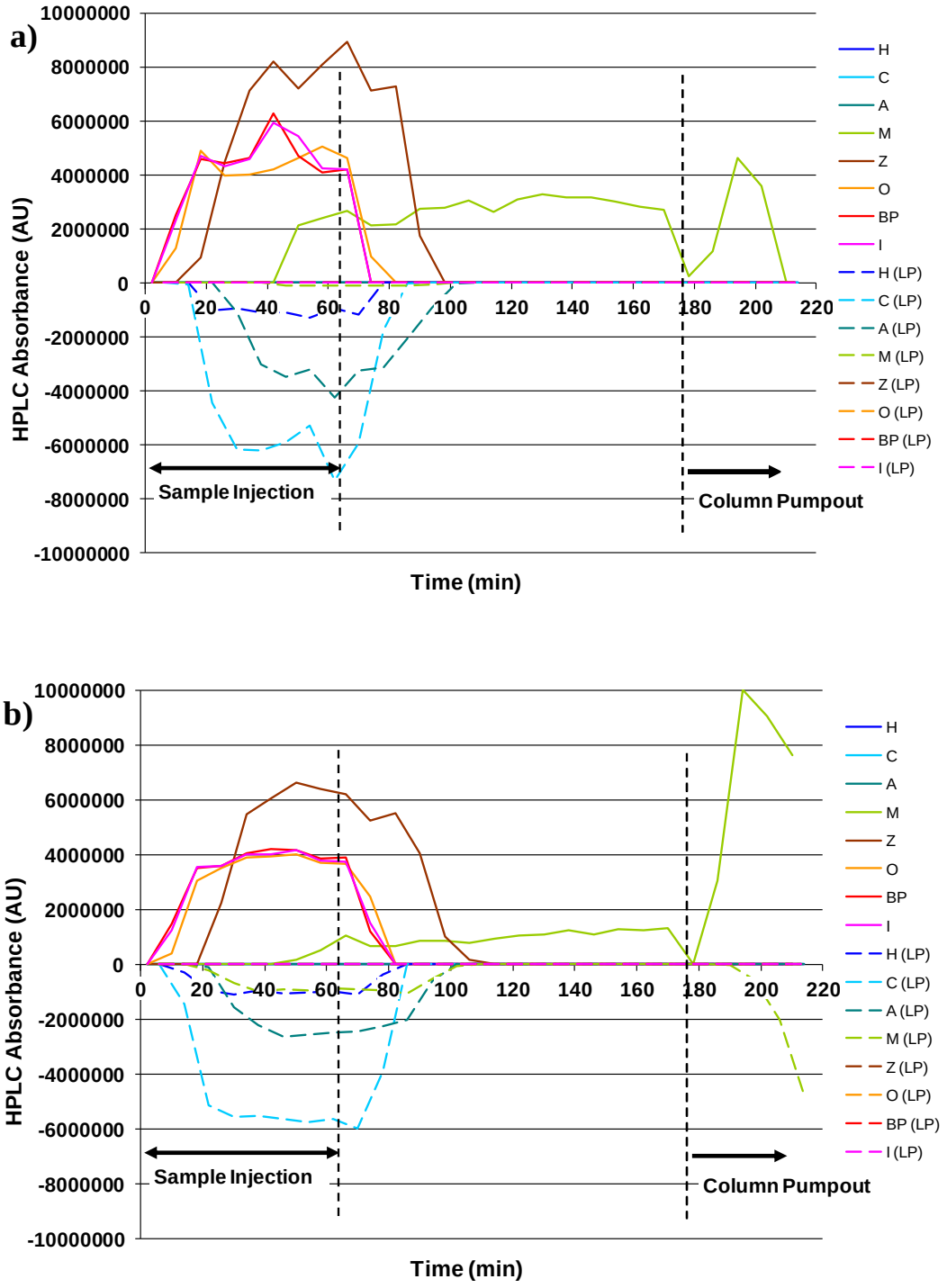


Figure 5.5.2: Fractogram constructed from HPLC peak areas of the solutes in each eluted fraction for the ICcE separation using Solvent system: HEMWat 17 at 30°C for the separation of eight compounds from a modified GUESSmix on a) DE-Spectrum semi-preparative columns, coil volume 143.5 mL, upper and lower phase flow rate 5.5 mL/min switched every 4 minutes, sample flow rate 0.86 mL/min, sample volume 55 mL, rotational speed 1600 rpm (240*g*) and b) DE-Midi preparative columns, coil volume 912.5 mL, upper and lower phase flow rate 35 mL/min, flow switched every 4.0 minutes, sample flow rate 5.5 mL/min, sample volume 352 mL, rotational speed 1400 rpm (240g); upper phase elution (solid line) upper domain; lower phase elution (dotted lines) lower domain.

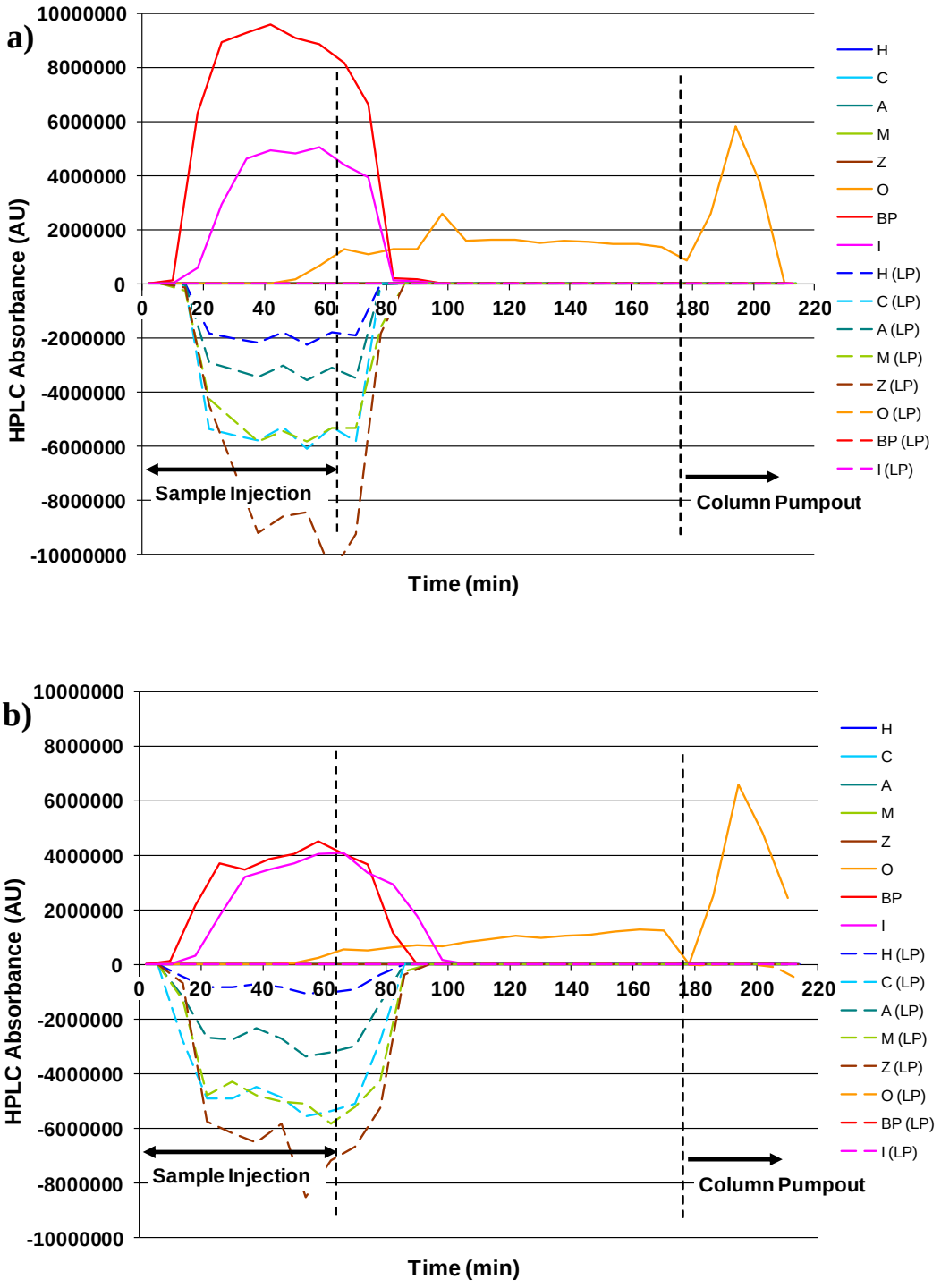


Figure 5.5.3: Fractogram constructed from HPLC peak areas of the solutes in each eluted fraction for the ICcE separation using Solvent system: HEMWat 23 at 30 °C for the separation of eight compounds from a modified GUESSmix on a) DE-Spectrum semi-preparative columns, coil volume 143.5 mL, upper and lower phase flow rate 5.5 mL/min switched every 4 minutes, sample flow rate 0.86 mL/min, sample volume 55 mL, rotational speed 1600 rpm (240*g*) and b) DE-Midi preparative columns, coil volume 912.5 mL, upper and lower phase flow rate 35 mL/min, flow switched every 4.0 minutes, sample flow rate 5.5 mL/min, sample volume 352 mL, rotational speed 1400 rpm (240*g*); upper phase elution (solid line) upper domain; lower phase elution (dotted lines) lower domain.

5.5.2) Modelling component separation of GUESSmix using the ICcE method

To confirm the elution order of the compounds were as would be predicted by the theory presented in Section 4.1, the time to maximum concentration of each compound for the data presented in Figure 5.5.1-3 were calculated. These times were compared to the elution profiles predicted by Equations 4.1.24-25.

Figure 5.5.4 shows the results for the semi-preparative DE-spectrum instrument with HEMWat 11 (Figure 5.5.4a), HEMWat 17 (Figure 5.5.4b) and HEMWat 23 (Figure 5.5.4c), while Figure 5.5.5 shows similar results for the preparative DE-Midi instrument.

Though the results compare reasonably well, with compounds eluting in the phase expected and close to the predicted elution time, the theory for both instruments predicts slightly longer retention times of the compounds within the columns than is seen experimentally.

Compounds which have relatively long retention times in the column for a given solvent system show larger shifts from ideal behaviour. For example, caffeine the HEMWat 11 lower phase, elutes earlier than would be expected for both scales of instrument as does salicylic acid in the HEMWat 17 upper phase. This implies there is increased band broadening occurring in comparison to classical elution due to the repeated cycling of the phases between normal and reversed phase.

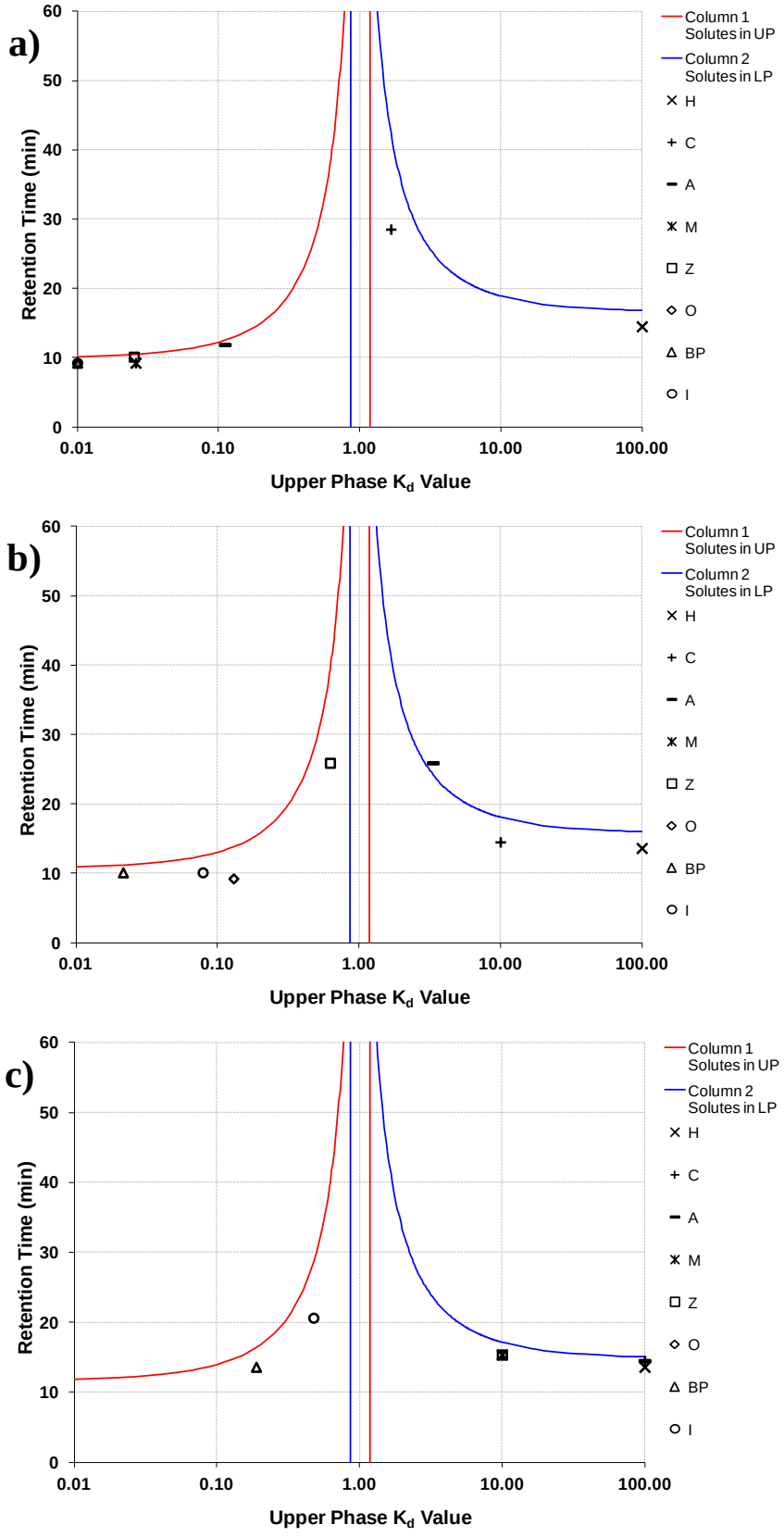


Figure 5.5.4: Retention times for modified GUESSmix compounds compared with theoretical prediction of elution times (Red line elution with UP, blue line elution with LP) for a) HEMWat 11, b) HEMWat 17 and c) HEMWat 23 phase systems using the semi-preparative DE-Spectrum instrument, $F_U = F_L = 5.5$ mL/min, $F_S = 0.86$ mL/min, $t_U = t_L = 4$ min

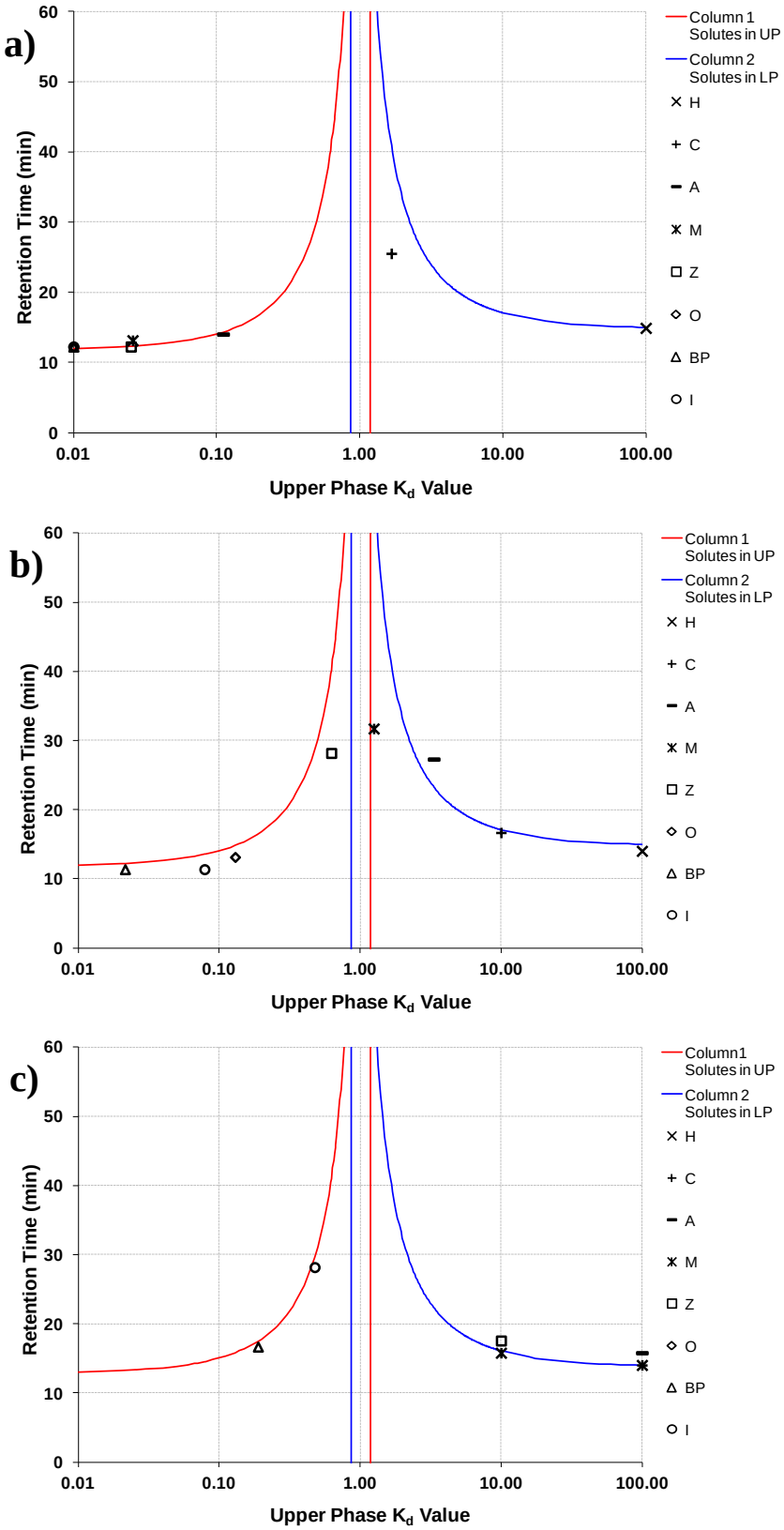


Figure 5.5.5: Retention times for modified GUESSmix compounds compared with theoretical prediction of elution times (Red line elution with UP, blue line elution with LP) for a) HEMWat 11, b) HEMWat 17 and c) HEMWat 23 phase systems using the preparative DE-Midi instrument, $F_U = F_L = 35$ mL/min, $F_S = 5.5$ mL/min, $t_U = t_L = 4$ min

5.5.3) Effects of loading and flow rate changes on the ICcE method

Figure 5.5.6 shows the effect of doubling upper and lower phase flow rates and Figure 5.5.7 the effect of additionally changing switching times while the sample loading concentration remained constant. The sum of eluant and sample flow was doubled from 35 mL/min (Figure 5.5.3b) to 70 mL/min (Figure 5.5.8), though the loading time was halved to keep loading mass the same. This result corresponds to a potential throughput of 1.1 Kg/day on a preparative CCC instrument when run continuously.

Figure 5.5.7 shows that adjusting the time cycle will determine the K_d value of the split point where compounds elute with the upper and lower phase flows. By reducing the switching time of the lower phase flow from 4.0 to 2.5 minutes, but keeping all other conditions the same as in the previous experiment, carvone which was retained in the column in the initial runs using HEMWat23 now elutes with the upper phase so the model mix is split into two streams separated between salicylic acid and carvone.

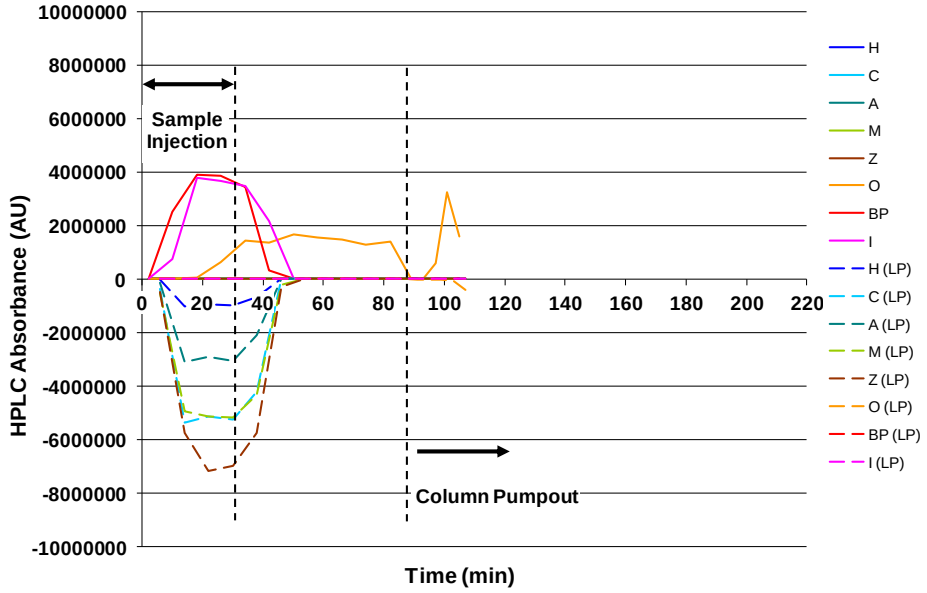


Figure 5.5.6: Fractogram constructed from HPLC peak areas of the solutes in each eluted fraction for the ICcE separation using Solvent system: HEMWat 23 at 30 °C for the separation of eight compounds from a modified GUESSmix on DE-Midi preparative columns, coil volume 912.5 mL, upper and lower phase flow rate 70 mL/min, upper and lower phase time 4.0 min per cycle, sample volume 365 mL, rotational speed 1400 rpm (240*g*); upper phase elution (solid line) upper domain; lower phase elution (dotted lines) lower domain.

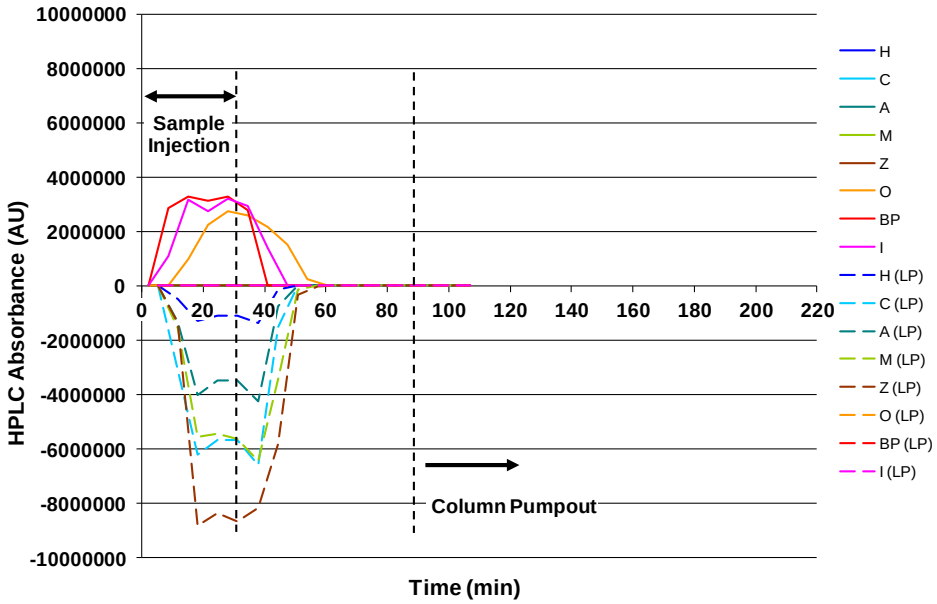


Figure 5.5.7: Fractogram constructed from HPLC peak areas of the solutes in each eluted fraction for the ICcE separation using Solvent system: HEMWat 23 at 30 °C for the separation of eight compounds from a modified GUESSmix on DE-Midi preparative columns, coil volume 912.5 mL, upper and lower phase flow rate 70 mL/min, upper phase time 4.0 min and lower phase time 2.5 min per cycle, sample volume 370 mL, rotational speed 1400 rpm (240*g*); upper phase elution (solid line) upper domain; lower phase elution (dotted lines) lower domain.

5.5.4) Scale up of ICcE to the 4.6 L Maxi pilot scale CCC instrument

The further scale up to the 4.6 L Maxi instrument which had first been tested by myself and published (See Section 2.3.2.3, Sutherland 2009) for a short 16 minute injection was confirmed by volumetrically scaling the experiment given in Figure 5.5.2 using HEMWat17 and the GUESSmix compounds. Intermittent operation successfully scaled to the 4.6 L instrument with a continuous 64 minutes loading achieved, compared with the 20 minute loadings in Sutherland 2009 (Figure 5.5.10). This longer loading time gave the compounds time to reach maximum concentration within the column before the loading was stopped. Due to the underperformance of the lower phase pump the flow was reduced therefore the results are were not directly comparable to those in figure 5.5.2 for the Spectrum semi-preparative and Midi preparative instruments. However, it was clear when compared to the expected compound elution times (Figure 5.5.9), from the model derived in Section 4.1, that the system ran as would be expected with salicin, caffeine and aspirin eluting with the lower phase and the remaining compounds eluting with the upper phase. This run gave a throughput of model mixture of 3.3 Kg/day and a solvent usage of 91 L/Kg of model mixture.

5.5.5) Conclusions on the scale up of ICcE using a model system

The robustness of the ICcE method as a continuous process across a range of instrument sizes, scaling-up from semi-preparative to pilot scale has been demonstrated and the method is shown to be applicable to a range of phase system polarities, from HEMWat 11 to 23, using the model GUESSmix. Continuous sample loading gives a throughput of 1.1 Kg/day at the preparative scale. The ability to trap a compound in the column is also confirmed while the adjustment of the switching times to control the elution point is confirmed. The GUESSmix compounds eluted as predicted by the model given in Section 4 based on the K_d values measured in static test tube experiments. Although the theory predicted longer retention times for the compounds retained within the columns, increased band broadening may occur in comparison to classical elution due to the repeated cycling of the phases between normal and reversed phase. In the following Section 5.6, to minimise this effect, a redesign of end fittings will allow a system where the switching between normal and reversed phase flow happens at the bobbin rather than on the bench so dead volume is not repeatedly pumped on and off the columns.

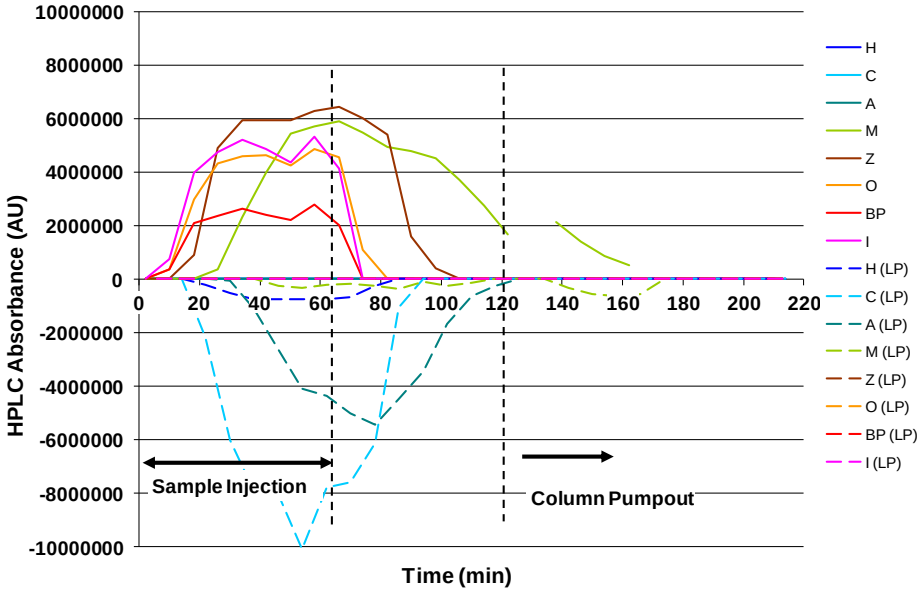


Figure 5.5.8: Fractogram constructed from HPLC peak areas of the solutes in each eluted fraction for the ICcE separation using Solvent system: HEMWat 17 at 30 °C for the separation of eight compounds from a modified GUESSmix on the Maxi pilot scale columns, coil volume 4.6 L, upper phase flow rate 193 mL/min and lower phase flow rate 157 mL/min switched every 4 minutes, sample flow rate UP = 37 mL/min, Sample flow rate LP = 28 mL/min, Sample volume 2.08 L, rotational speed 600 rpm (121*g*); upper phase elution (solid line) upper domain; lower phase elution (dotted lines) lower domain.

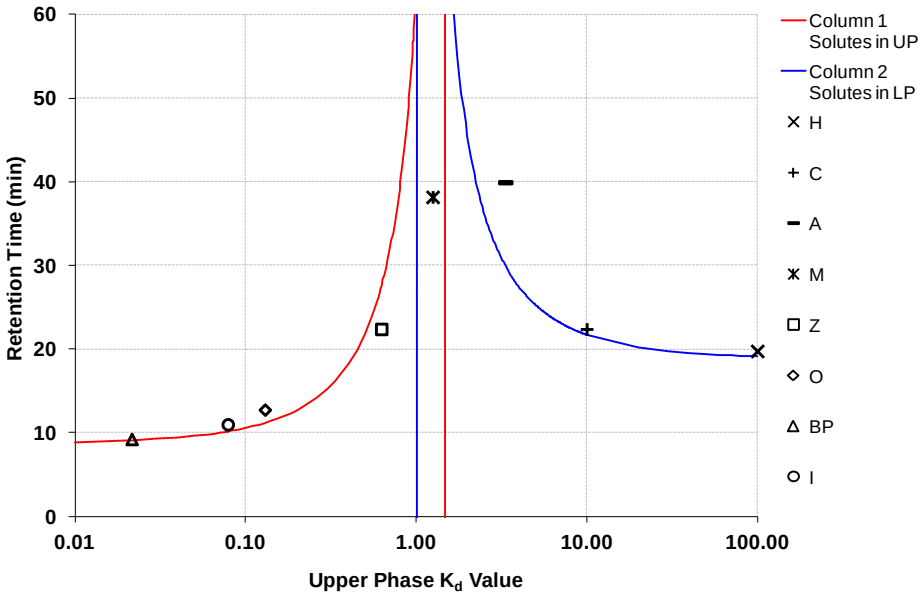


Figure 5.5.9: Retention times for modified GUESSmix compounds compared with theoretical prediction of elution times (Red line elution with UP, blue line elution with LP) for HEMWat 17 phase system using the 4.6 L Maxi pilot instrument, upper phase flow rate 193 mL/min and lower phase flow rate 157 mL/min, sample flow rate UP = 37 mL/min, sample flow rate LP = 28 mL/min, Sample volume 2.08 L, $t_U = t_L = 4$ min

5.6) Zero dead volume ICcE semi-preparative bobbins

The previous sections (5.4-5.5) demonstrated ICcE operated on a standard twin bobbin instrument with a single set of flying leads which create dead volume between switching cycles. The disadvantage of using a standard centrifuge design is that the dead volume in the flying leads has to be displaced and cleared at each cycle change. This results in the previous mobile phase (which is now stationary phase) being displaced back onto the bobbins, so delaying the separation process and potentially disrupting the equilibrium within the column. A slug of clean stationary phase is also forced into each collected fraction. To overcome this problem, a pair of bobbins was constructed by Dynamic Extractions with exactly the same column geometry, but with modified end fittings to allow two sets of flying leads to be connected at the ends of each bobbin. Therefore, the switching of the flows of mobile phase is taken onto the column resulting in no such displacement of phase in the flying leads as this dead volume is removed. In this Section the standard bobbin design (with phase switching outside of the centrifuge on the bench) will be referred to as "Switching on Bench – SB" and the purpose built ICcE bobbin design (with parallel flying leads and on-column phase switching) will be referred to as "Switching on Column – SC". The aims of this study are to:

- compare the standard bobbins (SB) with a single set of flying leads to purpose built ICcE Bobbins (SC) with a double set of flying leads
- demonstrate that ICcE can be operated with flying leads for the inlet and outlet of phases being fed directly to the column
- demonstrate the elimination of the displaced phase using on column switching (SC)
- demonstrate an improvement in retention time and reduction in peak width due to the reduced disturbance of the hydrodynamic equilibrium in the column using SC

5.6.1) Comparison of the SB and SC using the GUESSmix

The SC bobbins (Column 1 V_c = 94 mL, Column 2 V_c = 90 mL) were installed in a standard DE-Spectrum semi-preparative case. Figure 5.6.1 shows the inlet and outlet positions on the end fittings for one of the SC bobbins. In Figure 5.6.2 valves blocked the flow at positions marked with an (X) and recycled the phases at positions marked with an (O) as required to either flow upper phase along the upper flow line, for normal phase operation, or lower phase along the lower phase flow line, for reversed phase operation.

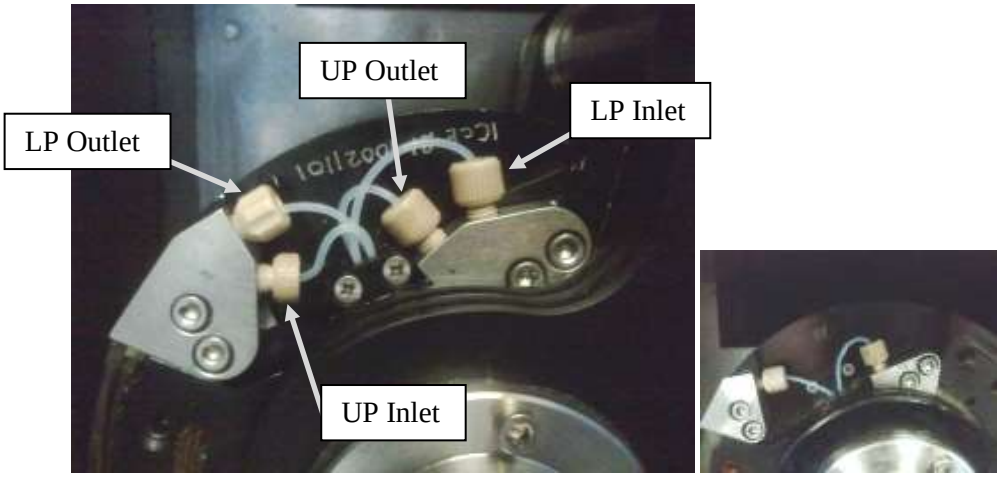


Figure 5.6.1: Inlet and outlet positions for the SC bobbins compared with standard SB bobbin (inset)

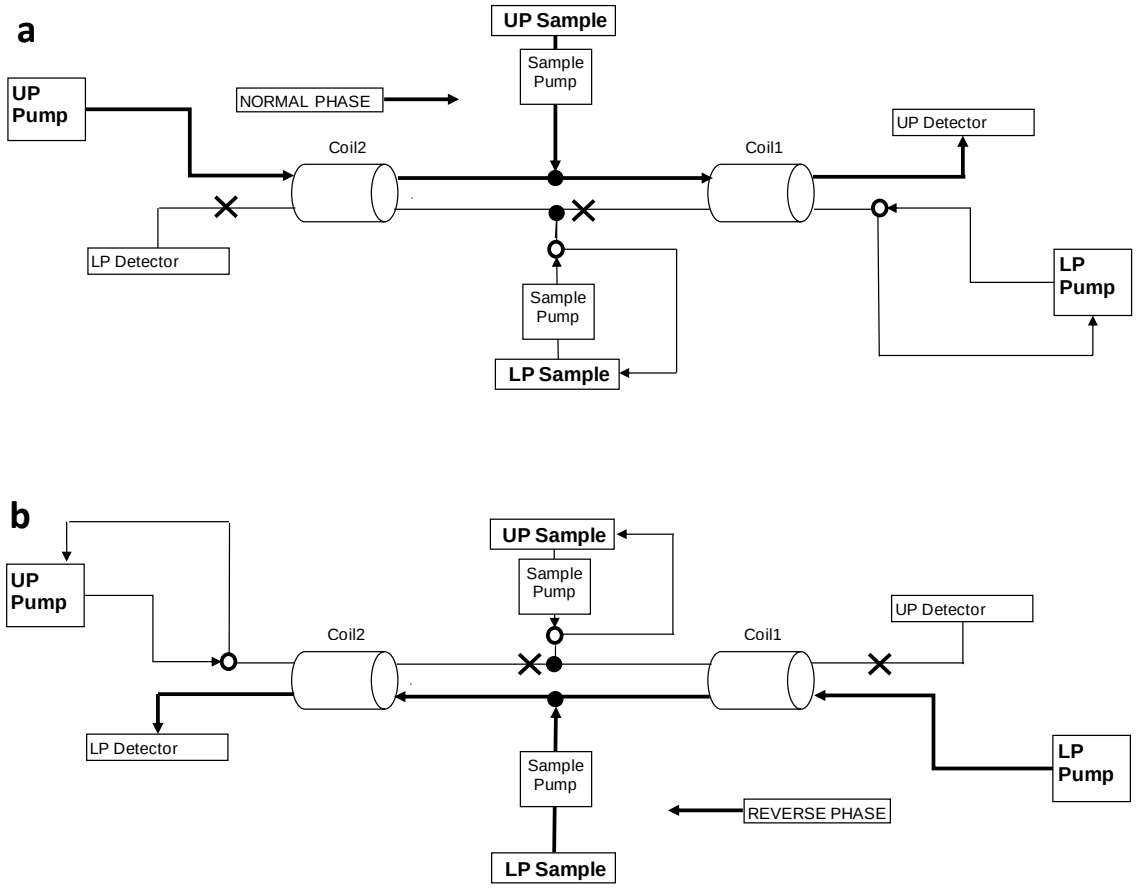


Figure 5.6.2: SC bobbins with on column switching for a) normal phase and b) reversed phase flow (bold lines indicate the active flow path)

The SC bobbins can be connected as standard bobbins for the comparison runs. When the bobbins are connected with standard flying leads the upper phase outlet, in Figure 5.6.1, is also the lower phase inlet and the lower phase outlet is also the upper phase inlet. These fittings are in the same position as inlets and outlets of a standard bobbin (see Figure 5.6.1 inset) while the other parallel connections are blanked off.

Table 5.6.1 gives the run conditions for the comparison runs. The same GUESSmix model sample solution, as in Section 5.5, was used. The upper and lower phases of the sample were separated and loaded through individual sample pumps.

Table 5.6.1: ICcE HPCCC method

Column	DE Spectrum HPCCC, semi-prep 184mL column, 1.6mm bore
Solvent systems	Hexane/Ethyl Acetate/Methanol/Water v/v 1:1:1:1, HEMWat17
Operation mode	Normal Phase, Upper phase as a mobile for 4 min @ 7.0 mL/min Reversed Phase, Lower phase as a mobile for 4 min @ 7.0 mL/min Repeated for 22 cycles
Method	1590 rpm (240g), 30 °C, ICcE
Sample Loading	70mg/mL model sample was made up in 50/50% UP/LP, loaded for first 8 cycles (64 minutes) @ 1.1 mL/min (caffeine (C), aspirin (A), coumarin (M), salicylic acid (Z), carvone (O) and ionone (I) at 10 mg/mL and biphenyl (BP) and salicin (H) at 5 mg/mL)

Figure 5.6.3a shows the fractogram for the new purpose built bobbins for on-column switching with the SC flying lead arrangement and Figure 5.6.3b shows the fractogram for the SB flying lead arrangement with switching on the bench. The compounds elute as would be expected from their distribution ratios. Biphenyl, ionone, carvone and salicylic acid eluting with the upper phase fractions, salicin, caffeine and aspirin eluting with the lower phase fractions and coumarin partially retained in the column and slowly eluting with the upper phase. These results are directly comparable to those seen with HEMWat17 in Section 5.5.1 previously (Figure 5.5.2). Both these runs were repeated to confirm the results.

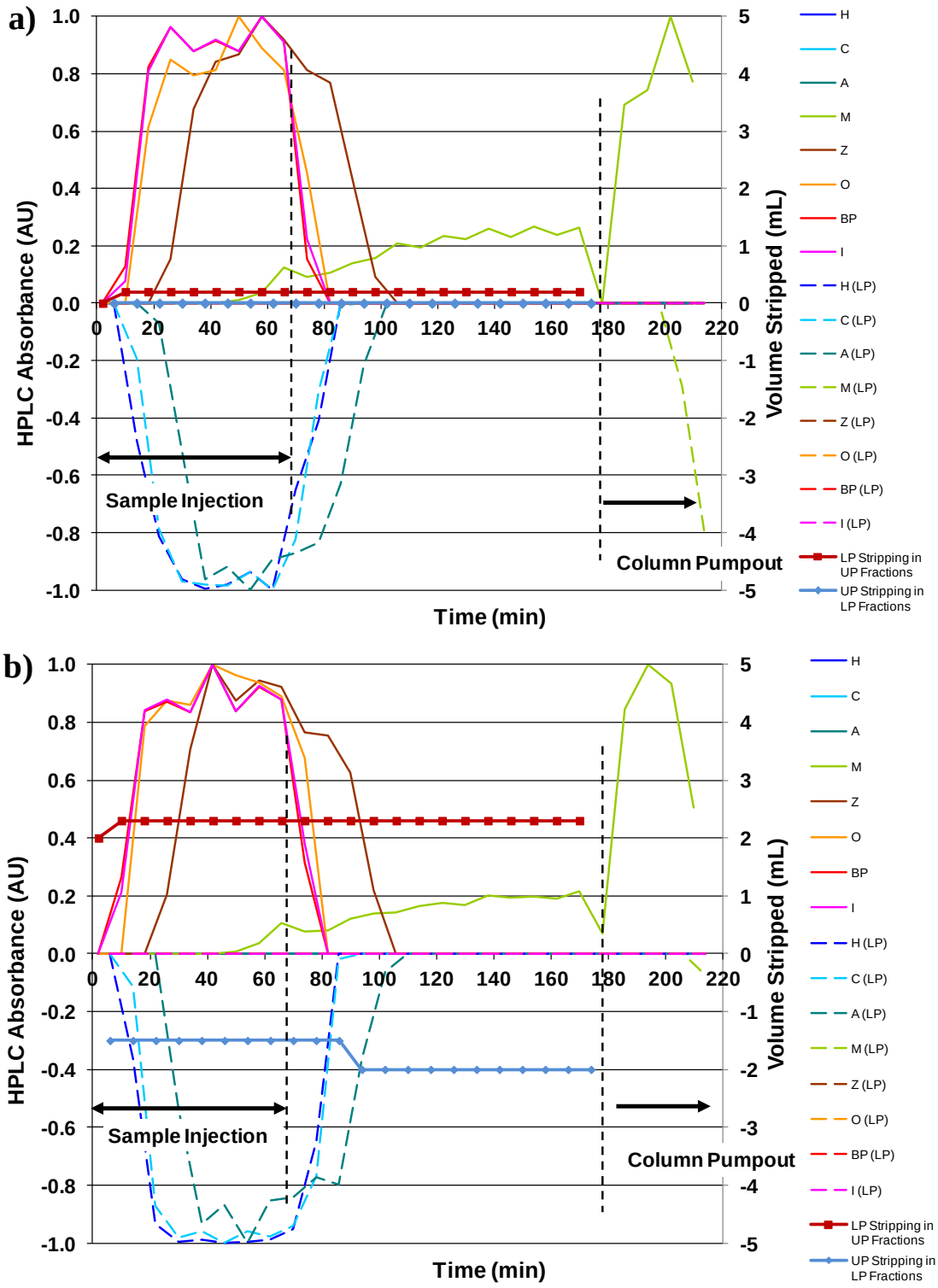


Figure 5.6.3: Fractogram constructed from HPLC peak areas of the solutes in each eluted fraction for the ICcE separation with a) SC and b) SB. Solvent system: HEMWat 17 at 30 °C for the separation of eight compounds from a modified GUESSmix on DE-Spectrum semi-prep columns, coil volume 194 mL, upper and lower phase flow rate 7.0 mL/min, upper and lower phase time 4.0 min per cycle, sample flow rate 1.1 mL/min, rotational speed 1590 rpm (240*g*); upper phase elution (solid line) upper domain; lower phase elution (dotted lines) lower domain.

While the fractograms are all comparable the stripping characteristics are very different. The stripping characteristics for the two modes of operation, given graphically in Figure 5.6.4, show the use of independent flying leads and on column switching has almost totally eliminated the displacement of the previous mobile phase with each switching cycle. For SC no upper phase is displaced and 0.2 mL of lower phase is displaced with each switching cycle. Unlike on-bench switching, where between 1.5 and 1.75 mL of upper phase is displaced and 2.3 mL of lower phase is displaced with each switching cycle. The amount of stripping is greater when running normal phase (so lower phase is stripped) than when running reversed phase.

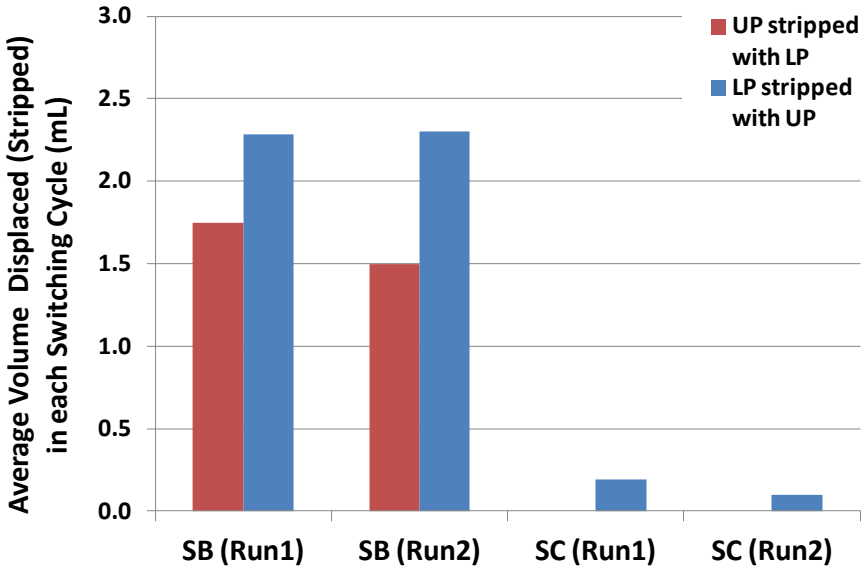


Figure 5.6.4: Average volume stripped in each switching cycle over 22 cycles using SC and SB on a DE-Spectrum semi-prep instrument with the conditions given in Figure 5.6.3

When using SC there is a slight reduction in the retention times of the compounds (Figure 5.6.5) of between 4 and 10% (6% on average), though ionone shows an unexplained slight increase, compared to the SB setup. This reduction can be explained as on-column switching allows the separation to carry on immediately after switching, whereas with standard flying leads the volume of phase in the flying leads must be displaced first reducing the time for separation. For these experiments, that volume was 2 mL, therefore at 8 mL/min there was a 15 second (7%) delay in each four minute half-cycle before mobile phase flows within the column, comparable to the shorter retention times observed.

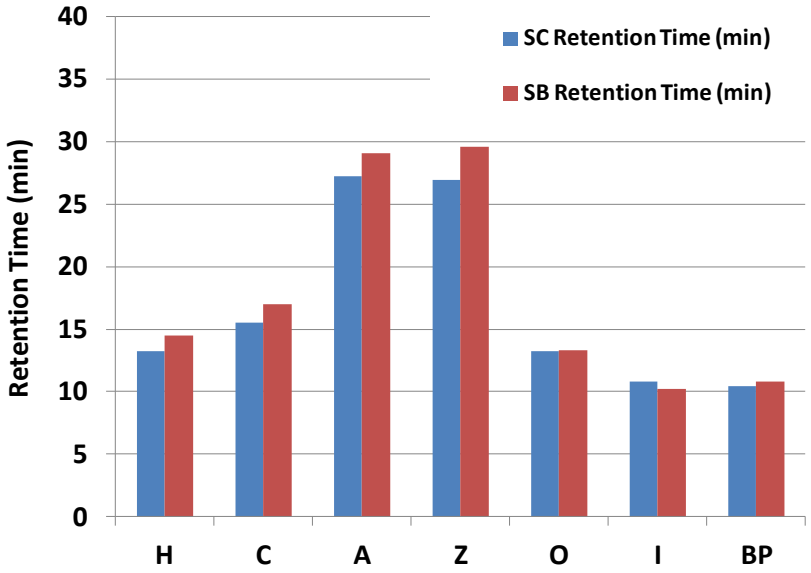


Figure 5.6.5: Retention time of compounds eluted from the columns using SC and SB regimes with the conditions given in Figure 5.6.3

The full width of the elution profiles (Figure 5.6.6) show a slight reduction in the profile width for SC. This can be partially explained by the slightly shorter retention times observed, however this also implies the equilibrium within the column was more stable throughout the run due to the reduced disturbance at switching. This effect should translate into a gain in resolution between the compounds separated between the upper and lower phase flows. Therefore, a further study using two compounds with a small alpha factor, to assess the ability of ICcE to separate them and any gain in resolution achieved, is discussed in the following section.

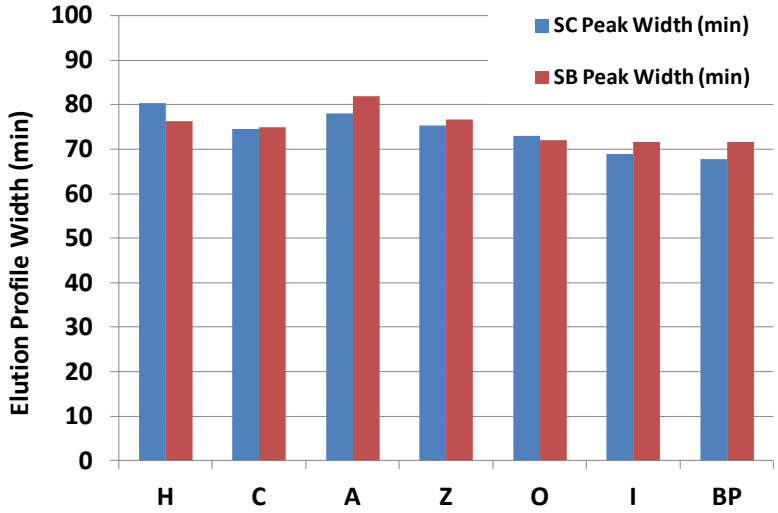


Figure 5.6.6: Full width of the elution profiles of the compounds eluting from the columns using SC and SB regimes with the conditions given in Figure 5.6.3

5.6.2) Comparison of the SB and SC using compounds with small alpha-factor

The comparison study in the previous section showed on column switching, for the inflow and outflow of phases, successfully eliminated the displaced phase seen when operating ICcE with SB. Further analysis of the results indicated a small shortening of retention time due to the elimination of the delay required to pump the previous mobile phase from the standard flying leads and some reduction of the peak widths, which will potentially provide improved resolution. The aim of this study was to confirm:

- A reduction in retention time due to the elimination of the pumping delay
- Improved resolution due to the reduced disturbance of the hydrodynamic equilibrium

A batch injection of ferulic acid and vanillin, dissolved in upper phase, was loaded between the two ICcE columns and the columns were then run intermittently for a number of cycles before eluting the two compounds isocratically with the upper phase. This will allow any changes in resolution and retention times to be assessed. Table 5.6.2 gives the run conditions.

Table 5.6.2: ICcE HPCCC method

Column	DE Spectrum HPCCC, semi-prep 184mL column, 1.6mm bore
Solvent systems	Hexane/Ethyl Acetate/Methanol/Water v/v 1:2:1:2, HEMWat14
Operation mode	Run1A (SB), Run1B (SC): Normal Phase (NP), Upper phase as a mobile for 2 min @ 5.0 mL/min Reversed Phase (RP), Lower phase as a mobile for 2 min @ 5.0 mL/min Repeated for 10 cycles, then elute in UP at 5.0 mL/min Run2A (SB), Run2B (SC): Normal Phase (NP), Upper phase as a mobile for 1 min @ 5.0 mL/min Reversed Phase (RP), Lower phase as a mobile for 1 min @ 5.0 mL/min Repeated for 20 cycles, then elute in UP at 5.0 mL/min
Method	1590 rpm (240g), 30 °C, ICcE
Sample Loading	1.24 mL solution containing 2.5 mg/mL vanillin and 2.5 mg/mL ferulic acid in HEMWat#14 upper phase

Figure 5.6.7 shows the UV chromatograms for run1A with SB (upper blue trace) and run1B with the SC (lower red trace) where 10 switching cycles occur before elution of the compounds in the upper phase. Retention times, peak widths and resolutions are given in Table 5.6.3.

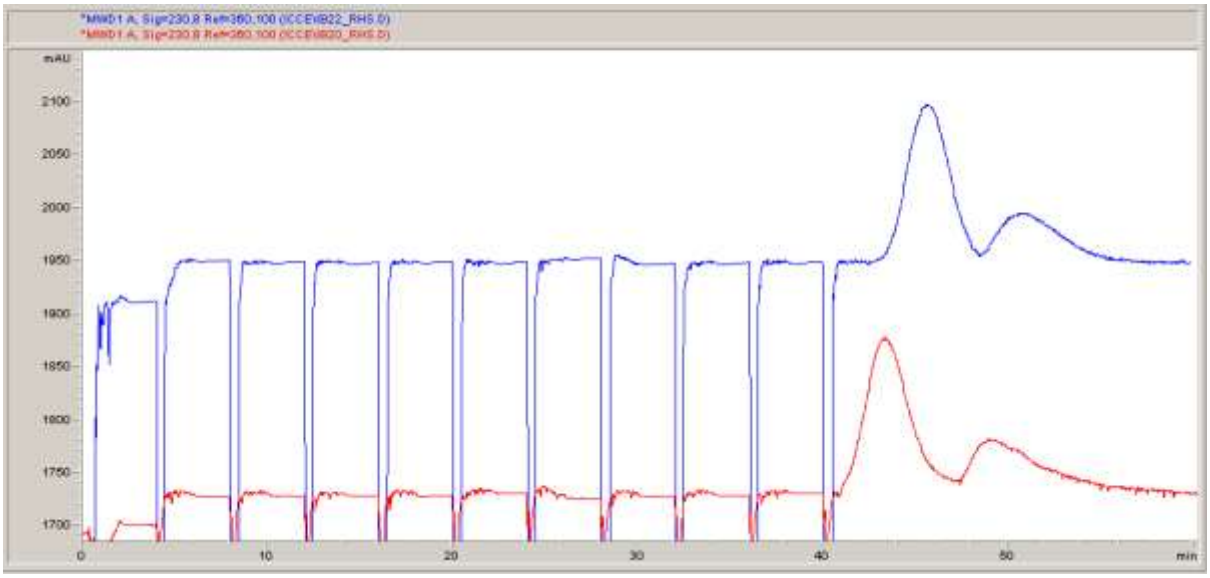


Figure 5.6.7: Blue upper UV trace: SB, red lower UV trace: SC, upper phase mobile for 2 min @ 5.0 mL/min then lower phase mobile for 2 min @ 5.0 mL/min repeated for 10 cycles, then elute in UP at 5.0 mL/min

The compounds elute as expected from their K_d values, vanillin (C_L/C_U = normal phase K_d= 0.45, calculated from HPLC data) eluting first and ferulic acid (K_d = 0.60, α=1.33) eluting second. The SC reduce the retention times from 45.6 to 43.3 minutes for vanillin and from 50.5 to 48.9 minutes for ferulic acid, compared to SB, while resolution is increases by 12% from 0.88 to 0.98. However, the resolution increase is driven by the larger difference in retention times seen with the SC, 5.6 minutes compared with 4.9 minutes. The peak widths with SC are in fact broader and some tailing of the peaks is also observed.

Table 5.6.3: Retention times and resolution of vanillin (V) and ferulic acid (F)

	10 Cycles		20 Cycles	
	SC	SB	SC	SB
RT_V	43.3	45.6	42.2	47.3
RT_F	48.9	50.5	47.9	51.1
T_F-T_V	5.6	4.9	5.7	3.8
W_V+W_F	11.4	11.1	12.6	10.4
Rs	0.98	0.88	0.90	0.74

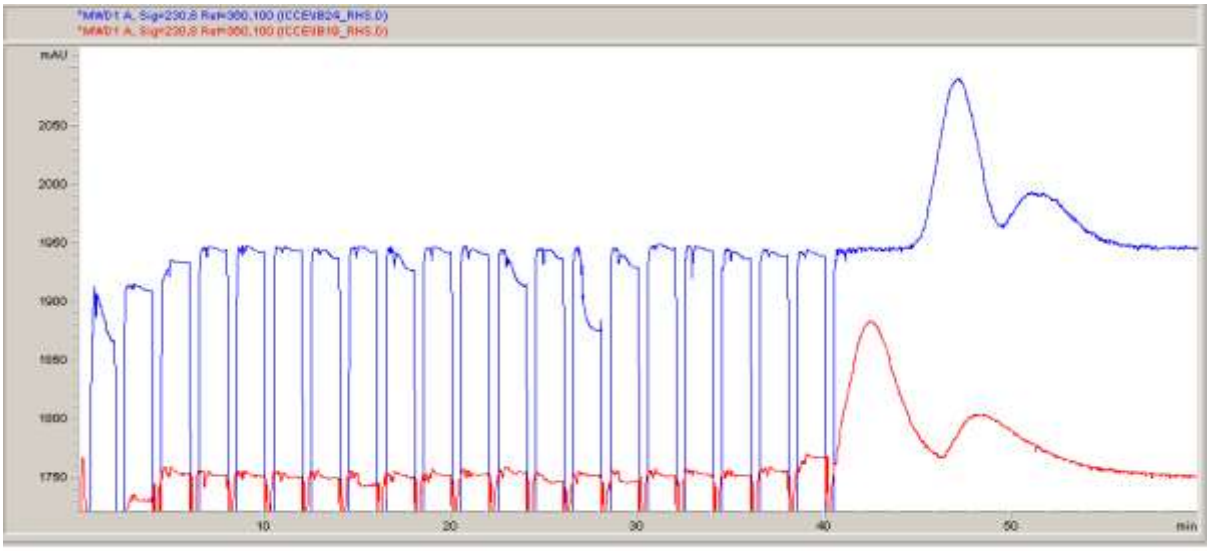


Figure 5.6.8: Blue upper UV trace: SB, red lower UV trace: SC, upper phase mobile for 1 min @ 5.0 mL/min then lower phase mobile for 1 min @ 5.0 mL/min repeated for 20 cycles, then elute in UP at 5.0 mL/min

Figure 5.6.8, showing the UV chromatograms for run2A and 2B where the number of switching cycles has been doubled to 20, with all other conditions kept the same. For SB, the increased rate of cycling reduces the difference between retention times of the two peaks from 4.9 minutes to 3.8 minutes, as the extra cycling increased the dead time while the previous mobile phase is cleared from the flying leads, so also reducing resolution from 0.88 to 0.74. However, with SC further peak broadening and tailing is observed, which also reduces resolution from 0.98 to 0.90. The observed peak broadening and tailing with SC may be caused by a part of the sample being trapped, held stationary, in one half of the central sections of the flying leads between each switching cycle. This should not matter for continuous processing as trapped sample will be similar to the next portion of sample to be injected. For a bolus injection a single flying lead between the columns could be used to eliminate the trapped sample, however this would lead to the columns becoming unbalanced over time as discussed in Section 5.4.1.

5.6.3) Comparison of the SB and SC using the GUESSmix - conclusions

Switching on the column was shown to operate for the inflow and outflow of phases. This mode successfully eliminated the displaced phase seen when operating ICcE with switching on the bench.

A shortening of retention time was seen due to the elimination of the delay required to pump the previous mobile phase from the standard flying leads. An improvement in resolution

from switching on the column has been observed. However, some peak tailing is seen with the SC flying leads, though the resolution is still improved due to the larger difference in retention times between the two peaks with SC.

To operate the columns in SC mode, both a bespoke column and extra valving is required, the complexity of which would need to be assessed against the benefits of cleaner fractions, the time savings and the potential improvement in resolution, though the bespoke column could be used for isocratic runs as required. Further, the use of four flying leads to move the phase switching to be on the column rather than on the bench, where only two flying leads are required per column, will reduce the lifetime of the flying leads. More wear occurs with four flying leads continuously twisting and untwisting over each other.

5.7) Comparison of the model ICcE separations with other applications using CCC

From Table 5.7.1, the throughput (3.3 Kg/day) and solvent usage (91 L/Kg of model mixture) achieved on the 4.6 L pilot Maxi CCC instrument (Section 5.5.4) compare favourably with recent published figures for both batch and other continuous methods. Looking in detail at this table the results, the resultant throughput and solvent usage figures are highly application dependant. Solvent usage for continuous processing ICcE and DFCCC methods are in the range 79 to 120 L/Kg of crude (Table 5.7.1a-e, g) except for the traditional Chinese medicine example (Table 5.7.1f) which contained only 3% of the target in the crude, with close running impurities) where the solvent usage increased to 388 L/Kg of crude. An isocratic separation of similar crude was even more costly, using 1460 L/Kg of crude at a throughput of only 0.08 Kg/day (Table 5.7.1m).

The solvent usage for most isocratic batch applications is higher than the continuous applications, above 350 L/Kg of crude, for all accept the highly polar and soluble glucoraphanin example (Table 5.7.1k) where sample concentrations of 500 g/L could be achieved which translated into much lower solvent usage of 76 L/Kg of crude and exceptionally good throughput of 6.6 Kg/day of crude on the 4.6 L Maxi instrument.

The application dependence of the throughput is further shown by the factor of nine between the throughput of a model system separation (1.1 Kg/day, Table 5.7.1b) compared to a very insoluble insecticide separation (0.12 Kg/day, Table 5.7.1j) on a similar sized preparative instrument. Therefore solubility, the effect of the crude on the two phase system

hydrodynamics and the alpha factor between the target and the unwanted impurities will all effect the throughputs and solvent usage values achievable, and therefore the ultimate economics of the process.

Table 5.7.1: Throughput of recent large scale continuous and isocratic batch separations using counter-current chromatography

Run	Instrument type	Method	Type of coils	Coil volume (ml)	Bore (mm)	Rotation speed (rpm)	*g* field	Phase system	Applications	Compounds separated	Normal phase flow rate (ml/min)	Reversed phase flow rate (ml/min)	Run time (min)	Loading (g)	Loading duration (min)	Crude throughput (Kg/day)	Solvent usage (L/Kg of crude)	Reference
a	Maxi CCC	**ICcE**	CCC	4600	10	600	121	HEMWat (1:1:1:1)	Model system	8 compounds GUESSmix	230	185	120	146	64	**3.3**	**91**	Section 5.5.4
b	DE-Midi CCC	**ICcE**	CCC	912	4	1400	240	HEMWat (1:1:1:1)	Model system	8 compounds GUESSmix	80	80	88	25.6	32	**1.1**	**100**	Section 5.5.3 (Hewitson et al. 2011)
c	Maxi-CCC	**ICcE**	CCC	4600	10	600	121	HEMWat (1:1.5:1:1.5)	Model system	Caffine, Vanillin, Naringenin, Carvone	250	250	90	40.5	20	**2.9**	**123**	(Sutherland et al. 2009)
d	DE-Midi CCC	**ICcE**	CCC	912	4	1250	192	HEMWat (1:1:25:1:1.25)	Model system	Caffine, Vanillin, Naringenin, Carvone	50	60	100	11.2	16	**1.0**	**79**	Section 5.4.3.1 (Hewitson et al. 2009)
e	DE-Midi CCC	**ICcE**	CCC	909	4	1250	192	HEMWat (2:1:2:1)	TCM - Extract of magnolia officinalis Rehd. et Wils.	Honokiol, Magnolol	25	25	120	30.0	120	**0.36**	**100**	(Peng et al. 2010)
f	DE-Midi CCC	**ICcE**	CCC	912	4	1250	192	HEMWat (1:1.5:1:1.5)	TCM Tripterygium Wilfordii Hook. F.	Triptolide, Peritassines A, Wilforigine, Wilforine	40	35	270	11.2	116	**0.14**	**388**	Section 5.4.3.2 (Hewitson et al. 2009)
g	Midi-CCC	**CCCE**	DFCCC	625	5	1000	123	HEMWat (3:1:2:2)	Pharmaceutical liquor	7 pharmaceutical components	30	30	90	20.1	40	**0.72**	**120**	(van den Heuvel et al. 2009)
h	Kromaton	**MDM**	CPC	200	1320 chambers	1800	83	Heptane:ACN	Model System	Acenaphthylene, Naphthalene	8	8	180	3.0	125	**0.03**	**333**	(Delannay et al. 2006)
i	DE-Maxi CCC	**Batch**	CCC	17080	10	600	121	HEMWat (4:1:4:1)	Polyketide-derived macrolides	Spinetoram J Spinetoram L	360	-	140	111	2.5	**1.1**	**733**	(DeAmicis et al. 2011)
j	DE-Midi CCC	**Batch**	CCC	912	4	1400	240	HEMWat (4:1:4:1)	Polyketide-derived macrolides	Spinetoram J Spinetoram L	42	-	81	7.0	2.5	**0.12**	**733**	(DeAmicis et al. 2011)
k	Maxi CCC	**Batch**	CCC	4600	10	600	121	Propanol:Acetonitrile:Ammoniumsulphate:Water (1:0.5:1.2:1)	Natual Product from broccolli seeds	Glucoraphanin, Glucoiberin	350	-	20	115	0.7	**6.6**	**76**	(Sutherland et al. 2007)
l	Maxi CCC	**Batch**	CCC	4600	10	600	121	HEMWat (1:0.4:1:0.4)	TCM - Extract of magnolia officinalis Rehd. et Wils.	Honokiol, Magnolol	600	-	16	43.0	0.3	**2.5**	**349**	(Chen et al. 2007)
m	DE-Midi CCC	**Batch**	CCC	912	4	1400	240	HEMWat (1:1.25:1:1.25)	TCM Tripterygium Wilfordii Hook. F.	Triptolide, Peritassines A, Wilforigine, Wilforine	25	-	25	1.25	1.0	**0.08**	**1460**	(Ye et al. 2008)

Therefore, to allow a more detailed assessment of the potential benefit of the ICcE method, a comparison to batch isocratic methods with respect to purity, yield, throughput and solvent usage is done in Section 6.2 using an actual API waste stream target supplied by GSK.

5.8) Conclusions on a route to continuous processing

The stationary phase retention of HEMWat phase systems 11 to 23 were shown to be similar across the semi-preparative, preparative and pilot scale of CCC instruments. For reversed phase, linear Du-plot behaviour was observed as described by Du et al (1999). However, in normal phase the Du-plot has an inflection point at all scales and across all polarity phase systems, which limits the maximum flow rate in normal phase. With non-polar phase systems the rate of decline of retention after the inflection point is shallow, however with more polar phase systems the retention declines rapidly with increased mobile phase flow rate. There is evidence that a limiting mobile phase linear velocity is reached within the column for normal phase and that the Reynolds numbers reach values where turbulence may occur.

When comparing the DFCCC and the ICcE it was clear that both methods were effective for use in continuous operation. However, the ICcE method gave stable phase volume ratio in the columns over time while for the DFCCC method the phase volume ratio was dependant on the back pressure applied at the centre outlet of the column. A small 0.1 bar change in back pressure gave a 4.9% change in the lower phase volume in the column.

At the present time, the ICcE method is seen as the preferred option for further continuous processing studies as the phase volume ratio was simple to setup and maintain, the separation point is controlled by flow rates, step time and phase system and the method can be run on well established commercial twin column HPCCC instruments which are available at a range of scales so the automation and scale-up of ICcE have potentially less barriers.

Scaling of the ICcE method was shown to be effective from the semi-preparative to the pilot scale using compounds from the GUESSmix model mixture, with throughput of 1.1 Kg of sample/day processed at the preparative scale. The theoretical model, in Section 4, predicted the elution of the GUESSmix compounds.

Finally, the column design was optimised by modification of the end-fittings, at the semi-preparative scale, to allow on column switching between normal and reversed phase

operation. This modification successfully eliminated the displaced phase seen in the eluted fractions with on-bench switching between normal and reversed phase, although some peak tailing was observed with these columns which can increase band broadening within the column.

Because the purity, yield, throughput and solvent usage are extremely application dependent the potential benefit of the ICcE method is assessed on three practical applications, one polar, one intermediate polarity and one non-polar in the following Chapter 6.

Chapter 6: Case studies – ICcE of industrial applications

The model separations with the GUESSmix across the solvent system polarity range given in the previous chapter showed the potential for ICcE as a continuous separation methodology for industrial users. Therefore, three practical applications were examined as demonstration samples for the ICcE method, a polar target of a waste stream from a senna seed pod extract (Section 6.1), an intermediate polarity API extracted from a waste stream (Section 6.2) and non polar compounds from a plant extract (Section 6.3).

6.1) Highly polar sennoside fractionation by ICcE

Sennosides are a highly effective natural laxative. The aim of this study was to produce pure fractions of two target sennosides from a crude milled waste biomass raw material which had been previously aqueous extracted and subjected to reverse osmosis, ultra-filtration and precipitation to selectively pre-concentrated the target compounds. ICcE was demonstrated to have the potential to separate the target sennosides. The aqueous extracted target compounds were actually highly polar; therefore the phase system chosen for the separations was significantly more polar than the HEMWat 11 phase system used with the Model GUESSmix. The highly polar alcohol/salt solution (propan-1-ol/12% w/w NaCl Solution 1:1 v/v) phase system was used, where the salt (NaCl) is preferentially soluble in the water so increasing the density of the water such that two phases form.

6.1.1) Determination of distribution ratios and modelling of the ICcE parameters

The static distribution ratios of the target sennoside compounds in the alcohol/salt solution phase system were measured at three crude concentrations and these results were input to the model given in Chapter 4 to select operating conditions for the ICcE runs and predict the elution of the target compounds.

For the propan-1-ol/NaCl phase system, crude material was dissolved in lower phase (2 mL) then sonnicated at 60C for 30 minutes, before adding upper phase (2 mL) and vortexing to mix. The pH was adjusted with either HCl or sodium hydroxide solution as required. 1 mL of each phase was transferred to HPLC vials, dried down and re dissolved in water for analysis on HPLC. The samples were analysed on a reversed-phase Phenomenex Prodigy C18 column (150 x 4.6 mm I. D. 3.5 µm) thermostated at 50 °C. The mobile phase was a

mixture of A (20% ACN solution containing 0.08% acetic acid) and B (acetonitrile) in a gradient program with a flow rate of 1 mL/min: 0-8.0 min 100% A, 8.0-9.0 min ramp from 0 to 95% B, 9.0-14.0 min hold at 95%B, 14.0-15.0 min ramp down from 95% to 0% B. The eluant was monitored using a DAD detector. The distribution ratio for a particular compound in normal phase was calculated as the ratio of the peak area in the lower phase to the peak area in the upper phase. The resultant distribution ratios are given in Table 6.1.1.

Table 6.1.1: HPLC peak areas for sennosides A and B in 1:1 propan-1-ol/12.5% NaCl solution phase system

Conc. of Crude in LP (mg/mL)	pH	Sennoside A				Sennoside B			
		UP	LP	Total	K_d (L/U)	UP	LP	Total	K_d (L/U)
5.7	5	1150546	721506	1872052	0.63	870058	676002	1546060	0.78
51	2-3	14925510	5841140	20766650	0.39	8748770	5751910	14500680	0.66
154	2-3	10821240	8363800	19185040	0.77	17192820	16956310	34149130	0.99

From Table 6.1 it can be seen that with increasing concentration of crude in the sample there is an increasing concentration of Sennoside B, however Sennoside A reaches a limiting solubility at 51 mg/mL, therefore this was set as the sample concentration of the crude sample for the ICcE run. The pH of the sample solution was known from previous work[1] to affect the solubility of the target sennosides, therefore the aim was to maintain the pH between 2 and 3 pH units.

Using the distribution ratios from the 50 mg/mL crude solution, the separation was modelled to retain sennoside B (K_d = 0.66), the more important target, in the column and wash all impurities way from this target, while at the same time elute sennoside A (K_d = 0.39) with the upper phase. Total loading required was 4.0 g, therefore a sample flow rate of 5 mL/min (at 50 mg/mL) was assumed and therefore the total loading time would be 16 minutes, the sample volume would be 80 mL and the switching times were equal at 4 minutes for both the normal and reversed stages. Further, assuming a relatively conservation factor for the practical flow rates of 2.5 times the optimal flow rate gave upper and lower phase flow rates of 18.2 and 29.2 mL/min respectively (Figure 6.1.1). The sample was therefore loaded for two full cycles and then washed for a further five cycles (40 minutes) to give a total run time of 56 minutes at which point the sennoside A peak should be eluting with the upper phase (Figure 6.1.2). The column can then be extruded to collect the sennoside B peak.

[1] L. Grudzień – Personal communications

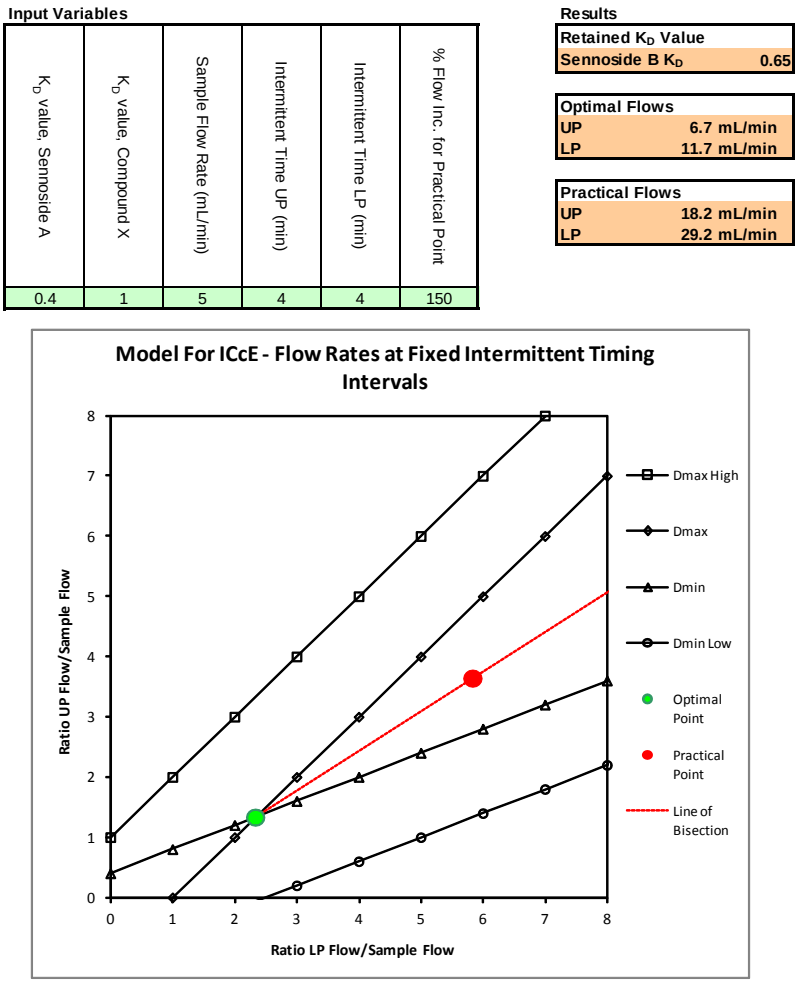


Figure 6.1.1: Model of ICcE separation of sennoside compounds. For equal switching times and flow rates of 18.2 mL/min for the upper phase and 29.2 mL/min for the lower phase sennoside B is retained in the column (red dot) while Sennoside A is eluted in the upper phase

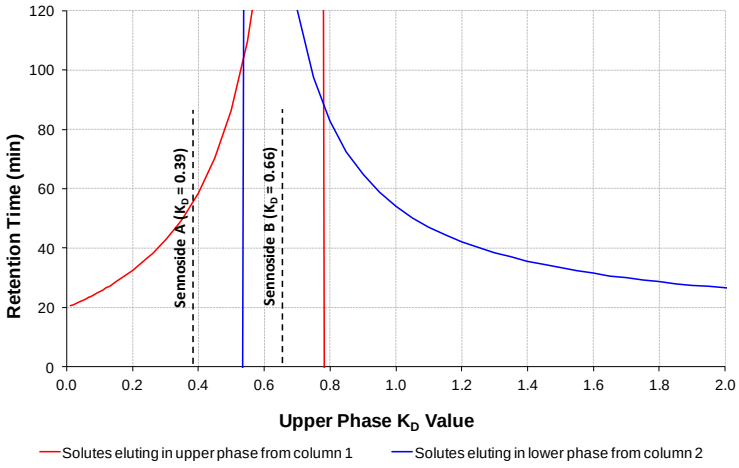


Figure 6.1.2: Model of the elution times for the ICcE separation of sennoside compounds for the conditions given in Figure 6.1. Sennoside B (K_d = 0.66) is retained in the column while Sennoside A (K_d = 0.39) will be eluted with the upper phase

6.1.2) ICcE of sennosides: methodology

10 L of the propan-1-ol/12% NaCl phase system was made up and the pH was adjusted to 2.1. The DE-Midi preparative instrument was used for the separation, using the procedures given in Section 3. The column was equilibrated at a flow rate of 36 mL/min in reversed phase to given phase retention of 54%. The sample of crude sennoside extract was made up in a mixture of upper and lower phase at a concentration of 50 mg/mL, then put in an ultrasound bath to dissolve, before filtering to remove suspended matter. It was loaded at 5 mL/min for two complete cycles. The upper phase flow rate was set at 18 mL/min and the lower phase flow rate was set at 30 mL/min, with equal switching times of 4 minutes for the upper and lower phase flow times of each cycle. Wash cycles were then run for 5 cycles, stopping the instrument at 56 minutes. The columns were emptied with compressed air from head-centre to tail periphery, fractionating them as 100 mL fractions.

6.1.3) ICcE of sennosides: results and discussion

Figure 6.1.3 gives the reconstructed HPLC fractogram (the HPLC peak area for the solutes in each eluted fraction with respect to time) for the separation of the target sennosides.

In Figure 6.1.4 the HPLC chromatograms for the fractions with the highest concentrations of the target sennosides (Fraction U7 for sennoside A and Fraction C8 for sennoside B) are compared to the original crude sample in the upper and lower phases.

As can be seen from Figure 6.1.3 and as predicted by the model Sennoside B is retained in the column with increased purity and sennoside A eluted with the upper phase fractions also with improved purity. Both compounds are slightly more in the upper phase fractions than was predicted by the model, as sennoside B is in the column on the upper phase side of the separation rather than right in the middle of the columns and sennoside A elutes earlier with the upper phase with a maxima at 50 minutes rather than the predicted 58 minutes. The total quantity of sennoside B is actually greater than the quantity of sennoside A in the eluted fractions, which was not expected from the initial HPLC analysis. This may be due to the pH used for the crude solution. Therefore further studies would need to look at the effect of pH on extraction efficiency for the target sennosides into this phase system.

From Figure 6.1.4d, the purity of sennoside B was greater than the target 80% with the only major impurity being the co-eluting peak at RT 3.1 minutes (peaks at RT 1.50, 11.5, 12.9 and 16.5 minutes are seen in the phase systems). Because of the nature of the process Sennoside A (Figure 6.1.4c) is still relatively impure, the main fractions in the upper phase containing both this target and the impurity with RT 14.5 minutes.

At present the purity and yield achieved for this separation are not good enough to use the process in an industrial environment. Further improvements to yield and purity could be achieved by increasing the wash cycle by either one or two steps to completely remove sennoside A before extruding the column, though this would be at the expense of increased solvent usage.

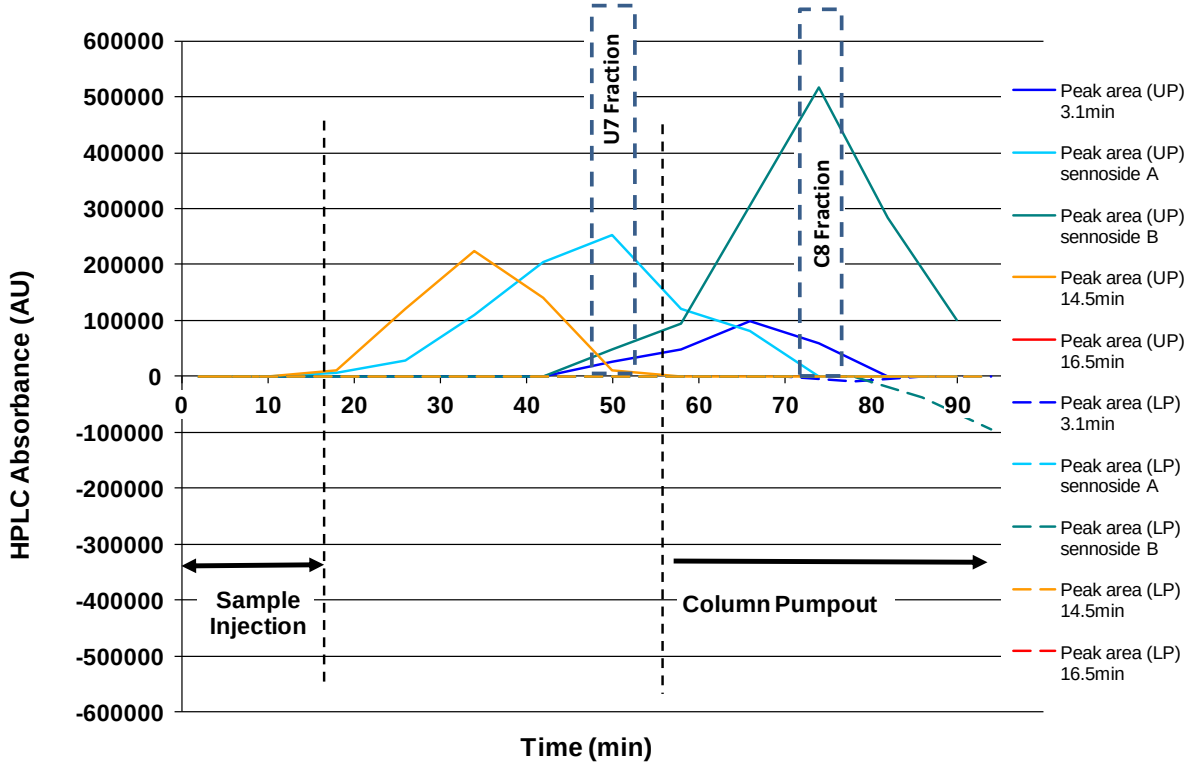


Figure 6.1.3: Fractogram constructed from HPLC peak areas of the solutes in each eluted fraction using the propan-1-ol/12% NaCl solution (1:1 v/v) solvent system at 30 °C for the separation sennoside targets on DE-Midi preparative columns, coil volume 912.5 mL, upper phase flow rate 18 mL/min and lower phase flow rate 30 mL/min, upper and lower phase time 4.0 min per cycle, sample volume 80 mL, rotational speed 1400 rpm (240g); upper phase elution (solid line) upper domain; lower phase elution (dotted lines) lower domain.

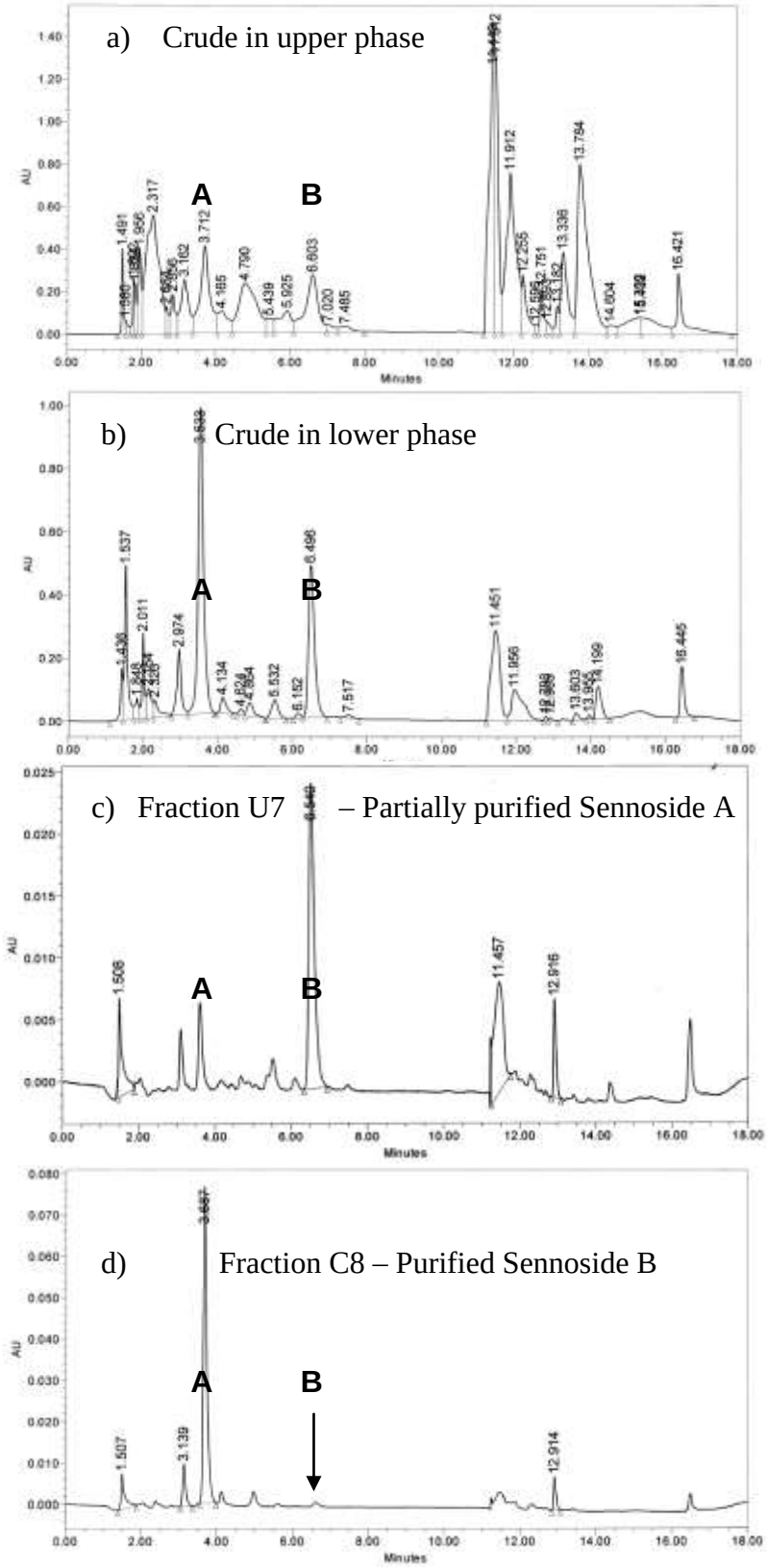


Figure 6.1.4: HPLC chromatograms for the crude sample in the a) upper and b) lower phases compared to enriched fractions with the highest concentrations c) sennoside A (retention time 6.6 min) and d) sennoside B (retention time 3.6 min) when using ICcE with the propan-1-ol/12% NaCl solution (1:1 v/v) solvent system at 30 °C on DE-Midi preparative columns, coil volume 912.5 mL.

6.2) Intermediate polarity API purification from a waste stream

GSK#14 is an intermediate polarity API which required isolation from a waste stream. The required product is present at 20% by HPLC peak area at 260nm however the actual content is approx 7.7% w/w. This sample is a residual from evaporation of the mother liquors (containing methanol, MIBK and toluene) although ultimately it may be desirable to isolate the material directly from the mother liquors. The compound structure is confidential, but contains amide and tertiary amine functional groups. The overall objective is to find an economical way to recover this material from the waste stream to increase the overall efficiency of the manufacturing process and to simplify and reduce the cost of waste disposal. Specifically, the potential benefits of ICcE with respect to throughput and solvent usage were compared to the best isocratic conditions achieved with a preparative CCC instrument. The target purity for the separation was greater than 95%, as a following crystallization step removed the remaining impurities to give an intermediate grade (IG) specification with all individual impurities below 0.05%.

6.2.1) Analytical method

All fractions were screened with the HPLC method given in Table 6.2.1. The HPLC chromatogram of the crude mother liquors (Figure 6.2.1) shows the target peak (RT 6.990 min) three major more polar impurities (RT 3.121, 5.026 and 6.560 min) and two major less polar impurities (RT 8.576 and 9.887 min) plus over 60 other minor impurities.

Table 6.2.1: HPLC method for GSK#14 material

Column	Symmetry C18 (75 x 4.6 mm, 3.5 µm), Waters, 40 °C
Mobile Phase	A: 0.2% aqueous formic acid and triethylamine at pH 3.5 B: MeCN
Method	0-8 min 25 to 55% B, 8.1-9.5 min 90% B, 9.5-9.6 min 90-25% B, 9.6-12 min 25% B
Flow Rate	1 mL/min
Detection	260 nm

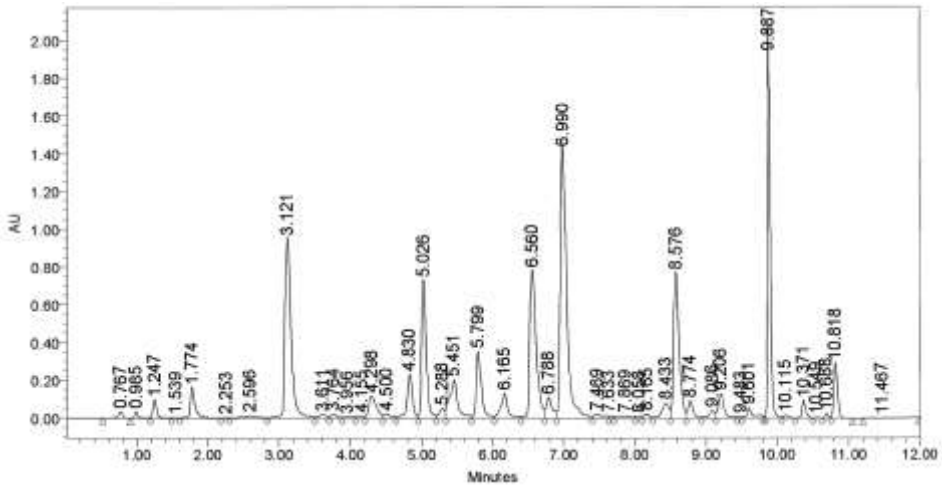


Figure 6.2.1: HPLC analysis of GSK#14 crude mother liquor (target RT = 6.990 min) by the method given in Table 6.2.1

6.2.2) Initial assessment of GSK14 by ICcE

Two ICcE methods, covering four scenarios were compared for purifying the target API from the mother liquor solution, with the most effective method then being fine tuned and compared directly to the isocratic scale-up results achieved separately.

- Method 6.1: With a first ICcE run all the impurities which were more polar than the target were stripped away in the lower phase, keeping the target and the non-polar impurities in the upper phase. The target with the non-polar impurities in the upper phase was then dried down and reprocessed by:
  - Either a second ICcE run which removed all the non-polar impurities with the upper phase and eluted the target with the lower phase fractions
  - Or a normal phase isocratic CCC run to purify the target
- Method 6.2: The phase system is selected to concentrate the target in the column and all the impurities are washed away with the upper and lower phases. The target is then:
  - Either eluted using isocratic normal phase CCC from the column
  - Or directly extruded to minimise the cycle time

6.2.2.1) Method 6.1 – Two runs to remove polar and non-polar impurities sequentially

Gravimetric analysis of the mother liquors crude solution showed them to have a total mass content of 65 mg/mL. When distributed in the HEMWat 17.5 phase system, which was used for the isocratic CCC studies[1], the crude material is 75% by mass in the lower phase and only 25% in the upper phase, therefore it was decided to strip away the bulk of the mass in the first ICcE step leaving the target in the upper phase with the remaining upper phase impurities for reprocessing in the second ICcE run to produce pure target material.

As it would be preferable to load the mother liquors directly, to avoid the need for a solvent switch step, this was tested for the initial run. The conditions used for these runs are given in Table 6.2.2. HEMWat 15 (Hexane:Ethyl acetate:Methanol:Water, 2:3:2:3) was used as this gave the correct distribution ratio to elute the target in the upper phase while removing the polar impurities with the lower phase. However all three runs failed to achieve successful separation as the loading of the mother liquors directly onto the columns containing the HEMWat 15 phase system caused the phase system to destabilise and become one phase.

In run 6.2.1 ICcE operated as expected for the first two cycles however after 19 minutes (in the third upper phase step) the column started to strip phase completely from both eluant streams. On emptying the column s they were found to contain only a single phase. The solvents in the mother liquors destroy the two phases in the column and the hydrodynamic equilibrium is lost so the solutions in the columns simple plug flow back and forth with the intermittent addition of upper and lower phase. In run 6.2.2 the loading of the mother liquors was reduced by 50 % (5 mL/min) to attempt to minimise the impact of the solvents in the mother liquors however the run destabilised in the same way. It was clear that the phase system would need reformulating to have the same solvents as the mother liquors to ensure the two phase system was not disrupted.

Table 6.2.2: Operating parameters for ICcE with mother liquor crude sample

Run Number	HEMWat Phase System	F_{UP}	F_{LP}	T_{UP}	T_{LP}	Sample Flow	Sample Conc.	Comments
		ml/min	ml/min	min	min	ml/min	mg/ml	
6.2.1	15	20	30	4	4	10	65	After 19 minutes (just over two cycles) the column started stripping and by the end of the third cycle the column was plug flowing back and forth with the intermittent addition of upper and lower phase. On emptying the column was found to contain only one phase.
6.2.2	15	20	30	4	4	5	65	The column became unstable in a similar way to run 6.2.1.

[1] TSB Final Project Technical Report: Theme2 – Generate a comprehensive applications portfolio

As the mother liquors could not be loaded directly the crude was dried down by evaporation to give a semi-solid which could then be redissolved in a 50/50% mix of the upper and lower phases of the solvent system to be used for the relevant ICcE run.

Table 6.2.3 gives the conditions for the initial ICcE run to remove all the more polar impurities leaving the target in the upper phase with the non-polar impurities. To elute the target in the upper phase fractions the HEMWat 17 phase system was used with flow rates of 25 mL/min for the upper phase and 30 mL/min for the lower phase and equal switching times of 4 minutes. The dried down crude mother liquors were loaded in a 50% mixture of upper and lower phases at three different concentrations, close to the original mother liquor concentration (69.2 g/mL) and twice (133 g/mL) and four times (278 g/mL) this value.

Table 6.2.3: Operating parameters to remove polar and non-polar impurities sequentially

Run Number	Mode	HEMWat Phase System	F_{UP}	F_{LP}	T_{UP}	T_{LP}	Crude Type	Sample Flow	Sample Conc.	Sample Inj. Vol.	Load time	Loading	Phase target eluted in	Target purity
			ml/min	ml/min	min	min		ml/min	mg/ml	ml	min	g		%
6.2.3	ICcE	17	25	30	4	4	Dried	10	69.2	425	40	29.41	UP	20
6.2.4	ICcE	17	25	30	4	4	Dried	5	133.1	600	120	79.86	UP	20
6.2.5	ICcE	17	25	30	4	4	Dried	5	278.2	600	120	166.92	UP	20
6.2.6	2nd ICcE adter Run 6.2.4	18	40	40	4	4	Dried pur.	10	20	440	40	8.8	LP	79
6.2.7	NP CCC	17.5	41				Dried pur.		212.5	40	1	8.5	UP	87
6.2.8	NP CCC	17.5	41				Dried pur.		112	50	1.2	5.6	UP	87

Run 6.2.3 demonstrated the principle of removing the lower phase impurities. 69% of the mass was stripped away with the lower phase. 31% of the mass was collected in the upper phase stream, including the target at 20% purity by HPLC (Figure 6.2.2). Run 6.2.4 repeats the previous run with doubled sample concentration but at half the flow rate, giving the same throughput but with a much longer sample loading time of 120 minutes. The target was successfully separated for a continuous loading time of two hours. The fractogram profile for the run is given in Figure 6.2.3. In Run 6.2.5 the sample concentration was again doubled to 278 mg/mL however this concentration destabilised the column. After 96 minutes the lower phase fractions eluted with a very dark concentrated interface layer implying the column had become saturated and steady state separation was not occurring.

The combined upper phase fractions containing the target and non-polar impurities were dried down for reprocessing to remove the impurities, either by a second ICcE run or by an isocratic normal phase CCC run. It was theorised that once the bulk of the mass had been removed with the initial ICcE run much higher loadings would be achievable for the cleanup.

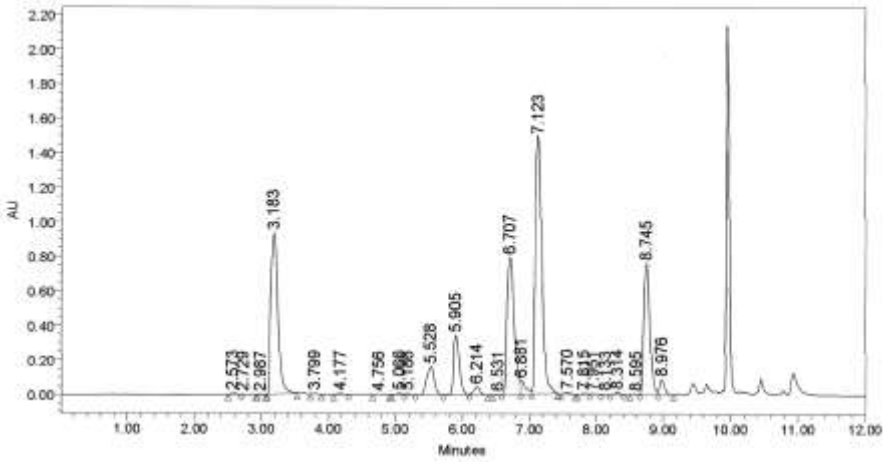


Figure 6.2.2: HPLC analysis of Run 6.2.4 combined upper phase fractions (Target RT 7.123 at 20% purity) by the method given in Table 6.2.1

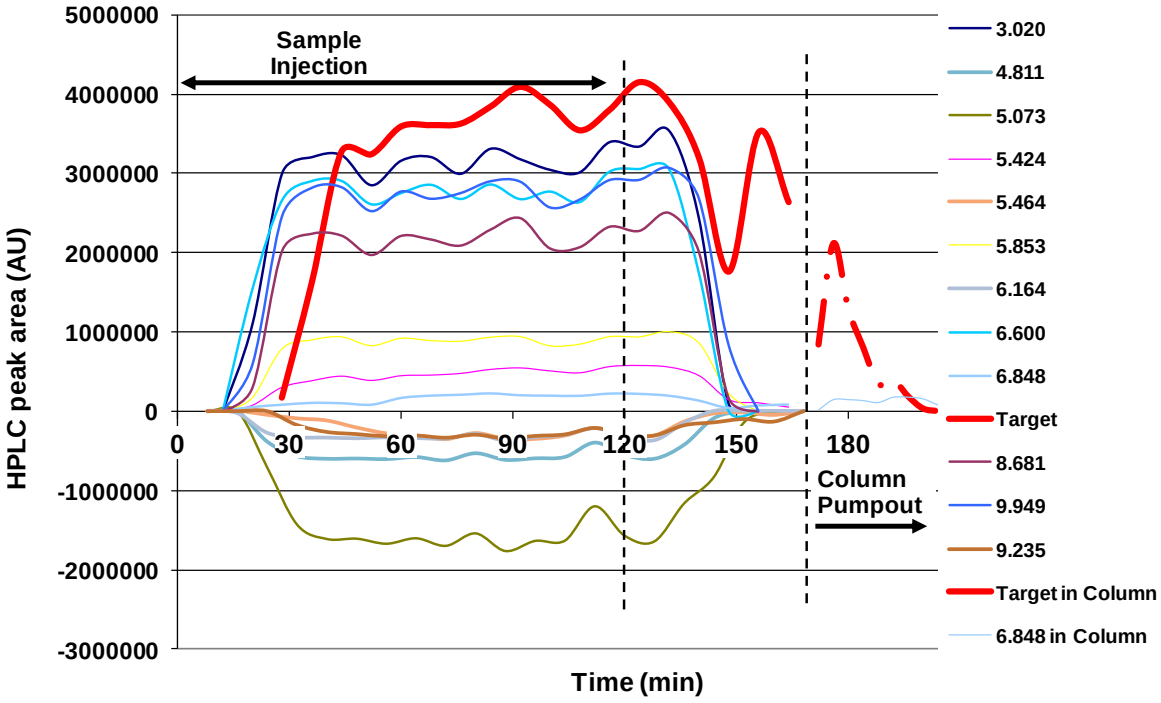


Figure 6.2.3: Fractogram constructed from HPLC peak areas of the solutes in each eluted fraction of the GSK#14 crude for the ICcE separation using Solvent system HEMWat 17 to concentrate the target in the upper phase fractions while stripping away the polar impurities in the lower phase. (30 °C on the DE-Midi preparative columns, coil volume 912.5 mL, upper phase flow rate 25 mL/min, lower phase flow rate 30 mL/min flow switched every 4.0 minutes, sample flow rate 5.0 mL/min, sample volume 600 mL, rotational speed 1400 rpm (240g); upper phase elution upper domain; lower phase elution lower domain)

In Run 6.2.6 the concentrated upper phase fractions were reinjected in an ICcE run using solvent system HEMWat 18 (Hexane:Ethyl acetate:Methanol:Water, 6:5:6:5) to separate the upper phase impurities and elute the target only with the lower phase. Figure 6.2.4a and b show examples of the HPLC chromatogram for the eluting fractions from the upper and lower phase fractions respectively at steady state (Cycle 5 after 40 minute run time). The four major non-polar impurities (RT 3.034, 6.704, 8.690 and 9.922 min) have been removed with the upper phase fractions. However, the purity of the target (RT 7.149 min) in the lower phase fractions is only 62% which is not acceptable for the required process. The target was slightly over retained in the column, therefore was not eluting with the lower phase quite as quickly as would have been expected from modelling. However, changing the operating parameters slightly to elute the target quicker would not increase the purity enough as the remaining polar impurities would continue to elute with the lower phase fractions contaminating the target. Figure 6.2.4c shows that combining all the fractions containing target from the lower phase elution of the run gave an estimation of 79% for the maximum expected purity achievable with this regime, with three minor impurities remaining (RT 5.570, 6.902 and 10.453 min). By this two-stage method loading is 39g/hr with solvent consumption of 0.054L/g for the first separation and 0.077L/g for the second separation.

In Run 6.2.7 the concentrated upper phase fractions containing the target were purified using a normal phase isocratic CCC run. As the bulk of the mass had been removed by the previous step it was theorised that a significantly higher loading would be possible for this CCC run so minimising the overall solvent usage and also achieving higher overall throughput. However using solvent system HEMWat 17.5, with a flow rate of 41 mL/min and a sample loading of 8.5 g (213 mg/mL), the column stripped stationary phase from the initial retention of 92% to a final retention of 56% (36% of column stripped). The purity of the peak eluted fraction was only 87% (Figure 6.2.5), which was below the target purity. This run was repeated at a lower sample loading (5.6 g) and concentration (112 mg/mL) in an attempt to reduce the stripping and so improve the stationary phase retention and overall purity however, similar stripping was observed, with the final retention being 58%. This shows that although over two thirds of the mass had been removed with the initial ICcE run the compounds which had the greatest effect on the column stability remained in the concentrated crude, therefore minimising any gain in throughput that could be achieved by this method.

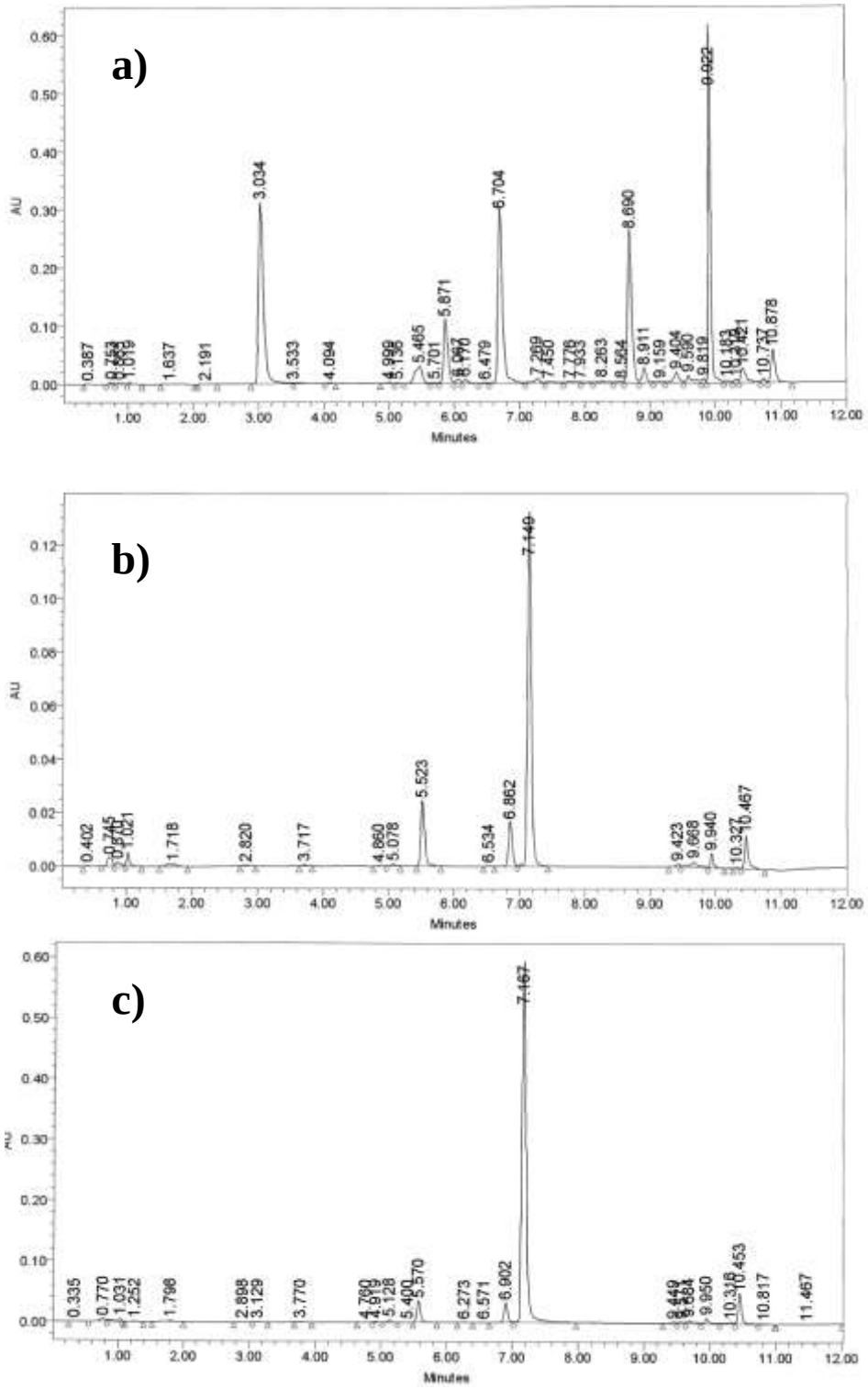


Figure 6.2.4: HPLC analysis of Run 6.2.6 steady state a) upper phase fraction from cycle five b) lower phase fraction from cycle five and c) all the combined lower phase fractions containing the target (Target RT 7.167 at 79% purity) of the GSK#14 sample with the upper phase impurities removed for the ICcE separation using solvent system HEMWat 18 to collect the target in the lower phase fractions while stripping away the non-polar impurities in the upper phase. (30 °C on the DE-Midi preparative columns, coil volume 912.5 mL, upper phase flow rate 40 mL/min, lower phase flow rate 40 mL/min flow switched every 4.0 minutes, sample flow rate 10.0 mL/min, sample volume 440 mL, rotational speed 1400 rpm (240*g*))

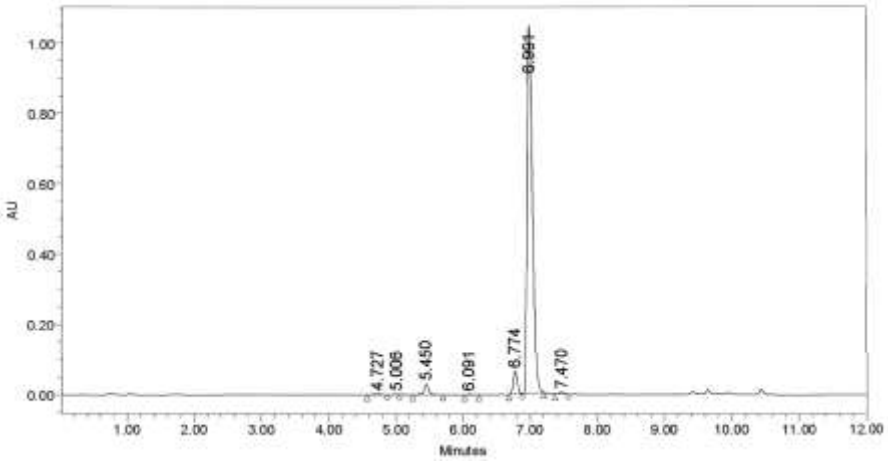


Figure 6.2.5: HPLC analysis of the top of the eluted target peak fraction for Run 6.2.7 where the concentrated upper phase fractions containing the target were purified using a normal phase isocratic CCC run using HEMWat 17.5. (30C on the DE-Midi preparative columns, coil volume 912.5 mL, flow rate 41 mL/min, sample mass 8.5 g at 213 mg/mL, rotational speed 1400 rpm, 240*g*)

6.2.2.2) Method 6.2 – Single ICcE run to concentrate the target in the column

The aim of this method will be to concentrate the target in the column and wash the non-polar and polar impurities away with the upper and lower phases respectively. The initial run conditions were set using the model described in Section 4 and the static partition coefficient value measured for the target (K_d=0.90) in the HEMWat 17.5 phase system. The run conditions for the ICcE runs are given in Table 6.2.4.

Table 6.2.4: Operating parameters to remove polar and non-polar impurities sequentially

Run Number	Mode	HEMWat Phase System	F_{UP}	F_{LP}	T_{UP}	T_{LP}	Crude Type	Sample Flow	Sample Conc.	Sample Inj. Vol.	Load time	Loading	Phase target eluted in
			ml/min	ml/min	min	min		ml/min	mg/ml	ml	min	g	
6.2.9	ICcE	17.5	27	30	4	4	Dried	4	134	220	56	29.5	UP/Column
6.2.10	ICcE	17.5	20	30	4	4	Dried	4	134	220	56	29.5	Column
6.2.11	ICcE	17.5	20	30	4	4	Dried	4	134	220	56	29.5	Column

For run 6.2.9 the target (29.5 g) was loaded for 56 minutes at a flow rate of 4 mL/min with upper and lower eluant flow times of 27 and 30 mL/min respectively. After the seventh and final loading cycle the remaining impurities were washed away with six further cycles with the aim of leaving the pure target in the column. From the fractogram shown in Figure 6.2.6 it can be seen that the target although largely retained in the column partially eluted with the upper phase. The reason for this discrepancy to the modelled elution was not fully understood at the time however later studies (Section 6.2.3) showed the static K_d value of the target to be highly sensitive to the alkane used in the phase system. A change between the

batches of hexane used for the K_d measurement and the ICcE may have effected the distribution ratio of the target. However, the combined pure fractions from the column, which was extruded with compressed air, had a purity of 97.9% with a 50% yield (Figure 6.2.6 inset).

This run was therefore repeated (Run 6.2.10) with the lower phase flow rate reduced to 20 mL/min to retain the target fully in the column. Because in the previous run the major impurities had been removed from the column by 88 minutes the number of wash cycles was also reduced from six to five for this run. From Figure 6.2.7 it can be seen that the target is now retained in the column with only a small fraction eluting with the upper phase fractions. The purity of the extruded column contents was 92.7% with a yield of 92%. The slight reduction in purity is caused by the removal of one wash cycle.

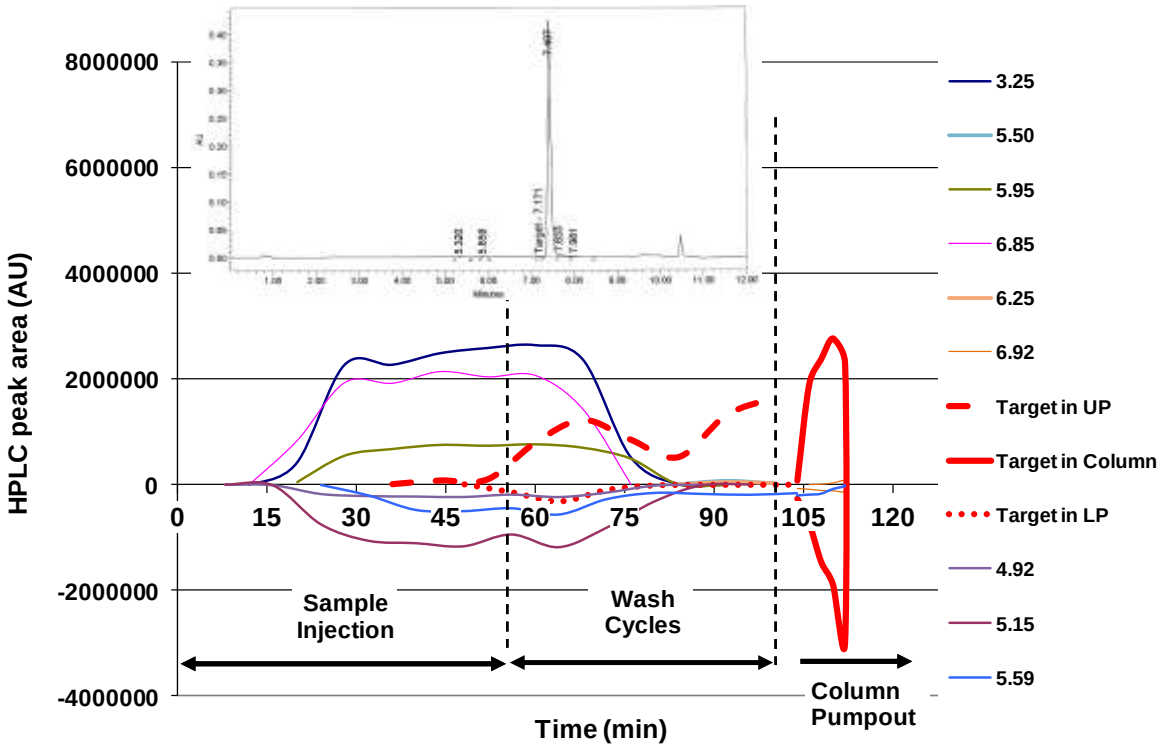


Figure 6.2.6: Fractogram constructed from HPLC peak areas of the solutes in each eluted fraction of the GSK#14 crude for the ICcE separation using Solvent system HEMWat 17.5 to concentrate the target in the column and strip away all impurities with the upper and lower phase streams. (30 °C on the DE-Midi preparative columns, coil volume 912.5 mL, upper phase flow rate 27 mL/min, lower phase flow rate 30 mL/min flow switched every 4.0 minutes, sample flow rate 4.0 mL/min, sample 29.5g in 220 mL, rotational speed 1400 rpm (240g); upper phase elution upper domain; lower phase elution lower domain). Inset HPLC of combined column fractions.

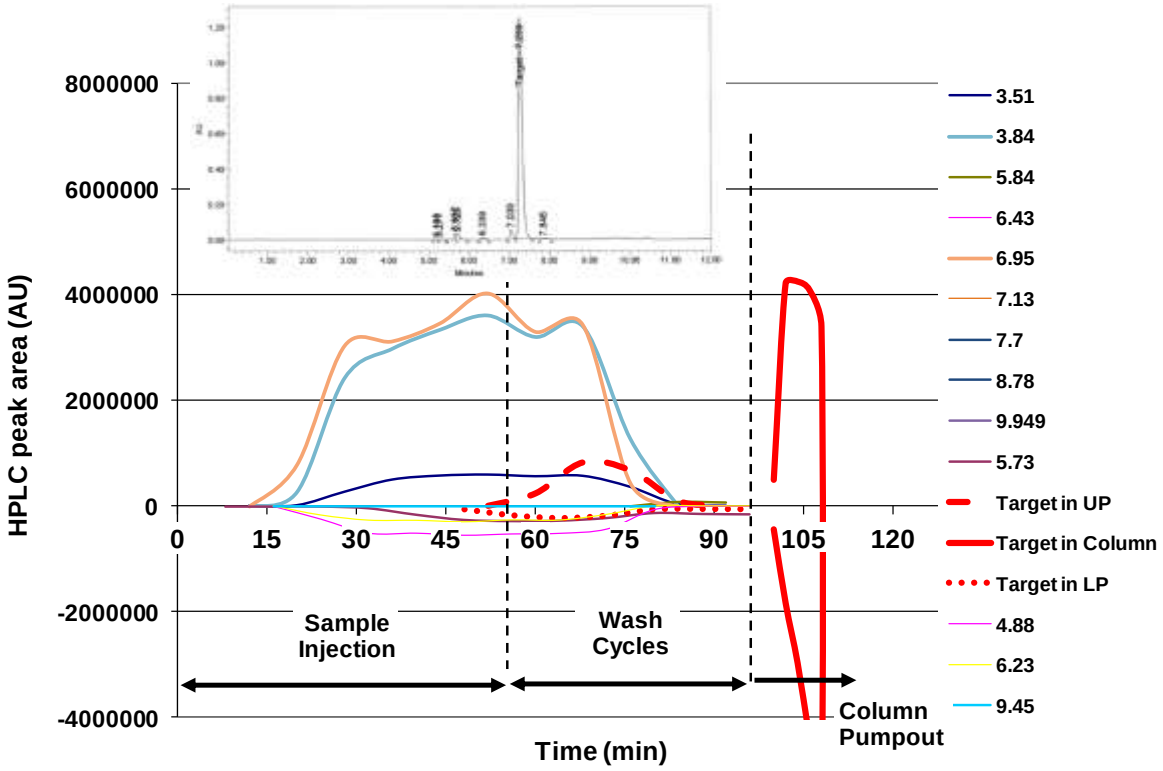


Figure 6.2.7: Fractogram constructed from HPLC peak areas of the solutes in each eluted fraction of the GSK#14 crude for the ICcE separation using Solvent system HEMWat 17.5. Run conditions as for Figure 6.2.6 except upper phase flow rate 27 mL/min, lower phase flow rate 30 mL/min flow.

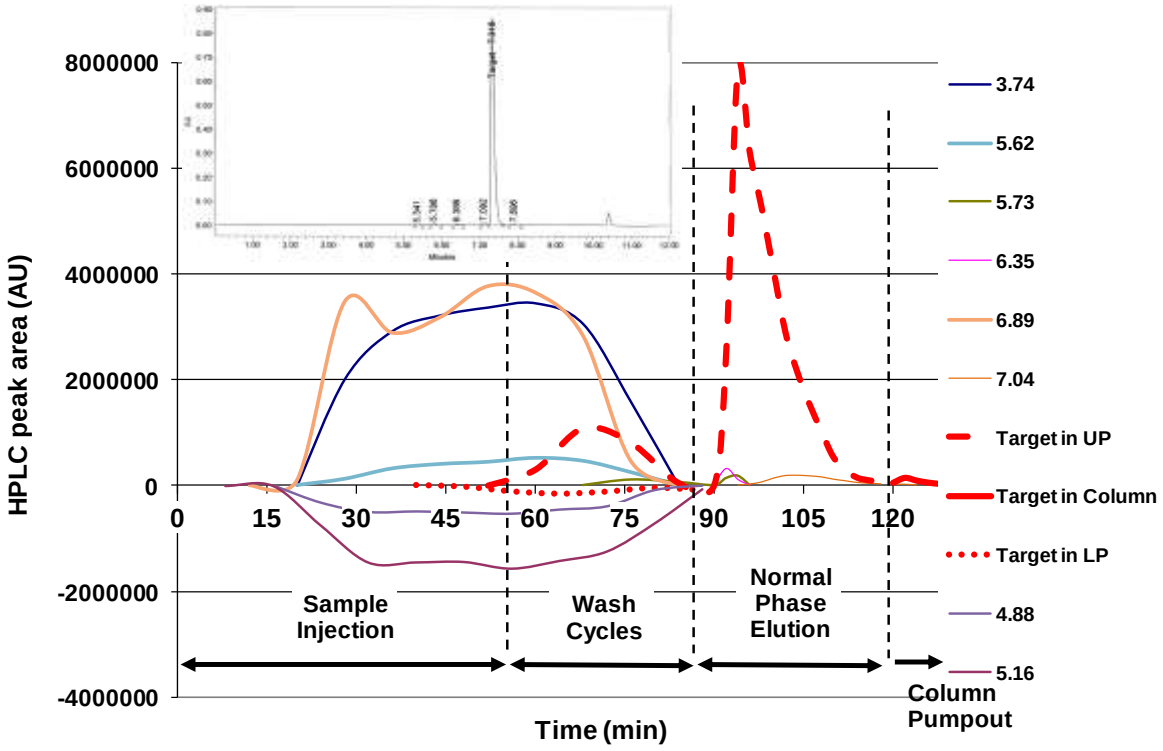


Figure 6.2.8: Fractogram constructed from HPLC peak areas of the solutes in each eluted fraction of the GSK#14 crude for the ICcE separation using Solvent system HEMWat 17.5. Run conditions as for Figure 6.2.7 except target eluted with upper phase at 40 mL/min.

For run 6.2.11 rather than extruding the column contents they were instead eluted with the upper phase at 40 mL/min after seven loading cycles and 4 wash cycles. This has the advantage of the eluted target fractions being only upper phase therefore reducing the cost of solvent removal, unlike the extruded column which is a mixture of upper and lower phase. The number of wash cycles was again reduced to try and keep the total run cycle time similar so the overall throughput for the system was matched to run 6.2.10. The purity of the combined eluted fractions was 95.4% and the yield was 82% (Figure 6.2.8 inset).

Comparing the two ICcE methods (6.1 and 6.2) it is clear that the second method (6.2) which concentrates the target in the column gives much improved purity by either option than the first methods options. Therefore Method 6.2 was compared to the separately produced isocratic CCC scale-up study produced as part of the TSB High Value Manufacturing programme, "Scalable Technology for the Extraction of Pharmaceuticals" (Grant No. TP14/HVM/6/I/BD506K).

6.2.3) Comparison of the ICcE method to the isocratic CCC preparative method

For the comparison to the isocratic CCC data determined previously a new batch of the GSK14 crude was supplied. The isocratic studies on the preparative Midi CCC were carried out at 1000 rpm (121*g*) as this data was also used to scale-up the isocratic method to the pilot scale 18L Maxi CCC instrument. Further, the use of heptane in the phase system makeup rather than hexane was requested due to health and safety requirements. Heptane is less toxic and has a higher flash point. Therefore the static partition coefficients were measured for the target in HEMWat phase systems made up with heptane. The phase system was made up both classically and using the tables provided by Garrard (2005).

Table 6.2.5: K_d values for GSK14 target in HEMWat phase systems made with heptane

Phase System	Alkane used	Phase System makeup	Crude Type	Target K_d	SD
17.5	Hexane	Classical	Old Crude	0.90	0.03
17.5	Heptane	Classical	New Crude	1.98	0.06
17.5	Heptane	Table	New Crude	2.12	0.11
17	Heptane	Table	New Crude	1.29	0.02
16	Heptane	Table	New Crude	0.39	0.01
15	Heptane	Table	New Crude	0.10	0.01

As can be seen from Table 6.2.5 the use of heptane in the phase system rather than hexane shifts the partition coefficient of the target from 0.90 to 1.98 for the HEMWat 17.5 phase system used previously. Ideally as the target is being concentrated in the column for the ICcE runs it would be good to have a partition coefficient near one so the flows and switching times are similar. Therefore for the following ICcE runs HEMWat 17 (1:1:1:1; with heptane as the alkane) was used which gave a partition coefficient of 1.29 for the target compound.

The experimental conditions for the isocratic runs are given in Table 6.2.6. and the isocratic results are summarised in Table 6.2.7.

Table 6.2.6: Experimental conditions for isocratic CCC runs with GSK14

Column	Dynamic Extractions Midi HPCCC, preparative 999.5mL column, 4mm bore
Solvent systems	Heptane/Ethyl Acetate/Methanol/Water (HEMWat 17.5 (12/11/12/11))
Operation mode	Normal Phase (NP), Upper organic phase as a mobile
Method	1000 rpm, 30 °C, isocratic, elution
Sample Loading	Sample was made up in aqueous stationary phase

Table 6.2.7: Summary of isocratic CCC results with GSK14

Run Number	Mode	HEMWat Phase System	Run Time	Cycle Time	Flow Rate	Sample Mass	Sample Conc.	Sample Inj. Vol.	Crude Throughput	Target purity @ 85% yield
			min	min	mL/min	g	mg/ml	ml	kg/day	%
6.2.12	Isocratic CCC	17.5	60	70	40	4	100	40	0.08	100.0
6.2.13	Isocratic CCC	17.5	39	49	62	9.9	198	50	0.29	97.0
6.2.14	Isocratic CCC	17.5	40	50	62	15	199	75.3	0.43	94.1
6.2.15	Isocratic CCC	17.5	45	55	62	20	200	100	0.52	90.3

From the data presented in Table 6.2.7 it can be seen that very high target purity (100% @ 85% yield or 99.8% at 100% yield) can be achieved if the flow rate, sample volume and sample concentration are minimised (Run 6.2.12). However this is at the expense of a relatively low throughput of 0.08 kg/day of crude material processed. Therefore, the mobile phase flow rate, sample concentration and injection volumes were increased to achieve cost effective throughput rates of over 0.4 kg/day, with the aim of keeping the target purity above 95% at a yield of 85%. An initial increase in flow rate to 60 mL/min and doubling of the sample concentration gave a throughput of 0.29 kg/day with high purity of 97% at 85% yield (Run 6.2.13). Further increases in the loading by increasing the injection volume to 15% of

coil volume (75 mL) and 20% (100 mL) gave good throughput of 0.43 and 0.52 kg/day respectively (Runs 6.2.14 and 6.2.15). The target purity at for Run 6.2.14 of 94.1% at 85% yield, was slightly below the purity aim of 95% however this was found to pass intermediate grade (IG) specifications on recrystallisation, therefore was acceptable as a process. The purity of 90.3% at 85% yield for Run 6.2.15 was also acceptable for this specific example after recrystallisation, therefore Runs 6.2.13-15 were selected for comparison to ICcE.

The aim of the ICcE runs was to produce results to compare the solvent usage, API purity, yield and purified throughput to the isocratic runs at similar crude throughput levels. The experimental conditions for the ICcE runs are given in Table 6.2.8 and table 6.2.9.

The ICcE runs concentrate the target in the column (Method 6.2). Runs 6.2.16-18 and 20 elute the target in normal phase, while Run 6.2.19 extrudes the target after the wash cycles.

Table 6.2.8: Experimental conditions for ICcE runs with GSK14 crude

Column	Dynamic Extractions Midi HPCCC, preparative 912.5 mL column, 4 mm bore
Solvent systems	Heptane/Ethyl Acetate/Methanol/Water (HEMWat 17 (1/1/1/1))
Operation mode	ICcE, Upper phase flow rate 26 mL/min, lower phase flow rate 20 mL/min, step switching time 4.0 minutes for both phases
Method	1000 rpm, 30 °C, ICcE
Sample Loading	Sample was made up in a 50%/50% mixture of upper and lower phase.

Table 6.2.9: ICcE operating parameters for GSK14 crude

Run Number	Mode	Sample Flow	Sample Conc.	Sample Inj. Vol.	Sample Load Time	Sample Loading	Wash Time	Wash UP Flow	Wash LP Flow	Elution Time	Elution Flow
		ml/min	mg/ml	ml	min	g	min	ml/min	ml/min	min	ml/min
6.2.16	ICcE	4	133	240	56	29.9	32	26	20	30	40
6.2.17	ICcE	4	133	240	56	29.9	32	26	20	30	40
6.2.18	ICcE	4	133	240	56	29.9	32	30	24	30	40
6.2.19	ICcE	4	133	240	56	29.9	32	26	20	7.5	200
6.2.20	ICcE	4	200	160	40	32.0	32	26	20	30	40

In Table 6.2.9, Runs 2.6.16-18 have a crude throughput of 0.34 kg/day and are comparable to isocratic Run 6.2.13. For these ICcE runs adjustments to the wash stage were made to understand the effect of the washing stage on the ICcE process. For Run 6.2.16 after sample loading the sample pumps were stopped, while in Run 6.2.17, after sample injection, the sample flow was replaced by upper and lower phase streams at the same flow rate and in Run 6.2.18 the sample pumps were stopped but the upper and lower phase eluant flows were

increased by the sample flow from 26 mL/min to 30 mL/min in the normal phase step and from 20 mL/min to 24 mL/min in the reversed phase step. Run 6.2.19, uses column extrusion rather than normal phase elution, has a crude throughput of 0.44 kg/day and is comparable to isocratic Run 6.2.14. Run 6.2.20 with increased sample concentration has a potential crude throughput of 0.51 kg/day and is comparable to isocratic Run 6.2.15.

From Figure 6.2.9, using the model from Section 4.1 where the target has a K_d value of 1.29, gave aim flow rates of 26.0 mL/min for the upper phase and 20.0 mL/min for the lower phase with matched four minute switching steps and a sample flow rate of 4.0 mL/min.

From Figure 6.2.10, the target should be trapped in the column, while impurities with K_d values below 0.98 elute with the upper phase fractions and impurities with K_d values above 1.68 elute with the lower phase fractions.

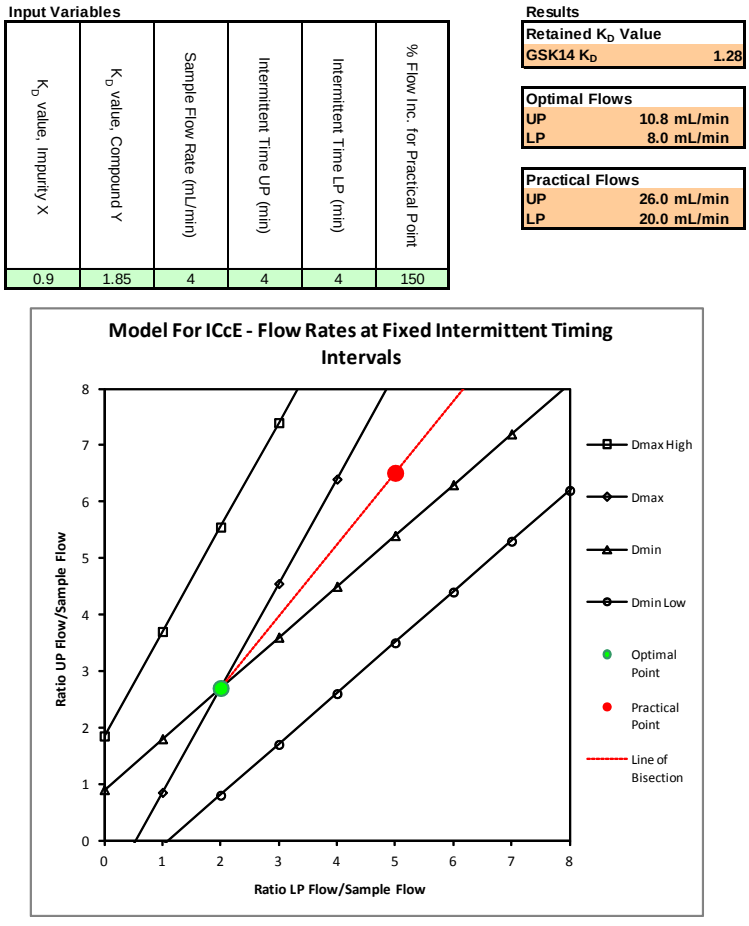


Figure 6.2.9: Model of ICcE separation of GSK14 target compound. For equal switching times and flow rates of 26.0 mL/min for the upper phase and 20.0 mL/min for the lower phase the target is retained in the column (red dot)

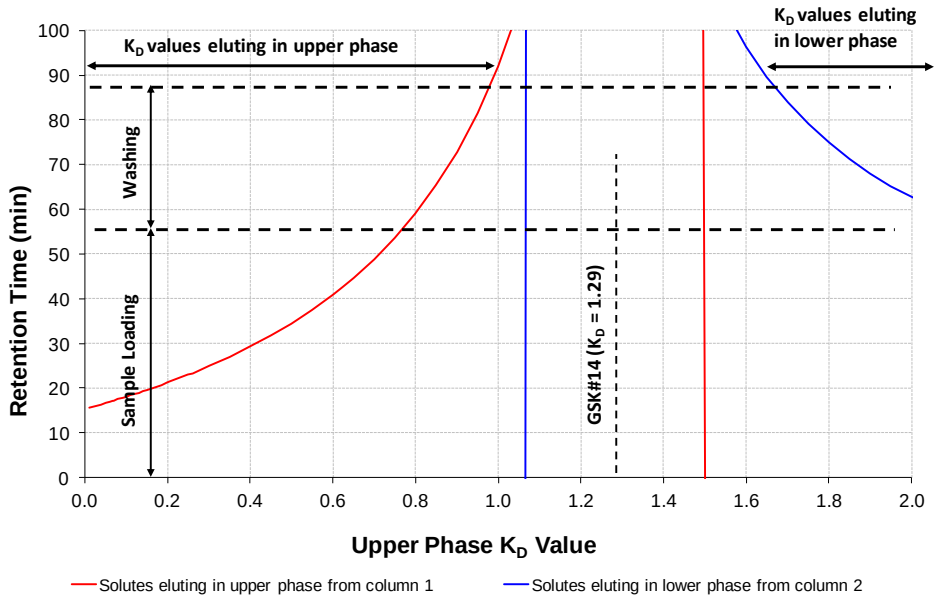


Figure 6.2.10: Model of the elution times for the ICcE separation of GSK14 waste stream material for the conditions given in Figure 6.1.9. Target compound GSK14 is retained in the column, while all compounds with K_d values below 0.98 are removed with the upper phase and all compounds with K_d values above 1.68 are removed with the lower phase

Figure 6.2.11 gives the elution profiles of the target compounds, reconstructed from the HPLC peak areas. For ICcE Run 6.2.16 the target is largely retained in the column (84.9%) before subsequent elution, with a small quantity (5.4%) of the target eluting with the upper phase ICcE cycle elution fractions and 9.7% eluting with the lower phase ICcE cycle elution fractions. The fractions eluted in the final normal phase elution step had a purity of 96.7%.

Adding fresh upper and lower phase at the sample inject point for the wash cycles (Run 6.2.17) caused less target to be retained in the column (73.4%) and therefore more to be eluted with the ICcE cycle elution fractions (12.3% target in the upper phase ICcE elution fractions, 14.3% in the lower phase fractions). The normal phase eluted column profile was also split into a double peak by the addition of eluant at the sample injection point. Further, the purity of the eluted fractions was reduced to 95.9% in comparison to the original method (Run 6.2.16).

However, increasing the wash phase eluant flows (Run 6.2.18) improved the purity of the eluted fractions to 97.2% yet retained 84.1% of the target in the column (5.5% target in the upper phase ICcE elution fractions, 10.4% in the lower phase fractions), which was comparable to run 6.2.16.

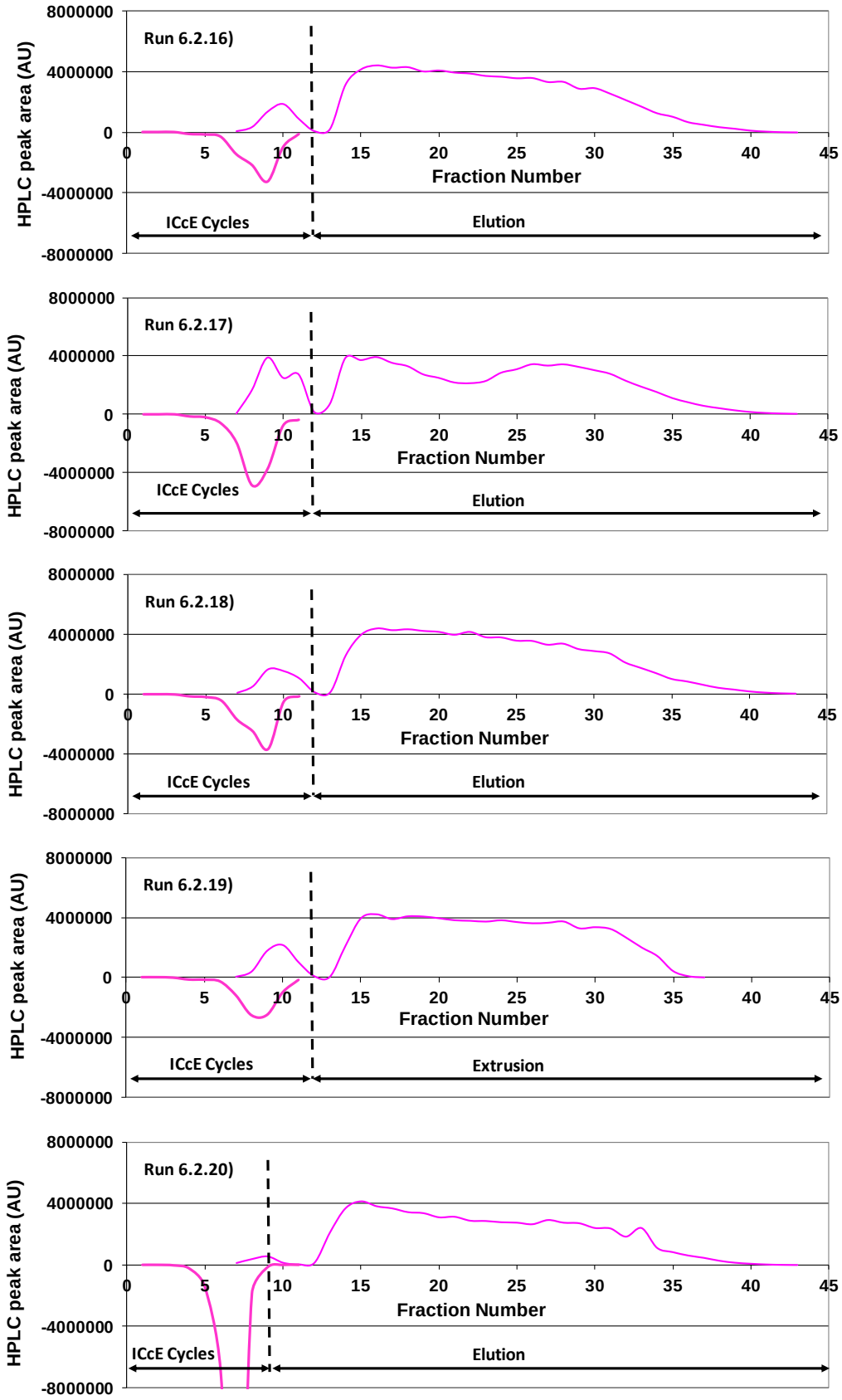


Figure 6.2.11: Fractogram constructed from HPLC peak areas of the GSK14 target (impurities not shown) in each eluted fraction for the ICcE separations given in Table 6.2.8

The normal phase elution profiles of the target for all three runs give relatively long tail so both increasing the run time and diluting the target, which will reduce overall throughput and increase solvent removal costs.

In Run 6.2.19, direct extrusion of the column at high flow rate (200 mL/min) gave a reduced run time and therefore higher throughput of crude material, however purity in the extruded fractions is reduced to 93.8%, though 84.3% of the target was retained in the columns before elution as for Runs 6.2.16 and 6.2.18.

In Run 6.2.20, increasing the sample concentration (200 mg/m L cf. 133 mg/mL), in an attempt to match the crude throughput of the best isocratic run (0.53 kg/day) caused the column hydrodynamics to become unstable and stripping of the target with the ICcE cycle lower phase elution fractions. 35.5% of the target was lost with the lower phase fractions and the injection was limited to only 40 minutes so the target crude throughput was not reached. Purity of the normal phase eluted fractions was 94.6%.

6.2.4) Purity, yield, throughput and solvent usage of isocratic CCC cf. ICcE

From Figure 6.2.12a, both the ICcE and isocratic methods are able to provide the target at greater than 95% purity. Run 6.2.18 for the ICcE method and Run 6.2.13 for the isocratic method are at comparable crude throughputs of 0.34 kg/day and 0.29 kg/day respectively. The isocratic run gives improved yield (Figure 6.2.12b) over the comparable ICcE run (84% versus 69% at the highest purity), though throughput of the recovered API (Figure 6.2.13a) is matched as the crude loading for the ICcE run was slightly higher (0.75 gAPI recovered/hr versus 0.78 gAPI recovered/hr respectively). Solvent usage (Figure 6.2.13b) is approximately two times higher for the isocratic run than the ICcE run which will introduce increased cost for consumables purchase and solvent handling to the isocratic method (6.0 L/gAPI recovered versus 3.2 L/gAPI recovered).

The overall lower yield of the ICcE runs was driven by the small loss of target in the ICcE cycle elution fractions which averaged 15.6% across the three ICcE runs. Minimising this loss will be dependent on controlling the band broadening within the column with successive switching cycles.

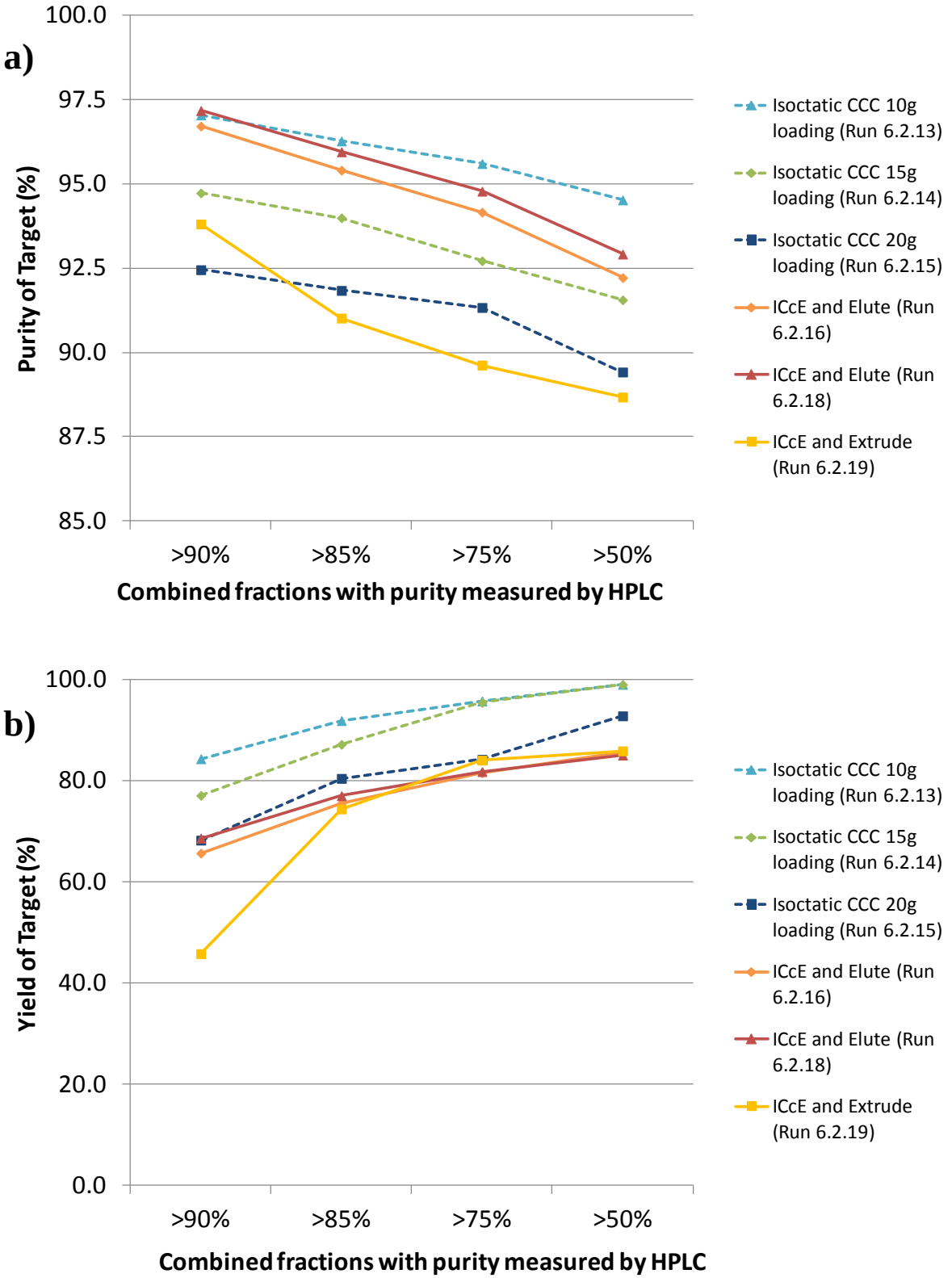


Figure 6.2.12: a) Purity and b) yield of GSK14 target calculated from HPLC peak areas of each eluted fraction with purity above 50, 75, 85 and 90% for the isocratic CCC and ICcE separations given in Table 6.2.5 and Table 6.2.8

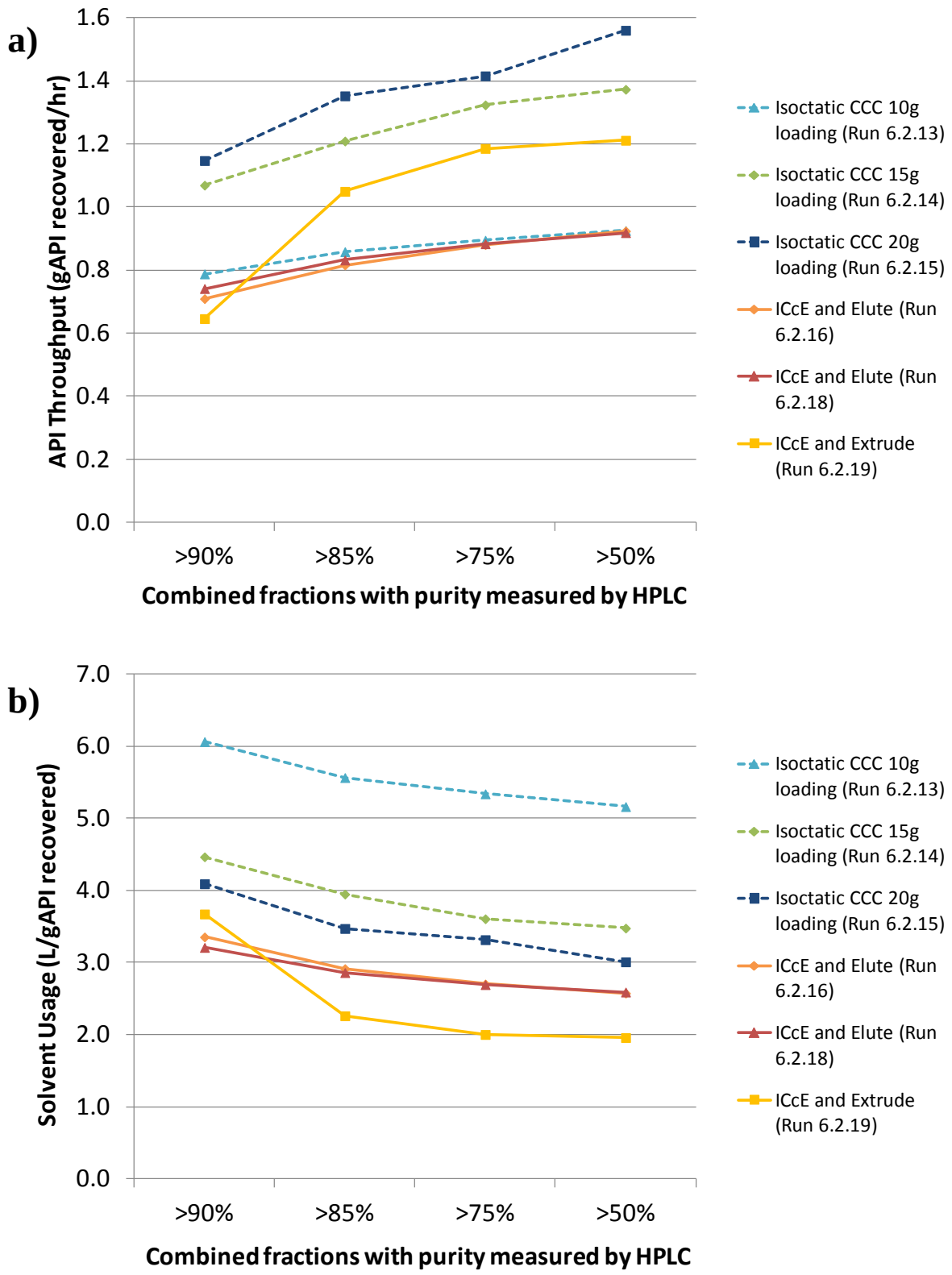


Figure 6.2.13: a) API throughput and b) solvent usage of GSK14 target calculated from HPLC peak areas of each eluted fraction with purity above 50, 75, 85 and 90% for the isocratic CCC and ICcE separations given in Table 6.2.5 and Table 6.2.8

For the isocratic runs, the throughput (Figure 6.2.13a) could be improved considerably by increasing the loading to 15g of crude material (Run 6.2.14) and 20 g of material (Run 6.2.15) and though these loadings destabilised the column hydrodynamics causing stripping of the stationary phase, acceptable (though reduced) purity and yield could be achieved. Because the ICcE method becomes unstable if the column is overloaded (Run 6.2.20) the throughput could only be increased by extruding the column to reduce the run time (Run 6.2.19). This ICcE run gave purity and yield comparable to the 20 g loading isocratic run, but with only 80% of the throughput (Figure 6.2.13a). However the isocratic run again has higher solvent usage, approximately1.6 times per gram of API recovered.

Overall, if the columns are run in hydrodynamic equilibrium the ICcE method gives improved solvent usage per gram of processed API at similar purity and throughput levels. However, if reduced purity specifications were acceptable, the isocratic method can be run in overload conditions which disrupt the hydrodynamic equilibrium of the column causing stripping of the stationary phase, which allows an increase in throughput by a factor of 1.6. Whereas, for the ICcE method, where the column must stay in hydrodynamic equilibrium so extruding the column was used to reduce the run time and therefore increased throughput by a factor of 1.3 times. In all cases the ICcE method uses less solvent per gram of API recovered (Figure 6.2.13b).

6.2.5) Conclusions on the purification of GSK14

The GSK14 target compound was successfully purified from mother liquors (65mg solids/mL with 7.7% w/w target in MeOH-MIBK mix) after these had been dried down and re-dissolved in phase system by the ICcE method where the target was trapped in the column, before elution in normal phase. The direct injection of mother liquors into a containing HEMWat phase system is not possible as it destroys two-phase structure in the column and separation doesn't occur. A two stage ICcE method to strip the upper phase and then the lower phase impurities gave reduced purity and was not seen as economic as the mother liquors cannot be loaded directly at present, therefore two solvent swaps are required to achieve purification.

The ICcE methods used at least 37% less solvent than the isocratic CCC methods. The isocratic method could achieve 23% higher overall throughput as lower purity than had originally been specified for the crude mixture was acceptable because close running

impurities could be readily removed by a subsequent crystallisation step. The isocratic column could therefore be run in overload conditions where the hydrodynamic equilibrium was destabilised, leading to stripping of stationary phase.

Realistically specifications for impurities may not be available initially and therefore development of the specification is likely to be an iterative process with a combination of information coming from, for example, CCC trials and crystallisation/chemistry development.

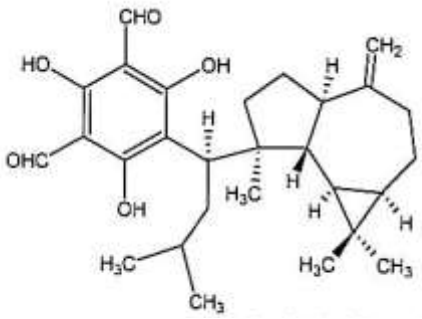
Comparing the isocratic and ICcE methods the final choice will be dependent on external cost factors, specifically the API value; the bulk solvent costs and the differing capex between two methods; which are not included in the comparison.

This work was published as a prize winning poster at SPICA2012 (14th International Symposium on Preparative and Industrial Chromatography and Allied Techniques, Brussels, Belgium, Appendix 1.2.1).

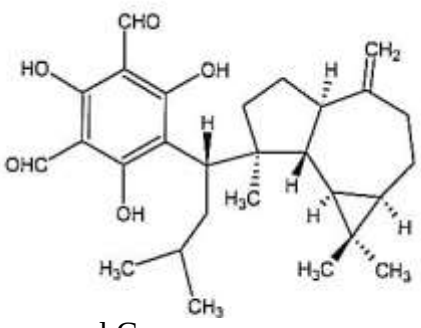
6.3) Separation of non polar macrocarpals by ICcE

Macrocarpals are phloroglucinoid based derivatives with an attached diterpene moiety that makes them relatively non polar. They are naturally occurring compounds present in the leaves of a variety of eucalyptus species (Eschler et al. 2000). The Institut de Recherche Pierre Fabre (IRPF) is studying these compounds for their potential to stop smoking. The purification of these compounds from a crude extract is difficult due to their closely related structures. The originally developed method was to use conventional isocratic and gradient HPCCC for the group purification of enantiomers macrocarpals C and G to a purity of greater than 85% (for the pair measured by HPLC relative peak area) from all other impurities in a crude extract, including enantiomers macrocarpals A and B, which are mono-oxygenated sesquiterpene analogs of macrocarpals C and G (Figure 6.3).

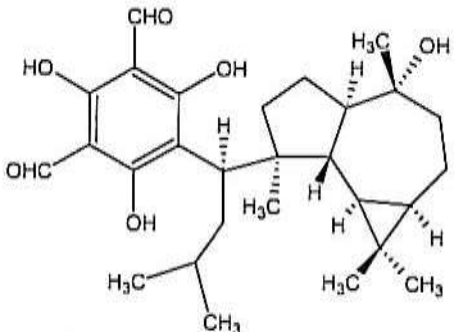
The aim of the study is to apply the ICcE method for the purification of macrocarpals C and G to further improve cost of the process by potentially increasing yield or reducing solvent usage in comparison to the earlier study using conventional HPCCC.



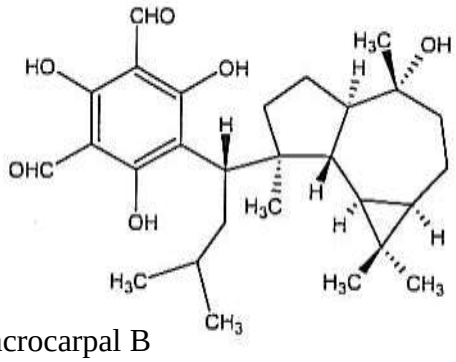
Macrocarpal C



Macrocarpal G



Macrocarpal A



Macrocarpal B

Figure 6.3: Enantiomeric macrocarpals C & G and A & B are non- or mono oxygenated phloroglucinoid sesquiterpene analogs

6.3.1) Methodology – HPLC analysis, K_d determination and CCC operation

The HPLC analysis of the crude extract, fractions and K_d determination was performed on a Waters Alliance separations module with Empower software connected to a Waters 2996 photodiode array (DAD) detector (210-800nm). Fractions were analysed on a reversed phase Waters X-Bridge C_{18} column (150 mm x 3.0 mm I.D., 3.5 µm) thermostatically controlled at 40 °C. Mobile phase was a mixture of A (Acetonitrile) and B (Water adjusted to pH 1.7 with TFA) in a gradient programme with a flow rate of 0.5 mL/min: 0–50 min ramp up from 55% to 95% A, 50-53 min hold 95% A, 53–54 min ramp down from 95% to 55% A and 54-60 min hold 55% A. Aliquots from the sample solutions (20 µL) were transferred to a HPLC vial and diluted 50 fold with methanol, while aliquots from the fractions of the CCC runs (50 µL) were diluted 20 fold with methanol.

As the application had potential for further scale-up, where classical phase system mixing becomes impractical, the upper and lower phases of the HEMWat phase systems used were made up separately using tables (Garrard 2005) with heptane, ethyl acetate, methanol and water. The static distribution ratios (K_d) of the target compounds and major impurities in the crude material were determined by dissolving 10 mg of crude material in 600 µL each of upper and lower phase in a HPLC vial and determining the concentration of the compounds in the upper and lower phases.

All studies used the semi-preparative DE-Spectrum instrument with the column rotating at 1600rpm (243*g*, volume of 143.5 mL, 1.6 mm bore PTFE tubing). For the isocratic and gradient runs, used for comparison, the column was initially filled with the stationary phase then the mobile phase was pumped to equilibrate the column. The sample solutions of the crude material were made up in lower phase of the chosen solvent system. The phase systems, flow rates, gradient details and sample loadings for the isocratic and linear gradient elution runs are given in Table 6.3.1. For the ICcE runs the methodology previously described in Section 6.2 was used. The sample as injected, for six ICcE cycles, with the aim of holding the target in the column. ICcE wash cycles were then used to remove the close running upper and lower phase impurities, before eluting the purified targets with a normal phase elution step. For both ICcE runs HEMWat 27 phase system was made up by tables, with the sample made up in a 50/50% mixture of upper and lower phase. The specific operating conditions are given in Table 6.3.2. The ICcE runs were done with the aim of

matching the crude throughput to that of the best conventional CCC runs and maintaining the target purity above 85%.

Table 6.3.1: Isocratic and linear gradient CCC operating parameters

Run Number	Mode	HEMWat Phase System	Eluant Flow	Sample load	Sample volume	Sample conc.
			mL/min	mg	mL	mg/mL
6.3.1	NP CCC	27	2.0	500	6.0	83
6.3.2	NP CCC	27	4.0	505	6.0	84
6.3.3	NP CCC	27	8.0	500	6.0	83
6.3.4	NP CCC	27	4.0	1000	6.0	167
6.3.5*	RP Linear Gradient	17-27	8.0	500	6.0	83

* Linear gradient: 0-6min HEMWat17; 7-46min linear gradient to HEMWat27; 47+ hold at HEMWat27

Table 6.3.2: ICcE operating parameters

Run number	6.3.6	6.3.7
F_{UP} (mL/min)	8.0	8.0
F_{LP} (mL/min)	9.0	8.0
T_{UP} (min)	4.0	4.0
T_{LP} (min)	4.0	4.0
Sample flow (mL/min)	0.66	0.66
Sample conc. (mg/mL)	125	125
Sample inj. vol. (mL)	32	32
Sample load time (min)	48	48
Sample load (mg)	4000	4000
Wash time (min)	56	136
Wash UP flow (mL/min)	8.0	8.0
Wash LP flow (mL/min)	9.0	9.0
UP elution time (min)	90	0
Elution flow (mL/min)	2.0	0

Table 6.3.3: K_d values of macrocarpals and major impurities in HEMWat 27

Target	$K_d=C_{UP}/C_{LP}$
Eucalyptine	0.08
Macrocarpal A	0.04
Macrocarpal E	0.07
Macrocarpal B	0.03
Macrocarpal L	0.05
U1	0.06
U2	0.14
U3	0.08
U4	0.29
Macrocarpal C	1.16
Macrocarpal G	1.08
U6	1.27
U7	1.19
U8	1.36
U9	1.52

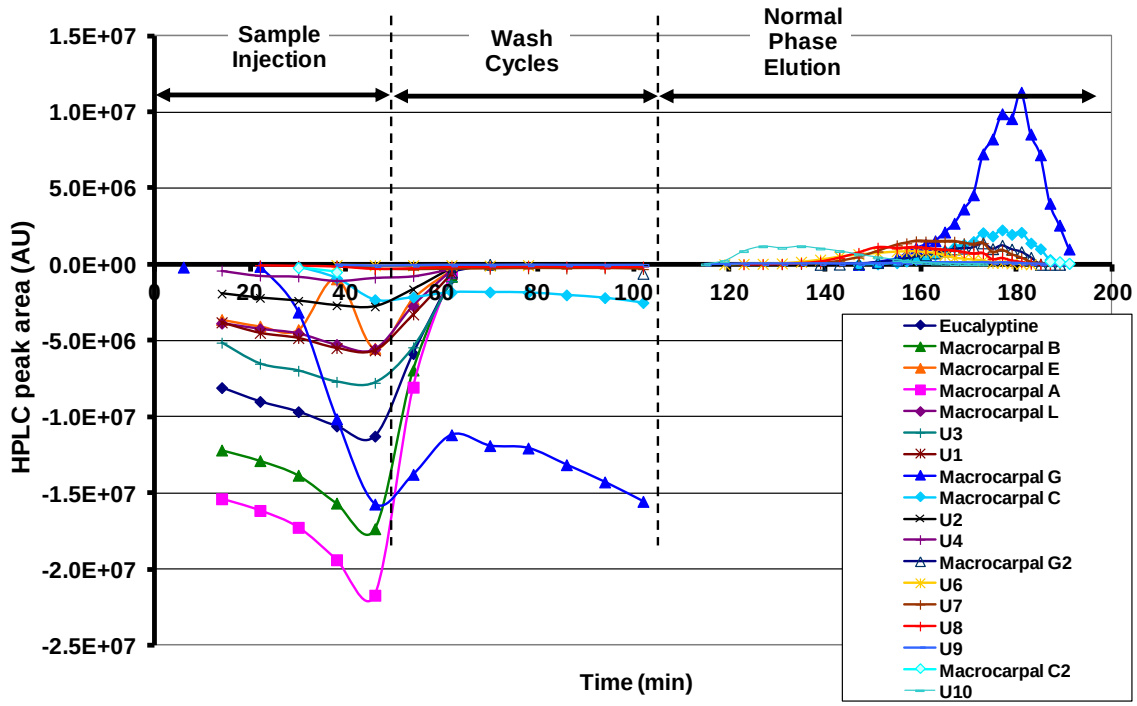


Figure 6.3.1: Fractogram constructed from HPLC peak areas of the solutes for the ICcE separation Run 6.3.6 with the macrocarpal crude; solvent system HEMWat 27, DE-Spectrum semi-prep columns, Vc = 143.5 mL, 30 °C, UP flow rate 8.0 mL/min, LP flow rate 9.0 mL/min flow switched every 4.0 minutes, sample flow rate 0.66 mL/min, sample volume 32 mL, UP elution flow rate 2.0 mL/min, rotational speed 1600 rpm (243g), upper phase elution upper domain, lower phase elution lower domain

6.3.2) Results and discussion

Based on the K_d values for the target group macrocarpals C and G (1.16 and 1.08 respectively, Table 6.3.3) initial conditions were set for a preliminary ICcE run (Run 6.3.6) with the aim of trapping the targets in the column before washing the impurities away and finally eluting the targets with the upper phase, as had been demonstrated previously with the GSK#14 waste stream application (Section 6.2.2.2).

As can be seen from the resultant fractogram, Figure 6.3.1, the targets were partially trapped in the column and eluted with the final upper phase elution step. However a proportion of the target eluted unexpectedly with the lower phase fractions.

The impurities eluted near where expected; eucalyptine, macrocarpals A, B, E and L, and unknown impurities U1-4 with K_d values significantly below one; all eluted with the lower phase elution and early wash stage fractions. The impurities U5-9, with K_d values slightly above the target (1.19-1.52) were trapped in the column and eluted on the front of the normal phase elution step before the major target started eluting.

Although the first ICcE separation was not ideal, comparison of the results for purity, yield, throughput and solvent consumption (Table 6.3.4) show a purity of 87.6% was achieved at a matched crude throughput (1237 mg/h compared with 1200 mg/h) for the best isocratic run (Run 6.3.3). For ICcE, solvent usage was only 0.32 L/g crude compared with 0.84 L/g crude for the isocratic run, though with a significantly reduced yield of 68.7% (Lower phase fractions 9-13 plus the normal phase eluted fractions containing C and G) compared with 88.6% yield for the isocratic run (Fractions 42-50).

It should be noted that the isocratic Run 6.3.4, which used a more concentrated sample, gave improved solvent usage of 0.43 L/g crude in comparison to Run 6.3.3, though with a reduced yield of 76.0%. The linear gradient method (Run 6.3.5) gave a very high yield of 97.4% though at a reduced throughput (500 mg/h) and greatly increased solvent usage (1.40 L/g crude). The linear gradient is of interest as it allows group macrocarpals A and B to be separated in addition to group macrocarpals C and G in single run. Macrocarpals A and B were eluted with a purity of 87.2% and yield of 97.4%. Therefore with the conventional CCC the run conditions can be adjusted to optimise for the most critical factor; be that yield, purity, solvent usage or the compounds required.

Due to the limited availability of crude material, all the fractions from the first ICcE run were combined and dried down under vacuum to reconstitute the crude material for a second ICcE run (Run 6.3.7). From the first run it was clear relatively pure macrocarpal C and G were eluting from the lower phase, therefore the aim was to enhance this in the second run. The lower phase elution flow was reduced to 8 mL/min to hold the targets in the column while the sample was loaded, then after 64 minutes once the lower phase impurities had eluted the lower phase flow was increased to 9 mL/min to speed up elution of the targets. The intermittent switching was continued to minimise the contamination of the targets by the remaining close running impurities, U5-9. These were extruded from the column at the end of the run at high flow.

In this run, as can be seen from the resultant fractogram and table (Figure 6.3.2, Table 6.3.4) the target concentration in the initial elution fractions was reduced compared to the previous run and the targets macrocarpals C and G eluted with the lower phase fractions as predicted. This successfully increased the yield to 87.6% from 68.7%, with purity and throughput at the same level as the previous run though the solvent usage increased to 0.50 L/g crude, due to the higher flow of the continued wash cycles relative to the normal phase elution. These results are comparable to the isocratic Run 6.3.3 and show a 1.7 fold reduction in solvent usage, similar to that seen with the GSK#14 waste stream application (Section 6.2.4).

Table 6.3.4: Conventional CCC purity, yield and solvent usage compared to ICcE

Run Number	Mode	elution time	cycle time	purity	yield	throughput	solvent usage
		min	min	%	%	mg/h	L/g crude
6.3.1	NP CCC	92	112	87.7	90.6	326	0.81
6.3.2	NP CCC	45	65	87.4	85.4	673	0.80
6.3.3	NP CCC	25	45	85.5	88.6	1200	0.84
6.3.4	NP CCC	51	71	87.2	76.0	1177	0.43
6.3.5	RP Linear Gradient	60	80	87.2	97.4	500	1.40
6.3.6	ICcE	194	214	87.6	68.7	1237	0.32
6.3.7	ICcE	208	228	88.0	87.6	1250	0.50

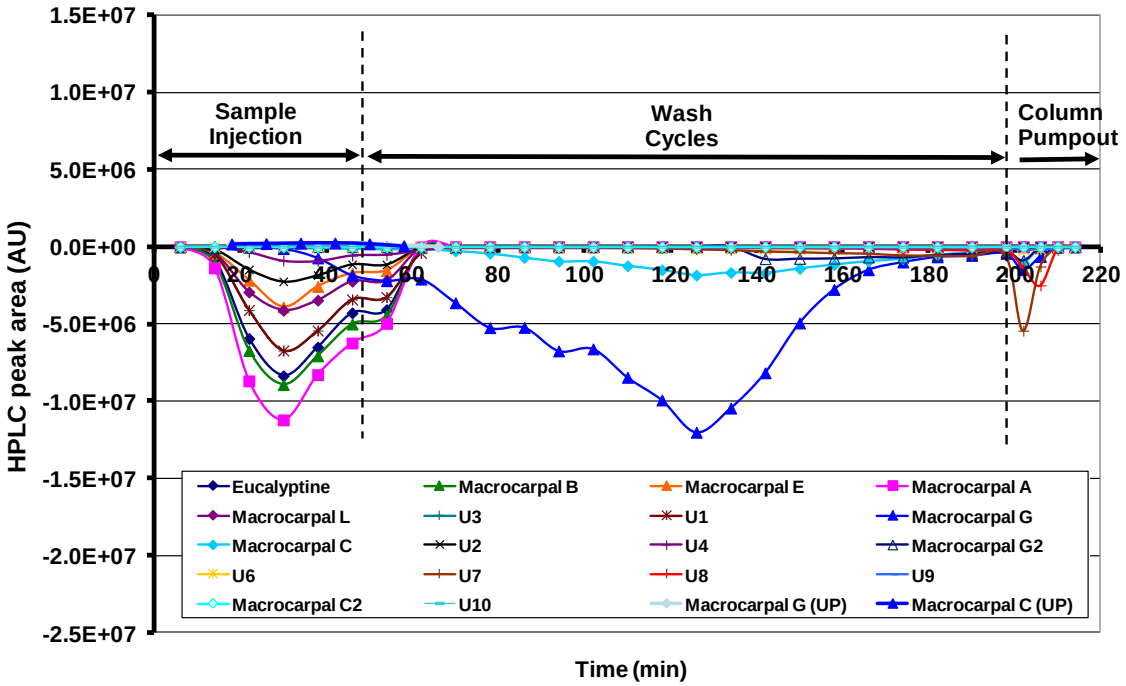


Figure 6.3.2: Fractogram constructed from HPLC peak areas of the solutes for the ICcE separation Run 6.3.7 with the macrocarpal crude; parameters as for Run 6.3.6 except the LP elution flow was reduced to 8.0 mL/min while the sample was loaded then the target compound was allowed to elute with the LP in the wash cycles

6.2.3) Conclusions on the purification of macrocarpals

Results for the ICcE run (Run 6.3.7) show a 1.7 times improvement in solvent usage over the best isocratic run (Run 6.3.3) with similar purity, yield and throughput profiles. However, it should be noted the conventional CCC methods can provide reduced solvent usage, though at a lower yield (Run 6.3.4). While the gradient elution method (Run 6.3.5) allows the purification of both the C & G and the A & B group macrocarpal enantiomers in a single run, though with increased run time and solvent usage. Further, it is difficult to recycle solvents from the gradient elution as composition of the mobile phase changes through the run. Both the gradient method and ICcE method elute the target in the lower phase which will increase the cost of recovery over the isocratic runs which elute the targets in the more volatile upper phase. This purification emphasises the flexibility of the different CCC approaches. In the end the approach chosen will depend on manufacturing priorities and a detailed cost benefit analysis, which is beyond the scope of this thesis.

Chapter 7: Conclusions and future work

ICcE has been demonstrated to be an effective method for the separation of a feed stream into two separate outlet streams around a given distribution ratio. Steady state operation was demonstrated on both model systems and a real world waste stream application, with operation reaching steady state equilibrium conditions and then being held in this condition. The ICcE method developed is competitive with the previously developed dual-flow CCC methods using bespoke DFCCC bobbins. The ICcE method can be using on standard commercially available CCC instruments which will allow for quicker adoption of the technology by industrial users.

Although initially developed as a steady-state equilibrium process, the ICcE method was found to be more effective as a non-equilibrium, trap and release method, with the available real world applications, which all contained target compounds in relatively low abundance. This flexibility of the process, to be used in either steady-state or non-steady state conditions, is an important advantage of the process.

The research questions asked in Chapter 1 have successfully been answered which have led to a number of key discoveries to ensure successful operation of both DFCCC and ICcE methods in steady-state equilibrium conditions. The specific progress of the field of liquid-liquid CCC instruments in an extraction mode is shown by looking at the original three research questions.

1) Can the liquid-liquid SMB type method be transferred from CPC to CCC instruments?

   In Chapter 5.4.3, for the first time, ICcE was demonstrated as a method for continuous processing and shown to operate at both the preparative and pilot scales. (Hewitson et al. 2009, Sutherland et al. 2009, Appendix 1.1.5, 6). The successful operation of ICcE opens up the possibility of a scalable continuous in-line all liquid flow down-stream processing unit, without the need for batch operation and the inherent delays and batch variability that would be seen.

2) Can ICcE be competitive with the DFCCC continuous process already developed on CCC instruments?

   ICcE was shown to be competitive with dual-flow CCC as a continuous processing method. For the ICcE method it has been discovered that to keep columns mechanically

balanced over time the volumes of the flying lead must be adjusted such that the displaced dead volumes are constant (Hewitson et al. 2011, Appendix 1.1.3). This is in comparison to DFCCC where to maintain a constant phase volume ratio in the column a specific back pressure must be applied to the centre outlet (Chapter 5.4.1). On balance the ICcE method was seen as a more robust and practical route to continuous processing as it can be operated using commercial instruments available at present with only minor changes to the ancillary valving of the system. Further the commercial instruments have significantly more robust flying lead lifetimes as only two flying leads are required for each bobbin with the ICcE system, whereas for the DFCCC system five flying leads are required on a single bobbin, reducing the lifetime of the flying leads due to the increased wear of the larger flying lead pack continuously twisting and untwisting around each other during operation. (Ignatova et al. 2011, Appendix 1.1.4).

3) If ICcE is competitive can it be scaled for preparative and pilot production on industrial applications?

   In Chapter 5.5, the ICcE method was successfully scaled using standard twin column CCC instruments from the semi-prep to pilot scale for the first time using a model sample mixture and using a range of polarities of phase system (Hewitson et al. 2011, Appendix 1.1.3). Further, the ICcE method has been successfully demonstrated on industrial applications for the first time for a natural product (Chapter 6.1) and a pharmaceutical waste stream (Chapter 6.2). In both cases the target was concentrated in the column and separated while the impurities were washed away, with at least a 1.6 times reduction in solvent usage in comparison to batch processing. Also, a final isocratic elution step has been demonstrated for extra purification from co-eluting impurities. The range of both equilibrium, continuous, and non-equilibrium, trap in column, operating modes for the use of ICcE gives greater freedom to achieve successful separation of a given target system, with improved solvent usage over conventional batch liquid-liquid chromatography.

4) How does the back pressure applied to the outlets of a DFCCC column affect the phase ratio within the column?

   In Chapter 5.4.1 for the first time the back pressure applied to the centre outlet of a DFCCC column to give constant phase volume ratio has been quantified. This allows for true control of the phase ratio within a DFCCC column when operated continuously. To

fully realise the back pressure control automated regulators and pressure measurement would be required on both the centre and periphery outlet of the DFCCC column.

5) How robust is the ICcE method to changes in phase system polarity?

In Chapter 5.5.1 the use of changes in phase system polarity to change the separation point in a range of different polarity compounds is demonstrated. Further, the robustness of the ICcE method to different polarity phase systems is seen in Chapter 5.3, although caution must be used in setting normal phase flow rates due to non linear Du plots observed at higher flow rates, especially with the more polar phase systems HEMWat 11 and 15.

In summary, the key novel contributions of the ICcE method as a new competitive method for continuous separation include:

- A model which gives a visual representation of the elution time of compounds from the ICcE method using static K_d values measured by HPLC (Chapter 4)
- For the DFCCC method, the back pressure applied to the centre outlet to give constant phase volume ratio has been quantified (Chapter 5.4.1)
- For the ICcE method, to keep columns mechanically balanced over time, the volumes of the flying lead must be adjusted such that the displaced dead volumes are constant (Chapter 5.4.2)
- The first use of the ICcE method for continuous processing and its scale-up using standard twin column CCC instruments (Chapter 5.5)
- A bobbin design which gave improved performance for ICcE was demonstrated (Chapter 5.6, Hewitson et al. 2013, Appendix 1.1.2)
- The ICcE method was demonstrated on industrial applications (Chapter 6)

The academic impact of the research is shown by the well cited papers published (Appendix 1.1), which have led to a number of further developments in the field, including the use of ICcE for the separation of the traditional Chinese medicine Honokiol (Peng et al. 2010) and improved modelling of the ICcE system (Kostanyan et al. 2013) and further development on CPC instruments (Hopmann et al. 2012).

While the industrial impact is demonstrated by the interest of the industrial partners in providing three applications (Chapter 6) which show reduced solvent usage over

conventional batch separation methods therefore opening up routes to reduced production costs, minimising waste streams which have high disposal costs, and improved overall product recovery.

The research developed in this thesis has opened a number of further avenues for study, which are now being progressed:

- The use of a set of dimensionless numbers to fully describe the inflection points seen in the Du-plots for normal phase operation (Chapter 5.3) will allow the prediction of the maximum flow in normal phase for both batch isocratic CCC and ICcE operation.
- The understanding of the pressure requirements to keep a stable phase ratio in the DFCCC column will allow the production of a fully automated DFCCC system with continuous pressure feedback control.
- Pressure fluctuations observed as part of the work to achieve stable phase volume ratio in the DFCCC instrument open up a new area of fundamental research to understand how these fluctuations effect the hydrodynamics of the system differently in normal and reversed phase operation.

The vision for future work related to the application of extraction methodology to both commercially available CCC instruments and DFCCC instruments is focused on the potential to produce instruments with higher throughput. Classical liquid-liquid extractors only have around 10 theoretical plates, whereas CCC instruments have between 200 and 500 theoretical plates, however liquid-liquid extractors have much higher throughputs. Future work needs to focus on optimisation of the CCC columns to balance the theoretical plates required for a given separation with the potential throughput that can be achieved. For example, shorter columns with wider bore will reduce theoretical plates and allow higher throughput.

For industry to take up the ICcE methodologies in this thesis a fully automated control system will be required, combining the model and automated valving with methods for solvent system determination for target molecules.

To summarise, the ICcE method has been developed as a continuous processing method for counter-current chromatography instruments. It can be used to split a feed stream into two component streams or for complex feed samples can be used as a trap method to enrich and purify a target compound with savings in solvent usage over traditional batch separations. ICcE is now available as another tool in the range of methods to operate CCC equipment.

Appendix1: Published outputs

Appendix 1.1) Published journal papers related to the subject of this thesis

1.1.1. Kostanyan, A. E., Ignatova, S., Sutherland, I. A., **Hewitson, P.,** Zakhodjaeva, Y. A., Erastov, A. A.; (2013) "Steady-state and non-steady state operation of counter-current chromatography devices"; Journal of Chromatography A, doi:10.1016/j.chroma.2013.08.100, **1314**, 94-105

1.1.2. **Hewitson, P**., Sutherland, I. A., Kostanyan, A. E., Voshkin A. A, Ignatova, S.; (2013) "Intermittent counter-current extraction - Equilibrium cell model, scaling and an improved bobbin design"; Journal of Chromatography A, doi:10.1016/j.chroma.2013.06.023, **1303**, 18-27

1.1.3. **Hewitson, P.,** Ignatova, S. and Sutherland, I.A.; (2011) "Intermittent counter-current extraction – Effect of the key operating parameters on selectivity and throughput"; Journal of Chromatography A, doi:10.1016/j.chroma. 2011.03.072, **1218**(36): 6072-6078

1.1.4. Ignatova, S., **Hewitson, P.** Mathews, B. and Sutherland, I.A., (2011) "Evaluation of dual flow counter-current chromatography and intermittent counter-current extraction", Journal of Chromatography A, doi:10.1016/j.chroma. 2011.02.032, **1218**(36): 6102-6106

1.1.5. Sutherland, I.A., **Hewitson, P.** and Ignatova, S. (2009). "Scale-up of counter-current chromatography: Demonstration of predictable isocratic and quasi-continuous operating modes from the test tube to pilot/process scale." Journal of Chromatography A, doi:10.1016/j.chroma.2009.03.040, **1216**(50): 8787-8792

1.1.6. **Hewitson, P.**, Ignatova, S., Ye, H., Chen, L. and Sutherland, I.A., (2009). "Intermittent counter-current extraction as an alternative approach to purification of Chinese herbal medicine." Journal of Chromatography A, doi:10.1016/j.chroma.2011.03.072, **1216**(19): 4187-4192

Appendix 1.2) Conference posters related to this thesis

1.2.1. Ignatova, S., **Hewitson, P**., Sutherland, I., Douillet, N., Thickitt, C., Johns, D., Wood, P., Freebairn, K.; "Pilot Scale Pharmaceutical Waste Stream Purification using High Performance Counter-current Chromatography as a Platform Technology"; SPICA2012 (14th International Symposium on Preparative and Industrial Chromatography and Allied Techniques, Brussels, Belgium); 30th Sep – 3rd Oct 2012 (2nd Prize (2/63))

1.2.2. **Hewitson, P.,** Sutherland, I., Ignatova, S., Douillet, N., Thickitt, C., Johns, D., Vilminot, E., Freebairn, K.; "Intermittent Counter-current Extraction for the Pharmaceutical Industry - Theory and Feasibility Study"; 1st RSC/ SCI Symposium on Continuous Processing and Flow Chemistry; GSK, Stevenage; 3rd – 4th Nov 2010

1.2.3. Ignatova, S., **Hewitson, P.** and Sutherland, I; "Evaluation of Dual Flow Counter-current Chromatography and Intermittent Counter-current Extraction"; CCC2010 (6th International Conference on Counter-current Chromatography, Lyon, France); 28th – 30th Jul 2010

1.2.4. **Hewitson, P.,** Ignatova, S., Ye, H., Chen, L., and Sutherland, I.A.; "Intermittent Counter-current Extraction as an Alternative Approach to Purification of Chinese Herbal Medicine" CCC2008 (5th International Conference on Counter-current Chromatography, Rio de Janeiro, Brazil); 26th – 29th Jul 2008

Appendix 1.3) Conference proceeding related to the subject of this thesis

1.3.1. **Hewitson, P.**, Sutherland, I. and Ignatova, S; "Continuous downstream processing using Intermittent Counter-current Extraction – Theory and feasibility studies"; AIChE2013 (2013 Annual Meeting – Global Challenges for Engineering a Sustainable Future, San Francisco, CA, USA); 3rd -8th Nov 2013

1.3.2. Ignatova, S., **Hewitson, P.**, and Sutherland, I.; "API Recovery From Pharmaceutical Waste Streams By High Performance Countercurrent Chromatography and Intermittent Countercurrent Extraction"; AIChE2013 (2013 Annual Meeting – Global Challenges for Engineering a Sustainable Future, San Francisco, CA, USA); 3rd -8th Nov 2013

1.3.3. Sutherland, I. A., **Hewitson, P.**, Thickitt, C., Douillet, N., Johns, D., Freebairn, K., Wood, P., Harris, G., Brown, R. and Ignatova, I; "Scalable Technology for the Extraction of Pharmaceuticals (STEP): progress on developing versatile small footprint technology for continuous processing at the lab scale up to 1 kg/day"; SPICA2012 (14th International Symposium on Preparative and Industrial Chromatography and Allied Techniques, Brussels, Belgium); 30th Sep – 3rd Oct 2012

1.3.4. **Hewitson, P.**, Sutherland, I., Wood, P. and Ignatova, S; "Intermittent counter-current extraction: Scale up and an improved bobbin design"; CCC2012 (7th International Conference on Counter-current Chromatography, Hangzhou, China); 6th-8th Aug 2012

1.3.5. Ignatova, S., **Hewitson, P.**, Wood, P., Douillet, N., Thickitt, C., Johns, D., Freebairn, K., Brown, R. and Sutherland, I; "API recovery from pharmaceutical waste streams by high performance counter-current chromatography and intermittent counter-current extraction"; CCC2012 (7th International Conference on Counter-current Chromatography, Hangzhou, China); 6th – 8th Aug 2012

1.3.6. Sutherland, I., **Hewitson, P.**, Janaway, L., Wood, P., Edwards, N., Rooke, D., Harris, G., Keay, D., Freebairn, K., Johns, D., Douillet, N., Thickitt, C., Mountain, M., Mathews, B., Brown, R. and Ignatova, S; "Scalable technology for the extraction of pharmaceuticals (STEP): Outcomes from a 3 year collaborative research programme"; CCC2012 (7th International Conference on Counter-current Chromatography, Hangzhou, China), 6th – 8th Aug 2012

1.3.7. Ignatova, S. Douillet, N., **Hewitson, P.,** Thickitt, C., Vilminot, E., Johns, D., Freebairn, K., Sutherland, I., Edwards, N., Harris, G., Janaway, L., Wood, P., Keay, D. and Mathews, B. (2011) "API Recovery from Pharmaceutical Waste Streams by High Performance Counter-current Chromatography (HPCCC) and Intermittent Counter-current Extraction (ICcE)"; PREP 2011 (International Symposium, Exhibit and Workshops on Preparative and Process Chromatography, Boston, USA); 10th – 13th Jul 2011

1.3.8. Ignatova, S., **Hewitson, P**., Sutherland, I.A., Douillet, N., Thickitt, C., Johns, D., Freebairn, K., Guzlek, H., Edwards, N.A., Wood, P.L., Janaway, L and Mathews, B.; "New ways to solve old problems: Purification of waste streams"; SPICA2010 (13th International Symposium on Preparative and Industrial Chromatography and Allied Techniques, Stockholm, Sweden); 12th – 15th Sep 2010

1.3.9. **Hewitson, P.,** Ignatova, S. and Sutherland, I.A.; "Intermittent counter-current extraction – Continuous processing for the pharmaceutical industry"; CCC2010 (6th International Conference on Counter-current Chromatography, Lyon, France); 28th – 30th Jul 2010

1.3.10. Ignatova, S. and **Hewitson, P.,** "Establishing the key operating parameters for continuous counter-current extraction", CCC2008 (5th International Conference on Counter-current Chromatography, Rio de Janeiro); 26th – 29th Jul 2008 – Presented by P. Hewitson

Appendix 1.4) External reports and articles related to this thesis

1.4.1. Sutherland, I. A., **Hewitson, P**., Thickitt, C., Douillet, N., Johns, D., Freebairn, K., Wood, P., Harris, G., Brown, R. and Ignatova, I; "Scalable Technology for the Extraction of Pharmaceuticals (STEP): Final Report"; Oct 2012 (CONFIDENTIAL – available on request)

1.4.2. Ignatova, S. (PI) and Sutherland, I. A. (Co-I); "Pierre Fabre Report Phases 3/4"; Sep 2011 (CONFIDENTIAL – available on request)

1.4.3. Beggin, A. and Garrard, I.; TSB High Value Chemicals Competition – Developing High Value Chemicals through Industrial Biotechnology; "The Isolation and Purification of a High Value Natural Product" Oct 2009 http://www.innovateuk.org

1.4.4. Keith Freebairn, K., Johns, D., Douillet, N., Thickitt, C., Vilminot, E., Mathews, B., Harris, G., Hewitson, P., Sutherland, I. and Ignatova, S.; "Chromatography…but not as we know it!" Chromatography Today and International Labmate; Feb 2011

1.4.5. Sutherland, I. A. (PI), Ignatova, S. (Co-I) and **Hewitson, P.** (Co-I); Pfizer Final Report; "Establishing the ground rules for continuous counter-current extraction and its scale up"; Jul 2009 (CONFIDENTIAL – available on request)

Appendix 1.5) Other journal papers related to CCC

1.5.1. Sutherland, I.A., Thickitt, C., Douillet, N., Freebairn, K., Johns, D., Mountain, C., Wood, P., Edwards, N., Rooke, D., Harris, G., Keay, D., Mathews, B., Brown, R., Garrard, I., **Hewitson, P.,** and Ignatova, S., (2013) "Scalable technology for the extraction of pharmaceutics: Outcomes from a 3 year collaborative industry/academia research programme", Journal of Chromatography A, doi:10.1016/j.chroma.2013.01.019, **1282**, 84-94

1.5.2. DeAmicis, C., Edwards, N. A., Giles, M, B., Harris, G., **Hewitson, P.,** Janaway, L., Ignatova, S.; (2011) "Comparison of preparative reversed phase liquid chromatography and counter-current chromatography for the kilogram scale purification of crude spinetoram insecticide", Journal of Chromatography A, doi:10.1016/j.chroma.2011.06.073, **1218**(36): 6122-6127

1.5.3. Sutherland, I., A., **Hewitson, P.,** Siebers, R., van den Heuvel, R., Arbenz, L., Kinkel, J. and Fisher, D.; (2011) "Scale-up of protein purifications using aqueous two-phase systems: comparing multilayer toroidal coil chromatography with centrifugal partition chromatography"; Journal of Chromatography A, doi:10.1016/j.chroma.2011.04.013, **1218**(32): 5527-5530

1.5.4. Sutherland, I.A., Ignatova, S., **Hewitson, P.,** Janaway, L., Wood, P., Edwards, N., Harris, G., Guzlek, H., Keay, D., Freebairn, K., Johns, D., Douillet, N., Thickitt, C., Vilminot, E. and Mathews, B., (2011) "Scalable Technology for the Extraction of Pharmaceutics (STEP): The transition from academic knowhow to industrial reality", Journal of Chromatography A, doi:10.1016/j.chroma.2011.01.016, **1218**(36): 6114-6121

1.5.5. Sutherland, I.A., **Hewitson, P**. and de Folter, J., (2011) "Toroidal coil chromatography: The effect of scale-up and "g" field on stage efficiency", Journal of Chromatography A, doi:10.1016/j.chroma.2010.12.090, **1218**(36): 6144-6147

1.5.6. Ignatova, S., Hawes, D., van den Heuvel, R., **Hewitson, P.** and Sutherland, I.A., (2010) "A new non-synchronous preparative counter-current centrifuge--the next generation of dynamic extraction/chromatography devices with independent mixing and settling control, which offer a step change in efficiency." Journal of Chromatography A, doi:10.1016/j.chroma.2009.10.055, **1217**(1): 34-39

1.5.7. Sutherland, I.A., **Hewitson, P.** and Ignatova, S. (2009). "New 18-l process-scale counter-current chromatography centrifuge." Journal of Chromatography A, doi:10.1016/j.chroma.2008.11.097, **1216**(19): 4201-4205

1.5.8. Guan, Y.H., Bourton, E.C., **Hewitson, P.,** Fisher, D. and Sutherland, I.A.; (2009) "Importance of Column Design for Readily Scaleable Separations of Protein using Aqueous Two Phase Systems in J-Type Centrifuges", Separation and Purification Technology, doi:10.1016/j.seppur.2008.07.016, **65**(1) 79–85

1.5.9. Sutherland, I.A., Audo, G., Bourton, E., Couillard, F., Fisher, D., Garrard, I., **Hewitson, P.** and Intes, O. (2008) "Rapid linear scale-up of a protein separation by centrifugal partition chromatography", Journal of Chromatography A, doi:10.1016/j.chroma.2008.02.092, **1190**(1-2), 57-62

Appendix 1.6) Other posters related to CCC

1.6.1. Murhandini, S., Garrard, I., Fisher, D., **Hewitson, P.** and Ignatova, S.; "Development of an Efficient Method for Production of α-Mangostin Reference Standard from Garcinia mangostana L. Rinds using Liquid Flow Processing"; SPICA2012 (14th International Symposium on Preparative and Industrial Chromatography and Allied Techniques, Brussels, Belgium); 30th Sep – 3rd Oct 2012

1.6.2. Deamicis, C. V., Edwards, N. A., Giles, M. B., Harris, G. H., **Hewitson, P.**, Ignatova, S., Janaway, L. and Wood, P. L.; "HPCCC offers significant advantages compared with RP-HPLC for a 1kg Spinetoram purification"; CCC2010 (6th International Conference on Counter-current Chromatography, Lyon, France); 28th – 30th Jul 2010

1.6.3. R. Siebers, Y.H. Guan, R. van den Heuvel, **P. Hewitson** and I.A. Sutherland; "Protein Separation using Aqueous Two-Phase Systems on Countercurrent Chromatography"; BPP2009 (15th International Conference on Biopartitioning and Purification, Uxbridge, UK); 15th – 18th Jun 2009

1.6.4. Guan, Y.H., Bourton, E.C., **Hewitson, P.,** Fisher, D. and Sutherland, I.A.; "Phase Separation and "Mixing" for Bioseparation using Aqueous Two-Phase Systems in Counter-Current Chromatography"; BPP2007 (14th International Conference on Biopartitioning and Purification, Lisbon, Portugal); 17th – 20th Jun 2007

Appendix 1.7) Other conference proceedings related to CCC

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1.7.2. Harris, G. H., De Amicis, C., Edwards, N.A., Giles, M.B., **Hewitson, P.,** Janaway, L. and Ignatova, S.; "Rapid scaleup of high performance countercurrent chromatography from bench to kilogram"; Planta Medica; **78**, 11, PJ104

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