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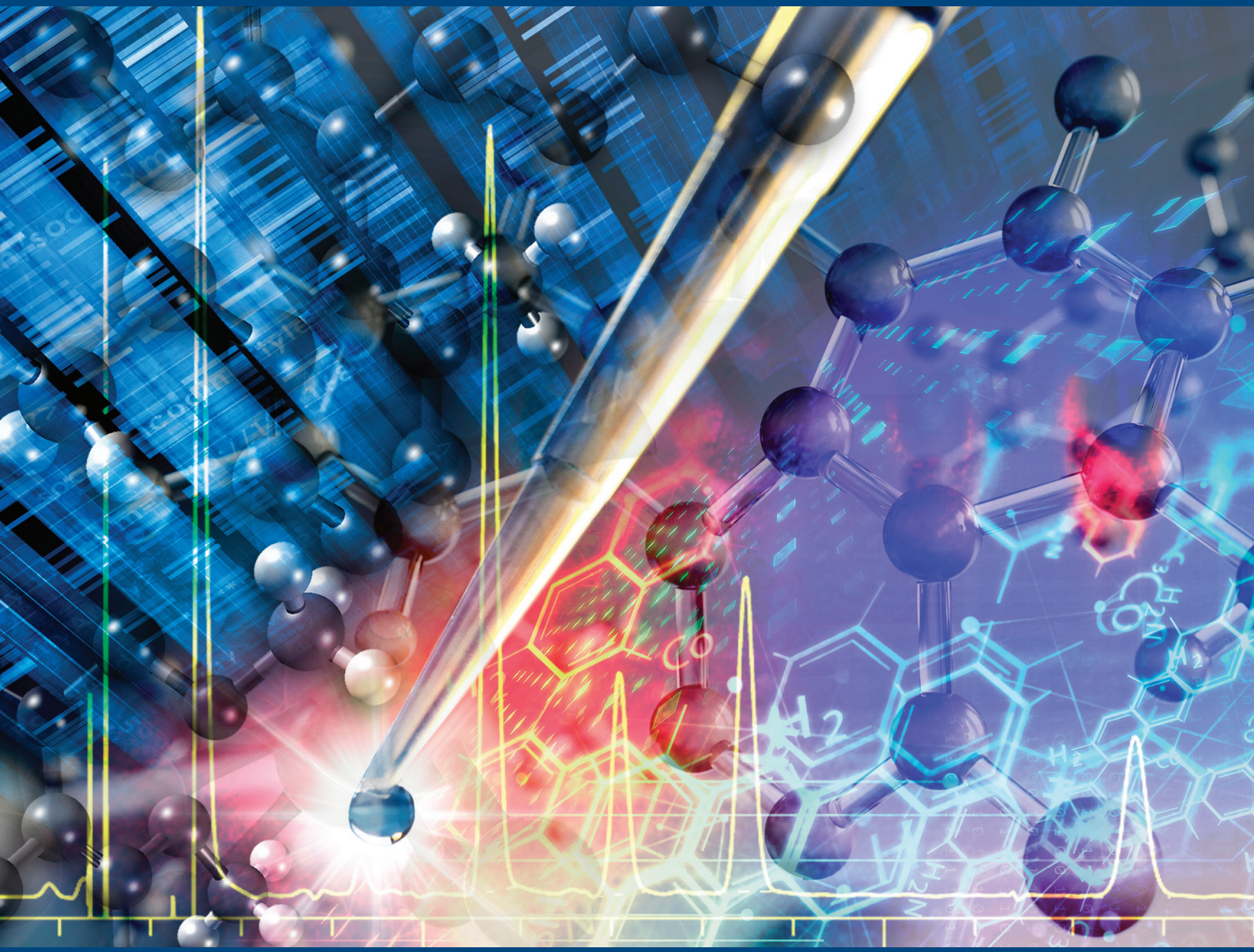

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Supercritical Fluid Chromatography in Bioanalysis–A Review

Laura Toribio | María Teresa Martín | José Bernal

Department of Analytical Chemistry, Faculty of Sciences, I. U. CINQUIMA, Analytical Chemistry Group (TESEA), University of Valladolid, Valladolid, Spain

Correspondence: Laura Toribio (ltoribio@uva.es)

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ABSTRACT

In the last decade, the instrumentation improvements in supercritical fluid chromatography (SFC) and the hyphenation to mass spectrometry (MS), have increased the SFC acceptance between scientists, becoming today a valuable tool in analytical chemistry. The unique selectivity, short analysis times, low consumption of organic solvents, and the greener mobile phase, have contributed to expanding its applicability which has led to an increase in the number of publications especially in the bioanalysis area. This work reviews the advantages and main applications of SFC in bioanalysis during the last 5 years. Fundamental aspects concerning mobile phase composition, stationary phase, hyphenation to MS as well as matrix effect have been discussed. Finally, the most relevant applications have been summarized.

1 | Introduction

Bioanalysis covers the analysis of biological or synthetic compounds, as well as their metabolites, in biological samples. It plays an important role in different areas such as metabolomics, the development of new pharmaceuticals, anti-doping control, and forensic analysis. Usually, low detection limits and fast analysis are required. The number of compounds to be analyzed is high and they can have different physicochemical properties, moreover, there is a broad variety of samples, all of which make bioanalysis a challenging task. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the gold standard technique for bioanalysis, usually employing reverse phase (RP) or hydrophilic interaction LC (HILIC) columns [1–3]. Nevertheless, there are some drawbacks related to the simultaneous analysis of highly polar and lipophilic compounds. Polar compounds show very little retention on RP columns and HILIC mode is not suitable for lipophilic compounds. On the other hand, there is an increasing concern about the sustainability of analytical processes and the reduction in the consumption of organic solvents, encouraging the development of strategies that comply with the principles of green analytical chemistry [4–7]. Using supercritical fluid chromatography (SFC) the greenness of an analytical method could be improved by using less toxic organic solvents and providing faster analysis. Dasilva et al. [8] developed a SFC method for the analysis and purification of over 40 α,α-diaryl primary amine mixtures. The proposed method was compared in terms of greenness to the high-performance LC (HPLC) counterpart and the results showed that the SFC method had an analytical method greenness score 30 times better than the HPLC one. However, this cannot be generalized, it depends on the compounds analyzed and the overall procedure.

In this context, SFC has attracted the attention of scientists as a complementary technique to LC or gas chromatography (GC) [9].

SFC is characterized by the use of a supercritical fluid as a mobile phase, which means a fluid that is maintained at a temperature

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and pressure above its critical values. The supercritical fluid most widely employed is CO_2 because its critical conditions are easily achievable (31°C and 73 bar), it is noninflammable, nontoxic, and it can be considered environmentally friendly. The CO_2 used in SFC is obtained from the atmosphere and thus there is no contribution to global warming. At present, it can be said that, in most cases, SFC separations are performed employing as mobile phase a mixture of CO_2 and a percentage of an organic solvent (organic modifier) to favor the elution of polar compounds. However, whatever the proportion of the organic modifier, the benefits of the chromatographic separation are maintained. Supercritical fluids possess lower viscosities than liquids and the molecular diffusivities of solutes are higher. This allows to use of high flow rates with low pressure drops and to obtain higher efficiencies in shorter analysis times compared with HPLC. Moreover, the consumption of organic solvents is lower. The retention behavior of the compounds in SFC is different from that in HPLC, which could be advantageous when analyzing complex mixtures. SFC is considered a complementary technique to HPLC and in some instances, both techniques can be used in parallel or multidimensional approaches. In addition, SFC separations can be performed at low temperatures enabling the analysis of thermally labile or nonvolatile compounds, which are difficult to analyze without derivatization using GC.

In the early years, the main application areas of SFC were chiral analysis and preparative separations [10–18]. It provided very good results, although the equipment limitations, in terms of sensibility and performance, restricted its use in routine analysis. Fortunately, the development of new equipment with improved performance, the possibility of employing columns packed with sub 2 μm particles as well as new stationary phases specially designed to be used in SFC, and the hyphenation to MS detectors, has renewed the interest in SFC separations. Moreover, its use has been expanded to other areas where high levels of accuracy and sensitivity are required, as is the case of bioanalysis [19]. The possibility of increasing the mobile phase polarity by adding an organic modifier or even low percentages of water opens the possibility of analyzing simultaneously compounds with a broad range of polarities. Thus, the number of studies showing the potential of SFC in bioanalysis has increased in the last few years.

This study reviews the principal applications of SFC in bioanalysis during the last five years.

2 | Mobile Phase and Stationary Phase Considerations

Due to the non-polar character of CO_2 (in terms of polarity is considered similar to hexane), it is necessary to add a miscible polar organic solvent (organic modifier) in order to elute polar compounds. Modifiers not only increase the polarity of the mobile phase but also the viscosity, the density, and they can also modify the interactions between the analytes and the stationary phase [20, 21]. Usually, the organic modifiers employed are alcohols (methanol, ethanol, or isopropanol); methanol is the most widely employed. In some cases, even using an organic modifier, polar or ionizable analytes are strongly retained on the stationary phase or elute with tailed peak shapes, in these cases, the use of additives is required. Traditionally, the additives employed were organic acids (trifluoroacetic acid [TFA], formic acid, etc.) or bases (triethylamine, diethylamine, etc.), but more recently the use of water, salts (ammonium formate or acetate), or ammonia have been described. Additives are added in a small percentage (0.1%–1%) to the organic modifier and they can act in different ways. They can modify the polarity of the mobile phase, the pH, and the ionization of the analytes and they can act as ion-paring reagents, but also they can be adsorbed on the stationary phase reducing the unspecific interactions of the analytes with residual polar groups of the stationary phase, and thus improving the peak shapes [22–24]. In addition, they can enhance the signal in the MS detector. As a general rule, acidic additives are employed for acidic analytes and basic additives for basic analytes, but when the analyte possesses acidic and basic functional groups, then mixtures of acidic and basic additives, or salts, are employed. When MS detectors are employed, an important aspect is additive compatibility. In these cases, volatile additives are mandatory being ammonium formate and ammonium acetate are increasingly employed for the analysis of ionizable or polar compounds [19]. A less popular additive ammonium fluoride has provided good peak shapes and enhanced MS signals in the analysis of polar compounds (including amino acids), even the mixture of ammonium formate and ammonium fluoride has shown better results than each individual additive. The problem was the low solubility of ammonium fluoride in alcohols, which limited its concentration to 1 mM [25]. In the last years, the use of water as an additive has increased in the bioanalysis area, especially for the analysis of highly polar compounds. Water is usually introduced with the organic modifier at a maximum percentage of 10%, due to problems of miscibility with CO_2, although Thurbide et al. [26] showed that when using isopropanol as an organic modifier the percentage of water could be higher, five times higher than using methanol. They proposed the use of 60% of the mixture of methanol/isopropanol/water (4:1:5) to elute polar compounds without increasing the column backpressure.

Nowadays, SFC is operated using a percentage of organic modifier that in some cases can be higher than the corresponding to CO_2 and even using gradients where at the initial conditions the modifier percentage is low (or even pure CO_2) and at the final conditions it is 100%. These wide gradients were first proposed by Bamba et al. [27] to simultaneously determine fat and water-soluble vitamins. The mobile phase state changed from supercritical to subcritical and liquid without any discontinuous transitions, and the term "unified chromatography" (UC) was used to refer to this mode. This has been subsequently used by other authors, with good results, in the analysis of polar or mixtures of polar and low polar compounds [25, 28, 29].

The stationary phases employed in HPLC can also be used in SFC. RPs such as C18, C8, or C30 are used for the separation of nonpolar compounds (lipids, carotenoids, etc.) [30], while normal phase (NP) columns such as propanediol-, amino-, or cyano-bonded silica as well as bare silica provide better selectivity for polar or slightly polar compounds and are the most frequently employed in the published achiral SFC methods [19]. Also, HILIC or mixed-mode stationary phases have been employed in SFC with satisfactory results [31, 32]. In fact, the diversity of available stationary phases for SFC is high. In some instances, secondary interactions of the analytes with residual silanol groups result in tailed peak shapes, which necessitates the use of additives. To

circumvent these problems, new stationary phases, specifically designed to be used in SFC, have been developed. The first one was 2-ethylpiridine bonded to silica, which provided good peak shapes for basic analytes without using additives [33]. Other stationary phases with different functionalities have been subsequently developed, such as aminophenyl-1-aminoanthracene, 2-picolylamine, or fluoro-phenyl [19].

Chiral separations are an area where SFC has shown all its potential. The chiral stationary phases (CSP) with the broadest applicability and the highest rate of success are those derived from polysaccharides, being the amylose tris(3,5-dimethylphenylcarbamate), and amylose tris(5-chloro-2-methylphenyl-carbamate) the most widely employed [34].

In the last 10 years, the development of a new generation of SFC instruments with lower dead volumes, based on the ultra-high-performance LC (UHPLC) technology, has favored the use of columns packed with sub 2 μm particles. These columns are shorter than the conventional ones and provide higher efficiencies in lower analysis times, which is an advantage when analyzing complex samples such as those studied in the bioanalysis area. Nevertheless, it should be noted that when using wide gradients of modifier, where at the final conditions the percentage of organic modifier is high, the pressure drop increases gradually due to the increase in the mobile phase viscosity, and the smaller the particle size, the greater the increase. Thus, the equipment should be designed to withstand high pressures, otherwise, the flow rate and/or back pressure should be reduced in order not to exceed the pressure limit of the system. Superficially porous particles, provide also high efficiencies and generate low-pressure drops when using high flow rates or high percentages of organic modifiers, nevertheless, their use in SFC is scarce.

Column selection is an important step in method development and column screening is usually performed to select the best stationary phase. For this purpose, several stationary phases with complementary selectivities are usually compared being the works of West and Lesellier of great value [31, 35, 36].

3 | MS Detection

Due to the levels of sensitivity and accuracy required in bioanalysis, the MS detector is the most frequently employed. Moreover, it is possible to obtain more and better information than with traditional ultraviolet-visible (UV-Vis) or diode array detectors. Similarly to LC-MS, electrospray ionization (ESI) is, nowadays, the most popular ionization source for SFC. In the early years, atmospheric pressure chemical ionization was preferred, as it can withstand high flow rates, and thus was considered suitable for SFC-MS. Nowadays, the development of new interfaces has made ESI the preferred due to its ability to ionize compounds over a wider range of polarities [37, 38].

The hyphenation SFC-MS can be performed in different ways and different interfaces have been developed, being the backpressure regulator always located before the MS detector. Only two of them are commercially available at present: split flow introduction or full flow introduction in the MS detector. In the first, a make-up fluid is added after the column (or the UV detector if used) but before the MS detector, and then the total flow is split before entering the MS detector. A small portion is directed to the MS detector and most of the flow is transferred to the back pressure regulator (BPR). This interface is commercialized by Waters and Agilent. In the second interface, the total flow is introduced into the MS detector; the make-up fluid is pumped after the column, and then the total flow is transferred to the BPR and then to the MS detector which is serially connected. This interface is commercialized by Shimadzu and Agilent. For an insight into the different types of interfaces, readers are referred to several reviews published on this topic [39–43].

The ionization mechanism in SFC/ESI-MS is different from LC/ESI-MS because of the different physicochemical properties of the mobile phase. In SFC, most of the mobile phase (the CO_2) is volatilized in the decompression process, and only a small amount of the organic solvent used as a modifier enters the MS interface. Thus, a gas/liquid mixture is introduced in the ESI capillary and the presence of CO_2 gas can increase the vaporization efficiency of the ionization process. On the other hand, several studies [44, 45] have described the formation of alkoxylcarbonic acid when CO_2 is mixed with alcohol. The presence of this compound could be the cause of the acidic character of the CO_2/methanol mobile phases [24], but also it could favor the ionization process in SFC/MS. Recently, Fujito et al. [46] studied the ionization mechanism in SFC/ESI-MS. They concluded that methoxylcarbonic acid was generated in CO2/methanol mixtures and it played an important role in the ion generation in positive mode because it acts as a proton donor. On the contrary, in negative mode, the methoxylcarbonic acid had a negative effect causing ion suppression. This effect could not be eliminated by adding ammonium acetate, an additive commonly employed to improve peak shape in SFC. It is important to note that this study revealed that the addition of ammonium acetate did not improve the sensitivity in either positive or negative modes and the best results, in terms of sensitivity, were obtained using methanol without ammonium acetate. Thus, the authors recommended that if the addition of salt is necessary to obtain a good separation, the concentration should be as low as possible to minimize ion suppression. The exceptions are the cases where ammonium adduct ions are used as ion precursors for detection, as is the case of lipid analysis [47]. Moreover, the methoxylcarbonic acid can further react with methanol-generating water, thus the ionization process could be influenced by the presence of water even when water-free mobile phases are employed [38, 48].

The decompression process can cause solute precipitation, which is avoided by introducing the make-up fluid before the flow splitting or the BPR. The make-up fluid can also improve the analyte ionization and thus enhance the method sensitivity [49]. Usually, the make-up solvent is the same organic modifier employed for the chromatographic separation and in some instances, an additive is added, but it depends on the compounds to be studied. It is important to optimize its composition and flow rate independently because the organic modifier and/or additive that provides good chromatographic separation does not necessarily favor the ionization of the analytes [50]. It is interesting to note that due to the acidic character of the CO_2/methanol mobile

phases (pH close to 5), the apparent pH in the spray ranges from 3.8 to 7.2 regardless of the use of acids, bases, or buffer additives in the make-up solvent [49]. This fact can reduce the ionization efficiency and sensitivity in ESI negative mode.

Considering the type of analyzer, the most widely used are single quadrupole (Q), triple quadrupole (QqQ), and quadrupole time of flight (Q-TOF) [38]. The selection depends on the type of compound and analysis. Q has the lowest sensitivity and resolution power but is the cheapest option. When higher sensitivity and selectivity are required, MS/MS analysis should be performed and QqQ or Q-TOF are the options. QqQ provides good results in the quantitative analysis of trace compounds in complex samples, due to its high sensitivity, high scanning speed, when working in single reaction monitoring mode, and wide dynamic range. Q-TOF is an alternative to QqQ, the high resolving power of TOF allows the resolution of interfering compounds with the same nominal mass and retention time as the analytes, improving the signal-to-noise ratio. Orbitrap is the most recently developed. It has high-resolution power, but it has scarcely been used in SFC-MS, which could be justified because it is more expensive and Orbitrap vendor does not provide SFC systems, thus the coupling is less straightforward, and home-made solutions need to be applied.

4 | Matrix Effect

In MS detection, the matrix effect is an important aspect to evaluate especially when using ESI sources. Matrix compounds can enhance or suppress the analyte ionization. Consequently, the method sensitivity and the quality of the data obtained are affected. The interfering matrix compounds depend on the type of sample analyzed and these compounds can affect not only the signal intensity but also the fragmentation and MS spectra obtained. Since some matrix compounds coelute with the target analytes, they can interfere with the ion fragmentation process. This can lead to the erroneous interpretation of results, because of the modification of the typical mass spectra patterns and the difficulty of performing database searching, especially when these matrix compounds are present at high concentrations and elute within the same retention window as the target compounds. Losacco et al. found that in the analysis of urine and plasma samples, MS/MS spectra obtained in ESI negative mode contained fewer matrix interferences than those obtained in positive mode [51]. Some interfering compounds have been identified as naturally present in biological samples, such as the case of phospholipids, creatinine, or metal clusters originating from alkaline ions [48, 52].

In the reviewed studies (last 5 years), the matrices more frequently analyzed are plasma, serum, and urine. The more conventional sample treatments are "dilute and shoot" for urine and protein precipitation for plasma and serum, although other strategies such as solid phase extraction (SPE) [53–56], solid-liquid extraction, or liquid-liquid extraction (LLE) [57–61] have also been applied. In some cases, analytes can be degraded during sample treatment; to avoid this problem other strategies based on online extraction procedures have been developed. The research group of Bamba [62] developed a method based on the online coupling of supercritical fluid extraction (SFE) and SFC. They used the SFE-SFC-MS/MS system commercialized by Shimadzu to analyze dried serum spots and determined 21 metabolites (four hydrophilic and 17 hydrophobic) used as biomarkers of cancer. The objective was successfully achieved and the results obtained were comparable to those obtained with the LC-MS/MS analysis of the serum samples. More recently, Jinn et al. [63] used the same SFE-SFC system to determine inflammation-related lipids, in brain tissues of depressed rats. They obtained high recoveries and sensitivities moreover, some lipids easily oxidizable such as docosahexaenoic acid, arachidonic acid (AA), hydroxy docosahexaenoic acids, and hydroxyeicosatetraenoic acids showed higher recoveries than using off-line LLE procedures.

Although there are no rules for the matrix effect, several authors have obtained a signal suppression for plasma matrices and a signal enhancement for urine samples [51, 64], being matrix effect more frequent in the positive mode than in the negative one. Other matrices such as sweat have also been analyzed using SFC-MS, in an attempt to determine metabolic biomarkers in non-invasive samples [65, 66]. In this case, the matrix effect was negligible.

5 | Applications

5.1 | Metabolomics and Lipidomics

Metabolomics involves the comprehensive analysis of metabolites in living organisms. It plays an important role in the development of personalized medicine and in the study of diseases, therefore there is a growing interest in this area, and the number of studies related to the application of chromatographic techniques, including SFC, has increased in the last years [67–69]. The compounds analyzed are small molecules, with molecular weights lower than 2 kDa and with a wide range of polarities and physicochemical properties, thus different chromatographic methods and/or techniques are required. LC-MS is the technique most widely applied [70, 71], combining RP and HILIC modes, but there is not a single platform that can perform the analysis of polar and nonpolar compounds simultaneously.

Although lipidomics has traditionally been classified under the scope of metabolomics, nowadays is considered an independent discipline. Metabolomics mainly focuses on polar and low-polar metabolites and lipidomics is devoted to the comprehensive analysis of all kinds of lipids. Usually, metabolomics and lipidomics analysis are performed separately.

SFC has been postulated as a good platform for developing a single method to analyze metabolites with a wide range of polarities. Applying the concept of UC, where the mobile phase changes from supercritical to subcritical and finally to liquid, Losacco et al. [51] studied the analysis of 597 metabolites including hydrophilic and hydrophobic ones. More than 66% were successfully identified but phosphorylated metabolites were difficult to detect. Later, the research group of Bamba developed a chromatographic method that combined UC and HILIC modes in the same run [72]. The aim was to expand the metabolome coverage of UC, enabling the analysis of highly polar compounds,

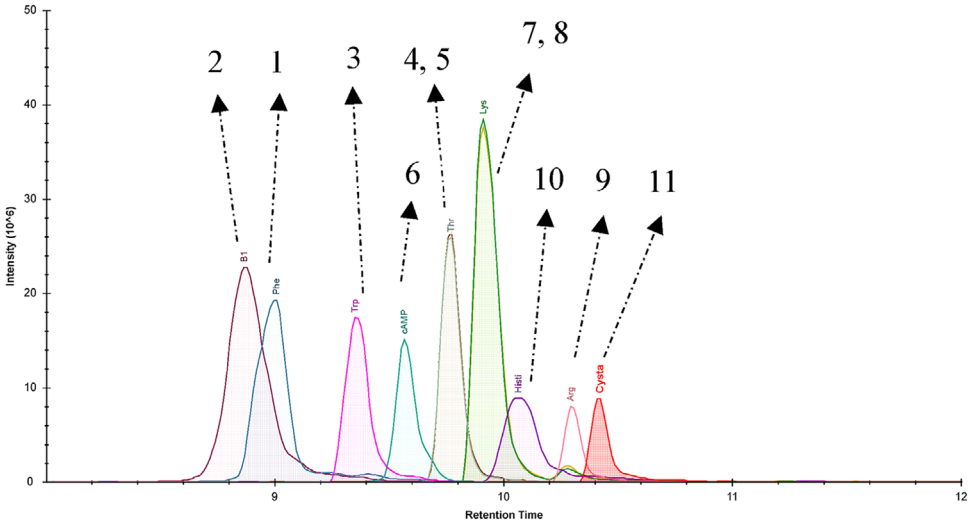

FIGURE 1 | Elution order of 11 reliable identified hydrophilic metabolites including B1 [1], phenylalanine (Phe, 2), tryptophan (Trp, 3), homo-serine (H-ser, 4), threonine (Thr, 5), adenosine cyclic monophosphate (cAMP, 6), lysine (Lys, 7), glutamine (Glu, 8), arginine (Arg, 9), histidine (Hisd, 10), and cystathionine (Cysta, 11) on Amide column using the optimized unified chromatography/hydrophilic interaction liquid chromatography (UC/HILIC) method. See [72] for chromatographic conditions. Reproduced with permission [72]. 2023 Elsevier.

such as phosphorylated metabolites. They applied a UC gradient followed by an HILIC-like gradient, using the same column (packed with a polar stationary phase, in this case, amide), chromatographic system, and in the same run. In the UC gradient the components were CO_2 and the organic modifier was a mixture of acetonitrile/methanol/water (60:38:2) with 10 mM ammonium formate. The percentage of organic modifiers increased from 2% to 100%. In the HILIC-like gradient, the aqueous phase was a 10 mM ammonium formate solution with 0.01% phosphoric acid, and the organic phase was the same as in the UC gradient. In the HILIC-like gradient, the percentage of the aqueous phase increased from 0% to 100% and the percentage of the organic phase decreased in the opposite way. Using this gradient, highly polar metabolites were eluted with better peak shapes (Figure 1).

Metabolome consists of a very large number of metabolites with different physicochemical properties, so is difficult to obtain a single extract containing all of them, and biphasic extraction systems are employed. The analytes are distributed between the aqueous (polar compounds) and the organic (lipophilic compounds) phases, which are analyzed separately with different chromatographic methods. Recently, Kozlov et al. [73] developed an SFC method, using a diol column, to simultaneously analyze metabolites with a broad range of polarities (polar compounds and lipids) in plasma samples. They used the same chromatographic system to perform lipidomic/metabolomic analysis without the need to change the stationary phase or the nature of the mobile phase. The organic modifier was a mixture of methanol/water (96:4) with 30 mM ammonium formate. The extracts were consecutively injected in the same column and from the same vial by adjusting the needle height. Two gradients of modifier were combined within the same analysis cycle, one of them from 1% to 50% for the first injection (lipid compounds) and the other from 20% to 100% for the second injection (polar compounds). In addition, a gradient of flow rate was employed to avoid overpressure problems. Lipids were separated into classes, according to their polarity, while the separation of polar compounds was more dependent on the structure. The analysis time was 24 min and allowed the identification of 39 metabolites without the need to separate the two phases after the sample extraction (Figure 2).

MS is the detection mode usually employed in metabolomics, with a high increase in tandem MS/MS modes with Q-TOF or QqQ analyzers. ESI sources are usually employed providing positive mode with a higher rate of success. The make-up solvent more frequently employed is methanol with a small concentration of an additive such as formic or acetic acids. Ammonium salts have also been used and in some instances, a mixture of ammonium formate and acetic acid has been employed to increase the method's sensitivity [74]. Concerning the mobile phase additive, the most widely used are ammonium formate and ammonium acetate, although in some instances TFA has also been described. The additive is selected according to the signal and peak shape obtained, but there is not a general rule, it depends on the type of compounds analyzed.

In the published studies (see Table 1), the achiral stationary phases employed are in most cases polar (diol, silica, and amino). The best overall performance in the analysis of metabolites with a wide range of polarities was achieved with the diol columns; nevertheless, pentafluorophenyl or 1-aminoanthracene-based columns provided good results in the analysis of vitamin D and its main metabolites [57, 58]. In some instances, CSP have been employed in achiral separations, that was the case of the analysis of eicosanoids [75] or the isomeric forms of some urolithin glucuronides [76]. In both cases, several stationary phases including achiral (diol, amino, phenyl, silica, etc.) and chiral ones (based on amylose and cellulose derivatives or Pirkle-type) were checked. In the analysis of 11 eicosanoids, including prostaglandins, leukotrienes, and thromboxanes, the best results were obtained with the chiral column Lux i-Amylose 3 achieving the baseline separation of all isobaric forms, which allowed the

TABLE 1 | Supercritical fluid chromatography (SFC) applications in metabolomics.

Ref.	Analytes	Sample	Column	Organic modifier	Other chromatographic conditions	Make-up solvent	Detector
[73]	Polar and lipid metabolites covering a wide range of polarities	Plasma	Torus Diol column (100 × 3.0 mm, 1.7 μm)	MeOH/water (96/4) with 30 mM ammonium formate Two gradient modes from 1% to 50% and from 20% to 100%	Flow-rate gradient from 1.6 to 0.8 mL/min 60°C, and 10.3 MPa	MeOH:water (98:2, v/v) with 10 mM ammonium formate. Gradient from 0.25 to 0.1 mL/min	Q-TOF, ESI Positive mode
[76]	Five urolithin glucuronides (urolithin A 3- and 8-glucuronide isourolithin A 3- and 9 glucuronide, and urolithin B 3-glucuronide)	Urine	(S, S) Whelk-O 1 (150 × 4.6 mm, 3.5 μm)	30% isopropanol with 0.1% trifluoroacetic acid	2.5 mL/min, 35°C, and 130 bar		UV-Vis (225 nm)
[100]	Nucleosides, biogenic amines, carbohydrates, amino acids, and lipids	Human plasma	ACQUITY UPC2 Torus Diol (100 × 3 mm, 1.7 μm)	MeOH with 30 mM ammonium acetate and 2% H_2O, in gradient mode from 5% to 100%	Flow rate gradient from 2.0 to 0.8 mL/min. 180 bar and 60°C	MeOH:water (95:5, v/v) with 0.1% formic acid. Gradient from 0.8 to 0.2 mL/min	Q-TOF, ESI positive mode
[53]	103 octadecanoid oxylipins	Human plasma	Waters Trefoil AMY1 column (3.0 × 150 mm, 0.5 μm)	MeOH:EtOH (8:2, v/v) with 0.1% acetic acid. Gradient from 5% to 30%	2.0 mL/min, 200 bar, and 35°C	MeOH with 5 mM ammonium acetate at 0.2 mL/min	QqQ, ESI negative mode
[65]	Purines (hypoxanthine, alantoine, xanthine and inosine) and small organic acids (lactic and pyruvic)	Human sweat	Torus 1-aminoanthracene (1-AA) for purines and Torus Diol for organic acids. both 3 × 100 mm, 1.7 μm)	MeOH (for purines) and MeOH:water (98:2, v/v) with 50 mM ammonium acetate (for organic acids) In both cases in gradient mode	1.5 mL/min, 150 bar, and 40°C (organic acids) 50°C (purines)	MeOH (1% formic acid) at 0.45 mL/min	Single Quadrupole ESI positive mode
[58]	12 vitamin D analogues(D_2, D_3 and seven hydroxylated metabolites	Human plasma	Torus 1-aminoanthracene (1-AA) (100 × 3.0 mm, 1.7 μm)	MeOH gradient from 2 to 15%	2.0 mL/min, 19.0 MPa, and 50°C	MeOH with 0.5% formic acid at 0.1 mL/min	QqQ, APCI positive mode
[75]	11 eicosanoids, including prostaglandins, leukotrienes, and thromboxanes	Blood cells	Lux i-Amylose 3 (2 × 150 mm, 3 μm)	IPA/ACN (7/3) with 0.1% formic acid. Gradient from 5% to 35%	1.2 mL/min 35°C and 100 bar	MeOH with 0.1% ammonia at 0.05 mL/min	Single Quadrupole ESI negative mode

(Continues)

TABLE 1 | (Continued)

Ref.	Analytes	Sample	Column	Organic modifier	Other chromatographic conditions	Make-up solvent	Detector
[51]	49 metabolites	Plasma and urine	Poroshell HILIC (100 × 3.0 mm–2.7 µm)	MeOH:water (95:5, v/v) with 50 mM ammonium formate and 1 mM of ammonium fluoride in gradient mode	0.9 mL/min, 105 bar, and 40°C	MeOH at 0.3 mL/min	Q-TOF, ESI positive and negative modes
[57]	Vitamin D3, vitamin D2, and their 25-hydroxy metabolites. Derivatized with 4-phenyl-1,2,3-triazoline-3,5-dione (PTAD)	Human milk	Acquity UPC2 Fluoro-Phenyl (3.0 × 100 mm, 1.7 µm)	MeOH:water (98:2, v/v) with 10 mM ammonium formate. Gradient from 0,5% to 50%	Flow rate gradient from 3.0 to 1.75 mL/min, 45°C, 128 bar	MeOH with 10 mM ammonium formate at 0.4 mL/min	QqQ, APCI positive mode
[77]	100 polar compounds comprising non proteinogenic amino acids, dipeptides tripeptides, nucleic acids, neurotransmitters niacin	Rat serum	CROWNPAK CR-I(+) (150 × 3 mm, 5 µm)	CO_2/MeOH/water/TFA in a 70/27/3/0.15 (v/v/v/v)	2.0 mL/min, 10 MPa, and 50°C	Not described	QTRAP, ESI positive mode
[101]	19 hormones from five classes (progestogens androgens, oestrogens and corticoids and peptide hormones)	Human plasma	Acquity UPC2 BEH (3.0 × 150 mm, 1.7 µm) for desulfated steroids hormones and CSH fluorophenyl (3.0 × 150 mm, 1.7 µm) for sulfated steroid hormones	MeOH:IPA (1:1, v/v) with 0.1% formic acid for desulfated steroid hormones; MeOH:water (97:3, v/v) with 10 mM ammonium acetate for sulfated steroid hormones Gradient from 2% to 17%	2.0 mL/min (desulfated steroid hormones) 1.5 mL/min (sulfated steroid hormones), 103.4 bar, and 40°C	MeOH with 0,1% formic acid at 0.2 mL/min	QqQ, ESI positive mode
[74]	Vitamin D2, vitamin D3 and four metabolites	Serum	PFP (4.6 × 250 mm, 3.5 µm)	MeOH. Gradient from 8% to 40%	1.5 mL/min, 40°C, and 100 bar	MeOH with 5 mM ammonium formate and 1% (v/v) formic acid	QqQ, ESI positive mode
[66]	Metabolic concentrations of lactate and pyruvate	Sweat and sebum from Human skin	Torus Diol (3 × 100 mm, 1.7 µm)	MeOH:water (98:2 v/v) with 50 mM ammonium acetate	1.5 mL/min, 50°C, and 15.0 MPa	MeOH with 1% acetic acid at 0.45 mL/min	Single Quadrupole ESI positive and negative modes

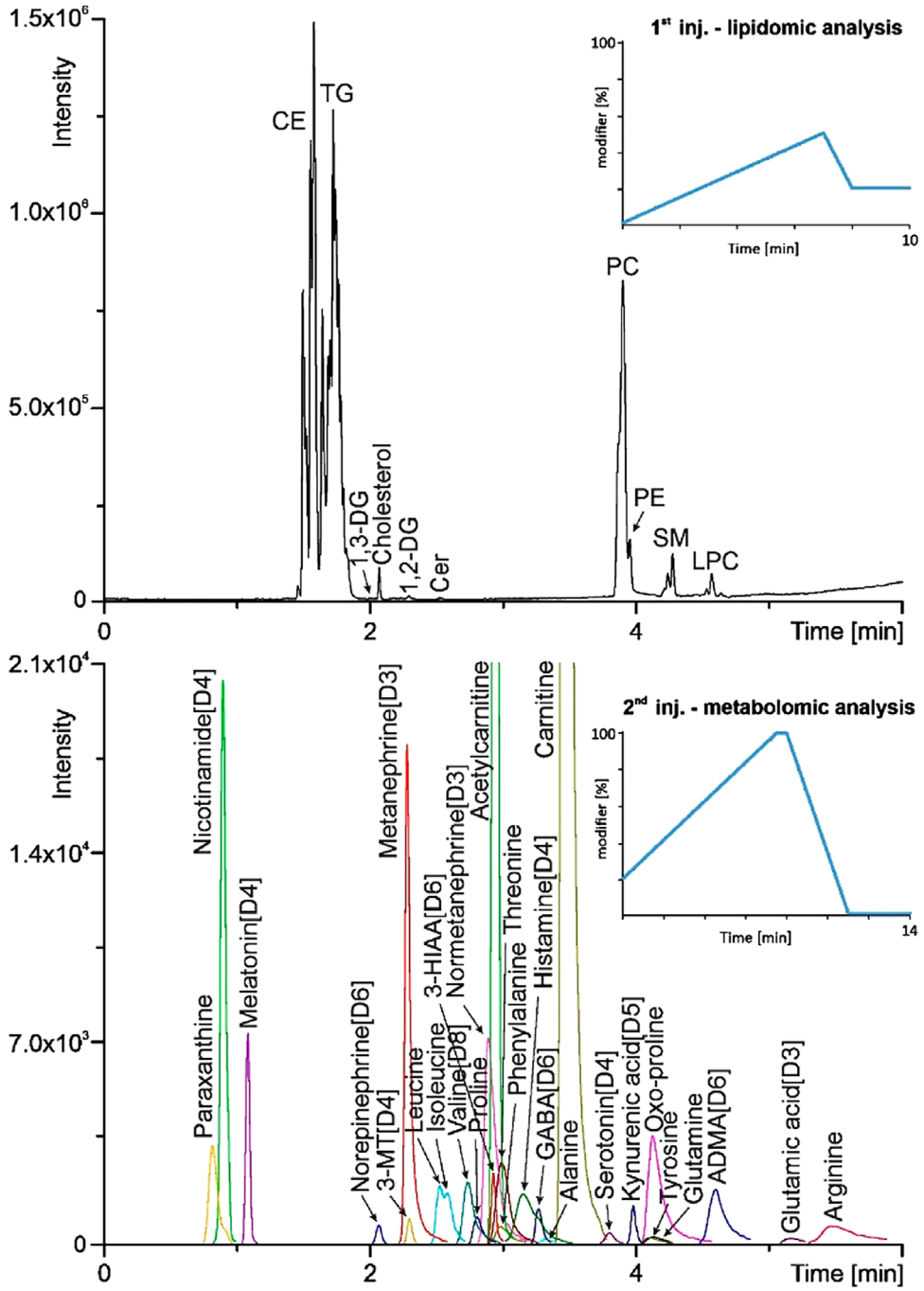

FIGURE 2 | Optimized single-platform supercritical fluid chromatography–mass spectrometry (SFC–MS) lipidomic/metabolomic analysis from an extraction vial using two consecutive injections from organic (top) and aqueous (bottom) layers after MTBE extraction of the plasma model sample. Conditions: Torus Diol column, 60°C, 10.3 MPa, modifier MeOH–water (96:4) with 30 mM ammonium formate, injection volumes: top—0.2 μL, bottom—1.0 μL; for flow rate and mobile phase gradients, see [73]. Reproduced with permission [73]. 2024 American Chemical Society.

use of a simple quadrupole mass spectrometer. The separation of five urolithin glucuronides was accomplished by using the (S, S) Whelk-O 1 column. The determination of all the isomers in urine samples was successfully achieved, which was not possible using HPLC methods. Konya et al. [77], also employed a chiral column, the CROWNPAK CR-I (+), for the screening of 100 polar metabolites with a wide range of polarities, including proteinogenic and nonproteinogenic amino acids, peptides, nucleic acids, etc. In this column, all the compounds were eluted in 10 min with excellent peak shapes and working in isocratic conditions with a low percentage (30%) of an organic modifier. Moreover, the pairs L-Gln/L-Lys and L-Ile/ L-Leu could be resolved. The method was applied to the analysis of rat serum with good repeatability of retention times and areas (relative standard deviations [RSDs] lower than 2.4% and 14.9%, respectively), and 43 polar metabolites were identified.

SFC has been traditionally employed in lipid analysis due to the nonpolar character of CO_2. Moreover, the possibility of tuning the polarity of the mobile phase by adding a polar modifier widens the

applicability range of SFC, making the simultaneous analysis of lipids with different polarities. The use of SFC in lipidomics was pioneered by the research group of Bamba [78]. They developed an SFC method for the simultaneous analysis of lipids including phospholipids, glycolipids, neutral lipids, and sphingolipids. The analysis was performed in less than 15 min using a cyano column (250 × 4.6 mm, 5 μm). Since then, SFC has been applied in targeted and untargeted lipidomic studies, providing very good results in lipid class separations [47, 79, 80]

Different kinds of columns have been employed for lipid analysis, including RP (C18) and NP columns (bare silica or silica functionalized with aminopropyl, propanediol, cyanopropyl, diethylamine, or 1-aminoanthracene groups). Normal phase columns separate lipids into lipid classes according to their polarity. In this case, retention time increases with the lipid polarity, and some kind of separation, based on the fatty acyl composition, can be achieved for individual lipids within one lipid class [79]. On the contrary, RP columns are more suitable for the separation of lipids according to their fatty acyl composition (alkyl chain length and degree of unsaturation). When complex mixtures of polar and non-polar lipids are analyzed using RP columns, the coelution of individual compounds from different lipid classes could happen [78]. Nevertheless, employing polar embedded C18 columns, such as Inertsil ODS-EP, polar lipids were separated based on not only their polarity but also their fatty acyl composition [81].

Yang et al. [82] identified 370 lipids in human plasma using an online two-dimensional SFC-RPLC (2D SFC-RPLC) system, coupled to QqQ-MS. Two 10-port, two-position valves were employed to connect the SFC and RP columns, and the mobile phase from the SFC column was evaporated by a vacuum pump when it flowed through the loops. Lipid classes were separated in the first-dimension SFC (on two Zorbax RX-SIL columns) according to their polarity, and individual lipids were further separated in the second-dimension RPLC (C8 column) according to their fatty acyl chains. Compared with an NP/RP 2D LC-MS method, previously developed by the same research group, the analysis time was significantly reduced using the 2D SFC/RPLC method (38 min vs. 170 min), and the limit of detection for TG (18:1/14:0/17:1), MG (17:0), and Cer (d18:1/12:0) were respectively 50-fold, tenfold, and tenfold lower. The capabilities of the method were demonstrated by the analysis of lipids in human plasma, from patients with breast cancer and healthy controls. Twenty potential lipid biomarkers for breast cancer were found.

Pseudotargeted lipidomic approaches using SFC have been developed recently. Yang et al. [60] applied UHPSFC-MS/MS to develop a pseudotargeted lipidomics method. Using UHPSFC-Q-TOF in MS^E mode, the tandem mass spectra of the lipids were acquired. Then the multiple reaction monitoring (MRM) transitions of the lipidome were defined and verified by UHPSFC-QqQ, which was used to establish the final method. With this approach, the number of lipids quantified was higher than in the targeted analysis. Moreover, working in MRM mode the sensitivity was higher than in untargeted approaches. Compared with pseudotargeted lipidomics methods based on UHPLC-MS/MS, isomer separation was improved, the analysis time was lower, and the sensitivity was higher, with detection limits in the range of 0.900–1.00 × 10^3 pg/mL. The proposed method was applied to the lipidomics study of the therapeutic effects of liquiritin on depression.

Unsaturated fatty acids (UFAs) play an important role in biological systems as they are essential in some cellular processes and the position of double bonds is important to understand their biological functions. Nevertheless, determining the double bond location in UFAs is challenging due to the structural similarities between the UFAs and the stability of these bonds. Usually, this determination is accomplished by including a chemical derivatization or ion-activation step, previously to the chromatographic analysis with MS/MS detection. Chen et al. [83] developed an SFC-Q-TOF-MS method to determine the position of the double bonds in UFAs. They designed a chromatographic system where a microreactor, based on the Paternò-Büchi reaction, was online connected to the SFC equipment (Figure 3). The derivatization took place after the column but before the MS detector. Different derivatization reagents were checked and benzaldehyde was finally selected. With this approach, they could identify different UFA isomers based on the position of the double bonds. The method was applied to the analysis of free FAs in human plasma. Twenty FAs, including seven saturated and 13 unsaturated were identified.

An important aspect of metabolomics and lipidomics is the possibility of comparing the quantitative data obtained with different systems and methodological approaches. In an attempt to harmonize the data obtained from lipidomic analyses, Chocholoušková et al. [84] performed an intra-laboratory comparison employing four different platforms: two Q-TOF mass spectrometers, from the same vendor, connected to HILIC-UHPLC and UHPSFC. They evaluated the quantitative differences in the lipidome analysis of 268 human plasma samples obtained from renal cell carcinoma patients and healthy volunteers. The number of lipids detected employing UHPSFC was higher than employing HILIC-UHPLC because non-polar lipid classes and ceramides elute in the void volume of the HILIC-UHPLC system. On the other hand, the lipid profile was similar in all the systems. The overall differences in lipidome quantitation using the different chromatographic modes, expressed as the mean RSD for all lipid species in all samples, was below 30% and could be reduced using the normalization to a reference sample with defined lipid concentrations (NIST standard plasma). Moreover, the results of the renal cell carcinoma diagnostic study were to a high degree comparable among all platforms, both for non-normalized and normalized data. Considering method validation, Wolrab et al. [85] found similarities between the results obtained using UHPSFC-MS and HILIC-UHPLC-MS, for the analysis of eight lipid classes in human plasma and serum, which reinforces the applicability of UHPSFC-MS in lipidomic quantitation of polar and non-polar lipids.

The applications of SFC in metabolomics and lipidomics, during the last 5 years, are summarized in Tables 1 and 2.

5.2 | Pharmaceuticals and Clinical Analysis

The pharmaceutical industry was one of the first to embrace SFC, especially for enantiomeric separations and preparative purposes [18, 86–89]. In most cases, SFC was applied to the quality control

TABLE 2 | Supercritical fluid chromatography (SFC) applications in lipidomics.

Ref.	Analytes	Sample	Column	Organic modifier	Other chromatographic conditions	Make-up solvent	Detector
[83]	Unsaturated fatty acids (UFAs)	Human plasma	Acquity UPC2 Torus 1-AA (100 × 3.0 mm, 1.7 μm)	Acetonitrile with 0.1% formic acid. Gradient from 4.5% to 10%	1.0 mL/ min, 130 bar, and 50 ∘C	Not described	Q-TOF MS, ESI in negative mode
[61]	104 types of triglycerides	Human plasma	Acquity UPC2 HSS C18 SB (3.0 × 100 mm, 1.7 μm)	MeOH:water (95:5, v/v) with 0.1% (w/v) ammonium acetate. Gradient from 0% to 50%	1.0 mL/min, 100 bar, and 50°C	MeOH:water (95:5, v/v) with 0.1% (w/v) ammonium acetate at 0.1 mL/min	QqQ, ESI in positive mode for triglycerides and in negative mode for fatty acids
[102]	396 and 95 lipids belonging to 14 lipid classes	Vertebrate gastrula	ACQUITY UPC2 Torus DEA column (3.0 × 100 mm, 1.7 μm)	MeOH:water (95:5, v/v) with 0.1% (wt/ v) ammonium acetate. Gradient from 1% to 65%	1.0 mL/min, 150 bar, and 50°C	MeOH:water (95:5, v/v) with 0.1% (wt/v) ammonium acetate at 0.2 mL/min	QqQ, ESI in positive mode
[63]	23 lipids	Rat brain tissue	Shim-pack UC-X RP (C18) (250 × 4.6 mm, 3 μm)	MeOH in gradient mode from 1% to 30 %	5.0 mL/min, 120 bar, and 40°C	Not used	QqQ, ESI in positive mode
[84]	176 lipid compounds	Human plasma	Viridis BEH (100 × 3 mm, 1.7 μm)	MeOH:water (99:1; v/v), with 30 mM of ammonium acetate. Gradient from 1% to 51%	1.9 mL/min, 180 bar, and 60 ∘C	MeOH:water (99:1; v/v), with 30 mM of ammonium acetate at 0,25 mL/min	Q-TOF, ESI in positive mode
[60]	758 lipid compounds	Rat plasma	Torus Diol (100 × 3.0 mm, 1.7 μm)	MeOH-water (99:1, v/v) containing 10 mM ammonium acetate. Gradient from 1% to 51%	1.8 mL/min, 160 bar, and 60°C	MeOH at 0.25 mL/min	Q-TOF MS, ESI in positive and negative modes
[79]	13 lipid classes	Salmon liver	Acquity BEH UPC2 (100 × 3 mm, 1.7 μm)	MeOH:water (99:1, v/v) with 30 mM ammonium acetate. Gradient from 1% to 50%	1.9 mL/min, 180 bar, and 50°C	MeOH:IPA:water (50:49:1, v/v/v) at 0.2 mL/min.	Q-TOF, ESI in positive mode
[103]	500 lipids species belonging to the six main classes of lipids including phospholipids, sphingolipids, free fatty acids, sterols, and fatty acyl-carnitines	Dried blood spots	ACQUITY UPC2 Torus diethylamine (DEA) (100 × 3.0 mm, 1.7 μm)	MeOH:water (98:2, v/v), with 20 mM of ammonium acetate. Gradient from 1% to 65%	1.2 mL/min, 150 bar, and 40°C	MeOH:water (95:5; v/v) at 0.1 mL/min	Q-TOF, ESI in positive and negative modes

(Continues)

TABLE 2 | (Continued)

Ref.	Analytes	Sample	Column	Organic modifier	Other chromatographic conditions	Make-up solvent	Detector
[85]	Eight lipid classes	Human plasma and serum	Viridis BEH column (100 × 3 mm, 1.7 µm)	MeOH:water (99:1) with 30 mM ammonium acetate. Gradient from 1% to 51%	1.9 mL/min, 180 bar, and 60°C	MeOH:water (99:1) with 30 mM ammonium acetate at 0.25 mL/min	Q-TOF, ESI in positive mode
[82]	370 lipid compounds	Human serum	SFC: two Zorbax RX-SIL columns (2.1 × 150 mm, 5 µm)HPLC: Poroshell 120 EC C8 column (2.1 × 50 mm, 2.7 µm)	First dimension SFC: MeOH:water (98:2 v/v) with 20 mM ammonium formate Second dimension RP-HPLC: MeOH with 10 mM ammonium formate. Both in gradient mode	SFC: 0.4 mL/min, 160 bar, and 40°C RP-HPLC: 0.6 mL/min, 40°C	MeOH with 5 mM ammonium formate	QqQ, ESI in positive mode
[104]	171 lipid species	Human blood	Viridis BEH column (100 × 3 mm,1.7 mm)	MeOH:water (99:1, v/v) with 30 mM of ammonium acetate. Gradient from 1% to 51%	1.9 mL /min, 180 bar, and 60°C	MeOH:water (99:1, v/v) with 30 mM of ammonium acetate at 0,25 mL/min	Q-TOF, ESI in positive mode
[59]	244 lipids in exosomes and 191 lipids in plasma	Human plasma and exosomes	Viridis BEH column (100 × 3 mm, 1.7 µm)	MeOH:water (99:1, v/v) with 30 mM of ammonium acetate. Gradient from 1% to 51%	1.9 mL/min, 180 bar, and 60°C	MeOH:water (99:1, v/v) with 30 mM of ammonium acetate at 0.25 mL/min	Q-TOF, ESI in positive mode
[105]	153 gangliosides	Swine brain	^{1}D SFC: Acchrom Diol (4.6 × 150 mm, 5 µm) ^{2}D RP-UHPLC: XAqua C18 (Acchrom, 2.1 × 150 mm, 5 µm)	SFC: MeOH with 0,1% trifluoroacetic acid. Gradient from 50% to 60%. RP: (A) 20 mM ammonium formate (pH=3) and acetonitrile (B). Gradient from 50% to 100%	SFC: 3.5 mL/min, 150 bar, and 30°C UHPLC: 0.2 mL/min, 30°C	Not described	Q-TOF, ESI in positive and negative mode
	12 lipid classes	Rabbit blood	ACQUITY UPC2 Torus diethylamine (100 × 3.0 mm, 1.7 µm)	MeOH:water (95:5, v/v) with 0.1% (w/v) ammonium acetate. Gradient from 1% to 65%	1.0 mL/min, 150 bar, and 50°C	MeOH:water (95:5, v/v) with 0.1% (w/v) ammonium acetate at 0.20 mL/min	QqQ, ESI in positive mode
[106]	15 lipids	Cell culture matrix	Shimpack UC-X RP column (250 × 4.6 mm, 5.0 µm)	Ethanol in gradient mode from 1% to 30%	5.0 mL/min, 400 bar, and 40°C	Not described	QqQ, ESI in positive mode

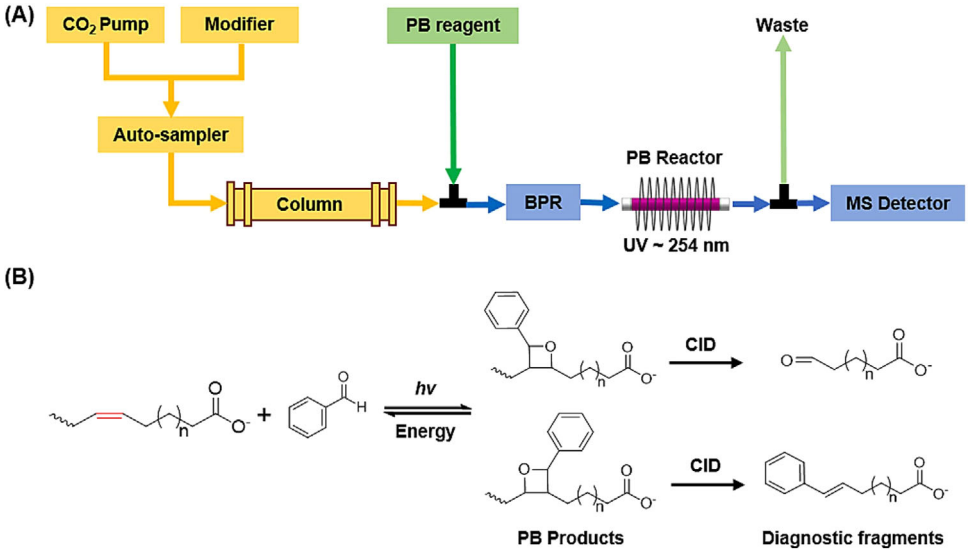

FIGURE 3 | Schematic diagram of SFC-PB-Q-TOF MS system for double bonds identification with benzaldehyde in unsaturated fatty acids (UFAs). (A) The experimental apparatus used for lipid identification. (B) Derivatization reaction scheme of double bonds with benzaldehyde and subsequent CID fragmentation. Reproduced with permission [83]. 2023 Elsevier.

of drug substances, such as to assess the purity of the active ingredients and to impurity profiling; but also SFC provided good results, at preparative or semipreparative scales, in the earliest stages of the development of a drug candidate. Nowadays, SFC is also applied to determine pharmaceuticals in biological samples for pharmacokinetics or toxicological studies.

The advantages described when using SFC methods are the short analysis times, resulting in higher throughput, the use of small injection volumes, which facilitates the analysis of samples for which only small quantities are available, and the possibility of injecting the extracts without the removal of the organic solvent, reducing the number of sample treatment steps.

Usually, the compounds studied are small molecules in plasma or serum samples and the method developed is applied to pharmacokinetics studies. An important aspect of the pharmacokinetic study of chiral drugs is the evaluation of chiral inversions in vivo and in vitro. Chen et al. [90] used SFC-MS/MS to investigate the possible chiral inversion of three chiral drug candidates. The enantiomeric separation was achieved on polysaccharide-based stationary phases with analysis times lower than 5 min (Figure 4). The successful results obtained demonstrated the utility of SFC-MS/MS in drug discovery. Other most complex molecules, such as monoclonal antibodies, have also been analyzed using SFC. The glycoform of a therapeutic monoclonal antibody (mAb) plays an important role in its pharmacokinetics and safety, but the complete analysis of the glycan structure is a challenging task due to its complexity. Haga et al. [91] developed an SFC-MS/MS method for glycan profiling and it was applied to the glycan structural analysis of mAbs. Sample treatment included the peracetylation of glycans, to increase their solubility in CO_2. The separation was performed on a Shim-pack UC-Phenyl (2.1 × 150 mm, 3 μm) column achieving the quantitative analysis of 102 glycan structures in 8 min. This is clearly an advantage compared to previously published works in which about 20 structures were detected in 45 min using LC-MS/MS. Moreover, the method sensitivity was high, with detection and quantification limits of 5 and 10 attomoles, respectively.

Table 3 summarizes the applications of SFC in pharmaceutical and clinical analysis during the last 5 years.

5.3 | Doping Control and Toxicological Analysis

The potential of SFC in doping and toxicological analysis has been also shown in the last few years. Considering doping analysis, one of the first applications was the use of chiral SFC to determine clenbuterol enantiomers in urine, to distinguish deliberate from accidental consumption [92]. Since then, the published SFC methods have increased progressively and have been recently reviewed [93]. Although both GC and UHPLC-MS/MS are the analytical techniques most broadly employed in doping analysis, SFC has provided satisfactory results, especially in the analysis of highly polar compounds that are poorly retained in RP-LC. On the other hand, SFC offers complementary separations to LC, which is useful not only for confirmation but also for screening analysis.

The use of SFC in routine anti-doping analysis has been studied in several works. In this way, Wuest et al. [94] developed an SFC-MS/MS method to determine 197 drugs and metabolites prohibited in sports. The column employed was based on 2-ethylpirydine and the modifier was methanol:water (96.5:3.5, v:v) with 5 mM ammonium acetate, delivered in a gradient mode (from 2 % to 62.5%). They analyzed more than 1000 urine samples using the "dilute and shoot" approach. The method was satisfactorily validated and the matrix effect was negligible. The results obtained proved that it was robust for routine anti-doping analysis. Moreover, compared with RP-LC-based methods, it provided similar performance for most of the compounds analyzed, being advantageous for the analysis of the

TABLE 3 | Supercritical fluid chromatography (SFC) applications in pharmaceutical and clinical analysis.

Ref.	Analytes	Sample	Column	Organic modifier	Other chromatographic conditions	Make-up solvent	Detector
[107]	Cinnarizine	Rat plasma	Torus 1-AA column (3.0 × 100 mm, 1.7 μm)	MeOH with 5 mM ammonium acetate. Gradient from 10% to 30%	1.5 mL/min, 200 bar, and 50°C	MeOH at 0.2 mL/min	QqQ, ESI positive mode
[108]	Atorvastatin, 2-hydroxy atorvastatin, and tangeretin	Rat plasma	ACQUITY Torus 1-AA column (100 × 3 mm, 1.7 μm)	MeOH with 0.2% formic acid. Gradient from 10% to 40%	1.0 mL/min, 200 bar, and 50°C	MeOH with 0.1% formic acid at 0.1 mL/min	QqQ, ESI positive mode
[56]	Pseudouridine and uridine	Human serum	Acquity UPC2 Torus DIOL column (2.1 × 50 mm, 1.7 μm)	MeOH with 5% water. Gradient from 2% to 60%	1.0 mL/min, 200 bar, and 50°C	MeOH with 0.1% formic acid at 0.2 mL/min	QqQ, ESI negative mode
[91]	Glycans from therapeutic monoclonal antibodies (bevacizumab, nivolumab, ramucirumab, rituximab, and trastuzumab	Therapeutic monoclonal antibodies	Shim-pack UC-Phenyl (2.1 × 150 mm, 3 μm)	MeOH. Gradient from 10% to 40%	1.0 mL/min and 40°C	MeOH with 0.1% ammonium formate at 0.1 mL/min	QqQ, ESI in positive mode
[109]	Naringenin and its carbamate prodrug	Rat plasma	ACQUITY UPC2TM BEH 2-EP (3.0 × 100 mm, 1.7 μm)	MeOH:water (99/1) with 0.2% formic acid. Gradient from 10% to 60%	1.0 mL/min, 170 bar, and 40°C	MeOH at 0,1 mL/min	QqQ, ESI in positive mode
[110]	Naproxen and esomeprazole	Dog plasma	ACQUITY UPC2TM BEH column (100 × 3 mm, 1.7 μm	MeOH. Gradient from 6% to 70%	1.5 mL/min, 200 bar, and 50°C	MeOH with 0.1% formic acid at 0.2 mL/min	QqQ, ESI in positive mode
[111]	Flupirtine and its acidic and basic metabolites	Human urine	Lux Amylose-2 (150 × 4.6 mm, 5 μm)	MeOH with 0.075% ammonia and 0.225% water. Gradient from 5% to 25%	4.0 mL/min, 150 bar, and 40°C	MeOH at 0.1 mL/min	Single quadrupole, ESI
[90]	Three chiral compounds	Plasma	Chiralpak AD-3, Chiralcel OJ-3and Chiralcel OZ-3 (150 × 3.0 mm, 3 μm)	MeOH with 0.1% ammonia. Gradient from 5% to 40%	3.0 mL/min, 100 bar, and 40°C	Not required	QTRAP, ESI positive mode
[112]	Nimodipine. IS: nitrendipine	Beagle plasma	Acquity UPC BEH2-EP (100 × 3.0 mm 1.7 μm)	MeOH with 0.5% formic acid and 5 mM ammonium acetate. Gradient from 10% to 40%	1.5 mL/min, 180 bar, and 50°C	Not described	QqQ, ESI in positive mode

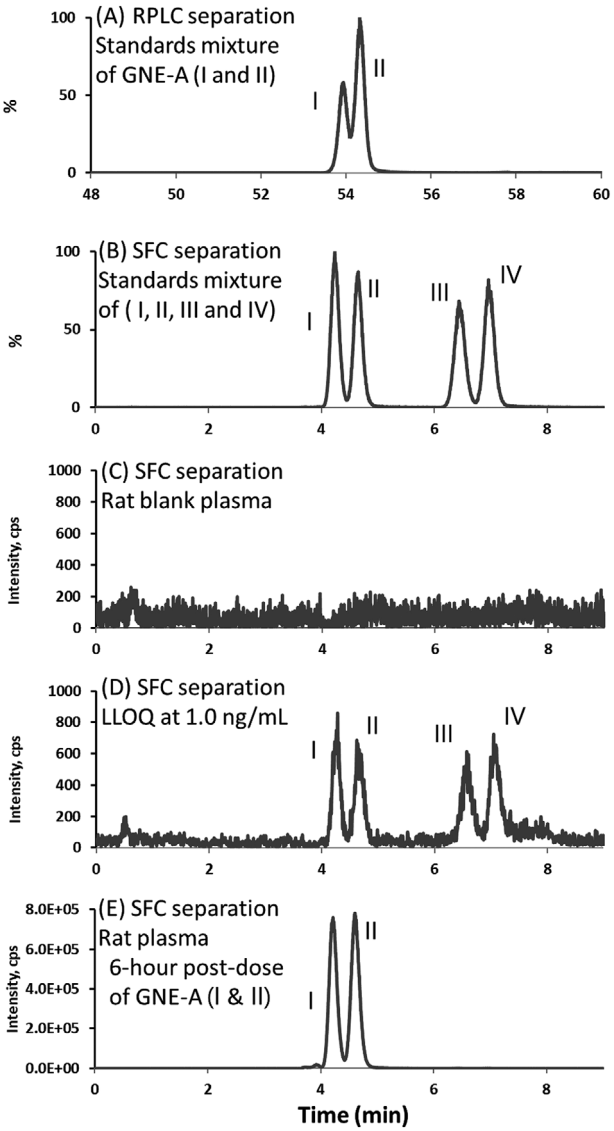

FIGURE 4 | Chiral analysis of GNE-A (mixture of I and II) and its epimer metabolites (III and IV). (A) Reversed-phase liquid chromatography-multiple reaction monitoring (RPLC-MRM) chromatogram of GNE-A (mixture of I and II) (B) Supercritical fluid chromatography (SFC)-MRM chromatogram of I, II, III, and IV from a mixture of reference standards. (C) SFC-MRM ion chromatogram of blank rat plasma. (D) Spiked plasma samples with 1.0 ng/mL for each diastereomer. (E) SFC-MRM ion chromatogram of a 6-h post-dosed selected plasma sample for a subject dosed with GNE-A. Reproduced with permission [90]. 2019 Elsevier.

early eluting LC-MS/MS compounds. Losaco et al. [95] studied the retention time variability of a UHPSFC-MS/MS analytical method for 51 doping compounds in standard solutions and in human urine samples, over four months. They employed three different stationary phases: Torus 2- Picolylamine, UPC2 Viridis BEH, and Acquity UPLC HSS C18 SB. The inter-moth RSD were 0.5% and 1.3% for the Torus 2-PIC column and Viridis BEH silica, respectively, similar to those obtained with UHPLC-MS/MS. On the contrary, using the HSS C18 SB column the RSD was higher. Despite this, the results showed the potential of the method for routine analysis.

Ion-exchange separations have scarcely been studied in SFC. Xhaferaj et al. [96] developed a SFC-MS/MS method to determine highly polar and ionic analytes in urine samples. They employed an ion-exchange stationary phase and a mixture of additives (water, ammonium formate, and formic acid) added to the organic modifier, thus the interaction mechanisms included ion-exchange and mixed mode. Method validation was satisfactorily performed and the quantification limits ranged from 0.005 to 2.5 mg/L. Considering the HPLC-MS/MS existing methods, the SFC-MS/MS method provided comparable results and the author considered the method suitable for anti-doping or forensic analysis.

In toxicological analysis, chiral separations play an important role because in some cases common drugs of abuse are also used therapeutically, this is the case of amphetamine. The prescribed drug is composed of one enantiomer while the illegal drug is racemic. In this case, the enantiomeric analysis is necessary to distinguish between legal and illegal intake. In other cases, the enantiomers of a drug can have different legal restrictions and also the development of enantiomeric methods of analysis is of utmost importance. Chiral SFC has been successfully applied to the enantiomeric analysis of chiral drugs in urine, serum, plasma, or post-mortem samples, obtaining high enantioresolutions in short analysis times (2–4 min) [54, 97–99]. Usually, sample treatment consists of an LLE or SPE extraction, but other newer approaches such as dried matrix spots using Whatman paper [97] or electromembrane extraction [99] have also been applied.

Table 4 summarizes the applications of SFC in doping and toxicological analysis during the last 5 years.

6 | Summary and Outlook

While LC or GC are the first choices for bioanalysis, the interest in SFC has increased in recent years owing to the advances in instrumentation, the hyphenation of MS, and the development of innovative methodologies. The commercialization of new stationary phases and the possibility of using columns packed with sub-2 μm particles have improved the chromatographic performance and reduced the analysis time, consequently increasing its use in bioanalysis applications, with UHPSFC showing similar performances to UHPLC. In addition, the possibility of analyzing, in the same run, compounds with a broad range of polarities is one of the main advantages. In this way, the use of water as an additive and the use of wide elution gradients where the percentage of organic modifier increases from a small value to 100%, applying UC conditions, have had an important impact on SFC applicability. Nowadays, SFC can be used to analyze very different compounds, from nonpolar to highly polar and even ionic ones. All of this, using the same instrument, column, and mobile phase components.

The matrix effect in SFC-MS has been reported to be similar to LC-MS. It depends on the type of compounds and sample to be analyzed and, obviously, on the sample treatment. Concerning sample treatment, another advantage of SFC in bioanalysis applications is that is not necessary to remove the organic solvent used for extraction. Frequently, the described sample treatment is very simple, like protein precipitation or “dilute and shoot”

TABLE 4 | Supercritical fluid chromatography (SFC) applications in doping control and toxicological analysis.

Ref.	Analytes	Sample	Column	Organic modifier	Other chromatographic conditions	Make-up solvent	Detector
[54]	Zopiclone (ZOP)	Human serum	Waters Trefoil CEL2 (50 × 3.00 mm, 2.5 μm)	MeOH with 20 mM ammonium formate. Isocratic 40%	2.0 mL/min, 103 bar, and 40°C	MeOH with 20 mM ammonium formate at 0.1 mL/min	Q-TOF, ESI in positive mode
[94]	197 drugs and metabolites prohibited in sports	Human urine	ACQUITY UPC2 BEH2-EP (100 × 3.0 mm, 1.7 μm)	MeOH:water (96.5:3.5, v/v) with 5 mM ammonium acetate. Gradient from 2% to 62.5%	1.4 mL/min, 150 bar, and 28°C	Not described	QqQ, ESI in positive and negative modes
[99]	Amphetamine enantiomers	Human urine	Chiralpak AD-3 (150 × 2.1 mm, 3.0 μm)	MeOH:IPA (1:1, v/v) with 0.1% ammonium hydroxide. Isocratic 6.5%	1.0 mL/min, 250 bar, and 10°C	IPA with 0.1% ammonium hydroxide at 0.3 mL/min	QqQ, ESI in positive mode
[97]	methadone(R and S MTD) and 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (R and S EDDP)	Post-mortem samples: blood, vitreous humor, etc.	Chiralpak IH-3 (150 × 3.0 mm, 3 μm)	MeOH:water (98:2, v/v) with ammonium acetate 20 mM. Gradient from 10% to 40%	0.4 mL/min, 150 bar, and 30°C	MeOH:water (98:2, v/v) with ammonium acetate 20 mM at 0.5 mL/min	QqQ, ESI in positive mode
[96]	Polar and ionic drugs	Human urine	SFC-SCX (sulfonic acid) (150 x 4.6 mm, 5 μm)	MeOH:water (95/5 v/v) with ammonium formate 20 mM and formic acid 15 mM. Gradient from 15% to 60%	Flow gradient from 2.0 to 2.5 mL/min, 170 bar, and 45°C	Not described	QqQ, ESI in positive and negative modes
[95]	51 doping agents	Human urine	BEH silica (100 × 3.0 mm, 1.7 μm). Torus 2-PIC (100 × 3.0 mm, 1.7 μm) HSS C18 (100 × 3.0 mm, 1.8 μm)	MeOH:water (98:2, v/v) with 20 mM ammonium formate Gradient: from 2% to 50%	1.2 mL/min, 120 bar, and 40°C	MeOH at 0.3 mL/min	QqQ, ESI in positive and negative modes
[113]	45 doping agents	Human urine	Waters UPC2 Torus DIOL (100 × 2.1 mm, 1.7 μm)	EtOH:water (99:1, v/v) with 10 mM ammonium formate. Gradient from 5% to 40%	1.2 mL/min, 150 bar, and 50°C	MeOH at 0.1 mL/min	QqQ, ESI in positive and negative modes
[98]	R/S-amphetamine	Human serum	Chiralpak AD-3 (150 × 2.1 mm, 3.0 μm)	MeOH:IPA (1:1, v/v) with 0.1% of ammonium hydroxide. Isocratic 6.5%	1.0 mL/min, 250 bar, and 10°C	IPA with 0.1% ammonium hydroxide at 0.3 mL/min	QqQ ESI in positive mode
[114]	Perflurooctane sulfonate (PFOS)	Human serum	Chiralpak QN-AX (150 × 4.6 mm, 5 μm)	IPA with 0.5% formic acid and 0.2% diethylamine Isocratic 30%	1.0 mL/min, 180 bar, and 32°C	Not described	QqQ, ESI in negative mode
[55]	34 perfluoroalkylated substances (PFASs PerPFASs)	human milk and food matrices	ACQUITY UPC2 Torus 2-PIC (100 × 3.0 mm, 1.7 μm)	MeOH with 20 mM ammonium acetate. Gradient from 10% to 30%	2.0 mL/min, 138 bar, and 50°C	MeOH 0.3 mL/min	QqQ, ESI in positive mode

approaches. Moreover, instruments where SFE is online coupled to SFC have also been commercialized.

We expect that the use of SFC in bioanalysis will increase in the future, as well as the studies of its suitability in routine analysis. Moreover, new instrumental improvements related to the ability to withstand high pressures, especially when using columns packed with sub–2 μm particles, and the reduction of the system void volume are required. This will provide faster and more efficient separations.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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