



The effect of increasing centrifugal acceleration/force and flow rate for varying column aspect ratios on separation efficiency in Counter-Current Chromatography

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ABSTRACT

Increasing column/tubing aspect ratio has been shown in a feasibility study to improve column efficiency when operating in reversed phase mode. This paper contains a thorough investigation on how increases in mobile phase flow and centrifugal force field affect stationary phase retention and column efficiency (as measured by the resolution between adjacent peaks) for columns wound with rectilinear tubing of different aspect ratio. The study uses a Mini CCC instrument operating from 1500 to 2100 rpm (126–246 g) to compare three columns with the same cross-sectional area but different aspect ratio – rectangular horizontal (force field perpendicular to the flat side – aspect ratio 3.125); square (aspect ratio 1.0) and rectangular vertical (flat side parallel with force field – aspect ratio 0.32). Columns are compared by measuring stationary phase retention, resolution and normalized resolution for 3 different mobile phase flow rates 2, 4 and 8 ml/min in both normal phase and reversed phase modes. The results with rectilinear tubing are compared to conventional circular tubing with the same cross-sectional area. The results show that resolution increases with aspect ratio and that at the highest aspect ratio the highest flow rate can maintain a high efficiency only if the highest g-field of 246 g is used. When comparing the rectangular horizontal tubing which gave the best results with conventional circular tubing with the same cross-sectional area a 45% improvement was found in reversed phase mode and a 51% improvement in normal phase mode over the conventional circular cross-section tubing. In other words, a rectangular horizontally wound bobbin with half the length of tubing can achieve the same result as a circular one. These are very significant results for halving separation times analytically or enabling designers to produce new instruments of the same capacity with a much-reduced size.

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1. Introduction

This study builds on the original feasibility study presented by Hewitson at the 9th International Counter-current Chromatography Conference (CCC2016) held in Chicago August 1–3, 2016 and recently published [1]. It introduces square tubing with an aspect ratio of 1.0 instead of the circular tubing used by Hewitson – so that a full range of aspect ratio can be investigated with rectilinear tubing. Conventional circular tubing is also tested so that improvements on existing technology can be made. This study extends the centrifugal force field from 126 to 246 g beyond the 181 g used by

Hewitson, doubles the flow rate range (halving the separation time) and investigates normal phase flow in addition to reversed phase flow as this is often the favoured operation mode for commercial applications.

Hewitson's study was inspired by two studies that investigated potential changes in tubing geometry in a conventional J-type coil planet centrifuges. These were by Degenhardt [2] who investigated rectilinear tubing with a small aspect ratio¹ difference and more recently by Englert [3] who showed promising results with crimped tubing.

There is scope for improving CCC efficiency by changing the aspect ratio of the tubing/column geometry for two reasons: 1) increases in interfacial mixing area and helix angle may lead to

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¹ Defined as the tubing internal width divided by the internal height where the width is measured perpendicular to the g field

increased mass transfer across the interface and better mixing efficiency and 2) more rectilinear geometry could lead to much more efficient use of potential bobbin winding space and produce longer,

more efficient columns. There are also other studies that investigate changes in column geometry but not using the more commonly used J-type centrifuge [4–12]

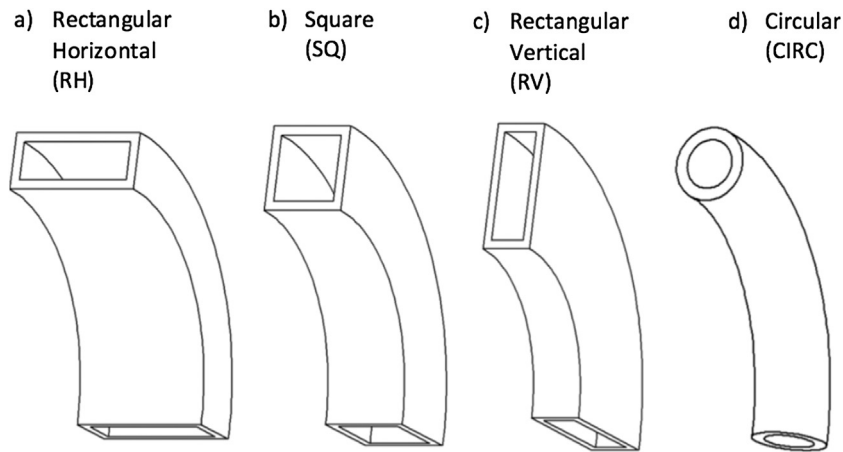


Fig. 1. Tubing cross-sections and notation used in this study: a) rectangular horizontal (RH), b) square (SQ), c) rectangular vertical (RV) and d) circular (CIRC).

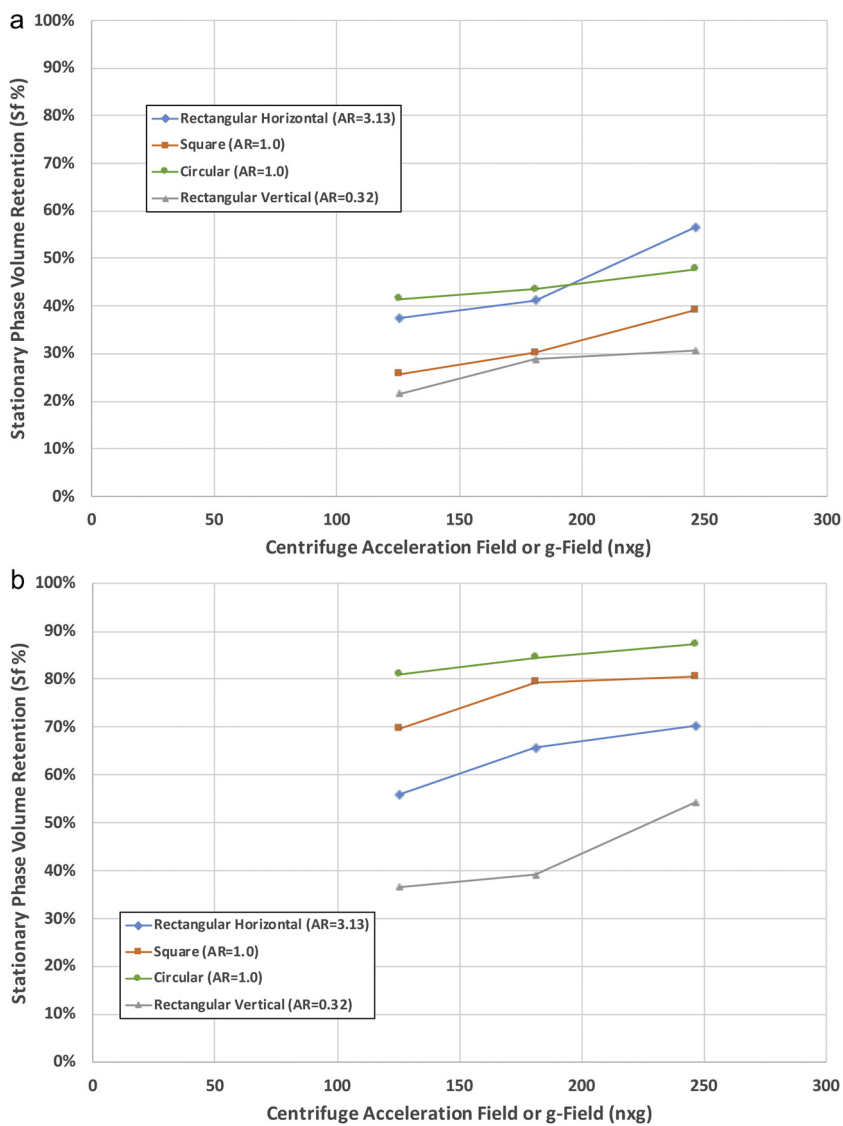


Fig. 2. The Variation of Stationary Phase Volume Retention (Sf) with Centrifuge Acceleration Field (g-field) for the Highest Flow Rate – 8 mL/min in a) RP Mode and b) NP Mode.

Hewitson [1] found that efficiency increased as aspect ratio increased. Degenhardt [2] found the opposite. With this thorough investigation, we intend to find out 1) which way aspect ratio affects efficiency; 2) whether improvements in efficiency are enhanced by high acceleration/force fields; 3) whether efficiencies can still be maintained when doubling flow rate to halve separation time and 4) see whether efficiency gains in reversed phase are also present in normal phase (NP).

2. Materials and methods

2.1. Experimental columns

Three experimental rectilinear columns were constructed, all wound on identical bobbins with the same internal and external dimensions. The first column (RH) has a rectangular section (Fig. 1a) with its wide section horizontal relative to the radial force field which is perpendicular to it ($h=0.8$ mm; $w=2.5$ mm; $AR=3.125$, $V_c=27.5$ mL); the second column (SQ) has a square section (Fig. 1b— $h=1.42$ mm; $w=1.42$ mm; $AR=1$; $V_c=22.2$ mL) and the third (RV) a rectangular section (Fig. 1c) with its wide section vertical ($h=2.5$ mm; $w=0.8$ mm; $AR=0.32$; $V_c=27.4$ mL). The cross-sectional area of all tubing is the same – 2.0mm^2 , but each

column has been wound to capacity so that the column volumes may vary.

Rectilinear cross-sectional tubing was manufactured with the above dimensions by Adtech Polymer Engineering Ltd (Stroud, Glos.) in the UK and by Hongfa (Chengdu, Sichuan Province) in China. Bobbins were constructed using 3D printing technology (Viper Si2 stereolithography printer with Accura Xtreme photopolymer resin, South Carolina, USA) in the Advanced Bioprocessing Centre at Brunel University London.

A conventional bobbin wound with circular cross-sectional area tubing (CIRC) of 1.6 mm internal diameter (Fig. 1d - area = 2.0mm^2 ; $V_c=23.9$ mL) is used for reference.

2.2. Apparatus

The columns were mounted on a Mini-DE CCC centrifuge (Tredgar, Wales, UK), with a rotational planetary radius of 50 mm and a β value ranging from 0.54 to 0.76. A Knauer K-1800 HPLC pump (Berlin, Germany) was used to pump solvent into columns. A Knauer K-2501 spectrophotometer with a preparative flow cell was operated at 254 nm to monitor the elution.

High performance liquid chromatography (HPLC) was performed on a Waters Alliance 2695 separation module (Empower software) connected to a Waters 2996 photodiode array

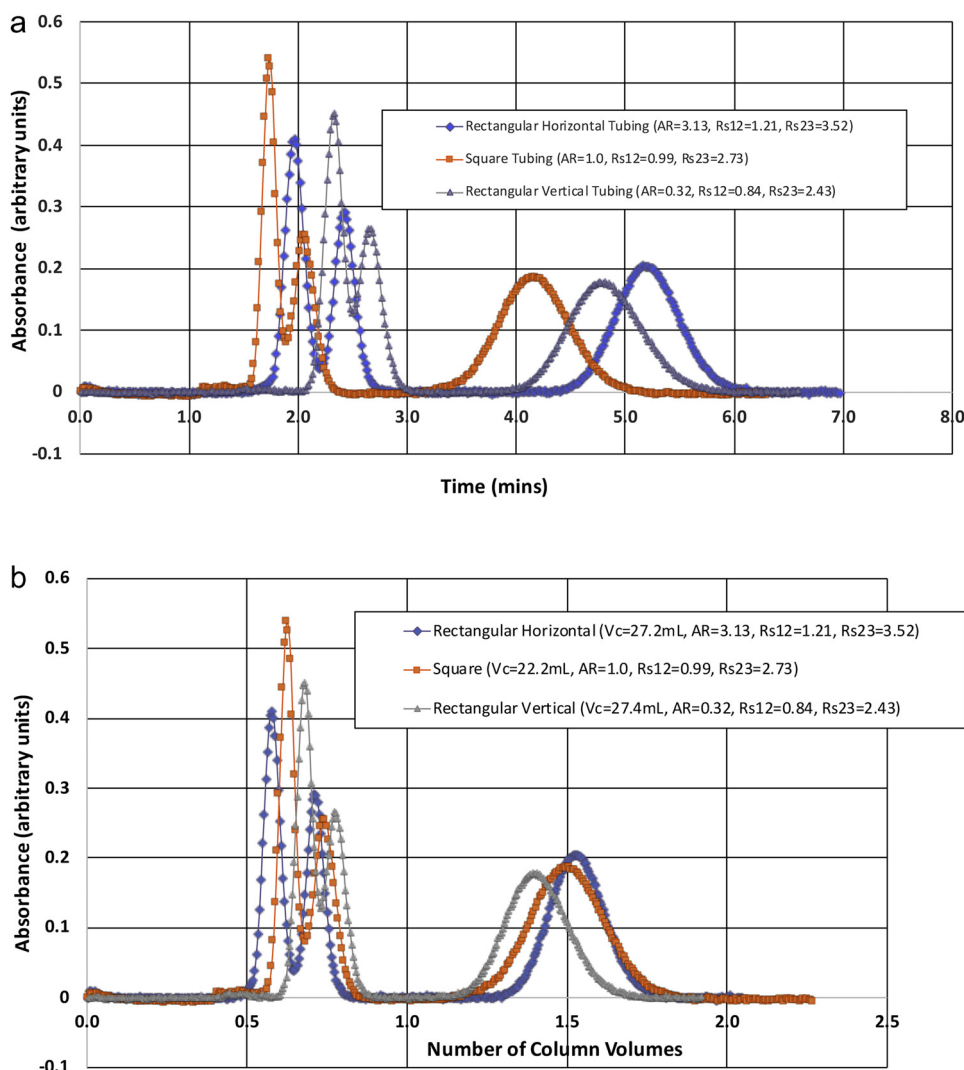


Fig. 3. Reversed Phase Chromatograms for Various Tubing Cross-Sectional Areas (RH/SQ/RV) - $F=8$ ml/min, $N=2100$ rpm plotted against a) Time and b) Column Volume (CV) - Run Time to $2^*CV=.6$ min.

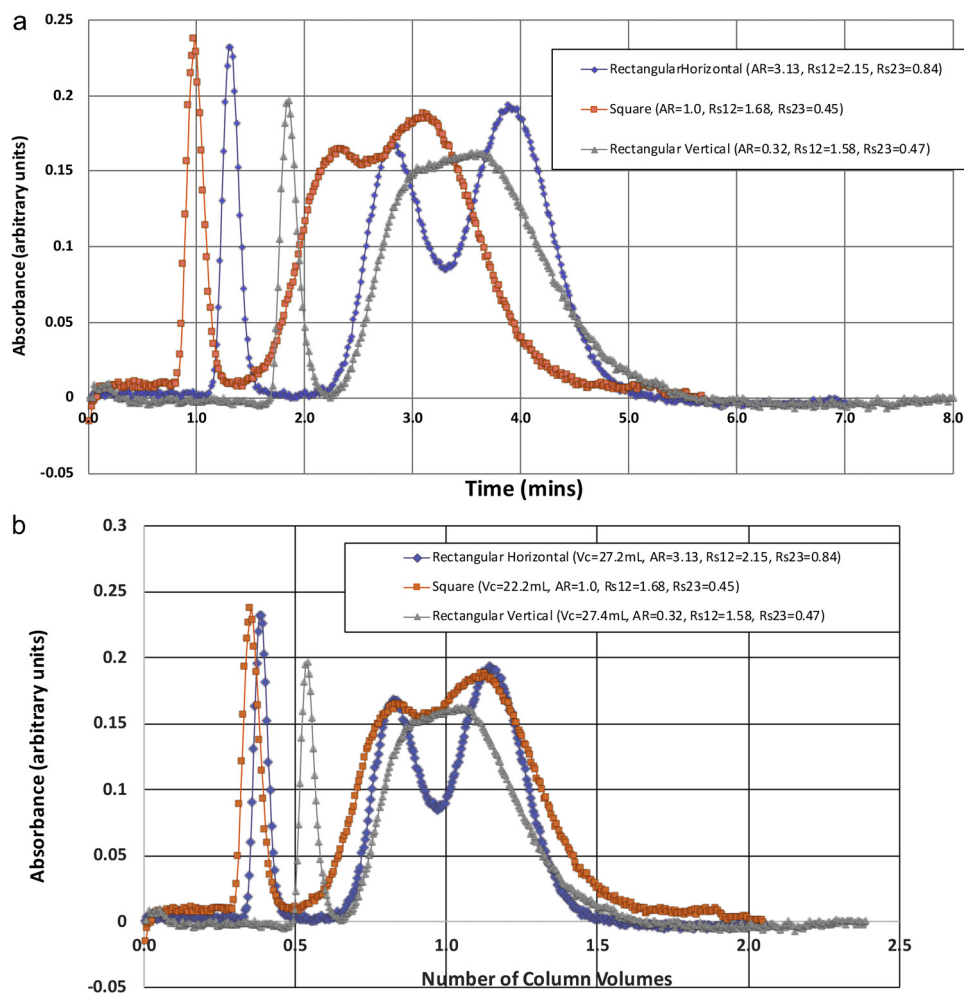


Fig. 4. Normal Phase Chromatograms for Various Tubing Cross-Sectional Areas (RH/SQ/RV) - $F = 8$ ml/min, $N = 2100$ rpm plotted against a) Time and b) Column Volume (CV) - Run Time to $2 \times CV = .6$ min.

(PDA) detector (210–400 nm) using a Sunfire C18 column (150 mm \times 4.6 mm I.D., 5 μ m) (Waters, Milford, MA, USA).

2.3. Reagents

The solvent system used for the two-phase flow experiments was a HEMWat system 17 [13] (Heptane/Ethyl Acetate/Methanol/Water - 1:1:1:1). The flow directions were either Reversed Phase (RP) (Centre to Periphery - $C > P$) or Normal Phase (NP) ($P > C$). All solvents were of analytical grade and for HPLC detection were HPLC grade from Fisher Chemicals (Loughborough, UK). HPLC water was purified by a Purite Select Fusion pure water system (Thame, UK).

2.4. Sample solution

The sample solution was Caffeine ($K_d = 0.03$, 2 mg/mL), Aspirin (acetylsalicylic acid, $K_d = 0.275$, 9 mg/mL) and Naproxen ((2S)-2-(6-methoxynaphthalen-2-yl)propanoic acid - $K_d = 1.376$, 4 mg/mL) for reversed phase (RP) and Biphenyl ($K_d = 0.03$, 4 mg/mL), Coumarin ($K_d = 0.71$, 1.5 mg/mL) and Salicylic Acid ($K_d = 1.12$, 6 mg/mL) for normal phase (NP). Each injection was 0.5 mL of 15 mg/mL for reversed phase (RP) and 0.5 mL of 11.5 mg/mL for normal phase (NP).

Salicylic Acid, Coumarin and Caffeine were purchased from Fisher Chemicals (Loughborough, UK). Biphenyl, Aspirin and Naproxen were purchased from Sigma-Aldrich (Gillingham, UK).

2.5. CCC separation procedure

The column was filled with the phase intended to be the stationary phase, then the rotor speed was set at 1500 rpm (126 g), 1800 rpm (181 g) or 2100 rpm (246 g), and the mobile phase pumped into the column to establish hydrodynamic equilibrium at either 2, 4 or 8 mL/min in either reversed phase (RP - $C > P$) or normal phase (NP - $P > C$) mode. Then the sample solution was injected and elution started, which was monitored with an UV detector at 254 nm. The volume of stationary phase eluted (V_e) was collected so that the volume retention of stationary phase (S_f) could be calculated in the usual way.

$$V_e = V_m + V_{in} + V_{out} \quad (1)$$

Where V_{in} and V_{out} are the volumes of the inlet and outlet flying leads respectively and V_m is the volume of mobile phase in the column. With V_m obtained from equation (1), S_f is calculated as follows:

$$S_f = V_s/V_c = (V_c - V_m)/V_c \quad (2)$$

Where V_s is the volume of stationary phase in the column and V_c is the total column volume.

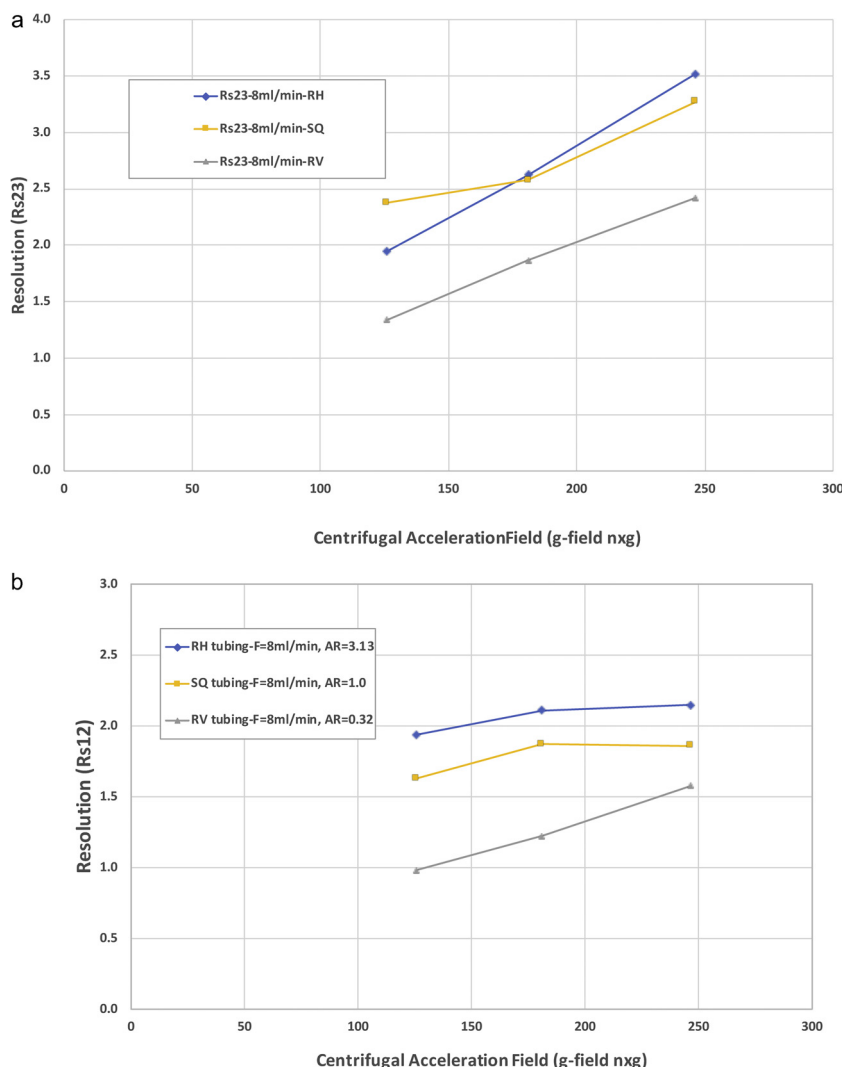


Fig. 5. Resolution (Rs) v. g-Field for Various Aspect Ratio (AR) Tubing at the Highest Flow Tested - 8 mL/min for a) RP Mode and b) NP Mode.

2.6. Measurement of efficiency

The resolution (Rs) between adjacent peaks was used to assess separation efficiency.

$$Rs_{12} = 2(t_2 - t_1)/(w_2 + w_1) \quad (3)$$

Where t_1 and t_2 are the times of elution of peak 1 and 2 and w_1 and w_2 are their baseline widths respectively.

In reversed phase (RP) mode, the resolution between the second and third peaks (Rs_{23}), Aspirin and Naproxen, were used. In normal phase (NP) mode the resolution between the first two peaks, Biphenyl and Coumarin, were used. As Caffeine in RP mode and Biphenyl in NP mode are $K_d=0$ marker peaks, it would be better to measure resolution between Coumarin and Salicylic Acid in NP mode, but they do not resolve well under low flow and low aspect ratio conditions, so Rs_{12} was used. However, under optimum conditions Rs_{23} could be easily measured and this was used to assess overall improvement in efficiency for NP mode when compared to circular tubing at the end of the study.

When assessing overall efficiency, differences in column volume were not taken into account as the maximum winding in a given bobbin volume was used. However, it was important to differentiate between gains due to improved packing efficiency and gains due to improvements in retention or mixing efficiency, so, where indicated, resolution results were normalized to correct for differences

in column length/volume using the method proposed by Du [14] where resolution is proportional to the square root of the length or volume difference assuming a constant cross-sectional area.

2.7. Calculation of g-field

The values of g-field given in this paper are based on the g-field measured at the centre of the planetary rotor ($R\omega^2$) where R is the rotational radius of the planetary axis and ω is the angular rotation of the main centrifugal rotor. The acceleration field measured at the centre and periphery of where the tubing is wound on the bobbin will be much larger as described by van den Heuvel and Konig [15]

2.8. Repeat experiments

Peak height variations were within 2–4%, whereas peak elution time/position was within $\pm 2\%$.

3. Results

3.1. The effect of aspect ratio on stationary phase retention

The variation of stationary phase volume retention (S_f) with g-field for reversed phase (RP) and normal phase (NP) for the highest flow rate of 8 mL/min is shown in Fig. 2a and b respectively. For

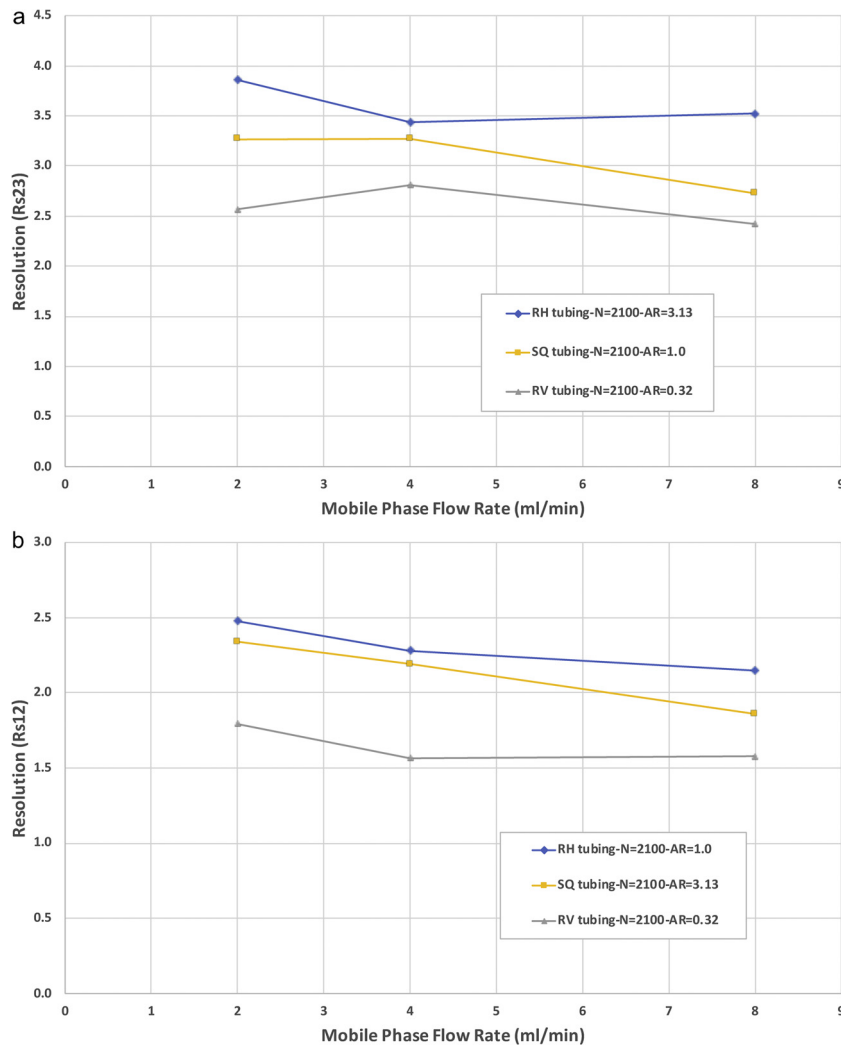


Fig. 6. Resolution (R_s) v. Mobile Phase Flow Rate for Various Aspect Ratio (AR) Tubing at the Highest g -Field Tested - 246 g at 2100 rpm for a) RP Mode and b) NP Mode.

reversed phase mode, high aspect ratio clearly gives better retention particularly at the highest g -level. The results confirm those obtained by Hewitson [1] that circular (CIR) gave slightly higher retention than rectangular horizontal (RH) at 181 g , but this situation is reversed at 246 g . Low aspect ratio rectilinear tubing gives poor retention. For normal phase mode, square tubing with an aspect ratio of 1.0 gives better retention, but by the time g -field has increased to 246 g , high aspect ratio RH tubing is giving better retention but still not as good as the circular tubing (CIR).

The variation of stationary phase volume retention for the range of mobile phase flow conditions is shown in Table 1 for the highest g -field where retentions were always higher. For Reversed Phase mode, rectangular horizontal (RH) gave the best retention results for all flows with the low aspect ratio rectilinear tubing (SQ & RV) performing badly. In normal phase mode, the square tubing (SQ) performed quite well and gave higher retention than RH and RV at low flow but at high flow was competitive with RH and not quite as good as CIR.

3.2. The effect of aspect ratio on the resolution for the highest flow and g -field

Chromatograms for the three different aspect ratios at the highest flow and g -field (8 mL/min and 246 g – 2100 rpm) in reversed phase mode (RP) are shown in Fig. 3a plotted against time. All separations

Table 1

Variation of Stationary Phase Volume Retention at $N = 2100$ rpm (246 g) for rectilinear tubing of different aspect ratio with mobile phase flow rate varying from 2 to 8 mL/min.

Reversed Phase (RP)					
Column	Column Vol (mL)	Aspect Ratio	Flow (mL/min)		
			2	4	8
RH	27.2	3.13	77.21%	68.75%	56.62%
SQ	22.2	1.00	63.96%	51.80%	39.19%
RV	27.4	0.32	45.26%	39.78%	30.66%
CIR	23.9	1.00	72.80%	62.34%	47.70%
Normal Phase (NP)					
Column	Column Vol (mL)	Aspect Ratio	Flow (mL/min)		
			2	4	8
RH	27.2	3.13	80.51%	72.43%	64.71%
SQ	22.2	1.00	84.23%	81.98%	66.22%
RV	27.4	0.32	68.98%	67.15%	56.20%
CIR	23.9	1.00	83.26%	78.66%	74.48%

are performed in less than 6 min and all give good resolution between caffeine, aspirin and Naproxen, but only the RH tubing gives near basely resolution for all compounds. As the columns have different volumes they are difficult to compare. Plotting the same

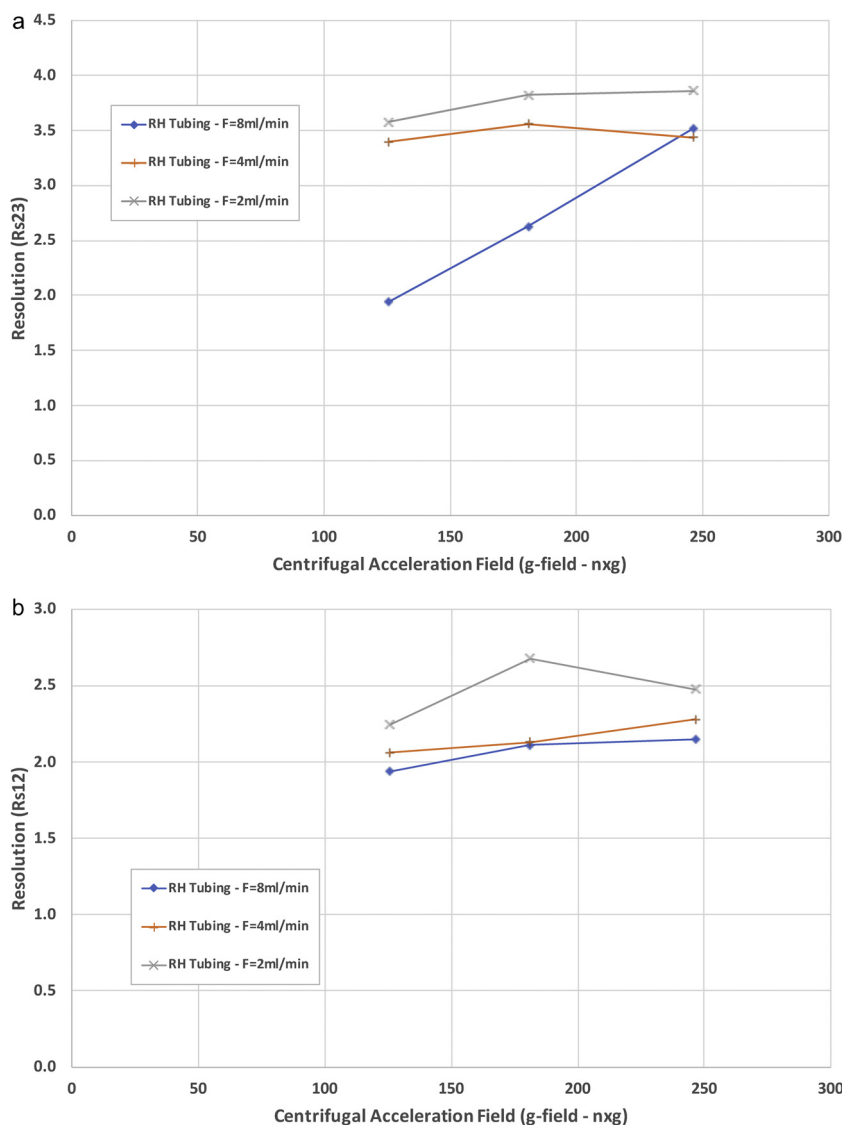


Fig. 7. Resolution (R_s) v. g -Field for various Flow Rates using the Highest Aspect Ratio Tubing (RH) for a) RP Mode and b) NP Mode.

results again number of column volumes (CV) in Fig. 3b, makes it much easier to see how stationary phase volume retention is affecting the results as the chromatograms are expanded or contracted about the $K_d = 1$ point depending on S_f .

Chromatograms for the three different aspect ratios at the highest flow and g -field (8 mL/min and 246 g –2100 rpm) in normal phase mode (NP) are shown in Fig. 4a and b plotted against a) time and b) number of column volumes. Ultimately, the approach taken to assess column efficiency for RP and NP mode was to measure the resolution between Aspirin and Naproxen (R_{s23}) and the resolution between Biphenyl and Coumarin (R_{s12}) respectively.

3.3. The variation of resolution (R_s) with g -field for various aspect ratio tubing at the highest mobile phase flow rate (8 mL/min) for both reversed phase and normal phase

Resolution versus g -field for various aspect ratio tubing is shown in Fig. 5a and b for RP and NP modes respectively. The reversed phase results show linear increases in resolution as g -field increases. The increases are most marked in RH tubing ($AR = 3.125$) where at the highest g -field (246 g) resolution or separation efficiency is 30–45% higher than the lower aspect ratio rectilinear tubing.

The normal phase results (Fig. 5b) also show increases in resolution with increases in g -field, but only the RV tubing shows a linear increase, while RH and SQ tubing appear to be plateauing as g -field increases. RH tubing ($AR = 3.125$) gave a resolution or separation efficiency 16–37% higher than the lower aspect ratio tubing.

3.4. The variation of resolution (R_s) with mobile phase flow rate for various aspect ratio tubing at the highest g -field (246 g –2100 rpm) for both reversed phase and normal phase

Resolution versus mobile phase flow rate for various aspect ratio tubing is shown in Fig. 6a and b for RP and NP modes respectively. The reversed phase results show that resolution or column efficiency can be maintained up to the highest flow rate of 8 mL/min and that the highest aspect ratio tubing shows the best performance with even a slight increase in resolution as flow is increased to the highest value.

The normal phase results show that resolution or column efficiency almost remains the same as mobile phase flow rate increases. This again suggests that the highest flow rate is best if throughput needs to be maximized for commercial purposes.

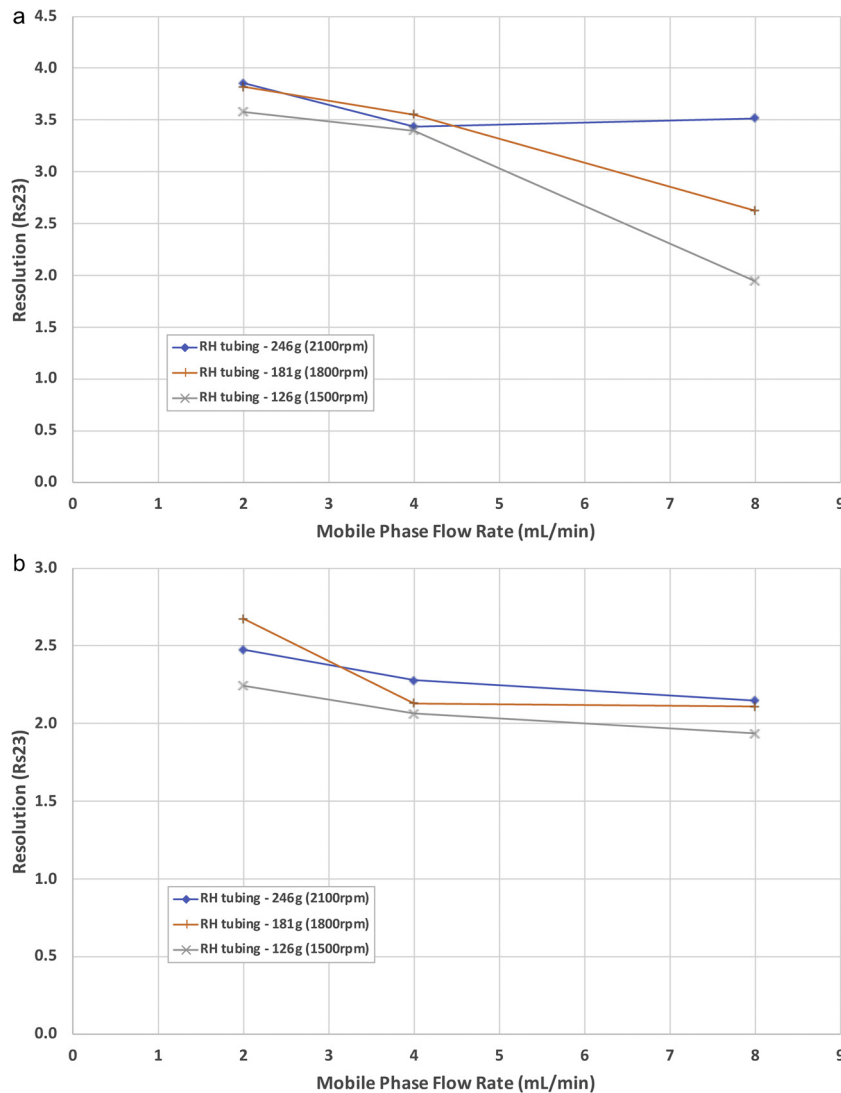


Fig. 8. Resolution (R_s) v. Mobile Phase Flow Rate for various g -Fields.

using the Highest Aspect Ratio Tubing (RH) for a) RP Mode and b) NP Mode

3.5. The variation of resolution with g -field for various mobile phase flow rates for the best tubing geometry – the highest aspect ratio rectangular horizontal tubing, for both reversed phase and normal phase

For reversed phase (Fig. 7a), increasing the g -field has little effect when flow rates are 2 and 4 mL/min. But for 8 mL/min there is a marked linear increase in resolution. This shows that a high g -field is essential if high throughput for commercial purposes is required. For normal phase, resolution or separation efficiency is not as sensitive to g -field changes as in RP mode. Nevertheless, the best resolutions for high flow is still a g -field of 246 g .

3.6. The variation of resolution with mobile phase flow rate for various g -fields for the best tubing geometry – the highest aspect ratio rectangular horizontal tubing, for both reversed phase and normal phase

The same data is plotted against mobile phase flow in Fig. 8a and b for RP and NP modes respectively. The reversed phase results (Fig. 8a) clearly show how resolution or column efficiency drops off as flow rate is increased – only at the highest g -field (246 g) is resolution preserved across the whole range. The normal phase

results, by contrast, show that resolution or column efficiency can be maintained across the flow range for all g -fields tested.

3.7. Analysis of resolution/column efficiency for rectilinear tubing of varying aspect ratio compared to conventional circular tubing for the highest flow rate and varying g -field

The variation of resolution with g -field for the highest flow is shown for all rectilinear aspect ratio tubing compared to conventional circular tubing of the same cross-sectional area in RP-mode in Fig. 9a, and the same results corrected for column length in Fig. 9b. The results show a 45% increase in resolution/column efficiency of the high aspect ratio RH column compared to the conventional circular one. This means that a conventional circular column would have to be wound 2.1 times longer to achieve the same resolution/efficiency. These results are uncorrected for differences in column volume, but are justified because the tubing has been wound in the same space for each column – the RH tubing having a better packing efficiency than the circular tubing.

When adjusting/normalising the resolution against the RH tubing ($V_c = 27.2$ mL) in Fig. 9b, there is still a 36% in resolution/column efficiency between the two columns, which means that 9% of the increased efficiency is due to improvements in packing density and 36% due to column efficiency.

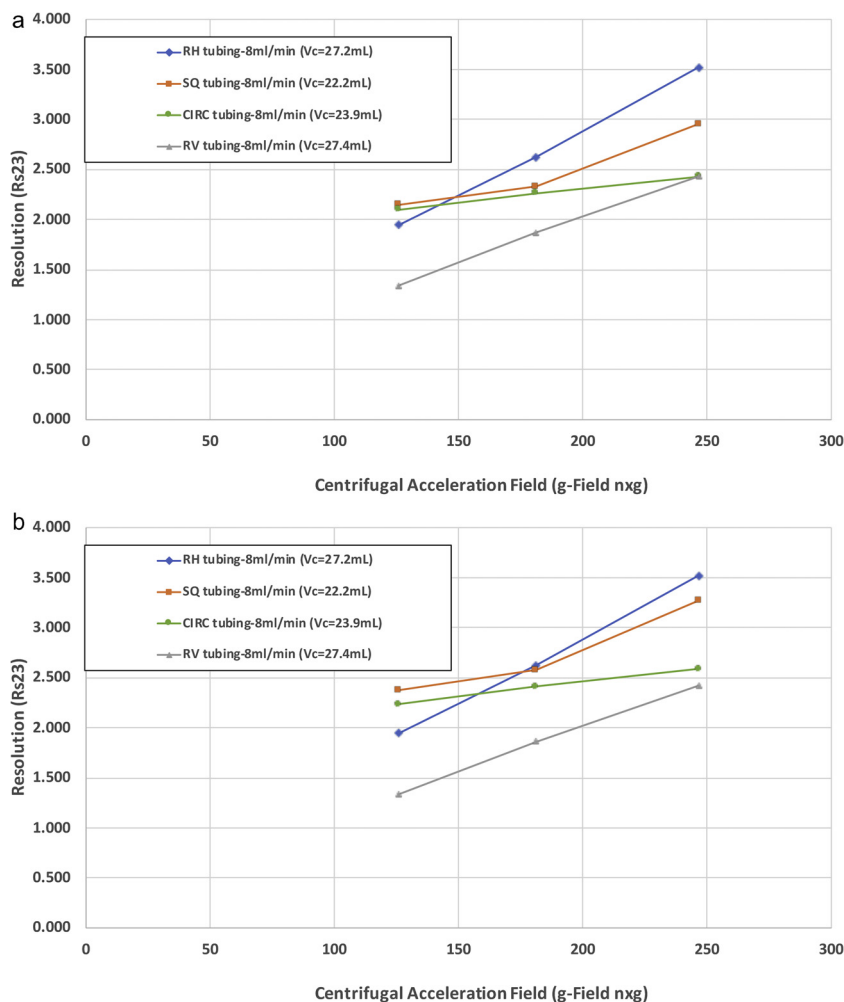


Fig. 9. Resolution (Rs) v. g-Field and Column Geometry for the Highest Mobile Phase Flow – 8 mL/min and RP Mode for a) Non-corrected and b) Corrected for $V_c = 27.2$ mL.

The equivalent results for normal phase are shown in Fig. 10a and b respectively for non-corrected and corrected for column length. In this case both resolution between Biphenyl and Coumarin (Rs_{12}) and Coumarin and Salicylic Acid (Rs_{23}) are given. The increase in resolution/column efficiency between the rectangular horizontal tubing with the highest aspect ratio (3.13) is 33% for Biphenyl and Coumarin (Rs_{12}) and 51% for Coumarin and Salicylic Acid (Rs_{23})

4. Discussion

In answer to the four questions posed at the end of the Introduction Section: 1) aspect ratio has a big effect on column efficiency – the highest aspect ratio of 3.13 giving the highest column efficiency with the rectangular horizontal tubing; 2) increases in g-field certainly produce massive increases in efficiency both in RP and NP mode; 3) when doubling flow rate from Hewitson's study from 4 to 8 mL/min, it was possible to maintain efficiency only at the highest g-field of 246 g. At lower g-field performance at 8 mL/min was much lower and 4) the gain in efficiency in RP mode of 45% was increased to 51% in NP mode. If the column length is doubled the increase in resolution will be only $\sqrt{2}$ or 1.42 a 42% increase. The increases in resolution measured are therefore extremely significant particularly when scaling up for production. Instruments could be half the size that they currently are for the same capacity.

At the outset of this study the hypothesis was that increasing aspect ratio would increase the interfacial area, leading to better mixing between the phases and improvements in separation efficiency. It was also thought that the increase in helix angle would create a larger Archimedean screw effect and lead to better stationary phase retention. With circular tubing, if stationary phase volume retention (S_f) is increased to 80–90% the potential area for mixing decreases, whereas for rectilinear tubing it does not.

In practice, the conventional circular tubing had the best retention except at the highest flow rate and g-field in reversed phase mode where S_f for RH tubing was 19% higher than for circular tubing of the same cross-sectional area. For normal phase, RH tubing had a retention 13% lower than circular tubing at the highest flow rate and g-field. It is not clear why there are not better retention results for the high aspect ratio tubing as it would be expected that the increase in aspect ratio would result a better Archimedean screw effect and better retention. However, increases in aspect ratio do increase the axial helix but at the same time decrease the radial helix angle. Perhaps radial helix angle is more important from the retention point of view. The differences in retention behavior between RP and NP mode suggest that the properties of the phase system (ie density, viscosity and interfacial tension) are having a big influence on fluid behavior. It will be important to extend this study to other phase systems across the hydrophobicity range to verify that these results can be generalised.

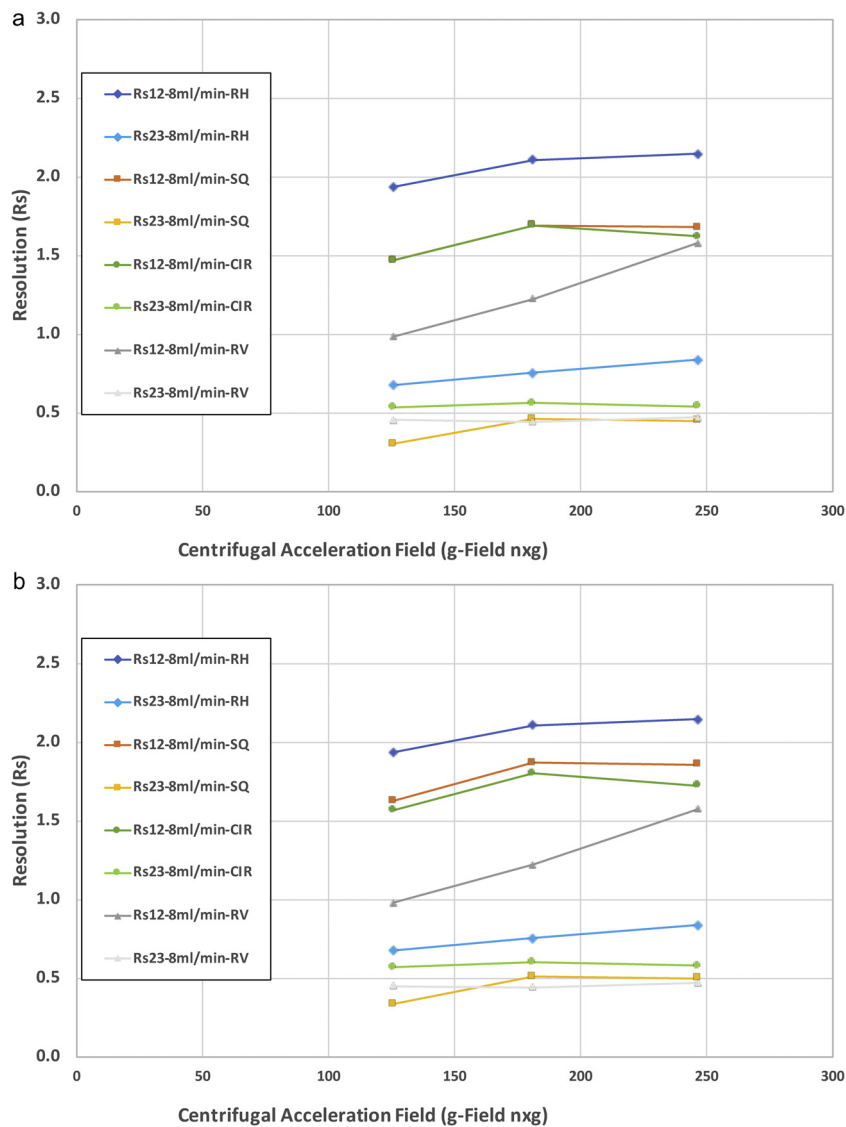


Fig. 10. Resolution (Rs) v. g-Field and Column Geometry for the Highest Mobile Phase Flow – 8 mL/min and NP Mode for a) Non-corrected and b) Corrected for $V_c = 27.2$ mL.

For optimum condition with the highest mobile phase flow and g-field, the fact that resolution/column efficiency is 45% higher in RP-mode and 51% higher in NP-mode suggests that the main factor affecting efficiency is interfacial area, particularly for NP-mode where efficiency would be expected to fall for RH-tubing due to the 13% reduction in retention (S_f) but the opposite happened in practice.

The results confirm those of Hewitson [1] and the principle that high aspect ratio tubing gives more efficient separations than low aspect ratio tubing, disagreeing with the results obtained by Degenhardt [2]. Comparing results in this paper with Hewitson's Resolution results at 181 g (1800 rpm) and 2 mL/min and 4 mL/min flow – the two sets of results are within $\pm 5\%$ of each other. Comparing retention results, RH and CIR tubing columns are within $\pm 5\%$ of each other but the RV tubing column in this paper has a retention 27% lower than Hewitson's RV column.

While this research looks extremely promising for designing new instruments with shorter separation times and greater capacity for a given size, research is still needed on 1) will high aspect ratio tubing be able to handle the high sample loading regimes managed with conventional circular tubing and 2) will it be scalable and work with larger cross-sectional area tubing and 3) will these results be upheld as different solvent phase systems are used.

5. Conclusions

This study has shown that increases in rectilinear tubing aspect ratio can give significant increases in column resolution/efficiency partly because of better packing efficiency of the tubing wound on the bobbin, but mainly due to increases in mixing efficiency. These improved column efficiencies are in the order of 50% for both reversed and normal phase modes which is equivalent to doubling the capacity or the length of a column. Furthermore, it was found at the highest g-field tested (246 g) that flow could be double those of previous studies which would lead to shorter separation times and a doubling of productivity. Increasing aspect ratio did increase stationary phase volume retention as expected, but rectilinear tubing did not perform as well as expected in comparison with circular tubing and so the results are more due to improvements in mixing efficiency than any improvements in stationary phase volume retention. If these results can be generalized to a range of different solvent systems; can be shown to be capable of increased sample loading and are shown to be scalable, then they will have a significant effect on new designs of CCC instruments in the future where large scale instruments that currently occupy a whole room could become bench size instruments.

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