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► **To cite this version:**

Caroline West. Recent trends in chiral supercritical fluid chromatography. Trends in Analytical Chemistry, 2019, 120, pp.115648 -. 10.1016/j.trac.2019.115648 . hal-03487279

HAL Id: hal-03487279

<https://hal.science/hal-03487279v1>

Submitted on 20 Jul 2022

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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 CO₂ 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 (CO₂) 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 β -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 CO₂ 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 μm 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 examples 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 μm
242 FPP (that were already quite an improvement over previously available 5 μm -FPP [40]),
243 coated polysaccharide phases were first introduced by Waters with 2.5 μm FPP (Trefoil
244 AMY1, CEL1 and CEL2) in 2014. Then immobilized polysaccharide stationary phases based
245 on 1.6 μm 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 μm
250 FPP [16,42,43]. Macrocyclic glycopeptide stationary phases based on sub-2 μm FPP were
251 proposed some years ago [44], but finally the authors preferred to develop these phases
252 commercially as 2.7 μm 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 μm 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% CO₂ 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.

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446

447 **Acknowledgements**

448 Caroline West is grateful for the support received by the Institut Universitaire de France
449 (IUF), of which she is a Junior Member.

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Figures

Figure 1. Achiral-chiral analysis of a pharmaceutical sample by sRPLCxSFC with gradient elution in ²D-SFC: ¹D-RPLC chromatogram for chemical purity (top); zoom on chiral compound peaks (middle) and ²D-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 CO₂, 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: CO₂/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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Conditions	1990	2005	2010	2017
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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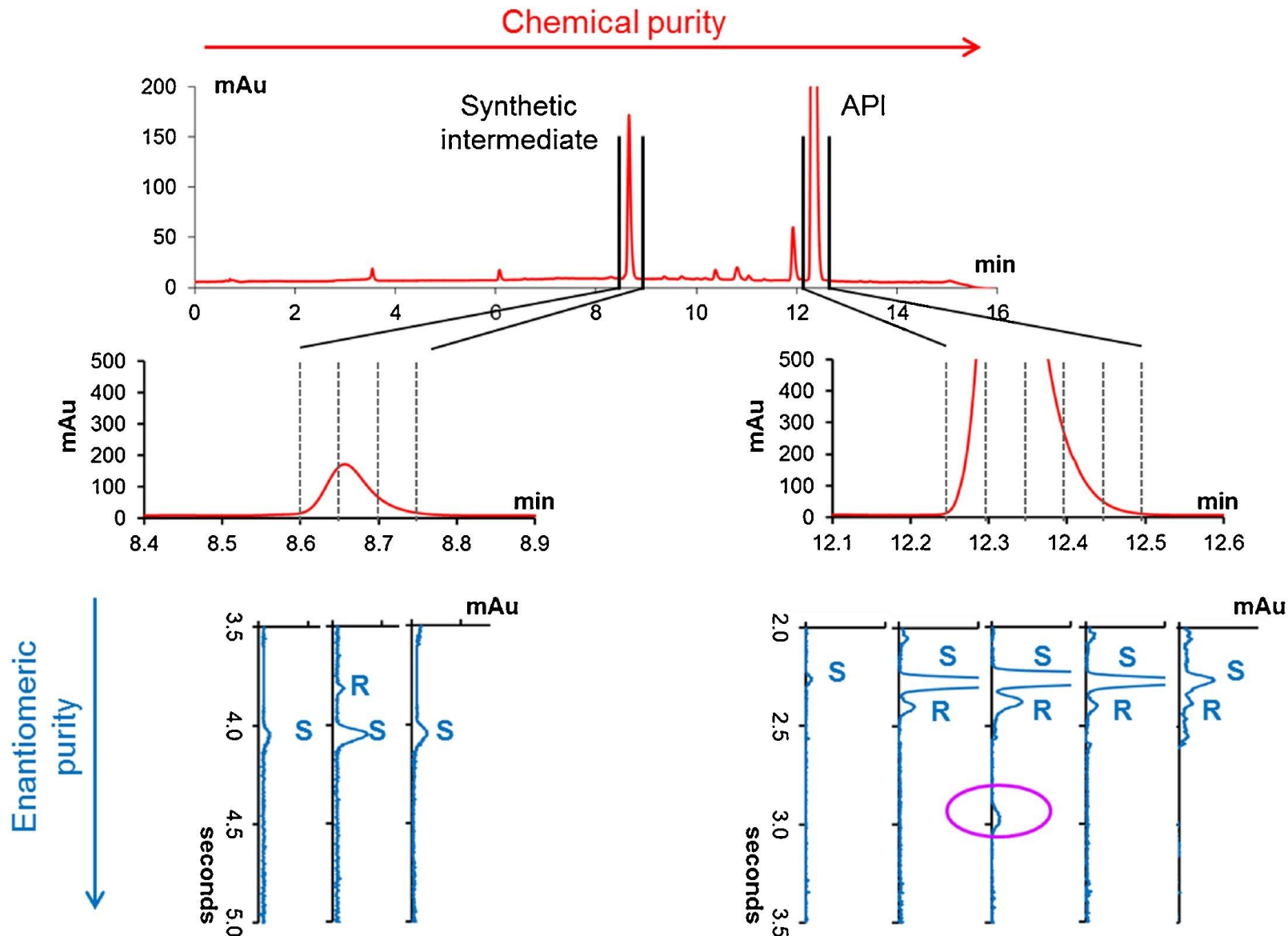


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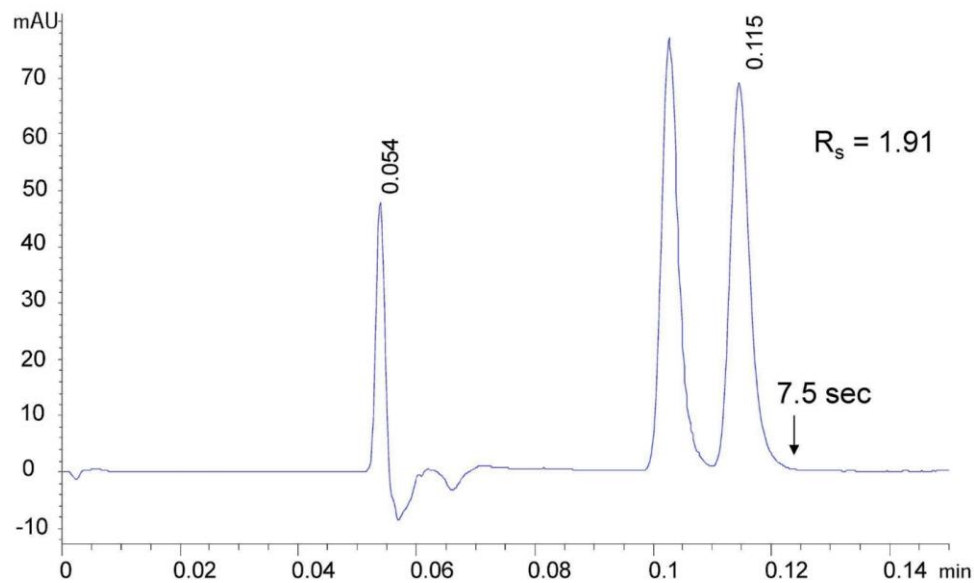


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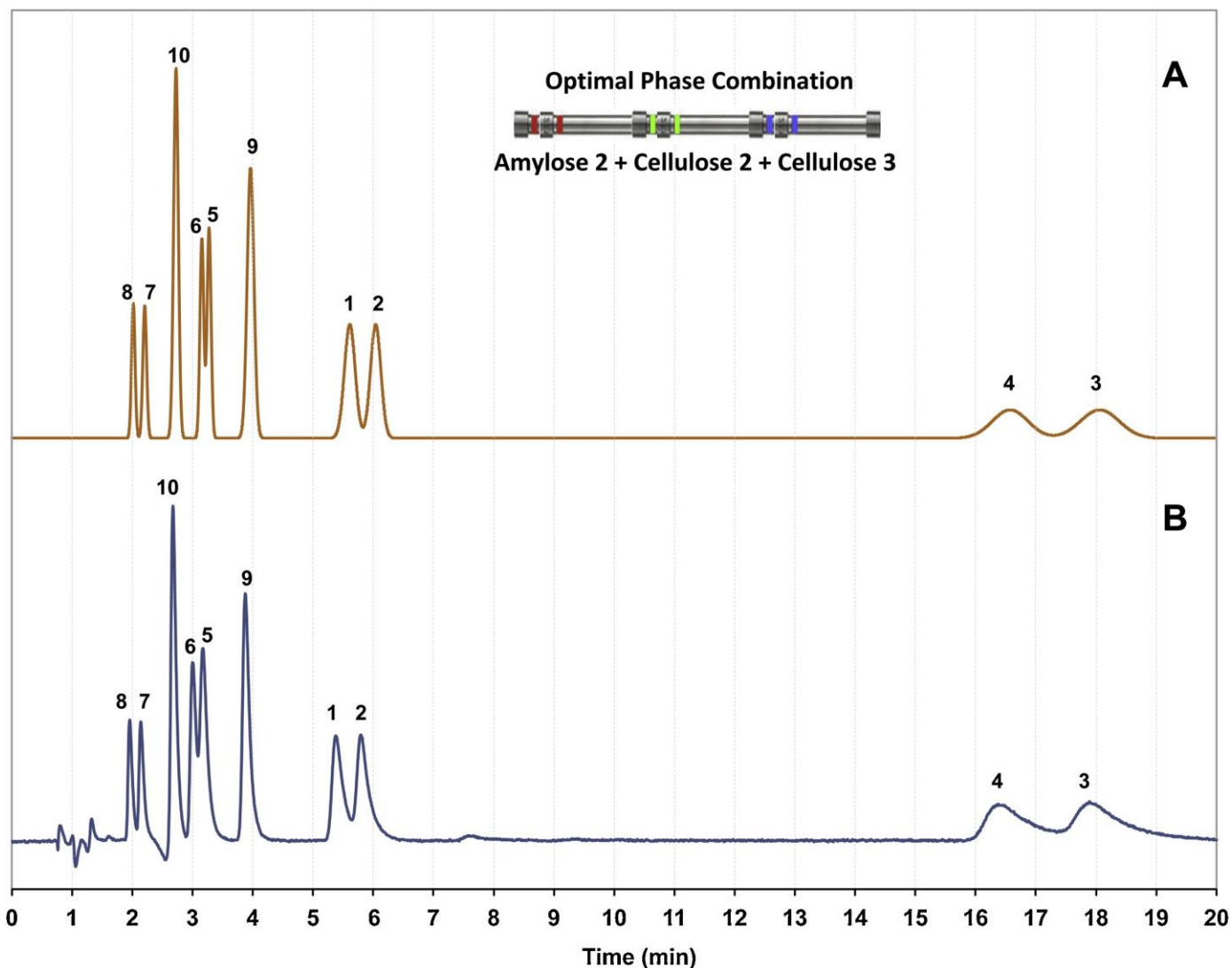


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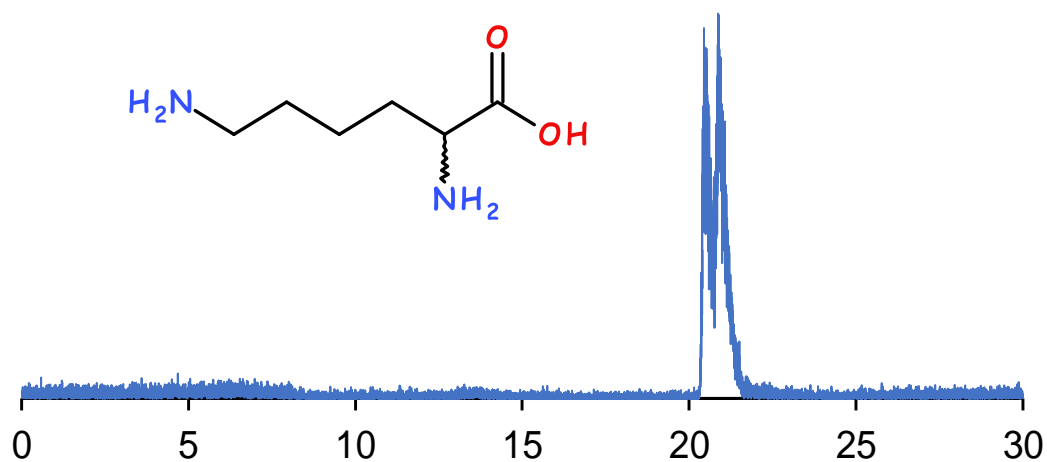


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INNOVATIONS IN CHIRAL SFC



Pharmaceuticals



Forensics



Natural products

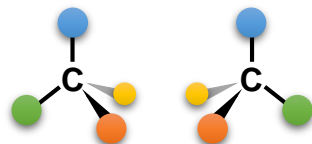


Agrochemicals

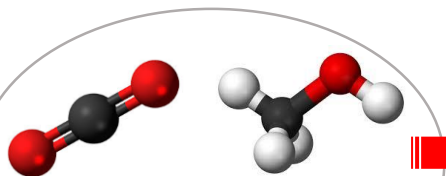


Cosmetics

Enantiomers

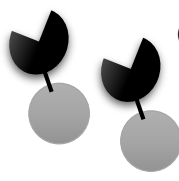


SFC mobile phase



CO_2 + co-solvent
in all proportions

High-efficiency chiral stationary phases



Chiral selector

Sub-2 μm fully porous particles

Chiral selector

Superficially porous particles

Mass spectrometric detection

Enantio-resolution

