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1 **Recent trends in chiral supercritical fluid chromatography**

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10

11 **Abstract**

12 This paper presents a review of the recent literature covering separations of enantiomers  
13 achieved with supercritical fluid chromatography (SFC) or subcritical fluid chromatography.  
14 First of all, the recent progress in SFC technology are explained in relation to chiral  
15 separations. Mobile phases and operating conditions mostly encountered in current practice,  
16 instruments and stationary phases are discussed. Some elements related to fundamental  
17 studies are also given. While the pharmaceutical industry was long the principal user of chiral  
18 SFC for preparative purposes, it is now more and more in use for analytical purposes and  
19 especially pharmacokinetics. Other application fields are now emerging like the  
20 determination of different degradation rates in chiral agrochemicals, forensics, natural  
21 products and cosmetics. This review does not aim at being comprehensive but rather  
22 provides a global view of current practice of chiral resolution with SFC.

23

24 **Keywords:** applications; chiral resolution; chiral stationary phase; enantiomer separation;  
25 supercritical fluid chromatography.

26

27 **Abbreviations**

28	AcN	Acetonitrile
29	APCI	Atmospheric pressure chemical ionization
30	CSP	Chiral stationary phase
31	DAD	Diode-array detection
32	ELSD	Evaporative light-scattering detection
33	ESI	Electrospray ionization
34	EtOH	Ethanol
35	HPLC	High-performance liquid chromatography
36	iPrOH	Isopropylalcohol
37	GC	Gas chromatography
38	MeOH	Methanol
39	MS	Mass spectrometry
40	SFC	Supercritical fluid chromatography
41	UHPLC	Ultra-high-performance liquid chromatography
42	UHPSFC	Ultra-high-performance supercritical fluid chromatography

43

## 44 **Introduction**

45

46 Chirality is an essential feature of the living as it is present in a large proportion of natural  
47 molecules, including proteins, carbohydrates and many lipid species. Most natural species  
48 bearing some chirality are actually homochiral, meaning that one of the stereoisomer(s) is  
49 largely prevailing over the other(s). Living organisms therefore display different biological  
50 responses to stereoisomers: bioactivity resulting from interaction to target receptors, ADME  
51 processes (absorption, distribution, metabolism and excretion) and environmental fate may  
52 all differ between the isomers. As a result, the ability to identify, quantify and purify  
53 stereoisomeric species (enantioresolution) is necessary to many application fields. This is  
54 most easily achieved with chromatographic methods using enantioselective or chiral  
55 stationary phases (CSP). Among the possible chromatographic methods, supercritical fluid  
56 chromatography (SFC) is one of the most effective, both at the analytical and preparative  
57 scales [1]. In the following, recent developments in this technology will be exposed, in  
58 relation to enantioresolution. Major application fields and emerging trends will be presented.

59

60

## 61 **Evolutions in the technology and practice of SFC in relation to** 62 **enantioresolution**

63

### 64 ***Operating conditions***

65 In early developments of SFC in the years 1960s' to 1980s', the fluids employed as mobile  
66 phases were true supercritical fluids, obtained when the temperature and pressure are both  
67 set above the critical values. The advantages of such fluids to perform chromatographic  
68 separations are inherent to their intermediate state between gases and liquids: diffusion  
69 coefficients and viscosity are intermediate between those of gases and liquids, while density  
70 is close to that of liquids. These features result in fast high-efficiency separations, which may  
71 be reached at higher flow-rate and lower pressure drops than in liquid-phase  
72 chromatography. Note that this feature may be useful not only at the analytical scale but also  
73 at the preparative scale, where a recent paper from DaSilva and co-workers demonstrated  
74 that high flow-rates were beneficial to obtain higher productivity and lower energy  
75 consumption [2]. The low viscosity and high diffusivity also mean that system equilibrium is  
76 reached very rapidly, ensuring rapid method development and the possibility to employ  
77 ballistic gradients of mobile phase composition (<1 min).

78 Initially, the mobile phases employed were essentially pure fluids like fluorocarbons,  
79 ammonia or carbon dioxide. The latter is now the preferred fluid due to several advantages:

80 low values of critical temperature and pressure (31°C, 7.3 MPa), non-flammable, non-  
81 explosive, relative inertness, available in large quantities with a sufficient purity at a low price,  
82 no generation of waste and low toxicity. The last two points are highly desirable features, in a  
83 context where ecological impact of chromatographic methods is being increasingly  
84 scrutinized. However, carbon dioxide has a low polarity. Increasing its elution strength  
85 through increase of density (that can be obtained with higher pressure and lower  
86 temperature values), allows the elution of only moderately polar species. To elute polar  
87 species, it is necessary to introduce co-solvents, most often a short-chain alcohol like  
88 methanol (MeOH), ethanol (EtOH) or isopropanol (iPrOH), or sometimes acetonitrile (AcN),  
89 in proportions that may vary from 1 to 99%, although the most frequent operating range is  
90 rather 5-50%. 2,2,2-Trifluoroethanol was sometimes advocated as an alternative co-solvent  
91 for unstable analytes that may react with alcohols [3,4]. On another hand, a small portion of a  
92 stronger solvent like dichloromethane was shown to favour solubility at the preparative scale  
93 to enhance productivity [5]. Solubility is indeed often the limiting factor in preparative SFC [6].  
94 Because the portion of solvent is usually rather low (typically about 20%), the solvent  
95 consumption at the preparative scale remains moderate compared to liquid-phase methods.  
96 Also, because CO<sub>2</sub> returns to the gaseous phase after the separation, collecting fractions in  
97 preparative separations is yielding smaller volumes of solvent to evaporate and less waste to  
98 be processed than is usual in liquid phase. This is a definite ecological and economical  
99 advantage.

100 This combination of a principal non-polar solvent (CO<sub>2</sub>) and a polar co-solvent makes the  
101 mobile phase similar to those employed in normal-phase high-performance liquid  
102 chromatography (HPLC), which was long the preferred chromatographic method employed  
103 for enantioresolution. The transposition from normal-phase HPLC to SFC thus seems quite  
104 natural, although it is not always successful.

105 Other components called “additives” are also frequently introduced in small concentrations in  
106 the co-solvent; they are acids, bases, salts or small proportions of water used to favour the  
107 elution of most polar and ionisable species. While the additive is usually significant in SFC to  
108 achieve good peak shapes of ionisable species, its effect on chiral separations is also  
109 through changes in selectivity. The substitution of one additive by the other is sometimes  
110 causing tremendous impact on altering enantioresolution.

111 Technically, direct enantioresolution is possible with a non-chiral stationary phase and chiral  
112 mobile phase additives (CMPA). While this method was sometimes employed in the past  
113 (see for instance [7,8], the preferred method is now, by far, the use of enantioselective  
114 stationary phases. Still, one recent attempt is worth mentioning for its originality. Thurbide  
115 and co-workers have demonstrated that an empty tubing could be filled with water that would  
116 set on the walls of the tubing, thereby creating a stationary phase. Analytes could then

117 partition between the carbon dioxide mobile phase and the water stationary phase. This  
118 concept was applied in several papers, but most particularly to enantioresolution with a  
119 CMPA like  $\beta$ -cyclodextrin or vancomycin [9]. This is however a rather isolated experiment. In  
120 the following, only enantioresolution with chiral stationary phases will be related.

121  
122 When CO<sub>2</sub> is mixed to the co-solvent and additives in binary or ternary mixtures, the critical  
123 point is displaced towards higher values of temperature and pressure. In most experiments,  
124 the operating pressure is maintained above the critical pressure, while the operating  
125 temperature remains moderate. For enantioresolution, it is often desirable to reduce the  
126 temperature well below the critical temperature as low temperature tends to favour  
127 enantioselectivity in “enthalpy-driven separations”. For example, a recent paper showed the  
128 improved resolution of ketamine enantiomers at low temperature (15°C) [10]. The fluid  
129 employed is thus not a supercritical fluid anymore but it may be called “subcritical fluid”.

130 Because the way to operate the instrument and develop methods is not any different, and  
131 because there is a continuum of properties when moving from gas to supercritical fluid and  
132 liquid, no distinction will be made in the following. The term “SFC” (supercritical fluid  
133 chromatography) is then applied to any separation method employing a mobile phase  
134 containing pressurized carbon dioxide, whatever the exact state of the fluid.

135 Apart from this specific effect of temperature, the effects of operating conditions on  
136 enantioselectivity are often found to be rather modest [11]. The effect on enantioresolution  
137 may however be significant, through variations of retention (especially with mobile phase  
138 composition) and band width (all parameters).

139 The basic principles and fundamentals of SFC were largely detailed in recent papers [12,13].

140

### 141 ***Instruments***

142 The first SFC experiment was demonstrated in 1962 [14] but the first chiral separation in  
143 SFC was done more than twenty years later in 1985 [15]. Initial developments, particularly in  
144 the years 1980s', were mostly done with open-tubular columns and instruments based on  
145 gas chromatography (GC) systems. From the early 1990s', packed-column SFC with  
146 columns and instruments based on HPLC systems took the lead. Modern instruments are  
147 mostly based on HPLC and ultra-high-performance liquid chromatography (UHPLC)  
148 systems, with a few additions to ensure adequate pumping of compressible fluids and back-  
149 pressure regulation. Following the UHPLC trend, ultra-high efficiency is obtained in ultra-  
150 high-performance SFC (UHPSFC) through reduced extra-column volumes, although further  
151 developments in this direction are still desirable [16,17]. The most recent developments  
152 clearly aim at improved robustness and sensitivity to satisfy the needs of regulated  
153 environments [18,19], and higher efficiencies to comply with the needs of high-throughput

154 and high-resolution methods [20]. For enantioresolution, sensitivity is especially important to  
155 be able to quantify low proportions of a minor enantiomer in a scalemic mixture. High-  
156 throughput is particularly useful when a large number of samples must be processed in a  
157 short time, which may be the case of high-throughput experimentation of organic chemistry  
158 reactions [21,22].

159 The trend to ultra-high efficiency often goes along with ultra-fast separations [17,20,23]. In  
160 this view, further developments of analytical instruments are highly desirable. Currently, the  
161 injection process is too slow, taking more time than would be required for very high-  
162 throughput separations. Miniaturization is also one way to reduce analysis time, especially in  
163 reducing column dimension as was first demonstrated by Regalado and Welch with 1 cm-  
164 columns to achieve separations in a few seconds [25]. With some modifications of the  
165 injection program, it is then possible to realize multiple injection in a single experimental run  
166 (MISER), as was demonstrated by Zawatzky and co-workers [24]. Employing short chiral  
167 columns to reduce analysis time to a few seconds, they achieved 96 chiral separations in  
168 about half an hour.

169  
170 A recent paper demonstrated the first on-chip SFC separation. This is technically demanding,  
171 especially to deliver accurate flow-rate and maintain adequate temperature in each portion of  
172 the system but the authors succeeded in this task. Some of the examples were done with  
173 enantioselective stationary phases (polysaccharide-type on 3  $\mu\text{m}$  silica particles), with  
174 enantioseparations achieved within 10 to 20 s. Further reducing the stationary phase particle  
175 size should yield even faster analyses. Future developments shall include the full  
176 miniaturization of all system components (pumping of fluids, back-pressure regulator), as the  
177 glass-chip proposed was limited to injection, on-column separation and detection.

178  
179 Recent instrument evolution in SFC also applies to detection. Indeed, the most popular  
180 detectors employed in modern SFC are also essentially the same as in HPLC. For two  
181 decades (1990s' and 2000s'), UV-visible or diode-array detection (DAD) and, to a lesser  
182 extent, evaporative light-scattering detection (ESLD) or charged-aerosol detection (CAD)  
183 were dominating all applications. Chiroptical detection (optical rotation or circular dichroism),  
184 which is often employed in chiral HPLC, is infrequently found in chiral SFC publications [26].  
185 More recently, the use of mass spectrometry (MS) with electrospray ionization (ESI) or  
186 atmospheric pressure chemical ionization (APCI) sources has been rising fast, thanks to the  
187 facilitated hyphenation of modern instruments [27,28]. The selectivity and sensitivity of MS  
188 are clearly opening the way to new application fields like bioanalysis, where low  
189 concentrations of metabolites are looked for, or when synthetic intermediates might be

190 chemically susceptible to the aqueous conditions used in reversed-phase HPLC separations  
191 [29]. However, sample preparation must often be adapted, as the aqueous phases employed  
192 in the most usual reversed-phase HPLC-MS methods are not necessarily appropriate to  
193 injection in the carbon dioxide-based mobile phase, especially at preparative scale [30].  
194 Rather than looking for appropriate dilution solvent, an interesting solution was proposed by  
195 Shimadzu with the on-line coupling of supercritical fluid extraction (SFE) to SFC. One of the  
196 first example demonstrating the interest of this device for bioanalysis was shown with the  
197 extraction of dried serum samples [31]. A broader view on the possibilities of this technology  
198 can be found in a recent review [32]. A recent example related to enantioresolution was  
199 shown with the SFE extraction of ketamine from dried urine and on-line chiral SFC  
200 separation on a polysaccharide stationary phase [33].

201  
202 The inclusion of SFC in two-dimensional systems was demonstrated by several academic  
203 and industry teams in the past decade, but was rarely applied to chiral separations. The  
204 interest in combining an enantioselective dimension to a non-enantioselective dimension is  
205 mostly found in compounds of pharmaceutical interest, as active pharmaceutical ingredients  
206 may contain both chiral and achiral impurities. An older paper from Zeng et al. had shown  
207 how heart-cut SFC-SFC could be useful in isolating a single stereoisomer, with a first  
208 dimension on achiral SFC and a second dimension on chiral SFC, for analytical or  
209 preparative purposes [34]. More recently, solely for analytical purposes, Iguiniz et al.  
210 demonstrated the interest of selective comprehensive achiral reversed-phase HPLC x chiral  
211 SFC to achieve a most complete impurity profiling of chiral drugs [35]. Indeed, this is usually  
212 achieved in two separate steps where the achiral impurities are determined with HPLC and  
213 chiral impurities with chiral HPLC or SFC. The proposed method was thus very attractive and  
214 looked promising. An example of this method applied to a real sample of active  
215 pharmaceutical ingredient containing impurities is presented in Figure 1. Also for in-vivo  
216 interconversion of stereoisomers, the first achiral HPLC dimension could be used as a  
217 desalting method for complex biological matrices prior to the chiral SFC method, as  
218 demonstrated in a paper from Goel et al. [36].

219  
220 Further details on analytical instruments can be found in other reviews. Improved semi-  
221 preparative SFC instruments are now also under development, following the initiative of  
222 several companies (designated as the “Enabling Technologies Consortium” [37]) having  
223 joined their efforts to establish a common wish-list of desirable features. This unprecedented  
224 process should be most efficient in achieving the best compromise to satisfy most  
225 requirements. Prototype instruments are now under evaluation.



226

## 227 ***Stationary phases***

228 In the past decades, a variety of enantioselective stationary phases have been made  
229 available to HPLC and SFC chromatographers. Although a single chiral selector may be  
230 capable to resolve all enantiomers of a complete synthetic route [38], a diversity of stationary  
231 phases is indeed necessary, as no universal stationary phase exists, which would be  
232 capable to resolve all enantiomeric pairs. The usual method development process then starts  
233 with an automated column screening, most often conducted with a variety of mobile phase  
234 compositions, identified as “generic conditions”, meaning to provide adequate elution and  
235 resolution for a majority of cases [39].

236 High efficiencies are also achieved with the help of stationary phases containing small fully  
237 porous particles (FPP) or superficially porous particles (SPP). Achiral stationary phases with  
238 such features have been made available more than a decade ago, but enantioselective  
239 stationary phases with such particles have only been very recently developed and made  
240 commercially available.

241 While most polysaccharide stationary phases were long available at no smaller than 3  $\mu\text{m}$   
242 FPP (that were already quite an improvement over previously available 5  $\mu\text{m}$ -FPP [40]),  
243 coated polysaccharide phases were first introduced by Waters with 2.5  $\mu\text{m}$  FPP (Trefoil  
244 AMY1, CEL1 and CEL2) in 2014. Then immobilized polysaccharide stationary phases based  
245 on 1.6  $\mu\text{m}$  FPP were introduced by Daicel (Chiralpak IA-u, IB-u, IC-u, ID-u, IG-u and IH-u).  
246 The kinetic evaluation of Chiralpak IA-u in SFC conditions has been evaluated by Berger  
247 [41]. An example chromatogram is shown in Figure 2, where baseline resolution of trans-  
248 stilbene oxide is obtained in 7.5 seconds on a 50 x 3.0 mm column.

249 A Pirkle-type stationary phase, Whelk-O1, is available from Regis Technologies on 1.8  $\mu\text{m}$   
250 FPP [16,42,43]. Macrocyclic glycopeptide stationary phases based on sub-2  $\mu\text{m}$  FPP were  
251 proposed some years ago [44], but finally the authors preferred to develop these phases  
252 commercially as 2.7  $\mu\text{m}$  SPP (VancoShell, TeicoShell, TagShell and NicoShell available from  
253 Azyp). The same manufacturer is also offering a cyclofructan-based SPP phase (LarihcShell-  
254 P). All are advocated for SFC use, appear to have interesting complementarity and good  
255 overall performance. For instance, the applicability of the TeicoShell and NicoShell stationary  
256 phases to SFC enantioresolution of active pharmaceutical ingredients was demonstrated in a  
257 recent paper [38]. In addition to high efficiency, SPP particles offer the advantage of low  
258 pressure drops, making it possible to increase flow rates or co-solvent percentage at higher  
259 values than would be possible on FPP stationary phases of identical dimensions. This should  
260 be most useful in two-dimensional systems where chiral SFC would be the second  
261 dimension, and would thus need to be conducted at a very fast rate for comprehensive

262 analysis. Other chiral SPP phases have been presented in publications, like one quinine-  
263 based chiral selector [45], or immobilized polysaccharides [46], which are not yet available  
264 commercially. It is expected that they will be more broadly developed and applied in the near  
265 future.

266  
267 Another interesting possibility in SFC to achieve high efficiencies (but usually not at a high  
268 speed) is to couple columns in-line, taking advantage of the low viscosity of the fluid that  
269 ensures moderate pressure drops. Another advantage of this method is that different  
270 stationary phases may be combined to make full use of complementary selectivities. While  
271 this concept has been applied in several occasions in the past, some rationale in its  
272 development was recently proposed by two different teams. First, Hegade and Lynen  
273 extended the concept of stationary phase-optimized selectivity, previously employed in  
274 reversed-phase HPLC and achiral SFC, to enantioselective SFC [47]. To use this method,  
275 the enantioselectivity is first assessed on individual short columns. Then chromatographic  
276 retention is predicted to define the best selection of stationary phases, combining small  
277 sections of columns. The concept was demonstrated for mixtures of 4 or 5 racemates.  
278 Predictive errors were greatly reduced when corrected to take account of internal pressure  
279 and when mobile phase additives were employed, reaching as low as 1-5%. An example  
280 comparing predicted and experimental chromatograms is presented in Figure 2. In another  
281 publication, the group of Lipka applied this concept to achieve semi-preparative purification  
282 of all four stereoisomers of an active pharmaceutical ingredient containing two chiral centres  
283 and also demonstrated very good predictive ability of the separation factors [48]. Both  
284 approaches are particularly relevant in the present context of pharmaceutical development,  
285 where drug candidates issued from chemical synthesis tend to contain more and more chiral  
286 features [49], in an attempt to “escape from flatland”, as planarity was often observed to be  
287 unfavourable to bioactivity.

288

## 289 **Fundamental studies**

290 The search for ideal enantioresolution conditions is often a pretext for examining separation  
291 mechanisms, for instance through Van't Hoff plots [50] or molecular modelling [10], especially  
292 when several compounds with small structural variations are available. Unfortunately, the  
293 conclusions are generally only applicable to the series of compounds examined, as  
294 enantioseparation mechanisms are very complex. This is particularly the case on  
295 polysaccharide stationary phases that are the most employed in chiral SFC applications.  
296 Some comparison to chiral HPLC separations in the normal-phase, reversed-phase or polar  
297 organic modes are sometimes proposed (see for example [50–52]). Comparing identical

298 column dimensions, it is often observed that SFC provides higher resolution-to-analysis time  
299 ratio than HPLC (typically 2- to 5-times improvement). Indeed, even when resolution is not  
300 higher, it is often obtained in a shorter experimental time.

301  
302 Preparative SFC in general, and preparative chiral SFC in particular, would deserve some  
303 more attention to achieve more effective scale up. Usually transferring a method from  
304 analytical to preparative scale presents no particular difficulty, if one considers the internal  
305 pressure to maintain it identical between the two experiments [53], and taking into account  
306 the real conditions of operation, especially the fact that analytical systems are usually  
307 volume-controlled while preparative systems are mass-controlled [54]. However, peak  
308 deformation may occur, which may be due to the co-solvent adsorption [55] or to viscous  
309 fingering and adsorption of the dilution solvent. Modifier-stream injection (eluting the sample  
310 with co-solvent prior to mixing with carbon dioxide) [56] and extraction-injection (injecting the  
311 samples as solids from an extraction cell) could be the best solutions to avoid solubility  
312 issues.

313

## 314 **Applications**

315

### 316 ***Pharmaceuticals***

317 The pharmaceutical industry and design of drug candidates in academic laboratories are  
318 both largely concerned by enantioresolution: firstly, to evaluate the enantiopurity of synthesis  
319 intermediates and active pharmaceutical ingredients at every stage of drug development and  
320 purify the desired stereoisomer when the observed purity is not considered sufficient;  
321 secondly to evaluate the fate of chiral drugs and notably isomer conversion during  
322 metabolism; thirdly, to obtain sufficient quantities of pure isomers to evaluate their bioactivity  
323 separately. Both analytical and preparative enantioresolution methods are required. For all  
324 these purposes, SFC is now most useful as it allows for fast method development, in  
325 accordance with the requirements of the industry [18]. Indeed, while HPLC has long been in  
326 the lead in pharmaceutical industries, the advantages of SFC have been recognized for  
327 many years, first at the preparative scale and more recently at the analytical scale [57]. This  
328 latter point is mostly due to the introduction of improved analytical SFC instruments and  
329 stationary phases, as detailed above. In particular, the progressive reduction in particle size  
330 of chiral stationary phases has permitted tremendous reduction in analysis time of the  
331 screening process, as previously discussed by Mattrey et al. [57] and evidenced in Table 1.  
332 Even when no chiral separation is required, a chiral stationary phase may be useful:  
333 basically, in any case when structurally similar analytes are encountered and especially

334 isomers (not only enantiomers), a chiral stationary phase may offer a better selectivity than  
335 some achiral stationary phases. For instance, Regalado and Welch used this strategy for  
336 difficult pharmaceutical compounds at both analytical and preparative scale [58].

337 In addition, the fast emergence of SFC applications in the field of bioanalysis [59], for  
338 instance for pharmacokinetics, is largely due to recent improvements in the hyphenation to  
339 mass spectrometry mentioned above. For instance, Camacho-Muñoz et al. measured the  
340 interconversion of the stereoisomers of an active pharmaceutical ingredient during  
341 metabolization processes. Another significant example of the interest of SFC-MS to analyse  
342 pharmaceutical compounds and their metabolites is the enantioresolution of pharmaceuticals  
343 as environmental pollutants in wastewater samples [60], a task that is also requiring most  
344 sensitive detection. In another example, Regalado et al. successfully applied  
345 enantioselective SFC to the separation and analysis of complex enantiomeric mixtures  
346 containing hydroxywarfarins obtained from incubation of warfarin with human liver  
347 microsomes [61]. Many other examples of chiral SFC applied to bioanalysis can be found in  
348 a recent review [62].

349 A broader view of preparative chiral SFC can be found in a recent review [63].

350 Open-access SFC systems are now implemented in academic and industry laboratories [64],  
351 for analytical or preparative purposes, revealing the interest of end-users and the facilitated  
352 operation of the instruments that has been achieved in recent years. Simplified platforms with  
353 well-selected complementary stationary phases [65] (preferably the least fragile ones like  
354 bonded-silica or polysaccharides immobilized on silica) offer the possibility to achieve  
355 enantioresolution in a majority of cases, while a minority of cases still require the intervention  
356 of a chromatography specialist.

357 More details on chiral SFC of pharmaceuticals can be found in a recent review [66], while  
358 other reviews relate the use of achiral and chiral SFC to pharmaceuticals [67,68].

359

### 360 ***Natural products***

361 Chiral fragrances are mostly resolved with gas chromatography, and supercritical fluid  
362 chromatography is rarely found competitive in this field. Only when some transfer to  
363 preparative scale is desired can SFC be found of some use. However, many natural  
364 products, which may be of interest in the context of traditional medicine or in the food  
365 industry, contain non-volatile chiral species that may be analysed with enantioselective SFC.  
366 The interest of sub-2  $\mu\text{m}$  FPP stationary phases was demonstrated with the  
367 enantioresolution of phytocannabinoids on a Whelk-O1 stationary phase [69]. Most  
368 particularly, one specific feature of this brush-type stationary phase is that it is available in  
369 two enantiomeric configurations: (R,R) or (S,S), allowing for reversed elution orders when

370 one column is replaced by the other. In this paper, this was cleverly employed to determine  
371 the enantiomeric excess of tetrahydrocannabinol in medicinal cannabis.

372 Another paper describes the enantioresolution of free amino acids in a traditional  
373 phytotherapeutic plant, Shepherd's purse [70]. Enantioseparation of 16 amino acids out of 19  
374 was achieved with Chiralpak ZWIX stationary phases, which are brush-type CSP containing  
375 quinine or quinidine-based zwitterionic ligands and are well known for their capability to  
376 resolve amino acids in HPLC. In this experiment, to achieve the retention and elution of all  
377 proteinogenic amino acids, from the least polar to the most polar, it was necessary to employ  
378 a wide elution gradient ranging from nearly 100% CO<sub>2</sub> to 100% co-solvent. Also, adequate  
379 peak shapes could only be obtained with the introduction of water and ammonium acetate  
380 (respectively 5% and 50 mM in the methanol co-solvent), especially for the most basic  
381 species. An example of enantioseparation obtained for one particularly difficult racemate, the  
382 basic lysine, is shown in Figure 3. Previous works [71,72] showing the enantioresolution of  
383 free amino acids in SFC did not necessarily employ mobile phase additives but had not  
384 succeeded in such a wide polarity range. This way of operating the SFC system, moving  
385 from supercritical fluid to liquid in a single experiment, was inspired by a seminal paper from  
386 the group of Bamba [73], and could be termed "unified chromatography" (UC).

387 In the field of natural products, sample complexity is high with many structurally similar  
388 compounds encountered. For instance, isomers are often numerous within one structural  
389 family encountered in one plant. Here again, as was mentioned for pharmaceutical  
390 compounds, to achieve a most complete separation, the use of a chiral SFC method is often  
391 found useful, even though no chiral samples is involved. For instance, such examples can be  
392 found in paper from Xin and co-workers [74,75] where a chiral preparative SFC method was  
393 used as a complementary purification to an achiral SFC method to simplify a complex plant  
394 extract or to resolve diastereomers. Epimeric pairs of triterpenoids, another example of  
395 diastereomeric pairs, were also resolved with SFC and a chiral stationary phase in the works  
396 of Qiao et al. [76].

397 With the fast rise of SFC use in the field of traditional Chinese medicine [59,77], it can be  
398 expected that other applications of chiral SFC to natural products will be seen in the near  
399 future.

400

#### 401 ***Agrochemicals***

402 A large portion of agrochemicals (about 30% [78]) bear one or more chiral centres. Racemic  
403 mixtures are still employed in most cases even when the bioactivity of the stereoisomers is  
404 known to differ significantly. To assess the individual bioactivity, chiral preparative methods  
405 are needed, while analytical methods are useful to assess the different degradation rate of

406 the isomers to determine their fate in the environment. In the recent years, many studies  
407 have been devoted to chiral SFC for this purpose, applied to different categories of  
408 agrochemicals that were looked for in food products (fruits, vegetables, tea or cereals),  
409 tobacco and soil samples. For instance, see [78–82]. Other examples are listed in other  
410 reviews on food analysis [83,84].

411

### 412 ***Forensics***

413 Other application fields also require enantioresolution where chromatographic separation is  
414 most useful. Chiral SFC has been recently implemented in the field of forensic toxicology,  
415 where the advent of designer drugs is requiring the development of complementary high-  
416 performance analytical methods including gas chromatography, HPLC and SFC [85]. Indeed,  
417 impurity profiling of drugs, and chiral drugs in particular is helpful in identifying the  
418 clandestine laboratories. For example, a recent paper showed the development of chiral SFC  
419 methods to analyse illicit samples of methamphetamine and its precursors [86]. Another  
420 paper identified enantiomeric excess of amphetamine in urine samples to differentiate  
421 between legal and illegal consumption [87]. Further examples of the application of (achiral  
422 and chiral) SFC-MS in the field of forensics can be found in another review [88].

423

### 424 ***Cosmetics***

425 The chromatographic analysis of cosmetic ingredients or formulations is rising slowly but  
426 steadfastly. Only rare examples are yet found concerning chiral SFC. For instance, Khater  
427 and West [89] had demonstrated the interest of SFC-MS for the direct enantioresolution of  
428 panthenol enantiomers, an active ingredient often found in many creams and lotions to  
429 favour skin hydration. In previous methods, it was necessary to derivatize the small polar  
430 molecules to achieve adequate retention and enantioresolution in gas chromatography or  
431 reversed-phase HPLC.

432

433

### 434 **Conclusion**

435 Although supercritical fluid chromatography had been well developed for chiral separations  
436 since the 1990s', the recent technological evolution in the instruments have been beneficial  
437 to achieve high-efficiency, high-throughput enantioresolution in regulated environments  
438 where methods need to be thoroughly validated. Novel stationary phases with small and/or  
439 superficially porous particles are especially useful in this respect. Also the progresses in  
440 hyphenation to mass spectrometry have opened the way to new application fields where  
441 sensitivity is desired (like bioanalysis and environmental analysis) or where accurate mass

442 detection is necessary in complex samples (like natural products). In other words, while  
443 chiral SFC was long a favourite for its benefits at the preparative scale, its merits at the  
444 analytical scale are finally recognized as well.

445

446

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451

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## Figures

**Figure 1.** Achiral-chiral analysis of a pharmaceutical sample by sRPLCxSFC with gradient elution in <sup>2</sup>D-SFC: <sup>1</sup>D-RPLC chromatogram for chemical purity (top); zoom on chiral compound peaks (middle) and <sup>2</sup>D-SFC chromatograms of the different consecutive fractions for enantiomeric purity (bottom). An impurity separated from active pharmaceutical ingredient in SFC is circled in pink. Reproduced from Iguiniz et al. [28] with permission from Elsevier.

**Figure 2.** Performance of the 50 × 3 mm, 1.6 μm ChiralPak IA-U column, with 0.1 μL injections of *trans*-stilbene oxide. 4.65 mL/min of 30% MeOH in CO<sub>2</sub>, 40 °C, 100 bar back-pressure. Pump pressure 592 bar. Reproduced from Berger [31], with permission from Elsevier.

**Figure 3.** Predicted (A) compared to experimental (B) chromatograms obtained with a combination of three chiral stationary phases for a mixture of 5 racemates. Conditions: CO<sub>2</sub>/ethanol/trifluoroacetic acid/isopropylamine (90/10/0.1/0.5% v/v) isocratic composition. Reproduced from Hegade and Lynen [38] with permission from Elsevier.

**Figure 4.** Resolution of lysine enantiomers on Chiralpak Zwix(+) (150 × 4.6 mm, 5 μm), with methanol co-solvent containing 50 mM ammonium formate and 5% of water. Gradient elution from 10% to 100% co-solvent, 25 °C, 150 bar, 0.5 mL/min, ESI (+) detection (m/z = 147). Adapted from Raimbault et al. [58], with permission from Springer.

783 **Tables**

784

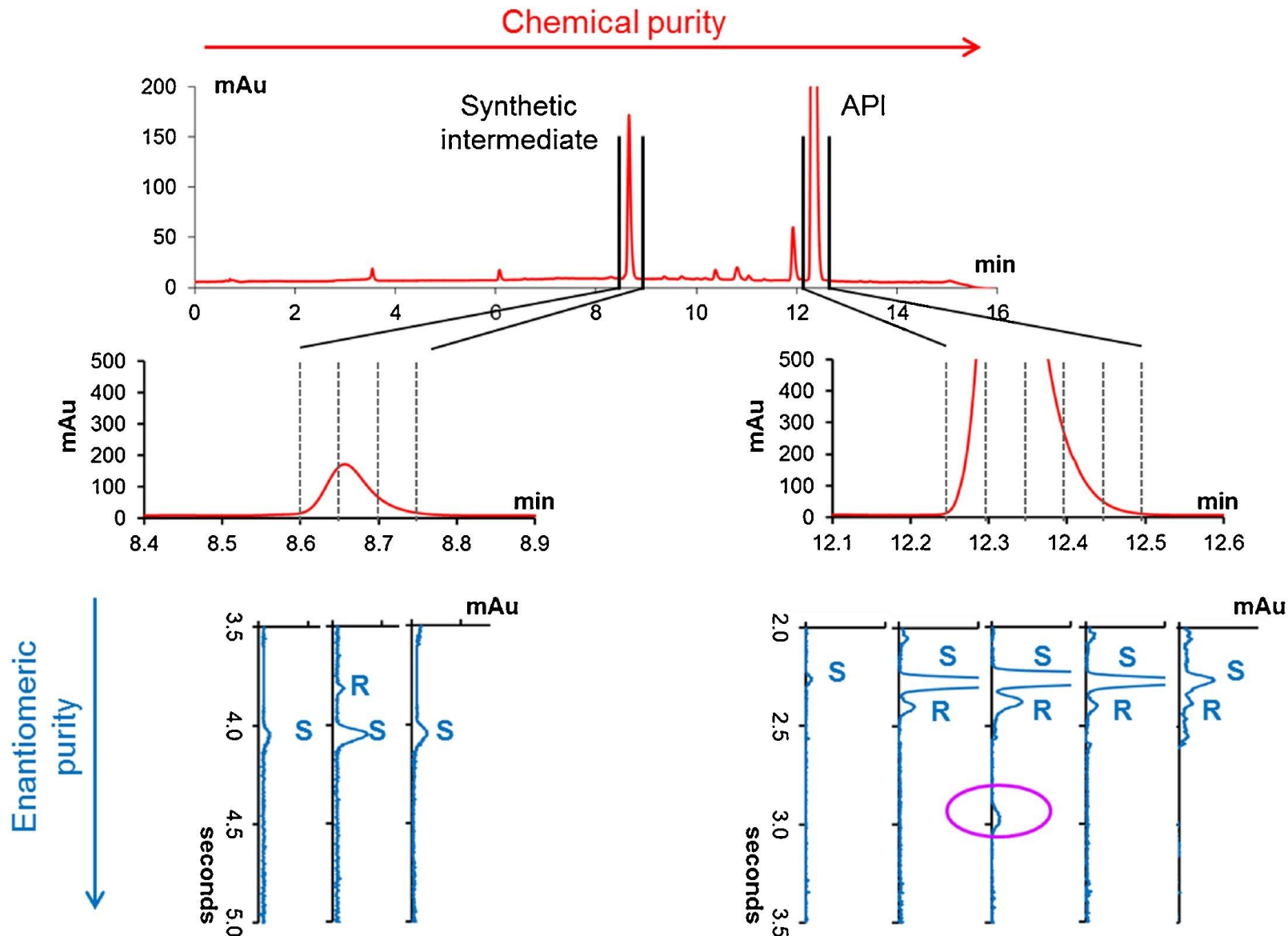
785 **Table 1.** Continuously improving chiral SFC screening for analytical method development.

786 Adapted from Mattrey et al. [47], with permission from Elsevier

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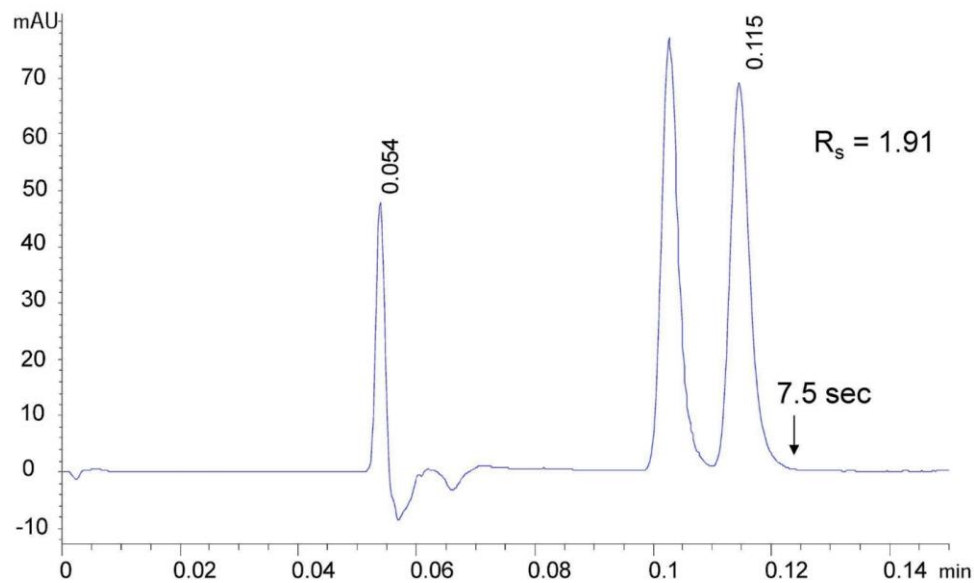
<b>Conditions</b>	<b>1990</b>	<b>2005</b>	<b>2010</b>	<b>2017</b>
Column size (mm)	250 x 4.6	250 x 4.6	150 x 4.6	50 x 3.0
Particle size (µm)	10	5	3	1.6
Flow rate (mL/min)	1.5	2	3	3
Approximate run time (min)	30	16	6	2

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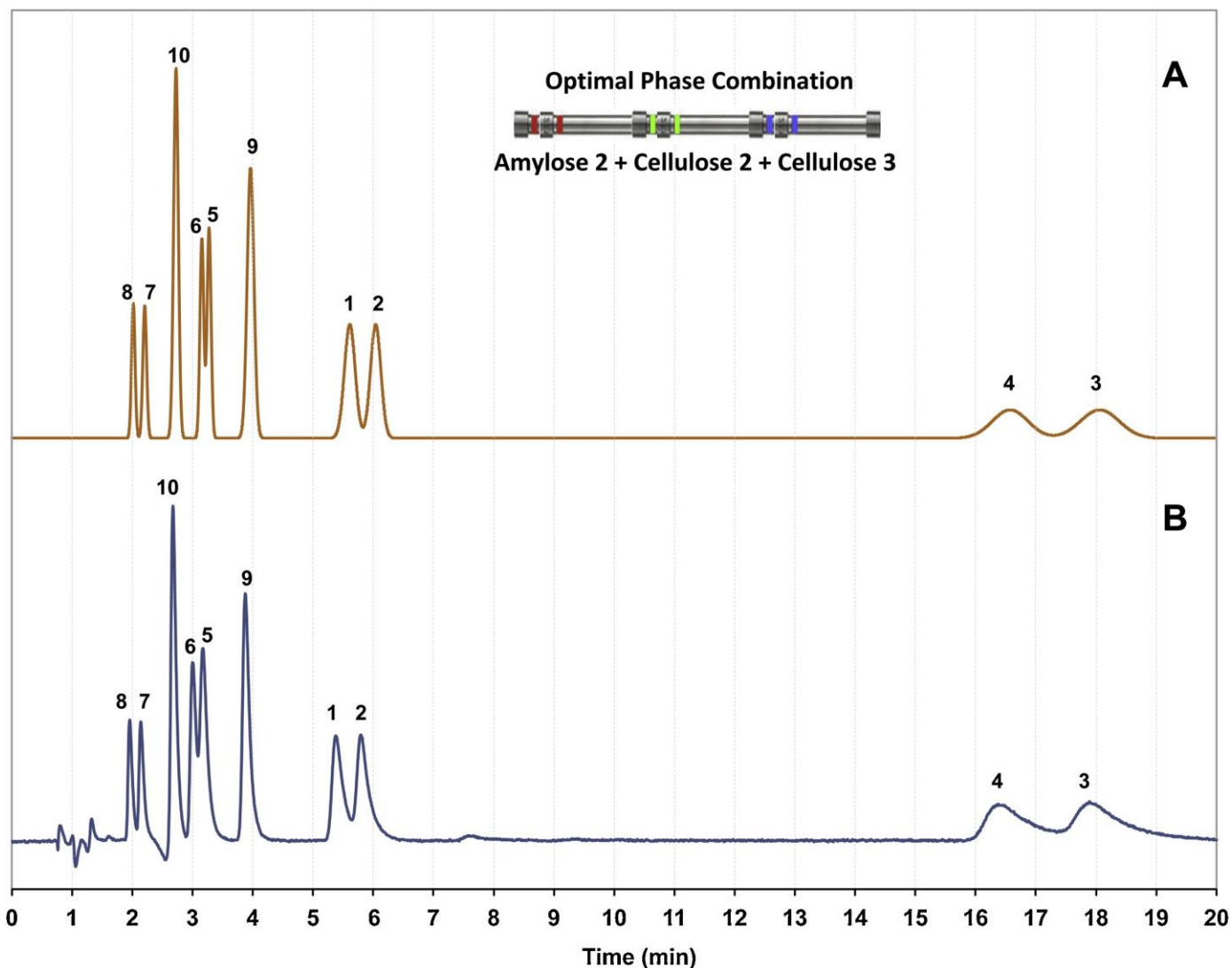


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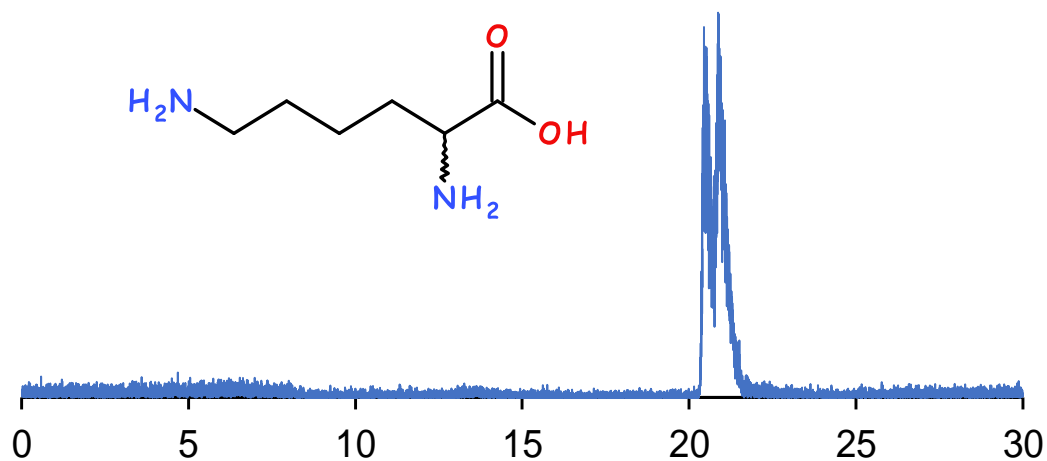




**Figure 2.** Performance of the 50 × 3 mm, 1.6 μm ChiralPak IA-U column, with 0.1 μL injections of *trans*-stilbene oxide. 4.65 mL/min of 30% MeOH in CO<sub>2</sub>, 40 °C, 100 bar back-pressure. Pump pressure 592 bar. Reproduced from Berger [31], with permission from Elsevier.



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# INNOVATIONS IN CHIRAL SFC



Pharmaceuticals



Forensics



Natural products

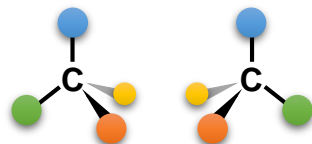


Agrochemicals

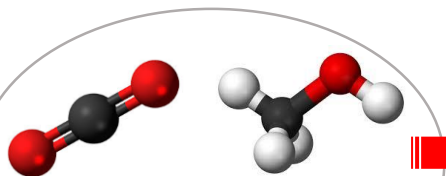


Cosmetics

*Enantiomers*

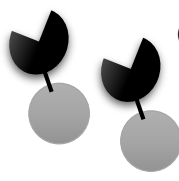


*SFC mobile phase*



$\text{CO}_2$  + co-solvent  
*in all proportions*

*High-efficiency chiral stationary phases*



Chiral selector

Sub-2  $\mu\text{m}$  fully porous particles

Chiral selector

Superficially porous particles

*Mass spectrometric detection*

*Enantio-resolution*

