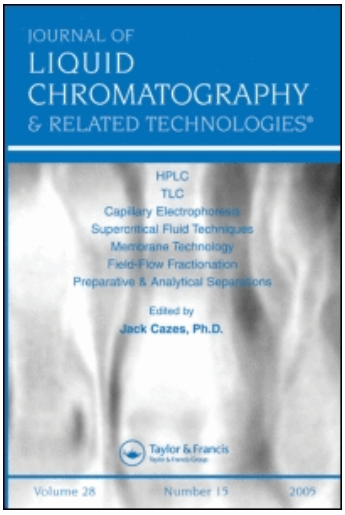

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OPTIMIZATION OF THE FRACTIONATION AND RECOVERY OF POLYKETIDE ANTIBIOTICS BY COUNTERCURRENT CHROMATOGRAPHY

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EXTRACTIONS AND PURIFICATIONS

OPTIMIZATION OF THE FRACTIONATION AND RECOVERY OF POLYKETIDE ANTIBIOTICS BY COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

The polyketide antibiotics are a large and diverse range of natural products exhibiting a wide range of antimicrobial activities. The biosynthesis of these compounds results in the formation of analogues of the main antibiotic that are structurally and chemically very similar. These pose a significant separation problem, particularly on a large scale. In this work, we have investigated the fractionation and recovery of erythromycin A (EA) from its analogues by countercurrent chromatography (CCC). In particular, we studied the effect of increasing mobile phase flow rate (2-10 $mL.min^{-1}$) and solute loading (0.1-1.0 g) on various chromatographic parameters, such as stationary phase retention, solute partition coefficients, and column efficiency, together with the estimated throughput of the process.

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Experiments were performed on a Quattro J-type coil planet centrifuge (94.3 or 101.1 mL PTFE coil, 800 rpm) using a quaternary phase system, comprising hexane/ethyl acetate/methanol/water (1.2/2.0/2.0/1.0 v/v). Under optimal conditions, at a mobile phase flow rate of 8 $mL.min^{-1}$ and an injected solute mass of 0.6 g, EA could be obtained with a purity and yield of 97% w/w and 100% w/w, respectively. The maximum solute throughput in this case was estimated to be 0.96 $Kg.(L_{coil}.day)^{-1}$, which represented a 17 fold increase over the starting conditions identified during earlier method development studies. The results provide an encouraging basis for the subsequent application of CCC technology to the separation of novel recombinant polyketides, currently being developed by combinatorial biosynthesis techniques.

INTRODUCTION

Countercurrent chromatography (CCC) is a form of liquid-liquid chromatography with the ability to separate molecules based on their selective partitioning between two non-miscible liquid phases.[1] The absence of any conventional solid support, as used in other chromatographic separations, eliminates problems of irreversible adsorption and degradation of the target compound. The latter may occur due to conformational changes as a result of binding interactions between the target compound and a solid phase matrix. These advantages, together with the ability of CCC to be linearly scaled from analytical to preparative scale with ease,[2] makes this separation technique an attractive process option. Numerous applications of CCC for the separation of both natural and synthetic products have been reviewed, and illustrate its flexibility as an effective separation tool.[1,3] In addition, there is continued interest in the application of CCC devices as a novel means of integrating reaction and separation steps.[4-6]

In this work, we focus on the fractionation of a commercial erythromycin preparation consisting of a number of structurally similar analogues of the main product, erythromycin A (EA). Erythromycin is a macrolide antibiotic (MWt = 732 Da), produced commercially by large scale fermentation of the actinomycete, *Sacchropolyspora erythrea*. Erythromycin is a member of a large and diverse range of natural compounds termed polyketides, which are produced as a result of the sequential polymerisation of coenzyme-A-activated carboxylic acids by a number of enzymes and carrier proteins, collectively called polyketide synthases (PKSs).[14] The fermentation process for erythromycin manufacture results in the formation of small quantities of erythromycins B (EB), C (EC), D (ED), E (EE), and F (EF) in addition to EA.[15] These are structurally and chemically very similar, differing in some cases by only a single hydroxyl group. Further acidic or

basic degradation products can sometimes be present in the final fermentation broth, such as the erythromycin enol ether (EEA) and anhydroerythromycin (AE). Similar impurity profiles can also be seen in the next generation of recombinant polyketides now being developed by combinatorial biosynthesis techniques.[7, 8]

A number of studies, specifically on the separation of antibiotics by CCC, have been previously performed.[9-13] These, however, have generally focused on analytical scale fractionations, operating at low mobile phase flow rates and low solute loading. Very few studies have addressed the issue of how solute throughput affects resolution and product recovery levels. These are obviously vital issues in relation to eventual process scale applications of CCC. In this paper, the optimization of solute throughput, while maintaining a satisfactory separation and recovery yield of erythromycin A, by increasing the mobile phase flow and/or solute loading is examined. The results provide an indication as to the most effective optimization strategy for the CCC separation of polyketide antibiotics, together with an indication of potential throughputs of processed material ($Kg.(L_{coil}.day)^{-1}$).

EXPERIMENTAL

Chemicals

Solvents used in CCC experiments were hexane, ethyl acetate, and methanol (purity >99.9%, Fisher Scientific, Loughborough, UK), and freshly deionized water (Purite). Acetonitrile used for HPLC analyses was also from Fisher Scientific, as were K_2HPO_4, H_3PO_4 and H_2SO_4. All solvents were degassed prior to use with compressed helium (BOC). The commercial preparation of the erythromycin base, together with the purified erythromycin standards used, were kindly donated by Abbott Laboratories (Chicago, USA).

CCC Instrumentation and Operation

The CCC machine used was a Quattro Labprep ("J" type design) (AECS Ltd, Bridgend, UK). This device was fitted with two equivalent bobbins, each consisting of an inner and outer coil. Each column was made from 3.2 mm o.d. 1.6 mm i.d. PTFE tubing spirally wound onto the bobbin from the center to the periphery in an anticlockwise manner. The total combined volume of both the inner and outer coils on each bobbin were 355 mL and 370 mL. Temperature was controlled at 30°C with an internal fan and a water-cooled jacket. The stationary and mobile phases were pumped using Dionex P580 high-pressure isocratic and

gradient pumps (Dionex, UK). Samples were injected through a Rheodyne 7725i valve fitted with either a 1 or 2 mL loop (Rheodyne, Rohnert Park, USA). Solutes in the mobile phase were detected using a Beckman programmable UV spectrometer (model 166). System Gold® software (Beckman Instruments Inc., CA, USA) was used to capture the data.

The biphasic solvent system used consisted of hexane/ethyl acetate/ methanol/water (1.4/2.0/2.0/1.0 v/v/v/v). This had been identified as a suitable solvent system for the fractionation of erythromycin from our previous method development studies.[16] Upon equilibration of this phase system, the pH of the aqueous phase (methanol and water with a small degree of ethyl acetate) was found to be 7.2. Dissolution of the erythromycin base in the aqueous phase at the concentrations used during this study (0.1 to 1 g) resulted in a shift in the pH to 9.7-10.

The CCC machine was operated in the reverse-phase mode, i.e., with an organic stationary phase. Using one of the outer coils with an internal volume of 101.1 mL for the loading studies (100–500 mg), or 94.3 mL ($\beta = 0.83$ to 0.86) for all other experiments, the upper organic phase was first pumped into the column at a flow rate of 2 mL.min^{-1} in a 'head' to 'tail' direction. Once filled with stationary phase, rotation of the bobbins was started in the 'reverse' direction. In this mode of rotation the head end of the coil is located at the center of the bobbin with the tail being at the periphery. When a rotational speed of 800 rpm had been reached, the lower aqueous mobile phase was pumped isocratically through the column at a constant rate, again in the 'head' to 'tail' direction.

Eluted stationary phase was collected in a graduated measuring cylinder and used to calculate the amount of stationary phase retained (S_f), taking into account the volume of the inlet and outlet leads. Once hydrodynamic equilibration of the phases in the column was achieved, i.e., no further stationary phase stripping occurred; the solute dissolved in the aqueous mobile phase was injected onto the column.

The measured backpressures during CCC operation were in the range 500-4000 kN.m^{-2}. The full range of experiments performed with regard to mobile phase flow rate, injection volume, and sample loading are summarised in Table 1. The eluent from the column was continuously monitored at 290 nm and fractions were also collected at 1 minute intervals for further off-line analysis.

Off-Line Analytical Techniques

Quantitative Determination of Total Erythromycin Concentration

Off-line analysis of the total erythromycin concentration in collected CCC fractions was performed using a colorimetric assay proposed by Ford *et al*.,[17] and

Table 1. Summary of Loading and Flow Rate Studies Together with Experimental Parameters

Conc. mg/mL	Injected Vol. mL	Mass g	Flow Rate mL/min	S_F % v/v	t_R (EA) min	K_{EA}	k'_{EA}	Effic. N Plates	Total Yield % w/w	EA Yield % w/w	EA Purity % w/w	Throughput Kg/L/day
100	1	0.1	2	85	19.0	0.3	1.12	338	100	100	100	0.058
200	1	0.2	2	84	22.5	0.3	1.09	334	98	100	100	0.113
300	1	0.3	2	84	22.5	0.3	1.00	328	88	92	100	0.146
400	1	0.4	2	84	20.0	0.23	0.98	267	98	95	93	0.225
500	1	0.5	2	84	21.0	0.24	0.91	218	93	90	92	0.239
100	2	0.2	2	84	20.0	0.3	1.59	254	100	90	92	0.120
200	2	0.4	2	84	21.0	0.33	1.72	210	97	93	95	0.191
300	2	0.6	2	84	17.4	0.24	1.25	164	87	88	98	0.260
400	2	0.8	2	84	17.5	0.24	1.26	129	90	92	98	0.350
500	2	1.0	2	84	17.2	0.24	1.23	118	89	89	98	0.430
100	1	0.1	4	75	13.5	0.38	1.14	118	80	91	100	0.086
100	1	0.1	6	66	10.0	0.4	0.78	64	74	81	100	0.110
100	1	0.1	8	63	8.5	0.46	0.70	81	77	87	100	0.130
100	1	0.1	10	56	7.3	0.5	0.65	87	80	87	96	0.158
300	2	0.6	8	59	7.1	0.29	0.36	23	100	100	97	0.960

further developed by Danielson *et al*.[18] The method is based on the reaction of concentrated sulphuric acid with the two sugar moieties attached to the main 14-membered ring of the erythromycin molecule. The reaction generates an intense yellow colour, which can be measured spectrophotometrically at 470 nm. All assays were performed in triplicate and the maximum coefficient of variance for this assay was 10%.

HPLC Analysis of CCC Fractions

HPLC analysis of collected CCC fractions was performed using a C-18 reverse phase column (150 × 4.6 mm i.d.), packed with 8 μm PLRP-S [poly (styrene-divinylbenzene)] particles having a 1000Å pore size (Polymer Laboratories, Church Stretton, UK). The column was connected to a Beckman HPLC system (Beckman Instruments Inc., CA, USA) comprised of an autosampler (model 507) and solvent pumps (model 126). Prior to HPLC analysis, 0.5 mL samples of collected CCC fractions were dried using a Speedvac SC100 (Life Science International, UK) at 40°C for 2 hours, or until the vial weight had stabilized. The dried fractions were then re-dissolved in 1 mL of methanol before being injected onto the HPLC column (20 μL). The mobile phase used consisted of 45% acetonitrile, 55% 10 mM dipotassium hydrogen phosphate (v/v) adjusted to pH 7 using a 1% H_3PO_4 solution, and was pumped isocratically at a flow rate of 1 $mL.min^{-1}$. The column temperature was 70 ± 0.1°C. Erythromycin analogues were detected by UV absorption at 215 nm and were identified from the known retention times of the various species present in the commercial erythromycin preparation. Quantification of the erythromycin concentration in collected fractions was achieved using calibration curves of peak area against injected concentration of the various erythromycin standards. All assays were performed in triplicate and the maximum coefficient of variance for this assay was 10%.

Calculation of Chromatographic Parameters

The formulae used to calculate the various chromatographic parameters associated with CCC technology are somewhat different to those for conventional HPLC and, hence, they are briefly summarized here.[1] An important hydrodynamic parameter is the volume of stationary phase retained in the column. Stationary phase retention is commonly represented as the stationary phase fraction or a percentage ($100.S_f$):

$$S_f = \frac{V_s}{V_c} \qquad (1)$$

where V_s is the volume (mL) of stationary phase retained in the column at hydrodynamic equilibrium, and V_c is the total coil volume (mL).

Conway[1] has previously described how the partition coefficient ($K = C_s/C_m$) can be calculated from a CCC chromatogram - the procedure adopted during this work. The retention factor, the most commonly used distribution coefficient term is defined as:

$$k = \frac{Q_s}{Q_m} \tag{2}$$

where Q_s is the quantity (mg) of solute in the stationary phase, and Q_m is the quantity (mg) of solute in the mobile phase. This is related to the partition coefficient by the phase volume ratio:[1]

$$k = \frac{C_s\ V_s}{C_m\ V_m} = K\ \frac{V_s}{V_m} = K\ \left(\frac{S_F}{1\ -\ S_F}\right) \tag{3}$$

Column efficiency, or the number of theoretical plates (N), was estimated using a formula based on the geometry of the normal curve, but using the peak width at one half the peak height, $W_{h/2}$ (Equation 4). This is less susceptible to error in drawing tangents and is more appropriate for use with peaks that exhibit significant tailing or that slightly overlap the adjacent peaks.[19] The quantities V_R, W_b, and $W_{h/2}$ are usually measured as distances (d_R and d_b) on the chromatogram. At a constant flow rate, the units of either time or volume on the horizontal axis of a CCC chromatogram will be proportional and, therefore, either can be used to provide a value for d_R and d_b:

$$N = 5.54\ \left(\frac{V_R}{W_b}\right)^2 = 5.54\ \left(\frac{d_R}{d_{h/2}}\right)^2 \tag{4}$$

When studying the effect of mobile phase flow rate on the fractionation of EA, due to the variations in the phase volume ratio another, more applicable, means of comparing column performance was required. It has been advocated that the effective theoretical plate number, N_{eff} (Equation 5), is a more accurate means of comparing column performance, as it incorporates the contribution of the phase volume ratio and varies less than N as a function of k:[19, 20]

$$N_{eff} = N\ \left(\frac{k}{k\ +\ 1}\right)^2 \tag{5}$$

Finally, throughput of the solute through the CCC machine (T) was estimated in terms of kilograms of purified erythromycin per litre of coil per day, and

was based on the continuous operation of a CCC instrument fitted with parallel columns:

$$T = \left(\frac{Q_s}{L_c}\right) \times N_{op} \quad (6)$$

where Q_s is the mass of recovered erythromycin (mg), L_c is the volume of the coil (L) used and N_{op} corresponds to the number of operational runs achievable per day.

RESULTS AND DISCUSSIONS

Effect of Mobile Phase Flow Rate on Stationary Phase Retention

Before examining the fractionation of erythromycin by CCC, it is necessary to first establish the degree of stationary phase retention over the range of column operating conditions to be investigated. The biphasic quaternary solvent system used in this work is based upon our earlier method development studies for erythromycin fractionation.[16] The densities of the two phases used are ρ_u = 0.657 g.cm^{-3} and ρ_l = 0.903 g.cm^{-3}. The kinematic viscosities of the two phases at 30°C are μ_u = 0.434 cP and μ_l = 1.087 cP, while the interfacial tension (σ) of the equilibrated two phase system was found to be 0.45 mN.m^{-1}.

Du *et al*.[20] and Sutherland[22] have both shown that there is a linear relationship between the square root of mobile phase flow and the degree of stationary phase retention. This was confirmed in the present study (Figure 1). Du *et al*.[21] have further shown that for a given phase system, only two retention studies are needed at different mobile phase flow rates in order predict the maximum mobile phase throughput for a given degree of stationary phase retention. Sutherland[22] has also shown that there is a linear relationship between the square root of linear velocity and mobile phase flow. For scale-up, this linear flow will ideally need to be as large as possible, with the volume of mobile phase present in the coil being as low as possible, i.e., large S_f, in order to achieve a high degree of resolution for a given process.

Composition of the Commercial Erythromycin Preparation

Prior to erythromycin fractionation by CCC, it was also necessary to accurately establish the composition of the feed material to be used. An analytical HPLC chromatogram of the commercial erythromycin preparation (2 mg.mL^{-1}) is shown in Figure 2. This chromatogram provides a qualitative and quantitative

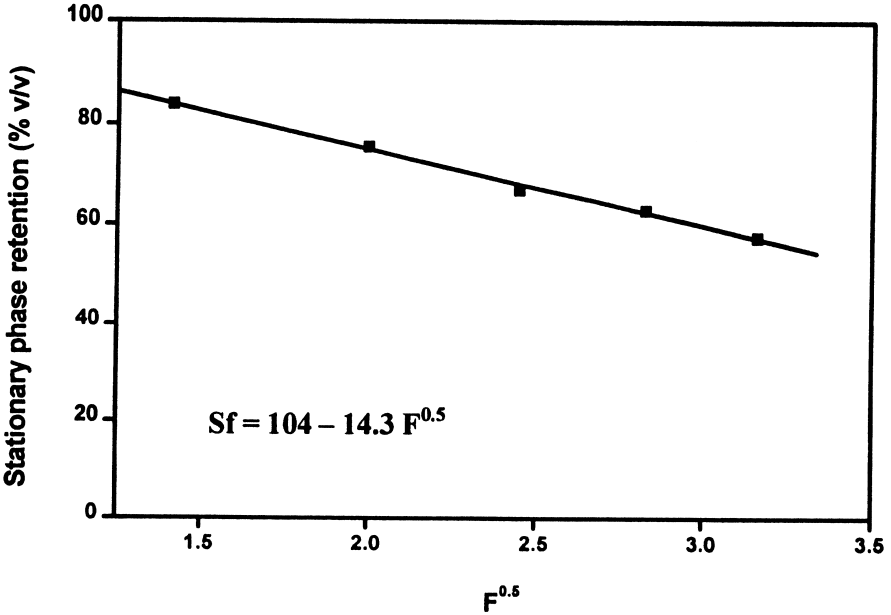


Figure 1. Percentage stationary phase retention against the square root of mobile phase flow rate (2-10 mL.min^{-1}). Solid line fitted by linear regression. Phase system consisted of hexane/ethyl acetate/methanol/water in the ratios 1.4/2.0/2.0/1.0 v/v. Experiments performed using a Quattro J-Type Coil Planet Centrifuge (94 mL coil, 800 rpm, 30ºC).

comparison with fractions analysed from CCC runs in terms of retention times and peak areas for the various erythromycin analogues. The main component of the feed material is clearly seen to be erythromycin A, which has a retention time of 5 minutes. The minor components present, i.e., EB, EC, pseudo-EEA, and the enol ether of EA, account for approximately 24% w/w of the injected solute.

Optimisation of Erythromycin Fractionation and Recovery

Effect of Mobile Phase Flow Rate

As mentioned earlier, our initial method development studies resulted in the identification of a quaternary solvent system comprised of hexane/ethylacetate/methanol/water (1.4/2.0/2.0/1.0 v/v) for the fractionation of erythromycin A

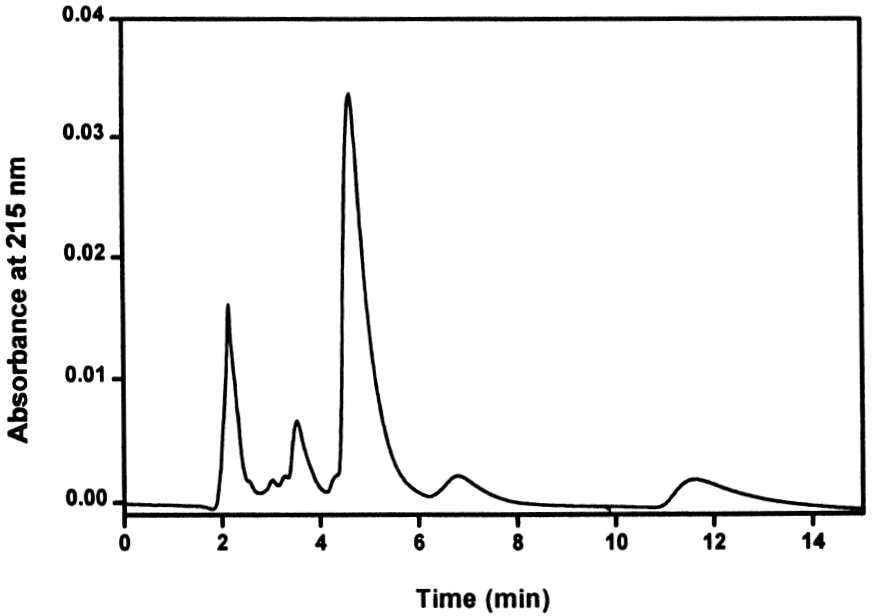


Figure 2. Analytical reverse phase HPLC chromatogram of a commercial erythromycin preparation. The retention times of the erythromycin analogues are as follows; EC = 3.5 min., EA = 5 min., EB and pseudo-EEA = 7 min., enol ether of EA = 11.5 min. The peak at 2.1 min. corresponds to the solvent front.

from its analogues.[16] The CCC system was operated in the isocratic mode, initially at a mobile phase flow rate of 2 mL.min^{-1} and a total injected solute mass of 100 mg (1 mL injection volume). The retention time of the main EA peak from off-line HPLC analysis was determined to be 19 minutes. As shown in Table 1, the partition coefficient (K_{EA}) and retention factor (k_{EA}) of the main component EA, in this case were 0.27 and 1.51, respectively. EA was shown to be recovered in the main peak of the CCC chromatogram with a purity of ~100% w/w and an estimated throughput of 0.058 Kg.(L_{coil}.day)$^{-1}$.

One operating strategy to increase the throughput of material through the CCC machine is to increase the mobile phase flow rate. The upper limit to which this is possible is determined by the hydrodynamics of the system, i.e., the decrease in stationary phase retention at higher flow rates and, consequently, the decrease in chromatographic resolution. In order to explore this operating limit we performed a series of separations at flow rates between 2 and 10 mL.min^{-1}. A typical CCC chromatogram showing the effect of increasing the mobile phase

flow rate to 8 mL.min^{-1} (S_f = 63%) on the separation time of EA can be seen in Figure 3. The UV trace is somewhat noisy, as is common in CCC fractionations monitored by this type of detection method.[1] This, in contrast to the chromatogram obtained by HPLC (Figure 2), makes it difficult to clearly distinguish between the various erythromycin peaks. It also makes calculation of chromatographic parameters like resolution and the separation factor difficult.

In order to overcome this limitation of the detection system and to identify the erythromycins present in each of the observed CCC chromatographic peaks, eluted fractions were collected every minute and analyzed individually by HPLC. Typical traces from the off-line HPLC analyses are shown in Figure 4. It is clear from Figure 4(b), that the main peak of the CCC chromatogram at 8.5 minutes contains primarily EA. The collected CCC fractions were further analyzed by the H_2SO_4 assay, which is specific to the carbohydrate residues attached to the macrolide ring of the erythromycins. These assays confirmed the retention times assigned to the various erythromycins and the mass of solute in each of the col-

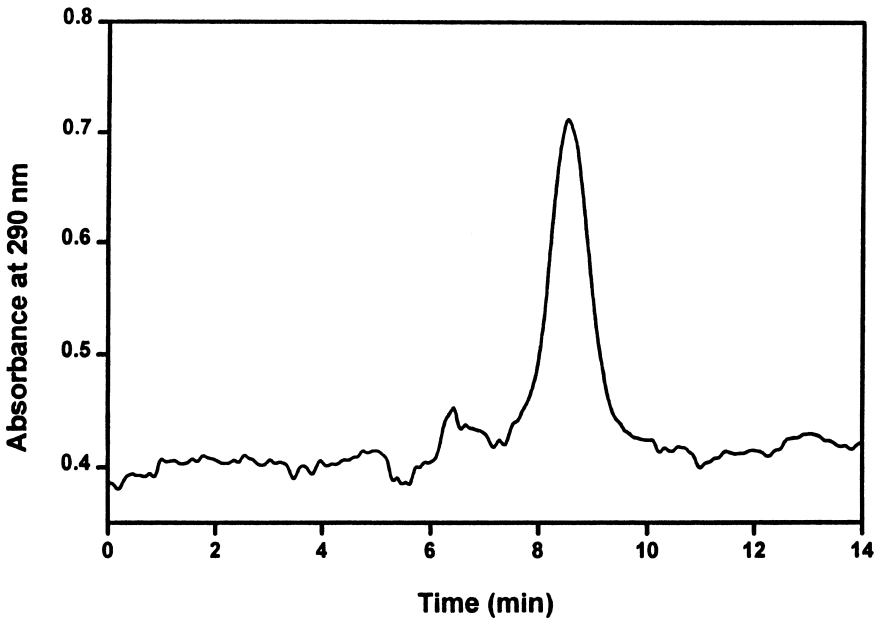
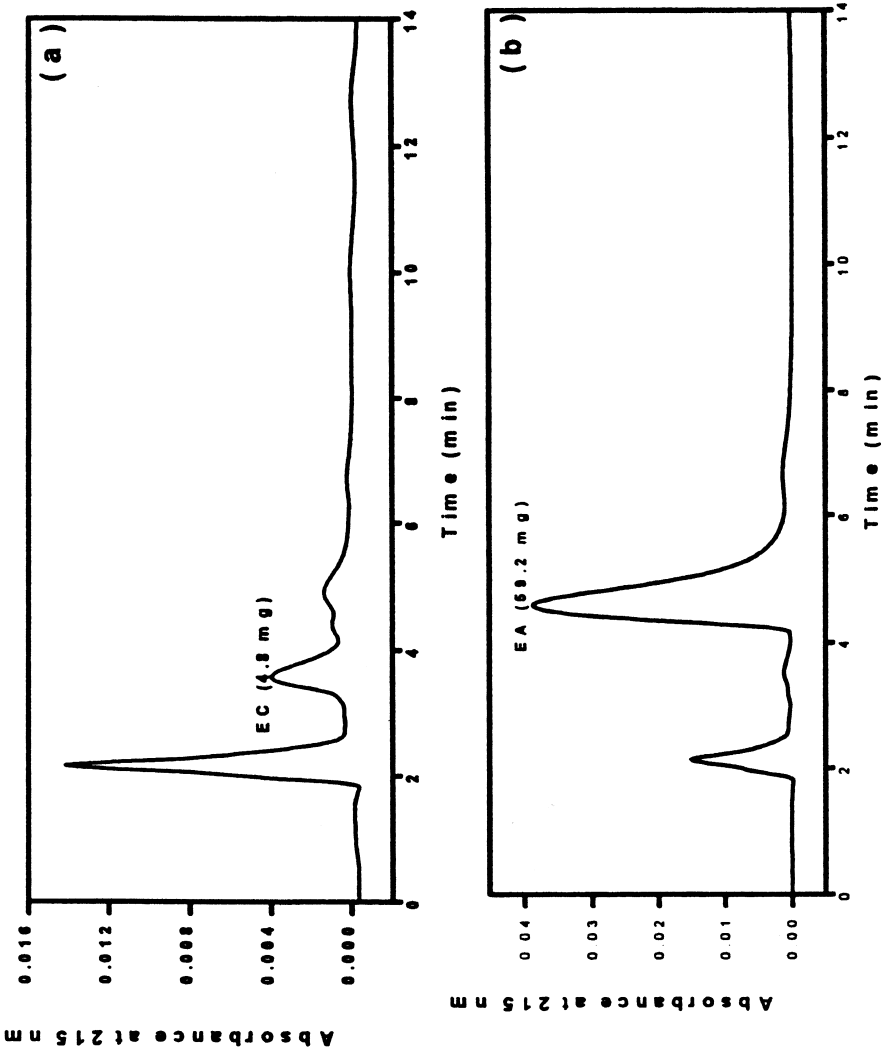


Figure 3. CCC chromatogram of erythromycin fractionation (100 mg) at a mobile phase flow rate of 8 mL.min^{-1} (S_F = 63% v/v). Retention time of the main EA peak was 8.5 minutes. Phases and CCC operational conditions as described in Figure 1.



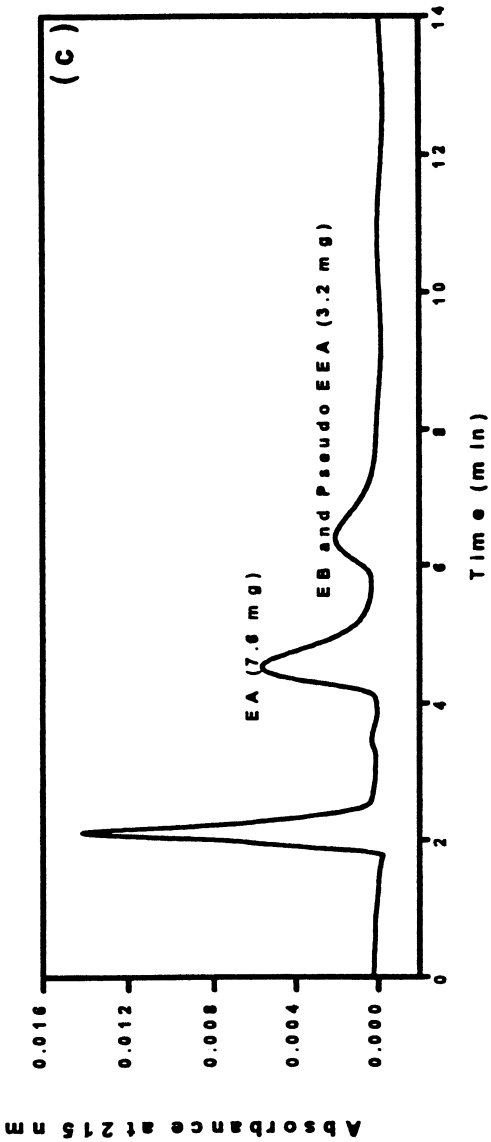


Figure 4. Analytical HPLC chromatograms of selected fractions collected from the CCC fractionation of erythromycin at a mobile phase flow rate of 8 mL.min^{-1} (Figure 3). Chromatograms (a)-(c) correspond to 1 mL mobile phase fractions collected after 8, 9, and 10 min., respectively.

lected fractions (the total solute mass balance determined by either HPLC or the H_2SO_4 assay agreed to within 10% for all CCC runs).

Table 1 summaries the effect of increasing mobile phase flow rate between 2-10 mL.min^{-1} on the solute partition coefficient, retention factor, column efficiency, total solute yield, and estimated throughput. It can be seen, that increasing the mobile phase flow rate decreases the retention time of EA from 19 minutes down to 7.3 minutes at a mobile phase flow rate of 10 mL.min^{-1}. Column efficiency initially decreased with increasing mobile phase flow, but was seen to gradually increase again at flow rates between 6 to 10 mL.min^{-1}. The observed decrease in the retention factor, k_{EA}, with increasing flow rate is to be expected, given the corresponding reduction in stationary phase retention and the fact that these terms are related, as shown in Equation 3. Increasing the mobile phase flow rate also reduced the total solute yield by approximately 20% w/w, but still provided a satisfactory separation of EA from its analogues (> 96% w/w purity at 10 mL.min^{-1}). The maximum estimated throughput in this case was 0.158 Kg.(L_{coil}.day)$^{-1}$.

Effect of Solute Loading

A second means of achieving a higher throughput of processed material is to increase the amount of solute injected onto the CCC column. In the current investigation, two loading strategies were considered due to the low solubility of the erythromycins in the aqueous mobile phase at the pH the chromatography was carried out (9.7-10). Firstly, solute concentration was increased in a fixed injection volume of 1 mL of the mobile phase. The solute concentrations thus ranged from 100–500 mg.mL^{-1}, the upper value being close to the solubility limit of erythromycin in the mobile phase used. An example CCC chromatogram can be seen in Figure 5(a), where the total mass of erythromycin injected was 300 mg at a mobile phase flow rate of 2 mL.min^{-1} (S_f = 79% v/v). The retention time of the main EA peak was again determined by off-line HPLC analysis to be 22.5 minutes, with a K_{EA} value of 0.30 and a k_{EA} value of 1.13. The off-line HPLC analysis of the fraction collected between 21 and 22 minutes (Figure 5(b)) shows the presence of only EA with a purity of ~100% w/w, and there was an overall recovery yield of 92% w/w. In Figure 5, the peaks observed in the CCC chromatogram from 11 to 18 minutes were found to consist of EC, while those from 18 to 21 minutes consisted of varying proportions of EC, EB, and pseudo-EEA, and a small amount of EA.

Table 1 again summaries the effect of increased solute concentration on K_{EA}, k_{EA}, EA purity, and EA yield, together with column efficiency and estimated throughput. It can be seen, that increasing the mass of solute loaded in this range

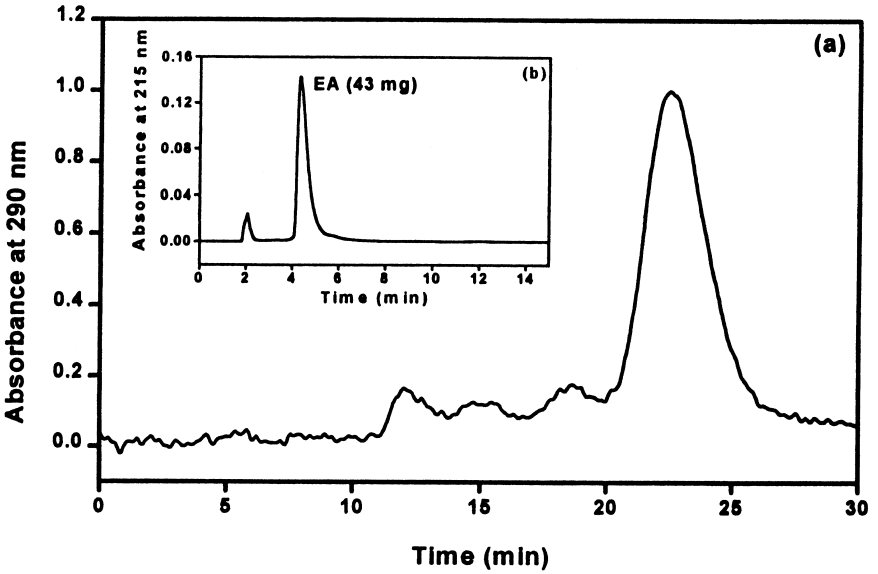


Figure 5. (a) CCC chromatogram of erythromycin fractionation (300 mg) at a mobile phase flow rate of 2 mL.min^{-1} (S_F = 79% v/v). Phases and CCC operation as described in Figure 1. Retention time of main EA peak was at 22.5 minutes. (b) The main peak fraction between 21-22 minutes analysed by HPLC shows the target compound EA at a purity of ~ 100% w/w.

(while keeping the injection volume constant) had only a slight effect on the retention time of the main EA. The increase in injected solute concentration also lowered column efficiency from 338 (100 mg.mL^{-1}) to 218 (500 mg.mL^{-1}). Column efficiency is known not to be directly dependent on S_f,[23] which remained virtually constant throughout. Although it was not possible to calculate resolution during these studies, Conway and Ito[23] have further shown that increasing S_f increases resolution by increasing the retention factor, k_{EA}. Therefore, in this work since the retention factor decreased as the mass injected increased, most notably at the higher solute loading concentrations, then so too would the resolution. This statement is supported by the off-line HPLC analysis of all the collected fractions from the CCC runs which, on closer inspection, show the merging of previously resolved peaks, especially the target solute EA at the higher solute loading concentrations. Despite this overlapping of peaks, the recovered yield and purity of EA remained high (> 90% w/w), with a maximum total solute

throughput estimated to be 0.239 Kg.(L_{coil}.day)$^{-1}$ at an injected concentration of 500 mg.mL^{-1}.

To further increase the amount of solute loaded onto the CCC column, a second loading strategy was used, which involved increasing the injection volume to 2 mL. In this way, up to 1g of solute could be injected onto the column. In principle, injection volumes could be increased further, but this is known to alter the hydrodynamic equilibrium of the phases and results in stationary phase stripping.[10] Such an operating strategy would be unwise in any industrial application of CCC technology. The results from these investigations are again summarized in Table 1. Increasing the mass of solute injected in this way, led to a decrease in the retention time of EA from 20 minutes (0.2g) to 17.2 minutes (1g). The partition coefficient (K_{EA}) also fell slightly as did the k_{EA} value peak at the higher loadings (600 – 1000 mg), i.e., the K values were no longer independent of solute concentration. Due to peak broadening and the reduced retention time, the column efficiency can also be seen to reduce from 254 to 118 theoretical plates with the increased mass injected. Despite this, a satisfactorily high yield (89% w/w) and purity (98% w/w) of the target solute, EA, was still achieved at the highest injected solute concentration. The maximum throughput for an injection of 1g of solute was estimated to be 0.43 Kg.(L_{coil}.day)$^{-1}$.

Optimization of the CCC Operation

Based on the above results, the final step was to combine the two operating strategies and determine the degree of separation and throughput achievable. The conditions chosen were a solute loading of 600 mg and a mobile phase flow rate of 8 mL.min^{-1}. The resultant CCC chromatogram is shown in Figure 6a. The presence of two main peaks can be seen between 5.5 and 8.4 minutes. The increased loading and flow rate used resulted in the erythromycin analogues EC, EB, and the target product EA to begin to coelute. Off-line HPLC analysis of the fraction corresponding to the main CCC peak between 7.1 and 8.1 minutes (Figure 6(b)), shows the presence of mainly EA (420 mg) with a very small amount of EB and pseudo-EEA (8 mg). The chromatographic parameters from this run are also summarized in Table 1. For a comparable fractionation at 8 mL.min^{-1}, it can be seen that the retention time of the main EA peak decreases when the solute loading increases from 0.2 g to 0.6 g. The increase in the solute loading further accounts for the reduction in the values of the partition coefficient (K_{EA}) and the retention factor (k_{EA}) (0.32 and 0.46, respectively). Despite the low calculated column efficiency of the column, a high degree of EA purity was achieved in the main fraction (97% w/w) with a maximum throughput estimated to be 0.96 Kg.(L_{coil}.day)$^{-1}$.

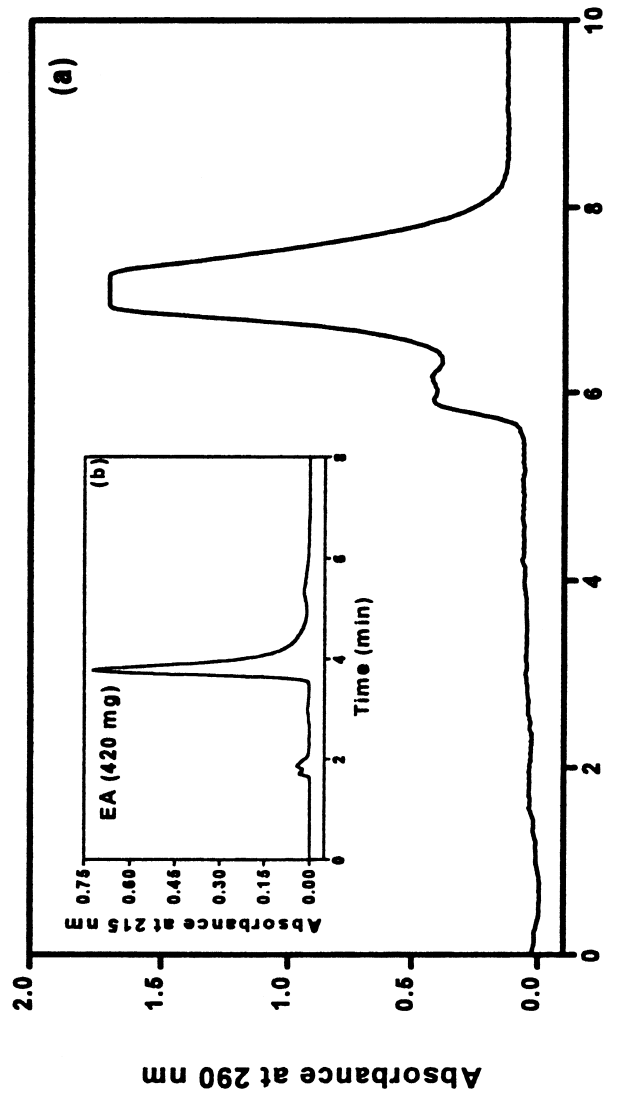


Figure 6. (a) CCC chromatogram of optimised erythromycin fractionation (600 mg) at a mobile phase flow rate of 8 mL.min^{-1} (S_F = 59% v/v). Phases and CCC operation as described in Figure 1. Retention time of main EA peak was at 7.1 minutes. (b) The main peak fraction between 7-8 minutes analysed by HPLC shows the target compound EA at a purity of 97% w/w.

Towards Process Scale Separations

Scale-up is a term used to describe an increase in the output of a target product from a given unit operation or process. In this work, we have examined the alteration of two process variables, the mobile phase flow rate and quantity of solute injected, to optimize the throughput of erythromycin through our CCC column. This led to an ~17 fold increase in throughput over the initial conditions. To increase throughput still further, it may be possible to perform multiple injections of the feed material onto the column. For this to be viable, detailed knowledge of the partition coefficients and, thus, the retention times of the various solutes would be required to develop a suitable injection schedule. A major consideration in this case, would be the critical level of any impurity build-up in the stationary phase before it is necessary to pump out and refill the CCC column. We speculate that this may increase throughput further by a factor of 2 to 3.

For further scale-up we could look at redesigning the CCC machine itself. This could be achieved by increasing either the column length or bore. Compared to conventional liquid chromatography, where solute separation or exchange occurs at the surface of the solid support, in CCC separation occurs throughout the entire volume of the stationary phase. This has the benefit of enabling the CCC column to have a higher capacity (loadability) than a comparable LC column. Therefore, increasing the volume by either increasing the column length or bore will increase the retention still further provided that the mechanical engineering issues can be overcome.

Finally, as with the scale-up of any operation, there are a number of other important factors that must be considered in parallel to the development of the process. In the case of large scale CCC these will include the safe design, installation, and operation of the rotating equipment, the minimization of explosion risks when using organic solvents, and the simplification of the solvent system used in order to aid recovery and recycling, and minimize the environmental impact of the process.

CONCLUSIONS

The study presented here, considered CCC as a process-scale separation technology for the high resolution fractionation of the natural polyketide antibiotic erythromycin A from its structurally similar analogues. The aim was to investigate how the optimization of the process, by increasing solute loading and the mobile phase flow rate, would impact on the achievable fractionation and throughput of processed antibiotic. A maximum estimated product throughput of 0.96 Kg.$(L_{coil}.day)^{-1}$, with a purity and yield of 97% w/w and ~100% w/w, respectively, was achieved at a solute loading of 0.6 g and a mobile phase flow rate of 8

$mL.min^{-1}$. This confirms the potential of CCC as a process scale purification operation, and provides encouraging results for future studies investigating the fractionation of both natural and recombinant polyketide antibiotics from real fermentation broths.

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