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Evaluation of dual flow counter-current chromatography and intermittent counter-current extraction

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ABSTRACT

The aim of this research is to compare two continuous extraction technologies, intermittent counter-current extraction (ICcE) and dual flow counter-current chromatography (DFCCC), in terms of loading and throughput using the GUESSmix, and show the advantages and disadvantages of the two methods. A model sample containing caffeine, vanillin, naringenin and carvone, with a total load of 11.2 g, was employed with a hexane–ethyl acetate–methanol–water (2:3:2:3) phase system to evaluate an ICcE method on a preparative (912 ml coil volume) DE-Midi instrument. While DFCCC was carried out on a specially designed preparative (561 ml coil volume) bobbin installed in a similar Midi instrument case. While similar throughputs of 7.8 g/h and 6.9 g/h were achieved for the ICcE and DFCCC methods respectively, ICcE was demonstrated to have a number of advantages over DFCCC.

1. Introduction

Pharmaceutical industries require fast methods for extraction, separation and purification of drugs and intermediates to minimise drug development time. Continuous counter-current chromatography (CCC) processing is seen as one method for achieving this. Therefore, it becomes essential to exploit further the advantages of the liquid stationary phase in CCC. Intermittent counter-current extraction (ICcE) is a quasi-continuous method where instead of one phase being held stationary by the gravitational field as in isocratic mode, the flow of the phases is alternated "intermittently" between reversed and normal phase so that the stationary phase also alternates. Quasi-continuous extraction, target compound enrichment [1] and preparative to pilot scale-up [2] have all been demonstrated for this method, using standard twin bobbin CCC instruments. Dual flow CCC (DFCCC), also described in the literature as continuous counter-current extraction (CCCE), is a method where the phases flow truly counter-current to each other "simultaneously". The method has been applied to the splitting of pharmaceutical liquors into two streams [3] using a specially constructed bobbin with three inlets (for the upper, lower phases and sample solution) and two outlets.

The possibility of separating a mixture by the introduction of the sample at the centre of a continuous column was first described by Craig and Craig in 1956 [4] using a bank of test tubes. A quasi-continuous extraction system for centrifugal partition chromatography (CPC) with sample introduced between bobbins was patented by Couillard et al. [5] and later this was successfully demonstrated using standard twin bobbin CCC instruments at both preparative and pilot scales by Hewitson et al. [1] and Sutherland et al. [2]. DFCCC was first described by Ito in 1985 [6] and a number of analytical studies have been presented since [7–9]. More recently the scale-up of this technology was achieved as a joint research programme between Brunel University and Pfizer [3].

This present study attempts to compare the two methodologies evaluating their similarities and operating benefits and difficulties by using a model sample system containing a mixture of four compounds (caffeine, vanillin, naringenin and carvone) from the GUESSmix [10] with hexane–ethyl acetate–methanol–water systems in continuous processing and target compound enrichment modes.

2. Experimental

2.1. Reagents and materials

All solvents used for ICcE and DFCCC were of analytical grade and for HPLC analysis were HPLC grade purchased from Fisher Chemicals (Loughborough, UK). Deionised water and HPLC water was purified from a Purite Select Fusion pure water system (Thame, UK). Compounds caffeine and umbelliferone were supplied by

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Fisher Chemicals, while naringenin and vanillin were supplied by Sigma–Aldrich (Gillingham, UK).

2.2. Preparation of the two-phase solvent systems and sample solutions

For the retention tests five solvent systems were used consisting of n-hexane, ethyl acetate, methanol and water with volume ratios of 1:4:1:4, 2:3:2:3, 1:1:1:1, 3:2:3:2 and 4:1:4:1 (HEMWat Systems 11, 15, 17, 19 and 23 respectively). All phase systems were made up classically by vigorously shaking them in a separating funnel and allowing them to equilibrate. This was repeated a second time. For the chosen solvent systems for separation of model compounds, upper and lower phases were made up separately as described in [11]. The sample solutions were prepared by dissolving compounds either in a 50%/50% mix of upper and lower phases or just in one of the phases.

2.3. Apparatus

Two types of the instruments were used in this research. The first is a Midi-HPCCC instrument (Dynamic Extractions, Slough, UK) setup for ICcE (Fig. 1a). The Midi-HPCCC has a rotor radius of 110 mm, tubing bore of 4 mm and two bobbins (columns) with a total capacity of 912 ml. The Midi can rotate up to a speed of 1400 rpm (241 × g), has a typical flow range of 10–100 ml/min and a mean β value of 0.75 where β is the ratio of planet to rotor radius.

The second instrument is a similar Midi-CCC case equipped with a specially designed preparative bobbin (supplied by Dynamic Extractions, Slough, UK). The column has a 5 mm bore and a total volume of 561 ml. Through special end terminals on the column it is possible to have both an inlet and an outlet connections at each end of the column. At each end, the inlet tube is extended for 1000 mm into the column through the special end terminal to establish the flow and prevent the introduced phase from flowing out the adjacent outlet (backflow) [6]. At the midpoint of the column a sample inlet is connected through a "T-junction". The column has a β-value range 0.54–0.83. The DFCCC set up is shown schematically in Fig. 1b.

For both ICcE and DFCCC, the Midi instruments were connected to two preparative Knauer K-1800 HPLC pumps (Berlin, Germany) and two Knauer K-2501 spectrophotometers with preparative flow cells. An analytical Knauer K-501 HPLC pump was used to inject the sample. For ICcE, two Knauer K-6 valves where required to allow flow in either normal or reversed phase through the system while for DFCCC two adjustable 0–100 psi compact back pressure regulators were fitted (Swagelok, Kings Langley, UK).

HPLC analysis was performed on a Waters Alliance 2695 separations module (Empower software) connected to a Waters 2996 photodiode array (DAD) detector (210–800 nm).

2.4. Determination of distribution ratios or partition coefficients

Upper phase (0.6 ml) and lower phase (0.6 ml) of each solvent system was dispensed into a HPLC vial. Model compound (2 mg) was added to the phase system. The vial was shaken vigorously until equilibrium had been established in both 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 diluted with methanol (1 ml) and analysed by HPLC. The distribution ratio/partition coefficient (K_d) of a particular compound was calculated as the ratio of peak area in the upper phase to the peak area in the lower phase.

2.5. ICcE operation

A detailed description of ICcE and maintaining the columns in balance is described in [12]. A summary follows here for clarity. ICcE was performed as described previously [1]. The mobile phase was flowed alternately, first in normal phase (upper phase mobile, from tail-periphery to head-centre) and then in opposite reversed phase (lower phase mobile, from head-centre to tail-periphery) direction. Switching between normal and reversed phase was carried out at regular time intervals. All liquid phases and CCC columns were thermostated at 30 °C. The empty columns were initially filled with upper phase. Hydrodynamic equilibration was established first in reversed phase mode with the lower (aqueous) phase as the mobile phase and upper (organic) phase as the stationary phase, with the aim of achieving 50%/50% upper to lower phase ratio in the columns. For the various phase systems, the ICcE method was run initially in normal phase for 4 min, then in reversed phase for 4 min. The eluted phase was collected and volumes measured to check the volume of phase displaced with each switching cycle. The volume of each phase remaining in the coils was measured.

2.6. DFCCC operation

All liquid phases and the DFCCC column were thermostated at 30 °C. The empty column was initially filled with upper phase at 100 ml/min from tail-periphery to head-centre, the column was rotated and all outlets were opened in sequence to purge any air. The rotation was then stopped and a given volume of the upper phase was then displaced by pumping in lower phase and measuring the eluant in a cylinder. The instrument was rotated at 1000 rpm and the back pressure regulators were set on the periphery and centre outlets. The flow of both the upper and lower phases were started. The phases eluted from the centre and periphery outlets were collected and the retention in the column was calculated from the displaced volumes.

For the separation runs, sample was loaded through the midpoint inlet at 5 ml/min. 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.

2.7. HPLC analysis of fractions

All samples were analysed on a reversed-phase Symmetry C18 column (75 mm × 4.6 mm I.D., 3.5 μm) temperature controlled at 30 °C. The mobile phase was a mixture of A (0.1% aqueous formic acid) and B (methanol) used in a gradient program with a flow rate of 1 ml/min: 0–2 min ramp up from 50 to 90% B, 2–5 hold 90% B. Eluant was monitored using a DAD detector.

3. Results and discussion

3.1. Phase ratios in the column

In classical CCC the higher the stationary phase retention the better separation efficiency. However, in ICcE stationary phase alternates and replenishes every time cycle. Whereas in DFCCC there is no stationary phase as both phases continuously flow counter-currently to each other. Therefore, a parameter such as a phase ratio (volume of upper phase in column/volume of lower phase in column) replaces stationary phase retention. Since ICcE can be employed on any standard two bobbin centrifuge the first question is what phase ratio should be used as a starting point. Most of the modern CCC instruments can deliver reasonable stationary phase retention of about 70% and higher. However, in the case of ICcE the situation is completely different. As mobile phase alternates between upper (UP) and lower (LP) phases the original

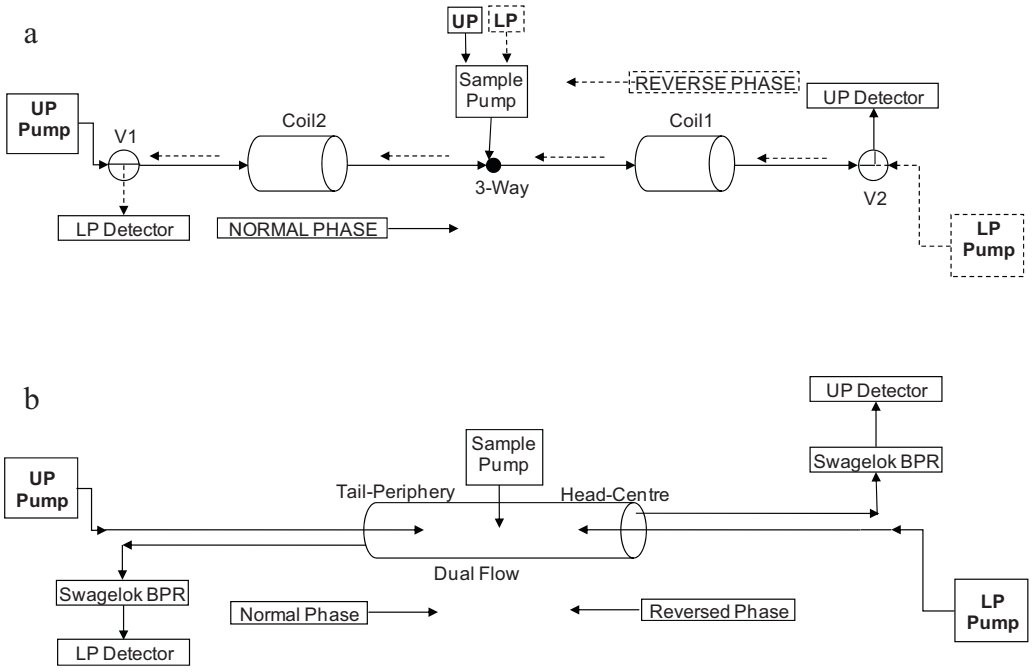

Fig. 1. Schemes of ICcE (a) and DFCCC (b) set up.

phase ratio will consequently change in time and might lead to bobbins become unbalanced [12]. Apart from this technical reason, any change in phase ratio will result in compounds eluting at unpredictable times. Therefore, it is logical to use 50%/50% phase ratio in the column as a starting point or at least keep it within the 40–60% range. It makes the process easier to model and control over time. Test results shown in Fig. 2 demonstrate the stable phase retention in the columns across a range of HEMWat phase system polarities. For HEMWat 11, 15, 17, 19 and 23 the upper and lower phase flow rates were equal to 40 ml/min with switching times of 4 min for both upper and lower phase time cycles. The lower phase retention was between 42% for HEMWat 15 and 55% for HEMWat 11. Increasing the flow of each phase from 40 ml/min to 80 ml/min for HEMWat 23 had practically no effect on the lower phase retention (from 48% to 45% respectively).

For DFCCC, both phases flow simultaneously and therefore, there is no issue with unbalanced bobbins. Even if the phase volume ratio varies between 10 and 90% a group separation is still possible because compounds will split between two phases according to their distribution ratios as has been demonstrated in [3]. Nevertheless, the phase ratio is important because it determines the column's loading capacity. Increasing sample flow rate or sample concentration may lead to solvent system overload. If the phase ratio is not optimal compound precipitation may occur inside the column if it has reached its solubility limit or compounds may elute from both ends of the column. Therefore, a 50%/50% or at least a 40–60% range of phase ratio will provide the best opportunity for high loading in the case of complex mixtures. However, achieving a 50%/50% phase ratio in DFCCC is not to be an easy task. Following the traditional approach, the column was first filled with the lower phase and then both phases were pumped counter-currently at equal flow rates of 35 ml/min. It took nearly 40 min to obtain a 40% UP/60% LP phase ratio (Fig. 3). In an attempt to shorten the equilibrium time the initial conditions were changed and the column was filled with UP and LP at different ratios. When the latter was in the 40–60% range, the system would displace about 5–10% of the lower phase within the first 5 min independently of the starting phase ratio. For example, in case of 50%/50% and 60%/40% as the initial ratio, the system first displaced 8% of the lower phase and then topped itself up to 48%. Moreover, the back pressure applied at the centre outlet (upper phase outlet) was adjusted manually to maintain the correct outlet flow rate. The best results were achieved with

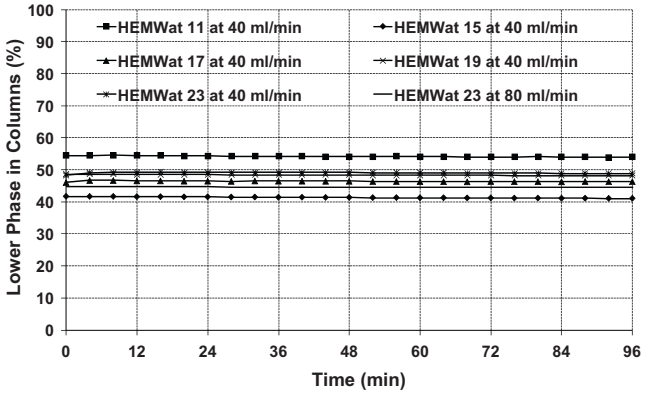

Fig. 2. ICcE phase retention in the columns from set up point for HEMWat 11, 15, 17, 19, 23 at 40 ml/min and 23 at 80 ml/min. Flow rates of both phases were equal.

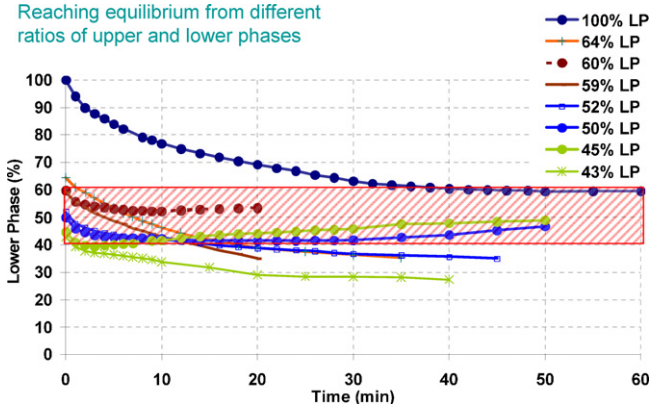

Fig. 3. DFCCC phase retention in the column from set up point for HEMWat 15 at 35 ml/min for each phase.

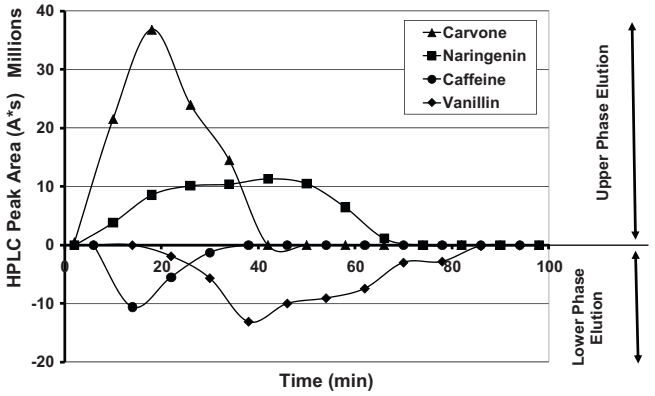

Fig. 4. Fractogram constructed from HPLC fraction analysis after each ICcE cycle for the separation of four compounds from the GUESSmix (caffeine (C), K_d = 0.09; vanillin (V), K_d = 0.55; naringenin (N), K_d = 1.25 and carvone (O), K_d = 7.4) [1]. Solvent system: HEMWat 16a; upper phase flow rate 50 ml/min; lower phase flow rate 60 ml/min; flow switched every 4 min; sample concentration: 50.0 g/l, sample volume: 224 ml; rotational speed: 1250 rpm; temperature: 30 °C.

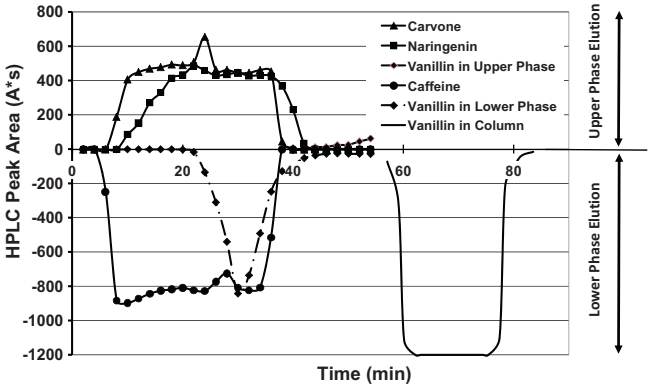

Fig. 5. Fractogram constructed from HPLC fraction analysis after DFCCC separation of four compounds from the GUESSmix (caffeine (C), K_d = 0.14; vanillin (V), K_d = 1.21; naringenin (N), K_d = 3.82 and carvone (O), K_d = 14.8). Solvent system: HEMWat 15; upper phase and lower phase flow rate 35 ml/min; sample concentration: 50.0 g/l, sample volume: 150 ml; rotational speed: 1000 rpm; temperature: 30 °C.

40% UP/60% LP as the initial ratio, which gave at the end 45%/55% phase ratio, with a back pressure of 72 psi set on the centre outlet and 54 psi set on the periphery outlet. However, even with manual pressure control the phase retention was seen to drift over the time. Continuous real-time flow measurement with automated feedback to the back pressure regulators would be required to maintain very stable phase retention.

3.2. GUESSmix sample separation

The feasibility of using ICcE for quasi-continuous extraction and target enrichment has been already demonstrated by the authors of the current paper [1]. The work was performed on a Midi-HPCCC instrument with the four compounds from the GUESSmix (caffeine, vanillin, naringenin and carvone) using the HEMWat 16a (4:5:4:5) phase system. The Midi ran at optimised conditions for splitting the sample mix into two streams gave 7.9 g/h throughput with the sample loaded alternatively in upper and lower phase over four cycles for a period of 16 min (Fig. 4). The target compound enrichment was demonstrated by retaining one of the target compounds with throughput of 3 g/h from herbal extract [1] and vanillin [2] with throughput of 6 g/h from model sample inside the column while the rest of compounds were washed away.

For comparison purposes, the same approach was applied to DFCCC using the same four compounds from the GUESSmix with HEMWat 15 (4:6:4:6). The latter was chosen as the original retention tests on DFCCC were carried out with this HEMWat system. After establishing equilibrium in the column at upper and lower phase flow rates equal to 35 ml/min, 7.4 g of sample solution in 150 ml of upper phase was injected in the middle of the DFCCC column within 30 min at 5 ml/min (Fig. 5). 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. Whereas vanillin (K_d = 1.21) partly eluted with lower phase and just started appearing in the upper phase when separation run was stopped. Yet the main part of vanillin fractions was retained in the column. The whole separation process was finished in just under 1 h, 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.

To split this four component sample into two streams using DFCCC, the flow rates of both phases were adjusted such that the upper phase flow was reduced to 20 ml/min and the lower phase flow was increased up to 50 ml/min. In this case the faster lower phase will push vanillin to elute earlier. Using the same procedure as described above, carvone and naringenin eluted with the upper phase while caffeine and vanillin eluted with the lower phase as expected (Fig. 6). Again the separation cycle time was 1 h, which gives 7.4 g/h throughput for sample processed. To maintain accurate flow, back pressure on centre outlet was manually adjusted throughout the run to achieve constant flow of 20 ml/min.

Based on the results presented and an analysis of the recent publications [1–3,12] a comparison of ICcE and DFCCC at preparative scale is summarised in Table 1. While the throughputs are similar, it can be seen that the set up for ICcE has a number of advantages currently over DFCCC: (1) it can be set up using commercially available instrumentation with the simple addition of some valves; (2) phase volume ratio is easy to set up and maintain; (3) once set up,

Table 1
Summary of comparison of ICcE and DFCCC.

Criteria	ICcE	DFCCC
Instrument	Any standard twin bobbin instrument	Requires a specialised column, the design of which at present is limited to 1000 rpm
Required phase ratio	Required running conditions can be setup within minutes	Column equilibration can take up to 1 h
Stability of phase retention	Once set up the 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 composition can be used to alter the split point

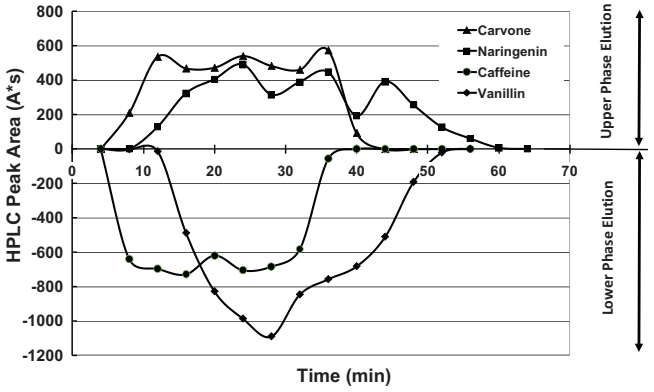

Fig. 6. Fractogram constructed from HPLC fraction analysis after DFCCC separation of four compounds from the GUESSmix (caffeine (C), K_d = 0.14; vanillin (V), K_d = 1.21; naringenin (N), K_d = 3.82 and carvone (O), K_d = 14.8). Solvent system: HEMWat 15; upper phase flow rate 20 ml/min; lower phase flow rate 50 ml/min; sample concentration: 50.0 g/l, sample volume: 150 ml; rotational speed: 1000 rpm; temperature: 30 °C.

the process is very stable and (4) the split point can be controlled in a number of different ways.

4. Conclusions

Both ICcE and DFCCC have been shown to be effective methods for continuous operation, giving very similar throughputs at the preparative scale. At the present moment ICcE has advantages over DFCCC as it is based on well established two-bobbin HPCCC instruments and shows more stable phase retention both during setup and once continuous sample injection is started. Therefore, ICcE can be easily automated and included as an additional running mode for a standard CCC set up which is currently available. However, DFCCC potentially has a great future for continuous processing, including flow chemistry. Further technical development of specialised bobbins and carefully arranged pressure control will help DFCCC development in the future.

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