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► **To cite this version:**

Marion Buret-Parendel, Karine Faure. Opportunities and challenges of liquid chromatography coupled to supercritical fluid chromatography. Trends in Analytical Chemistry, 2021, 144, pp.116422. 10.1016/j.trac.2021.116422 . hal-03326884

HAL Id: hal-03326884

<https://hal.science/hal-03326884>

Submitted on 26 Aug 2021

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1 Opportunities and challenges of liquid chromatography coupled to supercritical 2 fluid chromatography

3

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9

10 **Abstract:**

11 The analysis of complex samples is facilitated by the emergence of two-dimensional liquid
12 chromatography. Despite optimization efforts to reach tremendous peak capacities, the separation of
13 neutral compounds remains limited. Indeed, most combinations of chromatographic modes suffer
14 either from a lack of orthogonality or from serious solvent incompatibilities. The two-dimensional
15 separation involving a combination of liquid chromatography and supercritical fluid chromatography
16 seems to offer new opportunities for the separation of ionizable and neutral solutes. This review
17 highlights the orthogonality the combination can offer thanks to a wide range of stationary phases.
18 Injection effects occurring in SFC, that may drastically reduce the performances of online LC x SFC are
19 also discussed in details. Finally, despite net improvement in SFC instrumentation in the recent years,
20 instrumental limitations still have to be overcome. While this review highlights the potential of LC and
21 SFC combination and its complementarity with 2D-LC, it also demonstrates that its development
22 requires the joint efforts of researchers and instrumental suppliers before comprehensive LC x SFC
23 gets adopted by the scientific community.

24

25 **Keywords:**

26 Two-dimensional chromatography; liquid chromatography; supercritical fluid chromatography;
27 injection effects; neutral molecules

28

29 **Abbreviations:**

30 2D-LC: two-dimensional liquid chromatography.

31 SFC: supercritical fluid chromatography

32 ¹D, ²D: first and second dimension, respectively.

33 LC x LC: comprehensive 2D-LC

34 LC-LC, sLC x LC: 2D-LC in heart-cut mode and selective comprehensive mode, respectively

35 LC x SFC: two-dimensional chromatography with LC as the first dimension and SFC as the second
36 dimension; SFC x LC being the same combination but in the opposite order.

37 MS: mass spectrometry

38 HILIC: Hydrophilic interaction liquid chromatography

39 NPLC; RPLC: normal-phase LC; reversed-phase LC

40 SEC: size exclusion chromatography

41

42 **Highlights:**

- 43 • The separation of neutral molecules is limited in LC x LC
- 44 • Two-dimensional LC x SFC or SFC x LC combinations exhibit interesting orthogonality for the
- 45 separation of both ionizable and neutral molecules
- 46 • Injection effects in SFC are still under investigation
- 47 • Specific interfaces have been proposed but a commercial instrumentation is still lacking

48

1. Limitations of LC x LC for the separation of neutral compounds

Liquid chromatography (LC) is one of the most widely used analytical techniques. Its popularity lies in its remarkable versatility, owed to the great variety of its separation modes, the wide range of parameters available to refine selectivity, its robustness, reliability and compatibility with diverse detectors. However, when dealing with complex samples containing a high number of compounds, the separation power of LC is generally limited. In this case, two-dimensional liquid chromatography (2D-LC) has proven to be an efficient alternative [1]. Based on the difference in selectivity between the two dimensions, the technique can be applied either in heart-cut mode (noted LC-LC), in comprehensive mode (noted LC x LC), or even in comprehensive mode on a selected part of the first dimension separation (selective comprehensive mode, noted sLC x LC). Comprehensive LC x LC generates exceptionally high peak capacities compared to LC. Indeed, the theoretical peak capacity of a two-dimensional separation equals the product of peak capacities of each dimension. Nevertheless, this relationship only applies under certain ideal conditions, which are in practice, never met. The effective peak capacity is therefore lower due to i) undersampling effect, ii) non-ideal transfer between the two dimensions and iii) incomplete retention space coverage. These three negative effects have to be minimized in any 2D separation and much consideration has been brought to LC x LC optimization. Undersampling effect may be restrained by dividing each first dimension (¹D) peak into a sufficient number of fractions, though in online mode, it involves to minimize the duty cycle time of the second dimension (²D) separation. On the other hand, the negative impact of non-ideal transfer of the ¹D fractions to the ²D column, generating strong solvent effect or immiscibility issue has to be tempered, otherwise peak broadening or peak distortion can affect the ²D separation. Finally, the retention space coverage depends on the orthogonality between both dimensions. The maximization of orthogonality is of primordial importance and necessitates the selection of two systems whose selectivities towards the analytes are as uncorrelated as possible. This delicate selection is the starting point of any 2D separation development.

The combination of two dimensions involving both reversed-phase mechanism, RPLC x RPLC, constitutes the most widespread type of 2D-LC. This can be explained by the relatively good compatibility between the two dimensions, but also by the numerous advantages of RPLC in comparison with other LC modes. Among these ones, good understanding of separation mechanisms, repeatability, low re-equilibration time, compatibility with MS detection and applicability to a wide range of solutes can be mentioned. Coupling RPLC to RPLC modes is ideal for ionizable species as high orthogonality degree can be easily achieved by applying different pH in the two dimensions, hence changing the analyte ionization state [2]. However, when dealing with neutral species, pH cannot be used to modulate selectivity, leaving only low-impact parameters to generate orthogonality. The compounds retention between both dimensions are thus generally strongly correlated, no matter the conditions, although some non-conventional stationary phases like porous graphitic carbon, zirconium-based or polymeric organic-based stationary phases can bring some selectivity differences. This results in relatively poor orthogonality degree for RPLC x RPLC combinations used for the separation of neutral analytes, with retention space coverage usually not exceeding 60 % [3-5].

The combination of normal-phase liquid chromatography (NPLC) with RPLC allow the generation of high orthogonality degrees[6]. Tian et al. [7] and Fan et al. [8] both reported more than 70 % retention space coverage for neutral flavonoids in licorice and a traditional Chinese medicine extract, respectively. Such results are related to the very different retention mechanisms of NPLC and RPLC. However, the two modes are highly incompatible in terms of mobile phases, making their coupling very challenging. NPLC uses strongly apolar solvents such as hexane and heptane, which are not fully miscible with RPLC mobile phases, usually composed of water with acetonitrile or methanol. This leads to the formation of massive signal interferences in online NPLC x RPLC using UV detection [6, 7], whereas only non-aqueous RPLC can be set as first dimension as water adsorbs onto the NPLC

97 stationary phase and ruins the ²D separation. Complete removal of the ¹D mobile phase is hence
98 compulsory, either thanks to fraction evaporation in off-line mode [9] or vacuum-evaporation
99 interfaces [10]. Although very interesting in terms of orthogonality for neutral solutes, combining RPLC
100 to NPLC is therefore a tedious task.

101 Hydrophilic interaction liquid chromatography (HILIC) is a variant of NPLC, and its combination with
102 RPLC can theoretically achieve important orthogonality degrees [11]. While the retention in HILIC is
103 somehow limited for polar or ionizable species, HILIC mode provides an important surface coverage in
104 combination with RPLC, as recently illustrated for the 2D separation of peptides [12, 13], polyphenols
105 [14] or carbohydrates [15, 16]. Nevertheless, the two mobile phases suffer from important eluting
106 strength mismatch. Such contrast can result in severe injection effects, usually characterized by peak
107 distortions or even breakthrough phenomena, i.e. elution of a part of the analyte population at the
108 dead time while the other part elutes at the normal retention time, hence reducing the sensitivity of
109 the method. Very recently, alternatives have been proposed such as online dilution with or without
110 trapping columns, or with a specific interface known as active solvent modulation [1]. These recent
111 strategies decrease the eluting strength of the ¹D mobile phase prior injection into the second
112 dimension, hence significantly improving the compatibility of HILIC and RPLC. Unfortunately, the use
113 of a combination of HILIC and RPLC remains limited to ionisable or highly polar compounds.

114 Other combinations of LC chromatographic modes such as size exclusion chromatography (SEC) with
115 either RPLC or NPLC can apply to neutral compounds analysis, but their use is generally limited to
116 polymers and proteins characterization [17].

117

118 **2. Benefits of combining LC and SFC for the separation of neutral compounds**

119 While the analysis of ionizable or polar compounds can be investigated using RPLC x RPLC or HILIC x
120 RPLC, the separation of many neutral compounds remains challenging using LC x LC. To extend the
121 range of possibilities whatever the polarity, several papers suggest that the combination of LC and
122 supercritical fluid chromatography (SFC) could be worth investigating.

123 The first online coupling between LC and SFC appeared in the late 80s – early 90s and generally used
124 SEC in the first dimension with capillary SFC in the second one [18]. The main purpose was to find an
125 alternative to gas chromatography in a SEC x GC configuration for the characterization of non-volatile
126 and thermally unstable compounds. However, the interest in such LC-SFC combinations turned out to
127 be ephemeral as, to the best of our knowledge, none of them was reported in literature between mid-
128 90s and mid-2000s. This can be attributed to (i) the loss of interest in capillary SFC due to the low
129 polarity of pure supercritical CO₂, limiting the technique to hydrophobic compounds, and ii) to the lack
130 of popularity of packed column SFC during this period. Although developed in 1983, packed column
131 SFC was suffering, at that time, from poor robustness and was certainly not mature enough for two-
132 dimensional hyphenations [19].

133 Since then, great progress has been made in packed column SFC technology, making the technique a
134 relevant and reliable alternative to LC [20, 21]. Such advances allowed the re-emergence of LC and SFC
135 coupling, which, as illustrated in Table 1, found applications in various fields since 2008. While this
136 table aims at providing a complete overview on the subject, more details about the applications and
137 the used operating modes (comprehensive vs. heart-cut, online vs off-line) can be found in the recent
138 review from Kaplitz et al. [22]. Complementary to this publication, the following discussion does not
139 aim to review the experimental set up that have been designed so far but wishes to draw more
140 attention to the limitations that can divert the hyphenation of LC and SFC to express its full 2D
141 potential, namely orthogonality, injection effects and long 2D duty cycles.

Table 1: Applications of LC and SFC hyphenations reported from 2008 until June 2020. The (x) represents a comprehensive mode while the (–) represents a heart-cut mode.

	Application	Compounds	Coupling type	Mode	First dimension	Second dimension	Interface	Reference
Bioenergy	Aqueous extract of bio-oil	Small phenolic compounds	RPLC × SFC	Online	Hypercarb ACN/H ₂ O	Acquity UPC ² BEH-2EP ACN/MeOH (1:1)/CO ₂	Empty loops	[23]
	Depolymerized lignin	Small phenolic compounds	RPLC × SFC	Online	Zorbax Eclipse Plus C18 ACN/H ₂ O	Torus diol ACN/MeOH (4:1)/CO ₂	Packed loops	[24]
Food	Fish oil	Phenacyl esters of triaglycerols	Ag-SFC × RPLC	Online	Nucleosil + Ag ⁺ ions ACN/iPrOH (6:4)/CO ₂	Zorbax SB C18 ACN/H ₂ O	Packed loops + H ₂ O make-up flow	[25]
	Fish oil	Triaglycerols	Ag-SFC × NARPLC	Online	Nucleosil + Ag ⁺ ions ACN/iPrOH (6:4)/CO ₂	Chromolith RP-18 ACN/iPrOH	Packed loops + H ₂ O make-up flow	[26]
	Fish oil	Triaglycerols	Ag-SFC × NARPLC	Off-line	Nucleosil + Ag ⁺ ions ACN/iPrOH (6:4)/CO ₂	Zorbax SB C18 ACN/acetone		[26]
	Sweet bell peppers	Carotenoids, chlorophylls	SFC - RPLC	Off-line	Acquity HSS C18 SB EtOH/CO ₂	YMC C30 MeOH/MTBE/H ₂ O		[27]
	Red chilli pepper	Carotenoids	SFC × RPLC	Online	Ascentis ES Cyano MeOH/CO ₂	Acquity BEH C18 ACN/H ₂ O (8:2)/iPrOH	Packed loops + H ₂ O make-up flow	[28]
Lipidomics	Human plasma	Lipids	SFC - RPLC	Online	Zorbax RX-SIL MeOH + 2 % H ₂ O + AmF /CO ₂	Poroshell 120 EC C8 MeOH/H ₂ O + 10 mM AmF	Vacuum evaporation	[29]
	Swine brain	Gangliosides	SFC - RPLC	Off-line	Unitary diol MeOH + 0.1 % TFA/CO ₂	XAqua C18 ACN/H ₂ O + 20 mM AmF		[30]
Pharmaceuticals	Chiral drug	Active ingredient and impurities	RPLC – ch-SFC	Online	SunFire C18 ACN+ 0.05 % FA/H ₂ O + AmF	Chiralpak IC3 MeOH + 0.1 % NH ₄ OH/CO ₂	Packed loops	[31]
	Chiral drug	Active ingredient and impurities	RPLC × ch-SFC	Online	Acquity BEH C18 ACN/H ₂ O + MsOH	Chiralpak IC MeOH + 2 % H ₂ O/CO ₂	Empty loops	[32]
	Chiral drug	Active ingredient and metabolites	RPLC – ch-SFC	Online	X Bridge C18 ACN/H ₂ O+0.05 % FA	Chiralpak IB-3 EtOH + 0.1 % NH ₄ OH/CO ₂	Packed loops	[33]
Traditional chinese medicine	Ganoderma lucidum (fungus)	Secondary metabolites	NPLC × SFC	Online	Hypersil CN Hexane/iPrOH	Chromolith Flash C18 neat CO ₂	Empty loops	[34]
	Piper Longum L. (plant)	Amide alkaloids	SFC × RPLC	Off-line	Xamide MeOH/CO ₂	HSS T3 ACN/H ₂ O		[35]
	Fructus Arctii (dry fruit)	Lignans	SFC – RPLC	Off-line	Xamide MeOH/CO ₂	Unitary C18 ACN/H ₂ O+ 0.1 % FA		[36]
	Panax notoginseng (plant)	Triterpen saponins	SFC × RPLC	Off-line	Atlantis HILIC Silica MeOH/CO ₂	Poroshell EC-C18 ACN/H ₂ O		[37]
	Piper kadsura (plant)	Secondary metabolites	RPLC × SFC	Off-line	Unitary C18 MeOH/H ₂ O	Acquity UPC ² BEH MeOH/CO ₂		[38]
Venenum Bufonis	Bufadienolides	RPLC × SFC	Off-line	HSS T3 ACN/H ₂ O+ 0.1 % FAd	HSS C18 MeOH/CO ₂		[39]	
Miscellaneous	Lemon oil	Psolarens, coumarins	SFC × RPLC	Online	Princeton SFC CN silica EtOH/CO ₂	Zorbax SB C18 ACN/H ₂ O	Packed loops + H ₂ O make-up flow	[40]
	Blackberry sage fragrant oil		RPLC × SFC	Off-line	Luna C18 ACN/H ₂ O	Princeton SFC Amino ACN/CO ₂		[41]
	Tobacco smoke	Nitrosamines	SFC – RPLC	Online	Torus 1-AA MeOH + 0.2 % NH ₄ /CO ₂	Poroshell EC-C18 ACN/H ₂ O + 10 mM AmF	Packed loops + H ₂ O make-up flow	[42]

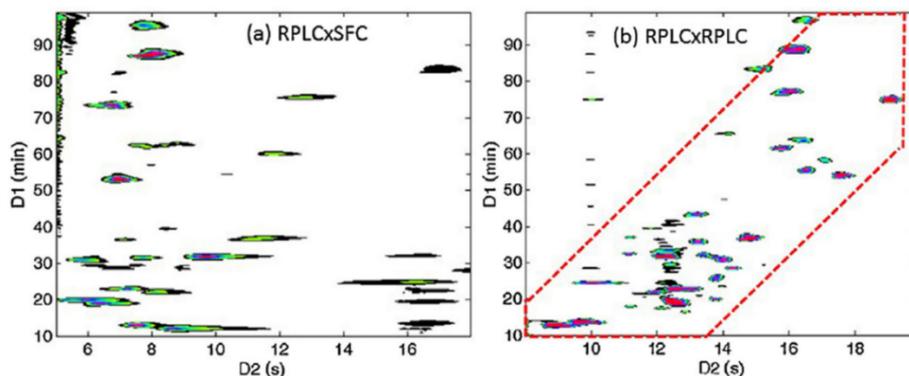
Ag-SFC: Silver-ion SFC, ch-SFC: chiral SFC, NARPLC: Non aqueous RPLC, AmF : ammonium formate ; FA : formic acid ; MsOH : methanesulfonic acid

2.1 High orthogonality potential between LC and SFC

145 The growing interest in coupling SFC to LC mainly stems from the great orthogonality potential
146 between the two techniques. As demonstrated by Lesellier and West [43], SFC offers a wide range of
147 selectivities largely influenced by the characteristics of the column material, including non-polar and
148 polar stationary phases. When compared to NPLC or HILIC, the use of polar stationary phases for SFC
149 result in generating similar interactions as in the aforementioned liquid based separation modes, but
150 for a wider range of solute polarity. In addition, intermediate selectivities, not available in LC, can be
151 obtained with moderately polar stationary phases such as aromatic phases or polar alkyl phases. It is
152 thus relatively straightforward to find non-correlated retention behaviors between LC and SFC, even
153 for non-ionizable species. It also implies that different selectivities, and therefore significant
154 orthogonality degrees, can be reached by coupling SFC to itself. This 2D-SFC strategy was
155 demonstrated by both Hirata and Thiebault's research groups using a silica column in one of the
156 dimensions and an octadecyl silica column in the other one [44, 45]. Despite requiring a third SFC for
157 transfer purpose and being limited to the use of neat CO₂ in the first dimension, the latest prototype
158 [45] looks similar to GC x GC and could also be worth exploring for the analysis of neutral compounds.

159 Regarding LC and SFC coupling reported in literature, researchers usually opted for combinations
160 implying hydrophobic and polar interactions, to generate maximal orthogonality. As shown in Table 1,
161 one of the dimensions is generally operated in RPLC, while the other one uses SFC with a polar
162 stationary phase. More atypical, Gao et al. [34] selected polar interactions in the LC dimension,
163 operated in NP mode with an heptane isopropanol gradient and hydrophobic interactions in the SFC
164 dimension, using an octadecyl (C18) column and neat CO₂ as mobile phase. Elsewhere, other types of
165 interaction sets have been successfully implemented. Such is the case, for instance, of RPLC with chiral
166 SFC [31-33] or RPLC with silver-ion SFC [25, 26], respectively allowing the introduction of chiral
167 interactions and specific interactions between the π electrons of the analytes and the silver ions of the
168 stationary phase. Other pairs of LC and SFC systems could certainly provide adequate orthogonality.
169 For instance, the use of aromatic phases in the SFC dimension could be worth investigating.

170 The high orthogonality degrees generated by these reported LC and SFC coupling was largely
171 demonstrated by the substantial retention space coverages obtained with neutral compounds. The
172 trend was even more obvious when a comparison with RPLC x RPLC coupling was performed. Using
173 online RPLC x SFC, Sarrut et al. [23] reached 100 % retention space coverage with an aqueous extract
174 of bio-oil, mainly composed of small neutral phenolic compounds. In contrast, the authors observed
175 only 65 % when using an optimized RPLC x RPLC method for the same sample. The difference between
176 the two coupling is clearly evidenced by Figure 1. The analytes are randomly spread throughout the
177 separation space in RPLC x SFC (Fig. 1a), while a significant diagonalization, typical of correlated
178 mechanisms between the two dimensions, is observed in RPLC x RPLC (Fig. 1b). Interestingly, while
179 SFC allows a better occupation of the retention space, the size of this retention space seems reduced
180 compared to RPLC, which affects the 2D peak capacity and so the overall effective peak capacity. This
181 phenomenon does not seem to represent a real limitation, as in the mentioned paper, the final
182 effective peak capacity obtained in RPLC x SFC was still higher than in RPLC x RPLC (620 vs 560).



183

184 *Figure 1: Comparison of (a) online RPLC x SFC and (b) online RPLC x RPLC separations of a bio-oil aqueous extract.*
 185 *The red dotted lines in b) delimit the retention space. Adapted from [23]*

186 Many other papers reported an efficient usage of the separation space with the coupling involving LC
 187 with SFC. Sun and co-workers [24] obtained a value of 79 % coverage for the online RPLC x SFC analysis
 188 of a lignin depolymerized sample, also containing small neutral phenolic compounds. François et al.
 189 [40] characterized a lemon oil sample, mainly composed of neutral psolarens and coumarins, by online
 190 SFC x RPLC and achieved a very low correlation factor $R^2 = 0.2$ between the retention data of the two
 191 dimensions, attesting again of a great space coverage. This is also the case for the online SFC x RPLC
 192 analysis of carotenoids in red chili peppers performed by Mondello's group [28], where a considerable
 193 spreading of the analytes over the 2D retention space was obtained. The independence between the
 194 retention mechanisms from both dimensions was further demonstrated by the elution pattern of the
 195 compounds. The carotenoids were separated according to the polarity of their chemical classes in the
 196 SFC dimension, while the RPLC dimension allowed, within the same chemical class, a separation based
 197 on the number of carbon atoms in the fatty acid chains. Similar behaviors were observed for the off-
 198 line SFC x RPLC characterization of gangliosides in swine brain [30] and triterpene saponins in medicinal
 199 herbs [46].

200

201 **2.2 Additional advantages of introducing SFC in one of the dimensions**

202 Although the main strength of coupling LC and SFC is the important orthogonality potential,
 203 desperately needed for the separation of neutral compounds, the properties of the SFC mobile phase,
 204 constituted of subcritical CO_2 , generally mixed with a small amount of organic co-solvent, provides
 205 other advantages. As an alternative to NPLC, the use of SFC involves mobile phases with a much lower
 206 toxicity and largely reduced re-equilibration times. Secondly, thanks to the decompression of
 207 subcritical CO_2 under atmospheric conditions, off-line SFC x LC coupling are much more convenient
 208 than off-line LC x LC hyphenations, when evaporation of the 1D fractions is required. Although a specific
 209 fraction collector is needed, the fractions can be quickly evaporated and with negligible energy costs
 210 compared to LC x LC coupling [35, 36]. Thirdly, recent works showed that, under optimal conditions,
 211 the sensitivity in electrospray mass spectrometry (ESI-MS) was significantly enhanced when using SFC
 212 rather than RPLC prior detection. This improvement is thought to be related to the minimal amount
 213 of water in the SFC mobile phase, facilitating the desolvation process in the ionization source compared
 214 to the hydro-organic mobile phase used in RPLC. Although this benefit remains at this time
 215 counterbalanced by injection effects (further detailed below), the use of SFC instead of RPLC as second
 216 dimension before MS detection could therefore become highly beneficial [47].

217 On the other hand, due to its high selectivity and ability to generate high throughput analyses, chiral
 218 SFC is progressively becoming the first choice for enantioseparations [48]. Its coupling with LC has an
 219 obvious interest for the complete characterization of both chiral and achiral compounds in complex

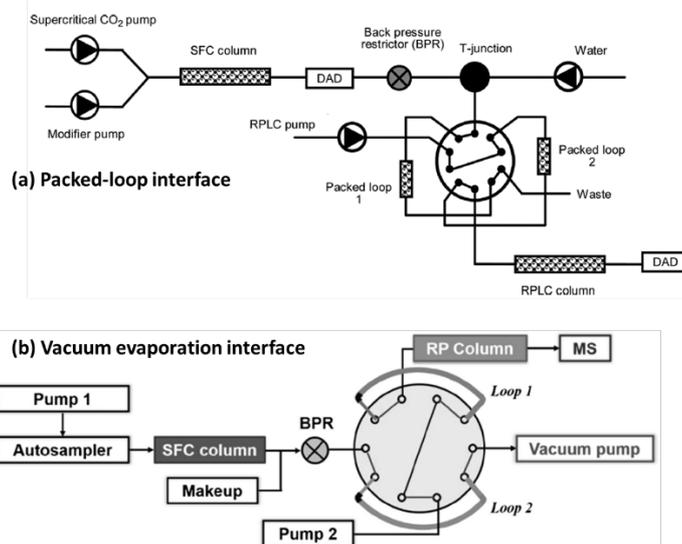
220 mixtures, such as chiral and achiral purities of active pharmaceutical ingredients [31-33]. In this case,
221 a heart-cut mode is carried out and only the unresolved enantiomeric mixture is sent to the chiral SFC
222 second dimension is limited to the. The limited number of modulations usually allows a longer ²D
223 analysis time that is beneficial to the resolution. Similarly, it is interesting to note that the hyphenation
224 of achiral SFC to chiral SFC is also promising for this type of application, as highlighted by Zeng et al
225 [49], despite being limited to the use of the same mobile phase in both separations for technical
226 reasons.

227 3. Main limitations of LC and SFC hyphenations

228 Although coupling LC with SFC seem very promising for the analysis of neutral compounds, some
229 limitations have been highlighted in literature. The implementation of online SFC × LC requires a
230 complex interface between the two dimensions, due to the nature of the SFC mobile phase. The
231 opposite combination LC × SFC suffers from serious injection effects, especially reported when the LC
232 dimension is operated in reversed-phase, and its potential in online mode is currently limited by
233 unsuitable commercial instrumentation.

234 3.1 Online SFC × LC: need for a complex interface

235 As previously mentioned, the high CO₂ content of the SFC mobile phase can be considered as an
236 advantage in off-line SFC x LC. On the contrary, when the coupling is operated in online mode, this
237 property becomes a constraint. Indeed, in the conventional 2D interfacing loops, the effluent is
238 depressurized, leaving the analytes in a spray of gaseous CO₂ containing co-solvent droplets. Specific
239 (non-commercial) interfaces are therefore necessary to properly collect the analytes and to eliminate
240 the expanded CO₂ that would be detrimental for the LC separation. Two types of home-made
241 interfaces have been reported in literature (Figure 2): packed-loop interfaces in combination with a
242 water make-up flow and vacuum solvent evaporation interfaces.



243
244 *Figure 2: Schematic diagram of online SFC × LC systems using a) a packed-loop interface with a water make-up*
245 *flow [40] and b) a vacuum evaporation interface [29].*

246
247 Packed-loop interfaces with water make-up flow (Fig. 2a) were first implemented for online SFC × RPLC
248 by Sandra's research group in three consecutive papers [25, 26, 40], and more recently by Donato et
249 al. [28] and Zhou et al. [42]. The addition of water to the SFC effluent before entering a loop filled with
250 non-polar stationary phase allows the efficient trapping of the analytes, and therefore prevents the
251 flow of expanded CO₂ from evacuating them through the waste line. This approach makes online SFC

252 × RPLC attractive as the analytes are transferred to the second dimension in a mixture of co-solvent
253 and water, usually compatible with the RPLC mobile phase. However, the method development is
254 burdensome regarding (i) the choice of packing material, as a good compromise must be found
255 between trapping efficiency and quick compound desorption, and (ii) the selection of the water make-
256 up flow, which shall not cause any damages to the SFC backpressure regulator. However, signal
257 interferences due to a non-complete evacuation of the remaining gaseous CO₂ before injection into
258 the second dimension were highlighted, despite decreasing the SFC flow rate or adding a water-rinsing
259 step of the loops [25, 40].

260 In parallel, Yang et al. [29] recently suggested a vacuum evaporation interface (Fig. 2b) for the online
261 SFC × RPLC analysis of lipids in human plasma. Similar to those used for online NPLC × RPLC, this
262 interface is based on the evaporation of the ¹D mobile phase prior the ²D separation. The SFC effluent
263 is completed with a make up flow (methanol + 5 mM ammonium formate) in the early stages of the
264 elution, then when reaching around 30 % co-solvent (methanol + 20 mM ammonium formate), the
265 make up flow is stopped. The effluent is collected in traditional empty loops but the latter are heated
266 in an oil bath and linked to a vacuum pump. While most of the non-volatile compounds remain in the
267 loops, the SFC mobile phase is evaporated. However, as for the previously described interface, the
268 instrumentation implementation and the associated method development are tedious. Several
269 parameters (e.g. oil bath temperature, make-up flow rate) must be optimized to limit the loss and
270 degradation of analytes between the two dimensions, while efficiently evaporating the gaseous CO₂.
271 Although the authors reported good recovery values for lipids, the use of this type of interface remains
272 limited to non-volatile compounds.

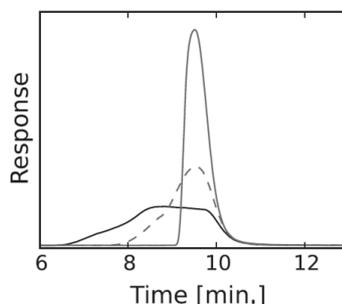
273

274 **3.2 Online LC x SFC: overcoming injection effects**

275 While online LC × SFC instrumental setup seems more straightforward than SFC x LC, the transfer of LC
276 fractions can easily lead to band broadening or peak deformation in the SFC dimension, impairing the
277 advantages of the 2D separation.

278 Indeed, the negative impact of the injection process already represents a great challenge in
279 unidimensional SFC. With the recent generation of SFC equipment and the increased use of sub-2µm
280 particles, the speed of SFC separation is improved, but the variance generated by the injection process
281 becomes even more critical. Indeed, the so-called injection effects can take place even at low or
282 moderate injection volumes. They mainly originate from the inevitable difference between the sample
283 diluent and the CO₂-based mobile phase, but also from the potential adsorption of the sample diluent
284 or the co-solvent onto the stationary phase. According to the nature of these physical phenomena,
285 different types of injection effects have been reported in literature. The most observed kind of
286 injection effects in SFC is the “strong solvent effect”, also called “plug effect”, and is due to a higher
287 eluting strength of the sample diluent compared to the mobile phase. If the diluent is weakly retained
288 compared to the analyte, the front molecules of the analyte plug are exposed to an environment rich
289 in diluent for a certain time, with an increase velocity, leading to distortions at the front of the peak .
290 The phenomenon is enhanced for low-retained solutes, spending an insufficient time in the column to
291 allow its compensation by molecular dispersion. Enmark and co-workers [50] highlighted this strong
292 solvent effect by comparing the peak shape obtained with three different diluents of increasing solvent
293 strength (toluene, ethanol and methanol) (Figure 3). Similar observations were made by Fairchild et al.
294 [51] and De Pauw et al. [52] using various other diluents, and they were in good agreement with the
295 systematic evaluation of diluents performed by Desfontaines et al. [53]. Their investigation revealed
296 that the best diluents had the lowest eluting strength: for polar stationary phases, diluents like
297 acetonitrile, methyl tert-butyl ether or cyclopentyl methyl ether were more suitable than methanol or
298 water, which generally caused peak distortion. In parallel of these experimental studies, the presence
299 of the strong solvent effect in SFC was also evidenced through theoretical considerations. Rajendran’s

300 research group [54] modeled the elution profile of an analyte dissolved in methanol and eluted with
301 a mobile phase composed of 87 % CO₂ and 13 % methanol. Only considering the local eluting strength
302 around the solute, their equilibrium dispersive (ED) model captured most of the experimental peak
303 shape, and especially the front peak deformations occurring at high injection volumes. This
304 demonstrated the major contribution of strong solvent effect to the observed peak deformations in
305 SFC. This effect was less visible in the oldest SFC instrument as the large external volumes between
306 injection point and column had the benefice of allowing a mixing of the sample with the initial mobile
307 phase composition.



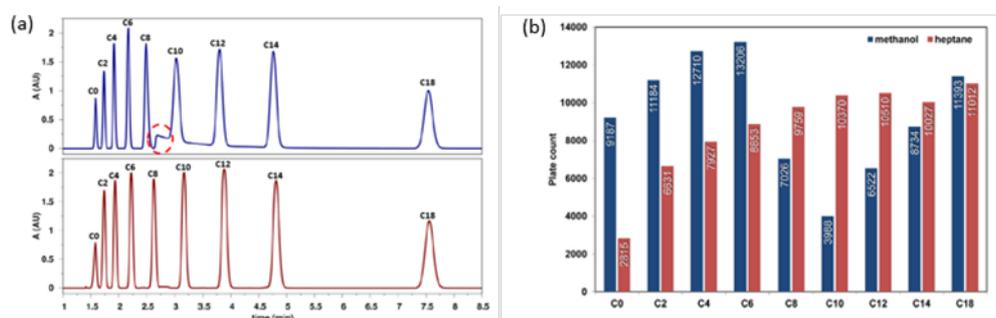
308
309 *Figure 3: Illustration of the strong solvent effect. Chromatograms of antipyrine dissolved in toluene (grey), ethanol*
310 *(dashed grey) and methanol (black). Diol stationary phase. Mobile phase CO₂/MeOH 90/10. Adapted from [50].*

311
312 Besides the strong solvent effect, viscous fingering has been identified as a potential source of peak
313 distortion even at moderate injection volume [55]. This effect is initiated by a difference in viscosity
314 between the mobile phase and the sample diluent and has been thoroughly studied in LC. Enmark and
315 co-workers experimentally visualised viscous fingering in LC experiments for which the viscosity ratio
316 between the mobile phase and the sample diluent was adjusted to match SFC conditions [50]. The
317 presence of viscous fingering in SFC was theoretically demonstrated by Rajendra et al. [56]. Through
318 hydrodynamic considerations, they describe viscous fingering in addition to the strong solvent effect
319 and were able to simulate most of the additional peak distortions that their previous equilibrium
320 dispersive model could not predict for large-volume injections. The study therefore confirmed the
321 contribution of viscous fingering to peak deformations and highlighted its interplay with the strong
322 solvent effect at high injection volumes.

323 In addition to differences between sample diluent and mobile phase, injection effects can also be
324 engendered by the adsorption of the diluent itself onto the stationary phase. Several SFC studies
325 revealed that the most commonly used sample diluents, such as methanol or acetonitrile, showed a
326 significant retention on various types of stationary phases [50, 57-59]. This behaviour can lead to a
327 competition between the analyte and the diluent to access the stationary phase, which in turn, can
328 result in unusual peak shapes. More precisely, its impact was found to be dependent on the relative
329 retention of the analyte compared to the diluent. Using an alkylamide column and neat CO₂ as mobile
330 phase, Redei et al. [59] compared the chromatograms of a series of n-alkylbenzenes dissolved in a
331 retained (methanol) and in a non-retained diluent (heptane) (Figure 4a). The retention of methanol
332 can be visualized at 200 nm (red circle). Surprisingly no strong solvent effect was noticed. This may be
333 due to the very small injection volume (2 μL) compared to the column size (4.6 x 150 mm). While the
334 observed efficiency increased along the series of homologous with heptane as a diluent, abnormal
335 efficiencies were recorded with methanol as a diluent, and this despite the limited injection volume.
336 As shown in Figure 4b, most of the n-alkylbenzenes eluting before methanol displayed sharpened
337 peaks, whereas for compounds that were more retained than methanol, the efficiency was much lower
338 than expected. The last eluting compound (octadecylbenzene), greatly retained compared to
339 methanol, was unaffected by the adsorption of the diluent. Supported by modelling based on

340 competitive isotherms, these observations were attributed to a displacement effect on less retained
341 compounds and to a tag-along effect on more retained compounds. The study was performed in neat
342 CO₂ to enhance the diluent adsorption, but most of the SFC separations require the addition of an
343 organic co-solvent (sometimes called modifier) besides CO₂ to facilitate solutes elution.

344



345

346 *Figure 4: Analysis of a series of n-alkylbenzenes (benzene, ethylbenzene, butylbenzene, hexylbenzene,*
347 *octylbenzene, decylbenzene, dodecylbenzene, tetradecylbenzene and octadecylbenzene) dissolved in methanol*
348 *(blue) and heptane (red), using an alkylamide column and a neat CO₂ mobile phase. (a) Chromatograms at 200*
349 *nm. The dotted red circle indicates the methanol signal. (b) Observed efficiencies for each compound. Adapted*
350 *from [59].*

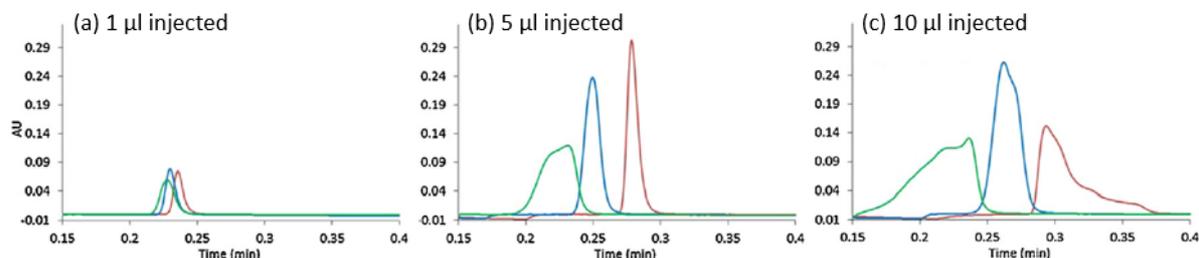
351 As previously mentioned, common co-solvents like methanol or acetonitrile, can adsorb onto SFC
352 stationary phases. In two subsequent papers, Glenne et al. [57, 58] showed that, when injecting high
353 solute concentrations and volumes in preparative SFC, a significant retention of the organic co-solvent
354 could also cause unexpected elution bands. These deformations were attributed to competition
355 between the solute and solvent molecules for the stationary phase sites. Nevertheless, the effect of
356 co-solvent adsorption in analytical conditions (i.e. low sample concentrations and volumes) was, to the
357 best of our knowledge, never reported in literature.

358 Injection effects in SFC were found to be enhanced as the injection volume increased, but depending
359 on the conditions, they could be observed for injection volumes as low as 0.5 % of the column dead
360 volume [51, 53]. Injection effects in the second dimension of LC × SFC can be even more challenging
361 than in unidimensional SFC, as the ²D injection volume and the composition of the transferred fractions
362 are both strongly related to the ¹D chromatographic conditions and cannot be tuned as one would
363 wish to.

364 Firstly, the injection volume is usually imposed by the ¹D flow rate and the sampling time between the
365 two dimensions, the use of loops at the interface prohibiting any split of the ¹D LC flow. To study the
366 impact of the transferred volume, Sarrut and co-workers [23] performed a preliminary 1D-SFC study
367 miming the second dimension of their RPLC × SFC system. A model compound was injected on the SFC
368 column, with different injection volumes and using three different diluents, representative of the RPLC
369 mobile phase composition throughout the ¹D gradient. As illustrated in Figure 5, depending on the
370 diluent nature, peak distortions could be observed from as low as 5 μL injection volume, corresponding
371 to only 5 % of the column dead volume. Injections representing 10 % of the column dead volume (i.e.
372 10 μL), systematically led to strong deformations, no matter the diluent nature. To minimize these
373 injection effects, the authors were therefore forced to limit the volume injected in the second
374 dimension. This led to a substantial dilution of analytes between the two dimensions and thereby,
375 affected the method sensitivity. The overall analysis time was also impacted: as the sampling time had
376 to be high enough to allow proper ²D separations, the reduction of the injection volume in the second
377 dimension necessarily implied a drastic decrease of the ¹D flow rate, which was set to its lowest
378 possible value (i.e. 10 μL/min). In addition, despite these precautions, the final RPLC × SFC contour-

379 plot, shown in Figure 1a, still revealed significant peak broadening in the SFC dimension, which reduced
380 the effective peak capacity. Hence reducing the volume transferred to the SFC dimension is a very
381 limited answer to injection effect-related band broadening.

382



383

384 *Figure 5: Effect of injected volume and diluent nature on peak shape. Solute: o-cresol. Injected volume: (a) 1 µl,*
385 *(b) 5 µl, (c) 10 µl. Diluent: ACN/H₂O 5:95 v/v (red), 50:50 v/v (blue), 95:5 v/v (green). Column BEH-2EP 50 mm x*
386 *2.1 mm. mobile phase: CO₂/MeOH/ACN 95:2.5:2.5 (v/v/v). Reproduced from [23]*

387 On the other hand, the composition of the transferred fraction cannot be easily manipulated in online
388 configuration, and usually corresponds to the ¹D mobile phase. In online NPLC × SFC, the ¹D mobile
389 phase, being water-free and usually containing low-polarity solvents, seems to have little impact on
390 the SFC separation. Using a mixture of hexane and isopropanol as ¹D mobile phase and neat CO₂ in the
391 second dimension, Gao et al. [34] did not mention any injection effects despite the significant volume
392 transferred to the second dimension (i.e. 34 % of the column dead volume). Only a decrease of
393 isopropanol solubility was suspected when the NPLC mobile phase contained the highest isopropanol
394 percentages. In contrast, in RPLC × SFC configuration, each of the components of the ¹D mobile phase,
395 usually composed of water with acetonitrile or methanol, are prone to generate detrimental injection
396 effects, whether from its high elution strength, its high viscosity or its adsorption on SFC stationary
397 phases.

398 To examine the impact of water on strong solvent effect in SFC, De Pauw and co-workers [52]
399 compared the observed efficiency obtained for low-retained and highly retained compounds on a polar
400 stationary phase. The mobile phase was composed of CO₂ with 8 % methanol and the sample diluents
401 were acetonitrile/water, with ratios ranging between 90/10 and 40/60. As the efficiency was not
402 decreasing with the increase of the percentage of water in the diluent, but showing, on the contrary,
403 local maxima for medium percentages of water, the authors concluded that the observed injection
404 effects were not entirely due to the most commonly observed strong solvent effect. Sarrut et al. [23]
405 and Sun et al. [24] confirmed these observations, by demonstrating that diluents rich in water did not
406 systematically worsened peak shape, but could on the contrary, be beneficial compared to acetonitrile-
407 rich diluents.

408 Peak broadening and distortion in online RPLC × SFC seem therefore to be the result of an interplay
409 between several physical phenomena that would deserve further investigation to better control their
410 impact on the separation.

411 While injection effects due to the transfer of hydroorganic eluents to SFC are not fully understood,
412 practical solutions have been sought to avoid, or at least to limit them. In off-line mode, fractions
413 collected from the first dimension can be evaporated and reconstituted in a suitable diluent before
414 injection in the second dimension [39]. However, dealing with injection effects in online mode is much
415 more complex. Iguiniz et al. [32] opted for a modification of the SFC mobile phase when using a chiral
416 stationary phase. While the analyte peak was strongly distorted at high injection volumes, it regained
417 a symmetrical peak shape by adding 2 % water to the co-solvent. The authors were therefore able to
418 inject 5 µL into the second dimension (1 % of the column dead volume) without any significant peak

419 deformation, against 2 μL with the initial mobile phase composition. Meanwhile, other researchers
420 investigated a different way to circumvent injection effects, based on a solvent switch thanks to
421 trapping columns at the interface between the two dimensions [24, 31, 33]. Traditional empty loops
422 at the interface are replaced by loops packed with stationary phase, onto which analytes are retained
423 while most of the mobile phase, unretained, is eliminated from the system. Unlike empty loops, this
424 approach allows a reduction of the volume of hydro-organic diluent injected in the SFC dimension, and
425 thereby a limitation of the injection effects, while enhancing sensitivity. Furthermore, analysis time is
426 shortened as higher flow rates can be used in the first dimension. Sun et al. [24] demonstrated the
427 potential of the technique by comparing its performance with an empty-loop interface. For the same
428 injected volume in the second dimension, the use of trapping column divided by two the analysis time
429 and allowed the detection of low-concentration analytes that could not be detected with the empty-
430 loop system. Nevertheless, the use of these packed-loop interfaces does not ensure a complete
431 elimination of the injection effects and present significant drawbacks. The stationary phase contained
432 in the cartridges must be carefully chosen to efficiently trap the analytes during the collection of the
433 RPLC effluent, whatever the matrix, while the cartridge volume, hence its capacity, remains limited to
434 the maximum volume to be injected to the second dimension [24, 31, 33]. Breakthrough may still
435 happen and a fast release of the focused fractions is required to limit sample dispersion at the
436 beginning of the second dimension. Even though this type of interface is an attractive way to limit the
437 injection effects, it remains complex to optimize.

438 Active solvent modulation consists in the mixing of the fraction eluting from the loop with the initial
439 mobile phase of the second dimension prior to injection in the ^2D column. This is a very effective way
440 to lower the elution strength in HILIC x RPLC combination [60], hence reducing solvent incompatibility.
441 This approach has never been attempted to LC x SFC setup. While this strategy could be of interest if
442 the amount of water in the ^1D fractions is limited, it cannot avoid water adsorption and immiscibility
443 of water-rich fractions in the CO_2 -rich mobile phase.

444 Although injection effects seem the main obstacle to the use of LC x SFC, there is no doubt that on-
445 going research efforts will identify solutions allowing the focusing of analytes at the head of the second
446 dimension, as they recently achieved for other 2D LC combinations.

447

448 **3.3 Instrumental limitations in online LC x SFC**

449

450 Despite injection effects, the coupling also suffers from instrumental limitations in online mode, mainly
451 due to the fact that no commercial setup is currently available. The main limitation of home-made LC
452 x SFC systems lies in the unsuitability of current commercial SFC instruments for ^2D analyses.

453 In order to reduce undersampling effect that may compromise the effective peak capacity of a
454 comprehensive 2D setup, the sampling time has to be minimal. It corresponds to the ^2D duty cycle,
455 including dwell time, column dead time, gradient time, and column re-equilibration. While SFC is often
456 claimed to run faster than LC, this is only true when a small proportion of co-solvent is used. Improving
457 the maximal pressure withstand by current instrumentation may accommodate mobile phase with
458 higher viscosity, hence fully exploit the speed potential of SFC as a second dimension. Due to large
459 mixing chambers, high dwell volumes are also found in current instruments: 440 μL for Waters UPC²
460 [61], 700 μL for Agilent Infinity I [19], 600 μL for Agilent Infinity II [32], against less than 100 μL for the
461 best UHPLC on the market. Here again, depending on the maximal attainable flow rate, these high
462 dwell volumes may induce large dwell times, reducing the time dedicated to the ^2D gradient. To be
463 noticed, Sarrut et al. [23] also faced software issues when an additional time was required for data
464 processing after each ^2D runs, further reducing the allowed time for gradient.

465 In addition, most SFC systems show important external volumes generating high extra-column band
466 broadening. A value of 85 μL^2 was experimentally determined for both Waters UPC² and Agilent Infinity

467 I systems [62], while UHPLC systems present values between 2 and 20 μL^2 [19]. This is mainly due to
468 the higher tubing volumes as well as non-optimal UV cell geometry in SFC instruments. To illustrate
469 this point, De Pauw et al. [52] showed that the extra-column band broadening in SFC was significantly
470 reduced when replacing the commercial UV cell by a state-of-the-art UHPLC UV cell and by decreasing
471 the tubing internal diameter. These extra-column band broadenings currently forbid the use of short-
472 length columns necessary in any second dimension. Nevertheless, progress is being made with the
473 latest Agilent model (Infinity II), for which a variance value around 20 μL^2 for extra-column band
474 broadening has been measured in our laboratory. On the one hand, reducing the external volume
475 between injection point and column has a beneficial effect on both dwell volume and external
476 dispersion, but on the other hand, it deprives of the opportunity of mixing the sample with the SFC
477 mobile phase of initial compositions and so lowering the solvent strength.

478

479 While there is room for instrumental improvement, developing SFC for a 2D configuration is definitely
480 not a straightforward process. But the complementarity of LC x SFC with 2D-LC combined with the
481 industrial needs are huge, as reflected by the recent call by the Enabling Technology Consortium [63]
482 will undoubtedly motivate instrument suppliers in the next years.

483

484 Conclusion and perspectives

485 The two-dimensional RPLC x RPLC and HILIC x RPLC techniques have demonstrated their massive
486 performances for the separation of complex mixtures of ionizable compounds. On the other hand, the
487 analysis of neutral species by 2D-LC currently constitutes a great analytical challenge. Among the
488 suitable combinations of chromatographic modes, the use of RPLC in both dimensions generates poor
489 orthogonality and the coupling of RPLC to HILIC, although attractive in terms of orthogonality, remains
490 limited to polar compounds. .

491 In this context, the hyphenation of liquid chromatography to supercritical fluid chromatography was
492 investigated, and successfully applied in various fields such as bioenergy, food and traditional
493 medicine. The major strength of coupling LC and SFC lies in their great orthogonality potential,
494 originating from the extremely wide range of selectivity available amongst SFC columns. In addition,
495 the introduction of SFC in one of the dimensions was found to bring considerable benefits, including a
496 reduction of the toxicity, a minimization of the times and costs dedicated to the evaporation of the
497 collected fractions (for off-line configurations using SFC in the first dimension), and highly selective
498 enantioseparations.

499 However, the technique is still in its early stages, and has several challenges ahead before it can be
500 considered as a real alter ego of to 2D-LC. While SFC x LC revealed major strengths compared to LC x
501 LC systems in off-line mode, its online mode remains for now a prototyping challenge. On the other
502 hand, the online LC x SFC configuration is limited by detrimental injection effects in SFC, particularly
503 observed when the first dimension is operated in RP mode. The hydro-organic RPLC mobile phase
504 generates significant injection effects, characterized by peak broadening and peak distortion in the SFC
505 dimension. These injection effects are not fully understood yet and seem to originate from a complex
506 interplay between several physical phenomena. The potential of LC x SFC to generate high peak
507 capacities is also restricted, in online mode, by unsuitable instrumentation.

508 Nonetheless, progress is being made with a view of fully exploiting the possibilities offered by the two-
509 dimensional separation involving LC and SFC. Design of interfaces for SFC x LC are still being
510 investigated and the issue of injection effects in RPLC x SFC is starting to be addressed, as some
511 approaches to limit their impact on separation have been recently proposed. Recent improvements
512 have also been made in SFC instrumentation. Further developments jointly from researchers and
513 suppliers could offer an attractive alternative for the separation of complex samples.

514

515 **Acknowledgements:** M. Burlet-Parendel thanks the French Ministry of Research (MESRI) for her PhD
516 grant.

517

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