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Intermittent counter-current extraction—Effect of the key operating parameters on selectivity and throughput

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ABSTRACT

Intermittent counter-current extraction (ICcE) has proved itself as a method for splitting compounds into streams and/or concentrating compounds in the column. In this paper a model mixture sample based on a modified GUESSmix (containing salicin, caffeine, aspirin, coumarin, salicylic acid, carvone, ionone and biphenyl) was separated into two eluant streams across a range of HEMWat phase system polarities from the polar system 11 through to non-polar system 23. ICcE could provide throughput of over 1 kg/day with this model sample, at the preparative scale, Changing the time cycle to adjust where the sample mixture is split into two streams was demonstrated. It is established that for the continuous running of ICcE, on a conventional twin bobbin counter-current chromatograph instrument, it is necessary to adjust the dead volumes of the flying leads to maintain similar phase retention in each column so the instrument does not become hydrodynamically and mechanically unbalanced due to the difference in densities between the upper and lower phases.

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1. Introduction

The unique nature if the liquid stationary phase in counter-current chromatography (CCC) allows great flexibility in operating methods [1–3]. The pharmaceutical industry requires high throughput continuous processes to reduce solvent usage and minimise separation times, but these processes need to be robust. Continuous counter-current extraction (CCCE) where phases are flowed truly counter-current to each other and the sample is loaded continuously to the centre of the column has been explored [4,5] but requires a bespoke column. Intermittent counter-current extraction (ICcE) is a relatively new method for hydrodynamic CCC [6]. The importance of this operational method is that a conventional twin-bobbin CCC instrument may be used by operating the columns in series alternating between normal phase and reversed phase operation with the sample injected continuously between the columns.

Historically, 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 was first introduced by Craig and Craig [7]. The concept of flowing initially in normal phase mode, then switching to reversed phase mode, with the sample injected at the beginning of the column, to improve the efficiency of a separation was first described by Zhang et al. [8]. This is described as dual-mode [1] and in a further development by Delannay et al. [9] a semi-continuous method for separating two components using a hydrostatic centrifugal partition chromatography (CPC), is termed multiple dual-mode (MDM). By loading a bolus of sample at the beginning of the column then running the system through an ascending and descending cycle before loading another bolus and repeating the cycle, the column length is effectively increased to give a better resolution, using only a single column. A similar principle but using a pair of CCC columns with injection between the columns and elution from one end was described as a "simulated dual-flow approach" by Dubant et al. [10]. A patent by Couillard et al. [11] describes a novel practical method for separating a crude sample into two component groups using a CPC device. The sample was loaded continuously between two columns consisting of serially connected chambers and the centrifuge was run alternately in ascending (less dense phase mobile) and then descending (more dense phase mobile) modes so splitting the sample into two groups which eluted from opposite ends of the system. An extension of dual-mode, as described above, is to repeatedly flow in normal and reversed phase. This is theoretically modelled by Kostanyan and Voshkin [12], as is controlled-cycle counter-current chromatography [13] where the flow of mobile phase is switched between the heavy and lighter phases, with a delay between the switching periods to allow for full equilibration. The current authors with Ye published the first results for separations with ICcE [6], where a sample was injected between a pair of CCC columns and the phases were alternately switched between normal and reversed phase on both a model mixture sample based on the GUESSmix proposed by Friesen and Pauli [14] and a representative Chinese

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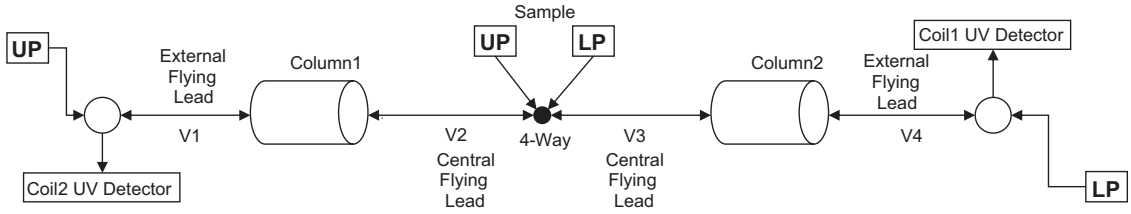

Fig. 1. ICcE method setup for a standard Midi instrument, V1 to V4 are the dead volumes of the four flying leads connecting the columns and the ancillary equipment.

herbal medicine, the herb *Tripterygium wilfordii* Hook. f. The authors [15] then demonstrated the scale up of the ICcE method from prep to pilot scale. Yang et al. [16] has proposed a theoretical model for the prediction of peak elution with ICcE for both hydrostatic and hydrodynamic systems, with separation of DNP-amino acids to confirm the predictions. Peng and Ye [17] recently compared ICcE and batch CCC for the separation of honokiol and magnolol, the main bioactive isomers in the traditional Chinese medicine "Houpu", showing approximately a 4× improvement in throughput.

To allow continuous running of the ICcE method it is important that the phase retention within the column is maintained over a reasonable continuous running period and that within the two columns the phase retention is similar so that the instrument does not become hydrodynamically and mechanically unbalanced.

This paper assesses the robustness of the phase retention within the CCC columns when using the ICcE method across a range of HEMWat phase systems polarities from the polar system 11 through to non-polar system 23 demonstrating the need for the correct flying lead dead volumes to maintain similar phase retention between the columns with extended operation.

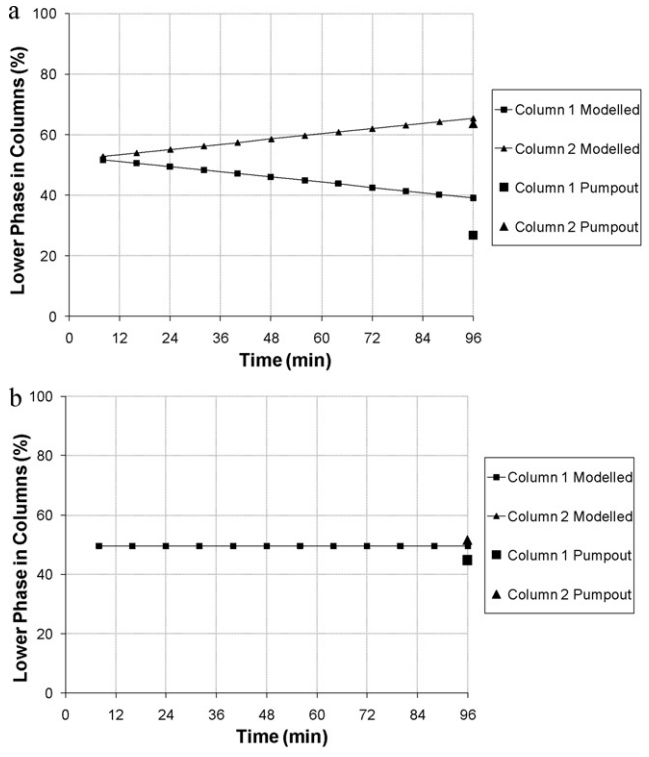

Fig. 2. (a) Un-balanced columns with unmatched volume flying leads, V1 = V4 ≠ (V2 + V3) and (b) balanced columns with matched volume flying leads, V1 = (V2 + V3) = V4, predicted and actual lower phase in columns after 96 min ICcE run; HEMWat system 23; upper phase and lower phase flow rate 40 ml/min; flow switched every 4 min; rotational speed: 1400 rpm; temperature: 30 °C.

Further, this study establishes the effectiveness of ICcE to successfully separate compounds of a sample into two eluant streams across a range of HEMWat phase system polarities. The specific compounds used for this study were a model sample mixture based on a modified GUESSmix proposed by Friesen and Pauli [14], containing salicin, caffeine, aspirin, coumarin, salicylic acid, carvone, ionone and biphenyl. Over the course of the study the sample mixture was successfully split by polar (HEMWat 11), intermediate (HEMWat 17) and nonpolar (HEMWat 23) phase systems at different points dependant on the polarity of the compounds. 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 which gave a throughput equivalent to 1.2 kg/day. Finally, the change of the splitting point by adjusting the flow switching times of the phases was experimentally demonstrated with ICcE for the first time.

2. Experimental

2.1. Reagents

Solvents used for ICcE were of analytical grade and for HPLC detection were HPLC grade from Fisher Chemicals (Loughborough, UK). Carvone, salicylic acid, coumarin and caffeine were also purchased from Fisher Chemicals. Biphenyl, ionone, aspirin and salicin were purchased from Sigma–Aldrich (Gillingham, UK). HPLC water was purified from a Purite Select Fusion pure water system (Thame, UK).

2.2. Apparatus

A high performance 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 was used to perform the intermittent counter-current extraction. The ICcE set up consisted of two preparative Knauer K-1800 HPLC pumps (Berlin, Germany) and two Knauer K-6 valves to

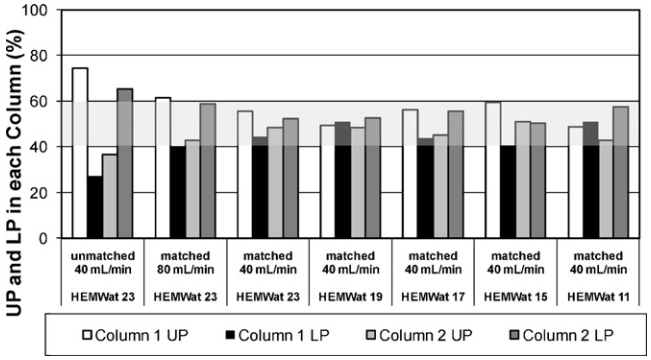

Fig. 3. Percentage phase in columns after 96 min ICcE run across a range of HEMWat systems; unmatched volume flying leads, V1 = V4 ≠ (V2 + V3); matched volume flying leads, V1 = (V2 + V3) = V4; upper phase and lower phase flow rates as indicated; flow switched every 4 min; rotational speed: 1400 rpm; temperature: 30 °C.

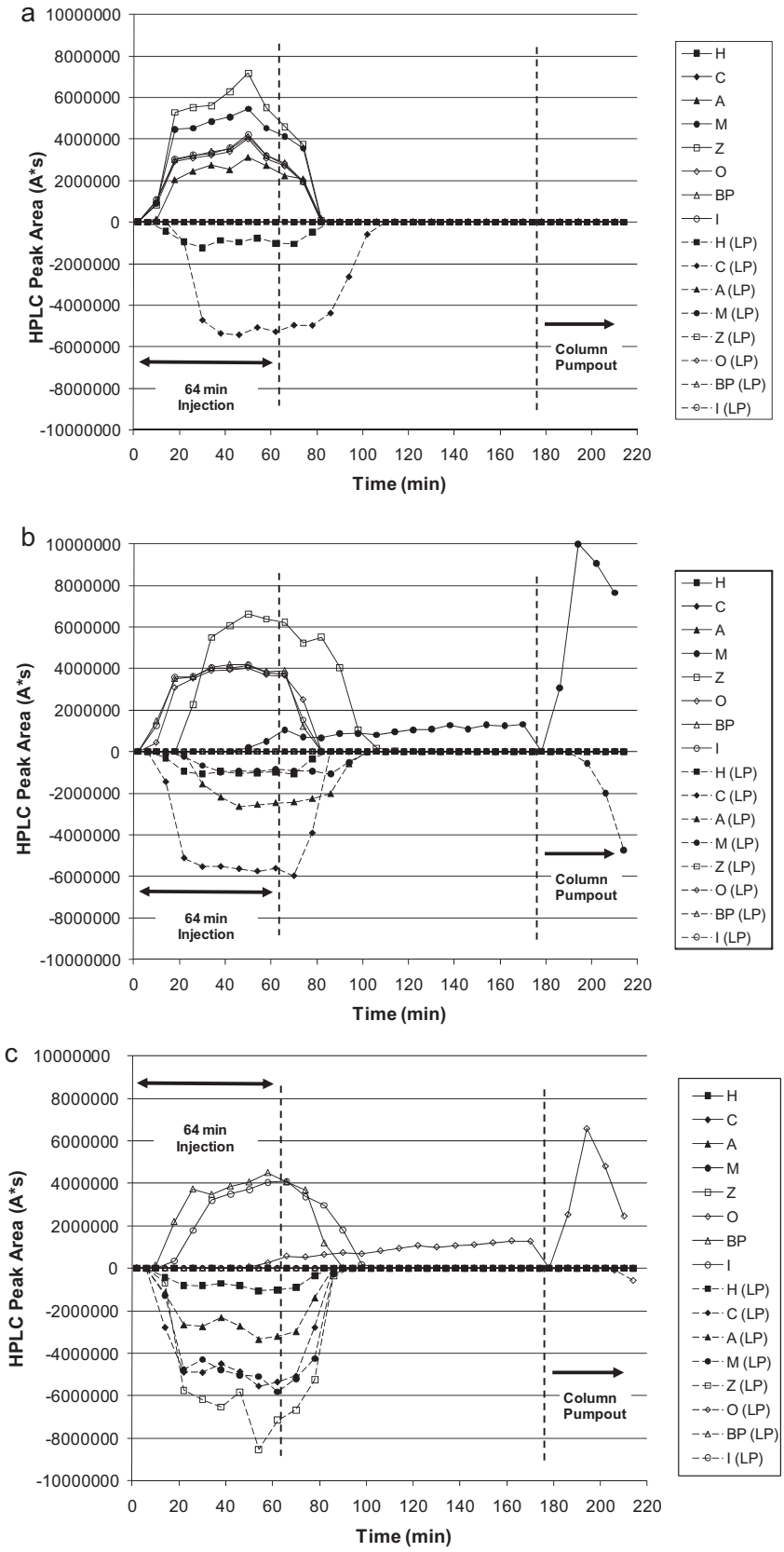

Fig. 4. Fractogram constructed from HPLC peak areas for the ICcE separation on a Midi preparative column for the separation of eight compounds from a modified GUESSmix. Solvent system: (a) HEMWat 11, (b) HEMWat 17, (c) HEMWat 23; upper phase flow rate 40 ml/min; lower phase flow rate 40 ml/min; flow switched every 4 min; sample concentration: 70.0 g/l, sample volume: 352 ml; rotational speed: 1400 rpm; temperature: 30 °C. Upper phase elution (solid line) upper domain; lower phase elution (dotted lines) lower domain.

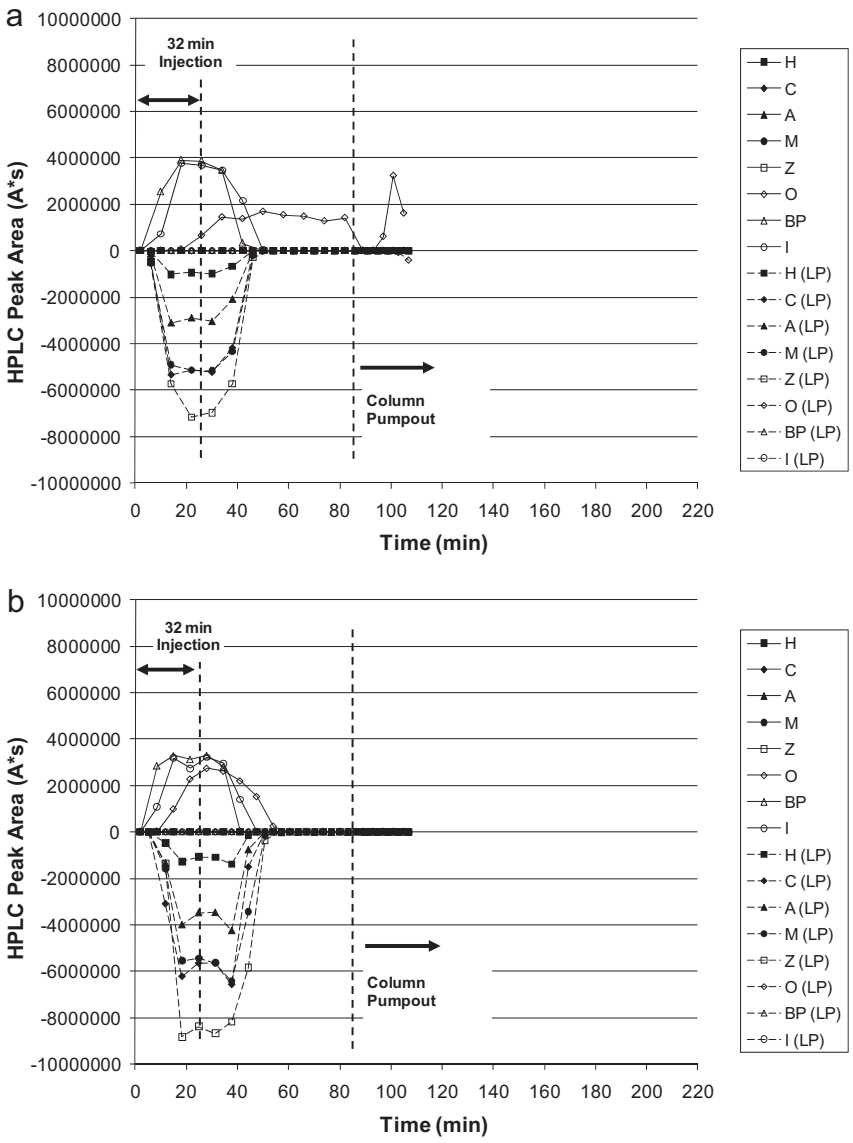

Fig. 5. Fractogram constructed from HPLC peak areas for the ICcE separation on a Midi preparative column for the separation of eight compounds from a modified GUESSmix. Solvent system: HEMWat 23; upper phase flow rate 80 ml/min; lower phase flow rate 80 ml/min; sample concentration: 70.0 g/l, sample volume: 383 ml; rotational speed: 1400 rpm; temperature: 30 °C. Upper phase elution (solid line) upper domain; lower phase elution (dotted lines) lower domain: (a) upper phase and lower phase flow switched every 4 min and (b) upper phase flow switched every 4 min and lower phase flow switched every 2.5 min.

allow flow in either normal or reversed phase through the system; an analytical Knauer K-501 HPLC pump to inject the sample; two Knauer K-2501 spectrophotometers with preparative flow cells.

HPLC was performed on a Waters Alliance 2695 separations module (Empower software) connected to a Waters 2996 photodiode array (DAD) detector (210–800 nm) using a Sunfire C_{18} column (150 mm × 4.6 mm I.D., 5 μm) (Waters, Milford, MA, USA).

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

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. The sample solutions were prepared by dissolving 2.5 g each of salicin and biphenyl with 5 g each of caffeine, aspirin, coumarin, salicylic acid, carvone and ionone in a 50%/50% mix of the upper and lower phase and then made up to a total volume of 500 ml.

2.4. Determination of distribution ratios or partition coefficients

Upper phase (0.5 ml) and lower phase (0.5 ml) was dispensed into a HPLC vial. Model compound (0.5 mg) was added to the phase system. 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.

2.5. ICcE and maintaining columns in balance

Intermittent counter-current extraction was performed as described previously [6]. The mobile phase was pumped alternately, first in normal phase (upper phase mobile, from tail-periphery to head-centre) and then in opposite reversed phase direction (lower phase mobile, from head-centre to tail-periphery). Switching between normal and reversed phase was carried out at regular time intervals.

For a Midi centrifuge the flying leads (V1 to V4 in Fig. 1) are identical lengths of polytetrafluoroethylene (PTFE) tubing with 3.2 mm O.D., 1.6 mm I.D., each being 2.5 m long with a volume of 5 ml. Therefore, at each switching interval 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 revered 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 the 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. The unbalanced columns will put excessive mechanical load on the instrument making long term continuous running impractical.

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 the bobbins should maintain their initial phase ratio. To achieve this scenario in practice the flying leads, 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 V2 + V3.

2.5.1. Filling the coils

All liquid phases and CCC columns were thermostatically controlled at 30 °C. The empty columns were initially filled with upper phase at a flow rate of 200 ml/min from tail-periphery to head-centre to remove the air in the column. Once filled, the columns were rotating at 1400 rpm to displace any remaining air inside the columns.

2.5.2. Establishing hydrodynamic equilibrium

Hydrodynamic equilibration was performed 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 by adjusting the rotational speed at a constant mobile phase flow rate of 80 ml/min with knowledge gained from a stationary phase retention versus rotational speed plot run at this flow (not shown). The rotational speed was set according to the phase system being used (HEMWat 11, 15, 17, 19 and 23; rotational speed 1400, 1400, 650, 575 and 550 rpm respectively) and the lower phase was flowed from head-centre to tail-periphery to equilibrate the system. When breakthrough occurred the rotational speed was increased to 1400 rpm (240 × *g*).

2.5.3. Operating conditions for phase retention stability within columns

For the various phase systems, the ICcE method was run initially in normal phase for 4 min, then in reverse phase for 4 min. The eluted phase was collected and volumes measured to check the volume of phase displaced with each switching cycle. This was repeated for 12 cycles at which point the flow and rotation were stopped. The columns were emptied separately by flowing nitrogen (4 bar) into the centre of the column while rotating at 200 rpm in reverse, placing the head of the column at the periphery. The volume of each phase remaining in the coils was measured.

2.5.4. Separation of GUESSmix compounds in different polarity phase systems

Using the ICcE method it should be possible to select a point on the polarity scale whereby a group of compounds is separated into two streams by changing the polarity of the phase system used for the separation. To confirm this, eight compounds from a modified GUESSmix were selected covering a wide range of polarities, from the highly polar salicin to the nonpolar biphenyl. The distribution ratios for these compounds are given in Table 1.

The system was initially run in normal phase with the upper phase mobile at 35 ml/min. After 4 min the flow was switched to reversed phase mode with the lower phase mobile at 35 ml/min. This cycle was then 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 min in total. At the end of the sample loading stage the eluant flows were increased to 40 ml/min to approximately maintain the same mean flow rate (i.e. previously it had been 35 + 5.5 ml/min) and continued for a further 14 cycles. For all runs the upper phase eluant was monitored with a UV detector set at 230 nm and the lower phase eluant was monitored at 270 nm. Fractions were collected every 4 min for analysis by HPLC. At the end of each run the columns were each emptied with nitrogen (4 bar), while rotating the coil in reverse at 200 rpm (placing the head at the periphery) and fractionated (100 ml fractions) for analysis by HPLC.

2.5.5. HPLC analysis of fractions

Model sample and ICcE fractions 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. Results and discussion

3.1. Modifications of flying lead length to maintain the columns in balance

It was observed that the length of the flying leads is an important factor to maintain the same phase ratio in the columns and therefore, keep them mechanically balanced. The latter is particularly relevant for pilot scale separations. For Midi columns with standard flying leads it was noted that there was a transfer of phases between the columns during separation due to the difference in dead volumes between the external flying leads connecting the columns to the ancillary equipment (V1 and V4) and the central flying lead section, connecting the two columns in series, V2 + V3. The predicted un-balance caused by the phase ratio mis-match between the columns and the actual ratio of phases observed after column pumpout are given in Fig. 2a.

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 35/64% in column 2 after 96 min.

Table 1
K_d values for HEMWat phase systems, 11, 17 and 23 in normal and reversed phase for the eight compounds from the modified GUESSmix. Compounds above dotted line elute with the lower phase, while those below elute with the upper phase. Compounds within the dotted boxes are predominantly retained in the columns.

		HEMWat 11		HEMWat 17		HEMWat 23	
		K_d NP	K_d RP	K_d NP	K_d RP	K_d NP	K_d RP
Salicin	H	100.0	0.0	100.0	0.0	100.0	0.0
Caffine	C	1.7	0.6	100.0	0.1	100.0	0.0
Aspirin	A	0.1	9.0	3.6	0.3	100.0	0.0
Coumarin	M	0.0	38.6	1.2	0.8	17.6	0.1
Salicylic Acid	Z	0.0	39.7	0.6	1.6	15.9	0.1
Carvone	O	0.0	100.0	0.1	7.7	1.1	1.0
Ionone	I	0.0	100.0	0.1	12.7	0.5	2.1
Biphenyl	BP	0.0	100.0	0.0	46.5	0.2	5.3

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 (Fig. 2b). After 96 min, 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.

These results were confirmed for different phase systems across the polarity range and the columns maintained their phase ratio in the range 40–60%, which would not cause un-balance between the columns (Fig. 3). It also confirmed that the protocol in Section 2.5.2 for setting up of the initial stationary phase retention to approximately 50% had been successful.

3.2. Separation of GUESSmix compounds with different phase systems across the polarity range

Fig. 4 shows reconstructed HPLC fractograms for three ICcE runs with the eight compounds from the modified GUESSmix. 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. With the polar HEMWat system 11 (Fig. 4a) the model mix is separated into two streams between the relatively polar caffeine and aspirin. Only salicin and caffeine eluted with the lower phase (the two compounds above the dotted line in Table 1) while the rest of the mixture eluted with the upper phase. With the intermediate polarity HEMWat system 17 (Fig. 4b) 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 elute with the upper phase. Whereas, with the non-polar HEMWat system 23 (Fig. 4c) 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. Note also that it is possible to concentrate up a target compound in the column as demonstrated for Coumarin with HEMWat 17 and Carvone for HEMWat 23 (see the dotted boxes in Table 1).

Fig. 5 shows the effect of doubling upper and lower phase flow rates (Fig. 5a) and changing switching times (Fig. 5b) while the sample loading remains approximately the same. The sum of eluant and sample flow was doubled from 40 ml/min (Fig. 4c) to 80 ml/min (Fig. 5a), though the loading time was halved to keep loading mass the same. This result corresponds to a potential throughput of over 1 kg/day on a preparative CCC instrument.

Recent ICcE modelling by Yang et al. [16] predicted 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 to 2.5 min, 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 (Fig. 5b).

4. Conclusions

It has been shown for ICcE on a conventional twin bobbin counter-current chromatograph instrument that it is necessary to adjust the dead volumes of the flying leads. This will maintain similar phase retention in each column so the instrument does not become mechanically unbalanced due to the difference in densities between the upper and lower phases. As a result, separation can be run continuously.

ICcE has proved itself as an effective method for splitting compounds into streams at a chosen point and/or concentrating compounds in the column. In this study a model mixture sample based on a modified GUESSmix (containing salicin, caffeine, aspirin, coumarin, salicylic acid, carvone, ionone and biphenyl) was separated into two eluant streams across a range of HEMWat phase system polarities. The potential for ICcE is to provide throughput, of over 1 kg/day, at a preparative scale and the ability to change the flow time cycle to control the splitting of a sample mixture was experimentally demonstrated for the first time.

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