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► **To cite this version:**

Elodie Bouju, Alain Berthod, Karine Faure. Scale-up in centrifugal partition chromatography: The “free-space between peaks” method. *Journal of Chromatography A*, 2015, 1409, pp.70-78. 10.1016/j.chroma.2015.07.020 . hal-01186354

**HAL Id: hal-01186354**

**<https://hal.science/hal-01186354>**

Submitted on 2 Sep 2020

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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,  $\Delta V_1$ , and with the large preparative one,  $\Delta V_2$ . 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  $\geq 10$ . 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  $\Delta V$  (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  $\Delta V$  is expressed in mL,  $\sigma$  is the volume standard deviation in mL and  $\Delta V_r$  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- $\sigma$  baseline width.. If one wishes to attain precision of 0.1‰, a 6- $\sigma$  baseline width can be  
 170 used [16]. Because standard deviation calculation is carried out from Gaussian peaks, the  $\Delta V$  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  $\Delta V$  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  $\Delta V$  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 $\Delta V$*

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  $\Delta V$  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  $4\sigma$ . 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  $\Delta V$  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  $\Delta V$  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  $\Delta V$  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  $\Delta V_1$  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  $\Delta V_1$  (Eq. (3)) between aspirin and coumarin was  $\Delta V_1 = 8.6$  mL.

248 Once  $\Delta V_1$  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  $\Delta V_2$ , 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  $\Delta V_2$  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  $\Delta V_2$  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  $\Delta V$  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  $\Delta V$  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  $\Delta V_r$  is directly proportional to  $Sf$ . So, it seems that the maximum  $Sf$  is desirable to  
339 obtain the maximum  $\Delta V_r$ , confirming the highest possible rotor rotation speed. Since high rpm also  
340 produces thinner peaks (higher efficiency), it reduces the  $\sigma$  values in eq. 3 also increasing the  $\Delta V_r$   
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  $\Delta V$  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  $\Delta V_r$  (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  $\Delta V$  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  $\Delta V$  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  $\Delta V$  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  $\Delta V$  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  $\Delta V_2$  volume is lower than  
368 the  $\Delta V_1$  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  $\Delta V$  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  $\Delta V$   
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  $\Delta V$  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",  $\Delta V$ , 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  $\Delta V$ ).
- 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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#### 419 **Acknowledgments**

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

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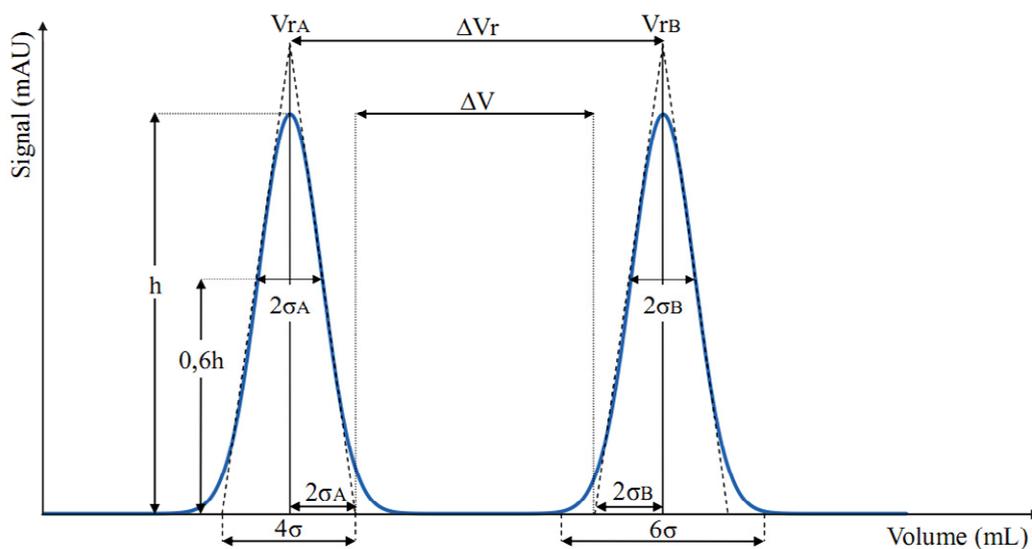
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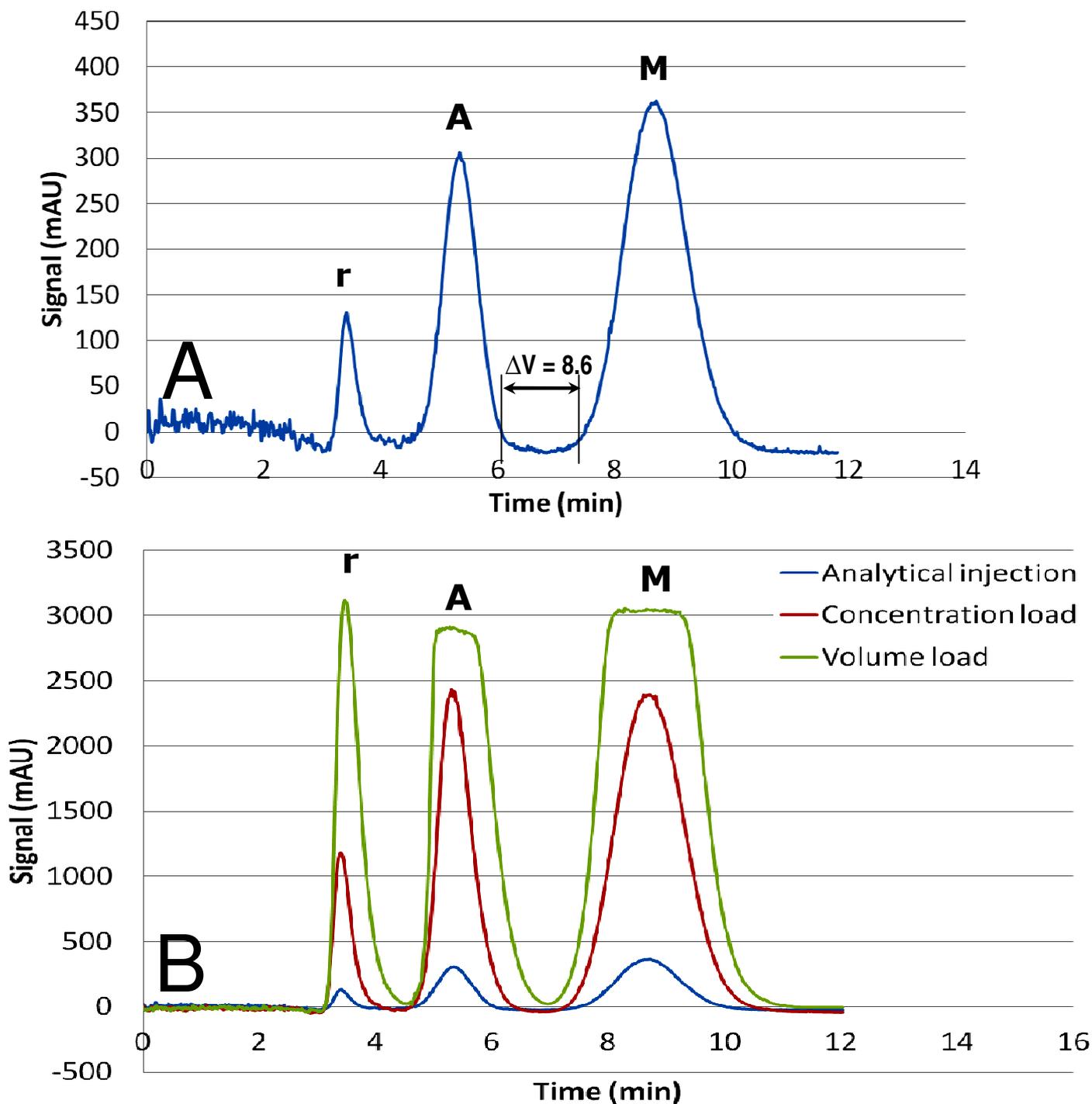
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**Fig. 1.** Representation of the separation of two Gaussian peaks illustrating the concept of "free space between peaks",  $\Delta V$ , compared to the retention difference,  $\Delta V_r$ , used in the definition of the chromatographic resolution.

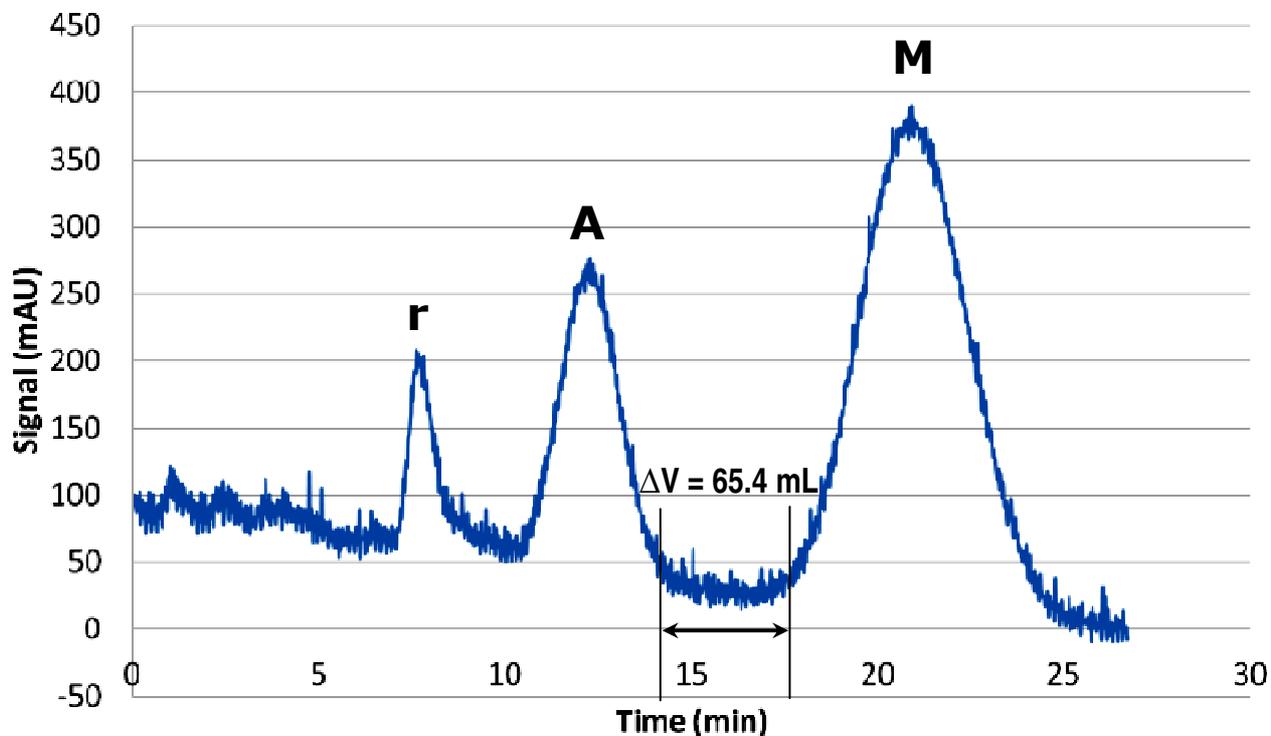
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500 **Fig. 2. A-** 35-mL rotor analytical injection of 0.36 mL (1% column volume) of 0.3 mg/mL new  
 501 coccine red (**r**), 8.0 mg/mL aspirin (**A**) and 3.3 mg/mL coumarin (**M**) in descending mode. **B-** 35-mL  
 502 rotor optimization injections: 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.

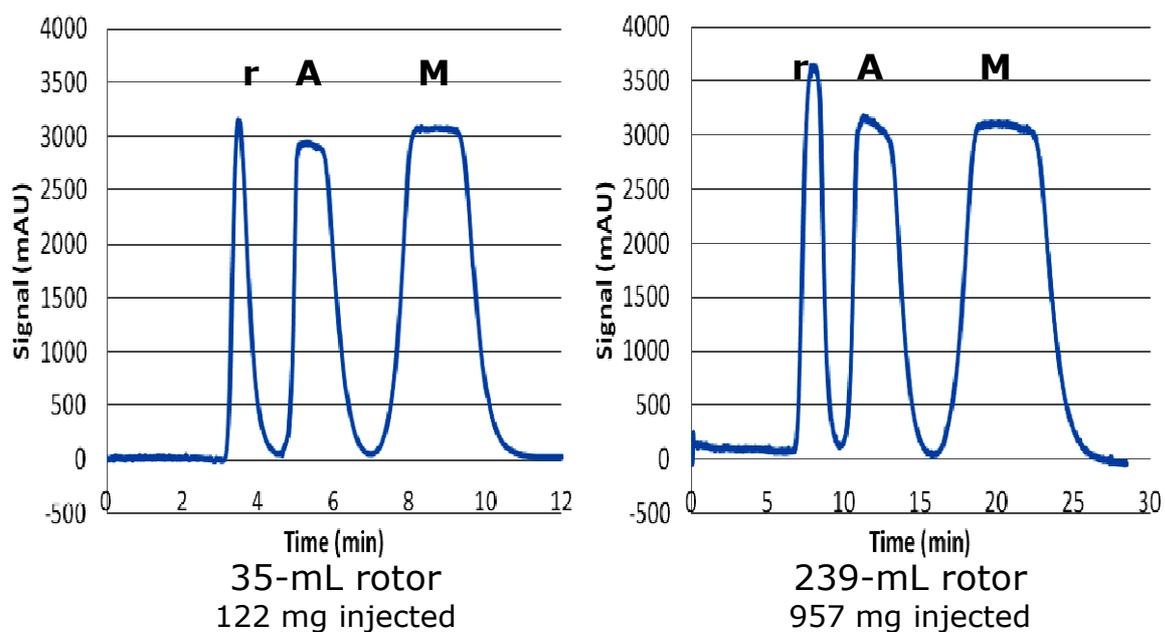
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**Fig. 3.** 239-mL rotor analytical injection of 0.3 mg/mL of new coccine red (**r**), 8.0 mg/mL aspirin (**A**) and 3.3 mg/mL coumarin (**M**). Column volume: 239 mL, injection volume 2.5 mL (1% column volume), injected mass: 29 mg, flow rate : 15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

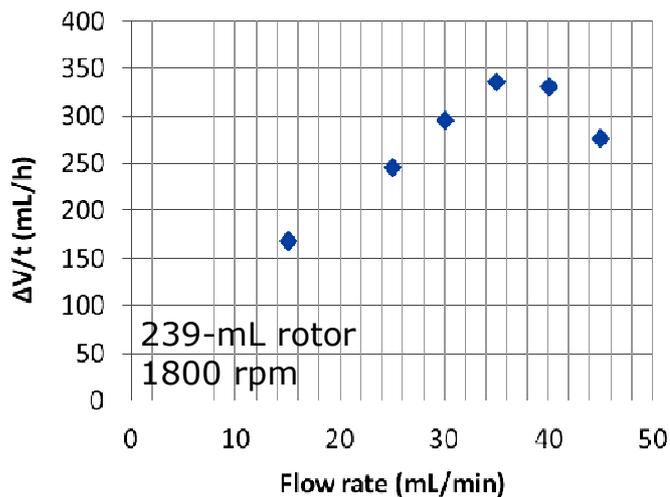
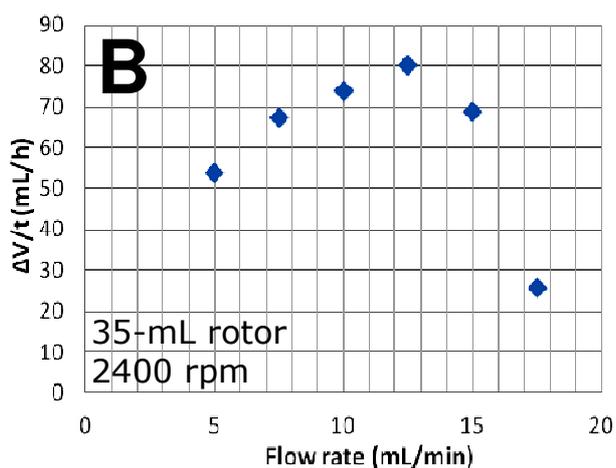
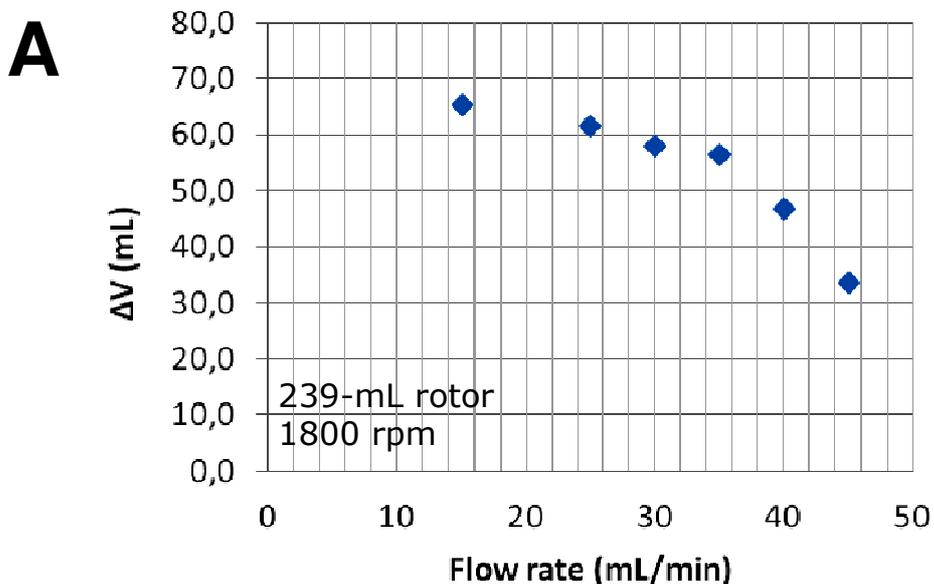
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549 **Fig. 4.** (left) 35-mL rotor preparative injections of 1.4 mL of the 2 mg/mL new coccine red (**r**), 60  
550 mg/mL aspirin (**A**) and 25 mg/mL coumarin (**M**) solution in descending mode, flow rate: 5 mL/min;  
551 (right) 239-mL rotor preparative 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.

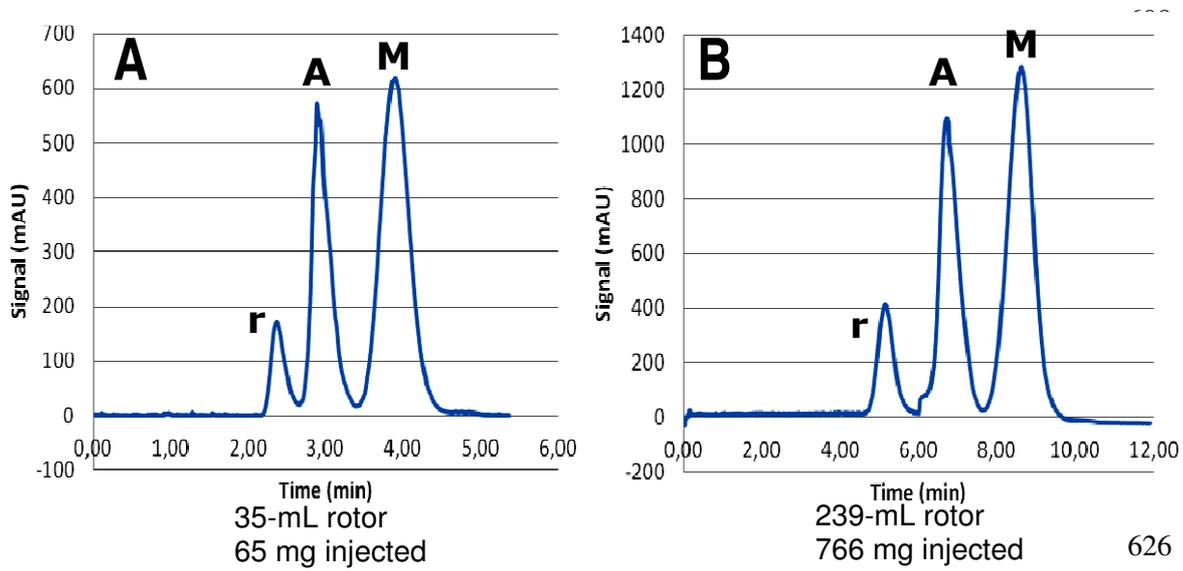
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**Fig. 5.** Effect of the flow rate on the "free-space between peaks" interval. **A-** Free-space between aspirin and coumarin peaks plotted versus the mobile phase flow rate at 1800 rpm on the 235-mL rotor (1 mL injection). **B-** Evolution of the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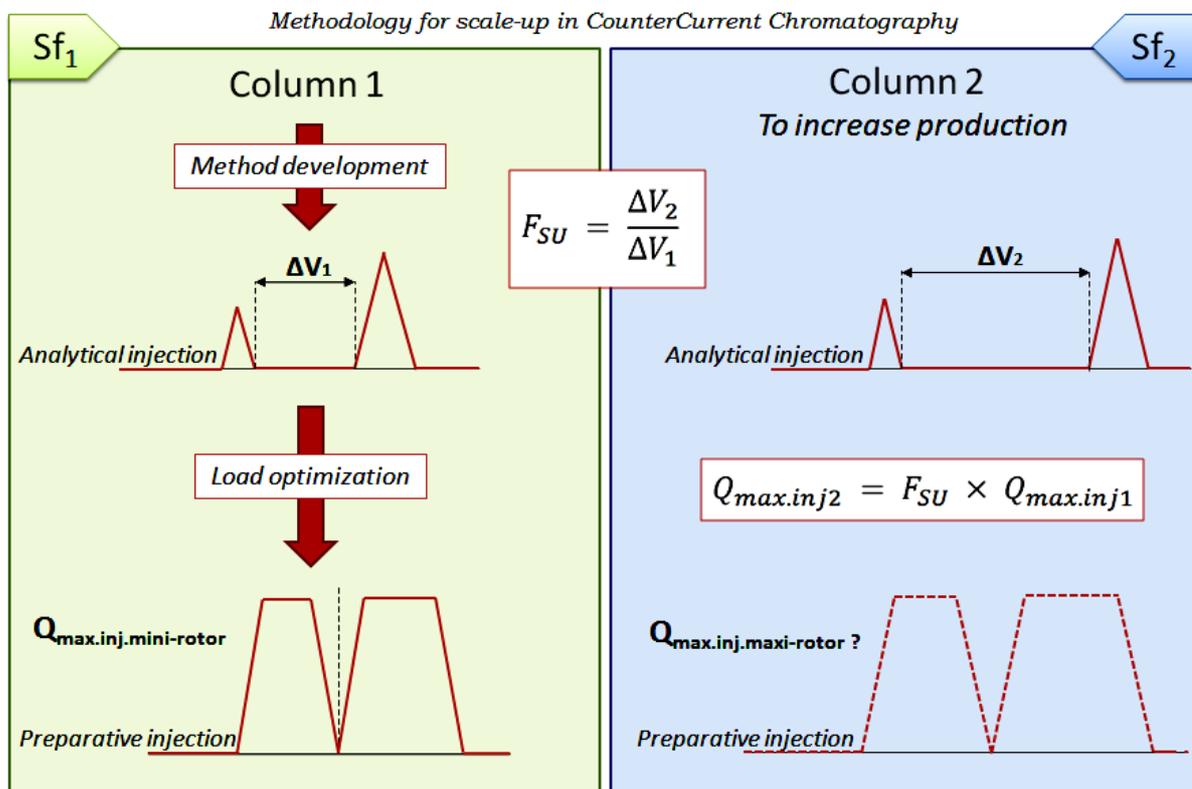


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628 **Fig. 6.** The "free-space between peaks" optimization of the productivity factor. Injections of 2.0  
629 mg/mL new coccine red (r), 60.0 mg/mL aspirin (A) and 25 mg/mL coumarin (M) solution in  
630 descending mode, system Arizona M, heptane/ethyl 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  $\Delta V$  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>Operating change transfer</i>		<i>Fig. 4 preparative transfer (mass load)</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>	
<b>V<sub>column</sub></b>	35 mL	<b>6.8</b>	239 mL	<b>1</b>	35 mL	<b>6.8</b>	239 mL	
<b>Flow rate</b>	5 mL/min	<b>3</b>	15 mL/min	<b>2.5</b>	12.5 mL/min	<b>2.8</b>	35 mL/min	
<b>Rotational speed</b>	2400 rpm	-	1800 rpm	-	2400 rpm	-	1800 rpm	
<b>Centrifugal field, g</b>	648 m/s <sup>2</sup>	-	365 m/s <sup>2</sup>	-	648 m/s <sup>2</sup>	-	365 m/s <sup>2</sup>	
<b>Pressure</b>	54 bar	-	54 bar	-	54 bar	-	52 bars	
<b>Stationary phase ratio, Sf</b>	50 %	-	52 %	-	28 %	-	42%	
<b>Free space between peaks, ΔV</b>	8.6 mL	<b>F<sub>SU</sub>= 7.6</b>	65.4 mL	<b>F<sub>SU</sub>= 0.56<sup>a</sup></b>	4.8 mL <sup>a</sup>	<b>F<sub>SU</sub>= 11.8</b>	56.6 mL	
<b>Maximum load, Q<sub>max, inj</sub></b>	122 mg	<b>7.8</b>	957 mg	<b>1.9</b>	65 mg	<b>11.8</b>	766 mg	
<b>Purification time</b>	11.3 min	<b>2.4</b>	27.4 min	<b>2.5</b>	4.5 min	<b>2.2</b>	10.1 min	
<b>Productivity</b>	648 mg/h	<b>3.2</b>	2095 mg/h	<b>1.3</b>	867 mg/h	<b>5.2</b>	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"  $\Delta V_2$  is smaller producing a  $F_{SU}$  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.

