Scale-up in Centrifugal Partition Chromatography: the "free-space between peaks" method

Elodie Bouju^{1,2}, Alain Berthod¹, Karine Faure¹

¹*Institut des Sciences Analytiques, UMR Université Lyon 1/ENS/CNRS 5280, 5 rue de la Doua, 69100 Villeurbanne, France.*

²*Kromaton Sarl, groupe Rousselet-Robatel, 42 Avenue Rhin et Danube, 07100 Annonay, France*

Abstract

Centrifugal Partition chromatography (CPC) is a purification technique using a biphasic liquid system. As a preparative separation technique, scale-up is of primary concern. Once the separation is optimized on a lab-scale instrument, the scale-up transfer is quite straightforward simply using the instrument volume ratio as the linear transfer factor, thanks to the absence of solid support. Such linear transfer underestimates the performances of large-scale CPC rotors that are usually better than that of small rotors. It means that more material than predicted by the linear estimation could be purified. A fully practical method based on experimental observations is proposed. The first step is to determine experimentally the free space volume available between the two peaks of interest working with the small analytical CPC instrument, ΔV_1 , and with the large preparative one, ΔV_2 . The second step is to determine on the small CPC instrument how much material can be loaded to reach the maximum mass load still giving the required purity and recovery ratio of the desired compound. Then, an accurate prediction of the maximal quantity of sample that the large-scale rotor can purify is simply obtained by multiplying the maximum mass load on the analytical CPC instrument by the free-space between peaks $\Delta V_2/\Delta V_1$ ratio. For demonstration purposes, the method is applied to the transfer of the CPC separation of a synthetic three-GUESS-compound mixture from a 35 mL-rotor to a semi-prep 239-mL rotor. The paper focuses also on operating conditions optimization depending on industrial production strategy (maximal load per run or maximal productivity).

Keywords

Centrifugal Partition Chromatography, Counter-Current Chromatography, scale-up, preparative chromatography, productivity.

1. Introduction

Counter-current Chromatography (CCC) is a liquid-liquid chromatographic technique, i.e. it requires only two immiscible liquid phases with no need for solid support [1, 2]. One of the liquid phases is the stationary phase, maintained in the column by centrifugal forces. The other one is the mobile phase, pumped through the stationary phase.

Since CCC development in the mid-1960s by Yoichiro Ito [3], numerous column designs were conceived. However, only two designs were developed and commercialized: the hydrodynamic and

hydrostatic systems [4]. Hydrodynamic systems are composed of Teflon tubing coiled on bobbins with two axes of rotation which generate variable centrifugal fields. On the contrary, hydrostatic systems, also named Centrifugal Partition Chromatographs (CPC), are composed of steel disks stacked in a rotor spinning around a single rotation axis (constant centrifugal field). Inside each disk, interconnected cells of different shape and/or volume are engraved.

Due to the liquid stationary phase, CCC has numerous advantages compared to classic solid phase chromatography techniques, such as higher load capacity and no solute infinite retention [5]. However, despite a relatively lower solvent consumption in CCC, method development in high capacity rotors is not economically viable yet. Thereby, manufacturers have recently introduced small columns for faster method development and optimization [6]. The purpose of these small volume instruments is to allow for the rapid development of the separation using minimal amounts of solvents and then transfer the optimized methods to higher column volumes for increased production: this transfer is named scale-up.

Up to now, CCC scaling-up is usually performed at constant stationary phase retention ratio. Once the method developed on a small column volume is optimized, a scale-up factor is used to estimate the conditions required to work with the higher column volume. With hydrodynamic systems, due to the tube configuration of the apparatus, the scale-up factor can be calculated according to the ratio of the columns volumes or the ratio of the tube sections. Luo [7] performed an analytical separation of four phenolic alkaloids using a 18-mL CCC column. He then transferred it on a 50-times larger 900-mL column. The strategy consists in increasing the flow rate and the solutes load by the same scale-up factor defined as the column volume ratio: $900/18 = 50$. Results showed almost the same resolution because the stationary phase retention ratios were the same in the two CCC columns. However, this is not always the case: would had the stationary phase retention been better on the preparative column, the resolution and the injected amount could have been higher. In hydrostatic systems, Sutherland [8] accomplished a scale-up example also using the ratio of the column volumes as the transfer factor. The myoglobin and lysozyme separation was optimized on a 500 mL hydrostatic column with an aqueous two-phase solvent system (ATPS). The transfer was performed on a 12.5 times larger column volume, i.e. 6.25 L working with a 12.5 times higher flow rate and injected protein amount. On this 6.25 L preparative separation, the protein resolution was greater than that observed for the 500 mL analytical separation: the sample load could have been increased significantly [8].

In these experiments, the scale-up factor based on the column volume ratio gives the flow rate and sample load to use on the larger column to have the same separation done in the same time. However, a better resolution is frequently observed with larger rotors, showing that a greater relative solute load could have been purified. Furthermore, it is often claimed that the scale-up factor must be determined for a similar stationary phase retention ratio in the small and large columns. This is not always feasible. In addition, it may be sometimes possible to work with higher stationary phase retention ratio in the larger rotors which might allow loading even more material on the preparative column.

The aim of this work is to develop a new practical scale-up methodology, in order to exactly predict the maximum loadable quantity in the large volume preparative rotor after optimization of a purification method on a small apparatus. This methodology will be developed on hydrostatic CPC columns but it should be adaptable to any device (hydrodynamic, hydrostatic) and with no parameter constrain. Several scaling-up issues will be covered: columns behavior, loading optimization, prediction of the maximal injectable load on the larger column and finally which column operating conditions should be specifically optimized to maximize productivity.

2. Experimental section

2.1 Chemicals

All reagents were of analytical grade. Methanol, heptane and ethyl acetate were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) as well as the three solutes new coccine red, aspirin and coumarin.

2.2 Instrumentation

The instrument is a hydrostatic apparatus model, FCPC-A from Kromaton Rousselet-Robatel (Annonay, France) with interchangeable columns (or rotors). The smaller analytical column has a volume of 35 mL whereas the semi-preparative rotor has a 239-mL volume.

A refrigerated circulator F10-C Julabo (Colmar, France) was used to cool down the CPC instrument flowing chilled water in the dedicated lines. A Spot Prep II integrated system from Armen Instruments (Saint-Avé, France) a division of Gilson USA, was used. This equipment is the assembly of a quaternary pump (flow rate from 5 to 250 mL/min, maximal pressure 230 bar), an automatic loop injection valve fitted on a 10 mL or a 50 mL sample loop, a UV/VIS spectrophotometer dual wavelength set up at 254 nm and 280 nm and a fraction collector. The Armen Glider Prep software installed in the integrated computer with touch-screen allows for full apparatus control and data acquisition.

2.3 Centrifugal Partition Chromatography process

The rotor to be used, either the 35-mL small rotor or the semi-prep 239-mL rotor, was installed inside the FCPC-A equipment and rinsed with the solvent system to be employed for the tests. The refrigerated circulator was regulated at 0°C to cool the FCPC-A chamber whose temperature would otherwise increase due to heat generated by rotary seals rotation.

All experiments were realized using a three-component mixture: new coccine red (r), aspirin (A) and coumarin (M). These three test solutes belong to the GUESS list of compounds defined by Friesen [9-11]. The GUESS list is a selection of 21 natural products of very different polarities, usually used for solvents systems polarity range determination. A stock solution was prepared in mobile phase with concentrations of 2 mg/mL new coccine red, 60 mg/mL aspirin and 25 mg/mL coumarin.

The selected solvent system for all experiments was the Arizona M system [12], composed of heptane, ethyl acetate, methanol and water, in proportion of 6/5/6/5 (v/v). This solvent system was used on descending mode (non-polar stationary phase). For a given experiment, the rotor was entirely filled with the upper stationary phase at 600 rpm to have a homogeneous solvent repartition in the rotor. Then the rotation was set up at the higher speed needed for appropriate stationary phase retention. The rotational speed is chosen to have: (i) the highest stationary phase retention during injection and analysis, (ii) no flooding phenomenon and (iii) good chromatographic efficiency [13-15]. For each column, it corresponds to the maximal rotational speed that will generate a mobile phase driving pressure lower than 80 bar, the rotary seal pressure limit. The rotation speed was 2400 rpm for the 35-mL rotor and 1800 rpm for the 239-mL rotor.

After the working rotational speed was set up, the lower aqueous mobile phase was pumped through the stationary phase in the descending mode until the equilibrium was reached, i.e. when only the

lower mobile phase came out of the column and the 254 nm UV signal baseline stabilized. The choice of the mobile phase flow rate had, amongst others, a large influence on the purification duration and the stationary phase retention ratio. The flow rate could be high to shorten purification time but increasing flow rate leads to decreasing stationary phase retention, so a compromise must be found.

The stationary phase retention S_f is calculated by the ratio of the stationary phase volume (V_s) and the column volume (V_c):

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

The stationary phase volume (V_s) is deduced from the overall column (V_c) using the mobile phase volume (V_m), measured by injection of the new coccine red, a non-retained compound on this solvent system:

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

For each rotor, flow rates are chosen to have at least a 50% stationary phase retention, $S_f = 0.5$, with short purification times (detection of the most retained compound in less than 30 minutes).

Two types of injections were performed: analytical injections and preparative injections. Analytical injections consisted in the injection of a sample volume not higher than 1% column volume and a low sample concentration giving a signal/noise ratio ≥ 10 . In this way, the peaks had Gaussian appearance (Fig. 1). To satisfying the analytical conditions, the stock solution was diluted 7.5 times to have concentrations $[r] = 0.3$ mg/mL, $[A] = 8.0$ mg/mL and $[M] = 3.3$ mg/mL. Preparative injections were load-optimized injections: the injected concentration is increased to have the maximal quantity that can be injected on the rotor in the given experimental conditions. The injected solution concentration was raised up to the solubility limit of the less soluble compound to remain in the linear range of the isotherm. It corresponds in our case to the injection of the above stock solution with $[r] = 2$ mg/mL, $[A] = 60$ mg/mL and $[M] = 25$ mg/mL.

3. Results and Discussion

3.1 Free-space between peaks and maximal load

The aim of a preparative separation is to charge the maximum mass of sample on the column still recovering the largest amount of one or several compounds at a specified purity. The loading is raised increasing both the sample concentration and injected volume. For this purpose the concept of free-space available for loading is commonly used.

The free-space ΔV (Fig. 1) between two neighbor solute peaks A and B in analytical conditions can be written by:

$$\Delta V = \Delta V_r - 2(\sigma_A + \sigma_B) \quad (3)$$

ΔV is expressed in mL, σ is the volume standard deviation in mL and ΔV_r is the difference between the retention volumes of the most retained solute and the less retained solute in mL used in the definition of the resolution factor (Fig. 1). Statistically, 95% of the solute molecules can be found

within the 4- σ baseline width.. If one wishes to attain precision of 0.1%, a 6- σ baseline width can be used [16]. Because standard deviation calculation is carried out from Gaussian peaks, the ΔV value can only be estimated in analytical injections. Indeed, preparative injections produce band broadening and subsequent non Gaussian peaks on which standard deviation cannot be determined easily.

For any chromatographic system working with solid stationary phase, the maximal column loadability $V_{\max, \text{inj}}$ is proportional to the free-space ΔV that is available between two analytical solutes peaks in the linear range of isotherms [17]. In CCC chromatographic systems with liquid stationary phase, partition isotherms are generally linear over a much wider range, since the limitations are solubility-linked. In this linear range, we hypothesized that:

$$Q_{\max, \text{inj}} \propto \Delta V \quad (4)$$

A successful scale-up consists in having the same separation with the lab scale equipment and with the industrial scale one. In this way, for a specific desired purity, if the maximal injected quantity is noted $Q_{\max, \text{inj}}$, this quantity can be determined working with a small analytical rotor as $Q_{\max, \text{inj}, 1}$. Next, it should be possible to predict the high $Q_{\max, \text{inj}, 2}$ load when working with a larger rotor.

Using eq. (4) for the two rotor size considered, the ratio of the maximum injectable quantities is equal to the free-space between peaks ratio on the two columns:

$$\frac{Q_{\max, \text{inj}, 2}}{Q_{\max, \text{inj}, 1}} = \frac{\Delta V_2}{\Delta V_1} \quad (5)$$

provided that (i) the solute isotherms are in the linear range, (ii) temperature and stationary phases retention are constant for a given rotor.

Using the two free-space between peak ΔV for the two columns, it is possible to define very simply the scale-up factor, F_{SU} , that will take into account the actual behavior of the two columns:

$$F_{SU} = \frac{\Delta V_2}{\Delta V_1} \quad (6)$$

In this way, knowing the maximal injection quantity in the 35-mL rotor, the maximal injection load in the 239-mL rotor can be directly deduced:

$$Q_{\max, \text{inj}, 2} = F_{SU} \times Q_{\max, \text{inj}, 1} \quad (7)$$

3.2 Relationship between resolution R_s and free-space between peaks ΔV

It is important to establish clearly our concept of free space between peaks since chromatographers always use the resolution factor, R_s . In preparative chromatography, the Gaussian definition of the resolution factor is not used because it is not representative of the loadability of the column due to the non-Gaussian character of the peak profiles. However, the relationship between R_s and ΔV can be established. The chromatographic resolution equation is classically:

$$Rs = \frac{\Delta V_r}{(W_A + W_B)/2} \quad (8)$$

In which W is the Gaussian peak width at peak base, equal to 4σ . The resolution equation can be rewritten as:

$$Rs = \frac{\Delta V_r}{2(\sigma_A + \sigma_B)} \quad (9)$$

As illustrated by Fig. 1, the free-space ΔV between two solutes A and B can be written as:

$$\Delta V = \Delta V_r - 2\sigma_A - 2\sigma_B = 2(\sigma_A + \sigma_B) \times (Rs - 1) \quad (10)$$

As shown in the above equation, if $Rs = 1$, $\Delta V = 0$. Thus, the ΔV concept can only be used for a resolution $Rs > 1$. However, eq. 10 is only valid with Gaussian peaks i.e. in analytical separations.

3.3 Free-space between peaks measurement and load optimization in the 35-mL rotor

In order to validate the proposed "free-space between peaks" method, a scale-up was realized between two CPC columns: an analytical 35 mL column and a larger, semi-preparative 239 mL column. The ratio of these two column volumes is 6.8. The tests were realized using a mixture of three GUESS compounds. New coccine red is a brightly red-colored unretained anionic compound that will be used for stationary phase retention determination. Aspirin and coumarin are two stable and easily UV detectable compounds that will allow for separation evaluation and ΔV calculation. These two last solutes were considered as the two model compounds for purification optimization aiming for 100% purity and 100% recovery i.e. baseline separations are required between aspirin and coumarin, even in preparative injections.

The first step was carried on the small 35-mL rotor. After method development (solvent system and operating conditions selection), an analytical injection of 1% column volume of the diluted solution of new coccine red, aspirin and coumarin was realized. The free-space between the aspirin and coumarin peaks was calculated for available space ΔV_1 estimation. Fig. 2A shows the analytical injection at 2400 rpm, 5 mL/min on the 35 mL mini-rotor. Under these conditions, the stationary phase retention ratio was 50% with a measured pressure of 54 bar. The partition coefficient of aspirin and coumarin between stationary and mobile phase were calculated as 0.55 and 1.52, respectively. The free-space ΔV_1 (Eq. (3)) between aspirin and coumarin was $\Delta V_1 = 8.6$ mL.

Once ΔV_1 is determined, the load optimization must be done with the same operating conditions. In conventional preparative chromatography, the load study is usually realized raising first the injected sample concentration and then the injected volume. In this way, dispersion is minimized [18, 19]. According to eq.

(5), the stationary phase retention ratio, Sf, must be the same during analytical injections and preparative injections. So, the injected quantity should be increased without causing any stationary phase loss which is frequently observed when loading more material in a CPC column [20, 21].

Fig. 2B shows three chromatograms of the loading study. The concentration load consists in injecting the stock solution while keeping the 1% column volume injection. The coumarin maximum solubility was reached and 31 mg GUESS sample was injected. The volume load was then increased until minimal baseline return between aspirin and coumarin (touching bands), hence having 100% purity and recovery. Table 1 shows the three solutes concentration, masses and injected volumes details during the different injections. The maximal mass that can be injected is noted $Q_{\max, \text{inj}1}(\text{35-mL rotor}) = 122 \text{ mg}$.

3.4 Analytical injection in the 239-mL rotor: free-space between peaks measurement and scale-up factor calculation

With the larger instrument, the first consists also in determining the free-space between peaks, ΔV_2 , in an analytical injection on the 239-mL rotor in order to deduce the scale-up factor F_{SU} between the two columns (eq. (6)) and to predict the maximal injectable quantity. The free-space ΔV_2 and thus the scale-up factor are directly calculated on the rotor experimental behavior observations. Therefore, it is not necessary to restrain operating parameters to identical gravitational field or stationary phase retention ratio as observed in the literature [7, 22, 23]. The proposed "free-space between peaks" method allows the user to choose rotational speed and flow rate conditions according to its own specifications (pressure, solvent costs, duration, reducing equipment wear).

For illustration purposes, we set up the 239 mL rotor rotation speed at 1800 rpm and the mobile phase flow rate at 15 mL/min. These settings correspond to our instrument pressure limit acceptable for the rotary seals. Under these conditions, the stationary phase ratio was 52% for a 54 bar pressure. As for the 35-mL rotor, a 1% column volume (2.4 mL) of the diluted mixture of the three GUESS compounds (0.3 mg/mL new coccine red, 8.0 mg/mL aspirin and 3.3 mg/mL coumarin) was injected on the 239 mL rotor (Fig. 3). The free-space ΔV_2 between aspirin and coumarin was calculated as 65.4 mL.

As required by eq. (6), the scale-up factor between the 35-mL rotor and the 239-mL rotor was calculated as $F_{\text{SU}} = 65.4/8.6 = 7.6$. Hence, the maximal injection load value on the semi-prep 239-mL instrument is calculated from eq. (7) as $Q_{\max, \text{inj}, \text{maxi-rotor}} = 7.6 \times 122 = 926 \text{ mg}$. We note that our F_{SU} scaling factor is 12% higher than the simple column volume ratio ($239/35 = 6.8$).

3.5 Comparing prediction and experimental results

The free-space between peaks method predicted a 926-mg maximal injectable quantity on the 239-mL rotor corresponding to an injection of 10.7 mL volume of the stock solution, and the developed chromatogram should be similar to that obtained on the small 35-mL rotor.

Fig. 4 shows the 926 mg preparative injection of 11 mL stock solution of the three GUESS solute mixture in comparison to the 122-mg preparative injection done with the analytical 35-mL instrument. The chromatograms are similar. A baseline return between the two peaks of interest is observed and the 100% purity and recovery criteria are both respected. Therefore, for a 6.8 column ratio volume, it has been possible to inject 7.6 times more on the larger 239-mL rotor without constraint on the operating conditions (Table 2) other than equipment constraints (maximum pressure on the rotary seals). Under these operating conditions and through this methodology, it was possible to increase the productivity by a factor 3.

If the simple experiments realized with a test mixture confirmed the hypothesis that the "free-space between peaks" ratio allows for the calculation of a scale-up factor with a loading study done on the analytical small instrument. This experimental scale-up factor permits to predict the quantity that should be injected to have the same separation on the larger preparative column, with the same purity and the same recovery, adapting the operating conditions to the equipment used. Nevertheless, this prediction has some limitations: the temperature must be controlled during all injections (both rotors) and the stationary phase ratios (S_f factors) must be maintained between the analytical and preparative injections on the same rotor. Indeed, if the temperature varies, the solutes partition coefficients will change distorting the free-space between peaks ΔV volume. Similarly, if the S_f ratio is different between the analytical and the preparative injection due to stationary phase different bleed, the solutes retention volumes will be different and the scaling-up will be compromised. Eventually, in some particular cases, it is not possible to determinate the ΔV value. If the resolution between the two peaks of interest is lower than 1, peaks overlap and there is no free-space between peaks and, obviously, no possible purification.

3.6 Best operating conditions for maximal load or for maximal productivity

One of the most important points before scaling-up a purification method is the operating conditions that must be set up in the higher volume column according to industrial objectives. There are two working strategies: the user can realize the purification in one large batch by loading a maximal sample quantity (maximal load per run) or can perform this purification in several smaller injections done faster to produce more quantity in less time (maximal productivity).

Two parameters can be adjusted with CPC columns: the rotor rotation speed and the mobile phase flow rate. Increasing the rotor rotation speed allows for a higher S_f and efficiency [13, 14]. Increasing the mobile phase flow rate shortens the purification time and increases productivity.

For maximum stationary phase retention ratio, S_f , and efficiency, N , the rotor rotation speed should be the highest possible, limited by the motor capabilities but also by the experimental driving pressure that should stay below 80 bar in order to safeguard the rotating seals. The retention volume of a compound, V_r , is given by:

$$V_r = V_m + K_D \cdot V_s \quad (11)$$

in which V_m and V_s are respectively the mobile phase volume (or dead volume) and the stationary phase volume. K_D is the partition coefficient of the compound between the mobile phase and the stationary phase. Thus, the difference between the retention volumes of two compounds is:

$$\Delta V_r = V_s \cdot \Delta K_D \quad (12)$$

Combining eqs (1) and (12), we obtain:

$$\Delta V_r = V_C \cdot S_f \cdot \Delta K \quad (13)$$

Eq. 13 shows that ΔV_r is directly proportional to Sf . So, it seems that the maximum Sf is desirable to obtain the maximum ΔV_r , confirming the highest possible rotor rotation speed. Since high rpm also produces thinner peaks (higher efficiency), it reduces the σ values in eq. 3 also increasing the ΔV_r difference. This is another reason to work at the highest possible rpm.

The second adjustable parameter is the mobile phase flow rate. Fig. 5A shows the ΔV evolution with flow rate variations, for a 1 mL injection of the diluted solution of new coccine red, aspirin and coumarin on the 239-mL rotor (Gaussian conditions). As the flow rate increased, the free space between the aspirin and coumarin peaks gradually decreased. Indeed, despite an efficiency increase, the faster flow of mobile phase expels some stationary phase reducing Sf , hence reducing ΔV_r (eq. 13). Thus, to load a maximal sample amount in a single elution run, it is better to apply a lower flow rate to have a higher free-space between peaks of interest. For the 239-mL rotor and the maximum 1800 rpm possible speed, a 15 mL/min flow rate was therefore appropriate to realize the maximal load in one purification run as shown by Fig. 3.

The free-space between peaks ΔV value can also be related to the duration t of the purification. The ratio $\Delta V/t$ will give an idea of the column productivity. Fig. 5B shows the $\Delta V/t$ evolution versus the mobile phase flow rate during analytical injections (Gaussian conditions) on the 35-mL and 239-mL rotors. The plots show that there is an optimum flow rate for which the $\Delta V/t$ ratio and thus the productivity are maximal. At flow rates lower than the optimum, the ΔV value is larger so it is possible to load a larger amount of sample but the time needed to complete the separation is long so that the productivity is poor. At flow rates higher than the optimal value, the stationary phase loss is so important that the column cannot be highly loaded so that the productivity is also poor. The best productivity in the practical cases presented was obtained at 12.5 mL/min flow rate and 2400 rpm for the 35-mL rotor and 35 mL/min and 1800 rpm for the 239-mL rotor.

A new scale-up is realized between the 35-mL and 239-mL rotor using these two optimized flow rates. First, the maximal load on the 35-mL rotor at 2400 rpm and 12.5 mL/min can be directly predicted by using our transfer method within the very same rotor but at different operating conditions. The ΔV value from the analytical injection (1% column volume and diluted solution) on the 35-mL column at 2400 rpm and 12.5 mL/min was measured as 4.8 mL. Comparing with the previous results at 5 mL/min, the ΔV ratio in these two flow rates conditions gives a transfer factor, F_{SU} , of 0.56 (Table 2 **Erreur ! Source du renvoi introuvable.**) a value lower than 1 because the ΔV_2 volume is lower than the ΔV_1 volume (eq. (6)). The maximal injectable quantity on the 35-mL rotor at 5 mL/min is only 65 mg at 12.5 mL/min. Following this prediction, 65 mg of the stock solution were injected on the 35-mL rotor at 2400 rpm and 12.5 mL/min flow rate producing the expected chromatogram with touching bands (Fig. 6A **Erreur ! Source du renvoi introuvable.**).

The maximal load on the 239-mL rotor at 35 mL/min can be calculated from any conditions tested on the 35-mL rotor provided that the "free-space between peaks" volume ΔV is measured on the 239-mL column. We chose to do the prediction with the 12.5 mL/min flow rate conditions listed in Table 2 **Erreur ! Source du renvoi introuvable.** for the small analytical 35-mL rotor with the measured ΔV value of 4.8 mL. Following the method, an analytical injection of the diluted solution on the 239-mL rotor at 1800 rpm and 35 mL/min returned a ΔV value of 56.6 mL which allows for the straightforward calculation of the scale-up factor $F_{SU} = 56.6/4.8 = 11.8$ (Table 2), a factor almost twice bigger than the simple column volume ratio. Multiplying this scale-up factor F_{SU} by the 65 mg maximal load on the 35-mL rotor allows to predict a 766 mg maximal load on the 239-mL rotor at 35 mL/min (Table 2). The Fig. 6B chromatogram shows the touching band baseline separation of the injected 766 mg material. The two chromatograms on the two different size instruments are similar (Fig. 6). Table 2 summarizes the results obtained for both rotors at maximal productivities. It is

pointed out that productivities were calculated only based on solute elution time. It might be interesting to calculate productivities while taking into account delays induced by stationary phase loads and equilibration durations.

The key point of this study is to demonstrate that the prediction of the maximal quantity that could be injected on rotors of different scales depends on the optimal operating conditions that themselves depend on the production strategy. We developed an example on two different instruments: a small 35-mL one and a larger 239-mL instrument with two different objectives: (i) to purify a batch by loading the maximal possible mass of sample in one run with the largest possible "free-space between peaks", ΔV , regardless of the duration of the purification or (ii) to optimize both the injected mass and the mobile phase flow rate so that several smaller injections can be done quickly, producing more purified material in less time, i.e. optimizing the productivity $\Delta V/t$ ratio.

In real cases, the optimal flow rate determination on the small rotor may not be needed because the aims of working on a small rotor are only method development and loading study. On the contrary, a large rotor must be used for increased productivity so it is best to work at the optimal flow rate. The determination of this optimal flow rate on the preparative instrument will require 4 or 5 analytical injections.

4. Conclusion

Fig. 7 gathers the essentials of the scaling-up method proposed for countercurrent chromatography instruments. Once the separation method is developed, a load optimization on a small and fast lab-scale rotor must be done. Next, the proposed "free-space between peaks" method requires only a single analytical injection on the large-scale production rotor to be able to predict the maximal productivity.

The method is only based on experimental observations; that is why it can be applied between any rotors or within a single rotor between operating conditions. If the transfer is possible at any operating conditions, provided that they give stable stationary phase retention and identical temperatures, it also indicates optimal operating conditions for batch or productivity strategies.

The method does have limitations:

- The stationary phase retention ratio, S_f , must be similar between analytical and preparative injections and should not change during runs.
- Resolution factors higher than 1 are required to have some space between peaks (positive ΔV).
- The method applies only to classical elution mode. Its uses in different modes such as the pH-zone refining mode or elution-extrusion have to be studied yet.

Acknowledgments

K.F. and A.B. thank the French Centre National de la Recherche Scientifique (CNRS, UMR 5280 ISA) for continuous support. E.B. thanks the Kromaton Rousselet-Robatel Company and the French Association Nationale de la Recherche et de la Technologie for a three-year CIFRE PhD grant.

References

- 427 1. A. Berthod, *Countercurrent chromatography: the support-free liquid stationary phase*.
428 Comprehensive analytical chemistry, Vol. 38 (Elsevier, 2002).
- 429 2. W. D. Conway, *Countercurrent chromatography: apparatus, theory, and applications*. (VCH,
430 1990).
- 431 3. Y. Ito, W. D. Conway, *High-speed countercurrent chromatography*. Chemical analysis (J.
432 Wiley, 1996).
- 433 4. A. Berthod, T. Maryutina, B. Spivakov, O. Shpigun, I. A. Sutherland, Countercurrent
434 chromatography in analytical chemistry (IUPAC Technical Report). *Pure Appli Chem.* **81**, 355-
435 387 (2009).
- 436 5. M. C. Menet, D. Thiebaut, Preparative purification of antibiotics for comparing hydrostatic
437 and hydrodynamic mode counter-current chromatography and preparative high-
438 performance liquid chromatography. *J. Chromatogr. A* **831**, 203-216 (1999).
- 439 6. K. Faure, N. Mekaoui, J. Meucci, A. Berthod, Solvent selection in countercurrent
440 chromatography using small-volume hydrostatic columns. *LC-GC North America* **31**, 132-143
441 (2013).
- 442 7. H. Luo, M. Peng, H. Ye, L. Chen, A. Peng, M. Tang, F. Zhang, J. Shi, Predictable and linear
443 scale-up of four phenolic alkaloids separation from the roots of *Menispermum dauricum*
444 using high-performance counter-current chromatography. *J. Chromatogr. B* **878**, 1929-1933
445 (2010).
- 446 8. I. A. Sutherland, G. Audo, E. Bourton, F. Couillard, D. Fisher, I. Garrard, P. Hewiston, O. Intes,
447 Rapid linear scale-up of a protein separation by centrifugal partition chromatography. *J.*
448 *Chromatogr. A* **1190**, 57-62 (2008).
- 449 9. J. B. Friesen, G. F. Pauli, GUESS—A generally useful estimate of solvent systems for CCC. *J.*
450 *Liq. chromatogr. Rel. Technol.* **28**, 2777-2806 (2005).
- 451 10. J. B. Friesen, G. F. Pauli, Rational development of solvent system families in counter-current
452 chromatography. *J. Chromatogr. A* **1151**, 51-59 (2007).
- 453 11. J. B. Friesen, S. Ahmed, G. F. Pauli, Qualitative and quantitative evaluation of solvent systems
454 for countercurrent separation. *J. Chromatogr. A* **1377**, 55-63 (2015).
- 455 12. A. Berthod, M. Hassoun, M. J. Ruiz-Angel, Alkane effect in the Arizona liquid systems used in
456 countercurrent chromatography. *Anal. bioanal. chem.* **383**, 327-340 (2005).
- 457 13. L. Marchal, A. Foucault, G. Patissier, J. M. Rosant, J. Legrand, Influence of flow patterns on
458 chromatographic efficiency in centrifugal partition chromatography. *J. Chromatogr. A* **869**,
459 339-352 (2000).
- 460 14. L. Marchal, J. Legrand, A. Foucault, Mass transport and flow regimes in centrifugal partition
461 chromatography. *AIChE j.* **48**, 1692-1704 (2002).
- 462 15. D. W. Armstrong, G. L. Bertrand, A. Berthod, Study of the origin and mechanism of band
463 broadening and pressure drop in centrifugal countercurrent chromatography. *Anal. Chem.*
464 **60**, 2513-2519 (1988).
- 465 16. E. Heftmann, *Chromatography: Fundamentals and techniques*. Chromatography:
466 Fundamentals and Applications of Chromatography and Related Differential Migration
467 Methods (Elsevier Scientific Publishing Company, 2004).
- 468 17. P. Gareil, R. Rosset, Analysis of the information in a preparative chromatogram for further
469 optimization of the operating conditions. *J. Chromatogr.* **450**, 13-25 (1988).

- 470 18. P. Gareil, C. Durieux, R. Rosset, Optimization of Production Rate and Recovered Amount in
471 Linear and Nonlinear Preparative Elution Liquid Chromatography. *Sep. Sci. Technol.* **18**, 441
472 (1983).
- 473 19. A. Berthod, D. W. Armstrong, Centrifugal Partition Chromatography. IV. Preparative Sample
474 Purification and Partition Coefficient Determination. *J Liq. Chromatogr.* **11**, 1187-1204 (1988).
- 475 20. L. Marchal *et al.*, Rational improvement of centrifugal partition chromatographic settings for
476 the production of 5-n-alkylresorcinols from wheat bran lipid extract: I. Flooding conditions—
477 optimizing the injection step. *J. Chromatogr. A* **1005**, 51-62 (2003).
- 478 21. L. Chen *et al.*, Rapid purification and scale-up of honokiol and magnolol using high-capacity
479 high-speed counter-current chromatography. *J. Chromatogr. A* **1142**, 115-122 (2007).
- 480 22. P. Wood *et al.*, Counter-current chromatography separation scaled up from an analytical
481 column to a production column. *J. Chromatogr. A* **1151**, 25-30 (2007).
- 482 23. H. Ye *et al.*, Preparative isolation and purification of three rotenoids and one isoflavone from
483 the seeds of *Millettia pachycarpa* Benth by high-speed counter-current chromatography. *J.*
484 *Chromatogr. A* **1178**, 101-107 (2008).
- 485 24. S. Chollet *et al.*, Methodology for optimally sized centrifugal partition chromatography
486 columns. *J. Chromatogr. A* **1388**, 174-183 (2015).
- 487
- 488

489
490
491
492
493
494

Fig. 1. Representation of the separation of two Gaussian peaks illustrating the concept of "free space between peaks", ΔV , compared to the retention difference, ΔV_r , used in the definition of the chromatographic resolution.

495
496
497

Fig. 2. A- 35-mL rotor analytical injection of 0.36 mL (1% column volume) of 0.3 mg/mL new coccine red (**r**), 8.0 mg/mL aspirin (**A**) and 3.3 mg/mL coumarin (**M**) in descending mode. **B-** 35-mL rotor optimization injections: analytical injection, concentration load and volume load. Solute concentrations, injected volumes and mass load are listed in Table 1. Column volume: 35 mL, descending mode, flow rate : 5 mL/min, system Arizona M, heptane/ethyl acetate/methanol/water 6/5/6/5 v/v, 2400 rpm, stationary phase retention: 50% , 54 bar.

506
507
508
509
511

512
513
514
515
516
517
518
519

Fig. 3. 239-mL rotor analytical injection of 0.3 mg/mL of new coccine red (**r**), 8.0 mg/mL aspirin (**A**) and 3.3 mg/mL coumarin (**M**). Column volume: 239 mL, injection volume 2.5 mL (1% column volume), injected mass: 29 mg, flow rate : 15 mL/min, rotational speed: 1800 rpm, stationary phase retention: 52%, P = 54 bar, descending mode, system Arizona M, heptane/ethyl acetate/methanol/water 6/5/6/5 v/v.

Fig. 4. (left) 35-mL rotor preparative injections of 1.4 mL of the 2 mg/mL new cocchine red (**r**), 60 mg/mL aspirin (**A**) and 25 mg/mL coumarin (**M**) solution in descending mode, flow rate: 5 mL/min; (right) 239-mL rotor preparative injection of 11 mL of the same solutions, flow rate : 15 mL/min, system Arizona M, heptane/ethyl acetate/methanol/water 6/5/6/5 v/v. All other experimental conditions are listed in Table 2.

Fig. 5. Effect of the flow rate on the "free-space between peaks" interval. **A-** Free-space between aspirin and coumarin peaks plotted versus the mobile phase flow rate at 1800 rpm on the 235-mL rotor (1 mL injection). **B-** Evolution of the ratio of the free-space ΔV between aspirin and coumarin over the time needed to complete the separation. Left: 35-mL rotor at 2400 rpm with 0.5 mL injections; right: 239-mL rotor at 1800 rpm with 1 mL injections.

Fig. 6. The "free-space between peaks" optimization of the productivity factor. Injections of 2.0 mg/mL new coccine red (r), 60.0 mg/mL aspirin (A) and 25 mg/mL coumarin (M) solution in descending mode, system Arizona M, heptane/ethyl acetate/methanol/water 6/5/6/5 v/v. **A-** 35mL rotor, injection volume: 0.75 mL and **B-** 239-mL rotor preparative. injection volume: 8.8 mL. All other experimental conditions are listed in Table 2.

Fig. 7. Sketch for the "free-space between peaks" methods. The scaling-up is based on the experimentally measured free-space ΔV between solute peaks with the same liquid system used on the analytical column (Column 1) and the larger volume preparative column (Column 2).

	Analytical injection	Concentration load	Volume load
New coccine red [r]	0.3	2.0	2.0
Aspirin [A]	8.0	25.0	25.0
Coumarin [M]	3.3	60.0	60.0
Inj. vol. (mL)	0.36	0.36	1.4
Inj. mass (mg)	4	31	122

Table 1. Solute concentrations in mg/mL, injected volumes and masses during analytical injection, concentration load and volume load as illustrated by Fig. 2.

	<i>Analytical reference</i>				<i>Operating change transfer</i>		
	<i>35-mL rotor</i>	<i>Ratio</i>	<i>239-mL rotor</i>	<i>Ratio</i>	<i>35-mL rotor</i>	<i>Ratio</i>	<i>239-mL rotor</i>
V_{column}	35 mL	6.8	239 mL	1	35 mL	6.8	239 mL
Flow rate	5 mL/min	3	15 mL/min	2.5	12.5 mL/min	2.8	35 mL/min
Rotational speed	2400 rpm	-	1800 rpm	-	2400 rpm	-	1800 rpm
Centrifugal field, g	648 m/s ²	-	365 m/s ²	-	648 m/s ²	-	365 m/s ²
Pressure	54 bar	-	54 bar	-	54 bar	-	52 bars
Stationary phase ratio, Sf	50 %	-	52 %	-	28 %	-	42%
Free space between peaks, ΔV	8.6 mL	F_{SV}= 7.6	65.4 mL	F_{SV}= 0.56^a	4.8 mL ^a	F_{SV}= 11.8	56.6 mL
Maximum load, Q_{max,inj}	122 mg	7.8	957 mg	1.9	65 mg	11.8	766 mg
Purification time	11.3 min	2.4	27.4 min	2.5	4.5 min	2.2	10.1 min
Productivity	648 mg/h	3.2	2095 mg/h	1.3	867 mg/h	5.2	4550 mg/h

a) In transferring from the 35-mL rotor run at 5 mL/min to the increased flow rate of 12.5 mL/min, the "free-space between peaks ΔV₂ is smaller producing a F_{SV} factor lower than 1.

Table 2. Data for the "free-space between peaks" method using the three GUESS solute test mixture. Operating conditions of the Fig. 4 scale-up example, including productivity capacities of the two columns for the model Guess mixture used.