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

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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

Abstract

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

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

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

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

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

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

2. Polarity and size features of the target biomolecules

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

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

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

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

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

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

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

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

3. Mobile phase composition

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

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

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

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

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

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

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

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

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

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

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

comprising methanol, 5% water and 0.2% ammonium hydroxide was employed, with gradient ranging from 5 to 50% co-solvent. The combined use of water and NH_4OH were suggested to cause chaotropic effects favoring the elution of a variety of analytes including nucleobases, nucleosides, nucleotides and some peptides (linaclotide 1523 Da and gramicidin S 1882 Da). In a separate study from the same group [23], the authors found that some proteins like bradykinin and insulin could be recovered to their original higher-order structure after SFC purification, while others like ubiquitin, cytochrome C and apomyoglobin had permanently lost their higher-order structure. The co-solvent was a 3:1 mixture of methanol and acetonitrile, added with 5% water and 0.2% trifluoroacetic acid. The elution gradient ranged from 35 to 70% co-solvent in CO_2 , which is covering both the usual SFC and EFLC domains. Another recent paper from Govender and co-workers [21] presented the purification of biosynthesized human insulin with SFC. The co-solvent was methanol comprising 5% water and 0.2% trifluoroacetic acid, thus identical to the Schiavone et al. [23] mobile phase. The purified insulins retained their bioactivity, suggesting that the higher-order structure was indeed retained.

On another hand, Wang and Olesik also relate the elution of intact proteins [22] in EFLC mode starting from a hydrophobic interaction chromatography (HIC) method, and progressively introducing CO_2 in the 70:30 methanol-ammonium acetate buffer mobile phase. In addition to improved chromatographic efficiency, the introduction of CO_2 was shown to improve ESI-MS detection through facilitated desolvation. However, the claim was on intact molecular weight, suggesting that no permanent reaction had damaged the primary structure, but no information was given on preservation of higher-order structure.

4. Stationary phase nature, particle type and dimension

The range of stationary phases available for packed column SFC and EFLC was always large, as any stationary phase designed for liquid chromatography can also be used with CO_2 in the mobile phase. The nature of stationary phases has however greatly expanded in the recent years as not only reversed-phase [90,91] and normal-phase [92] stationary phases are possible but also the most recent stationary phases designed for HILIC mode [93] and for mixed-mode liquid chromatography [94] and several novel stationary phases especially designed for SFC use [95]. The diversity of available stationary phases is an interesting feature of SFC and EFLC because it means that different selectivities are available to achieve a given separation. The role of a stationary phase is two-fold: it must interact with the target analytes to (i) retain them and (ii) separate them. It is customary in SFC method development to start developing a method by screening several stationary

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

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

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

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

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

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

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

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

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

5. Detection issues and hyphenation to mass spectrometry

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

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

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

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

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

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

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

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

6. Applications areas

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

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

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

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

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

7. Conclusion and perspectives

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

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

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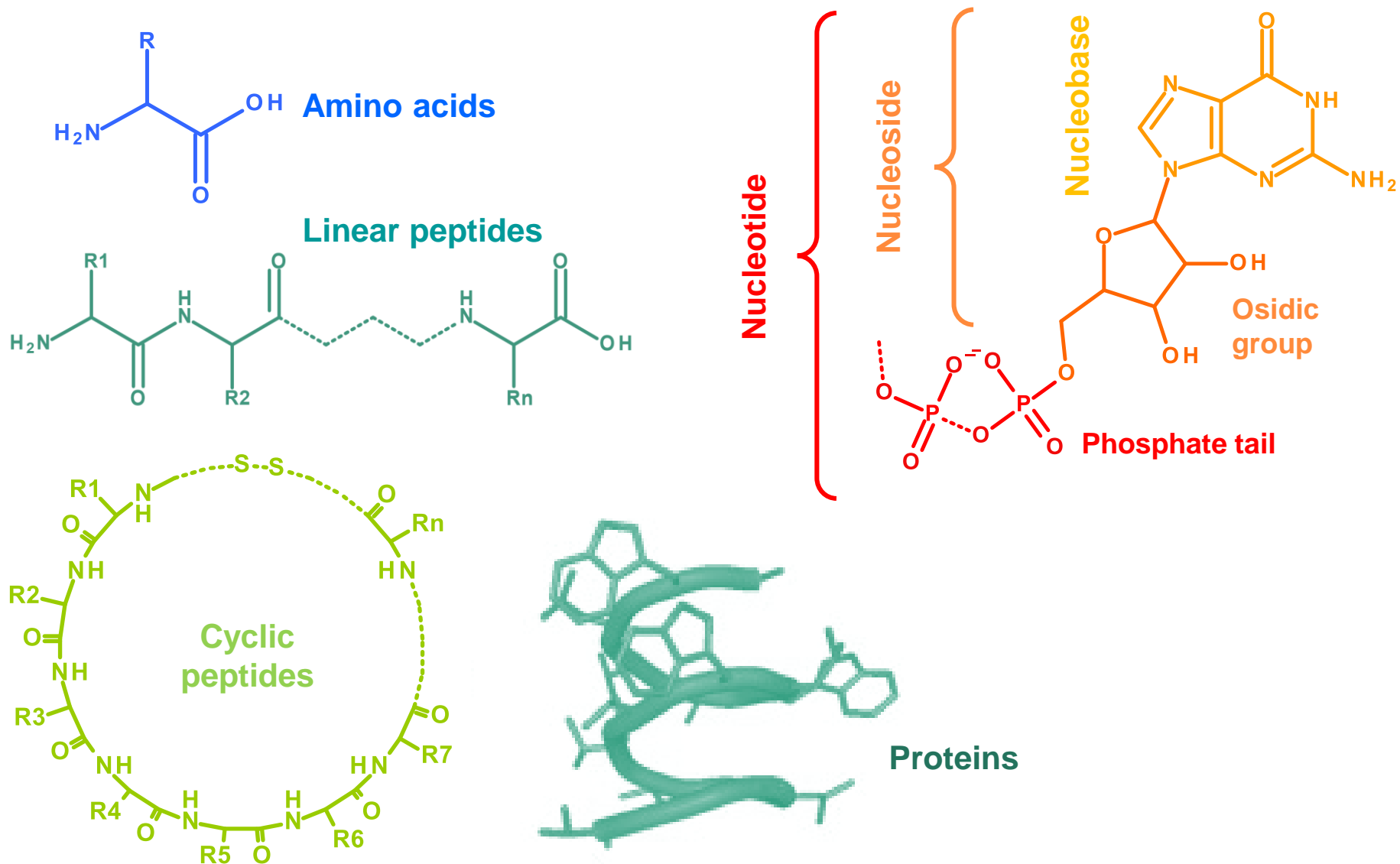


Figure 1. Generic and representative structures of the target molecules reported in the literature reviewed in this paper.

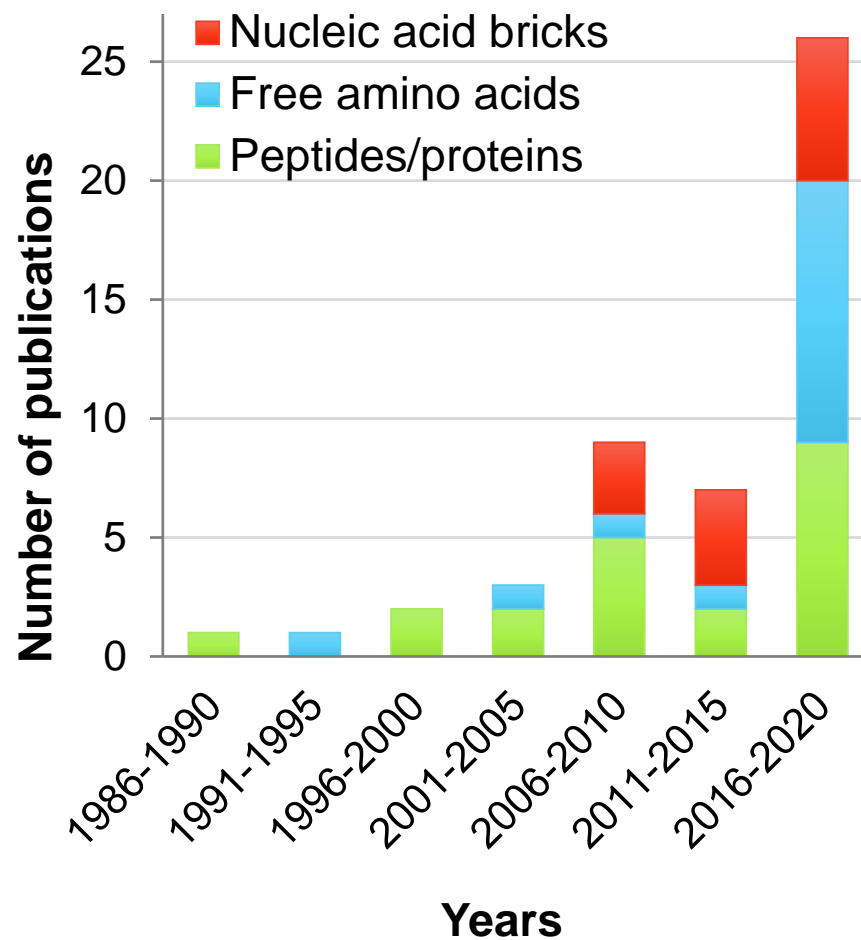


Figure 2. Published papers related to SFC or EFLC analysis of free amino acids, peptides, proteins and nucleic acid bricks (nucleobases, nucleosides and nucleotides).

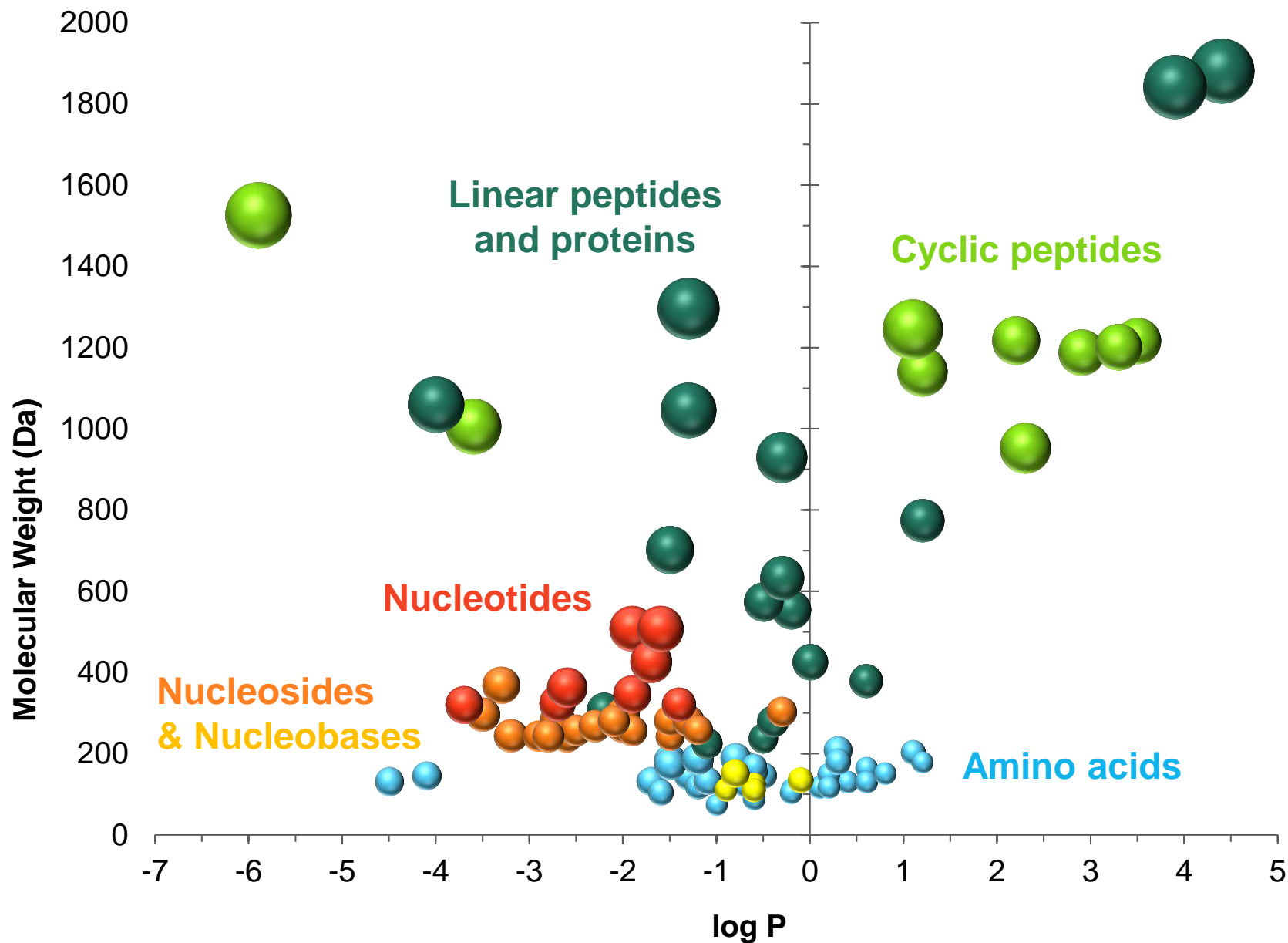


Figure 3. Molecular weight and polarity features related to biomolecules previously analyzed with SFC or EFLC. The bubble size reflects the topological polar surface area (TPSA). The three descriptors were computed with MolDesc freeware (<http://moldesc.icoa.fr/>).

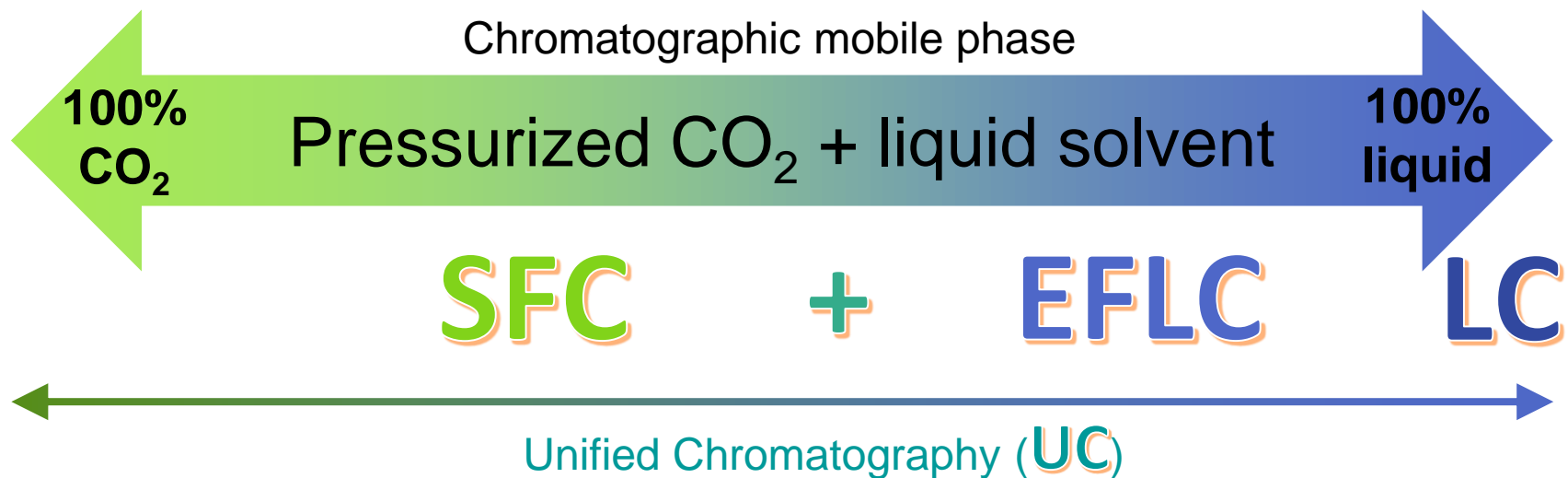


Figure 4. Usual operating domains of SFC, EFLC and LC. Unified chromatography (UC) operates with large elution gradients covering a wide range of mobile phase compositions.

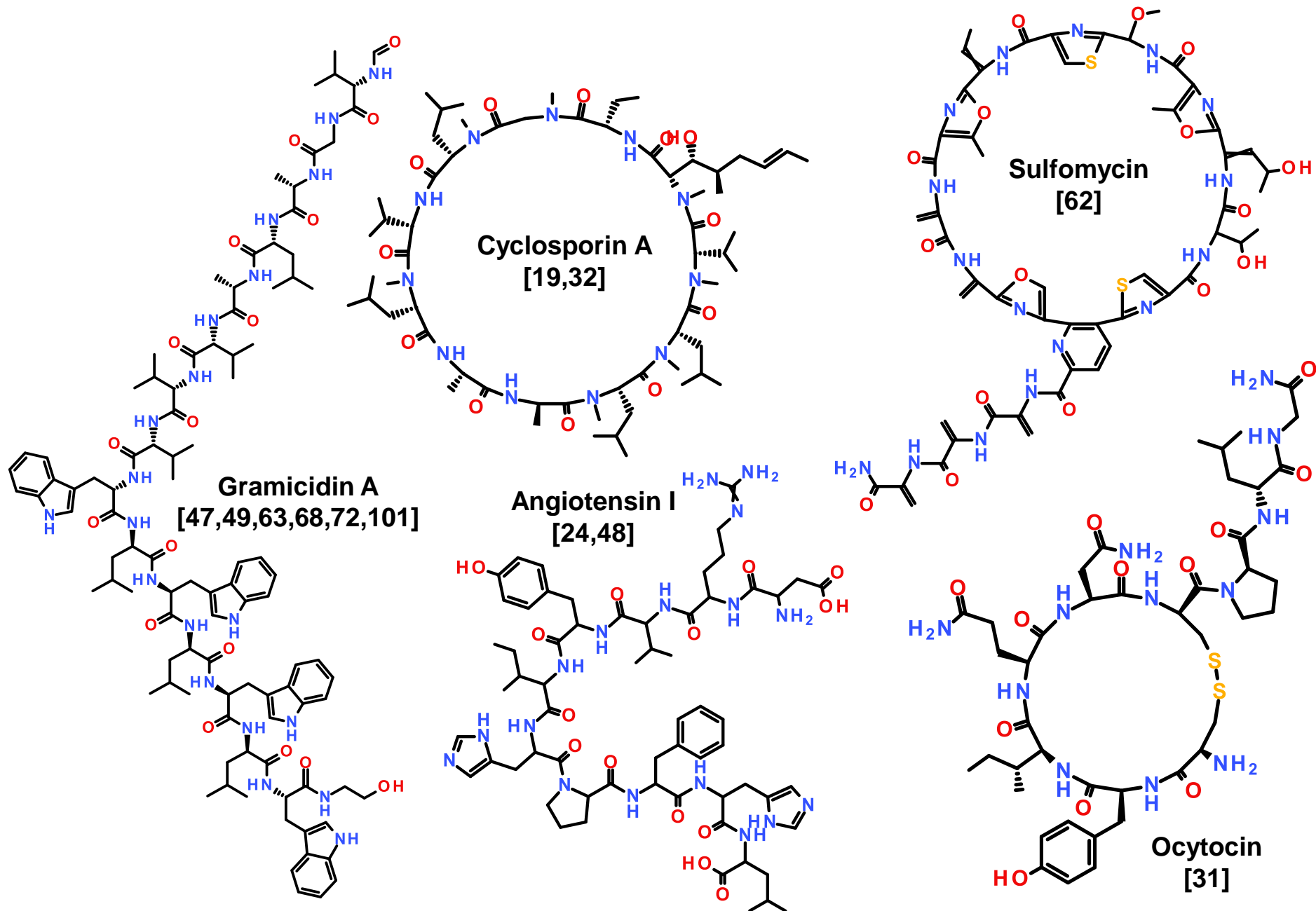
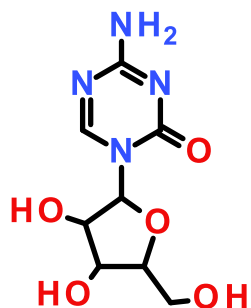
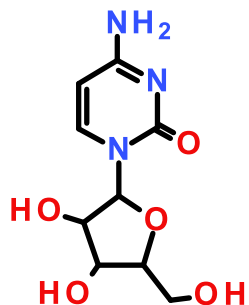


Figure 5. Sample peptides analyzed with SFC and EFLC

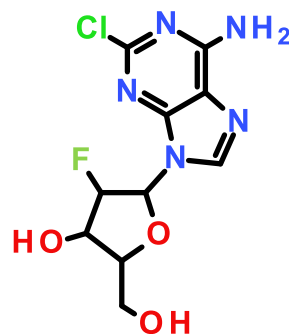
Azacytidin [57]



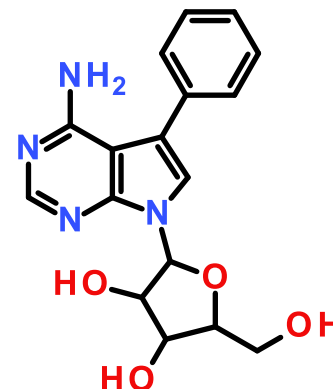
Cytarabin [64]



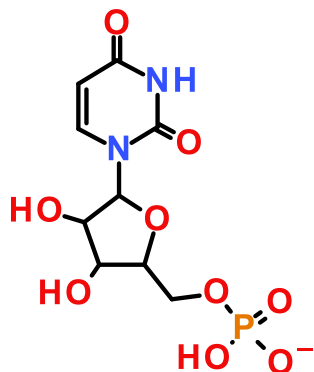
Clofarabin [103]



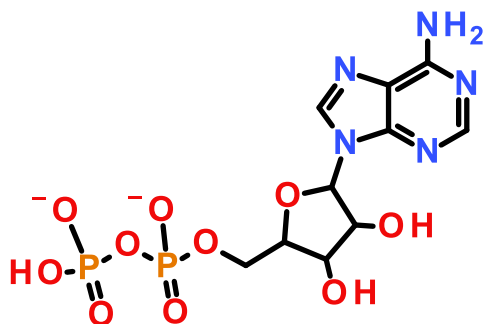
7-phenyl-7-deazaadenosine [30]



Uridin monosphate [77]



Adenosin diphosphate [69]



Inosin triphosphate [66]

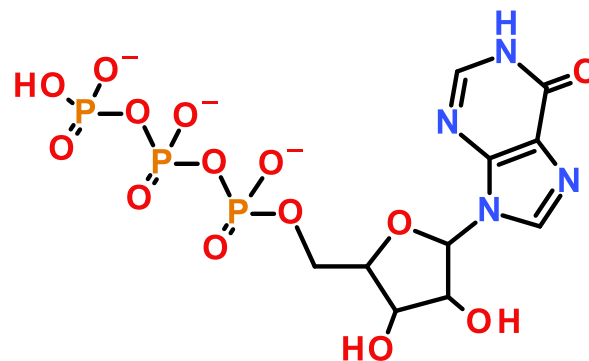


Figure 6. Sample nucleosides and nucleotides previously analyzed with SFC and EFLC

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

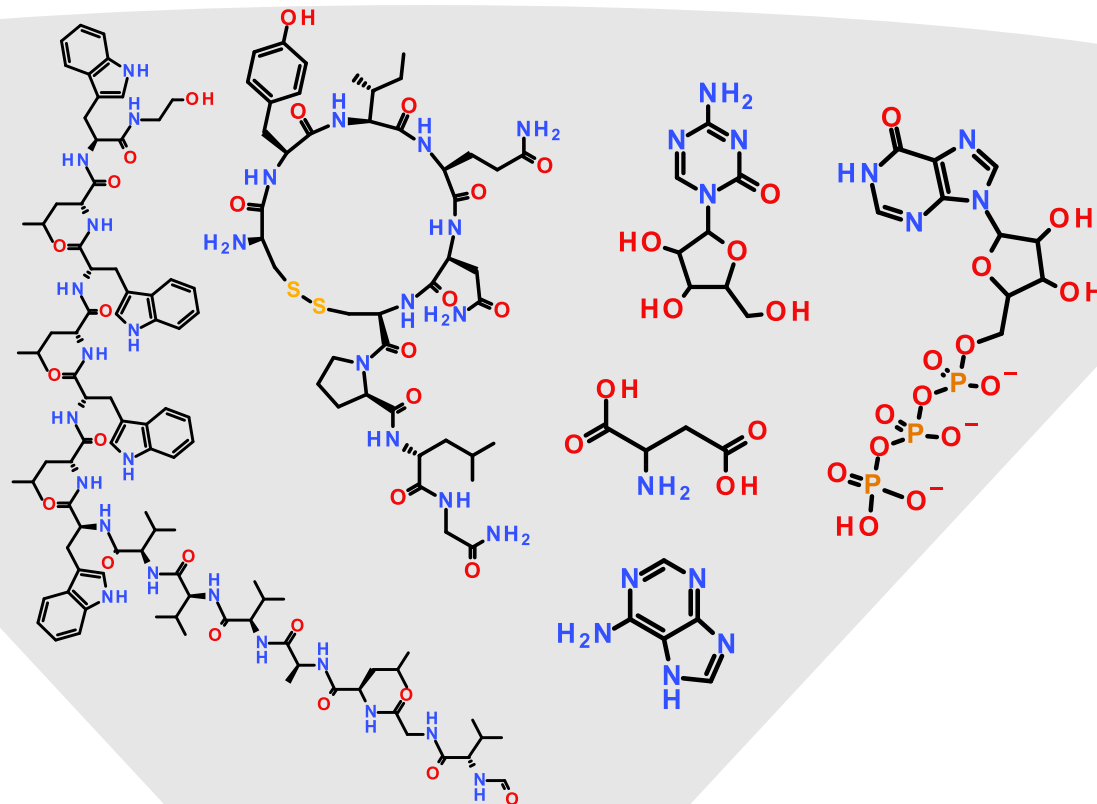
Reference	Year	Samples	Stationary phase nature	Mobile phase co-solvent	Detection
[40]	1992	Standards	Diol	Methanol with water , triethylamine and pyridine	UV (225 nm), ELSD
[60]	2005	Standards	Chiral polysaccharides	Ethanol with 0.1% ethanesulfonic acid	UV
[80]	2008	Standards	Polystyrene-divinylbenzene	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); Oxytocin (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 (≈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 (≈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 (≈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 (≈1200 Da)	Synthetic peptides	Silica, Pyridine, Diol, C18	Methanol with 5% water and 0.2% TFA	ELSD, ESI-MS
[32]	2016	Cyclic peptides	Cyclosporins (≈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 (≈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