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1 Contribution of supercritical fluid chromatography to serially coupling columns for chiral and
2 achiral separations.

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25 Prediction models

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Abstract

Serially-coupled columns are a useful alternative to achieve a desired chromatographic separation when a single column has failed. Compared to gas chromatography which is limited to volatile compounds, and liquid chromatography's limitation by the high pressure drop over serial columns, supercritical fluid chromatography (SFC) appears to be an interesting alternative method. The SFC mobile phase, containing an elevated percentage of carbon dioxide, has a low viscosity and a high diffusivity compared to the liquid mobile phases. Consequently, higher flow rates can be implemented which reduce the time of analysis and allow the use of several columns in series to increase separation efficiency without any impact on pressure. The unique mobile phase used in SFC, composed of carbon dioxide and a small amount of co-solvent, also facilitates the coupling of complementary stationary phases which increases the range of possible selectivity. Thus, improved efficiency and selectivity together offer the promise of high-resolution separations. On the other hand, because the fluid is compressible, increasing the column length will change the mobile phase density, increasing its eluotropic strength and decreasing the retention time. While this feature can be seen as an advantage, it is also the major challenge of serially-coupling columns as it can be difficult to predict the outcome of the separation. This review depicts the historical milestones of the contribution of SFC to serially-coupled, packed columns, for both achiral and chiral separations. It covers a period from the 1960's to date and describes the evolutions of the concept and how the main drawbacks can be tackled. A section is devoted to applications, illustrating the potential of this concept to successfully achieve the most challenging separations.

52

53 **1. Introduction**

54 While supercritical fluid chromatography (SFC) has been known for nearly 50 years [1], since
55 it was first described by Klesper *et al.* in 1962 [2], its success was not immediate – indeed, SFC
56 was initially derided by some as "science-fiction chromatography" [3-5]. SFC has nevertheless
57 begun to carve out a niche for itself for different reasons. Firstly, it is relatively non-polluting
58 as CO₂ is nontoxic, nonflammable, and a renewable resource and only small amounts of
59 hazardous solvents are used in SFC. Notably in the increasingly active field of chiral
60 chromatography, SFC is a useful alternative to HPLC which consumes large volumes of alkane
61 and organic solvents as mobile phases at the analytical scale, though particularly at the
62 preparative scale [6]. Lastly, the higher flow-rates possible in SFC lead to lower energy
63 consumption, higher productivity and shorter equilibration times compared with HPLC [7].
64 Thanks to major improvements in instrument technology, SFC has completely caught up with
65 LC in the remaining fields where it was lagging behind. For instance, robustness at the
66 analytical scale in a regulatory environment, but also when coupled with mass spectrometry
67 when sensitivity is desired. The reader is encouraged to consult two recent reviews by C. West
68 [8; 9] for better insight into recent progress in SFC instrumentation and stationary phases, in
69 both chiral and achiral separations. SFC has consequently been applied in many areas – for
70 example food, fossil fuels, polymers, bioactive compounds, agrochemicals, forensics,
71 cosmetics – but it remains the most active in the natural products field, encompassing fragrances
72 and pharmaceuticals.

73 In early developments of SFC, the mobile phase employed was essentially pure carbon dioxide
74 at its supercritical state, *i.e.* when the temperature and pressure are both above the critical
75 values. Nowadays, and in most of the coupling applications, the mobile phase is almost always
76 composed of supercritical carbon dioxide with some small amount of organic modifier, shifting

77 equilibria to the subcritical, *i.e* the operating pressure is above the critical value, but the
78 temperature remains below. In any case, the viscosity of super/subcritical fluid is much lower
79 than a liquid, thus reducing pressure drop along the length of column(s). As a consequence, the
80 idea of serially connecting two or more columns has naturally emerged. In 1993, Berger and
81 Wilson conducted an experiment to counteract the repeated assertions in the literature that
82 efficiency in SFC packed columns had a practical upper limit of 20,000 plates [10]. In a famous
83 article they serially coupled 11 columns without increasing the pressure drop (around 160bar).
84 Different separation examples such as for forty polycyclic aromatic hydrocarbons (PAHs)
85 separation, different herbicides, Brazilian lemon oil, chimney extract or gasoline were
86 presented. The concept of serially coupling columns was not new, however, and had been
87 published in 1985 by the team of P. Sandra in gas chromatography [11], and even in the early
88 1960's by Maier and Karpathy [12] and Hildebrand and Reilly [13]. In 1994, work by Järemo
89 *et al.* explored the contribution of the pressure drop and flow-rate to analyte retention, on
90 coupled capillary columns in supercritical fluid chromatography [14]. In 1996, Pirkle and
91 Welch applied this concept for chiral separation to liquid chromatography [15]. Along with
92 two-dimensional chromatography (2D-chromatography) [16], or the possibility of using chiral
93 stationary phases for achiral separation implemented by Regalado *et al* [17], tandem-columns
94 offer a way to achieve challenging separations thanks to high-resolution, avoiding the necessity
95 of a specific device, and with shorter analysis times. In addition, as will be presented here in
96 the applications section, the possibility of serially coupling two columns can be also exploited
97 at the preparative scale.

98 Hereafter the state of the art and contributing developments in this concept will be presented,
99 along with the major applications in different fields.

100 **2. Part I Serially coupling columns in supercritical fluid chromatography: state-of-the-art**

101 **2.1 Modification of existing device**

102 If the setup of tuning a coupled-column arrangement is simple and easy to implement, one must
103 be aware that while the two columns are plugged in the tandem, each of them cease to be
104 unavailable for a single use unless they are dismantled. To overcome this inconvenience, Welch
105 *et al.* have modified a Berger SFC by replacing the existing software-controlled solvent selector
106 valve by a second, high-pressure, six-position column selector valve, followed by some minor
107 modifications in plumbing. This change leads to the arrangement illustrated in Figure 1 and
108 allows for a software-controlled selection of twenty-five different coupling columns and ten
109 different single column arrangements [18]. This home-made device was implemented for the
110 screening of tandem column SFC system for the separation of complex mixtures of
111 stereoisomers, and another multicomponent mixture consisting of an achiral starting material
112 and the racemic product. Not much attention has been paid to the column order until this work
113 by Welch. A little later, work by Poole [19] also highlighted that tandem columns' separation
114 performance may depend on the order in which the columns are coupled.

115 **2.2 Rational method development**

116 The first benefit of tandem columns (two or more) is to achieve high efficiencies thanks to the
117 low viscosity of the fluid, ensuring a moderate pressure drop. The second advantage is the
118 possibility to connect both achiral and chiral columns in series, or even columns with different
119 polarities, to create unique selectivity [20]. This feature is possible in SFC because it uses a
120 carbon dioxide-based mobile phase whatever the stationary phase, while in HPLC the concept
121 of coupling stationary phases to achieve the desired efficiency and/or selectivity is somewhat
122 restricted by the columns' greater or lesser compatibility with the mobile phase, as well as the
123 pressure drop caused by long columns.

124 As one can see in the applications part of this review, finding the best tandem combination for
125 a given separation was, for a long time, done by trial-and-error with the number of trials for
126 n available columns being equal to $n \times (n - 1)$. This approach is obviously cumbersome, and

127 recently two teams have rationalized and formalized the prediction of retention times for a given
128 combination of columns. The first was the team of Wang, from Bristol-Myers-Squibb
129 Company, who considered the back pressure and the column order, and the other is the team of
130 Lynen, who considered the average internal pressure.

131 In 2013, Wang *et al.* studied the effect of the backpressure on the separation of enantiomers on
132 ten chiral stationary phases (CSP) [21]. They determined two empirical, quantitative pressure-
133 retention relationships, which can be used to predict retention time on a pair of columns:

$$134 \quad t_0 = aP^2 + bP + c \quad \text{Eq (1)}$$

135 where the fitted coefficients a , b , and c depend on each column, and:

$$136 \quad k_{P_i} = k_{P_0} e^{d(P_i - P_0)} \quad \text{Eq (2)}$$

137 where k_{P_i} is the retention factor at any pressure P_i , k_{P_0} is the experimental retention factor at
138 the fixed experimental outlet pressure P_0 , and d is a column constant. The last equation provides
139 a way to calculate retention time at different pressures based on a single measurement at P_0 ,
140 avoiding the necessity to perform the injection at the desired pressure. For each of the ten
141 columns the constants a , b , c and d were determined.

142 In 2014, the same team from Bristol-Myers-Squibb published a second work on this topic [22].
143 They highlighted the pivotal role of column order in any column pair, and how the two
144 configurations result in different pressures at the column heads. They showed that if a CSP_A is
145 placed first, it experiences higher back pressure (P_u) than the downstream column CSP_B (P_d).
146 The pressure difference between (P_u) and (P_d) is the pressure drop over the downstream column
147 CSP_B. The overall retention time, t_{AB} is the addition of the retention times on both columns at
148 corresponding back pressure $t_{AB} = t_{A,P_u} + t_{B,P_d}$ where t_{A,P_u} is the retention time on the
149 upstream column CSP_A at higher outlet pressure (P_u), and t_{B,P_d} is the retention time on the
150 downstream column CSP_B at lower back pressure (P_d). When columns CSP_A and CSP_B are

151 swapped, the overall retention time t_{BA} changes into $t_{BA} = t_{B,P_u} + t_{A,P_d}$. This results in two
152 different chromatograms for the same sample as a function of the column order.

153 In 2019, Akchich *et al.* experimented with the mathematical model developed by Wang *et al.*
154 [22] to separate four diastereoisomers which they could not resolve, despite testing many
155 different columns individually [23]. The accuracy between the simulated retention times and
156 the experimental ones was lower than 3.82%. In addition, they calculated the constants a , b , c
157 and d for two supplementary chiral columns the Lux Cellulose-2 and the Lux Amylose-2 (from
158 Phenomenex) allowing implementation of experimental tandems after mathematical prediction
159 of retention times. Figure 2 presents an example for a compound bearing two asymmetrical
160 carbons on a tandem consisting of the two Lux columns in two configurations. It is worth noting
161 that these works are of utmost importance because they allow anyone to calculate simulated
162 retention times thanks to the empirical equations defined above, to select the adequate tandem,
163 once the experiments are done on each individual column, all without the need of buying a
164 software or complex installation as simple polyether ether ketone (PEEK) tubing is sufficient.
165 The only limit is the type of CSP for which the constants are available.

166 The second approach was that of Lynen, who developed the concept of stationary-phase
167 optimized selectivity in liquid chromatography in isocratic mode (SOS-LC), and later in
168 gradient mode. They have extended this prediction model to SFC (SOS-SFC) using various
169 achiral stationary phases [24] sold in a commercial kit POPLC Basic Kit 250-5. A first step
170 consists of measuring the retention factors of the sample compound on each individual
171 stationary phase, and inputting the data to the following equation:

$$172 \quad k_{A,B,C,D,E} = \frac{k_A \phi_A + k_B \phi_B + k_C \phi_C + k_D \phi_D + k_E \phi_E}{\phi_A + \phi_B + \phi_C + \phi_D + \phi_E} \quad \text{Eq (3)}$$

173 where k_A , k_B , k_C , k_D and k_E correspond to the retention factors of a compound obtained from the
174 basic measurements on the five individual stationary phases and ϕ_A , ϕ_B , ϕ_C , ϕ_D and ϕ_E
175 represent the lengths of the individual column segment in a combined column. Then, the

176 chromatographic retention is calculated by the software (predicted) for all possible
177 combinations of serially-coupled column segments to define the best selection of stationary
178 phases, combining small sections of columns. The combinations were ranked in decreasing
179 order of selectivity of the critical pair and shortest analysis time. However, as stated in the
180 abstract, the issue of working with compressible fluids is the dependency of the solute retention
181 upon mobile-phase density, complicating linear extrapolation of retention on a single column
182 length to different coupled-column lengths, even when a constant outlet pressure is set by the
183 backpressure regulator (BPR). But this can be overcome by working at a constant average
184 mobile phase density (and hence at a constant average pressure in the column), rather than at a
185 constant back pressure. Two approaches can be implemented: in the isopycnic one, the flow
186 rate and average pressure in the column are kept constant by lowering the outlet pressure set by
187 the BPR for the longer columns, such that identical average pressures are obtained in all
188 experiments; in a second, variable-flow rate approach, the flow rate is reduced for longer
189 columns such that identical inlet and outlet pressures are obtained for all column lengths. An
190 example is presented in Figure 3 for the optimization of the separation of 15 steroids. The first
191 chromatogram (A) depicts the software-predicted chromatogram with the best combination of
192 segments of stationary phases. The second chromatogram (B) represents the experimental
193 chromatogram obtained under constant flow-rate and outlet pressure, called the traditional
194 approach, implying a variable inlet pressure. Chromatograms (C) and (D) are obtained under
195 the isopycnic and variable flow-rate approaches, respectively. In this paper, the influence of the
196 segment order appears less relevant than expected. It is noteworthy that the predictions cease
197 to be accurate for combinations longer than 150mm in total. A second paper from 2019 applied
198 this concept to chiral separations with 5cm length chiral stationary phases [25]. Predictive errors
199 were reduced when the internal pressure was considered, and ranged between 1 to 5%.

200 The challenges encountered when two or more columns are being optimized in liquid
201 chromatography are the following: a high pressure drop, a longer analysis duration and different
202 mobile phase composition (required for normal-phase and reversed-phase chromatography)
203 when columns with polar or apolar, chiral or achiral stationary phases are coupled. In contrast,
204 SFC has none of the above. The carbon dioxide-based mobile phase has a low viscosity
205 compared to the liquid mobile phase in LC, resulting in only a moderate pressure drop when
206 the column length is extended. No difference in mobile phase exists, whatever the stationary
207 phase, since it is composed of a major percentage of carbon dioxide with a minority organic co-
208 solvent added. Lastly, the expected increase in analysis time is not proportional to the column
209 length. This phenomenon is specific to SFC and rather complex. Indeed, a rise of the column
210 length, without any changes of other parameters, causes an increase of the internal pressure
211 because the fluid is compressible. When the internal pressure rises, the mobile phase density
212 increases, thus the solvent strength increases and the phase ratio changes. Consequently,
213 retention times resulting from increased column length are usually lower than would be
214 expected based on the number of columns. These features have been clearly summarized in the
215 Figure 4. However, working at constant average internal pressure overcomes this issue in
216 prediction models.

217 **3. Part II Serially-coupling columns in supercritical fluid chromatography: applications**

218 **3.1 Lipids (ceramides, carotenoids and natural products)**

219 Lipids are a challenging class of components to separate in SFC because of their low retention
220 and the high number of structurally-related compounds present in the samples. Extending the
221 column length is the obvious strategy for separating such a complex mixture.

222 3.1.1 Higher column length for higher efficiency

223 In 2000, Gaudin *et al.* [26] investigated the interest of using multiple coupled columns in SFC
224 for separating apolar ceramides, and established a retention chart which represents the

225 relationship between the retention and the compounds' structures. These hydrophobic
226 compounds are classically analyzed by either gas chromatography or non-aqueous reversed
227 phase LC. As none of the five different single columns tested in SFC were able to separate the
228 25 ceramides, up to seven identical stationary phases of 250 cm length were serially coupled,
229 with five columns being the best combination. If the paper is investigative, it must be
230 highlighted that seven serially-coupled columns is among the highest number reported since
231 the 1990s paper of Berger [10]. Thanks to the low viscosity of the mobile phase, this 1.75 meter
232 column length is possible without any increase of the pressure drop. In an article published in
233 2021, Abrahamsson *et al.* [27] described the separation of 8 carotenoids and applied it to their
234 quantification in *Scenedesmus almeriensis* from microalgae extract obtained by supercritical
235 fluid extraction. Carotenoids are usually analyzed by conventional HPLC using C18 or C30
236 columns and chloroform or methyl-tert-butyl ether mobile phases. But due to the high pressure
237 drop of the C30 stationary phase, the analysis time tends to be rather long and the method
238 appears as not sustainable. Once again, the challenge remains the separation of compounds
239 which are isomers of each other and hence very similar in structure. Two columns coupled in
240 series were optimized, and the separation was conducted in less than 10 minutes.

241 Modern Ultra High Performance SFC (UHPSFC) has also entered the tandem-columns theatre,
242 further extending the already high efficiency values. In the following papers, E. Lesellier's
243 group explore the potential of columns packed with superficially porous particles (SPP),
244 gaining in efficiency by connecting a high number of them without increasing pressure drop.

245 In a paper published in 2014, thirteen fatty acids were separated and quantified after
246 identification in vegetable oils (argan or rapeseed) [28]. These compounds are usually separated
247 in non-aqueous reversed phase LC with either one or two coupled columns, but the separation
248 of several critical pairs remains difficult. In this paper, ten different stationary phases were
249 tested. The methodology consisted of two steps. Briefly, the first step was to optimize the

250 operating conditions using three identical Kinetex C18 columns coupled in series. The second
251 step used an additional fourth column of the same type and consisted of choosing, among ten
252 different SPP C18 bonded phases, another column to be connected with the four Kinetex C18
253 columns (60 cm). The Derringer function was then applied (consisting of normalizing the values
254 of any parameter, between 0 and 1) to various parameters: theoretical plate number, separation
255 index for critical pairs of peaks, the analysis duration and the total peak number. Finally, by
256 coupling four Kinetex C18 and one Accucore C18 columns (75 cm total column length) a full
257 separation of triglycerides with a high efficiency was achieved. The chromatogram obtained in
258 those conditions is depicted in Figure 5. It is worth mentioning that this paper was a continuation
259 of a previous one, published in 2012, which achieved 120,000 theoretical plate, by coupling
260 four 150 mm SPP columns [29].

261 It can be seen that to achieve high efficiency in the shortest analysis time, fully porous, sub-two
262 micron particles (FPP) and superficially porous sub-three micron particles (SPP) seem to be
263 comparable in recently reported articles dealing with tandem-columns. However, while the
264 former can reach high chromatographic efficiency with acceptable pressure drops, allowing the
265 use of longer column lengths, the latter induces ultra-high pressure drops which limit the
266 possible column length, mainly because of the upper-pressure limit of current commercial
267 systems.

268 3.1.2 Application of the constant average internal pressure approach

269 In section 2.2, we described the usefulness of working at a constant average internal pressure
270 with different column lengths, using either an isopycnic approach or by a variable flow-rate
271 approach. A paper by E. Lesellier, dealing with the separation of cis/trans isomers of β -
272 carotenes was reported in 2003 [30], and presents a third possibility. In this work the use of
273 monolithic columns to avoid modifications to the fluid density in the first column set in the
274 chain was demonstrated through the calculation of k and α as a function of monolithic column

275 length. The different chromatographic columns tested individually were all octadecyl bonded
276 silicas. Because of their complementarity, the YMC Pack Pro C18 and the Chromolith RP 18e
277 were chosen for the experiment, where the YMC column was coupled with between one and
278 six Chromolith columns. The best separation was obtained by coupling one YMC to five
279 Chromolith columns, resulting in a total column length of 75 cm. The predicted retention factors
280 were in good agreement with those obtained experimentally, with acceptable accuracy.

281 **3.2 Pharmaceuticals**

282 For a long time, the classical method developed for analysis of pharmaceuticals was reverse-
283 phase LC. Nowadays, samples encountered in drug development or during pharmaceutical
284 testing are quite complex. For instance, they may consist of the analyte of interest (often chiral)
285 in a complex matrix-related protein. Impurity profiling of synthetic drugs is also often
286 implemented and requires the separation of structurally similar molecules ranging in polarity.

287 3.2.1 Greater column length for higher efficiency

288 The team of P. Sandra demonstrated the usefulness of coupling five cyanopropyl silicagel
289 Zorbax SB-CN columns to achieve 100,000 separation plates to resolve 17 diverse
290 pharmaceutical compounds [31]. In 2001 Roston *et al.* developed a method for an in-house drug
291 substance impurity profile thanks to multiple columns resulting in a 2 meters length [32]. One,
292 four, six and eight Supelcosil LC-CN columns were involved in the experiments. The highest
293 number of detected impurities (eleven) was obtained with six serially-coupled columns. In this
294 application paper, it is noteworthy that eight serially-coupled columns, with a column length of
295 2 meters, is the highest number reported since the 1993 paper by Berger.

296 3.2.2 Utility of complementary selectivity

297 Alexander *et al.* [33] demonstrated the utility of Luna silica/Chiralcel OD-H (tandem, for
298 profiling cinnamitrile and hydrocinnamitrile mixture. Indeed, the combination of an
299 achiral and a chiral column permitted the determination of the enantiomeric/diastereoisomeric

300 composition of the final product, as well as the remaining *Z/E* isomers present from the starting
301 compound, in only one step and in short time. Ultra-High Performance SFC has also been
302 explored for active pharmaceutical ingredients. Recently, West *et al.* published a work dealing
303 with impurity profiling of synthetic drugs [34]. This conceptual work did not intend to merely
304 develop a method for a given drug candidate, but rather to explore the interest of coupling two
305 columns to develop a generic tool for moderately complex samples containing synthetic drugs
306 and impurities. To achieve this goal twenty-five active pharmaceutical ingredients were chosen
307 to compare on single-column and tandem-columns systems (including the effect of column
308 order) through comparison of selectivity, efficiency, capability for impurity profiling and
309 sensitivity. Two columns of excellent complementarity were selected: an ACQUITY UPC2
310 HSS C18 SB column and a Nucleoshell HILIC column. Considering all the criteria, one of the
311 tandem-methods provided better results than a single column approach, and considering peak
312 efficiency alone the C18-HILIC tandem seemed the most promising.

313 **3.3 Preparative scale**

314 For a long time, SFC has dominated over LC for preparative purposes, mainly thanks to the low
315 environmental impact of the technique. This has been well covered in reviews by E. Francotte
316 [35] and Speybrouck *et al.* in 2016 [20] and 2021 [36]. Performing preparative-scale
317 purifications with serially coupled columns may seem less obvious, but has nevertheless been
318 reported in literature.

319 3.3.1 Preparative scale on analytical dimensions columns

320 In 2013, Zhao *et al.* separated six 25 (*R/S*)-spirostanol saponin diastereomers from the
321 *Trigonella foenum-graecum* L. seed, by serially coupling two Chiralpak IC immobilized
322 columns [37]. Among them, three isomeric mixtures were further purified into their 25
323 respective *R* and *S* isomers. Various experiments were investigated on Sunfire Silica (250 and
324 150 × 4.6 mm i.d; 5.0 μm) columns serially coupled to give 400 mm total length, or with

325 Chiralpak IC (250 and 150 × 4.6 mm i.d; 5.0µm) columns (also serially coupled with 400 mm
326 total length). This last approach, with the two chiral columns, was used to perform stacked
327 injections preparative separation. It is worth noting here that these columns were used with their
328 analytical dimensions and particle size at preparative scale thanks to their high loading capacity.

329 3.3.2 Utility of complementary selectivity

330 In 2005, Barnhart *et al.* coupled a Chiralpak AD and a Chiralcel OD columns to purify 1mg of
331 a pharmaceutical compound with four stereoisomers [38]. This baseline separation was not
332 achievable on either of the single columns with their different selectivity. The columns were
333 semi-preparative ones (21 mm internal diameter). The originality of the work rests in their
334 obtaining the four individual stereoisomers through a multi-step purification prior to the
335 development of the tandem-preparative method. The elution order was then studied as a
336 function of the co-solvent type on each single column, and in the tandem arrangement,
337 highlighting the possibility of changing selectivity and peak elution order to obtain the desired
338 stereoisomers.

339 The other article was reported by M. D. Ventura from Amgen Inc. [39]. Various pharmaceutical
340 compounds were chosen to illustrate the potential of achiral-chiral column pairs for purifying
341 racemic target molecules from achiral impurities. The apparatus employed to screen the achiral-
342 chiral tandem, was similar to that used by Welch *et al.* (in Figure 1) for automated coupling of
343 analytical chiral columns. An interesting example is the comparison between a preparative
344 purification done in two-steps and on performed using a coupled-columns system. Racemate
345 diperodon mixed with caffeine was chosen. On a single immobilized-cellulose (3,5)-
346 dimethylphenyl carbamate column, the caffeine peak mostly overlaps with the front edge of the
347 first eluting enantiomer. The collected fraction 1 required evaporation after collection and
348 another preparative process to separate caffeine from enantiomer 1 of diperodon. In the tandem

349 system, caffeine elutes before the two peaks of diperodon and the coupled system took 53
350 minutes less than the two-stage process.

351 3.3.3 Tandem-columns: an advanced solution for scale-up difficulties

352 One of the challenges of preparative enantiopurification is the transposition of the method from
353 analytical to preparative scale, which is not always straightforward. In 2009, Ali *et al.* from
354 Regis Technologies Inc., published a tandem preparative column method to routinely deliver
355 multikilogram quantities of a pharmaceutical candidate [40]. Indeed, while a high-resolution
356 value was obtained on the RegisCell analytical column (4.6mm i.d.), this separation was lost
357 on the preparative RegisCell column (30mm i.d.), whatever the co-solvent used, as the
358 compound did not scale-up linearly and productivity was low at 1g/h. While this was improved
359 to a rate of 2g/h using stacked injection, this compound remained a bad candidate for scale-up.
360 To overcome this issue, a Whelk-O1 (S, S) (50mm i.d.) was coupled to the RegisCell column
361 (particle size was not specified) and stacked injections yielded a 6g/h productivity rate on this
362 column pairing.

363 Tandem-column preparative SFC thus offers both cost and time savings and is a proven robust
364 method for gram to multi-kilogram scale separation projects.

365 **4. Conclusion**

366 Serially-coupled columns have real and proven used for the separation of complex mixtures of
367 structurally similar compounds, with applications including lipids present in natural products
368 and pharmaceuticals – in particular the enantio- and diastereo-isomers of compounds with more
369 than one asymmetrical center or impurity profiling when achiral and chiral separations are
370 required simultaneously. Separations are facilitated through increased resolution resulting
371 from: i) the higher number of plates obtained by increasing the length of the stationary phase;
372 and ii) increased versatility in selectivity through combining different stationary phases.

373 This enhanced selectivity, resulting from the almost countless possible combinations of
374 different stationary phases, is a feature worthy of keeping up to date with. Two points must be
375 considered: the first results from the internal pressure change in the first column, leading to
376 very different chromatograms depending of the column order, but which can be overcome by
377 the use of a monolithic second column or by adjusting the outlet pressure or the flow-rate; the
378 second point is that the selectivity provided by a given column for a given separation must be
379 complementary to that provided by the other column, requiring a good knowledge of column
380 selectivity.

381 The particularity of SFC tandem-column systems with high resolution separation is the ease
382 with which different stationary phases can be assembled to take advantage of their
383 complementary selectivity and extended length, without the technical constraints and issues of
384 two-dimensional methods. If SFC is already a mature technique for the coupling of columns in
385 series, further developments for their automated optimization or in pushing the upper pressure
386 limits are nevertheless desirable, notwithstanding advances in column and mobile phase
387 chemistry that have already permitted the analysis of biomolecules that were thought to be
388 difficult or even impossible to separate by SFC [41].

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394 *4756 words and 41 references*

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Figure captions

524 **Figure 1**

525 Schematic illustration of tandem column SFC modification made to Mettler-Toledo Berger
526 Analytical SFC Instrument. Both valve A and B are under independent software control.
527 Reprinted from [18] with permission from Wiley.

528 **Figure 2**

529 Overlay of predicted and experimental chromatograms obtained for compound **1** on LC-2//LA-
530 2 and LA-2//LC-2 column tandems with 20% methanol and a flow-rate of 3 mL/min, column
531 temperature of 40°C and 120 bar outlet pressure, $\lambda = 210$ nm. Reprinted from [23] with
532 permission from Elsevier.

533 **Figure 3**

534 Predicted and experimental chromatograms on the combined stationary phases: 20 mm C18 SH
535 2 + 110 mm C18 EPS 2 + 140 mm CN 2 + 230 mm C30.

536 (A) Predicted chromatogram.

537 (B) Experimental traditional chromatogram: flow rate 1.3 mL/min; outlet pressure 150 bar;
538 average pressure > 161 bar.

539 (C) Experimental isopycnic chromatogram: flow rate 1.3 mL/min; outlet pressure variable;
540 average pressure 161 bar.

541 (D) Experimental variable chromatogram: flow rate variable; outlet pressure 150 bar; average
542 pressure 161 bar.

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544

545 **Figure 4**

546 Theoretical consequences of coupling columns in supercritical fluid chromatography.

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548 **Figure 5**

549 UV (blue) and ELSD (red) chromatograms for rapeseed oil with the optimal selected

550 chromatographic system (60 cm Kinetex C18 + 15 cm Accucore C18). Reprinted from [28]

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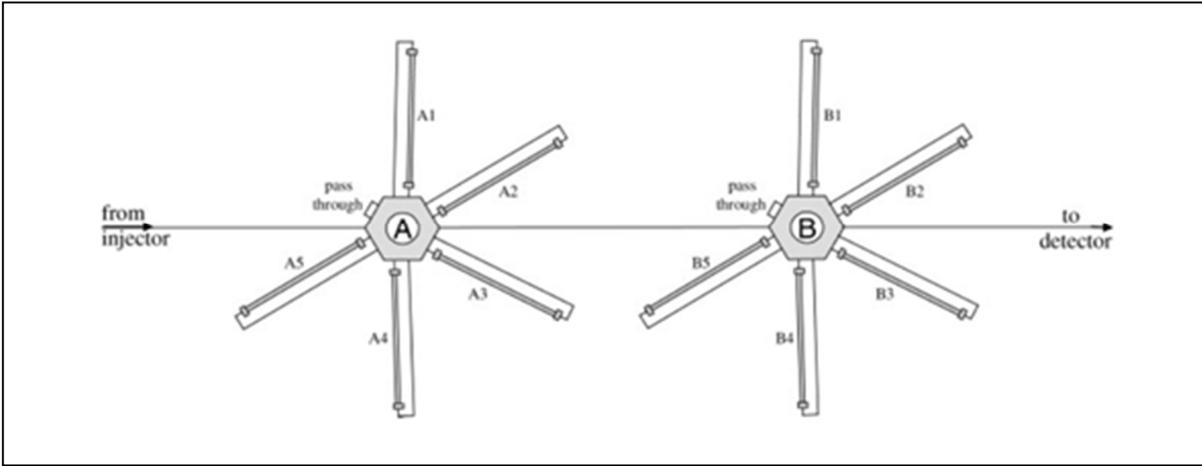


Figure 1

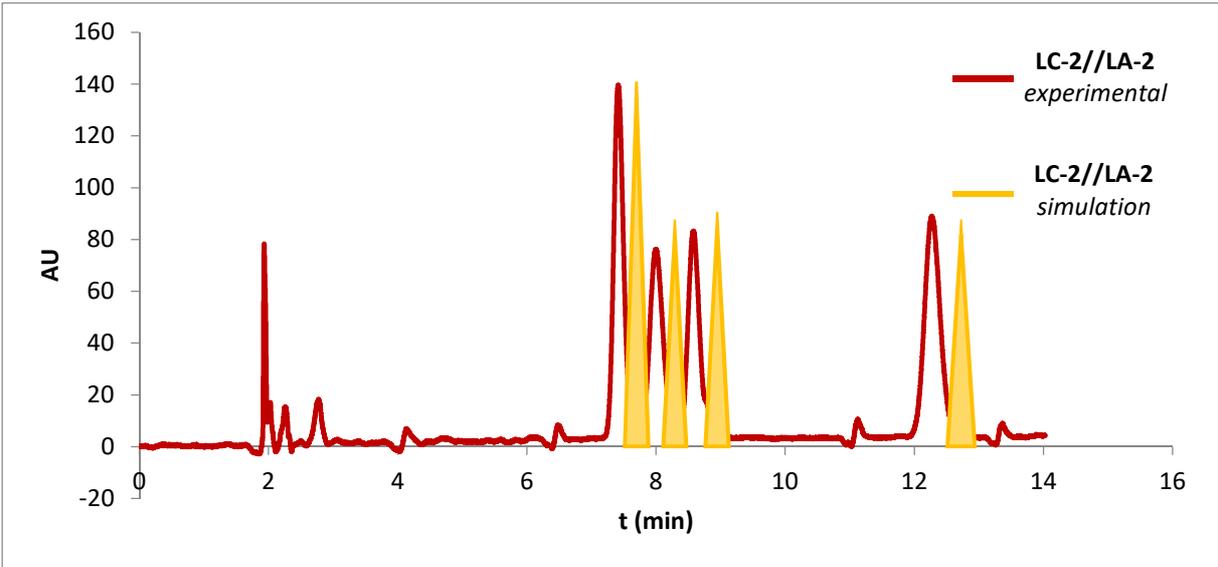
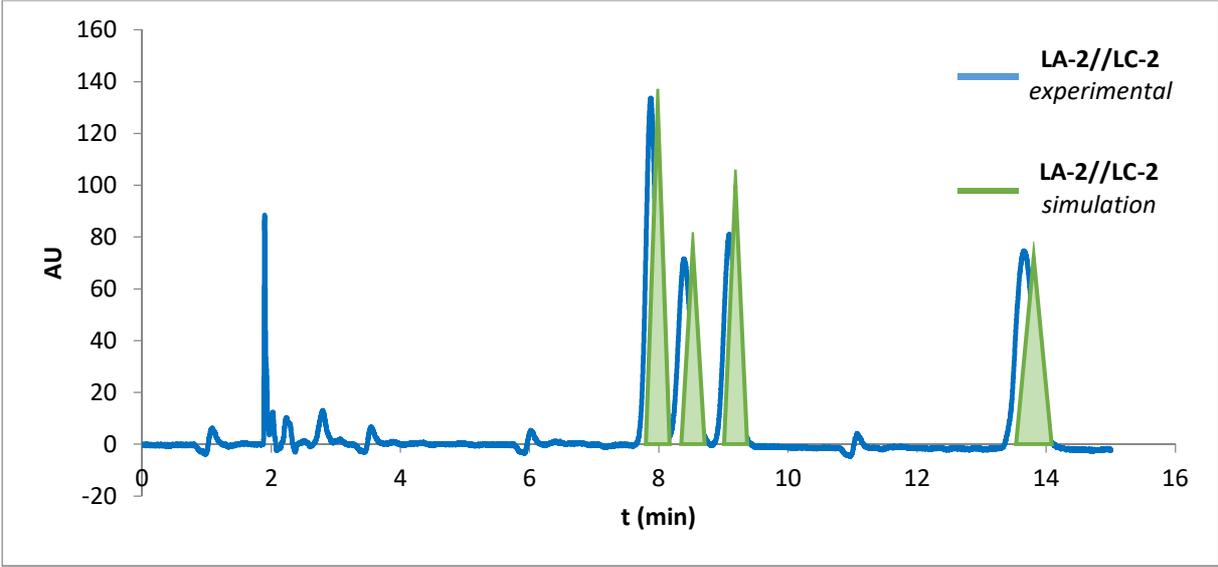


Figure 2

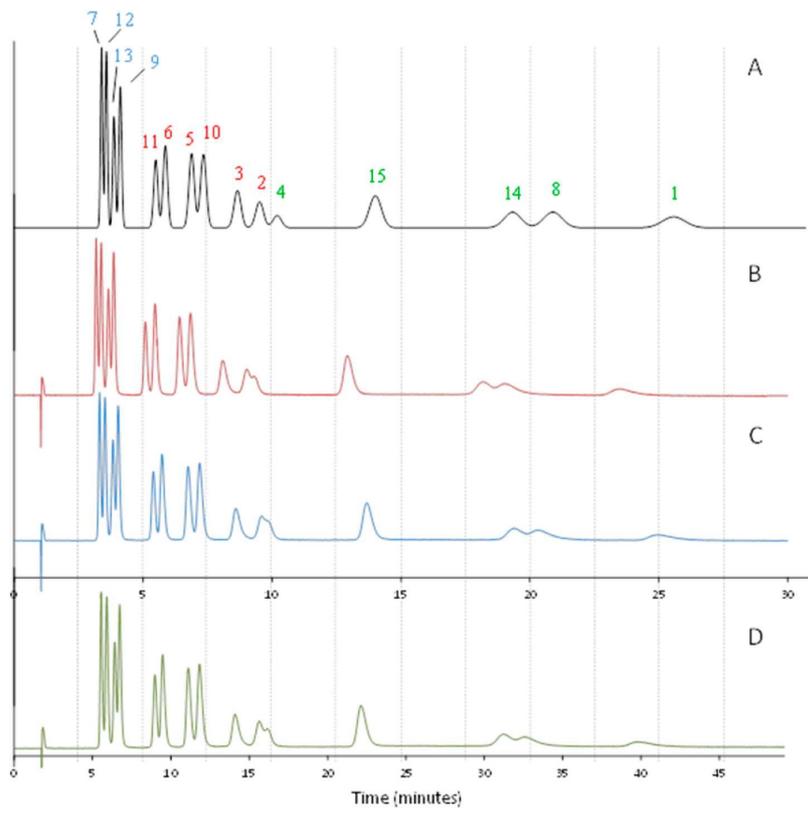


Figure 3

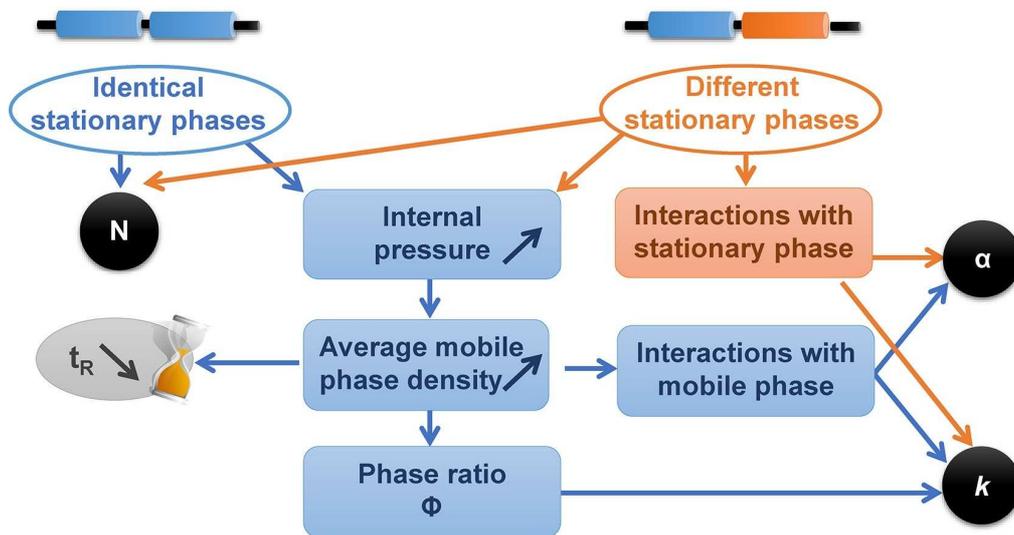


Figure 4

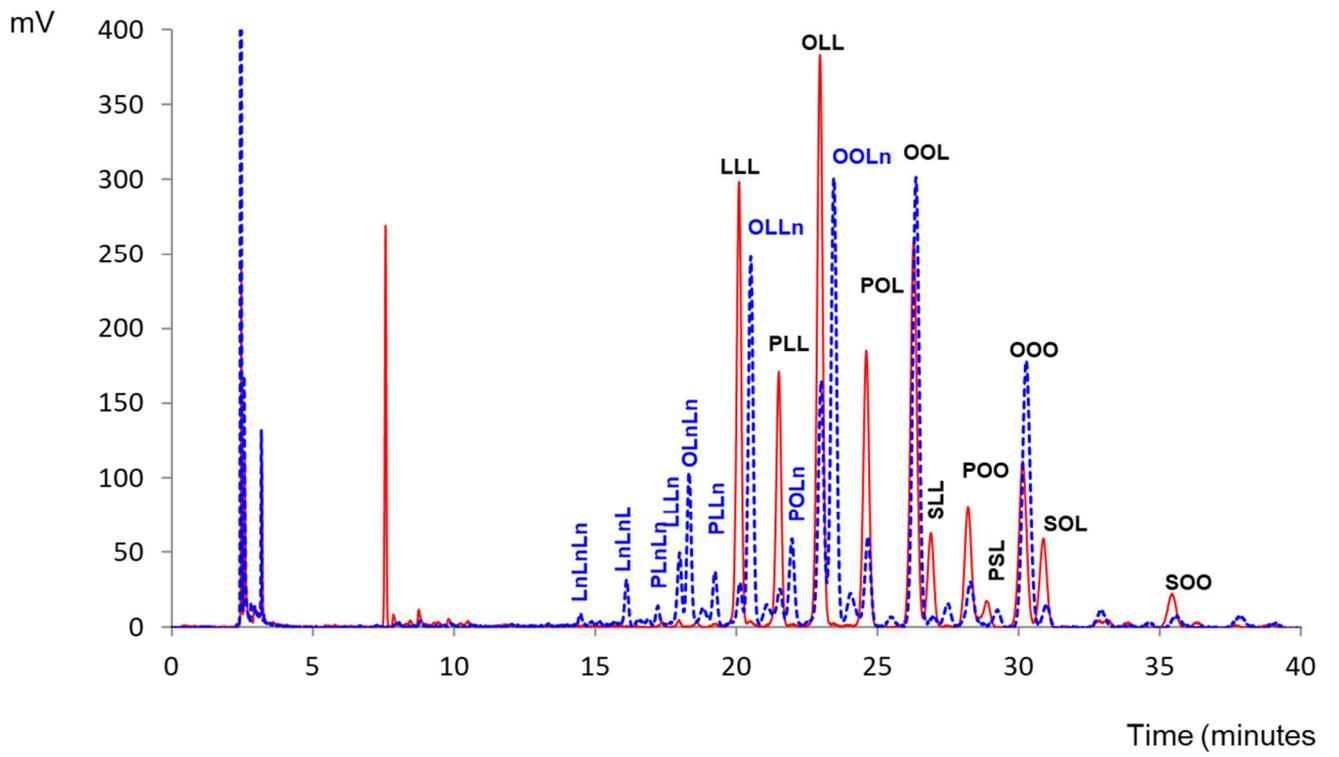


Figure 5

Table 1 References corresponding to various applications requiring serially coupled columns

Studied compounds	Mobile phase and conditions	Best number of columns	Coupled stationary phases	Reference
Lipids (ceramides, carotenoids, natural products)				
ceramides with dihydrosphingosine and saturated fatty acid sphingosine and saturated fatty acid phytosphingosine and saturated fatty acid sphingosine and α -hydroxy fatty acid sphingosine and unsaturated fatty acid	6% MeOH, 31°C, 130bar, at 3.2 mL/min	5	Kromasil UB 225 (250 × 4.6 mm, i.d., 5 μ m)	[26]
Canthaxanthin, violaxanthin, lutein, zeaxanthin, β -carotene, neoxanthin, astaxanthin, echinenone	From 10 to 25% MeOH, 32°C, 120bar, at 5 mL/min	2	1 SunFireC18 (250 × 4.6 mm, i.d; 5 μ m) and 1 Viridis SFC silica 2-ethylpyridine (250 × 4.6 mm, i.d.; 5 μ m).	[27]
Myristic, palmitic, palmitoleic, margaric, margaroleic, stearic, oleic, linoleic, linolenic, arachidic, gadoleic, behenic and lignoceric acids	12% (MeOH/ACN 1/9), 17°C, 100bar (flow-rate is stated to limit the inlet pressure)	5	4 Kinetex C18 (150 × 4.6 mm, i.d; 2.6 μ m) and 1 Accucore C18 (150 × 4.6 mm, i.d; 2.6 μ m)	[28]
<i>cis</i> and <i>trans</i> isomers of β -carotenoids	1% MeOH and 9% ACN, 25°C, 150bar, at 3 mL/min	6	5 Kromasil, YMC Pack Pro C18 (250 × 4.6 mm i.d., 5 μ m) and 1 Chromolith RP 18e (100 × 4.6 mm i.d., 5 μ m)	[30]

Studied compounds	Mobile phase	Best number of columns	Coupled stationary phases	Reference
Pharmaceuticals				
Cortisone, estradiol, progesterone, testosterone, 17-methyltestosterone, caffeine theophylline, thymine, uracil fenopufen, ketoprofen, naproxen, sulfadimethoxine sulfamerazine, sulfamethizole, sulfamethoxazole, sulfaquinoxaline	10% (MeOH/ACN 3/1 0.5% TFA and 0.5% DIPA), 40°C, 100bar, at 2 mL/min	5	Cyanopropyl silicagel Zorbax SB-CN (250 × 4.6 mm, i.d; 5 μm)	[31]
SC-65872, SC-65822, SC-66276, deoxybenzoin, SC-66285, SC-66414, SC-70730	(MeOH/0.5%IPA) from 98 to 30%, from -32 to 45°C, 120bar, at 3 mL/min	6	Supelcosil LC-CN (250 × 4.6 mm, i.d; 5 μm)	[32]
Cinnamionitrile and hydrocinnamionitrile enantiomers and diastereoisomers mixture and Z/E isomers from starting material	From 5 to 15% MeOH, 30°C, 120bar, at 1 mL/min	2	1 Luna silica (150 × 4.6 mm, i.d; 5 μm) and 1 Chiralcel OD-H (150 × 2.0 mm, i.d; 5 μm)	[33]
25 active pharmaceutic ingredients *	(MeOH/20 mM NH ₄ OH/2% H ₂ O) from 5 to 50%, 25°C, 150bar, at 0.8 mL/min	2	1 ACQUITY UPC2 HSS C18 SB (100 × 3.0 mm, 1.8 μm fully porous silica) and 1 Nucleoshell HILIC (100 × 3.0 mm i.d, 2.7 μm superficially porous silica)	[34]

*compound not depicted

Studied compounds	Mobile phase	Best number of columns	Coupled stationary phases	Reference
Preparative scale				
Six 25 (<i>R/S</i>)-spirostanol saponin diastereomers	30% MeOH, 40°C, 120bar, at 3.5 mL/min	2	1 Chiralpak IC (250× 21 mm i.d, 5 μm) and 1 Chiralpak IC (150× 21 mm i.d, 5 μm)	[37]
Four stereoisomers from a pharmaceutical compound*	10% (EtOH/2-PrOH 50/50), 40°C, 120bar, at 55 mL/min	2	1 Chiralpak AD (250× 21 mm i.d, 5 μm) and 1 Chiralcel OD (250× 21 mm i.d, 5 μm)	[38]
Chlormezazone and chrysine mixture	25% (MeOH/20mM NH ₃) at 4 mL/min	2	1 Amylose tris [(<i>S</i>)-α-methylbenzylcarbamate] and 1 HILIC (cross-linked Diol)	[39]
Racemate and achiral impurity mixture*	20% (MeOH/20mM NH ₃) at 4 mL/min	2	1 Imidazole and 1 immobilized amylose 3,5-dimethylphenyl carbamate (100 × 4.6 mm i.d.)	
Caffeine and dipiperdon mixture	23% (MeOH/20mM NH ₃), 20°C, 100bar, at 150 g/min	2	1 immobilized cellulose 3,5-dimethylphenyl carbamate (250 × 30 mm i.d.) and 1 pyridyl amide (150 × 30 mm i.d.)	
Two enantiomers from a pharmaceutical compound*	24% EtOH at 200g/min	2	1 Whelk-O1 (<i>S, S</i>) (250 × 50 mm i.d.) and 1 RegisCell (250 × 30 mm i.d.)	[40]

*compound not depicted