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

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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

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

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

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

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

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

86

87 **2. Experimental section**

88

89 *2.1 Chemicals*

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

93

94 *2.2 Instrumentation*

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

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

106

107 *2.3 Centrifugal Partition Chromatography process*

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

112 All experiments were realized using a three-component mixture: new coccine red (r), aspirin (A) and
113 coumarin (M). These three test solutes belong to the GUESS list of compounds defined by Friesen [9-
114 11]. The GUESS list is a selection of 21 natural products of very different polarities, usually used for
115 solvents systems polarity range determination. A stock solution was prepared in mobile phase with
116 concentrations of 2 mg/mL new coccine red, 60 mg/mL aspirin and 25 mg/mL coumarin.

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

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

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

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

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

136

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

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

140

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

143 Two types of injections were performed: analytical injections and preparative injections. Analytical
144 injections consisted in the injection of a sample volume not higher than 1% column volume and a low
145 sample concentration giving a signal/noise ratio ≥ 10 . In this way, the peaks had Gaussian appearance
146 (Fig. 1). To satisfying the analytical conditions, the stock solution was diluted 7.5 times to have
147 concentrations $[r] = 0.3$ mg/mL, $[A] = 8.0$ mg/mL and $[M] = 3.3$ mg/mL. Preparative injections were
148 load-optimized injections: the injected concentration is increased to have the maximal quantity that
149 can be injected on the rotor in the given experimental conditions. The injected solution concentration
150 was raised up to the solubility limit of the less soluble compound to remain in the linear range of the
151 isotherm. It corresponds in our case to the injection of the above stock solution with $[r] = 2$ mg/mL,
152 $[A] = 60$ mg/mL and $[M] = 25$ mg/mL.

153

154 3. Results and Discussion

155

156 3.1 Free-space between peaks and maximal load

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

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

163

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

165

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

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

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

179

$$181 \quad Q_{\max.inj} \propto \Delta V \quad (4)$$

182

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

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

$$190 \quad \frac{Q_{\max.inj,2}}{Q_{\max.inj,1}} = \frac{\Delta V_2}{\Delta V_1} \quad (5)$$

195

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

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

200

$$201 \quad F_{SU} = \frac{\Delta V_2}{\Delta V_1} \quad (6)$$

202

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

205

$$206 \quad Q_{\max.inj,2} = F_{SU} \times Q_{\max.inj,1} \quad (7)$$

207

208 *3.2 Relationship between resolution R_s and free-space between peaks ΔV*

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

214

215

216

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

217

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

220

221

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

222

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

224

225

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

226

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

229

230 3.3 Free-space between peaks measurement and load optimization in the 35-mL rotor

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

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

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

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

255 Fig. 2B shows three chromatograms of the loading study. The concentration load consists in
256 injecting the stock solution while keeping the 1% column volume injection. The coumarin maximum
257 solubility was reached and 31 mg GUESS sample was injected. The volume load was then increased
258 until minimal baseline return between aspirin and coumarin (touching bands), hence having 100%
259 purity and recovery. Table 1 shows the three solutes concentration, masses and injected volumes
260 details during the different injections. The maximal mass that can be injected is noted $Q_{\max, \text{inj}1}(\text{35-mL}$
261 $\text{rotor}) = 122 \text{ mg}$.

262

263 3.4 Analytical injection in the 239-mL rotor: free-space between peaks measurement and scale-up 264 factor calculation

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

273 For illustration purposes, we set up the 239 mL rotor rotation speed at 1800 rpm and the mobile
274 phase flow rate at 15 mL/min. These settings correspond to our instrument pressure limit acceptable
275 for the rotary seals. Under these conditions, the stationary phase ratio was 52% for a 54 bar pressure.
276 As for the 35-mL rotor, a 1% column volume (2.4 mL) of the diluted mixture of the three GUESS
277 compounds (0.3 mg/mL new coccine red, 8.0 mg/mL aspirin and 3.3 mg/mL coumarin) was injected
278 on the 239 mL rotor (Fig. 3). The free-space ΔV_2 between aspirin and coumarin was calculated as
279 65.4 mL.

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

284

285 3.5 Comparing prediction and experimental results

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

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

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

311

312 *3.6 Best operating conditions for maximal load or for maximal productivity*

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

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

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

325

$$326 \quad V_r = V_m + K_D \cdot V_s \quad (11)$$

327

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

331

$$332 \quad \Delta V_r = V_s \cdot \Delta K_D \quad (12)$$

333

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

335

$$336 \quad \Delta V_r = V_C \cdot S_f \cdot \Delta K \quad (13)$$

337

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

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

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

361 A new scale-up is realized between the 35-mL and 239-mL rotor using these two optimized flow
362 rates. First, the maximal load on the 35-mL rotor at 2400 rpm and 12.5 mL/min can be directly
363 predicted by using our transfer method within the very same rotor but at different operating conditions.
364 The ΔV value from the analytical injection (1% column volume and diluted solution) on the 35-mL
365 column at 2400 rpm and 12.5 mL/min was measured as 4.8 mL. Comparing with the previous results
366 at 5 mL/min, the ΔV ratio in these two flow rates conditions gives a transfer factor, F_{SU} , of 0.56 (Table
367 **2Erreur ! Source du renvoi introuvable.**) a value lower than 1 because the ΔV_2 volume is lower than
368 the ΔV_1 volume (eq. (6)). The maximal injectable quantity on the 35-mL rotor at 5 mL/min is only 65
369 mg at 12.5 mL/min. Following this prediction, 65 mg of the stock solution were injected on the 35-mL
370 rotor at 2400 rpm and 12.5 mL/min flow rate producing the expected chromatogram with touching
371 bands (Fig. 6A**Erreur ! Source du renvoi introuvable.**).

372 The maximal load on the 239-mL rotor at 35 mL/min can be calculated from any conditions tested
373 on the 35-mL rotor provided that the "free-space between peaks" volume ΔV is measured on the 239-
374 mL column. We chose to do the prediction with the 12.5 mL/min flow rate conditions listed in Table
375 **2Erreur ! Source du renvoi introuvable.** for the small analytical 35-mL rotor with the measured ΔV
376 value of 4.8 mL. Following the method, an analytical injection of the diluted solution on the 239-mL
377 rotor at 1800 rpm and 35 mL/min returned a ΔV value of 56.6 mL which allows for the
378 straightforward calculation of the scale-up factor $F_{SU} = 56.6/4.8 = 11.8$ (Table 2), a factor almost twice
379 bigger than the simple column volume ratio. Multiplying this scale-up factor F_{SU} by the 65 mg
380 maximal load on the 35-mL rotor allows to predict a 766 mg maximal load on the 239-mL rotor at 35
381 mL/min (Table 2). The Fig. 6B chromatogram shows the touching band baseline separation of the
382 injected 766 mg material. The two chromatograms on the two different size instruments are similar
383 (Fig. 6). Table 2 summarizes the results obtained for both rotors at maximal productivities. It is

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

387 The key point of this study is to demonstrate that the prediction of the maximal quantity that could
388 be injected on rotors of different scales depends on the optimal operating conditions that themselves
389 depend on the production strategy. We developed an example on two different instruments: a small
390 35-mL one and a larger 239-mL instrument with two different objectives: (i) to purify a batch by
391 loading the maximal possible mass of sample in one run with the largest possible "free-space between
392 peaks", ΔV , regardless of the duration of the purification or (ii) to optimize both the injected mass and
393 the mobile phase flow rate so that several smaller injections can be done quickly, producing more
394 purified material in less time, i.e. optimizing the productivity $\Delta V/t$ ratio.

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

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401 **4. Conclusion**

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

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

411 The method does have limitations:

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

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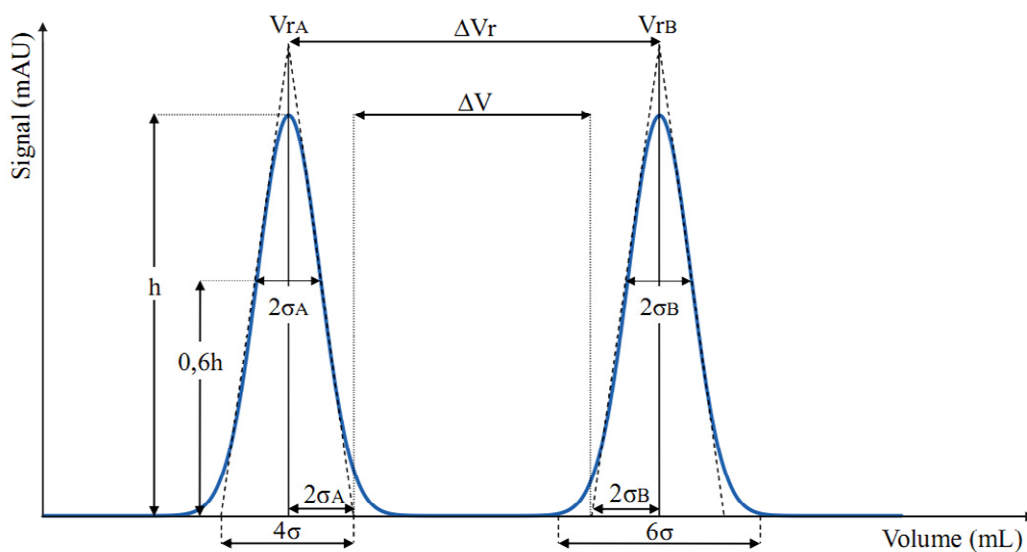
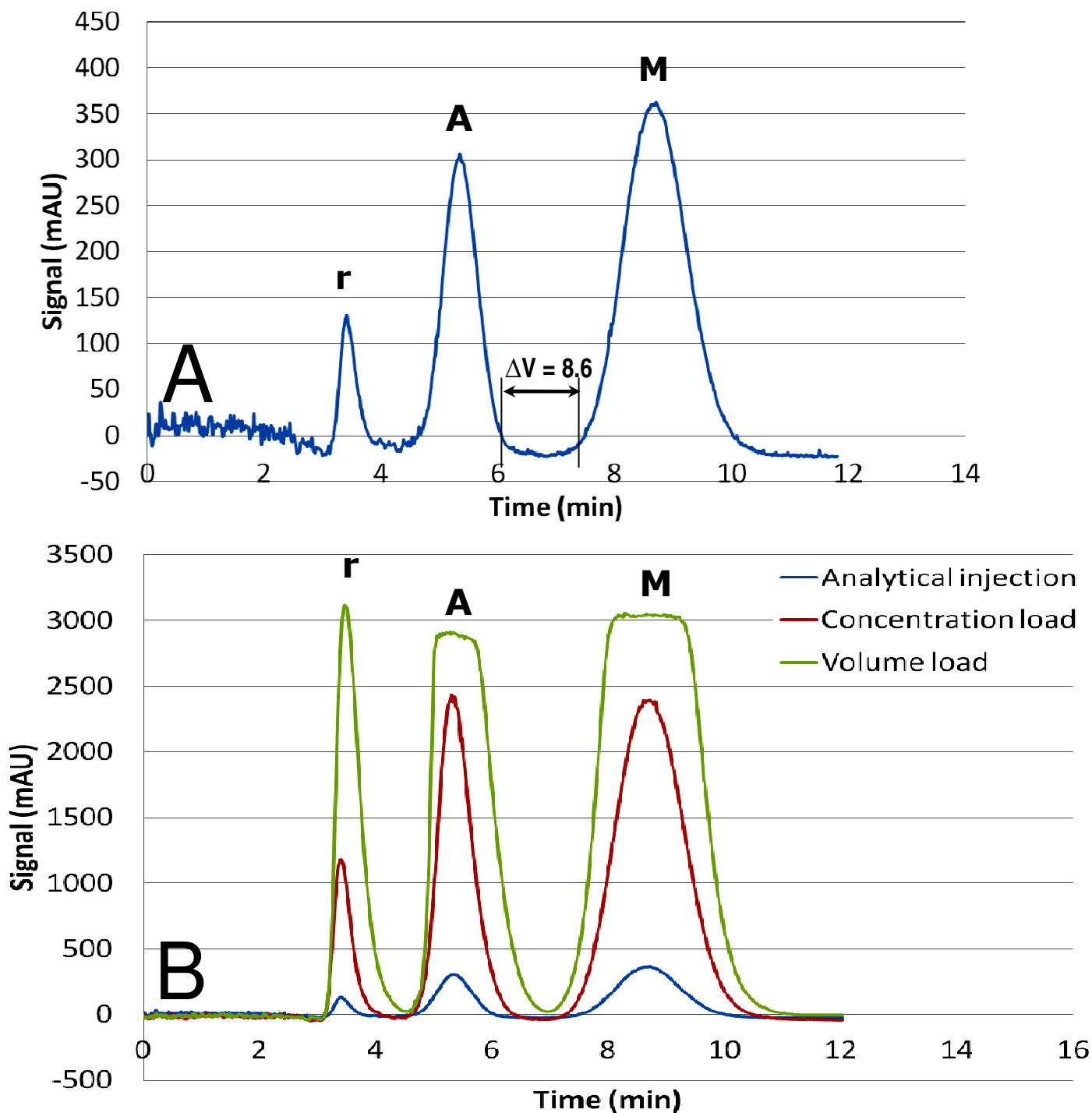


Fig. 1. Representation of the separation of two Gaussian peaks illustrating the concept of "free space between peaks", ΔV , compared to the retention difference, ΔV_r , used in the definition of the chromatographic resolution.

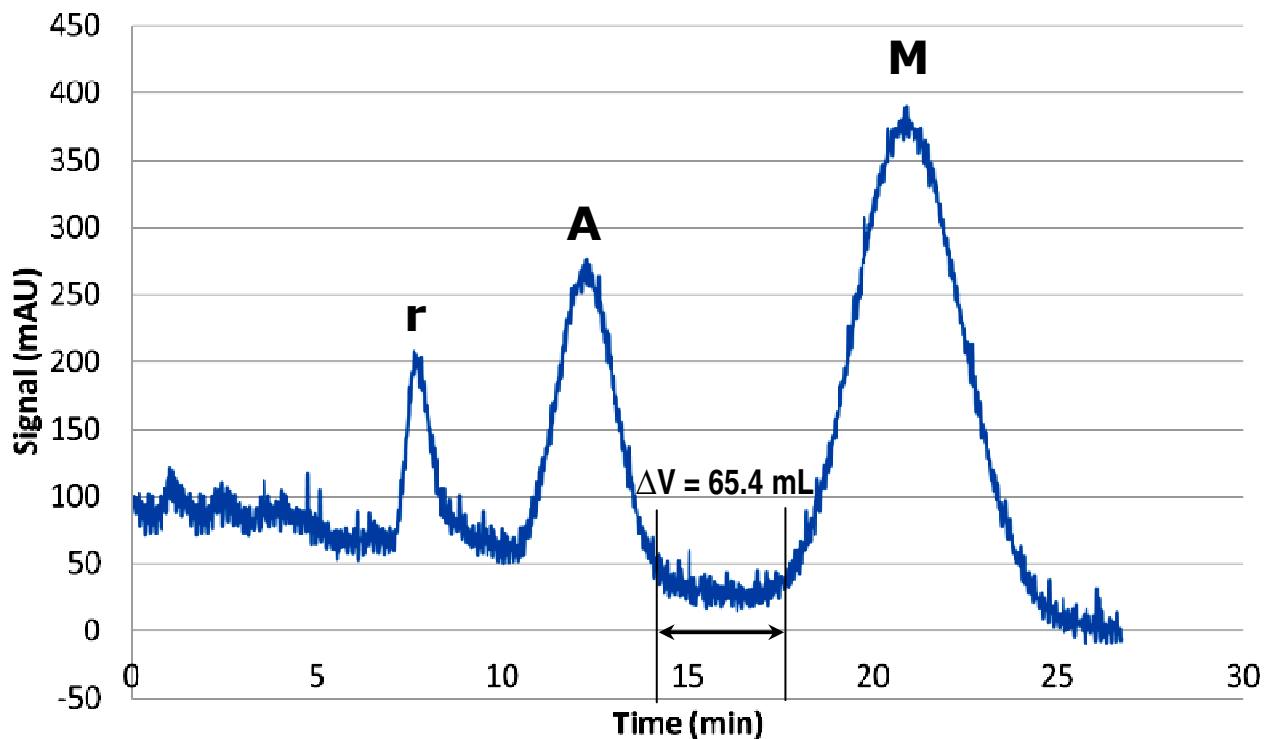
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500 **Fig. 2. A-** 35-mL rotor analytical injection of 0.36 mL (1% column volume) of 0.3 mg/mL new
 501 coccine red (**r**), 8.0 mg/mL aspirin (**A**) and 3.3 mg/mL coumarin (**M**) in descending mode. **B-** 35-mL
 502 rotor optimization injections: analytical injection, concentration load and volume load. Solute
 503 concentrations, injected volumes and mass load are listed in Table 1. Column volume: 35 mL,
 504 descending mode, flow rate : 5 mL/min, system Arizona M, heptane/ethyl acetate/methanol/water
 505 6/5/6/5 v/v, 2400 rpm, stationary phase retention: 50% , 54 bar.

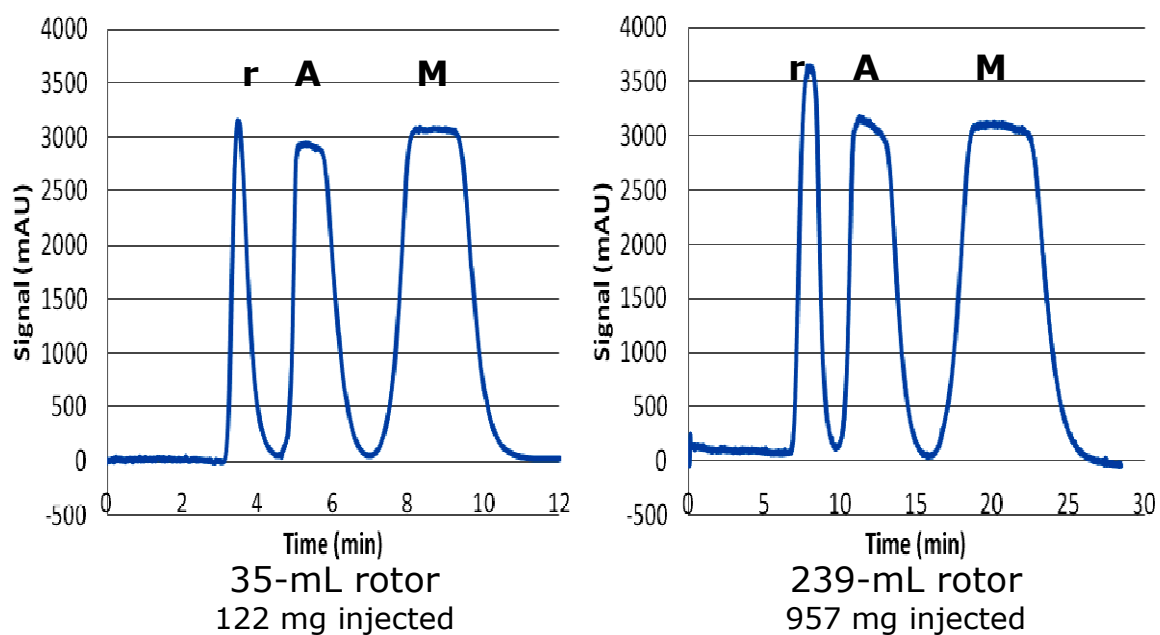
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Fig. 3. 239-mL rotor analytical injection of 0.3 mg/mL of new coccine red (**r**), 8.0 mg/mL aspirin (**A**) and 3.3 mg/mL coumarin (**M**). Column volume: 239 mL, injection volume 2.5 mL (1% column volume), injected mass: 29 mg, flow rate : 15 mL/min, 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.

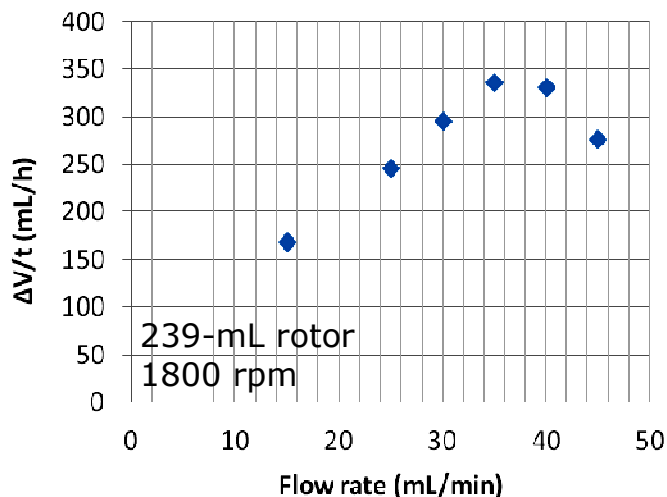
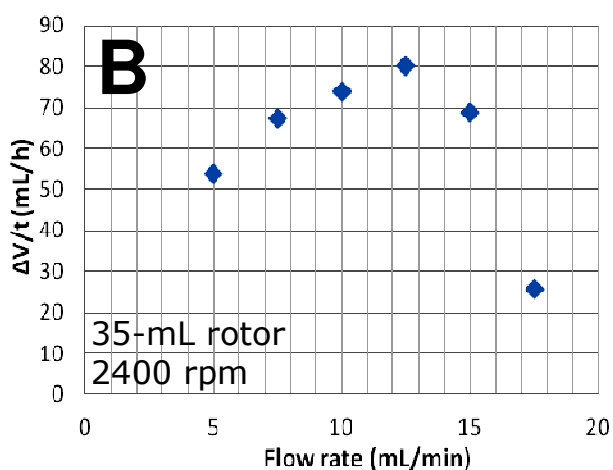
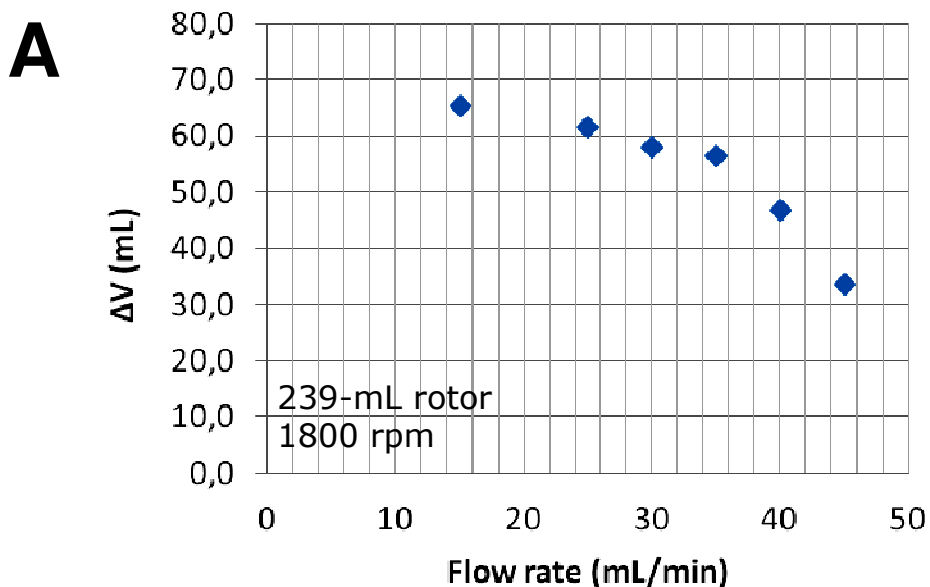
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549 **Fig. 4.** (left) 35-mL rotor preparative injections of 1.4 mL of the 2 mg/mL new coccine red (**r**), 60
550 mg/mL aspirin (**A**) and 25 mg/mL coumarin (**M**) solution in descending mode, flow rate: 5 mL/min;
551 (right) 239-mL rotor preparative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

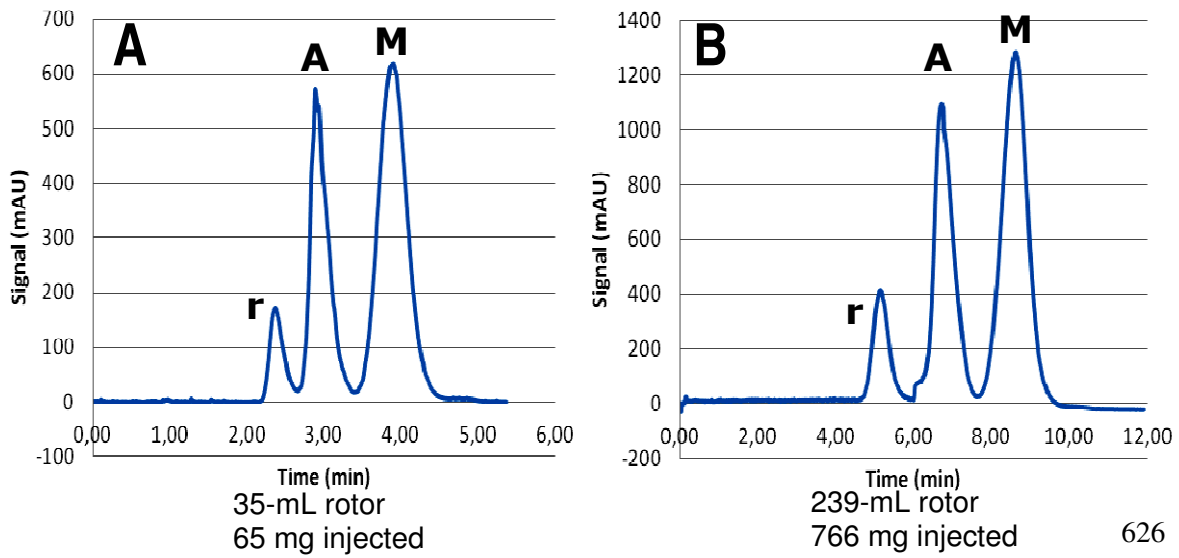
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Fig. 5. Effect of the flow rate on the "free-space between peaks" interval. **A-** Free-space between aspirin and coumarin peaks plotted versus the mobile phase flow rate at 1800 rpm on the 235-mL rotor (1 mL injection). **B-** Evolution of the ratio 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.

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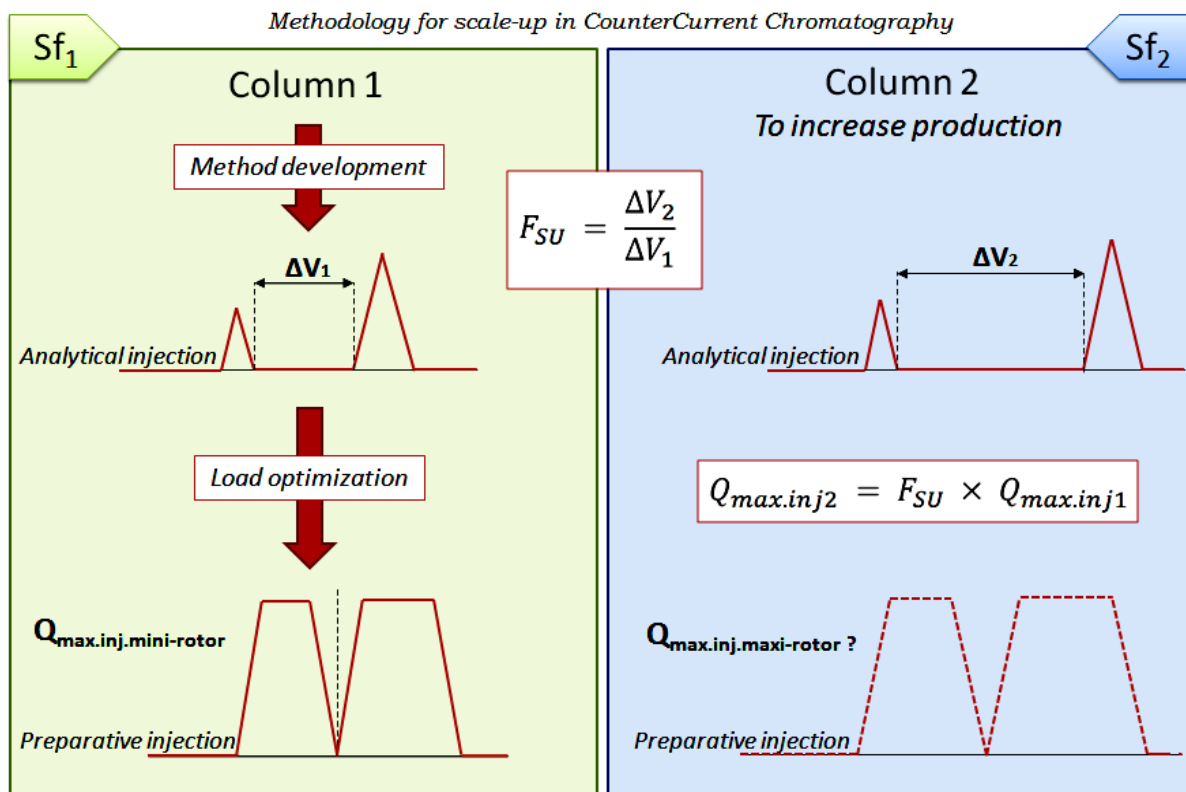


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628 **Fig. 6.** The "free-space between peaks" optimization of the productivity factor. Injections of 2.0
629 mg/mL new coccine red (r), 60.0 mg/mL aspirin (A) and 25 mg/mL coumarin (M) solution in
630 descending mode, system Arizona M, heptane/ethyl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

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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).

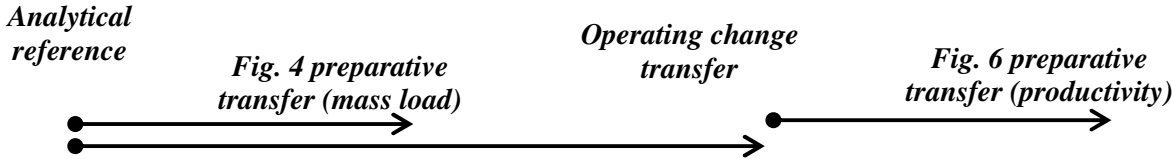
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| | Analytical injection | Concentration load | Volume load |
|---------------------|----------------------|--------------------|-------------|
| New coccine red [r] | 0.3 | 2.0 | 2.0 |
| Aspirin [A] | 8.0 | 25.0 | 25.0 |
| Coumarin [M] | 3.3 | 60.0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

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

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| | <i>Analytical reference</i> | | <i>Fig. 4 preparative transfer (mass load)</i> | | <i>Operating change transfer</i> | | <i>Fig. 6 preparative transfer (productivity)</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_{SU}= 7.6 | 65.4 mL | F_{SU}= 0.56^a | 4.8 mL ^a | F_{SU}= 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 | |

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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" ΔV_2 is smaller producing a F_{SU} factor lower than 1.

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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.

