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1 **Chromatographic analysis of biomolecules with pressurized**
2 **carbon dioxide mobile phases – A review**

3

4 Jérémy Molineau¹, Maria Hideux², Caroline West^{1*}

5 1. University of Orleans, ICOA, CNRS UMR 7311, rue de Chartres, BP 6759; 45067
6 Orléans, France

7 2. Institut de Recherches Servier, 11 rue des Moulineaux, 92150 Suresnes, France

8

9 Corresponding author:

10 caroline.west@univ-orleans.fr

11 tel: +33 (0) 238 49 47 78

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14 **Highlights**

15

16 • Supercritical fluid chromatography and enhanced fluidity liquid chromatography

17 • Free amino acids, peptides, proteins and nucleic acid bricks

18 • Mobile phases of CO₂ and co-solvent, comprising water and additives

19 • Detection issues and hyphenation to mass spectrometry

20 • Applications in foodomics, metabolomics, clinical and pharmaceutical areas

21

22

23

24 **Abstract**

25

26 Biomolecules like proteins, peptides and nucleic acids widely emerge in pharmaceutical
27 applications, either as synthetic active pharmaceutical ingredients, or from natural products
28 as in traditional Chinese medicine. Liquid-phase chromatographic methods (LC) are widely
29 employed for the analysis and/or purification of such molecules. On another hand, to answer
30 the ever-increasing requests from scientists involved in biomolecules projects, other
31 chromatographic methods emerge as useful complements to LC. In particular, there is a
32 growing interest for chromatography with a mobile phase comprising pressurized carbon
33 dioxide, which can be named either (i) supercritical (or subcritical) fluid chromatography
34 (SFC) when CO₂ is the major constituent of the mobile phase, or (ii) enhanced fluidity liquid
35 chromatography (EFLC) when hydro-organic or purely organic solvents are the major
36 constituents of the mobile phase. Despite the low polarity of CO₂, supposedly inadequate to
37 solubilize such biomolecules, SFC and EFLC were both employed in many occasions for this
38 purpose. This paper specifically reviews the literature related to the SFC/EFLC analysis of
39 free amino acids, peptides, proteins, nucleobases, nucleosides and nucleotides. The
40 analytical conditions employed for specific molecular families are presented, with a focus on
41 the nature of the stationary phase and the mobile phase composition. We also discuss the
42 potential benefits of combining SFC/EFLC to LC in a single gradient elution, a method
43 sometimes designated as unified chromatography (UC). Finally, detection issues are
44 presented, and more particularly hyphenation to mass spectrometry.

45

46 **Keywords:** amino acids; enhanced fluidity liquid chromatography; nucleosides; nucleotides;
47 peptides; proteins; supercritical fluid chromatography

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1. Introduction

Biomolecules or biological molecules are molecular species present in living organisms of the fauna, the flora and microorganisms. Four principal categories of macromolecular biomolecules are usually distinguished: carbohydrates, lipids, proteins and nucleic acids. Smaller molecules include primary and secondary metabolites. In this paper we will focus on amino acids, their oligomers and polymers (peptides and proteins), and on nucleic acid constituents like nucleobases, nucleosides and nucleotides (Figure 1). Peptides may be linear or cyclic, where the ring structure can be formed by an amide bond (“peptide bond”) or other stable bonds like disulfide [1]. Peptides and proteins differ in size and structure, in that peptides are usually smaller, with an undefined three-dimensional structure, while proteins are larger and with a defined, rather stable folding. However, there is no universally accepted limit value in the number of amino acid residues to distinguish between peptides and proteins. Nucleotides are the basic constituents of nucleic acids like DNA and RNA, and may be decomposed in (i) a nucleobase (purine or pyrimidine) and (ii) an osidic group, together forming a nucleoside, and (iii) a phosphate tail containing one, two or three phosphate groups linked to the 5' oxygen of the osidic group. The structures of the natural biomolecules may also be modified for specific purposes with other functional groups, as is the case with nucleoside antiviral drugs [2]. Indeed, in the recent years, peptide (linear or cyclic [1,3]), protein and nucleic acid biomolecules have largely emerged in the pharmaceutical arena, either as synthetic active pharmaceutical ingredients (API) [4,5] or from natural ingredients [6,7] as food products [8] or traditional Chinese medicine (TCM) [9]. The fast emergence of these types of bioactive molecules is setting new challenges to analytical chemists in general and chromatographers in particular.

Liquid chromatography is the most common method to analyze and purify such biomolecules [7,10,11], preferably in the reversed-phase mode (RPLC) but also in the hydrophobic interaction (HIC) and hydrophilic interaction (HILIC) modes, ion-exchange chromatography (IEX) and size-exclusion chromatography (SEC). Beside liquid chromatography, capillary electrophoresis (CE), and microfluidics are other favorite analytical methods for biomolecules.. Supercritical fluid chromatography (SFC), employing pressurized carbon dioxide as a major constituent of the mobile phase, is another possible alternative that would deserve more attention in this field [12]. In fact, the low viscosity and high analyte diffusivity allow for high flow rates together with high efficiency, thus the resolution per unit time is usually excellent with SFC. In addition, SFC is also advantageous at the preparative scale as it allows significant reductions in solvent consumption compared to liquid-phase preparative separations, and produces fractions that are more concentrated. As a result, the solvent cost, solvent evaporation time and waste disposal costs are all reduced compared to

97 LC methods. For this reason, preparative SFC is considered a green purification method.
98 Interestingly, the benefits of pressurized carbon dioxide in the mobile phase are maintained
99 whatever the proportions introduced in a liquid mixture. Traditionally, when the proportion of
100 CO₂ is the largest, the method is called subcritical or supercritical fluid chromatography
101 (SFC). When the proportion of CO₂ is the smallest, the method is rather called enhanced
102 fluidity liquid chromatography (EFLC). Aside habits, there are no fundamental reasons to
103 dissociate the two methods [13] and they will both be discussed in the following. While
104 already an old technique, with the first experiments dating from 1962 [14], SFC has faced a
105 rapid surge of interest in the last decade, thanks to the introduction of improved instruments
106 by several manufacturers. Before that time, the benefits of SFC were principally recognized
107 at the preparative scale and for enantioresolution [15–17]. A variety of application fields that
108 were previously little investigated with SFC are now being re-explored [18]. Biomolecules
109 analysis and purification to serve pharmaceutical and clinical research is one such field. As
110 appears in Figure 2, the number of papers published on this topic may have been scarce
111 during several decades but has been rising in the recent years. Although the total number of
112 them remains modest, there is clearly an interest for alternative methods in this field. When
113 analyzing biomolecules with SFC, difficulties are expected due to solubility issues of
114 potentially large and polar species (see section 2 below). In addition, conformational changes
115 may occur for proteins when exposed to carbon dioxide, as will be further detailed below.
116 The changes occurring nowadays in the way the technique is operated, typically with
117 increasing portions of co-solvent and water in the pressurized carbon dioxide mobile phase
118 are however raising new expectation.

119 The first example of peptide analysis in SFC was published in 1988 [19], where a cyclic
120 undecapeptide Cyclosporin A (1201.8 Da), was analyzed with SFC hyphenated to mass
121 spectrometry (MS). Further attempts at analyzing such biomolecules initially concentrated on
122 lipophilic peptides and proteins, while recent attempts also concern more polar species, as
123 will be detailed in section 2.

124 This paper reviews most significant articles related to SFC and EFLC analysis and
125 purification of free (underivatized) amino acids, peptides, proteins, and building blocks of
126 nucleic acids. It is not intended to be exhaustive but rather to spotlight trends and specific
127 features. First, the polarity and size issues related to these biomolecules is discussed, to
128 better relate them to the choice of stationary and mobile phases that were employed for their
129 analysis. Detection issues are also discussed, with a particular focus on MS hyphenation.

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131

132 **2. Polarity and size features of the target biomolecules**

133

134 To understand the chromatographic features of biomolecules analysis, it is useful to
135 examine their structures first. In Figure 1, the generic structures of the target molecules
136 related in this paper are presented. In addition, we have examined the structures of the
137 molecules previously analyzed with SFC and EFLC in terms of size and polarity. For this
138 purpose, structural descriptors were computed for the molecules mentioned in the literature
139 reviewed with MolDesc, an open-source software (<http://moldesc.icoa.fr/>). Three descriptors
140 were retained to discuss the relation to chromatographic analysis: molecular weight (MW),
141 octanol-water partition coefficient (log P) and the topological polar surface area (TPSA). Note
142 that, as any computed descriptors, they may not be entirely accurate, but they should be
143 good enough to compare the features of the target molecules. They are summarized in
144 Figure 3, where MW is plotted versus log P value, and bubble size is related to TPSA. Only
145 the larger proteins and peptides are not represented in this figure, as it would have flattened
146 all other molecules in an apparently small area at the bottom of the figure and because the
147 computation of polarity features for such molecules has little meaning, as will be further
148 discussed below. For instance, reports of large peptides and proteins with molecular weights
149 as high as 3.7 kDa (issued from peptic digestion of myoglobin [20]), 5.8 kDa (biosynthesized
150 human insulin [21]), 13.7 kDa (ribonuclease A [22]), 17.6 kDa (myoglobin [23]), 66.5 kDa and
151 80.3 kDa (bovine serum albumin and transferrin [24]) were excluded from this figure but are
152 however discussed in the following sections.

153 Amino acids are all small molecules and are thus placed at the bottom of Figure 3 (small
154 blue bubbles). Apart from the acid and amine functions, which may both be ionized
155 depending on operating conditions, most amino acids possess a side chain (residue), which
156 may be hydrophobic (hydrocarbon chain or aromatic group), hydrophilic bearing no ionizable
157 function (like hydroxyl, thiol or amide functions), acidic thus possibly bearing a negative
158 charge, or basic thus possibly bearing a positive charge. According to these varied residues,
159 amino acids may be slightly hydrophobic (on the right of the figure) or hydrophilic (on the left
160 of the figure). Note that several papers relating the analysis or purification of derivatized
161 amino acids (for instance methyl esters, carboxybenzyl esters [25,26] or dansyl esters [27])
162 will not be described in this review. Derivatization is usually facilitating the elution of amino
163 acids in reducing their polarity, and often facilitating their chiral resolution.

164 When linear peptides are considered, some ionizable functions should remain at both
165 ends, if the terminal amine and carboxylic functions are unmodified with protective groups.
166 When cyclic peptides are considered, there are not necessarily any pendant amine or
167 carboxyl functions as they may have served to form the ring, unless some side chains are
168 attached to the ring. Thus cyclic peptides are often observed to be less polar than linear
169 peptide chains. This can be observed in Figure 3. Most cyclic peptides (light green bubbles)
170 are situated in the right portion of the figure, with positive log P values, while most linear

171 peptides (dark green bubbles) are situated in the left portion of the figure, with negative log P
172 values. According to log P values, two polar cyclic peptides were reported, Linaclotide and
173 Oxytocin (on the left of Figure 3), and two non-polar linear peptides or proteins were
174 reported: the membrane proteins Gramicidin A and B.

175 However, as in any other molecule, the overall polarity of a peptide expressed by log P
176 value is the result of the positive contributions of hydrophobic residues and the negative
177 contributions of hydrophilic residues. When the two are counterbalanced, the overall log P
178 value may be close to zero, although a significant number of polar groups could be present.
179 It is thus useful to observe a second polarity parameter, like the topological polar surface
180 area (TPSA), which sums the individual contributions of all polar groups in a molecule, and is
181 not counterbalanced by hydrophobic groups. In Figure 3, TPSA is represented by bubble
182 size. Then it appears that all peptides reported, whatever their log P values, have significant
183 TPSA values that should at least result from the peptide bonds (amide functions), whatever
184 the residues. For instance, the membrane proteins Gramicidin A and B have some of the
185 largest TPSA values (large bubbles), even though the overall polarity (expressed by log P) is
186 low (around 4). In addition, in their stable conformation, the linear gramicidins are folded in
187 helices with hydrophobic rings directed outside the helix, with little exposed polar surface.
188 The stable conformation of proteins may or may not be preserved during SFC and EFLC
189 analysis [22,23]. It is thus difficult to conclude on the exposed polarity of such molecules
190 during the analysis. Actually, chromatographic retention on a polar stationary phase may be
191 the best indicator of the exposed polarity, as was demonstrated by Goetz et al. [28] for cyclic
192 peptides: longest retention of peptides on a polar stationary phase in SFC conditions was
193 indeed observed to be related to lower membrane permeability. Clearly, log P and TPSA are
194 both insufficient to assess the polarity of large flexible molecules [29].

195 Nucleotides are also visible in Figure 1, with a representative structure based on a
196 guanine nucleobase. The purine and pyrimidine structure of the five nucleobases (adenine,
197 guanine, cytosine, thymidine and uracil) are all small and polar as they appear at the bottom
198 of Figure 3, with negative log P values. Adding the pentose ring to form a nucleoside is
199 slightly increasing molecular weight but also significantly increasing polarity, as the bubbles
200 representing nucleosides are slightly higher and on the left. Finally, introducing a phosphate
201 tail is also causing increased molecular weight, is not greatly affecting log P values but
202 significantly increasing TPSA values (bigger red bubbles in Figure 3). Nucleic acid chains
203 should then be most polar structures, in terms of log P or TPSA.

204 From Figure 3 and the above discussion, we can conclude that the target molecules
205 reviewed in this paper constitute a very heterogeneous group, with a variety of polarities and
206 sizes, mostly from 100 to 2000 Da, or even higher for some bigger peptides and proteins not
207 represented in the figure. While all target molecules bear polar functional groups (expressed

208 by TPSA), be they charge-neutral or ionizable, their overall polarity expressed by log P varies
209 in a great range. As a consequence, it is unlikely that one generic chromatographic method
210 would work for all of them. The different choices of stationary phases and mobile phases
211 previously selected to achieve their analysis or purification, presented in Tables 1, 2 and 3,
212 will be detailed in the following sections.

213

214 **3. Mobile phase composition**

215

216 CO₂ has little polarity and is usually compared to hexane. This comparison is not entirely
217 fair as CO₂ has some polarity features (local dipole moments, polarizability) and reactivity.
218 However, a neat CO₂ mobile phase in usual SFC operating conditions (temperature around
219 30°C, back-pressure of 11-18 MPa) is unlikely to solvate any of the target biomolecules.
220 When charge-neutral species are considered, mixing pressurized CO₂ to a polar organic
221 solvent, like the most frequently used short-chains alcohols [18], is sufficient to achieve good
222 solubility. Methanol is usually the preferred co-solvent observed in SFC literature, and the
223 specific papers related to our target molecules are no exception, as methanol was observed
224 in above 80% of the articles. A few papers mention other co-solvents, at least at the stage of
225 method development: ethanol [30–32], isopropanol or acetonitrile [20,23,33–35], the latter
226 being often introduced in mixtures with methanol. The co-solvent increases the mobile phase
227 polarity [36] and viscosity, modifies the mobile phase density [37], introduces other possible
228 interactions between the mobile phase and the analytes and adsorbs onto the stationary
229 phase [38], thereby also changing the possible interactions between the analytes and
230 stationary phase [39].

231 For the most polar charge-neutral or ionized species, two options are usually considered.

232 First, (one or more) acidic, basic or salt additives are frequently employed. For instance,
233 the second oldest example of SFC analysis of our target biomolecules (1992) reported the
234 achiral analysis of free proteinogenic amino acids with SFC-UV [40], and was mostly focused
235 on adjusting the mobile phase composition with additives mixtures to elute the analytes with
236 satisfying peak shapes. In general, additives nature and concentration is explored early on in
237 method development, to determine adequate composition to achieve elution of the analytes
238 with good peak shapes, taking account of detector issues. Unlike liquid-phase
239 chromatography, the mobile phase in SFC and EFLC cannot be easily buffered because it is
240 not possible to measure pH* precisely. As a result, the acid and base ionizable functions are
241 not easily controlled. It was shown that the combined presence of CO₂ and an alcohol co-
242 solvent induces some acidic character in usual SFC [41,42] and EFLC [43] conditions due to
243 the formation of methoxycarbonic acid, typically between pH* 4 and 6, depending on the
244 proportion of water in the mobile phase, which is further acidifying the fluid through the

245 formation of carbonic acid. It was also shown that the introduction of additives could modify
246 the apparent pH^* , with strong acids further reducing the apparent pH^* down to 1-2, and
247 bases only moderately increasing it to pH^* 6-7 [41,42]. Salts like ammonium acetate could
248 act as stabilizers in gradient elution modes. However, we can only conjecture on the exact
249 ionization state of the target molecules as their exact pK_a values in such conditions are
250 unknown. Whenever ionic groups are present, the use of additives serves not only in
251 changing the apparent pH^* and ionization state of the analytes, but may also contribute to
252 the formation of ion pairs between the analyte and additive ions to favor their elution.
253 Furthermore, the additive can adsorb on the stationary phase to reduce strong interactions
254 between an ionic analyte and polar groups of the stationary phase [42,44]. Other benefits of
255 the co-solvent and additives may be found in detection issues, particularly when hyphenation
256 to mass spectrometry is desired [34,45]. This will be further detailed in the section related to
257 detection issues. From a practical point of view, it is customary to use acidic additives for
258 acidic analytes and basic additives for basic analytes, but the contrary is also observed when
259 ion-pairing may help in favoring the elution [46]. When the analytes are large and complex,
260 and possess several ionizable groups with acidic and basic functions, an acid and a base are
261 sometimes used together, or salts like ammonium acetate. Examining the literature related to
262 SFC/EFLC analysis of our target biomolecules, the most often cited additive to elute the
263 target biomolecules was trifluoroacetic acid (TFA) [21,23,24,30,40,42,46–57]. Other acids
264 like formic acid [20,33,40,58,59] or methane- and ethane-sulfonic acids [31,53,60,61] were
265 also mentioned in several occasions. Isopropylamine, a basic additive that is frequently used
266 in SFC analysis of basic drugs, appeared only in a few papers [30,46,51,62], suggesting that
267 a basic additive may not be the best in this case. Ammonium acetate
268 [22,34,48,51,54,56,58,63–65] and ammonium formate [33,34,52,53,59,66,67], which were
269 both frequently employed in recent SFC methods [18], were mentioned many times. More
270 recently, ammonium hydroxide has become more and more popular in SFC analysis of basic
271 drugs and was found to be equally useful for amino acids, peptides and proteins
272 [45,52,58,68]. In older papers, several other exotic additives were employed, while in the
273 past decade, the additives employed in SFC/EFLC analyses have mostly been standardized,
274 largely favoring the additives that are compatible to mass spectrometric detection. Still a few
275 original proposals can be found, like ammonium fluoride to achieve the elution of most polar
276 amino acids in SFC [66], or sodium phosphate for nucleotides in EFLC [69].

277 Secondly, some portion of water can also be introduced in the mobile phase. Although
278 already an old practice [70,71], this was somewhat forgotten for several years, only to
279 reappear most significantly in the last decade. Water may be present in small concentrations
280 – typically 2 to 5% – and then acting like a polar additive [72], or in large concentrations
281 (typically above 10%) and then acting as a modifier [73]. Because CO_2 and water are not

282 miscible in all proportions, large concentrations of water can only be introduced in ternary
283 compositions of CO₂-solvent-water. Such mobile phase compositions are most often
284 encountered in EFLC experiments. Note that an EFLC method may be developed by
285 introducing carbon dioxide in any sort of liquid mobile phase: either from organic solvents
286 used in a normal-phase LC method [74] or SEC [75], or from buffer-organic mixtures used in
287 RPLC [76], HILIC [35,69,77], HIC [22] and IEX [33] modes. The proportion of water possibly
288 introduced in a CO₂-solvent mixture depends on (i) the operating conditions of temperature
289 and pressure, (ii) the proportions of CO₂ and solvent and (iii) the nature of the solvent.
290 Ternary diagrams of such mixtures are scarcely available [78,79] but would be most useful to
291 develop further analytical and preparative methods for the mostly polar target biomolecules.
292 For instance, Li and Thurbide [80] showed that using some isopropanol in conjunction with
293 methanol as co-solvent was favorable to the introduction of larger proportions of water,
294 thereby allowing the elution of most polar species like free amino acids without any other
295 additive.

296 Additives and water may also have combined effects. For instance, Taylor had
297 demonstrated that the combined use of water and ammonium acetate was more powerful in
298 achieving the elution of nucleobases with good peak shapes than the single use of either one
299 or the other [81]. Recently, Regalado and co-workers discussed the chaotropic effects of
300 ammonium hydroxide combined with water in a CO₂-methanol mobile phase when eluting
301 peptides [68]. In fact, in many papers, the additives mentioned above were used in
302 conjunction with water [21,23,33,46,54–56,61,64–66,68,69,82]. Taking aside the synergistic
303 effect that water may have with an additive, it may also favor the additive solubility, both in
304 the pressurized CO₂ mobile phase and after the decompression to avoid additive
305 precipitation in the instrument.

306
307 Whenever a wide range of analyte polarities is encountered, the analyses are preferably
308 conducted in gradient elution mode. With the evolutions in SFC practice, the proportion of co-
309 solvent introduced in the carbon dioxide mobile phase has been increasing more and more.
310 In the early years, only neat fluids were employed (not only CO₂ but also ammonia,
311 fluorocarbons, etc.) Back in the years 1980s, when it was found that introducing a co-solvent
312 brought more benefits in expanding the range of analytes amenable to the technique than
313 the expected drawbacks of increased viscosity, only a few percents (1-5%) of solvent were
314 employed. In the years 1990s, with the advent of packed column SFC over capillary SFC, the
315 typical range of solvent proportion extended to 1-20%, then increased further in the years
316 2000s. In the years 2010s, the maximum amount of co-solvent encountered in most SFC
317 papers was rather around 60%, which makes the fluid closer to a liquid than a supercritical
318 fluid, although it does not change the way to operate the system.

319 As appears in Figure 4, SFC is usually operating in the low portion of solvent area, while
320 EFLC usually works in the large portion of solvent area. While no fundamental nor practical
321 limit exists between the two [13], the distinction is rather issuing from different approaches:
322 SFC historically started with neat fluids, among which was neat CO₂. A co-solvent was later
323 introduced to expand the polarity limits of soluble analytes. At the other end of the solvent
324 axis, EFLC started with LC conditions, where CO₂ was introduced to reduce the viscosity of
325 the mobile phase, thereby providing improved efficiencies and the possibility to use higher
326 flow rates. In addition, the EFLC mobile phase should be a greener technique compared to a
327 fully liquid mobile phase as the consumption of solvent should be reduced [24]. Olesik and
328 co-workers are the main explorers of the EFLC concept, particularly at the analytical scale.
329 Specifically on biomolecules, they have successfully applied the method to nucleotides and
330 nucleosides [35,69,77], amino acids [33] and proteins [22,24]. In addition, Bennett and co-
331 workers [83] have brought EFLC to purification settings by introducing a guide to scaling up
332 from analytical to preparative separations.

333
334 In 2014 [84], Bamba and co-workers proposed to use the full range of co-solvent
335 proportion, starting with a mobile phase containing nearly 100% CO₂ and ending the gradient
336 elution at 100% hydro-organic composition. Because the first portion of the chromatogram
337 was SFC, then crossing the EFLC area and ending in liquid chromatography conditions, the
338 method was called “unified chromatography” (UC), a term previously used in similar
339 experiments joining gas chromatography, SFC and LC [85,86]. Most modern instruments are
340 perfectly capable to operate these wide gradients, with some instruments being clearly
341 designed for easy switching between SFC and LC operation. UC was found most useful to
342 achieve the elution of all proteinogenic free amino acids, from the most hydrophobic to the
343 most hydrophilic [53,61]. Guillaume and co-workers also used the same method to elute
344 metabolites with a wide range of polarities, including free amino acids [66] and nucleosides
345 [87]. EFLC and UC approaches might be a part of the answer to achieve the analysis of a
346 wider range of biomolecules.

347
348 Finally, because many of the target molecules possess amine functions (amino acids,
349 peptides, proteins), a few words of caution should be said about the possible reactions
350 occurring between CO₂ and amines. Indeed, CO₂ is believed to react with some amines,
351 possibly forming carbamic acids or ammonium carbamates [88]. However, this reaction is
352 reversible so the original amine is usually found after CO₂ depressurization [89]. This may
353 not be an issue for smaller, unstructured molecules like amino acids and peptides, but may
354 be a problem for 3D-stable structures like proteins, if the amine reaction causes permanent
355 unfolding of the protein. In a paper from Regalado and co-workers [68], a co-solvent

356 comprising methanol, 5% water and 0.2% ammonium hydroxide was employed, with gradient
357 ranging from 5 to 50% co-solvent. The combined use of water and NH₄OH were suggested
358 to cause chaotropic effects favoring the elution of a variety of analytes including
359 nucleobases, nucleosides, nucleotides and some peptides (linaclotide 1523 Da and
360 gramicidin S 1882 Da). In a separate study from the same group [23], the authors found that
361 some proteins like bradykinin and insulin could be recovered to their original higher-order
362 structure after SFC purification, while others like ubiquitin, cytochrome C and apomyoglobin
363 had permanently lost their higher-order structure. The co-solvent was a 3:1 mixture of
364 methanol and acetonitrile, added with 5% water and 0.2% trifluoroacetic acid. The elution
365 gradient ranged from 35 to 70% co-solvent in CO₂, which is covering both the usual SFC and
366 EFLC domains. Another recent paper from Govender and co-workers [21] presented the
367 purification of biosynthesized human insulin with SFC. The co-solvent was methanol
368 comprising 5% water and 0.2% trifluoroacetic acid, thus identical to the Schiavone et al. [23]
369 mobile phase. The purified insulins retained their bioactivity, suggesting that the higher-order
370 structure was indeed retained.

371 On another hand, Wang and Olesik also relate the elution of intact proteins [22] in EFLC
372 mode starting from a hydrophobic interaction chromatography (HIC) method, and
373 progressively introducing CO₂ in the 70:30 methanol-ammonium acetate buffer mobile
374 phase. In addition to improved chromatographic efficiency, the introduction of CO₂ was
375 shown to improve ESI-MS detection through facilitated desolvation. However, the claim was
376 on intact molecular weight, suggesting that no permanent reaction had damaged the primary
377 structure, but no information was given on preservation of higher-order structure.

378
379

380 **4. Stationary phase nature, particle type and dimension**

381

382 The range of stationary phases available for packed column SFC and EFLC was always
383 large, as any stationary phase designed for liquid chromatography can also be used with
384 CO₂ in the mobile phase. The nature of stationary phases has however greatly expanded in
385 the recent years as not only reversed-phase [90,91] and normal-phase [92] stationary
386 phases are possible but also the most recent stationary phases designed for HILIC mode
387 [93] and for mixed-mode liquid chromatography [94] and several novel stationary phases
388 especially designed for SFC use [95]. The diversity of available stationary phases is an
389 interesting feature of SFC and EFLC because it means that different selectivities are
390 available to achieve a given separation. The role of a stationary phase is two-fold: it must
391 interact with the target analytes to (i) retain them and (ii) separate them. It is customary in
392 SFC method development to start developing a method by screening several stationary

393 phases with complementary selectivities, to identify a good starting point for optimization.
394 Judging from the above discussion on the biomolecules polarity, it appears that many
395 different stationary phase chemistries could be useful as our target molecules comprise both
396 polar and non-polar molecules. In addition, the molecules requiring some separation may
397 have very different structural differences. For instance, when nucleobases must be resolved,
398 polar interactions are necessary to distinguish between small polar analytes with subtle
399 differences, thus a polar stationary phase is necessary. When large hydrophobic peptides
400 must be separated, a non-polar stationary phase may be a best candidate.

401
402 As most of them possess a chiral center, underivatized amino acids were often analyzed
403 on enantioselective stationary phases. The most cited phases were polysaccharide-based
404 stationary phases [60,96], which are most popular in enantioselective SFC. Other possible
405 enantioselective phases are *Cinchona* alkaloid-based zwitterionic stationary phases, which
406 were specifically designed for amino acid enantioresolution in LC [53,61], macrocyclic
407 glycopeptide stationary phase [42,97] and crown-ether stationary phase [55], which are both
408 also known to perform well for amino acids in liquid phase.

409 When no enantioresolution was desired and only intra-class separation of free amino
410 acids was looked for, a variety of achiral stationary phases were used. Diol-type stationary
411 phases are most often cited [34,45,67,82], starting from the seminal paper from Camel and
412 co-workers [40]. Toribio and co-workers proposed to couple a chiral glycopeptide stationary
413 phase to an achiral diol phase to resolve two pairs of enantiomers (phenylalanine and
414 tyrosine) in a single analysis with SFC-UV [97]. *Cinchona* alkaloid-based zwitterionic
415 stationary phases also allowed a most complete separation of all proteinogenic amino acids
416 from each other, together with enantioresolution for most of them [53]. Interestingly, the four
417 isobaric leucine enantiomers and isoleucine enantiomers could all be resolved [61]. Finally,
418 other achiral stationary phases cited for the SFC/EFLC analysis of free amino acids are
419 phases normally used in HILIC mode, like the anionic polysulfoethyl stationary phase [33],
420 the zwitterionic sulfobetaine-bonded stationary phase [66], bare silica gel [66] or amide-
421 bonded stationary phase [54].

422 For peptides and proteins, where chiral separation is rarely desired, achiral stationary
423 phases with pyridine ligands were the most often cited stationary phases [21,23,46,48,50–
424 52,72,98]. 2-Ethylpyridine is a stationary phase that was designed specifically for SFC at the
425 beginning of the years 2000s by Princeton Chromatography, and found an immediate
426 success for basic drugs because it provided nice peak shapes without the need of mobile
427 phase additives, supposedly due to hydrogen bonding between residual silanol groups and
428 the pyridine nitrogen atom [99]. Other variations include a 4-ethylpyridine ligand [51,52] or
429 pyridyl-amide. A polymeric version of 4-ethylpyridine, poly(4-vinylpyridine) (P4VP) was also

430 cited [68]. 2-Picolylamine, appearing in the latest publications [23,72], is a most recent
431 version of 2-ethylpyridine, which was introduced to limit retention loss through silyl ether
432 formation [100]. Other polar stationary phases used for peptides and proteins include again
433 diol-type stationary phases [46,51,52,62], bare silica gel [20,32,46,72] and amide bonding
434 [24], but also cyanopropyl-bonded silica [49,63]. Non-polar and moderately polar phases,
435 which are less frequently encountered in SFC applications, also appear in a few instances.
436 For example, aromatic phases like pentafluorophenyl (PFP) [21] and polystyrene-
437 divinylbenzene (PS-DVB) [31,47,80,101] were mentioned in a few occasions, starting with
438 the first paper reporting SFC analysis of both cyclic and linear peptides [31]. Finally, alkyl-
439 type stationary phases were also mentioned: octadecylbonded-silica (ODS) was used in the
440 first report of cyclic peptide analysis (Cyclosporin A) [19] and later to resolve isomeric 12-mer
441 peptides [46]. Polybutyl and polypentyl phases normally used in HIC mode (hydrophobic
442 interaction chromatography) were recently employed to analyze intact proteins in EFLC [22].

443 To those less familiar with the technique, it may be surprising to observe that there
444 seems to be little rationale behind the use of one column or another as very different
445 stationary phases were found to be useful for the same purpose by different users. For
446 instance, the cyclic peptides of cyclosporins were analyzed on an ODS phase [19] or on bare
447 silica gel [32]. Gramicidin, a membrane protein comprising several members, was also
448 analyzed by several different authors on different stationary phases: cyanopropyl-bonded
449 silica [49,63], PS-DVB [47,101], bare silica gel [72] or pyridine phases [68,72]. Different pairs
450 of 12-mer isomeric peptides (about 1100 Da) were successfully resolved on a variety of
451 stationary phases including ODS, bare silica gel or 2-ethylpyridine [46]. Actually, it is often
452 observed in SFC that several very different stationary phases can be successful for the same
453 separation problems, although different elution orders would be observed [102].

454 The case of nucleic acid bricks is different because, as appeared in Figure 3,
455 nucleobases, nucleosides and nucleotides are all polar, with negative log P values. Thus
456 retention and selectivity in that case is favored by polar stationary phases with bare silica
457 [58,59,64], diol [30,56,58,67], amide [35,58,69,77] and pyridine-type [30,56,65,103] being
458 equally cited.

459 The improved instruments for analytical SFC introduced at the beginning of the years
460 2010 tended towards higher efficiency through reduced dead volumes and detector cells.
461 Concomitantly, there has been a trend to high efficiency packed columns, either through
462 small fully porous particles (FPP) with sub-2 μm diameter, or through superficially porous
463 particles (SPP) with sub-3 μm diameter. In the recent papers, sub-2 μm FPP stationary
464 phases appear [24,65,103]. However, when a large elution gradient is desired, the viscosity
465 of the mobile phase at the end of the gradient is high. Because small particles also generate
466 high pressure drops, not all SFC instruments can sustain the high pressure. Indeed, some

467 instruments are still limited to 400 bars inlet pressure (Waters, Jasco), while others can reach
468 600 bars (Agilent) or 660 bars (Shimadzu). In that case, the flow rate and/or back-pressure
469 must be reduced to avoid reaching the upper pressure limit. Another option is to favor
470 columns packed with SPP stationary phases [66].

471

472 **5. Detection issues and hyphenation to mass spectrometry**

473

474 Packed-column SFC and EFLC make use of the same detectors as LC, namely UV-
475 visible or diode-array detection (DAD), evaporative light scattering detection (ELSD), Corona
476 charged-aerosol detection (CAD) and mass spectrometry (MS). The latter is preferably
477 equipped with LC-type ionization source, namely electrospray ionization (ESI), atmospheric
478 pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI).

479 Among all these detectors, not all are equally useful for the target biomolecules. Amino
480 acids, their oligomers and polymers mostly lack of chromophores, thus UV and DAD
481 detection are of little use, unless the desired detection limit is not too low [54]. Bigger
482 peptides and proteins and those comprising aromatic residues may however be easier to
483 detect with UV at 210 or 280 nm. Because the nucleobases are good chromophores, UV and
484 DAD detection is appropriate for the nucleobases, nucleosides, nucleotides and nucleic
485 acids.

486 While UV detection is placed before the back-pressure regulator, ELSD and CAD are
487 placed after the BPR. Only a few studies explored ELSD possibilities in SFC [40,46,96] or in
488 EFLC [33] for underivatized amino acids and small peptides.

489 Because it provides additional information on analyte structure, mass spectrometry is
490 certainly the most desirable detection mode hyphenated to SFC/EFLC instruments,
491 especially now that it is easier to access in most laboratories, with simpler instruments
492 available at a moderate cost. Hyphenation of SFC/EFLC to MS may be done several ways
493 [104,105]. First, flow splitting may be done – or not – prior to entering the MS, with one
494 portion of the column effluent entering the MS and the other being directed towards the back-
495 pressure regulator (BPR) and waste or fraction collection [106]. When the chromatographic
496 flow rate is high, the flow is preferentially divided because the ionization sources cannot all
497 cope with the large volume expansion caused by CO₂ depressurization. However, this can
498 affect sensitivity for mass-sensitive detectors. As the largest portion of SFC instruments sold
499 are based on flow-splitting configuration, this is quite logically the most often cited in the
500 literature related to biomolecules. However, when the chromatographic flow rate is low, the
501 whole effluent can be directed to the MS, which is then placed right after the BPR. Second, a
502 make-up fluid may be introduced prior to entering the MS, and prior to the flow splitting, when
503 flow splitting is desired. The interest of a make-up fluid is to avoid analyte precipitation upon

504 the cold CO₂ depressurization and to introduce ionization enhancers prior to entering the MS
505 source. However, the make-up fluid may cause further dilution of the column effluent, thereby
506 may be reducing sensitivity for concentration-sensitive detectors. Deconvoluting the positive
507 and negative effects of the make-up fluid is difficult, so the variations observed are not
508 always in accordance with intuition [107].

509 The composition of make-up fluid is most often methanol, comprising a small proportion
510 of water (1 to 10%) and one acidic or salt additive. This additive is sometimes the same as
511 the one employed in the mobile phase [45,54], sometimes different [55,61] because the
512 composition favoring good chromatography is not necessarily the same as the composition
513 favoring good ionization. A supposedly generic composition and flow rate are often adopted
514 and their impact was rarely studied [45]. When large elution gradients are employed,
515 especially in UC experiments, a constant flow rate of make-up fluid could be inadequate.

516 The choice of ionization source, which depends on analyte size and polarity, has a
517 significant impact on the intensity of molecular and fragment ions detected. ESI and APCI
518 were employed in almost all studies. Wolrab et al. [34] compared ESI and APCI sources
519 specifically for SFC-MS/MS analysis of amino acids and related compounds. They concluded
520 that ESI seemed to be the best choice for the least polar amino acids. However, for polar
521 amino acids and non-polar amino acids with an additional heteroatom as well as acidic
522 amino acids, APCI seemed the best choice. In other words, the preferred ionization source
523 will be greatly dependent on analyte, even within one molecular family.

524 In terms of analyzers, some recent investigations were done with triple-quadrupole
525 [34,45] or quadrupole-time-of-flight [22,24,65,82] analyzers, which can provide precise
526 structural information the target analytes. When complex matrices are present, or high
527 molecular weight biomolecules are considered, high resolution is necessary.

528

529 **6. Applications areas**

530

531 Due to the limited number of papers published to date, applications only articulated
532 around three themes. First, food products were the topic of a few papers. Sánchez-
533 Hernández et al. [97] coupled chiral and achiral stationary phases to control the quality of
534 food supplements and confirm the absence of enantiomeric impurities of free amino acids
535 with UV detection. Raimbault et al. [61] used a single chiral stationary phase for quantitative
536 assessment of free branched amino acids in food supplements with single-quadrupole MS
537 detection. Huang and co-workers [54] determined 11 free amino acids in several varieties of
538 tea with an achiral stationary phase and single-quadrupole MS detection. The method was
539 fully validated. Ashraf-Khorassani et al. [62] focused on depsipeptides, which are peptides

540 with some amides functions replaced by esters. The authors developed a quantitative
541 extraction method for some growth-promoting animal feed additives, and analyzed them with
542 SFC-UV. The target molecules were the major components of alexomycin like sulfomycin
543 (see structure in Figure 5) and promothiocin.

544 Secondly, human/animal [34,45,66,67,87] or plant [82] metabolomics studies are also
545 concerned with biomolecules separations in SFC/EFLC or UC. Liquid-phase chromatography
546 hyphenated to mass spectrometry is currently dominating the metabolomics scene. However,
547 because the samples are complex and the metabolites are extremely varied in terms of
548 structures and polarities, the potential of other methods to offer complementary information
549 to other existing techniques is of great interest, as exposed by Shulaev and Isaac [108].
550 Wolrab et al. [34] focused on MS detection of free amino acids and related compounds in
551 human serum. Amino acids, peptides, and nucleosides were all included in SFC-MS
552 methods from Sen and co-workers [67], and from Akbal and Hopfgartner [45]. The latter
553 compared the results to those of RPLC and HILIC. Many metabolites previously included in
554 the method development were identified in human urine within fast SFC-MS runs. In a
555 different study, Desfontaine et al. [66] focused on a most generic method to analyze a wide
556 range of metabolite polarities, including most polar amino acids as well as non-polar lipids.
557 For this purpose, a wide UC elution gradient was favored, and was later applied to real
558 samples of plasma and urine [87]. Similarly, Grand-Guillaume and co-workers [82] developed
559 a widely applicable SFC-MS/MS method including a great variety of plant metabolites, from
560 the most polar free amino acids to the least polar lipids and terpenes. They later
561 demonstrated the interest of combining LC-MS/MS and SFC-MS/MS to acquire a most
562 complete knowledge on plant extracts [109].

563 Finally, analysis and purification of biomolecules for pharmaceutical and clinical applications
564 were the most abundant among applied studies. Therapeutic peptides may contain a large
565 number of impurities requiring identification and elimination, where chromatographic and
566 electrophoretic methods are most useful [110]. Method development for peptides and
567 proteins were well exposed in several papers [21,23,28,52,63,68,72,98]. Typical examples of
568 therapeutic peptides are presented in Figure 5, like gramicidin, cyclosporin, angiotensin and
569 ocytocin. As mentioned above, proteins set particular problems in that the ternary structure
570 should be preserved in purification methods. Free amino acids were analyzed in a traditional
571 medicinal plant with SFC-MS [53]. Nucleobases and nucleosides were also searched in
572 ginseng, a traditional Chinese medicine [58]. Other synthetic nucleosides were the topic of
573 clinical studies: azacitidine, clofarabine and cytarabine (see structures in Figure 6) were
574 analyzed with SFC along with their impurities following ICH guidelines [57,64,103]. For
575 instance, cytarabine was quantified in mouse plasma and the SFC-MS/MS method was
576 compared to LC-MS/MS [64]. Finally, Pirrone et al. showed an interesting application of SFC-

577 MS for impurity fate mapping of a nucleoside drug substance, where reversed-phase liquid
578 chromatography failed to deliver the desired answers due to keto-enol tautomerization in
579 aqueous conditions [111].

580

581

582 **7. Conclusion and perspectives**

583

584 As appeared in this review, amino acids, peptides, proteins and nucleic acid bricks are
585 widely diverse molecules in terms of sizes and polarities. It comes as no surprise that no
586 consensus stationary phase and mobile phase compositions have emerged for their
587 SFC/EFLC analysis. Significant diversity in stationary phase chemistry and mobile phase
588 composition was observed, although recent papers mostly cite polar stationary phases and
589 wide elution gradients comprising some portion of water in the co-solvent, along with
590 additives. Rather than a source of confusion, this diversity should be perceived as a great
591 potential for successful method development and optimization. MS proves to be an essential
592 tool for structure identification but UV detection still comes in handy for quantitative analysis
593 of samples with low complexity, or when sensitivity is not an issue. This is typically the case
594 in quality control of pharmaceuticals, food products or extracts of natural products.

595 Applications of biomolecules with SFC/EFLC clearly deserve more attention than is
596 currently the case. At the analytical scale, the complementary information that can be
597 obtained when comparing to most often used RPLC methods should justify the investment.
598 At the preparative scale, the time and solvent economy are well worth the effort. Finally,
599 unified chromatography (UC) slowly emerges with very promising capacities shared from
600 both LC and SFC/EFLC technologies.

601

602 **References**

603

- 604 [1] S.-H. Joo, Cyclic Peptides as Therapeutic Agents and Biochemical Tools, *Biomol. Ther.* 20 (2012)
605 19–26. <https://doi.org/10.4062/biomolther.2012.20.1.019>.
- 606 [2] S. Mahmoud, S. Hasabelnaby, S. Hammad, T. Sakr, Antiviral Nucleoside and Nucleotide Analogs:
607 A Review, *J. Adv. Pharm. Res.* 2 (2018) 73–88. <https://doi.org/10.21608/aprh.2018.5829>.
- 608 [3] D. Gang, D. Kim, H.-S. Park, Cyclic Peptides: Promising Scaffolds for Biopharmaceuticals, *Genes.*
609 9 (2018) 557. <https://doi.org/10.3390/genes9110557>.
- 610 [4] K. Fosgerau, T. Hoffmann, Peptide therapeutics: current status and future directions, *Drug*
611 *Discov. Today.* 20 (2015) 122–128. <https://doi.org/10.1016/j.drudis.2014.10.003>.
- 612 [5] M. Sherman, L. Contreras, Computational approaches in design of nucleic acid-based
613 therapeutics, *Curr. Opin. Biotechnol.* 53 (2018) 232–239.
614 <https://doi.org/10.1016/j.copbio.2017.12.001>.
- 615 [6] J. Koehbach, D.J. Craik, The Vast Structural Diversity of Antimicrobial Peptides, *Trends*
616 *Pharmacol. Sci.* 40 (2019) 517–528. <https://doi.org/10.1016/j.tips.2019.04.012>.
- 617 [7] S.-S. Tang, Z.H. Prophan, S.K. Biswas, C.-F. Le, S.D. Sekaran, Antimicrobial peptides from
618 different plant sources: Isolation, characterisation, and purification, *Phytochemistry.* 154 (2018)
619 94–105. <https://doi.org/10.1016/j.phytochem.2018.07.002>.
- 620 [8] J.M. Lorenzo, P.E.S. Munekata, B. Gómez, F.J. Barba, L. Mora, C. Pérez-Santaescolástica, F.
621 Toldrá, Bioactive peptides as natural antioxidants in food products – A review, *Trends Food Sci.*
622 *Technol.* 79 (2018) 136–147. <https://doi.org/10.1016/j.tifs.2018.07.003>.
- 623 [9] M. Liu, Y. Wang, Y. Liu, R. Ruan, Bioactive peptides derived from traditional Chinese medicine
624 and traditional Chinese food: A review, *Food Res. Int.* 89 (2016) 63–73.
625 <https://doi.org/10.1016/j.foodres.2016.08.009>.
- 626 [10] K. Sandra, M. Moshir, F. D'hondt, K. Verleysen, K. Kas, P. Sandra, Highly efficient peptide
627 separations in proteomics, *J. Chromatogr. B.* 866 (2008) 48–63.
628 <https://doi.org/10.1016/j.jchromb.2007.10.034>.
- 629 [11] C. Temporini, R. Colombo, E. Calleri, S. Tengattini, F. Rinaldi, G. Massolini, Chromatographic
630 tools for plant-derived recombinant antibodies purification and characterization, *J. Pharm.*
631 *Biomed. Anal.* 179 (2020) 112920. <https://doi.org/10.1016/j.jpba.2019.112920>.
- 632 [12] E. Lesellier, C. West, The many faces of packed column supercritical fluid chromatography - A
633 critical review, *J. Chromatogr. A.* 1382 (2015) 2–46.
634 <https://doi.org/10.1016/j.chroma.2014.12.083>.
- 635 [13] S. Olesik, Enhanced-Fluidity Liquid Chromatography: Connecting the Dots Between Supercritical
636 Fluid Chromatography, Conventional Subcritical Fluid Chromatography, and HPLC, *LC-GC N. Am.*
637 33 (2015) 24–30.
- 638 [14] E. Klesper, A.H. Corwin, D.A. Turner, High-pressure gas chromatography above critical
639 temperature, *J. Org. Chem.* 27 (1962) 700–706. <https://doi.org/10.1021/jo01049a069>.
- 640 [15] C. West, Recent trends in chiral supercritical fluid chromatography, *TrAC Trends Anal. Chem.*
641 120 (2019) 115648. <https://doi.org/10.1016/j.trac.2019.115648>.
- 642 [16] C. West, Enantioselective Separations with Supercritical Fluids - Review, *Curr. Anal. Chem.* 10
643 (2014) 99–120. <https://doi.org/10.2174/1573411011410010009>.
- 644 [17] D. Speybrouck, E. Lipka, Preparative supercritical fluid chromatography: A powerful tool for
645 chiral separations, *J. Chromatogr. A.* 1467 (2016) 33–55.
646 <https://doi.org/10.1016/j.chroma.2016.07.050>.
- 647 [18] C. West, Current trends in supercritical fluid chromatography, *Anal. Bioanal. Chem.* 410 (2018)
648 6441–6457. <https://doi.org/10.1007/s00216-018-1267-4>.
- 649 [19] H.T. Kalinoski, B.W. Wright, R.D. Smith, Chemical ionization mass spectra of high molecular
650 weight, biologically active compounds produced following supercritical fluid chromatography,
651 *Biol. Mass Spectrom.* 15 (1988) 239–242. <https://doi.org/10.1002/bms.1200150409>.

- 652 [20] M.R. Emmett, S. Kazazic, A.G. Marshall, W. Chen, S.D.-H. Shi, B. Bolaños, M.J. Greig,
653 Supercritical Fluid Chromatography Reduction of Hydrogen/Deuterium Back Exchange in
654 Solution-Phase Hydrogen/Deuterium Exchange with Mass Spectrometric Analysis, *Anal. Chem.*
655 78 (2006) 7058–7060. <https://doi.org/10.1021/ac060693n>.
- 656 [21] K. Govender, T. Naicker, S. Baijnath, A. Amichund Chuturgoon, N. Sheik Abdul, T. Docrat, H.
657 Gerhardus Kruger, T. Govender, The development of a supercritical fluid chromatography (SFC)
658 based method for the purification of biosynthesised human insulin, *J. Chromatogr. B.* (2020)
659 122126. <https://doi.org/10.1016/j.jchromb.2020.122126>.
- 660 [22] Y. Wang, S.V. Olesik, Enhanced-Fluidity Liquid Chromatography–Mass Spectrometry for Intact
661 Protein Separation and Characterization, *Anal. Chem.* 91 (2019) 935–942.
662 <https://doi.org/10.1021/acs.analchem.8b03970>.
- 663 [23] N.M. Schiavone, R. Bennett, M.B. Hicks, G.F. Pirrone, E.L. Regalado, I. Mangion, A.A. Makarov,
664 Evaluation of global conformational changes in peptides and proteins following purification by
665 supercritical fluid chromatography, *J. Chromatogr. B.* 1110–1111 (2019) 94–100.
666 <https://doi.org/10.1016/j.jchromb.2019.02.012>.
- 667 [24] R. Bennett, S.V. Olesik, Protein separations using enhanced-fluidity liquid chromatography, *J.*
668 *Chromatogr. A.* 1523 (2017) 257–264. <https://doi.org/10.1016/j.chroma.2017.07.060>.
- 669 [25] L.M. Nogle, C.W. Mann, W.L. Watts, Y. Zhang, Preparative separation and identification of
670 derivatized β -methylphenylalanine enantiomers by chiral SFC, HPLC and NMR for development
671 of new peptide ligand mimetics in drug discovery, *J. Pharm. Biomed. Anal.* 40 (2006) 901–909.
672 <https://doi.org/10.1016/j.jpba.2005.08.034>.
- 673 [26] C.M. Kraml, D. Zhou, N. Byrne, O. McConnell, Enhanced chromatographic resolution of amine
674 enantiomers as carbobenzyloxy derivatives in high-performance liquid chromatography and
675 supercritical fluid chromatography, *J. Chromatogr. A.* 1100 (2005) 108–115.
676 <https://doi.org/10.1016/j.chroma.2005.09.017>.
- 677 [27] J.A. Blackwell, R.W. Stringham, J.D. Weckwerth, Effect of Mobile Phase Additives in Packed-
678 Column Subcritical and Supercritical Fluid Chromatography, *Anal. Chem.* 69 (1997) 409–415.
679 <https://doi.org/10.1021/ac9608883>.
- 680 [28] G.H. Goetz, L. Philippe, M.J. Shapiro, EPSA: A Novel Supercritical Fluid Chromatography
681 Technique Enabling the Design of Permeable Cyclic Peptides, *ACS Med. Chem. Lett.* 5 (2014)
682 1167–1172. <https://doi.org/10.1021/ml500239m>.
- 683 [29] G. Caron, G. Ermondi, Molecular descriptors for polarity: the need for going beyond polar
684 surface area, *Future Med. Chem.* 8 (2016) 2013–2016. <https://doi.org/10.4155/fmc-2016-0165>.
- 685 [30] O. Kozlov, Z. Kadlecová, E. Tesařová, K. Kalíková, Evaluation of separation properties of
686 stationary phases in supercritical fluid chromatography; deazapurine nucleosides case study,
687 *Microchem. J.* 150 (2019) 104137. <https://doi.org/10.1016/j.microc.2019.104137>.
- 688 [31] J.A. Blackwell, R.W. Stringham, Effect of Mobile Phase Components on the Separation of
689 Polypeptides Using Carbon Dioxide-Based Mobile Phases, *J. High Resolut. Chromatogr.* 22
690 (1999) 74–78. [https://doi.org/10.1002/\(SICI\)1521-4168\(19990201\)22:2<74::AID-
691 JHRC74>3.0.CO;2-9](https://doi.org/10.1002/(SICI)1521-4168(19990201)22:2<74::AID-JHRC74>3.0.CO;2-9).
- 692 [32] Y. Shao, C. Wang, A. Apedo, O. McConnell, Rapid Separation of Five Cyclosporin Analogs by
693 Supercritical Fluid Chromatography, *J. Anal. Sci. Methods Instrum.* 06 (2016) 23–32.
694 <https://doi.org/10.4236/jasmi.2016.62004>.
- 695 [33] M.J. Beres, S.V. Olesik, Enhanced-fluidity liquid chromatography using mixed-mode hydrophilic
696 interaction liquid chromatography/strong cation-exchange retention mechanisms, *J. Sep. Sci.*
697 38 (2015) 3119–3129. <https://doi.org/10.1002/jssc.201500454>.
- 698 [34] D. Wolrab, P. Frühauf, C. Gerner, Direct coupling of supercritical fluid chromatography with
699 tandem mass spectrometry for the analysis of amino acids and related compounds: Comparing
700 electrospray ionization and atmospheric pressure chemical ionization, *Anal. Chim. Acta.* 981
701 (2017) 106–115. <https://doi.org/10.1016/j.aca.2017.05.005>.

- 702 [35] J.W. Treadway, G.S. Philibert, S.V. Olesik, Enhanced fluidity liquid chromatography for
703 hydrophilic interaction separation of nucleosides, *J. Chromatogr. A.* 1218 (2011) 5897–5902.
704 <https://doi.org/10.1016/j.chroma.2010.12.059>.
- 705 [36] J.F. Deye, T.A. Berger, A.G. Anderson, Nile Red as a solvatochromic dye for measuring solvent
706 strength in normal liquids and mixtures of normal liquids with supercritical and near critical
707 fluids, *Anal. Chem.* 62 (1990) 615–622. <https://doi.org/10.1021/ac00205a015>.
- 708 [37] T.A. Berger, Clarifying the relationship between density and viscosity of methanol/carbon
709 dioxide mixtures used in supercritical fluid chromatography, *Chromatogr. Today.* Feb.-Mar.
710 (2019) 28–31.
- 711 [38] E. Glenne, H. Leek, M. Klarqvist, J. Samuelsson, T. Fornstedt, Systematic investigations of peak
712 deformations due to co-solvent adsorption in preparative supercritical fluid chromatography, *J.*
713 *Chromatogr. A.* 1496 (2017) 141–149. <https://doi.org/10.1016/j.chroma.2017.03.053>.
- 714 [39] C. West, E. Lesellier, Effects of mobile phase composition on retention and selectivity in achiral
715 supercritical fluid chromatography, *J. Chromatogr. A.* 1302 (2013) 152–162.
716 <https://doi.org/10.1016/j.chroma.2013.06.003>.
- 717 [40] V. Camel, D. Thiébaud, M. Caude, M. Dreux, Packed column subcritical fluid chromatography of
718 underivatized amino acids, *J. Chromatogr. A.* 605 (1992) 95–101. [https://doi.org/10.1016/0021-](https://doi.org/10.1016/0021-9673(92)85032-O)
719 [9673\(92\)85032-O](https://doi.org/10.1016/0021-9673(92)85032-O).
- 720 [41] C. West, J. Melin, H. Ansouri, M. Mengue Metogo, Unravelling the effects of mobile phase
721 additives in supercritical fluid chromatography. Part I: Polarity and acidity of the mobile phase,
722 *J. Chromatogr. A.* 1492 (2017) 136–143. <https://doi.org/10.1016/j.chroma.2017.02.066>.
- 723 [42] A. Raimbault, C. West, Effects of high concentrations of mobile phase additives on retention
724 and separation mechanisms on a teicoplanin aglycone stationary phase in supercritical fluid
725 chromatography, *J. Chromatogr. A.* 1604 (2019) 460494.
726 <https://doi.org/10.1016/j.chroma.2019.460494>.
- 727 [43] D. Wen, S.V. Olesik, Characterization of pH in Liquid Mixtures of Methanol/H₂O/CO₂, *Anal.*
728 *Chem.* 72 (2000) 475–480. <https://doi.org/10.1021/ac9905219>.
- 729 [44] C. West, E. Lemasson, Unravelling the effects of mobile phase additives in supercritical fluid
730 chromatography. Part II: Adsorption on the stationary phase, *J. Chromatogr. A.* (2019).
731 <https://doi.org/10.1016/j.chroma.2019.02.002>.
- 732 [45] L. Akbal, G. Hopfgartner, Supercritical fluid chromatography–mass spectrometry using data
733 independent acquisition for the analysis of polar metabolites in human urine, *J. Chromatogr. A.*
734 1609 (2020) 460449. <https://doi.org/10.1016/j.chroma.2019.460449>.
- 735 [46] M.A. Patel, F. Riley, M. Ashraf-Khorassani, L.T. Taylor, Supercritical fluid chromatographic
736 resolution of water soluble isomeric carboxyl/amine terminated peptides facilitated via mobile
737 phase water and ion pair formation, *J. Chromatogr. A.* 1233 (2012) 85–90.
738 <https://doi.org/10.1016/j.chroma.2012.02.024>.
- 739 [47] K.B. Thurvide, J. Zhang, Separation of linear gramicidins using carbon dioxide-containing mobile
740 phases, *Anal. Bioanal. Chem.* 382 (2005) 1227–1233. [https://doi.org/10.1007/s00216-005-](https://doi.org/10.1007/s00216-005-3270-9)
741 [3270-9](https://doi.org/10.1007/s00216-005-3270-9).
- 742 [48] J. Zheng, J.D. Pinkston, P.H. Zoutendam, L.T. Taylor, Feasibility of Supercritical Fluid
743 Chromatography/Mass Spectrometry of Polypeptides with Up to 40-Mers, *Anal. Chem.* 78
744 (2006) 1535–1545. <https://doi.org/10.1021/ac052025s>.
- 745 [49] X. Zhang, M. Scalf, M.S. Westphall, L.M. Smith, Membrane Protein Separation and Analysis by
746 Supercritical Fluid Chromatography–Mass Spectrometry, *Anal. Chem.* 80 (2008) 2590–2598.
747 <https://doi.org/10.1021/ac702319u>.
- 748 [50] D. Tognarelli, A. Tsukamoto, J. Caldwell, W. Caldwell, Rapid peptide separation by supercritical
749 fluid chromatography, *Bioanalysis.* 2 (2010) 5–7. <https://doi.org/10.4155/bio.09.165>.
- 750 [51] M.A. Patel, F. Riley, J. Wang, M. Lovdahl, L.T. Taylor, Packed column supercritical fluid
751 chromatography of isomeric polypeptide pairs, *J. Chromatogr. A.* 1218 (2011) 2593–2597.
752 <https://doi.org/10.1016/j.chroma.2011.03.005>.

- 753 [52] M. Ventura, Advantageous use of SFC for separation of crude therapeutic peptides and peptide
754 libraries, *J. Pharm. Biomed. Anal.* (2020) 113227. <https://doi.org/10.1016/j.jpba.2020.113227>.
- 755 [53] A. Raimbault, M. Dorebska, C. West, A chiral unified chromatography–mass spectrometry
756 method to analyze free amino acids, *Anal. Bioanal. Chem.* (2019).
757 <https://doi.org/10.1007/s00216-019-01783-5>.
- 758 [54] Y. Huang, T. Wang, M. Fillet, J. Crommen, Z. Jiang, Simultaneous determination of amino acids
759 in different teas using supercritical fluid chromatography coupled with single quadrupole mass
760 spectrometry, *J. Pharm. Anal.* 9 (2019) 254–258. <https://doi.org/10.1016/j.jpba.2019.05.001>.
- 761 [55] L. Miller, L. Yue, Chiral separation of underivatized amino acids in supercritical fluid
762 chromatography with chiral crown ether derived column, *Chirality*. (2020) chir.23204.
763 <https://doi.org/10.1002/chir.23204>.
- 764 [56] M. Ashraf-Khorassani, L.T. Taylor, Subcritical fluid chromatography of water soluble
765 nucleobases on various polar stationary phases facilitated with alcohol-modified CO₂ and water
766 as the polar additive, *J. Sep. Sci.* 33 (2010) 1682–1691. <https://doi.org/10.1002/jssc.201000047>.
- 767 [57] V.N.R. Ganipisetty, B. Ravi, Ch.R. Reddy, R. Gurjar, P. Manoj, R.V. Nadh, G. dev Gudipati,
768 Supercritical fluid (CO₂) chromatography for quantitative determination of selected cancer
769 therapeutic drugs in the presence of potential impurities, *Anal. Methods*. 7 (2015) 1092–1097.
770 <https://doi.org/10.1039/C4AY02368D>.
- 771 [58] Y. Huang, T. Zhang, Y. Zhao, H. Zhou, G. Tang, M. Fillet, J. Crommen, Z. Jiang, Simultaneous
772 analysis of nucleobases, nucleosides and ginsenosides in ginseng extracts using supercritical
773 fluid chromatography coupled with single quadrupole mass spectrometry, *J. Pharm. Biomed.*
774 *Anal.* 144 (2017) 213–219. <https://doi.org/10.1016/j.jpba.2017.03.059>.
- 775 [59] A. dos Santos Pereira, A.J. Girón, E. Admasu, P. Sandra, Green hydrophilic interaction
776 chromatography using ethanol–water–carbon dioxide mixtures, *J. Sep. Sci.* 33 (2010) 834–837.
777 <https://doi.org/10.1002/jssc.200900791>.
- 778 [60] R.W. Stringham, Chiral separation of amines in subcritical fluid chromatography using
779 polysaccharide stationary phases and acidic additives, *J. Chromatogr. A.* 1070 (2005) 163–170.
780 <https://doi.org/10.1016/j.chroma.2005.02.044>.
- 781 [61] A. Raimbault, A. Noireau, C. West, Analysis of free amino acids with unified chromatography-
782 mass spectrometry—application to food supplements, *J. Chromatogr. A.* (2019) 460772.
783 <https://doi.org/10.1016/j.chroma.2019.460772>.
- 784 [62] M. Ashraf-Khorassani, L.T. Taylor, J.G.D. Marr, Analysis of the sulfomycin component of
785 alexomycin in animal feed by enhanced solvent extraction and supercritical fluid
786 chromatography, *J. Biochem. Biophys. Methods.* 43 (2000) 147–156.
787 [https://doi.org/10.1016/S0165-022X\(00\)00053-1](https://doi.org/10.1016/S0165-022X(00)00053-1).
- 788 [63] B. Bolaños, M. Greig, M. Ventura, W. Farrell, C.M. Aurigemma, H. Li, T.L. Quenzer, K. Tivel,
789 J.M.R. Bylund, P. Tran, C. Pham, D. Phillipson, SFC/MS in drug discovery at Pfizer, La Jolla, *Int. J.*
790 *Mass Spectrom.* 238 (2004) 85–97. <https://doi.org/10.1016/j.ijms.2003.11.021>.
- 791 [64] Y. Hsieh, F. Li, C.J.G. Duncan, Supercritical Fluid Chromatography and High-Performance Liquid
792 Chromatography/Tandem Mass Spectrometric Methods for the Determination of Cytarabine in
793 Mouse Plasma, *Anal. Chem.* 79 (2007) 3856–3861. <https://doi.org/10.1021/ac062441s>.
- 794 [65] L. Laboureur, V. Guérineau, S. Auxilien, S. Yoshizawa, D. Touboul, Profiling of modified
795 nucleosides from ribonucleic acid digestion by supercritical fluid chromatography coupled to
796 high resolution mass spectrometry, *J. Chromatogr. A.* 1537 (2018) 118–127.
797 <https://doi.org/10.1016/j.chroma.2017.12.020>.
- 798 [66] V. Desfontaine, G.L. Losacco, Y. Gagnebin, J. Pezzatti, W.P. Farrell, V. González-Ruiz, S. Rudaz, J.-
799 L. Veuthey, D. Guillarme, Applicability of supercritical fluid chromatography – mass
800 spectrometry to metabolomics. I – Optimization of separation conditions for the simultaneous
801 analysis of hydrophilic and lipophilic substances, *J. Chromatogr. A.* 1562 (2018) 96–107.
802 <https://doi.org/10.1016/j.chroma.2018.05.055>.
- 803 [67] A. Sen, C. Knappy, M.R. Lewis, R.S. Plumb, I.D. Wilson, J.K. Nicholson, N.W. Smith, Analysis of
804 polar urinary metabolites for metabolic phenotyping using supercritical fluid chromatography

805 and mass spectrometry, *J. Chromatogr. A.* 1449 (2016) 141–155.
806 <https://doi.org/10.1016/j.chroma.2016.04.040>.

807 [68] J. Liu, A.A. Makarov, R. Bennett, I.A. Haidar Ahmad, J. DaSilva, M. Reibarkh, I. Mangion, B.F.
808 Mann, E.L. Regalado, Chaotropic Effects in Sub/Supercritical Fluid Chromatography via
809 Ammonium Hydroxide in Water-Rich Modifiers: Enabling Separation of Peptides and Highly
810 Polar Pharmaceuticals at the Preparative Scale, *Anal. Chem.* 91 (2019) 13907–13915.
811 <https://doi.org/10.1021/acs.analchem.9b03408>.

812 [69] M.C. Beilke, M.J. Beres, S.V. Olesik, Gradient enhanced-fluidity liquid hydrophilic interaction
813 chromatography of ribonucleic acid nucleosides and nucleotides: A “green” technique, *J.*
814 *Chromatogr. A.* 1436 (2016) 84–90. <https://doi.org/10.1016/j.chroma.2016.01.060>.

815 [70] D. Pyo, S. Lee, Addition of water in carbon dioxide mobile phase for supercritical fluid
816 chromatography, *Bull. Korean Chem. Soc.* 20 (1999) 405.

817 [71] F.O. Geiser, S.G. Yocklovich, S.M. Lurcott, J.W. Guthrie, E.J. Levy, Water as a stationary phase
818 modifier in packed-column supercritical fluid chromatography : I. Separation of free fatty acids,
819 *J. Chromatogr. A.* 459 (1988) 173–181. [https://doi.org/10.1016/S0021-9673\(01\)82025-2](https://doi.org/10.1016/S0021-9673(01)82025-2).

820 [72] M. Enmark, E. Glenne, M. Leško, A. Langborg Weinmann, T. Leek, K. Kaczmarek, M. Klarqvist, J.
821 Samuelsson, T. Fornstedt, Investigation of robustness for supercritical fluid chromatography
822 separation of peptides: Isocratic vs gradient mode, *J. Chromatogr. A.* 1568 (2018) 177–187.
823 <https://doi.org/10.1016/j.chroma.2018.07.029>.

824 [73] D. Wen, S.V. Olesik, Chromatography of substituted benzoic acids with methanol–water–
825 carbon dioxide mixtures, *J. Chromatogr. A.* 931 (2001) 41–52. [https://doi.org/10.1016/S0021-](https://doi.org/10.1016/S0021-9673(01)01209-2)
826 [9673\(01\)01209-2](https://doi.org/10.1016/S0021-9673(01)01209-2).

827 [74] S.T. Lee, S.V. Olesik, Normal-phase high-performance liquid chromatography using enhanced-
828 fluidity liquid mobile phases, *J. Chromatogr. A.* 707 (1995) 217–224.
829 [https://doi.org/10.1016/0021-9673\(95\)00250-Q](https://doi.org/10.1016/0021-9673(95)00250-Q).

830 [75] H. Yuan, I. Souvignet, S.V. Olesik, High-Performance Size Exclusion Chromatography Using
831 Enhanced-Fluidity Liquid Mobile Phases, *J. Chromatogr. Sci.* 35 (1997) 409–416.
832 <https://doi.org/10.1093/chromsci/35.9.409>.

833 [76] Y. Cui, S.V. Olesik, Reversed-phase high-performance liquid chromatography using enhanced-
834 fluidity mobile phases, *J. Chromatogr. A.* 691 (1995) 151–162. [https://doi.org/10.1016/0021-](https://doi.org/10.1016/0021-9673(94)00878-D)
835 [9673\(94\)00878-D](https://doi.org/10.1016/0021-9673(94)00878-D).

836 [77] G.S. Philibert, S.V. Olesik, Characterization of enhanced-fluidity liquid hydrophilic interaction
837 chromatography for the separation of nucleosides and nucleotides, *J. Chromatogr. A.* 1218
838 (2011) 8222–8230. <https://doi.org/10.1016/j.chroma.2011.09.037>.

839 [78] S.T. Lee, T.S. Reighard, S.V. Olesik, Phase diagram studies of methanol-H₂O-CO₂ and
840 acetonitrile-H₂O-CO₂ mixtures, *Fluid Phase Equilibria.* 122 (1996) 223–241.
841 [https://doi.org/10.1016/0378-3812\(96\)03038-5](https://doi.org/10.1016/0378-3812(96)03038-5).

842 [79] S.V. Olesik, Physicochemical properties of enhanced-fluidity liquid solvents, *J. Chromatogr. A.*
843 1037 (2004) 405–410. <https://doi.org/10.1016/j.chroma.2004.04.001>.

844 [80] J. Li, K.B. Thurbide, A comparison of methanol and isopropanol in alcohol/water/CO₂ mobile
845 phases for packed column supercritical fluid chromatography, *Can. J. Anal. Sci. Spectrosc.* 53
846 (2008) 59–65.

847 [81] L.T. Taylor, Packed column supercritical fluid chromatography of hydrophilic analytes via water-
848 rich modifiers, *J. Chromatogr. A.* 1250 (2012) 196–204.
849 <https://doi.org/10.1016/j.chroma.2012.02.037>.

850 [82] A. Grand-Guillaume Perrenoud, D. Guillarme, J. Boccard, J.-L. Veuthey, D. Barron, S. Moco,
851 Ultra-high performance supercritical fluid chromatography coupled with quadrupole-time-of-
852 flight mass spectrometry as a performing tool for bioactive analysis, *J. Chromatogr. A.* 1450
853 (2016) 101–111. <https://doi.org/10.1016/j.chroma.2016.04.053>.

854 [83] R. Bennett, M. Biba, J. Liu, I.A. Haidar Ahmad, M.B. Hicks, E.L. Regalado, Enhanced fluidity liquid
855 chromatography: A guide to scaling up from analytical to preparative separations, *J.*
856 *Chromatogr. A.* 1595 (2019) 190–198. <https://doi.org/10.1016/j.chroma.2019.02.017>.

- 857 [84] K. Taguchi, E. Fukusaki, T. Bamba, Simultaneous analysis for water- and fat-soluble vitamins by
858 a novel single chromatography technique unifying supercritical fluid chromatography and liquid
859 chromatography, *J. Chromatogr. A.* 1362 (2014) 270–277.
860 <https://doi.org/10.1016/j.chroma.2014.08.003>.
- 861 [85] D. Tong, K.D. Bartle, A.A. Clifford, R.E. Robinson, Unified chromatograph for gas
862 chromatography, supercritical fluid chromatography and micro-liquid chromatography, *The*
863 *Analyst.* 120 (1995) 2461. <https://doi.org/10.1039/an9952002461>.
- 864 [86] D. Ishii, T. Takeuchi, Unified Fluid Chromatography, *J. Chromatogr. Sci.* 27 (1989) 71–74.
865 <https://doi.org/10.1093/chromsci/27.2.71>.
- 866 [87] G.L. Losacco, O. Ismail, J. Pezzatti, V. González-Ruiz, J. Boccard, S. Rudaz, J.-L. Veuthey, D.
867 Guillaume, Applicability of Supercritical fluid chromatography–Mass spectrometry to
868 metabolomics. II–Assessment of a comprehensive library of metabolites and evaluation of
869 biological matrices, *J. Chromatogr. A.* 1620 (2020) 461021.
870 <https://doi.org/10.1016/j.chroma.2020.461021>.
- 871 [88] C.A. Eckert, C.L. Liotta, D. Bush, J.S. Brown, J.P. Hallett, Sustainable Reactions in Tunable
872 Solvents, *J. Phys. Chem. B.* 108 (2004) 18108–18118. <https://doi.org/10.1021/jp0487612>.
- 873 [89] H. Fischer, O. Gyllenhaal, J. Vessman, K. Albert, Reaction Monitoring of Aliphatic Amines in
874 Supercritical Carbon Dioxide by Proton Nuclear Magnetic Resonance Spectroscopy and
875 Implications for Supercritical Fluid Chromatography, *Anal. Chem.* 75 (2003) 622–626.
876 <https://doi.org/10.1021/ac020527p>.
- 877 [90] C. West, E. Lesellier, Characterization of stationary phases in subcritical fluid chromatography
878 by the solvation parameter model: I. Alkylsiloxane-bonded stationary phases, *J. Chromatogr. A.*
879 1110 (2006) 181–190. <https://doi.org/10.1016/j.chroma.2006.01.125>.
- 880 [91] C. West, E. Lesellier, Characterisation of stationary phases in subcritical fluid chromatography
881 with the solvation parameter model: IV. Aromatic stationary phases, *J. Chromatogr. A.* 1115
882 (2006) 233–245. <https://doi.org/10.1016/j.chroma.2006.02.050>.
- 883 [92] C. West, E. Lesellier, Characterisation of stationary phases in subcritical fluid chromatography
884 with the solvation parameter model: III. Polar stationary phases, *J. Chromatogr. A.* 1110 (2006)
885 200–213. <https://doi.org/10.1016/j.chroma.2006.01.109>.
- 886 [93] C. West, S. Khater, E. Lesellier, Characterization and use of hydrophilic interaction liquid
887 chromatography type stationary phases in supercritical fluid chromatography, *J. Chromatogr. A.*
888 1250 (2012) 182–195. <https://doi.org/10.1016/j.chroma.2012.05.008>.
- 889 [94] E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West, Mixed-Mode Chromatography—A
890 Review, *LC-GC Eur.* 30 (2017) 22–33.
- 891 [95] C. West, E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, An improved classification of stationary
892 phases for ultra-high performance supercritical fluid chromatography, *J. Chromatogr. A.* 1440
893 (2016) 212–228. <https://doi.org/10.1016/j.chroma.2016.02.052>.
- 894 [96] E. Lipka, A.-E. Dascalu, Y. Messara, E. Tsutsqiridze, T. Farkas, B. Chankvetadze, Separation of
895 enantiomers of native amino acids with polysaccharide-based chiral columns in supercritical
896 fluid chromatography, *J. Chromatogr. A.* 1585 (2019) 207–212.
897 <https://doi.org/10.1016/j.chroma.2018.11.049>.
- 898 [97] L. Sánchez-Hernández, J.L. Bernal, M.J. del Nozal, L. Toribio, Chiral analysis of aromatic amino
899 acids in food supplements using subcritical fluid chromatography and Chirobiotic T2 column, *J.*
900 *Supercrit. Fluids.* 107 (2016) 519–525. <https://doi.org/10.1016/j.supflu.2015.06.027>.
- 901 [98] J. (Jenny) Qian Cutrone, X. (Stella) Huang, E.S. Kozlowski, Y. Bao, Y. Wang, C.S. Poronsky, D.M.
902 Drexler, A.A. Tymiak, Tiered analytics for purity assessment of macrocyclic peptides in drug
903 discovery: Analytical consideration and method development, *J. Pharm. Biomed. Anal.* 138
904 (2017) 166–174. <https://doi.org/10.1016/j.jpba.2017.01.056>.
- 905 [99] A. Cazenave-Gassiot, R. Boughtflower, J. Caldwell, L. Hitzel, C. Holyoak, S. Lane, P. Oakley, F.
906 Pullen, S. Richardson, G.J. Langley, Effect of increasing concentration of ammonium acetate as
907 an additive in supercritical fluid chromatography using CO₂–methanol mobile phase, *J.*
908 *Chromatogr. A.* 1216 (2009) 6441–6450. <https://doi.org/10.1016/j.chroma.2009.07.022>.

- 909 [100] J.N. Fairchild, D.W. Brousmiche, J.F. Hill, M.F. Morris, C.A. Boissel, K.D. Wyndham,
910 Chromatographic Evidence of Silyl Ether Formation (SEF) in Supercritical Fluid Chromatography,
911 Anal. Chem. 87 (2015) 1735–1742. <https://doi.org/10.1021/ac5035709>.
- 912 [101] J. Zhang, K.B. Thurbide, Direct analysis of gramicidin double helices using packed column
913 supercritical fluid chromatography, J. Chromatogr. A. 1101 (2006) 286–292.
914 <https://doi.org/10.1016/j.chroma.2005.10.008>.
- 915 [102] C. West, S. Khater, M. Khalikova, E. Lesellier, A classification of columns for SFC use, The
916 Column. 10 (2014) 2–8.
- 917 [103] V.N.R. Ganipisetty, J. D, G. dev G, R. Bandari, M. P, D. D, V.N. R, Development of supercritical
918 fluid (carbon dioxide) based ultra performance convergence chromatographic stability
919 indicating assay method for the determination of clofarabine in injection, Anal. Methods. 5
920 (2013) 7008–7013. <https://doi.org/10.1039/C3AY41561A>.
- 921 [104] D. Guillarme, V. Desfontaine, S. Heinisch, J.-L. Veuthey, What are the current solutions for
922 interfacing supercritical fluid chromatography and mass spectrometry?, J. Chromatogr. B. 1083
923 (2018) 160–170. <https://doi.org/10.1016/j.jchromb.2018.03.010>.
- 924 [105] A. Tarafder, Designs and methods for interfacing SFC with MS, J. Chromatogr. B. 1091 (2018) 1–
925 13. <https://doi.org/10.1016/j.jchromb.2018.05.003>.
- 926 [106] A. Grand-Guillaume Perrenoud, J.-L. Veuthey, D. Guillarme, Coupling state-of-the-art
927 supercritical fluid chromatography and mass spectrometry: From hyphenation interface
928 optimization to high-sensitivity analysis of pharmaceutical compounds, J. Chromatogr. A. 1339
929 (2014) 174–184. <https://doi.org/10.1016/j.chroma.2014.03.006>.
- 930 [107] L. Akbal, G. Hopfgartner, Effects of liquid post-column addition in electrospray ionization
931 performance in supercritical fluid chromatography–mass spectrometry, J. Chromatogr. A. 1517
932 (2017) 176–184. <https://doi.org/10.1016/j.chroma.2017.08.044>.
- 933 [108] V. Shulaev, G. Isaac, Supercritical fluid chromatography coupled to mass spectrometry – A
934 metabolomics perspective, J. Chromatogr. B. 1092 (2018) 499–505.
935 <https://doi.org/10.1016/j.jchromb.2018.06.021>.
- 936 [109] A. Grand-Guillaume Perrenoud, D. Guillarme, J. Veuthey, D. Barron, S. Moco, Combining the full
937 potential of UHPSFC-QToF/MS and UHPLC-QToF/MS to improve the workflow efficiency of both
938 plant metabolic profiling and natural bioactive discovery, Planta Med. 81 (2016) S1–S381.
939 <https://doi.org/10.1055/s-0036-1596250>.
- 940 [110] M. D’Hondt, N. Bracke, L. Taevernier, B. Gevaert, F. Verbeke, E. Wynendaele, B. De Spiegeleer,
941 Related impurities in peptide medicines, J. Pharm. Biomed. Anal. 101 (2014) 2–30.
942 <https://doi.org/10.1016/j.jpba.2014.06.012>.
- 943 [111] G.F. Pirrone, R.M. Mathew, A.A. Makarov, F. Bernardoni, A. Klapars, R. Hartman, J. Limanto, E.L.
944 Regalado, Supercritical fluid chromatography-photodiode array detection-electrospray
945 ionization mass spectrometry as a framework for impurity fate mapping in the development
946 and manufacture of drug substances, J. Chromatogr. B. 1080 (2018) 42–49.
947 <https://doi.org/10.1016/j.jchromb.2018.02.006>.

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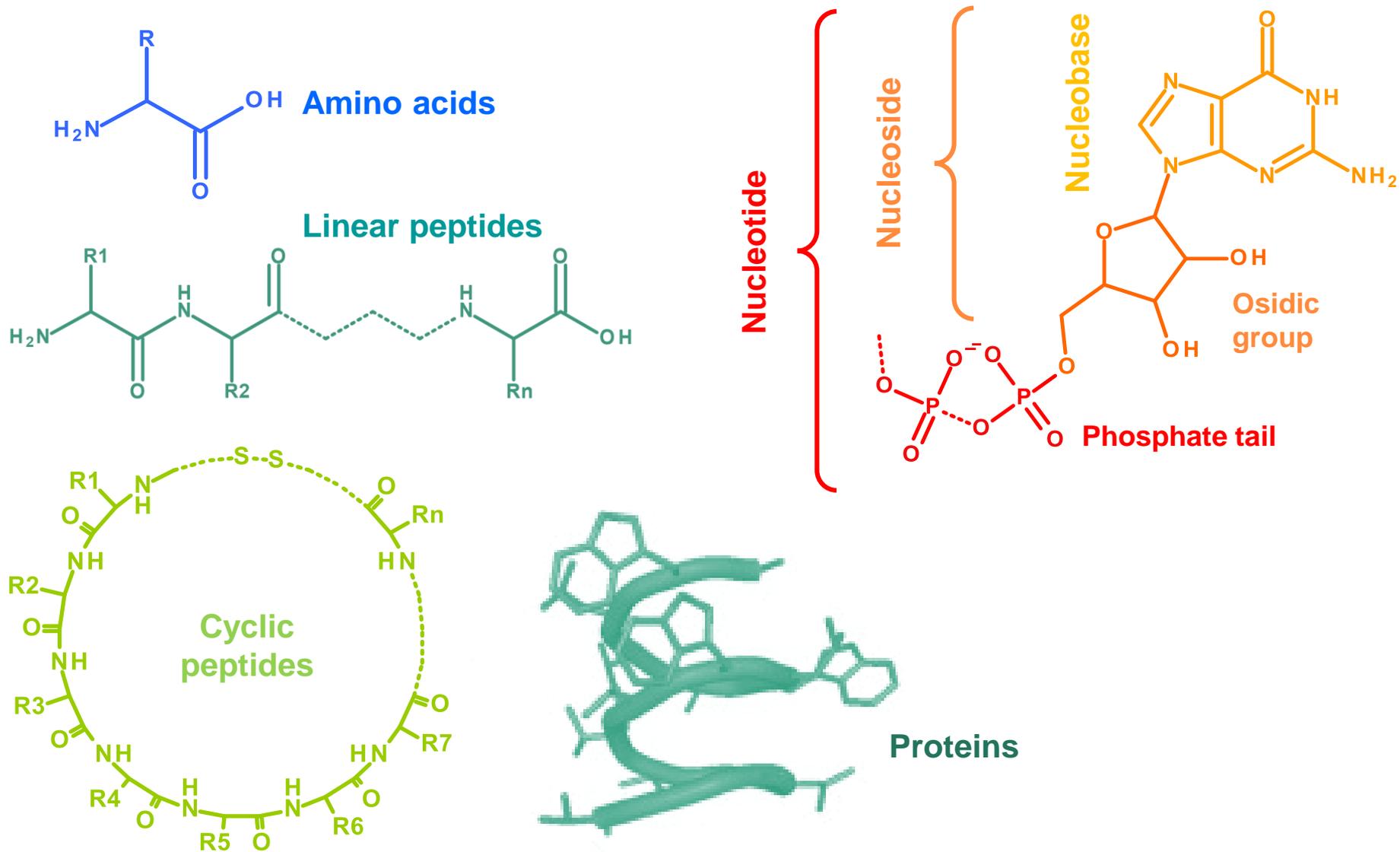


Figure 1. Generic and representative structures of the target molecules reported in the literature reviewed in this paper.

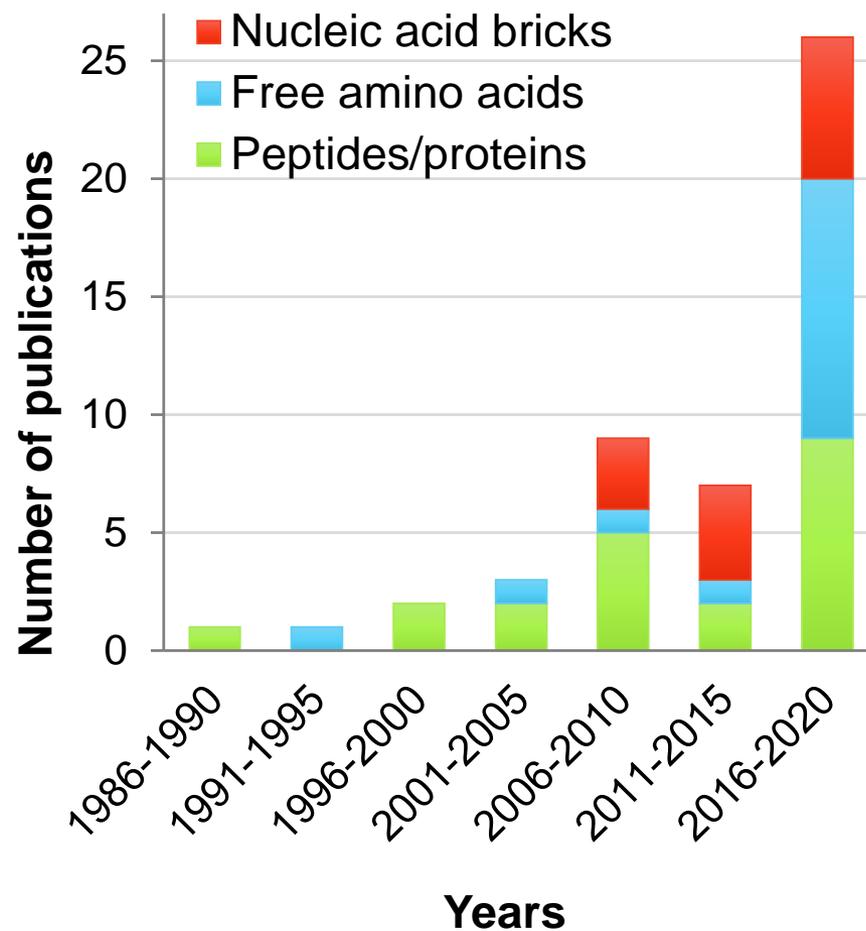


Figure 2. Published papers related to SFC or EFLC analysis of free amino acids, peptides, proteins and nucleic acid bricks (nucleobases, nucleosides and nucleotides).

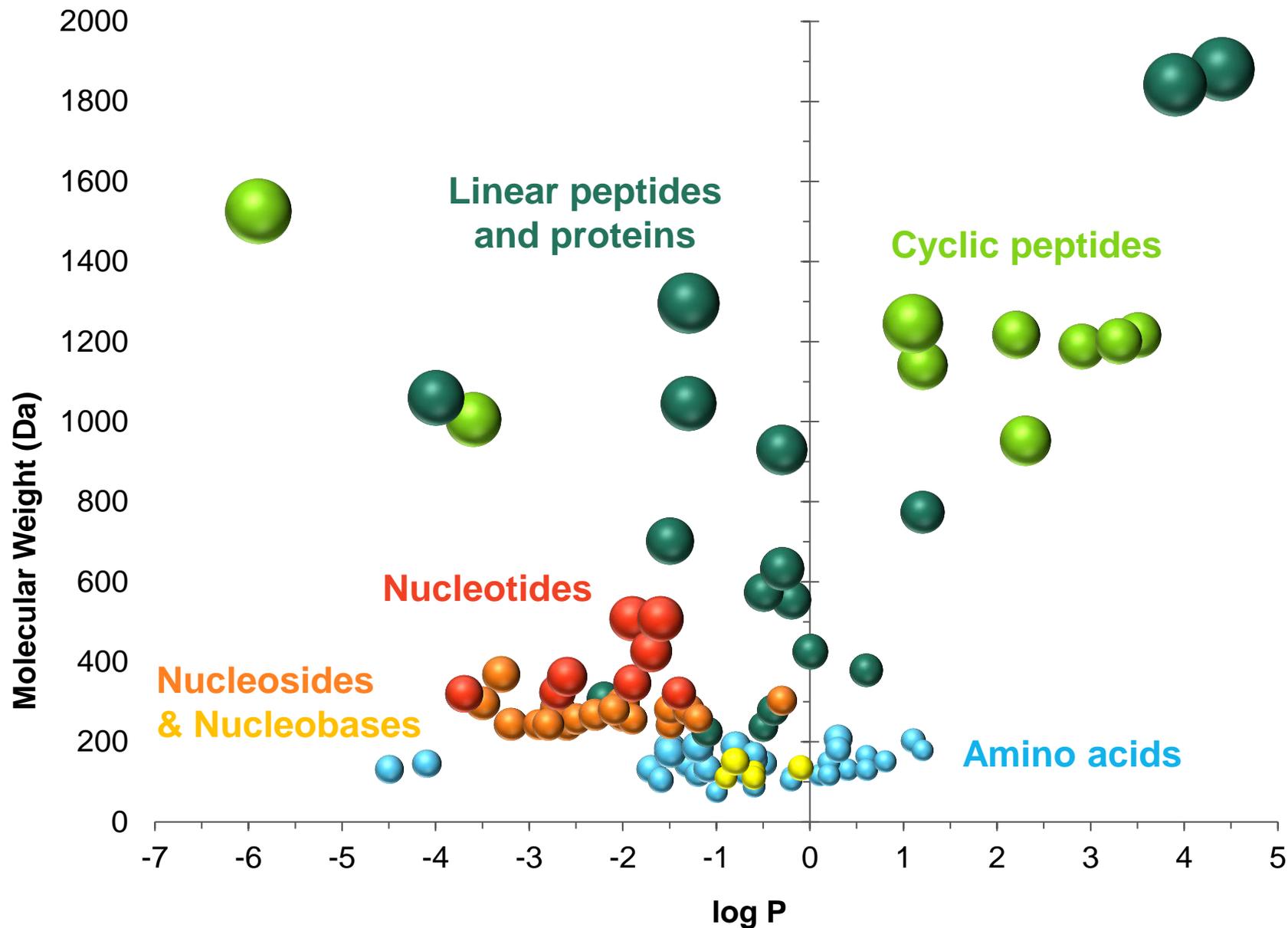


Figure 3. Molecular weight and polarity features related to biomolecules previously analyzed with SFC or EFLC. The bubble size reflects the topological polar surface area (TPSA). The three descriptors were computed with MolDesc freeware (<http://moldesc.icoa.fr/>).

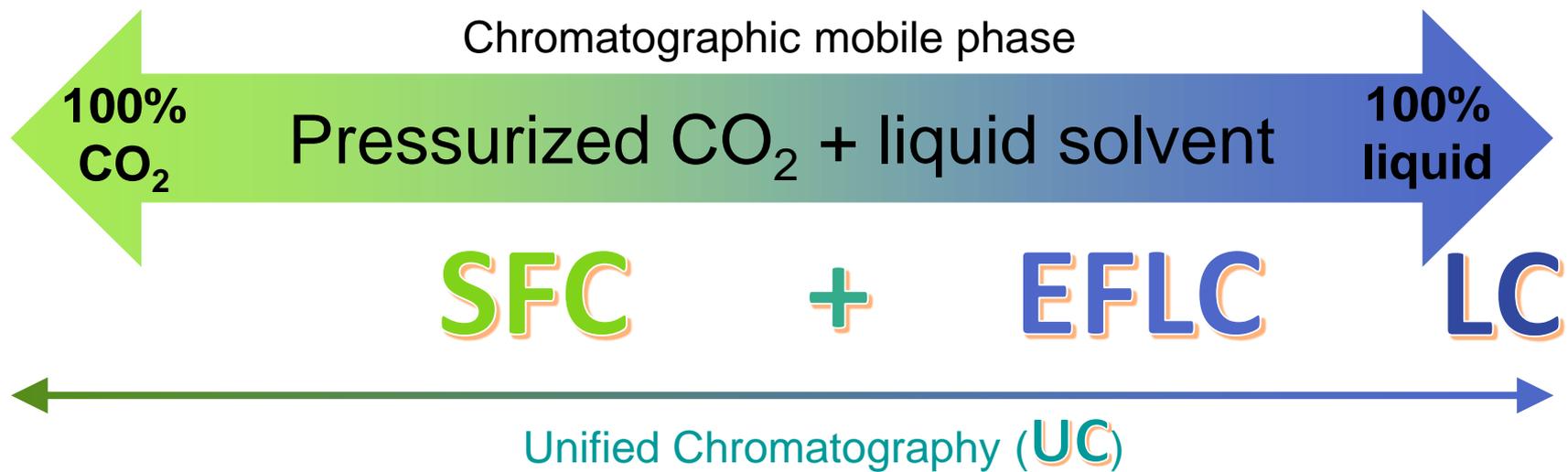


Figure 4. Usual operating domains of SFC, EFLC and LC. Unified chromatography (UC) operates with large elution gradients covering a wide range of mobile phase compositions.

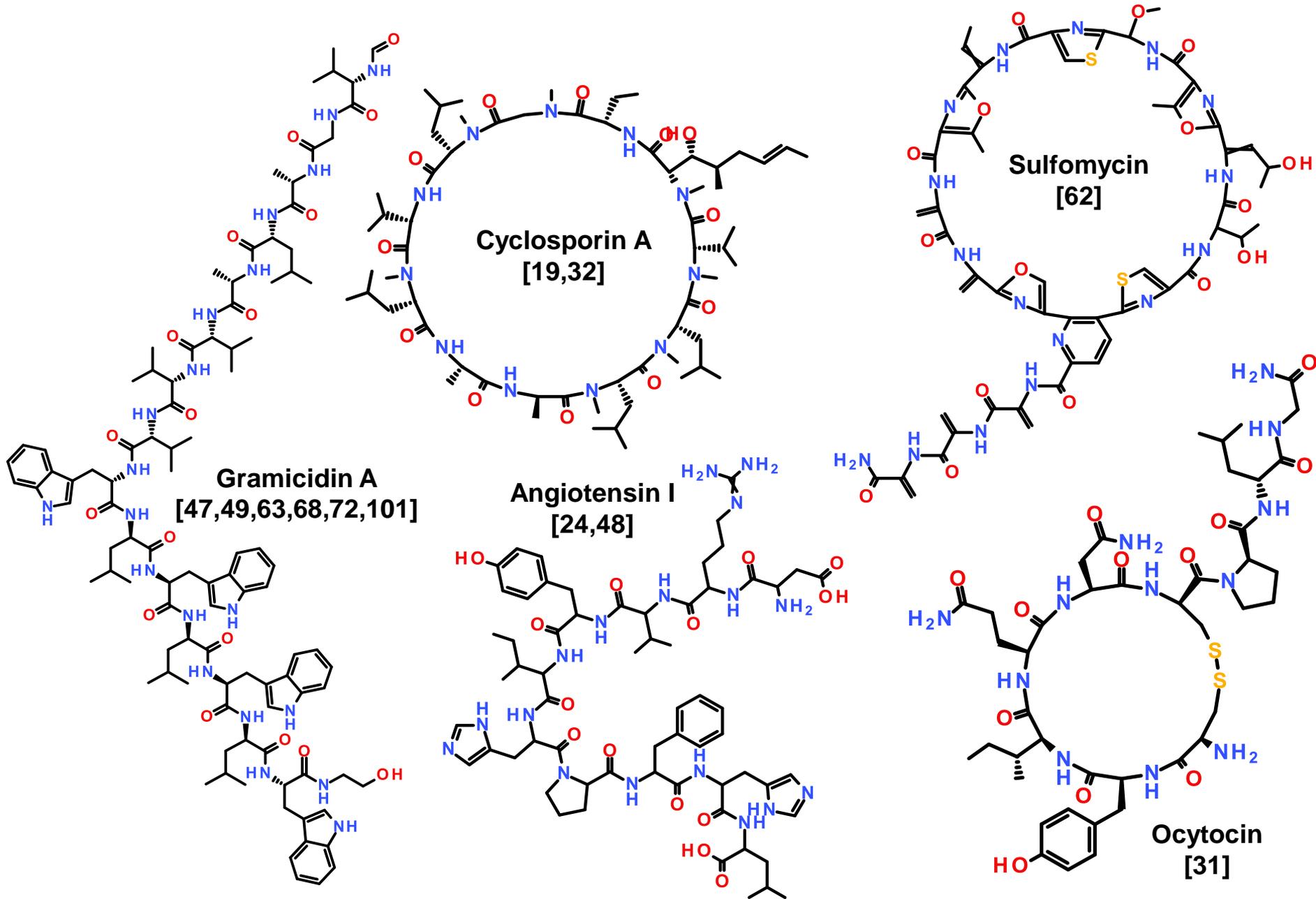
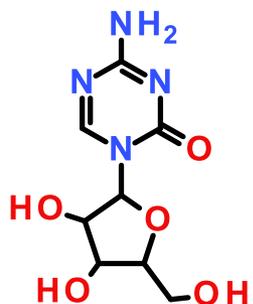
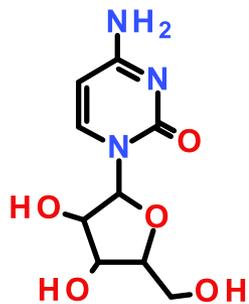


Figure 5. Sample peptides analyzed with SFC and EFLC

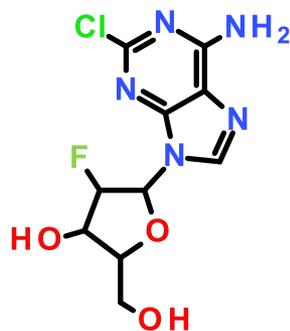
Azacytidin [57]



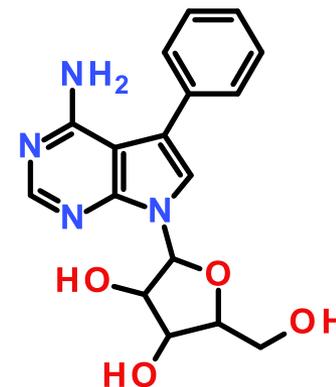
Cytarabin [64]



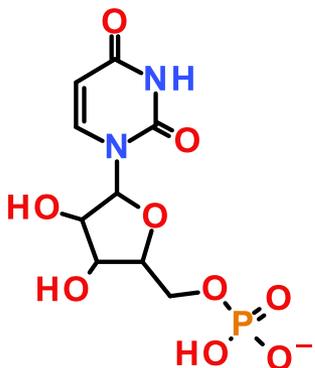
Clofarabin [103]



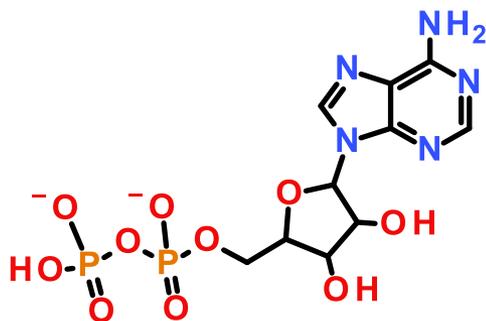
7-phenyl-7-deazaadenosine [30]



Uridin monosphat [77]



Adenosin diphosphat [69]



Inosin triphosphat [66]

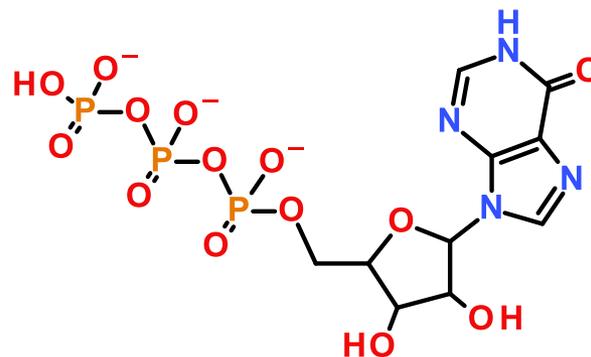


Figure 6. Sample nucleosides and nucleotides previously analyzed with SFC and EFLC

Table 1. Published SFC/EFLC methods to analyze free amino acids

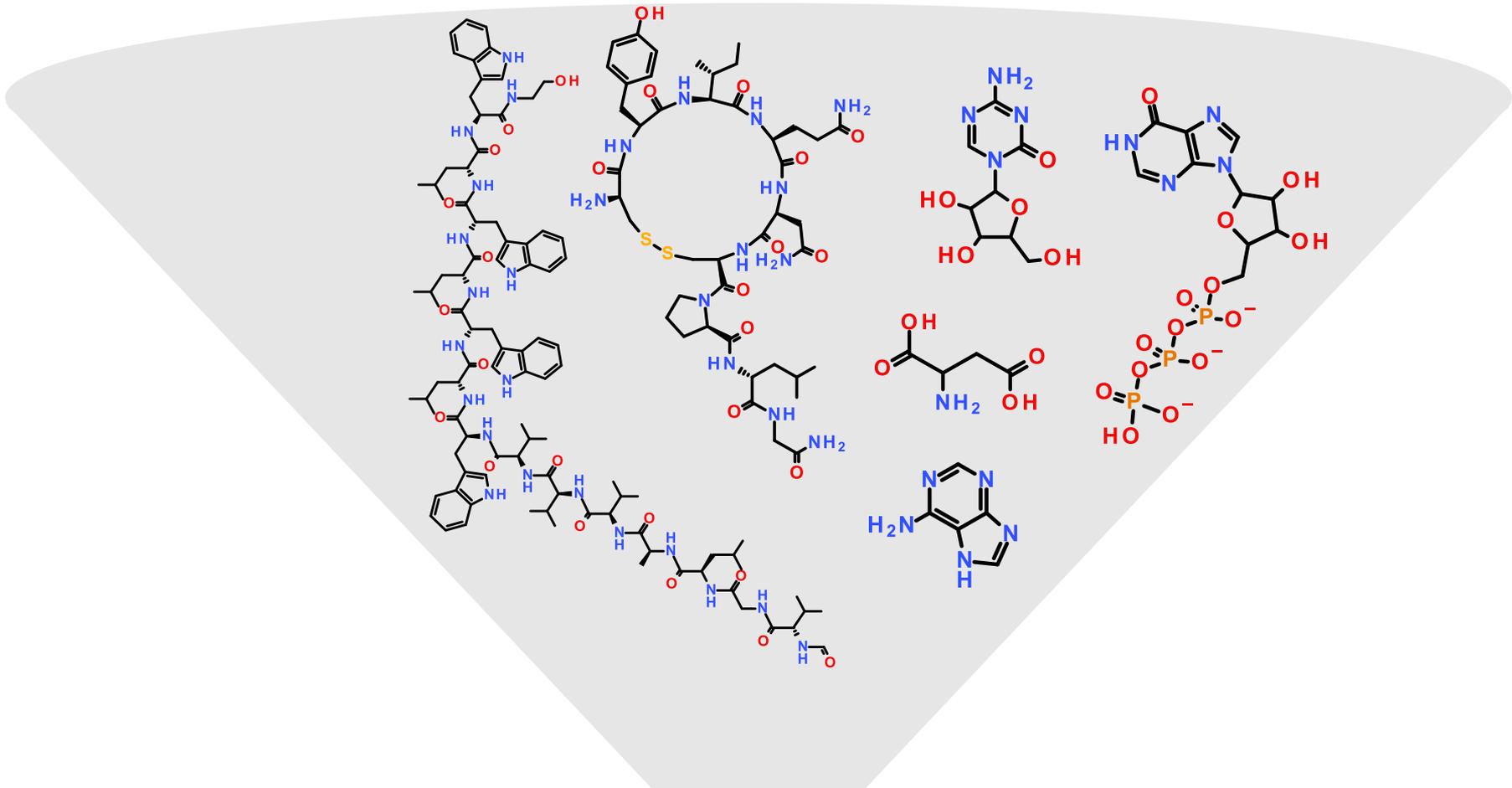
Reference	Year	Samples	Stationary phase nature	Mobile phase co-solvent	Detection
[40]	1992	Standards	Diol	Methanol with water , triethylamine and pyridine	UV (225 nm), ELSD
[60]	2005	Standards	Chiral polysaccharides	Ethanol with 0.1% ethanesulfonic acid	UV
[80]	2008	Standards	Polystyrene-divinylbenzene	Methanol-isopropanol-water	UV
[33]	2015	Standards	Polysulfoethyl	Acetonitrile or methanol, with 20% buffer (15 mM ammonium formate / formic acid, pH 3)	ELSD
[67]	2016	Standards and human urine	Diol	Methanol with 20 mM ammonium formate	DAD and ESI(+/-)-MS/MS
[82]	2016	Standards & plant extracts	Diol, C18, 2-ethylpyridine	Methanol with 2% water and 10 mM ammonium formate	ESI(+/-)-MS/MS
[97]	2016	Standards and food supplements	Diol and chiral macrocyclic glycopeptide	Methanol with 10% water	UV (200 & 220 nm)
[34]	2017	Standards and human serum	Diol	Methanol with 1% water and ammonium formate	ESI(+)-MS/MS & APCI(+)-MS/MS
[66]	2018	Standards	Silica and sulfobetaine	Methanol with 5% water, 50 mM ammonium formate and 1 mM ammonium fluoride	ESI(+/-)-MS/MS
[54]	2019	Standard and tea leaves	Amide	Methanol with 5% water, 5 mM ammonium acetate and 0.4% trifluoroacetic acid	ESI(+)-MS
[53]	2019	Standards and plant extract	Chiral zwitterionic <i>Cinchona</i> alkaloid	Methanol with 5% water and 50 mM ammonium acetate	ESI(+)-MS
[61]	2019	Standards and food supplements	Chiral zwitterionic <i>Cinchona</i> alkaloid	Methanol with 2% water and 20 mM methanesulfonic acid	ESI(+)-MS
[55]	2020	Standards	Chiral crown-ether	Ethanol with 5% water and 0.8% trifluoroacetic acid	ESI-MS
[45]	2020	Standards and human urine	Diol	Methanol with 10 mM ammonium hydroxide	ESI(+/-)-MS/MS

Table 2. Published SFC/EFLC methods to analyze peptides and proteins
TFA is trifluoroacetic acid; TFE is trifluoroethanol

Reference	Year	Biomolecule type	Example biomolecules with molecular weight	Samples	Stationary phase nature	Mobile phase co-solvent	Detection
[19]	1988	Cyclic peptide	Cyclosporin A (1202 Da)	Standards	C18	Methanol	CI(+)-MS
[31]	1999	Linear & cyclic peptides	Leucine enkephalin (555 Da); Ocytocin (1006 Da); Bradykinin (1060 Da)	Standards	Divinylbenzene	Ethanol with 50 mM heptadecanesulfonic acid	UV (210 & 254 nm)
[62]	2000	Cyclic depsipeptides	Sulfomycin (1244 Da); Promothiocin (953 Da)	Standards and animal feed	Diol	Methanol with 0.2% isopropylamine	UV (254 & 320 nm)
[63]	2004	Linear peptides and proteins	Gramicidin D mixture (\approx 1800 Da); Met-Enkephalin (573 Da); Angiotensin II (1045 Da)	Standards	Diol	Methanol with 0.5% TFE, or with 0.2% water, 10 mM ammonium acetate and 0.4% isopropylamine	ESI-MS
[47]	2005	Linear proteins	Gramicidin D mixture (\approx 1800 Da)	Standards	Polystyrene-divinylbenzene	Methanol	UV (220 & 280 nm)
[48]	2006	Linear peptides	Bradykinin (1060 Da); Sauvagine (4600 Da); Urotensin II (1389 Da)	Standards	2-Ethylpyridine	Methanol with 13 mM TFA	ESI(+)-MS
[101]	2006	Linear proteins	Gramicidin D double helices	Standards	Polystyrene-divinylbenzene	n-Pentanol	UV (220 nm)
[20]	2006	Linear peptides	Myoglobin fragments (longest fragment 3742 Da)	Myoglobin pepsin digest	Silica	Methanol-Acetonitrile-Water-Formic acid (40:40:19:1)	ESI-MS
[49]	2008	Linear peptides and proteins	Cytochrome C tryptic digest (longest fragment 965 Da); Bacteriorhodopsin (24 kDa); PS II Core proteins (up to 39 kDa)	Standards and spinach leaves	Cyano	Methanol with 0.5% TFE, or with 0.1% TFA; or methanol-chloroform-formic acid (4:4:1)	UV (220 & 280 nm), ESI(+)-MS
[50]	2010	Linear peptides	Peptides with 2 to 8 residues (1042 Da)	Standards	2-Ethylpyridine	Methanol with 0.2% TFA	UV
[51]	2011	Linear peptides	12-mer peptides (\approx 1200 Da)	Synthetic peptides	Pyridine, Diol, Amino	Methanol with 0.2% TFA, or with 0.2% isopropylamine, or with 10 mM ammonium acetate	ESI-MS
[46]	2012	Linear peptides	12-mer peptides (\approx 1200 Da)	Synthetic peptides	Silica, Pyridine, Diol, C18	Methanol with 5% water and 0.2% TFA	ELSD, ESI-MS
[32]	2016	Cyclic peptides	Cyclosporins (\approx 1200 Da)	Standards	Silica	Ethanol with water	UV (220 nm)
[24]	2017	Proteins	Ribonuclease A (13.7 kDa); Bovine serum albumin (66.5 kDa); Transferrin (80.3 kDa)	Standards	Amide	Methanol-water (80:20) with 0.1% TFA	UV (215, 254 & 270 nm), ESI-MS/MS
[96]	2017	Cyclic peptides	Macrocyclic peptides	Synthetic peptides	2-Ethylpyridine	Methanol with 0.1% diethylamine	UV (220 nm)
[72]	2018	Linear proteins	Gramicidin D mixture (\approx 1800 Da)	Standards	Silica	Methanol with water (up to 8.7 w/w%)	UV, ESI-MS
[68]	2019	Linear and cyclic peptides, nucleosides	Gramicidin S (1141 Da); Linaclotide (1525 Da)	Standards and synthetic peptides	Pyridine	Methanol with 5% water and 0.2% ammonium hydroxide	UV, ESI-MS
[23]	2019	Proteins	Insulin (4679 Da); Ubiquitin (8565 Da); Myoglobin (17.6 kDa)	Standards	2-Picolylamine	Methanol-Acetonitrile (3:1) with 5% water and 0.2% TFA	circular dichroism UV (220 nm)
[22]	2019	Proteins	Ribonuclease A (13.7 kDa); Lysozyme (14.3 kDa)	Standards	Polybutyl, Polypentyl	Methanol-water with 1M ammonium acetate	UV (280 nm), ESI-MS/MS
[52]	2020	Linear peptides	Leucine enkephalin (555 Da); Angiotensin II (1045 Da); hydrophilic peptides up to 41-mer	Standards and synthetic peptides	Pyridine, Diol	Methanol with 0.1% TFA and 0.1% ammonium hydroxide	ESI-MS
[21]	2020	Proteins	Human insulin (5808 Da)	Standard and biosynthesized	Pentafluorophenyl	Methanol with 5% water and 0.2% TFA	UV (220 nm)

Table 3. Published SFC/EFLC methods to analyze nucleic acid bricks
DBN is 1,5-diazabicyclo [4.3.0] non-5-ene

Reference	Year	Biomolecule type	Samples	Stationary phase nature	Mobile phase co-solvent	Detection
[64]	2007	Nucleoside	Standards and mouse plasma	Silica	Methanol with 1% water and 11 mM ammonium acetate	APCI(+)-MS/MS
[59]	2010	Nucleobases	Standards	Silica	Ethanol with 20 mM ammonium formate - formic acid buffer (pH 3)	UV (254 nm)
[56]	2010	Nucleobases	Standards	Pyridine	Methanol with 5% water and 5 mM ammonium acetate	UV
[35]	2011	Nucleosides	Standards	Amide	Methanol-buffer or acetonitrile-buffer (90:10) with 20 mM sodium acetate - acetic acid (pH 4.4.)	UV (262 nm)
[77]	2011	Nucleosides and nucleotides	Standards	Amide	Methanol-buffer (90:10) with 75 mM ammonium phosphate, 5 mM DBN and 0.2 M NaCl	UV (262 nm)
[103]	2013	Nucleoside	Standards and pharmaceutical formula	2-Ethylpyridine	Methanol	UV (254 nm)
[57]	2015	Nucleoside	Standards and pharmaceutical formula	Chiral polysaccharide	Methanol with 0.25% TFA	UV
[69]	2016	Nucleosides and nucleotides	Standards	Amide	Methanol-buffer with 40 mM sodium phosphate (pH 2.65)	UV (270 nm)
[58]	2017	Nucleobases and nucleosides	Standards and plant extracts	Silica	Methanol with 5 mM ammonium acetate	ESI(+)-MS
[65]	2018	Nucleosides	Standards and digested tRNA of <i>E.coli</i>	2-Picolylamine	Methanol with 2% water and 10 mM ammonium acetate	ESI(+)-MS/MS
[111]	2018	Nucleosides	Synthetic drugs and their impurities	Chiral polysaccharide	Ethanol	ESI(+/-)-MS
[30]	2019	Nucleosides	Synthetic deazapurine nucleosides	Diol	Ethanol	UV (235 & 254 nm)



Chromatographic mobile phase

100%
CO₂

Pressurized CO₂ + liquid solvent

100%
liquid

SFC

+

EFLC