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Review

Supercritical fluid chromatography – Mass spectrometry in metabolomics: Past, present, and future perspectives

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ABSTRACT

Metabolomics, which consists of the comprehensive analysis of metabolites within a biological system, has been playing a growing role in the implementation of personalized medicine in modern healthcare. A wide range of analytical approaches are used in metabolomics, notably mass spectrometry (MS) combined to liquid chromatography (LC), gas chromatography (GC), or capillary electrophoresis (CE). However, none of these methods enable a comprehensive analysis of the metabolome, due to its extreme complexity and the large differences in physico-chemical properties between metabolite classes. In this context, supercritical fluid chromatography (SFC) represents a promising alternative approach to improve the metabolome coverage, while further increasing the analysis throughput. SFC, which uses supercritical CO_2 as mobile phase, leads to numerous advantages such as improved kinetic performance and lower environmental impact. This chromatographic technique has gained a significant interest since the introduction of advanced instrumentation, together with the introduction of dedicated interfaces for hyphenating SFC to MS. Moreover, new developments in SFC column chemistry (including sub-2 µm particles), as well as the use of large amounts of organic modifiers and additives in the CO_2-based mobile phase, significantly extended the application range of SFC, enabling the simultaneous analysis of a large diversity of metabolites. Over the last years, several applications have been reported in metabolomics using SFC-MS – from lipophilic compounds, such as steroids and other lipids, to highly polar compounds, such as carbohydrates, amino acids, or nucleosides. With all these advantages, SFC-MS is promised to a bright future in the field of metabolomics.

1. Introduction

Metabolomics, first introduced in the early 2000s, consists of the comprehensive analysis of metabolites (*i.e.*, low-molecular weight molecules, typically ≤1 kDa) within a biological system, including intermediate and end-products of the metabolism [1,2]. Together with other –omics approaches (*e.g.*, genomics, transcriptomics, and proteomics), metabolomics plays an essential role in the implementation of personalized medicine in modern healthcare. By providing a snapshot of the physiological processes currently taking place in an organism, metabolomics brings an important and unique insight into disease etiology [3,4]. In the last two decades, an increasing number of applications have been reported in the literature, showing its relevance in biomarker discovery, pathway elucidation, identification of novel drug

Abbreviations: **APCI**, atmospheric pressure chemical ionization; **API**, atmospheric pressure ionization; **BPR**, backpressure regulator; **cSFC**, capillary column supercritical fluid chromatography; **CE**, capillary electrophoresis; **EMA**, European Medicines Agency; **EP**, ethylpyridine; **ESI**, electrospray ionization; **GC**, gas chromatography; **GHB**, gamma-hydroxybutyrate; **HILIC**, hydrophilic interaction chromatography; **HMDB**, Human Metabolome Database; **HRMS**, high-resolution mass spectrometry; **LC**, liquid chromatography; **MS**, mass spectrometry; **NIST**, National Institute of Standards and Technology; **NMR**, nuclear magnetic resonance; **NPLC**, normal-phase liquid chromatography; **pSFC**, packed-column supercritical fluid chromatography; **RPLC**, reversed-phase liquid chromatography; **SFC**, supercritical fluid chromatography; **UC**, unified chromatography; **UHPLC**, ultra-high pressure liquid chromatography; **UHPSFC**, ultra-high performance supercritical fluid chromatography; **UV/Vis**, ultraviolet/visible.

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targets, disease prognosis, and treatment individualization [5–12].

The metabolome is remarkably complex, as highlighted by the more than 110,000 annotated metabolites listed in the latest version of the Human Metabolome Database (HMDB 4.0) [13]. Such a wide diversity in structures and chemical properties requires state-of-the-art analytical techniques capable of separating, detecting, characterizing, and – ultimately – quantifying metabolites. Metabolites are typically analyzed using nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS)-based techniques, the latter being usually combined with liquid chromatography (LC), gas chromatography (GC), and capillary electrophoresis (CE). Among those techniques, ultra-high pressure liquid chromatography (UHPLC)-MS is considered the gold standard due to its great sensitivity, high throughput, and important resolving power resulting in a relatively broad metabolome coverage [14,15]. However, a comprehensive analysis of the metabolome can be realistically envisioned only when using a combination of different chromatographic modes or orthogonal analytical techniques, due to the large differences in physico-chemical properties observed between different metabolite classes. Indeed, optimal experimental conditions can largely differ between metabolite classes depending on their polarity.

The success of metabolomics-based studies also relies on the number of samples analyzed, as a higher sample size results in an increased statistical power, which in turns leads to a higher probability of obtaining significant results. A sufficient statistical power is extremely important in metabolomics to obtain biologically or clinically relevant results, especially in human studies, due to the large inter- and intra-individual variability. Large-scale metabolomics studies are therefore frequently required, leading to the inclusion of hundreds of subjects in the study and the collection of hundreds or thousands of samples. This highlights the need for (ultra) high-throughput approaches, which enable the analysis of one sample within a couple of minutes, also reducing the overall costs associated to the analysis.

One of the major challenges in metabolomics therefore relies on the development of analytical approaches that can enable both high throughput analysis and broad metabolome coverage. Based on these two features, supercritical fluid chromatography (SFC)-MS represents one of the most promising alternative techniques in metabolomics. SFC uses a mobile phase composed of a mixture of a supercritical fluid (typically CO_2) and an organic modifier. Using an adequate gradient and suitable additive(s) at low percentages (*e.g.*, water, salts), the simultaneous analysis of metabolites with large differences in their polarities can be achieved. Moreover, due to the inherent composition of the mobile phase (*i.e.*, low viscosity and high diffusivity), higher mobile phase velocities can be used without significant loss of optimum efficiency and at a lower backpressure, theoretically allowing for (ultra) high-throughput analysis. Finally, by using a high proportion of CO_2 in the mobile phase – a non-toxic and recyclable fluid, SFC is more respectful of the principles of green analytical chemistry than LC-based approaches [16].

SFC has made a remarkable comeback in the early 2010s since the commercialization of improved instruments resulting in better robustness and reproducibility, followed by the development of dedicated columns packed with sub-2 µm particles (ultra-high performance supercritical fluid chromatography, UHPSFC). Moreover, significant technological improvements have been carried out over the last few years, with the development of different interfaces to successfully hyphenate SFC with MS [17]. Altogether, these improvements have fostered the use of SFC-MS in a broader range of bioanalytical applications. SFC-MS holds many promises in the field of metabolomics, as highlighted by the exciting applications reported in recent years in the field of lipid profiling, polar metabolome analysis, and chiral metabolomics.

2. Supercritical fluid chromatography

Depending on the temperature and pressure, a compound may exist in the gas, liquid, or solid state. When increasing both the temperature and pressure until their respective critical point, the substance enters in the so-called supercritical state [18]. As the physical properties of the fluids show gradual instead of abrupt changes when reaching this supercritical state, it is seen as a continuous phase. Supercritical fluids exhibit particular properties, which make them very attractive in separation sciences, *i.e.*, a density close to that of liquids, and a viscosity and diffusivities close to those of gases, respectively [19].

2.1. Technological evolutions over the years

When first introduced to the community more than 50 years ago, SFC did not raise much attention, as GC was already a well-established techniques with commercially available instrumentation [20]. The first important milestone was the introduction of open-tubular capillary column SFC (cSFC) by Milton and co-workers in the 1980s (Fig. 1) [21]. Due to the rather poor flexibility offered by this system, cSFC using neat CO_2 was mainly limited to the petrochemical industry and relatively rapidly replaced by packed-column SFC [20]. Packed-column SFC (pSFC), also initially introduced in the 1980s and inspired by LC separation principle, became more popular in the 1990s as it demonstrated its applicability to a broader range of compounds when using organic modifiers in the mobile phase. Initially, pSFC separations were performed using LC columns. The years 2000s saw the commercialization of the first dedicated pSFC stationary phases such as the 2-ethylpyridine (2-EP) phase, which was designed for the analysis of basic compounds.

The most important pSFC breakthrough occurred in 2012 with the commercialization of (i) hybrid SFC/UHPLC instruments, offering improved backpressure regulation, continuous flow rate, and automation capabilities; and (ii) ultra-high performance supercritical fluid chromatography (UHPSFC) systems, enabling the use of modern SFC columns packed with sub-2 µm fully porous particles and sub-3 µm superficially porous particles, resulting in an increased separation efficiency and enabling a higher mobile phase velocity without compromising the efficiency [20,22].

A large diversity of stationary phases dedicated to pSFC analysis have been commercialized over the last decade. pSFC analyses can be carried out in both normal and reversed-phase conditions. In reversed-phase mode, typical stationary phases include C_{18}, phenyl, cyanopropyl, and pentafluorophenyl. In normal phase conditions, polar columns such as bare silica, cyanopropyl, diol or amino columns are usually used. Normal phases are well suited for pSFC analysis due to the non-polar nature of CO_2. Polar hydrophilic interaction chromatography (HILIC)-type stationary phases, such as nitrogen-containing phases (amine or triazole-type), neutral phases (amide and alcohol-type), or silica phases are also suitable for pSFC analysis [23].

In general, all stationary phases developed for LC are also suitable for pSFC analysis, including chiral phases based on immobilized polysaccharides, cyclodextrins, macrocyclic glycopeptides, and “Pirkle”-type phases (with the exception of some protein-based columns since aqueous conditions are required for the analysis). Specific stationary phases dedicated for pSFC include 2-EP, 4-EP, pyridine amide, propylacetamide, aminophenyl, and pyridyl amide, which lead to improved peak shapes and decreased peak tailing (especially for basic compounds). More recently, novel stationary phases such as 2-picolylamine or 1-amino-anthracene, have been developed to provide additional selectivity and address the so-called silyl ether formation, which is a major contributor to retention shifts and alteration of selectivity over time [24]. This silyl ether formation is also reduced when using modern phases with high-density bonding [25] or when adding small amount of water in the mobile phase [26]. Fig. 2 displays a Spider diagram classifying a large diversity of commercially available SFC phases based on the adjusted linear solvation energy relationship model [27,28].

Due to its low critical parameters, *i.e.*, $T_c = 31$ °C and $P_c = 74$ Bar, and favorable properties, CO_2 is by far the most used solvent in SFC [29]. In order to maintain the pressure in the column and keep the CO_2-based

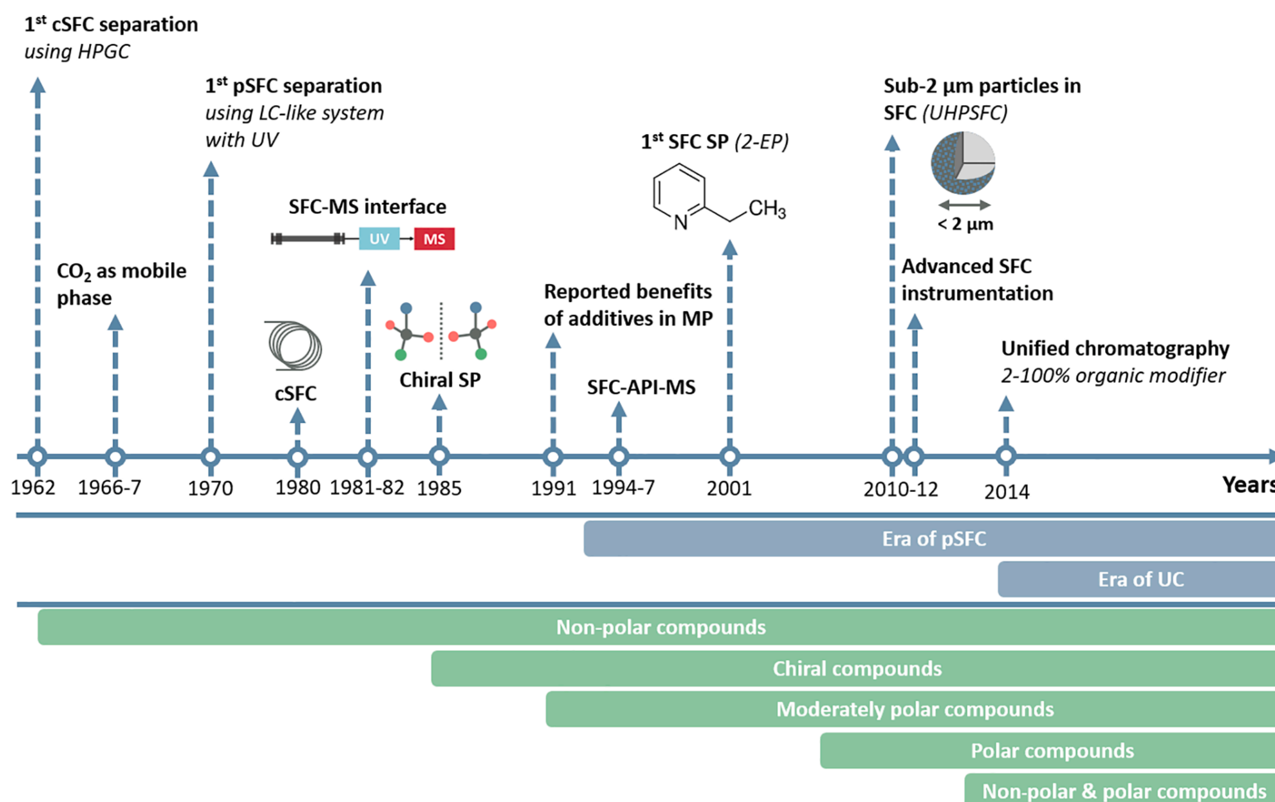

Fig. 1. Chronological developments of SFC between 1962 and 2014. cSFC, capillary column supercritical fluid chromatography; pSFC, packed-column supercritical fluid chromatography; MP, mobile phase; SP, stationary phase; UC, unified chromatography; API, atmospheric pressure ionization; EP, ethylpyridine; UHPSFC, ultra-high performance supercritical fluid chromatography. Adapted from [20] with permissions.

mobile phase in supercritical (or subcritical) state, SFC instruments are equipped with a backpressure regulator (BPR), which plays a crucial role in the experimental reproducibility. The solvation power of CO_2, a non-polar solvent, is often not sufficient for the elution of more polar compounds, which requires the use of organic modifiers and other additives in the mobile phase. Organic modifiers enable the retention and elution of polar compounds using different mechanisms, *i.e.*, (i) modification of the mobile phase polarity, (ii) change in the mobile phase density, (iii) block the active sites on the stationary phase, (iv) modifications of stationary phase characteristics through sorption of the organic modifier, (v) increase in the net volume of the stationary phase, and (vi) formation of clusters showing different distribution properties [24]. However, adding a proportion of organic modifier in the mobile phase also affects the critical parameters, shifting the supercritical conditions to a higher pressure and temperature. With a high percentage of organic modifier, the critical parameters are often too difficult to reach with current instruments. In this case, the mobile phase is not considered as a true supercritical fluid, but more likely as a subcritical fluid [30]. Some of the most common organic modifiers used in modern SFC include methanol, ethanol, isopropanol, and acetonitrile.

Despite the presence of organic modifiers in the mobile phase, strong acids and bases typically remain very difficult to elute in SFC. In this case, small amounts (mM/low-percent levels) of additives such as organic acids, bases, buffers, and water, can be added to the mobile phase. Generally, basic and acid additives are used for basic and acid compounds, respectively. Albeit, such additives may not be needed for the analysis of acidic compounds due to the acidic nature of CO_2 in methanol [31]. The addition of organic modifiers and additives not only allows for the retention and elution of strong acids and bases, but is also beneficial for the selectivity, efficiency, and peak shape. With such flexibility in the composition of the mobile phase, a wide range of compounds with large differences in physico-chemical properties can be analyzed within one SFC run using the same system and stationary phase, as illustrated in Fig. 3. This flexibility is typically not possible with other chromatographic techniques, illustrating the promising role that SFC can play in metabolomics, where analytical techniques allowing for the most comprehensive analysis of the metabolome are urgently needed [32].

2.2. *Hyphenation to mass spectrometry*

Detectors conventionally used in LC are also suitable for pSFC detection, which are either placed before (*i.e.*, pre-decompression detectors, such as UV/Vis and fluorescence) or after (*i.e.*, post-decompression detectors, such as evaporative light scattering detector) the BPR. Similar to other chromatographic techniques, those detection modes are often not sufficiently sensitive and selective for bioanalytical applications, where MS detection becomes essential. The first applications of SFC-MS were reported in the 1970s, using cSFC instruments combined to MS *via* GC-type ionization sources such as electron impact and chemical ionization. Today, commercial SFC-MS instruments are all based on pSFC and the coupling is performed using atmospheric pressure ionization (API) sources, mainly electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), with a dedicated interface.

2.2.1. *Supercritical fluid chromatography – mass spectrometry: interfaces*

The CO_2-based eluent must be depressurized before entering the ionization source in a controlled manner to maintain the chromatographic separation and achieve adequate MS detection [33–35]. Moreover, as the CO_2 decompresses, the density of the fluid decreases, leading to evaporation of CO_2 and potentially causing analyte precipitation. Therefore, a dedicated interface is needed to ensure a successful coupling between SFC and MS [36]. Five main interfaces have been

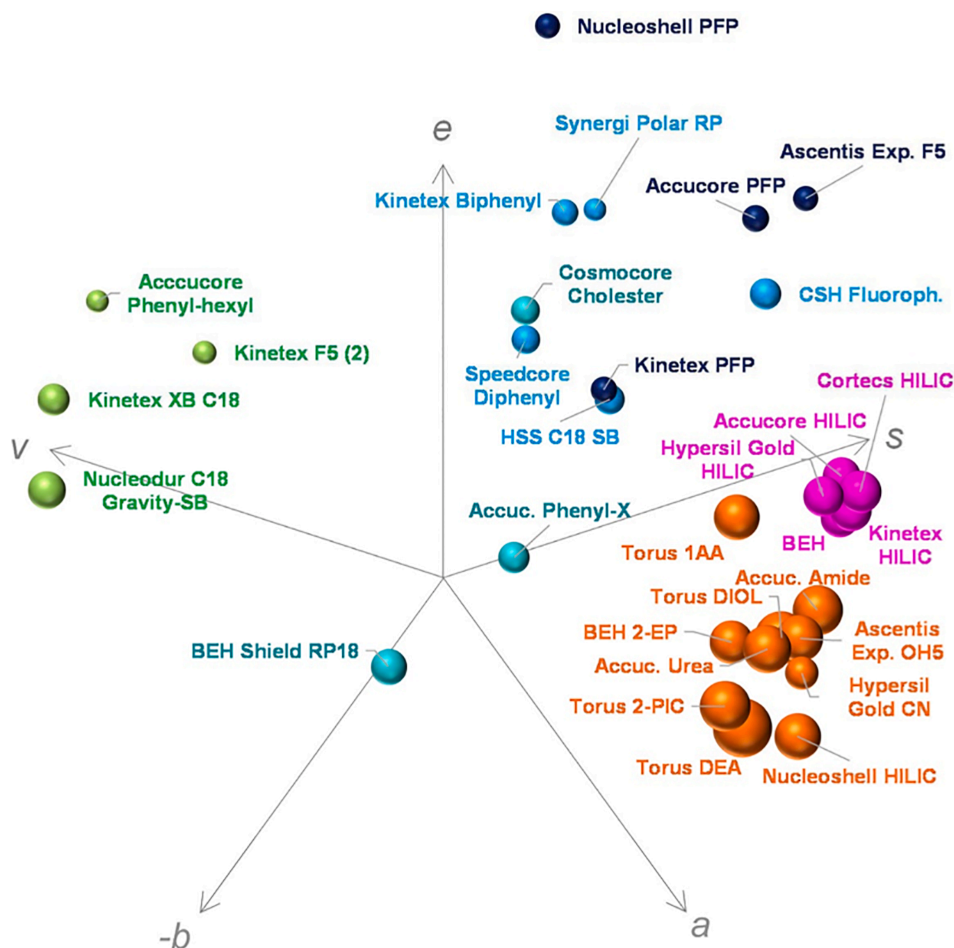

Fig. 2. Spider diagram based in the improved linear solvation energy relationship model with 7 descriptors, with retention data using 85 neutral species and 24 ionic species, and 31 different columns. Colors are used to indicate which columns cluster together. The most non-polar stationary phases lie in the center-left area, including most of C_{18} phases as well as phenyl-type columns. On the other hand, the polar stationary phases are presented in the center-right and lower right areas. The fluorophenyl (PFP and F5) columns are grouped in the upper area. See [27] for more explanations on the linear solvation energy relationship model. Reprinted from [27] with permissions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

developed so far, namely, (i) direct coupling; (ii) pre-UV and BPR splitter without sheath pump; (iii) pressure control fluid; (iv) pre-BPR splitter with sheath pump; and (v) BPR and sheath pump with no splitter, all displayed in Fig. 4 [34]. Among those five interfaces, two are currently commercially available, namely, the pre-BPR splitter with sheath pump interface (Fig. 4D) and the BPR and sheath pump with no splitter interface (Fig. 4E).

In the pre-PBR splitter with a sheath pump interface (Fig. 4D), the UV detector is located directly after the column and before the BPR. An added sheath liquid is mixed with the SFC effluent after the UV cell and introduced into the MS system. A proportion of the flow is directed towards the MS, while the remaining proportion goes to the BPR. The presence of the sheath liquid prevents the analyte precipitation during the decompression of CO_2. Moreover, this SFC-MS interface is heated to compensate for the cooling effect during CO_2 decompression [37]. Since the BPR is located downstream of the system, the chromatographic separation efficiency is maintained [38]. This interface has been reported to be reproducible and sensitive, enabling both qualitative and quantitative analysis [37,38].

In the BPR and sheath pump with no splitter interface (Fig. 4E), which is the latest interface commercialized, the column effluent is mixed with an added sheath liquid before the BPR. Unlike the previously described interface, this allows for the injection of the entire effluent in the MS system, which makes it better suited for APCI. The BPR and sheath pump with no splitter interface being relatively recent, few applications have been reported in the literature so far [39,40].

Among the possible commercially available interfaces, it remains difficult to determine the ideal configuration in terms of sensitivity and potential matrix effects. Indeed, different MS instruments are used with these interfaces, which hampers a direct comparison between the different interfaces [35]. Comparative studies using the same MS instrument (enabling both full-flow and split-flow configurations) are therefore still needed.

2.2.2. *Supercritical fluid chromatography – mass spectrometry: applicability in bioanalysis*

The sensitivity obtained with SFC-MS has been reported to be generally comparable to LC-MS, using either ESI or APCI, or even higher, depending on the experimental conditions and the analytes of interest [41–43]. The potential sensitivity gain observed with SFC-MS is also strongly dependent on the MS instrument (instrument generation and source design), the chromatographic conditions, and the composition of the sheath liquid [35,44–46]. As an example, a comprehensive study compared the performance observed for the analysis of a set of doping agents in urine using SFC-MS and LC-MS with two MS instruments (triple quadrupole mass analyzer) from the same vendor, but of a different generation [45,46]. With the first-generation MS instrument, *ca.* 27% of the doping agents showed a similar sensitivity between SFC-MS and LC-MS, while 65% of the analytes showed a better sensitivity using SFC-MS. However, this gain in sensitivity was more limited when using the relatively modern instrument, where 46% of the substances showed a similar sensitivity between SFC-MS and LC-MS, while 38% led to a better sensitivity using SFC-MS [46].

Matrix effects, *i.e.*, the alteration of the ionization efficiency by co-eluting molecules, are known as the Achilles heel of LC-MS and represent an important aspect during development and validation of bioanalytical methods. Matrix effects are particularly relevant in metabolomics as they can negatively affect the accuracy, precision,

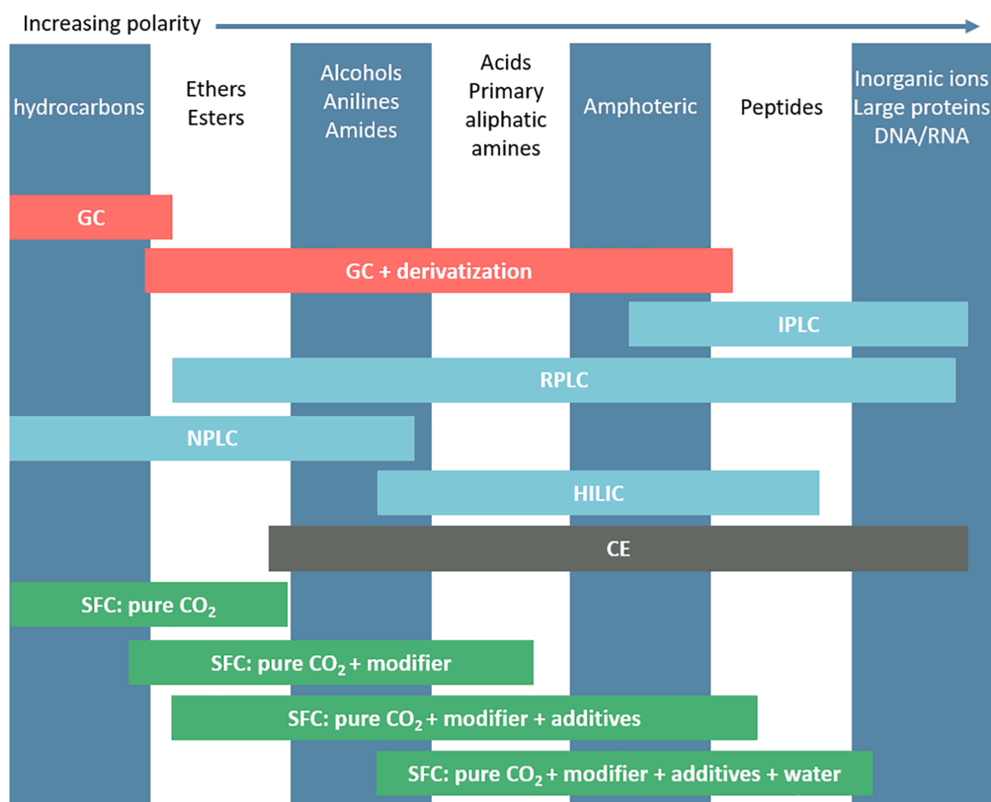

Fig. 3. Overview of the application range of supercritical fluid chromatography (SFC) compared with gas chromatography (GC), ion-pairing liquid chromatography (IPLC), reversed-phase liquid chromatography (RPLC), normal-phase liquid chromatography (NPLC), hydrophilic interaction liquid chromatography (HILIC), and capillary electrophoresis (CE). Adapted from [24].

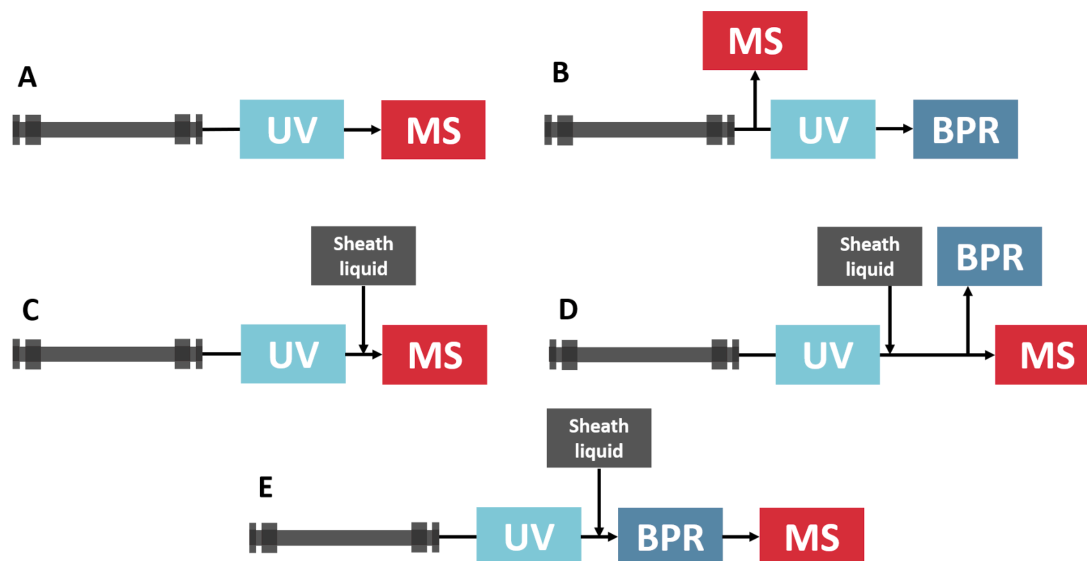

Fig. 4. Schematic overview of the five developed SFC-MS interfaces. A. Direct coupling interface; B. Pre-UV and BPR splitter without sheath pump interface; C. Pressure control fluid interface; D. Pre-BPR splitter with sheath pump interface; and E. BPR and sheath pump with no splitter interface. Commercially available instruments are equipped either with a pre-BPR splitter with sheath pump interface or a BPR and sheath pump with no splitter interface. BPR, backpressure regulator. Adapted from [17] with permissions.

linearity, and sensitivity of the analysis. Limited research on the matrix effects in SFC-MS has been published so far, but the comparative studies available have reported that SFC-MS is also affected by matrix effects, however to a different extent than LC-MS depending on the matrix and the application. For instance, a lower number of analytes prone to matrix effects in urine have been reported using SFC-MS, *i.e.*, *ca.* 30% *vs.* 50% using LC-MS [46,47]. SFC-MS has also shown to be less prone to ion enhancement than LC-MS, *i.e.*, 7% versus 65%, respectively; which has been observed for different biological matrices [48]. Among the possible interferents leading to ion suppression in SFC-MS, metal ion clusters – mainly K^+-, Mg^{2+}-, and Na^+-clusters – deserve a particular attention [35,49]. Indeed, alkali metal ions (naturally abundant in many

biological matrices and glassware) have shown to form clusters in combination with the alcohol modifier and make-up solvent, leading to possibly severe matrix effects *via* ion suppression [49]. Common strategies used to minimize matrix effects in LC-MS are therefore also recommended with SFC-MS, including the use of suitable isotopically-labelled internal standard, improved sample clean-up, a different ionization source, or adjustement of the mobile phase composition.

3. Applications to metabolomics

As emphasized in the previous sections, SFC-MS represents an attractive analytical tool in bioanalysis, offering an orthogonal separation selectivity, high kinetic performance, low pressure drop, low consumption of organic solvents, similar or better sensitivities than LC-MS, and the possibility of analyzing a wide number of compounds with large differences in their physico-chemical properties. The growing interest of the metabolomics community for this technique is therefore not surprising and reflected in the increased number of applications reported in

Table 1
Lipid profiling and lipidomics applications using SFC-MS.

Analytes	Matrix	Stationary phase	Critical pressure and temperature	Mobile phase composition	Detection	Publication year
Phospholipids	Dried plasma spots	PC HILIC (250 × 4.6 mm, 5 µm)	35 °C, 10 MPa	**A:** CO_2 **B:** MeOH + 0.1% ammonium formate	ESI-QqQ/MS	2012 [51]
Carotenoids and epoxy carotenoids	Human serum	Merck Puroshere RP-18e (250 × 4.6 mm, 5 µm)	35 °C, 10 MPa	**A:** CO_2 **B:** MeOH + 0.1% ammonium formate	ESI-QqQ/MS	2012 [52]
Oxidized phosphatidylcholine isomers	Mouse liver	Princeton 2-EP (250 × 4.6 mm, 5 µm)	35 °C, 10 MPa	**A:** CO_2 **B:** MeOH + 0.1% ammonium formate	ESI-QqQ/MS	2012 [53]
Phospholipids, lysophospholipids and sphingolipids	Mouse liver tissue	Inertsil ODS-4 (250 × 4.6 mm, 5 µm)	37 °C, 10 MPa	**A:** CO_2 **B:** MeOH + 0.1% formic acid	ESI-QqQ/MS	2013 [74]
Phosphatidylcholines and phosphatidylethanolamines	Mouse plasma	Inertsil ODS-EP (250 × 4.6 mm, 5 µm)	35 °C, 10 MPa	**A:** CO_2 **B:** MeOH + 0.1% ammonium formate	ESI-Orbitrap-FT/MS	2013 [57]
Bile acids conjugates	Rat serum	ACQUITY UPLC BEH Amide (100 × 3.0 mm, 1.7 µm)	70 °C, 13.8 MPa	**A:** CO_2 **B:** MeOH:H_2O (95:5, *v/v*) + 0.2% ammonium formate and 0.1% formic acid	ESI-QqQ/MS	2013 [54]
Plasma lipoproteins	Rabbit plasma	Inertsil ODS-4 (250 × 4.6 mm, 5 µm)	35 °C, 10 MPa	**A:** CO_2 **B:** MeOH + 0.1% ammonium formate	ESI-Q-Orbitrap-MS	2015 [55]
Steroid glucuronides and sulfates	Urine	**1.** ACQUITY UPC^2 BEH (100 × 3.0 mm, 1.7 µm) **2.** ACQUITY UPC^2 BEH 2-EP (100 × 3.0 mm, 1.7 µm)	**1.** 40 °C, 13 MPa **2.** 50 °C, 13 MPa	**A:** CO_2 **B:** MeOH:H_2O (95:5, *v/v*) + 30 mM ammonium formate	ESI-QqQ/MS	2015 [75]
Lipid profiling	Porcine brain	ACQUITY UPC^2 BEH (100 × 3.0 mm, 1.7 µm)	60 °C, 12.4 MPa	**A:** CO_2 **B:** MeOH:H_2O (99:1, *v/v*) + 30 mM ammonium formate	ESI-IM/qTOF/MS	2015 [76]
Phosphatidylcholines	Dried serum spots	Inertsil diol (150 × 2.1 mm, 3 µm)	40 °C, 10 MPa	**A:** CO_2 **B:** MeOH + 0.1% formic acid	ESI-QqQ/MS	2017 [40]
Oxylipins	Human plasma	ACQUITY UPC^2 Torus 1-AA (100 × 3.0 mm, 1.7 µm)	50 °C, 12.4 MPa	**A:** CO_2 **B:** MeOH + 0.1% acetic acid	ESI-IM/qTOF/MS	2017 [59]
Steroids and steroid sulfates	Academic standards	**1.** Zorbax Rx-Sil Rapid Resolution HT (3.0 × 100 mm, 1.8 µm) **2.** ACQUITY UPC^2 Torus DIOL (150 × 3.0 mm, 1.7 µm) **3.** ACQUITY UPC^2 BEH 2-EP (100 × 3.0 mm, 1.7 µm)	50 °C, 15 MPa	**A:** CO_2 **B1:** IPA **B2:** MeOH **B3:** MeOH	ESI-QqQ/MS	2018 [77]
Lipid profiling	Rabbit plasma	ACQUITY UPC^2 Torus diethylamine (100 × 3.0 mm, 1.7 µm)	40 °C, 10.3 MPa	**A:** CO_2 **B:** MeOH + 0.1% ammonium formate	ESI-QqQ/MS	2018 [78]
Arachidonic acid and derivatives	Human plasma	ACQUITY UPC^2 Torus 2-PIC (100 × 3.0 mm, 1.7 µm)	40 °C, 10.3 MPa	**A:** CO_2 **B:** MeOH + 0.1% formic acid and 10 mM ammonium acetate	ESI-QqQ/MS	2018 [79]
Lipid profiling	Porcine artery extract	ACQUITY UPC^2 Torus DIOL (100 × 3.0 mm, 1.7 µm)	50 °C, 11 MPa	**A:** CO_2 **B:** MeOH + 10 mM ammonium formate	ESI-qTOF/MS	2019 [80]
Lipid profiling	Human plasma	Two Zorbax RX-SIL (150 × 2.1 mm, 5 µm)	40 °C, 16 MPa	**A:** CO_2 **B:** MeOH:H_2O (500:10, *v/v*) + 20 mM ammonium formate	ESI-QqQ/MS	2020 [62]
Lipid profiling	Human plasma and serum	Viridis BEH column (100 × 3.0 mm, 1.7 µm)	60 °C, 11 MPa	**A:** CO_2 **B:** MeOH + 10 mM ammonium acetate and 1% water	ESI-qTOF/MS	2020 [61]
Lipid profiling and lipoprotein fractions	Rabbit serum	ACQUITY UPC^2 Torus diethylamine (100 × 3.0 mm, 1.7 µm)	50 °C, 10.3 MPa	**A:** CO_2 **B:** MeOH:H_2O (95:5, *v/v*) + 0.1% ammonium formate	ESI-QqQ/MS	2020 [81]

Abbreviations: 1-AA, 1-aminoanthracene; 2-PIC, 2-picolylamine; IPA, *2*-propanol; ACN, acetonitrile; ESI, electrospray ionization; EtOH, ethanol; FT, Fourier transform; IM, ion mobility; MeOH, methanol; Q, simple quadrupole; qTOF, quadrupole time-of-flight; QqQ, triple quadrupole.

the literature since the introduction of advanced commercial instruments in 2012. Due to the non-polar nature of supercritical CO_2, the early applications of SFC-MS in metabolomics were mostly focusing on the analysis of lipids and rather hydrophobic compounds. Modifying the polarity of the mobile phase with organic modifiers led to the first applications of SFC-MS to the analysis of the polar section of the metabolome. In recent years, a clear shift towards the simultaneous analysis of metabolites displaying a wide polarity range has been observed, fostered by the versatility offered by SFC-MS when using additives such as water or/and salts in the mobile phase (Fig. 3). Moreover, with the possibility offered by the composition of the make-up liquid to tune the ESI-MS response, SFC-MS appears very interesting in dealing with the large concentration dynamic ranges observed in metabolomics [35]. Finally, SFC-MS is also considered a worthy alternative for chiral analysis, offering a high throughput and suitable separation between metabolite enantiomers – which have been gaining more attention in the field of metabolomics in recent years.

3.1. Lipid profiling and lipidomics

Lipids play multiple roles in the physiology of a living organism, such as energy source, cell compartmentalization, and signaling. Lipids have also shown to be involved in a large diversity of pathological processes including cancer, inflammation, diabetes, and other metabolic diseases. They comprise a wide range of compounds with various structures divided into eight groups according to the LIPIDMAPS classification system (http://www.lipidmaps.org/), namely, fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides.

Table 1 lists the bioanalytical applications where SFC-MS has been used for lipid profiling and lipidomics. Compared to RPLC-based approaches where most of the studies report the use of C_{18} columns and water/acetonitrile mixtures as mobile phase, lipidomics methods using SFC-MS show a large diversity of stationary phases (*e.g.*, diol, bare silica, C_{18}, C_{30}, 2-EP, amino, *etc.*) and organic modifiers (*e.g.*, ethanol, methanol, isopropanol, and mixtures thereof) depending on the targeted lipid class [50].

The group of Bamba and co-workers has played a trailblazing role in demonstrating the promising use of SFC-MS in targeted and non-targeted lipid profiling with multiple applications reported in the last decade, including phospholipids profiling in dried plasma spots [51], carotenoids and epoxy carotenoids in serum samples [52], oxidized phosphatidylcholine isomers in mouse liver [53], bile acids conjugates in rat serum samples [54], and plasma lipoprotein fractions in rabbit plasma [55]. Besides groundbreaking applications, they have also demonstrated that further technological developments in SFC-MS, notably an improved sample injection system [56] or the on-line coupling with supercritical fluid extraction [40] could lead to a very powerful analytical tool for quantitative lipidomics and biomarker discovery.

Conventional and modern analytical approaches used in lipidomics include direct injection MS (referred to as *shotgun lipidomics*), RPLC-MS, normal-phase liquid chromatography (NPLC)-MS, and HILIC-MS. In RPLC, lipids are separated based on their fatty acyl moieties; isomers within the same lipid class are separated, but isomers from different classes may therefore co-elute. Conversely, in NPLC and HILIC, the separation of lipids is based on their polar head-group; isomers in the same lipid class therefore elute at the same time. Bamba and co-workers showed that with a careful optimization of the experimental conditions and using a single C_{18} column, SFC-MS enabled the separation of polar lipid species based on both their fatty acyl moieties but also their polar head groups, which remains unachieved by other chromatographic methods so far [57].

The complementarity between different state-of-the-art separation methods has also been demonstrated by the group of Holčapek and co-workers [58–60]. In recent comparative studies, they investigated the performance of UHPSFC-MS *vs.* HILIC-MS for the analysis of human plasma and serum, as well as tumor tissue and erythrocytes of kidney cancer patients after systematic method optimization and validation [58,61]. Overall, UHPSFC-MS showed some relevant general advantages over HILIC-MS, such as a shorter analysis time and a slightly higher robustness. More importantly, both non-polar and polar lipid classes were successfully separated using UHPSFC-MS unlike HILIC-MS, as illustrated in Fig. 5. Quantitative results obtained by the validated methods were also correlated with the expected concentrations in a standard reference material (NIST plasma). The most relevant disadvantage of the UHPSFC-MS method was a slightly lower precision in the retention times, with small shifts of retention times observed over a long period. These comprehensive studies, performed with a solid methodology, represent a further step towards a wider acceptance of UHPSFC-MS in high-throughput and quantitative metabolomics and lipidomics.

Due to the complementary information obtained by the different chromatographic modes in terms of lipid coverage, it seems logical to envision combining SFC with other chromatographic options using multidimensional approaches. Yang *et al.* very recently reported the development of a two-dimensional SFC × RPLC method combined with a triple quadrupole MS instrument for the analysis of lipids in plasma samples for the screening of biomarker candidates in breast cancer [62]. With the developed setup, lipids were first separated based on their class in the first dimension, prior to their separation within each class in the second dimension. The developed 2D method was also more than four times faster than the reference RPLC × NPLC method. However, such a multidimensional approach involving SFC and LC remains mostly an academic exercise and is hardly applicable to a large cohort of patients, due to its inherent complexity.

3.2. Characterization of the metabolome: from hydrophobic to hydrophilic metabolites

Since the advent of the first commercial instruments, SFC-MS has been predominantly used for the separation of relatively non-polar metabolites, while polar metabolites remained overlooked. This may be explained by the general idea that a high proportion of CO_2 in the mobile phase would result in poor performance for the analysis of polar metabolites. However, this misperception has significantly evolved in the last decade when the community realized that SFC-MS is also well-suited for the analysis of more polar compounds *via* the addition of small amounts of water and/or salts in the organic modifier, as well as the use of gradients with a higher proportion of organic modifier, *i.e.*, up to 70–100% (Fig. 3) [63]. Table 2 lists the applications reported in the literature where SFC-MS has been used for the analysis of polar metabolites in biosamples.

A relevant study in this field has been conducted by Sen *et al.* who investigated the performance of UHPSFC-MS for the analysis of medium and high-polarity metabolites ($-7 \leq \text{Log P} \leq 2$) from different classes in urine samples [64]. The method development included the screening of twelve different stationary phases, various additives in the CO_2-methanol-based mobile phase, and three different temperatures. The new generation of stationary phases specifically designed for SFC, particularly the Diol chemistry, lead to the best results in terms of peak shape, peak capacity, resolution, and robustness. Moreover, a significant enhancement in chromatographic performance was observed for the majority of the stationary phases tested when polar additives such as ammonium salts were added. Ammonium salts generally resulted in lower peak widths and higher overall resolution than the other additives for basic metabolites. However, the addition of water or alkylamines to the organic modifier was only beneficial for some polar metabolites, *i.e.*, caffeine, cytosine, and uridine. Finally, the optimized method consisted of a Diol stationary phase and a mobile phase composed of 5% water and 20 mM ammonium formate. No significant differences in terms of chromatographic performance were observed in the range of temperatures screened, *i.e.*, 30–45 °C, probably due to the fact that the

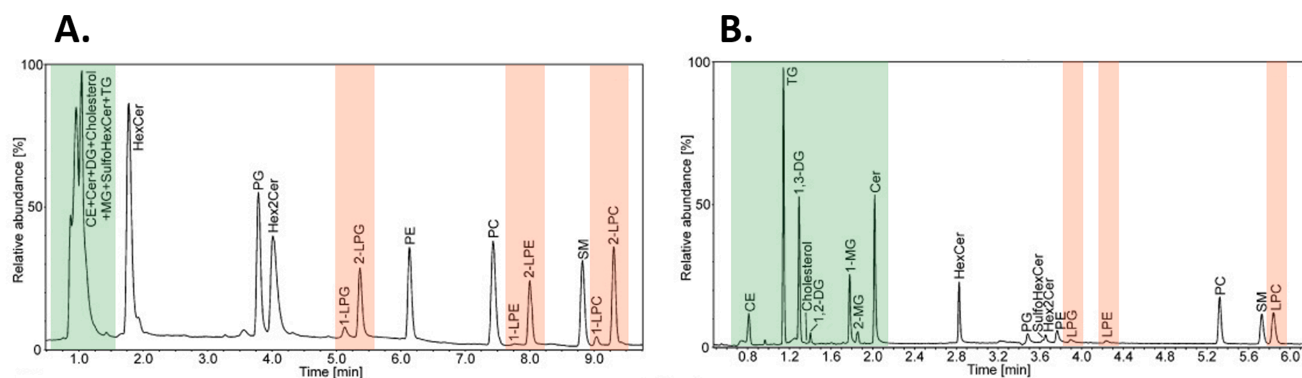

Fig. 5. Chromatograms observed with the analysis of the lipid internal standards. A. UHPLC-HILIC-MS using an Acquity UPLC BEH HILIC column (150 × 2.1 mm, 1.7 µm, Waters); B. UHPSFC-MS using an Acquity BEH UPC^2 column (100 × 3 mm, 1.7 µm, Waters). Non-polar lipid classes (MG, DG, TG, Cer, cholesterol, and CE) elute in the void volume in HILIC-MS due to the absence of a polar functional group (A., green highlight). With UHPSFC-MS (B.), both non-polar and polar lipid classes are well-retained, allowing for the separation between non-polar acylglycerol classes (MG, DG, and TG) and within those classes (*e.g.*, the positional isomers 1-MG and 2-MG, green highlight). On the other hand, positional isomers of more polar lyso-lipids are baseline resolved with HILIC-MS (A., red highlight) but not with UHPSFC-MS (B., red highlight). CE, cholesterol ester; TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol; Cer, ceramides; PC, phosphatidyl choline; PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; SM, sphingomyelin; LPC, lysophosphatidyl choline; LPG, lysophosphatidyl glycerol; LPE, lysophosphatidyl ethanolamine; LPA, lysophosphatidic acid; HexCer, hexosylceramide; Hex2Cer, dihexosylceramide; SHexCer, sulfohexosylceramide. See [58] for the detailed experimental conditions. Adapted from [58] with permissions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Analysis of hydrophilic and hydrophobic metabolites using SFC-MS and unified chromatography.

Analytes	Matrix	Stationary phase	Critical pressure and temperature	Mobile phase composition	Detection	Publication year
Fat- and water-soluble vitamins	Academic standards	ACQUITY UPC^2 C18SB (100 × 3.0 mm, 1.8 µm)	40 °C, 12.4 MPa	**A:** CO_2 **B:** MeOH + 0.1% formic acid	ESI-QqQ/MS	2014 [68]
Medium- and high-polarity compounds	Human urine	**1.** ACQUITY UPC^2 BEH 2-EP (100 × 3.0 mm, 1.7 µm) **2.** ACQUITY UPC^2 Torus DIOL (100 × 3.0 mm, 1.7 µm)	55 °C, 13.8 MPa	**A:** CO_2 **B1.1:** MeOH + 5% H_2O **B1.2:** MeOH + 20 mM ammonium formate **B2.1:** MeOH + 5% H_2O **B2.2:** MeOH + 20 mM ammonium formate	ESI-MS	2016 [64]
Amino acids	Human serum	Luna HILIC (150 × 3.0 mm, 3 µm)	40 °C, 15 MPa	**A:** CO_2 **B:** MeOH + ammonium formate and H_2O or ammonium acetate	ESI-MS/MS and APCI-MS/MS	2017 [82]
Set of representative metabolites (-6 ≤ cLogP ≤ 11)	Academic standards	ACQUITY UPC^2 BEH 2-EP (100 × 3.0 mm, 1.7 µm)	40 °C, 12 MPa	**A:** CO_2 **B:** MeOH:H_2O (95/5, *v/v*) + 50 mM ammonium acetate and 1 mM ammonium fluoride	ESI-QqQ/MS	2018 [69]
Polar and ionized drugs, and respective metabolites	Human urine	SFC-SCX (150 × 4.6 mm, 5.0 µm)	45 °C, 17 MPa	**A:** CO_2 **B:** MeOH:H_2O (95/5, *v/v*) + 20 mM ammonium formate and 15 mM formic acid	ESI-QqQ/MS	2020 [65]
Set of representative metabolites (Sigma Metabolite Library of Standards)	Academic standards	Poroshell HILIC (100 × 3.0 mm, 2.7 µm)	40 °C, 10.5 MPa	**A:** CO_2 **B:** MeOH:H_2O (95/5, *v/v*) + 50 mM ammonium acetate and 1 mM ammonium fluoride	ESI-QqQ/MS	2020 [70]
Set of representative doping agents	Spiked human urine	**1.** UPC^2 BEH silica (100 × 3.0 mm, 1.7 µm) **2.** ACQUITY UPC^2 Torus 2-PIC (100 × 3.0 mm, 1.7 µm) **3.** ACQUITY UPLC HSS C18 SB (100 × 3.0 mm, 1.7 µm)	40 °C, 12 MPa	**A:** CO_2 **B:** MeOH:H_2O (98/2 *v/v*) + 20 mM ammonium formate	ESI-QqQ/MS	2020 [26]

Abbreviations: 2-PIC, 2-picolylamine; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionisation; MeOH, methanol; IM, ion mobility; Q, simple quadrupole; qTOF, quadrupole time-of-flight; QqQ, triple quadrupole.

investigated temperature range was very narrow.

The new generation of dedicated UHPSFC stationary phases are typically leading to improved performance, but conventional columns have also demonstrated to be fully suited for modern bioanalytical challenges. As an example, an SFC-MS/MS method using an ion-exchange stationary phase has been developed for the analysis of polar and ionized drugs as well as their respective metabolites, including gamma-hydroxybutyrate (GHB), gamma-butyrolactone, GHB-glucuronide, ethyl sulfate, ethyl glucuronide, meldonium, and gamma-butyrobetaine [65]. The separation conditions were optimized using various modifier compositions as well as different stationary phase chemistries. The best chromatographic performance, especially related to the separation of the critical zwitterionic pair meldonium and gamma-butyrobetaine, were obtained using a strong cation exchange

column and a mobile phase gradient containing up to 60% of organic modifier. As a proof of concept, the optimized method was applied to the analysis of spiked human urine, demonstrating the applicability of such approach for screening purposes in forensic toxicology.

As the modifier composition in the mobile phase can range from 0 to 100%, the mobile phase composition in SFC-MS offers a remarkable flexibility in the compounds solubility and elution. However, increasing the concentration of organic modifier in the mobile phase results in an increase in the critical pressure and temperature. The mobile phase may therefore change from a supercritical to subcritical state, ultimately ending in the liquid state. Shifting the physical state of the mobile phase without phase separation by controlling temperature and backpressure is known as *unified chromatography* (UC), a concept described more than 50 years ago by Giddings and first practically applied in chromatography by Takeuchi *et al.* in 1987 [66]. UC is a modern chromatographic approach which opens the door to the simultaneous and comprehensive analysis of polar and non-polar compounds using one single SFC-based method [67].

The recent applications showing the simultaneous analysis of polar and non-polar compounds using SFC-MS or UC-MS are listed in Table 2. Most of the applications reported so far have focused on the analysis of standards solutions and not been applied to real-life cases yet. However, these studies are relevant as they pave the way towards the use of SFC-MS and UC-MS in metabolomics. A relevant example is the analysis of vitamins, commonly divided into fat- and water-soluble vitamins, which show a wide polarity range, *i.e.*, $-2.1 \leq$ Log P ≤ 10.1, making their analysis challenging. Bamba and co-workers used a gradient ranging from almost 100% CO_2 to 100% methanol together with a C_{18}SB column, resulting in the separation of 17 fat- and water-soluble vitamins in less than 4 min within one single run [68].

The potential power of UC in human metabolomics has been further investigated by Guillarme and co-workers with the analysis of metabolites from different classes showing a wide polarity range, *i.e.*, $-6 \leq$ Log P ≤ 11 [69]. In this systematic study, different stationary phase chemistries were screened in combination with a mobile phase gradient ranging from 2 to 100% organic modifier in CO_2 for the separation of 57 representative metabolites. HILIC-type columns, *i.e.*, bare silica and silica bonded with a zwitterionic ligand, led to the best results in terms of selectivity and peak shapes, as 60% of the model metabolites were detected with a Gaussian peak shape. On the other hand, the C_{18}-based column led to inferior performance, with the majority of compounds (61%) showing distorted peaks shape. The chromatograms obtained for a set of metabolites of different polarities using the optimized UC conditions are shown in Fig. 6**.** In a recent follow-up study, the potential metabolite coverage obtained with such UHPSFC-high-resolution mass spectrometry (HRMS)-based UC method was investigated with the analysis of the *Sigma Metabolite Library of Standards*, which encompasses 597 metabolites [70]. With a gradient ranging from 2 to 100% organic modifier using a Poroshell HILIC column, 66% of the metabolites were detected, including very polar metabolites such as amino acids, nucleosides, and carbohydrates, as well as hydrophobic metabolites such as steroids and lipids (Fig. 7). Carbohydrates, organic acids, amino acids, quaternary amines, sulphates/sulfonated metabolites, and nucleosides are typically difficult to detecte with classic UHPSFC methods and were all eluted with a relatively high percentage of methanol, showing the relevance of UC. However, inferior performance was reported for phosphate-containing metabolites and nucleotides, explained by a possible precipitation (especially in presence of high proportion of CO_2), adsorption of phosphorylated compounds on the walls and frits, or lack of elution of those compounds still retained in the column. It is worth mentioning that a careful attention was paid to the composition of the injection solvent, as the initial solubilization solvent, *i.e.*, a mixture of MeOH/water 95:5 (*v/v*), led to very poor detectability of hydrophobic compounds. Dichloromethane was selected as a suitable alternative, leading to sufficient dissolution of lipophilic compounds, while providing good peak shape due to its aprotic characteristics. This highlights the importance of investigating the composition of the injection solvent when using such approach to real-life bioanalytical applications.

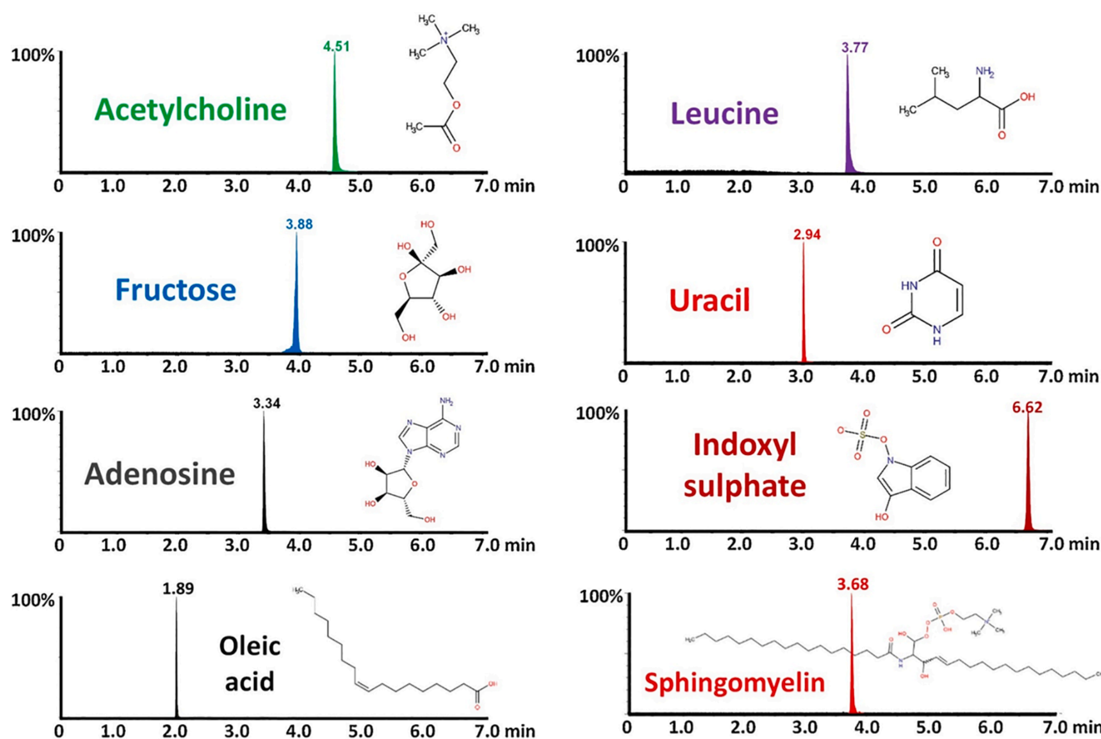

Fig. 6. SFC-MS/MS chromatograms obtained for several representative polar and non-polar metabolites using unified chromatography gradient conditions. Mobile phase: CO_2 + 10 mM ammonium formate in MeOH/water (95:5, *v/v*). Column: Nucleoshell HILIC 100 × 3.0 mm, 2.7 µm. Mobile phase temperature of 40 °C and backpressure of 120 bar. Reprinted from [69] with permissions.

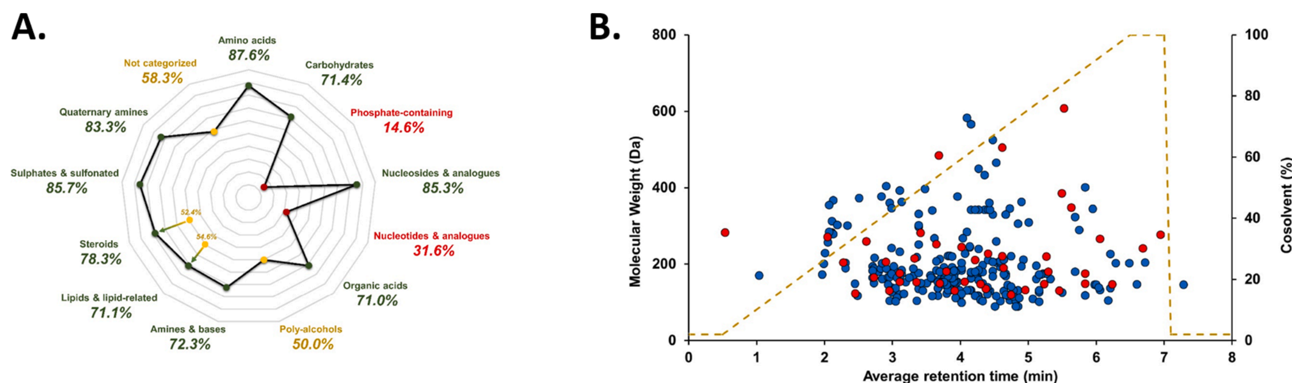

Fig. 7. Metabolite coverage observed with unified chromatography based on UHPSFC-HRMS. A. Spider graph illustrating the proportion of metabolites detected within each class from the Sigma Metabolite Library of Standards. B. Scatter plot displaying the detected metabolites according to their average retention time, molecular weight, and proportion of cosolvent needed for their elution. Blue dots, metabolites detected from the library; red dots, metabolites from the selected experimental set. See [70] for the detailed experimental conditions. Adapted from [70]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Chiral analysis

Chirality has a significant impact on nature, as two enantiomers frequently show relevant differences in their biological activities. Indeed, enantiomers and their respective metabolites can have substantial differences in their pharmacological and toxicological properties, highlighting the need for suitable analytical approaches for chiral analysis. SFC has long been used in industrial settings for the separation of enantiomers as preparative techniques and remains predominantly used in the pharmaceutical industry for the enantioseparation/purification of drug enantiomers and their metabolites. Table 3 lists the different applications of SFC-MS for the analysis of chiral metabolites analysis in various biological matrices. Chiral SFC-MS typically relies on the same chiral stationary phases than those used in LC-MS; however with a drastically improved throughput.

In addition to the different potency and toxicity between two drug enantiomers, chirality is an important concept in forensic toxicology, as the determination of the drug enantiomeric ratio can inform about drug consumption. A relevant example is amphetamine, where *d*-amphetamine is present as a single enantiomer in some pharmaceutical products (*e.g.*, Dexedrine, used in the treatment of narcolepsy and attention deficit disorder with hyperactivity), resulting in the detection of *d*-amphetamine only in blood, while ingestion of street amphetamine (synthetized racemic mixture) leads to the detection of both enantiomers. SFC-MS has been used to assess the enantiomeric ratio of amphetamine in urine samples from drug consumers [71]. A successful separation between both enantiomers was observed with a Chiralpak AD-3 column with the addition of cyclohexylamine. The developed SFC-MS method showed sufficient limits of detection, limits of quantitation, precision, recovery, and accuracy for clinical applications.

A chiral SFC-MS method has also been reported for the analysis of ketamine enantiomers. Ketamine is a racemic mixture of (*R*)-ketamine and (*S*)-ketamine, which both present different physiological activities. Indeed, (*R*)-ketamine is a less potent antagonist of the *N*-methyl-*D*-aspartate receptor (*ca.* four times) but show more marked antidepressant-like effects in animal models of major depressive disorder. In a recent study by Hoftstetter et al., an on-line supercritical fluid extraction-SFC-MS (SFE-SFC-MS) method was developed to determine ketamine enantiomers and their metabolites in dried urine [39]. With this method, 20 μL of urine only were needed for the analysis, with limit of quantitation as low as 0.5 ng/mL. Additionally, the within- and between-day accuracy and precision were within the acceptance limits, *i.e.*, ±15%. The developed method was the first SFE-SFC-MS methods to be successfully validated according to the European Medicines Agency (EMA) guidelines, demonstrating its applicability to modern bioanalytical challenges.

Overall, these examples of bioanalytical applications illustrate the role that chiral SFC-MS may play in metabolomics. Despite being an important aspect in clinical metabolomics due to the anticipated difference in biological activities between two metabolite enantiomers, the

Table 3
Chiral applications in metabolomics using SFC-MS.

Analytes	Matrix	Stationary phase	Critical pressure and temperature	Mobile phase composition	Detection	Publication year
Oxcarbazepine and chiral metabolites	Beagle dog plasma	Trefoil CEL2 (150 × 3.0 mm, 2.5 μm)	50 °C, 23 MPa	**A:** CO_2 **B:** MeOH	ESI-MS/MS	2016 [83]
GSK 1278863 and metabolites	Human plasma	Chiralpak AD-H (150 × 4.6 mm, 5 μm)	35 °C, NA	**A:** CO_2 **B:** 2-propanol:ethyl acetate:formic acid (80:20:0.5, *v/v*)	ESI-MS/MS	2016 [84]
Ketamine and metabolites	Dried urine	Lux amylose-2 (150 × 4.6 mm, 5 μm)	30 °C, 16.3 MPa	**A:** CO_2 **B:** 2-propanol + 0.075% NH_3	ESI-Q-MS	2018 [39]
R/S-amphetamine	Urine	Chiralpak AD-3 (150 × 2.1 mm, 3 μm)	30 °C, 11.03 MPa	**A:** CO_2 **B:** 2-propanol + 0.2% cyclohexylamine	ESI-QqQ/MS	2018 [71]
GNE-A, GNE-B, GNE-C	Mouse, rat, and dog plasma	**1.** Chiralcel OZ-3 (150 × 3.0 mm, 3 μm) **2.** Chiralpak AD-3 (150 × 3.0 mm, 3 μm) **3.** Chiralcel OJ-3 (150 × 3.0 mm, 3 μm)	40 °C, 10 MPa	**A:** CO_2 **B:** MeOH + 0.1% NH_4OH	ESI-IT-MS	2019 [85]

Abbreviations: ESI, electrospray ionisation; IT, ion trap; MeOH, methanol; Q, simple quadrupole; QqQ, triple quadrupole.

separation between optical isomers currently remains overlooked in a large majority of metabolomics-based studies.

4. Conclusions and perspectives

Since the introduction of advanced instrumentation in 2012, SFC has made a significant comeback. The technological improvements carried out over the last years have led to the commercial availability of sophisticated BPRs, SFC-MS interfaces, and a large diversity in column chemistries dedicated to SFC analysis. Moreover, the commercialization of columns packed with fully porous sub-2 μm particles and the associated improved chromatographic performance has fostered the use of SFC-MS in bioanalysis and metabolomics, with UHPSFC showing similar or even better kinetic performance compared with UHPLC.

Both sensitivity and matrix effects have been reported to be relatively equivalent between SFC-MS and LC-MS. The sensitivity in SFC-MS has shown to be significantly influenced by the MS instrumentation (*i.e.*, instrument generation and source design), the chromatographic conditions, and the composition of the make-up liquid. Similar to LC-MS, matrix effects depends on the targeted analytes, the composition of the matrix, and the sample pre-treatment. Besides conventional matrix interferents, alkali metal ions present in biosamples deserve a particular attention in SFC-MS as they can form clusters in combination with the alcohol modifier and make-up solvent, leading to severe ion suppression.

While the earliest application of SFC were tailored towards the analysis of non-polar compounds and chiral separations, SFC has been increasingly used for the analysis of more polar compounds. Indeed, the use of highly organic mobile phases and additives have extended the applications of SFC-MS to the analysis of a wide range of chemically-diverse metabolites with different polarities. This remarkable flexibility represents an essential feature of SFC-MS, enabling the simultaneous analysis of a large diversity of metabolites using the same instrument, chromatographic column, and mobile phase composition.

The first inter-laboratory study assessing the performance of SFC-MS for the determination of pharmaceutical impurities (which involved almost all of the major SFC players in academia, industry, and vendor demo labs) has demonstrated that SFC-MS shows similar or even better repeatability and reproducibility than state-of-the-art LC-MS methods, recognizing this method on equal merit to other chromatographic techniques [72]. Although SFC-MS is not expected to fully substitute other chromatographic or electrophoretic techniques, such study and the growing number of bioanalytical applications support the major role that SFC-MS can play in metabolomics. The most promising approach in this field is certainly UC-MS, or, in other words, the use of an "LC separation technique that happens to use liquid CO_2 as its primary mobile phase" [73]. Moreover, due to the orthogonality of SFC separation mechanisms to other liquid-phase separation techniques, major developments in multi-dimensional chromatography are expected, where SFC is incorporated as one or more of the dimensions.

In metabolomics-based SFC-MS, the method development remains quite complex due to the myriad of column chemistries available and the flexibility offered by the composition of the mobile phase (*i.e.*, type of organic modifier, nature and concentration of additive(s), and composition of the gradient). Although the prediction of the separation behavior remains challenging in SFC-MS and UC-MS, the increasing number of comprehensive and well-designed studies comparing a wide range of stationary phase chemistries and additives provide the scientists with a solid starting point for method optimization.

Additional improvements in the instrumentation and stationary phases are required to further promote the use of SFC-MS in metabolomics. First, metabolomics would benefit from a larger number of dedicated stationary phase chemistries leading to an improved peak symmetry for basic metabolites, which remains a bottleneck with the current phases commercially available. Moreover, the upper pressure limit of the currently commercially available UHPSFC instruments is set at 400–660 bar, which is a significant limitation when using high proportions of organic modifiers with columns equipped with sub-2 μm particles. Higher upper pressure limits are therefore required to allow for the use of higher mobile phase flow rates and, in turn, the possibility for (ultra)-fast UHPSFC analysis. Finally, efforts should be put in significantly decreasing the extra-column variances on current instruments, to fully benefit from the advantages provided by sub-2 μm particles.

UC opens new avenues in metabolomics due to the increased number of metabolites that can be detected in one single run. Nevertheless, the analytical steps occurring prior to the separation and detection of metabolites should not be overlooked. Indeed, similar to other chromatographic or electrophoretic approaches, it remains challenging to find a suitable injection solvent that allows for the solubilization of metabolites with different polarities, without compromising the separation efficiency. A careful optimization of the composition and the volume of the injection solvent is therefore recommended during the method development. Moreover, the sample pre-treatment should be as generic as possible to limit the discrimination towards one metabolite class, while still providing an efficient sample clean-up to avoid or lower the potential matrix effects.

The potential of SFC-MS for untargeted metabolomics and lipidomics still remains largely unexplored, as very few applications has been reported in the literature so far – even none for untargeted metabolomics. Moreover, due the aforementioned pressure-related instrumental limitations, the throughput of UHPSFC observed in practice is clearly below that which could be achieved in theory.

If the next generation of UHPSFC-MS instruments tackles the current aforementioned technological bottlenecks – and as long as tomorrow's young analytical scientists are adequately trained, SFC-MS is certainly promised to a bright future in metabolomics.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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