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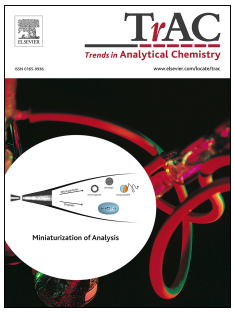
How to cite

LOSACCO, Gioacchino Luca, VEUTHEY, Jean-Luc, GUILLARME, Davy. Supercritical fluid chromatography – Mass spectrometry: Recent evolution and current trends. In: TrAC Trends in Analytical Chemistry, 2019, vol. 118, p. 731–738. doi: 10.1016/j.trac.2019.07.005

This publication URL: https://archive-ouverte.unige.ch/unige:121682
Publication DOI: 10.1016/j.trac.2019.07.005

Accepted Manuscript



Supercritical Fluid Chromatography – Mass Spectrometry: Recent Evolution And Current Trends

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PII: S0165-9936(19)30051-2

DOI: https://doi.org/10.1016/j.trac.2019.07.005

Reference: TRAC 15594

To appear in: *Trends in Analytical Chemistry*

Received Date: 6 February 2019

Revised Date: 11 June 2019

Accepted Date: 8 July 2019

Please cite this article as: G.L. Losacco, J.-L. Veuthey, D. Guillarme, Supercritical Fluid Chromatography – Mass Spectrometry: Recent Evolution And Current Trends, *Trends in Analytical Chemistry*, https://doi.org/10.1016/j.trac.2019.07.005.

SUPERCRITICAL FLUID CHROMATOGRAPHY – MASS SPECTROMETRY: RECENT EVOLUTION AND CURRENT TRENDS

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KEYWORDS:

Supercritical fluid chromatography; interfaces; matrix effects; sensitivity; doping agents; metabolomics

HIGHLIGHTS:

- A detailed summary on SFC-MS interfaces is given, with emphasis on current issues and potential solutions.
- Differences between LC-MS and SFC-MS in terms of matrix effects generated are highlighted.
- Sensitivity under SFC-MS has been demonstrated to be comparable to what it can be reached in LC-MS conditions.
- Applications for SFC-MS are shifting towards the analysis of compounds with increasing polarity and analytes available in complex matrices.

ABSTRACT

Supercritical fluid chromatography (SFC) has recently experienced renovated impulse from research groups. Its hyphenation to mass spectrometers (MS) proved to be of significant importance in catalysing interest from researchers. In contrast to liquid chromatography (LC), the coupling of SFC-MS requires the use of an interface in order to deal efficiently with the decompression of supercritical CO_2 and possible precipitation issues of samples while entering the ionization chamber. The most common SFC-MS interfaces employ an additional sheath pump that reduces sample precipitation. However, there are still issues in dealing with the CO_2 decompression phenomenon, with different solutions being given. Matrix effects (MEs) under SFC-MS have proved to be quite different from those generally observed in LC-MS, with ion suppression being the main form of ME. Nonetheless, SFC-MS is capable of reaching comparable sensitivity values to LC-MS, and in some cases performing even better. Several applications have been recently developed for SFC-MS, spacing from the analysis of plant extracts, biological matrices for anti-doping and forensic purposes, as well as highly polar compounds such as carbohydrates and endogenous metabolites.

1. Introduction

The use of mass spectrometers (MS) as a detector hyphenated to chromatographic separation has known an incredible growth, in the recent years, thanks to its high versatility, sensitivity, and range of possible applications [1, 2]. Considering the diversity of MS analyzers present on the market (i.e. single and triple quadrupole, time of flight, ion trap, Orbitrap and hybrid instruments), it is possible to perform qualitative and quantitative analysis at very high sensitivity [3-5], as well as to generate elevated MS resolution between compounds having very similar mass-to-charge values [6, 7].

One of the most successful marriages between chromatography and mass spectrometry is now represented by the hyphenation of liquid chromatography (LC) with MS [8-10]. This coupling became possible thanks to the development of atmospheric pressure ionization (API) sources such as electrospray ionization (ESI) [11, 12] and atmospheric pressure chemical ionization (APCI) [13]. Besides LC, other separation techniques have also been successfully hyphenated to MS, including gas chromatography (GC) [14] capillary electrophoresis (CE) [15], and supercritical fluid chromatography (SFC) [16, 17]. SFC was initially developed during the 1960s and regained the attention of several research groups starting from the 1980s [18], but the interest remained limited to chiral separation [19] and preparative chromatography [20], due to a lack of robustness and sensitivity of the instrumentation [21]. Since 2012, a new generation of SFC instruments was introduced on the market. These new systems possess various desirable features, such as i) reduced system volume and a relatively high upper-pressure limit, compatible with columns packed with sub-2 µm particles, ii) improved robustness and iii) easy MS hyphenation [22]. This allows SFC to transition into ultra-high performance supercritical fluid chromatography (UHPSFC) [18], in a similar way to what has been witnessed with LC since 2004, with a consequent increase in terms of interest and publications being made. A quick analysis made on the main research platforms currently available shows a gradual but constant increase in articles which have keywords such as “supercritical fluid chromatography” or

"SFC", in the period from 2012 (343 publications) till 2018 (634 publications). This chromatographic technique has the peculiarity of employing a supercritical (or often subcritical) mobile phase, thanks to the use of carbon dioxide in its supercritical state as major constituent [18, 23]. In modern UHPSFC, carbon dioxide is always mixed with an organic modifier, usually methanol, which ensures the complete elution of compounds from low to high polarity [23]. Some salts (i.e., ammonium formate, ammonium hydroxide…), as well as acids (i.e. formic acid, trifluoroacetic acid…) or bases (i.e. ammonium hydroxide, diethyl- and triethylamine…), and a small amount of water could also be added to the mobile phase to improve method repeatability and peak shapes of ionizable substances. Finally, the use of a supercritical mobile phase presents several advantages in chromatography, including a minor environmental impact compared to organic solvents such as *n*-hexane or *n*-heptane, low viscosity, high diffusion coefficients, and high density, thus enabling SFC to combine the advantages of LC and GC [18, 23].

The aim of this review was to describe the latest developments related to the hyphenation of UHPSFC and MS, highlighting some advantages that this technique can offer in contrast with the current state-of-the-art techniques. First, a detailed description of the UHPSFC-MS interfaces available on the market will be provided, including some potential issues related to the use of a supercritical fluid. Secondly, the influence of the make-up solvent nature and the evaluation of matrix effects will be assessed. Then, a comparison of achievable sensitivity in UHPSFC-MS and UHPLC-MS will be performed. Finally, an overview of some relevant applications that have been developed in the last few years will also be given.

2. UHPSFC-MS interfaces

The hyphenation of UHPSFC with MS is not as straightforward as with LC/UHPLC instruments. Indeed, supercritical fluids possess much higher compressibility than liquids, that needs to be controlled, particularly when the fluid is not anymore under the backpressure control [24]. Indeed, when the pressure is released, analytes can precipitate before entering the MS instrument. Besides the regulation of backpressure, the interface

should also help to improve the ionization yield in ESI, particularly when the mobile phase is composed of a high proportion of CO_2. Lastly, the chromatographic integrity (retention, selectivity, and efficiency) should also be maintained when MS detection is used. For all these reasons, the providers of SFC instruments have developed several interface schemes over the years (Fig. 1), able to solve these different issues [24, 25].

The most common interface available on the market is known as the "*pre-BPR splitter with sheath pump"*, commercialized by Waters and Agilent [25] (see Fig. 1D). This interface consists of two zero-dead-volume (ZDV) T-unions linked in series, allowing the addition of a make-up solvent from a sheath pump (first ZDV T-union) and the use of an active-backpressure regulator (ABPR) (second ZDV T-union), to direct only a limited part of the flow-rate into the MS ionization source, while the remaining part goes to the waste. This interface offers the obvious advantage of reducing the possible precipitation of samples in the mobile phase. In addition, thanks to the flexible BPR regulation and the presence of the sheath pump, it also allows sending a highly suitable mobile phase flow rate and composition to the ESI sources, thus producing an excellent sensitivity [25].

The second available interface, among the most popular ones, is called *"BPR and sheath pump with no splitter"*, commercialized by Shimadzu and Agilent (see Fig. 1E). In this configuration, there is only one ZDV T-union, used to deliver the make-up solvent. This interface, which does not possess flow-splitting, is well suited for APCI-MS, which is a mass flow dependent device, since it delivers the entire sample to the MS [24]. Moreover, the last tubing entering into the ionization source passes through the BPR, which is heated at a relatively high temperature (around 50°C), to limit decompression cooling phenomenon and solute precipitation. Until now, this interface has been rarely employed for real applications, and therefore its advantages and drawbacks are still not well identified [25].

A remark has to be done on the Agilent SFC-MS interface, since it is the only one that allows the user to choose between the "*pre-BPR splitter with sheath pump"* and the *"BPR and sheath pump with no splitter"* configurations.

Besides these two interfaces, there are also a few other solutions that have been described for hyphenating UHPSFC and MS, but they present some major issues, making them inferior to the ones previously described. More details on the different interfaces currently available can be found in a recently published review from our group [25].

When hyphenating UHPSFC and MS, several important issues need to be considered [24-26]. In the two previously described interfaces, the BPR module is located before the MS. Therefore, there is no control over the mobile phase state entering the ionization chamber. In this part of the setup, the CO_2 is not under the influence of the BPR and should decompress endothermically, which leads up to different problems [24]. First, the decompression, followed by a drop in the temperature at the connector level, increases the risk of analyte precipitation [24, 25]. Moreover, the addition of the make-up solvent, necessary to replace decompressed CO_2, might be insufficient to ensure the solubility of the samples, leading to possible precipitation issues [27]. Another issue related to the uncontrolled CO_2 decompression is the possible peak broadening that has been previously reported [24]. This phenomenon could be attributed to different factors: the temperature drop is certainly one of them, since it increases solvent viscosity and thus reduces analytes diffusion coefficient [24]. In addition, considering that there is no pressure and temperature control in the tubing located after the BPR, phase separation is most likely to occur between the liquid organic modifier and gaseous CO_2 [24, 25, 27]. As described elsewhere [24], to better understand the influence of the phenomena described above, it is advised to follow the vapor-liquid equilibrium (VLE) curves for CO_2+methanol mixtures. Different situations can be foreseen: the flow patterns can greatly change, with the formation of CO_2 bubbles of different diameters based on the volume ratios between the gas and the liquid. This, consequently, affects the linear velocity of the flow entering the MS. Linear velocity is also influenced by the change in surface tension, viscosity and other parameters which are not behaving as expected [24]. The phase separation can be another potential problem that should not be underestimated,

177 especially since it might lead to more severe issues such as band broadening or even loss of
178 the chromatographic separation.

179 **3.** Different solutions have been found to tackle these drawbacks observed in the SFC-
180 MS setup. For the precipitation issue, the addition of a sheath pump, which
181 continuously delivers a make-up solvent (i.e., methanol, methanol + buffer, methanol +
182 small amount of water), was found to be a good solution [27]. Indeed, the delivery of a
183 methanol-rich solvent strongly limits the precipitation of polar compounds, without
184 sacrificing too much SFC and MS performance. The addition of a make-up solvent,
185 however, may lead to the insurgence of another potential problem: a dilution factor can
186 appear which could negatively affect MS sensitivity, especially on concentration-
187 dependent ionization sources such as ESI [25, 27-29]. Contrary to what could be
188 expected, the dilution factor remains always reasonable, whatever the mobile phase
189 and make-up conditions, thanks to the use of the active BPR [27, 30]. Regarding the
190 management of CO_2 decompression, a solution is to modify the interface [6, 31]. One
191 key parameter is obviously the temperature that needs to be controlled, to avoid phase
192 separation. As described in more details elsewhere [24], heating is not always the best
193 choice. The use of combined isenthalpic and isopycnic plots, for mixtures of
194 CO_2/methanol with fixed compositions, clearly highlight that cooling, instead of heating,
195 should be preferred [24]. Indeed, the analysis of these plots definitely indicates that, by
196 lowering the temperature, it is possible to avoid the area in which phase separation
197 occurs for a greater range of pressure values [24]. The temperature reduction,
198 therefore, translates into a wider range of the CO_2 decompression. Density, also, does
199 not change, which therefore translates in much fewer precipitation issues of several
200 compounds, which were soluble with a high-density mobile phase. The other
201 parameter is the interface geometry; indeed, changing the geometry of the capillaries
202 used in the interface (i.e., length, inner diameter, etc.) can be an easy solution to
203 maintain a constant mobile phase density. Only two papers, however, [6, 31] describe

the evaluation of a new capillary restrictor for ESI interfaces, able to reduce the pressure drop in the connector. According to the authors, the new interface design has brought a more stable ESI spray, positively affecting the peak shapes and repeatability, thus allowing a better quantification of the compounds that have been tested [6, 31]. **Matrix effects in SFC-MS vs. LC-MS**

UHPLC coupled to ESI-MS and tandem ESI-MS/MS instruments, is one of the most successful analytical techniques for the analysis of endogenous and exogenous compounds in complex matrices, such as urine, plasma or plants extract [32-34]. However, when analyzing biological matrices, it is important to consider the possible enhancement or suppression of analytes signals in the ionization stage by compounds that are present in the matrix, and co-elute with the investigated compounds [35, 36]. This effect, better known as the matrix effect (ME), negatively affects the quantification of substances present in such matrices. Indeed, a signal suppression or enhancement of targeted substances has an obvious impact on LODs/LOQs and may increase variability on peak areas. Therefore, validation of the analytical method can become challenging. Since the retention mechanism in SFC on polar stationary phases (mostly polar interactions) is orthogonal to LC (mainly hydrophobic interactions), coelution of investigated compounds and substances contained within the matrix may be very different. Therefore, UHPSFC-ESI-MS(/MS) can be considered as a useful strategy to minimize or at least modify the impact of ME, in comparison with UHPLC-ESI-MS(/MS) [37].

In the last 3-4 years, there has been an increasing number of studies dealing with the application of UHPSFC-MS for the analysis of biological matrices [38-42]. Urine has been by far the most widely used matrix, due to its relative easiness of collection and sample treatment. In the case of urine, ME is mainly due to the presence of polar compounds such as urea, creatinine, glucuronic acid, uric acid, etc., as well as salts. Svan *et al.* [36] have recently made a systematic comparison of ME between RPLC-MS and SFC-MS, using 11 representative drugs in urine samples. In their study, ME was evaluated using the post-

column infusion matrix profiles approach. To explain the differences observed in terms of ME, the authors first described the modification in separation profiles of matrix components between the two chromatographic techniques. Indeed, compounds generating ME in urine, which are highly polar, are eluted quite early in RPLC conditions, while they are strongly retained on SFC conditions and lately eluted thanks to the increasing concentration of the polar organic modifier in the mobile phase [36]. The differences, however, are not limited only to the separation profiles. In fact, under SFC conditions, there is a clear predominance of the ion suppression phenomenon, whose origin was further investigated in a follow-up paper [43]. In RPLC, both types of MEs (ion suppression and enhancement) co-exist, depending on the investigated analyte [36]. A second paper [37] correlated MEs obtained in RPLC and SFC using two different sample preparation methodologies (non-selective and selective), and the Matuszewski's approach was used as the ME evaluation. The conclusions reached by both authors were similar, clearly stating that signal suppression is the major type of ME in SFC for urine [37]. Moreover, SFC has proved to give less ME than RPLC in all experiments with urine samples [37]. This statement is further confirmed in other papers, where ME was found to be quite low in SFC-MS conditions [38, 44, 45].

While using plasma, however, the situation seems to be different. Indeed, the ME generated by plasma for around 40 representative drugs in SFC and RPLC [37] gave unexpected results. Higher signal suppression was observed in RPLC *vs.* SFC with the selective sample preparation methodology (solid phase extraction, SPE). However, the impact of ME was also highly dependent on the selected column chemistry in SFC [37]. In another study, the use of protein precipitation (PP) for plasma sample brought results that are similar to urine, with signal suppression being more common in SFC [36]. A third paper dealing with the application of SFC for the determination of three major antiepileptic drugs in plasma reports the level of ME around 95-100%, with only one compound subjected to slight signal suppression, stating therefore that SFC does not present issues with ME in plasma [46]. To draw some reliable conditions on ME for plasma samples, there is, however, a need for more

experimental results and discussion, due to the limited number of applications reported with human plasma under SFC conditions. In addition, it is also important to keep in mind that ME may be highly dependent on the geometry of the electrospray ionization source.

4. Achievable sensitivity in SFC-MS vs. LC-MS

SFC has always been considered as a well-suited technique for MS detectors, thanks to the hybrid nature of the mobile phase, and the use of organic solvents (mostly methanol) with higher volatility than water, thus positively influencing the ionization process, especially in ESI mode. The recent introduction of modern and reliable UHPSFC-MS systems allowed to experimentally prove some of the potential benefits of SFC over LC. Indeed, as shown in [28, 47-50], excellent values for LODs and LOQs were met, with LOD values often down to below 1 ppb [28]. However, SFC-MS does not systematically provide a clear advantage over LC-MS in terms of sensitivity. Indeed, it was found that, while with the older generation of MS instruments, SFC generally provides a higher sensitivity than LC, with the more recent mass spectrometers, SFC and LC were found to give very close results (Fig. 2) [51]. This observation was explained by the use of improved ionization sources on the more recent MS instruments, making them more able to handle higher proportion of water [51]. As an example, it was found that, out of 43 anabolic agents tested in human urine, LC provided a sensitivity level equal to 0.1 ng/mL for 98% of the analyzed compounds, while in SFC this percentage was reduced to 76% [52]. A similar result was obtained for vitamin D metabolites, with worse LLOQs in SFC than LC [53]. The main reason for these negative results is related to the limited injection volume in SFC. Indeed, it is well known that a lower injection volume has to be used in SFC vs. LC, especially when using polar and polar protic solvents such as methanol or water as the injection solvents [53-55], which should obviously negatively affect sensitivity. Moreover, different column geometries are generally used in LC and SFC (2.1 mm and 3.0 mm as internal diameters, respectively), which could further increase the dilution factor in SFC and reduce achievable sensitivity [52].

As previously discussed, there is a need to use a make-up solvent to couple SFC with MS. This means that users have the possibility to modify the mobile phase composition before entering MS detection, so that the ionization process can be enhanced, especially in ESI mode. Some authors have recently demonstrated how the addition of either small quantities of water or the use of additives/buffers in the make-up pump, increased the MS signals, thus improving sensitivity [28, 48]. Using a wide range of endogenous steroids, the authors screened different buffers/additives in the make-up solvent, finding that either pure ammonium fluoride or ammonium fluoride mixed with formic acid in the solvent, can greatly improve ionization efficiency in ESI mode for steroids. In another work, it has been highlighted how the make-up solvent can positively influence the ionization of protease inhibitors in ESI conditions, with a simple tuning of its composition [27]. The authors have concluded that, while in LC, the mobile phase composition is not easily modifiable to enhance MS performance, the necessary addition of the make-up solvent in SFC can generate large MS signals increases, also allowing the possibility of considering post-column derivatization to improve further MS detection [27].

5. Applications of SFC-MS

As already observed for SFC-UV, there has been a constant and impressive increase in the number of new applications recently developed in SFC-MS.

An important field of application is the analysis of natural products. Indeed, there have already been developments and successful implementations in the past, however, now the constantly growing use of high resolutions MS instruments (HRMS), hyphenated not only to LC but also to SFC, has pushed the latter even further in this area. Besides the analysis of lipophilic compounds including lipids in plants [56-59], there is an interesting and growing trend, namely the analysis of compounds with increasing polarity, such as monosaccharides [60], saponins [61] and flavonoids [62]. Other natural compounds are also being analyzed under SFC-MS, such as plant metabolites with interesting potential as drugs (Fig. 3) [63, 64]. A specific category, which also attracts attention, is cannabinoids; indeed, the use of this

class of compounds is rapidly increasing, in both medical and forensic applications [65-67]. Today, SFC-MS can be considered as a complementary technique to LC-MS, with an interesting ability in obtaining resolution of positional isomers and diastereomers, with a high degree of orthogonality to LC [68]. Moreover, the methods developed in SFC-MS also fit well with quality control requirements of real-life cannabis samples analysis [49], thanks to an easier sample preparation phase and a robust, fast and generic analytical method [49].

A second application area that is being under constant development is the implementation of SFC-MS in the forensic and anti-doping control analysis. Indeed, there has been an important number of papers recently released and focusing on several classes of compounds: amphetamines [45, 69, 70], stimulants and sympathomimetic drugs [51, 71, 72] or anabolic agents and steroids [44, 54, 73, 74] (Fig. 4). Researchers involved in the field of anti-doping analysis are now testing new analytical techniques (such as SFC-MS), to find possible advantages to the current state of the art represented by LC-MS. Furthermore, SFC is not only being used as an analytical method but also employed in the sample preparation stage [74], with the aim to replace older methods employed in the sample treatment. Obviously, SFC-MS methods that wish to be employed in anti-doping laboratories also have to be validated. This aspect is being currently investigated by several authors, with a growing number of publications [38, 39, 45, 47, 49, 75] showing that the validation procedure in SFC-MS yields similar, if not even better results than LC-MS. Indeed, during different validation processes of SFC-MS methods, it was found that SFC-MS manages to provide better results in terms of identification, reproducibility, precision and accuracy when compared to LC-MS [47, 75]. These findings are extremely important in establishing SFC-MS itself as a technique that is compatible with regulated bioanalytical laboratories.

Another arising trend in SFC-MS applications is the analysis of hydrophilic and highly hydrophilic compounds under subcritical conditions [40, 76]. SFC has been historically considered as a substitute technique to normal phase LC, and therefore, it has been mostly used for the analysis of compounds with low to medium polarity. However, thanks to the

development of innovative strategies, such as the addition of small amount of water and/or salts in the organic co-solvent, as well as the use of gradient conditions up to 70-100% organic modifier, the range of analyzable molecules can be extended to molecules possessing log *P* values below 0 [76]. Thanks to this new possibility, SFC-MS is now shifting towards the analysis of compounds that classically fall under the domain of HILIC-MS. As example, SFC-MS is now increasingly employed in the field of metabolomics, [76] in particular for the analysis of amino acids [40, 77] and carbohydrates [76, 78]. In addition, due to the high versatility of SFC-MS, it can be successfully employed for the simultaneous analysis of both hydrophilic and lipophilic molecules, from carbohydrates to lipids in metabolomics [76] (Fig. 5), from water to fat-soluble vitamins in food [79], and from highly hydrophilic to lipophilic trace organic compounds in environmental samples [80]. As more applications involving the use of SFC-MS with polar and highly polar compounds are arising, it can be stated that SFC-MS has now become a well-suited technique not only for lipophilic compounds, but also for those analytes whose polarity falls between $-2 < \log P > 2$. A recent review on the latest applications developed in SFC-MS for natural products, food and environmental analysis as well as bioanalysis and metabolomics is now available [81].

In contrast to LC, SFC instruments also offer the possibility to have an online extraction unit linked to the chromatographic system (online SFE-SFC). It is now commercially available and has recently been successfully employed in analytical laboratories in different areas, from the metabolic profiling of drugs metabolites in human urine [38] to the determination of carotenoids and apocarotenoids in human blood [82], and the analysis of polycyclic aromatic hydrocarbons in soil [83]. In these different studies, the authors highlight the very low sample amounts requirement, possibility to achieve fast analysis and how it has been possible to validate those methods [38, 82, 83]. This type of online SFE-SFC instrument, although it still needs to be more deeply characterized, in particular in terms of connections between the extraction, separation, and detection [83], possesses an impressive potential for the analyses

where sample preparation stages can be time-consuming and do not provide sufficient yields.

6. Conclusions

The hyphenation of SFC to MS has undoubtedly known an impressive growth in the last five years. The development of several SFC-MS geometry interfaces has enabled to couple both systems, as well as a discrete handling of the supercritical fluid once the mobile phase is not under the influence of the APBR module. There are, however, still aspects that necessitate to be thoroughly covered to understand the influence of the CO_2 decompression and how to better solve issues related to this phenomenon.

SFC-MS is increasingly being used to analyze compounds present in biological matrices, from urine to plasma, as well as natural substances available in plant extracts. Matrix effects due by biological samples and their impact on the MS signal and performance have been demonstrated to be quite different from what was observed in LC-MS conditions, offering a good complementarity between those two techniques. Nonetheless, there is still a need to further investigate this aspect, with additional sample preparation approaches and different matrices.

ME also impacted sensitivity in a different way than LC-MS, due to the higher probability of signal suppression, rather than enhancement under SFC-MS conditions. Sensitivity, in general, was found to be in several cases at the same level as in LC-MS, if not even higher. However, there are problems related to the limited injection volume and higher dilution factors that, sometimes, make it difficult for SFC-MS to reach LOQ and LOD values obtained in LC-MS.

Finally, the investigation of the most recent applications clearly shows that SFC-MS is moving towards the analysis of small molecules with increasing polarity. This translates in an increasing overlap with RPLC and HILIC. Indeed, the impressive flexibility of SFC in analyzing compounds within an extremely wide polarity range is probably one of the main

390 interests behind this technique. Its complementarity to RPLC drives an increasing number of
391 research groups, and analytical laboratories are starting to use it, to tackle challenging
392 separations achieved under LC-MS conditions.

393

7. References

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Figure captions

Figure 1: Representations of the five most common 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, (E) *"BPR and sheath pump with no splitter"* interface. Reprinted from *J. Chromatogr. B*, Vol. *1083*; D. Guillarme, V. Desfontaine, S. Heinisch, J.-L. Veuthey; What are the current solutions for interfacing supercritical fluid chromatography and mass spectrometry?, pp 160-170 [ref 25]. Copyright 2018, with permission from Elsevier.

Figure 2: A comparison of sensitivity between two different triple quadrupole platforms, i.e., Modern MS/MS device, namely Waters Xevo TQ-S (A) and old-generation MS/MS device, namely Waters TQD (B) in UHPSFC–MS/MS and UHPLC–MS/MS modes. Data used for this comparison were taken from [62]. Reprinted from *Anal. Chim. Acta*, Vol. *853*; L. Nováková, M. Rentsch, A. Grand-Guillaume Perrenoud, R. Nicoli, M. Saugy, J.L. Veuthey, D. Guillarme; Ultra high perfomance supercritical fluid chromatography coupled with tandem mass spectrometry for screening of doping agents II: Analysis of biological samples; pp 647-659 [ref 50]. Copyright 2015, with permission from Elsevier.

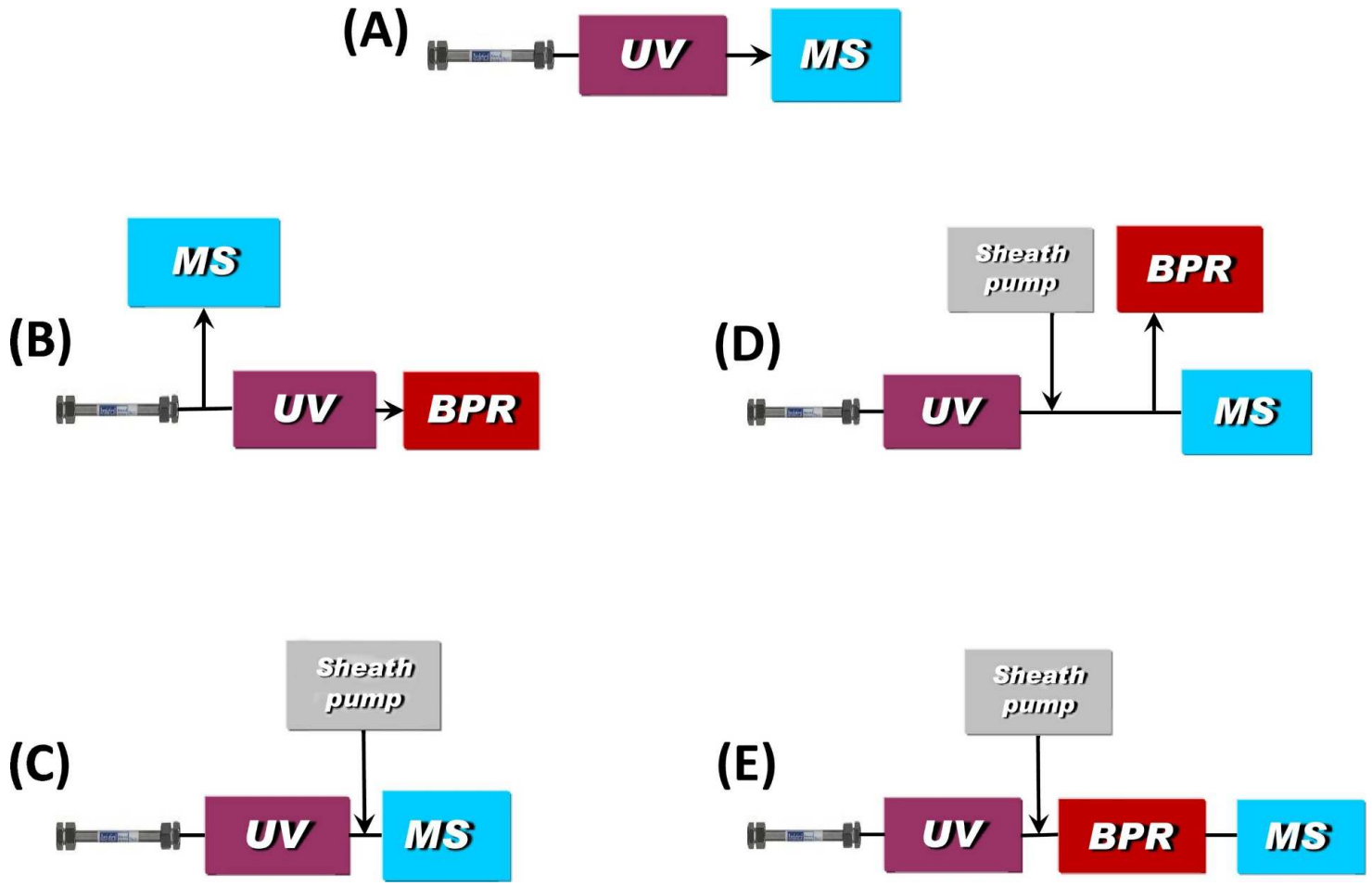
Figure 3: Example of chromatograms (UHPSFC-QqToF-MS traces) obtained on Diol column, highlighting chemically diverse compounds with Log P, H-bond capability and molecular mass respectively: carotenoid zeaxantin (10.92, 2, 568.43 Da); alkaloid sparteine (2.84, 2, 234.21 Da); triterpenoid lupeol (10.46, 2, 426.39 Da); the iridoid gentiopicroside (−3.03, 13, 356.11 Da); saponin ginsenoside-Rd (3.38, 30, 946.55 Da); diterpenoid paclitaxel (3.95, 19, 853.33 Da). Reprinted from *J. Chromatogr. A*, Vol. *1450*; A. Grand-Guillaume Perrenoud, D. Guillarme, J. Boccard, J.-L. Veuthey, D. Barron, S. Moco; Ultra-high performance supercritical fluid chromatography coupled with quadrupole-time-of-flight mass

spectrometry as a performing tool for bioactive analysis; pp 101-111 [ref 60]. Copyright 2016, with permission from Elsevier.

Figure 4: Chromatograms of nine steroids and related metabolites for injection of urine spiked at 10 ng/mL in UHPSFC-MS/MS.). Reprinted from *J. Chromatogr. A*, Vol. *1451*; V. Desfontaine, L. Novakova, F. Ponzetto, R. Nicoli, M. Saugy, J.L. Veuthey, D. Guillarme; Liquid chromatography and supercritical fluid chromatography as alternative techniques to gas chromatography for the rapid screening of anabolic agents in urine; pp 145-155 [ref 51]. Copyright 2016, with permission from Elsevier.

Figure 5: Chromatogram obtained for the simultaneous injection of tricosanoic acid and raffinose, using acetonitrile/water (50:50) as sample diluent and unified chromatography gradient conditions. Reprinted from *J. Chromatogr. A*, Vol. *1562*; V. Desfontaine, G.L. Losacco, Y. Gagnebin, J. Pezzatti, W.P. Farrell, V. González-Ruiz, S. Rudaz, J.-L. Veuthey, D. Guillarme; Applicability of supercritical fluid chromatography – mass spectrometry to metabolomics. I – Optimization of separation conditions for the simultaneous analysis of hydrophilic and lipophilic substance; pp 96-107 [ref 72]. Copyright 2018, with permission from Elsevier.

Figure 1


(A)
UV
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UV
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MS

Figure 2

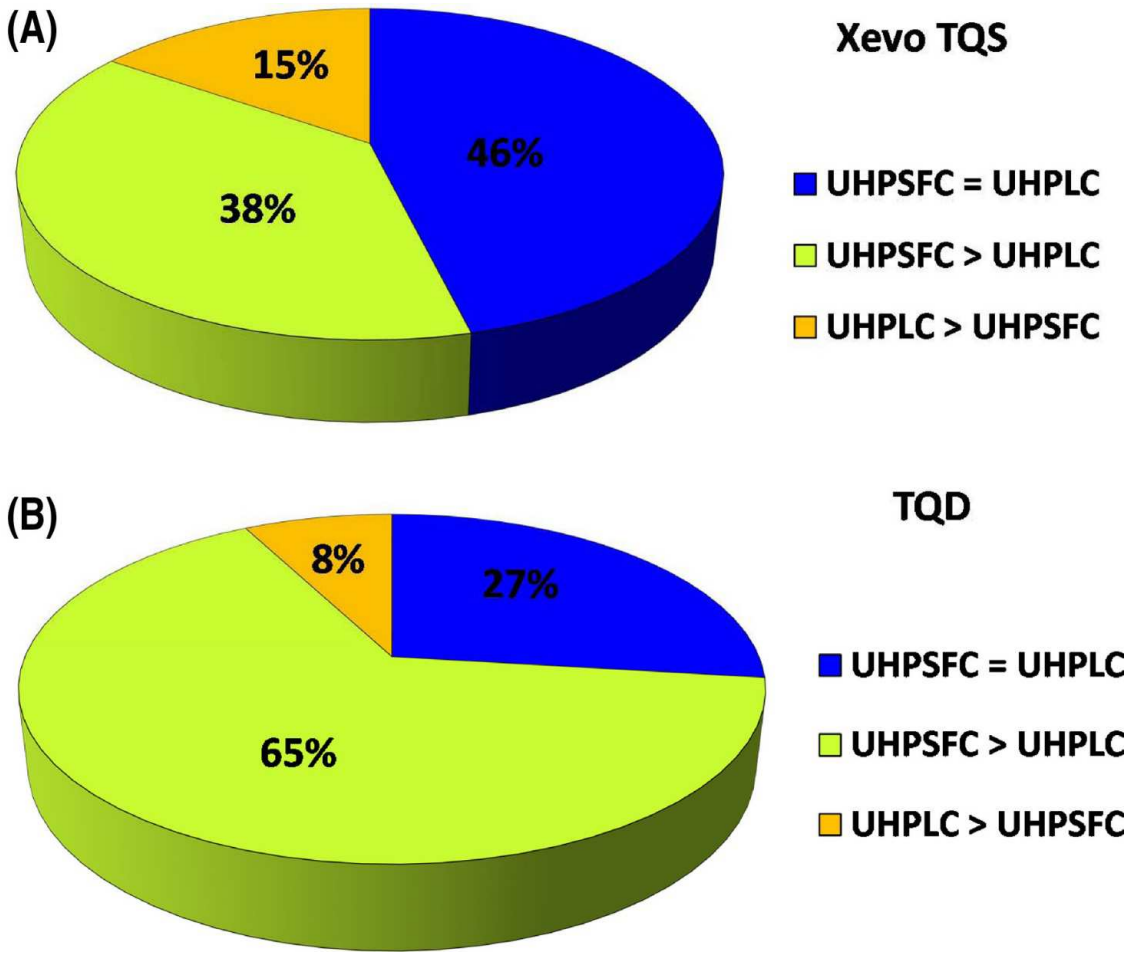
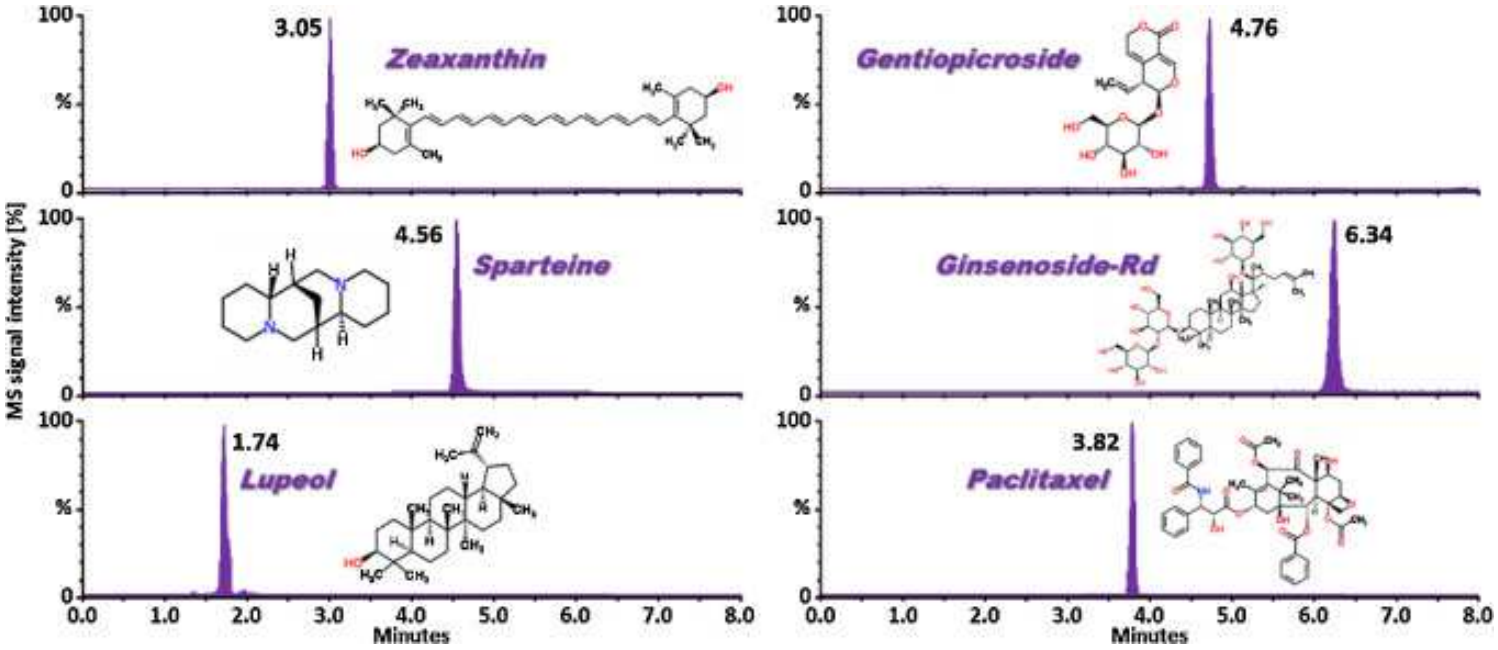


Figure 3


MS signal intensity [%]
100
%
0
3.05
Zeaxanthin
4.76
Gentiopicroside
4.56
Sparteine
6.34
Ginsenoside-Rd
1.74
Lupeol
3.82
Paclitaxel
0.0
1.0
2.0
3.0
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5.0
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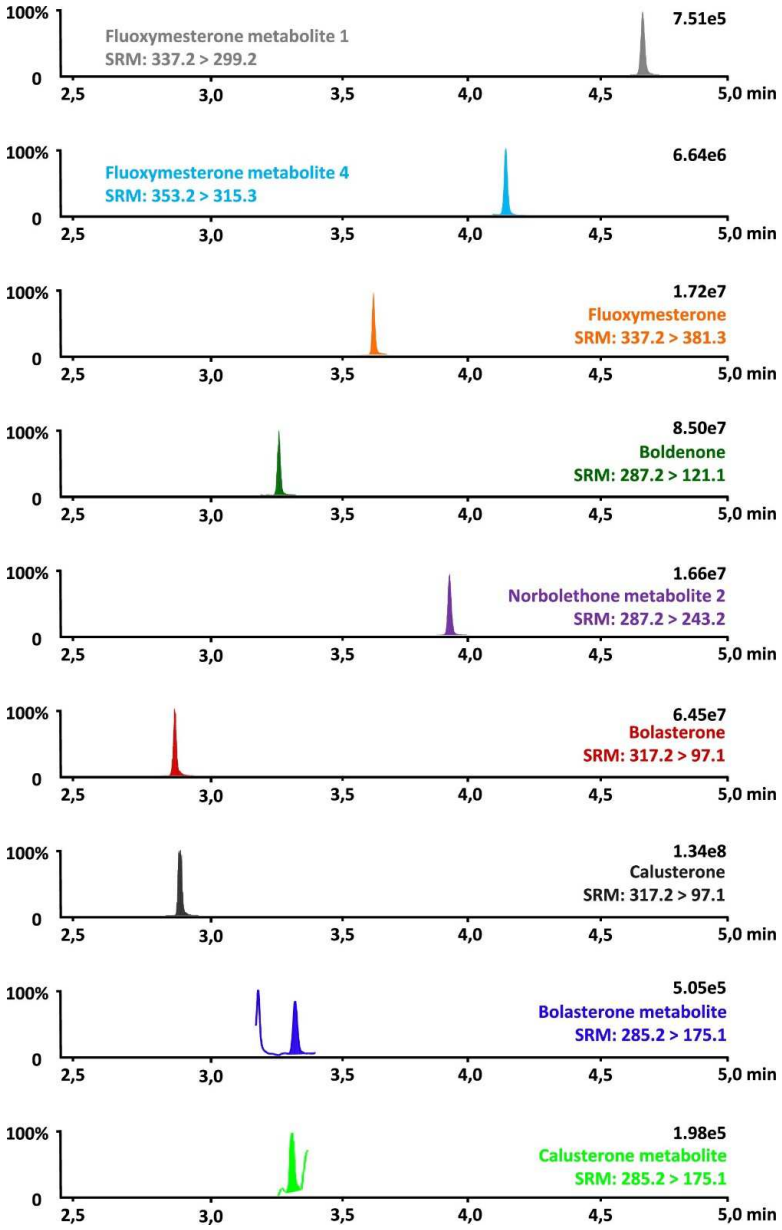
8.0
Minutes

Figure 4


100%
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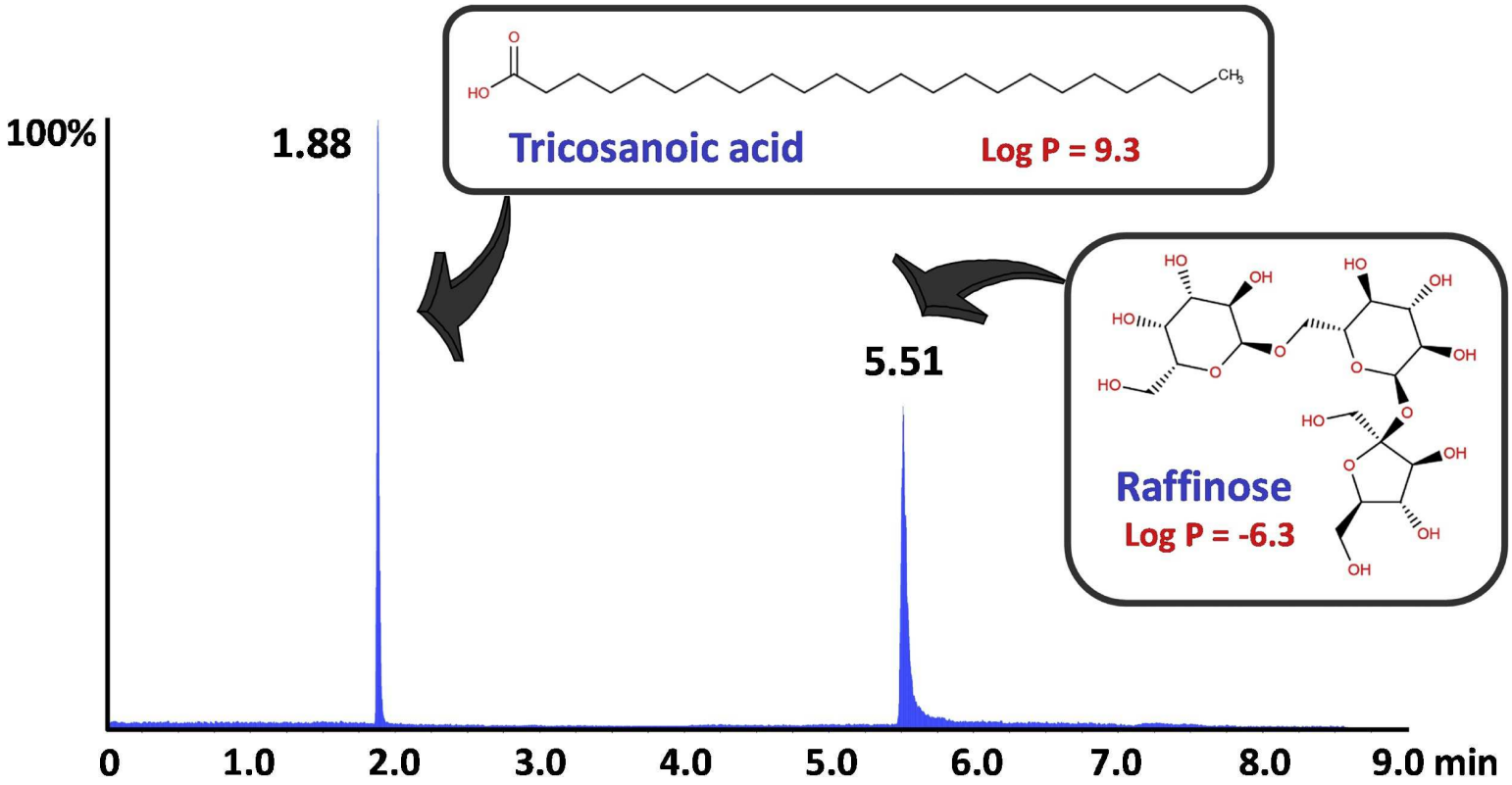
Fluoxymesterone metabolite 1
SRM: 337.2 > 299.2
7.51e5
2,5
3,0
3,5
4,0
4,5
5,0 min
Fluoxymesterone metabolite 4
SRM: 353.2 > 315.3
6.64e6
1.72e7
Fluoxymesterone
SRM: 337.2 > 381.3
8.50e7
Boldenone
SRM: 287.2 > 121.1
1.66e7
Norbolethone metabolite 2
SRM: 287.2 > 243.2
6.45e7
Bolasterone
SRM: 317.2 > 97.1
1.34e8
Calusterone
SRM: 317.2 > 97.1
5.05e5
Bolasterone metabolite
SRM: 285.2 > 175.1
1.98e5
Calusterone metabolite
SRM: 285.2 > 175.1

Figure 5


100%
1.88
5.51
Tricosanoic acid
Log P = 9.3
Raffinose
Log P = -6.3
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0 min