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Metamorphosis of supercritical fluid chromatography: A viable tool for the analysis of polar compounds?

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ABSTRACT

Recent developments in supercritical fluid chromatography (SFC) have highlighted the applicability of this technique for the analysis of highly polar compounds. The combination of polar stationary phases and CO_2-based mobile phases with an increasing presence of liquid co-solvent (up to 100%) has enabled to further expand the application field of SFC towards a variety of samples such as polar endogenous metabolites, plant extracts, water-soluble vitamins, pesticides, sugars, peptides and so on. In this evolution, a key role was played by the addition of up to 5–10% of water in the liquid co-solvent. Moreover, the presence of water enabled higher concentrations of additives, up to 75–100 mM in some cases. These improved conditions were fundamental in expanding the applicability range of SFC. Overall, SFC has demonstrated its evolution into a mature technique capable of offering a true alternative to liquid chromatography for the analysis of polar compounds.
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1. Introduction

Analytical laboratories are involved in a constant effort to develop their workflow, as they are facing with the need to analyze increasingly complex samples. In the pharmaceutical environment, scientists are often required to perform assays on compounds showing quite different physical-chemical properties (e.g. size, lipophilicity, ionizable groups …), going from small synthetic drugs to peptides and proteins [1–3]. Obviously, as the samples of interest show such different characteristics, the analytical technique has to be adapted. Liquid chromatography (LC) is highly versatile and has risen as one of the most employed approaches to analyze a wide variety of compounds [3]. Thanks to its various modes of operation, LC can successfully guarantee the analysis of samples covering log P values ranging from 10 to −10 (Fig. 1). Therefore, lipids, steroids, synthetic drugs, nucleosides, sugars, peptides and proteins have been successfully analyzed by using the LC mode that fits best to each analyte category [4–7].

While LC can guarantee a suitable retention for an impressive range of samples, it also shows some drawbacks. Normal phase LC (NPLC) employs toxic solvents (e.g. hexane, dichloromethane, etc.), therefore it is not considered environmentally friendly [8,9]. Reversed phase LC (RPLC) shows an excellent robustness and ease of transferability among different laboratories, but it is unsuitable to analyze highly polar substances [10,11]. Hydrophilic interaction chromatography (HILIC) was developed to better retain the latter, but long equilibration times are often needed [10]. Moreover, both techniques employ large amount of organic solvents, thus they cannot be considered as green analytical strategies [12]. Therefore, analysts have begun to shift their interest towards alternative solutions. Among them, supercritical fluid chromatography (SFC) and its modern version known as ultra-high performance supercritical fluid chromatography (UHPSFC) could be attractive [13,14]. UHPSFC employs a mixture of supercritical carbon dioxide ($scCO_2$) mixed with polar organic modifier, in most cases methanol (MeOH), using columns packed with sub-2μm particles. As highlighted in Fig. 1, UHPSFC can be considered a valid alternative to NPLC and RPLC for apolar and mildly polar substances [14]. UHPSFC methods have been generally developed following some specific guidelines. As an example, pure methanol was often chosen as the liquid co-solvent, especially when samples with limited polarity were analyzed. Gradient profiles with a maximum percentage of approximately 35–40% modifier mixed with supercritical CO_2 were constantly used. However, many analysts have recently begun to evaluate UHPSFC for hydrophilic analytes such as polar metabolites or water-soluble vitamins. In 2014, a work published by Taguchi et al.

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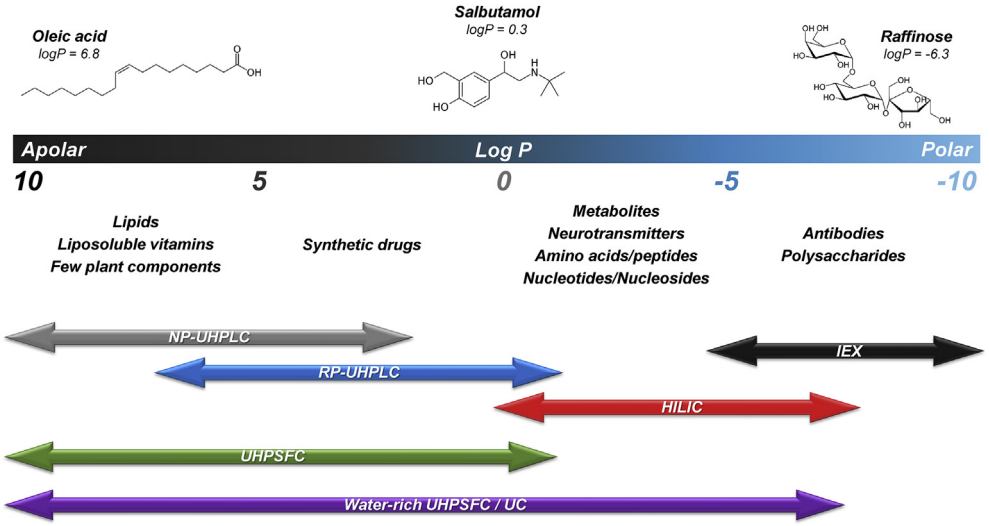

Fig. 1. Polarity range covered by different LC techniques (NPLC, RPLC, HILIC and IEX), compared to UHPSFC and water-rich UHPSFC/UC.

illustrated how the combined use of a water-rich modifier with a gradient profile up to 100% in the organic modifier, also known as "Unified Chromatography" (UC), enabled the simultaneous analysis of water soluble and liposoluble vitamins in UHPSFC [15]. Hence, the use of water-rich UHPSFC or UC conditions can potentially expand the polarity range of UHPSFC, allowing its use as an alternative not only to NPLC and RPLC, but also to HILIC (Fig. 1).

The aim of this review is to analyze and describe the latest trends in UHPSFC, focusing on its potential implementation with highly polar substances. A description of how UHPSFC mobile phase has evolved to guarantee the elution of such compounds is given, as well as on different gradient profiles which have arisen in recent years. Afterwards, a focus on the choice of the stationary phase is provided, highlighting the possible advantages of UHPSFC over UHPLC, as well as potential drawbacks and limitations. Finally, a detailed overview of the latest applications developed for a variety of compounds such as polar biomolecules, plant extracts, polar metabolites and pesticides is given, focusing also on how UHPSFC has evolved in a potential choice for multidimensional systems coupled to UHPLC techniques.

2. Evolution of UHPSFC mobile phase

2.1. Use of water-rich organic modifier

The implementation of water in SFC is not a new trend. Research groups initially tested water as an alternative to polar small alcohols to increase the polarity of $scCO_2$-predominant mobile phase [16]. However, its poor miscibility with supercritical CO_2 discouraged analysts to employ it as a pure modifier, but rather in combination with organic solvents such as methanol or ethanol [17,18]. In recent years, the use of water-rich modifier has started to become more preponderant. Various percentages of water, from 1% up to 7–8% mixed with methanol, have been used in UHPSFC methods for the analysis of various substances, such as polar metabolites [19–23], doping agents [20,24–26], plant extracts [27–29] as well as peptides and proteins [30–32]. Water became an increasingly employed additive also for chiral UHPSFC separations [33–36]. Applications involving UHPSFC to analyze strongly retained substances on the polar stationary phase, such as hydrophilic analytes, was sometimes difficult to develop in the past. Indeed, the "standard" choice of the organic modifier was often based on the use of pure methanol [37–39]. Such co-solvent, however, could not guarantee a sufficient eluent strength for the UHPSFC mobile phase, as the compounds are too strongly retained by the stationary phase. Therefore, either long time analysis or, in the worst cases, no elution at all of highly polar substances is observed [33,34]. The use of water in the organic modifier has enabled a remarkable increase in the elution strength of the UHPSFC mobile phase. As the extension of the gradient profile increases the presence of the modifier, and therefore water, a stronger competition between the stationary and mobile phases for the sample arises [34,40]. Consequently, a reduction of analysis time can be observed in most cases (Fig. 2), and the elution of strongly retained compounds becomes possible [34,35]. Another benefit to add water in the SFC mobile phase is to obtain sharper and more symmetrical peak shapes [28,41,42] (Fig. 2). Furthermore, the use of water as a simple additive greatly improves the reproducibility of UHPSFC analytical methods in some cases. It was indeed recently demonstrated how its systematic use contributed to generate low retention times variability for a set of doping agents in biological matrices across an extended timeframe (up to four months), as well as to reduce the inter-laboratory variability for the same UHPSFC method [24,25]. The authors claimed that the constant exposure of the stationary phase to water helped minimizing the formation of methyl-silyl ethers on the free silanols, a phenomenon which was proved to be detrimental in UHPSFC causing potential changes in the elution profile [43].

2.2. Use of high concentration of additives

The systematic implementation of water in the organic modifier was not the only development concerning UHPSFC mobile phases in recent years. Another feature allowing the analysis of highly polar molecules in UHPSFC has been the use of additives, such as trifluoroacetic acid (TFA), ammonia, ammonium formate (AmF) and acetate (AmAc), at relatively high concentrations (equal or above 20 mM) [42,44,45]. While acidic and basic additives, available under their liquid form, never presented miscibility issues regardless of their concentration, there was a limit in the concentration reachable for AmF and AmAc in the organic modifier, when using pure methanol. The addition of water to MeOH ensured, as expected, a much-improved solubility of these salts, thus allowing their use at higher concentrations. As demonstrated in recent

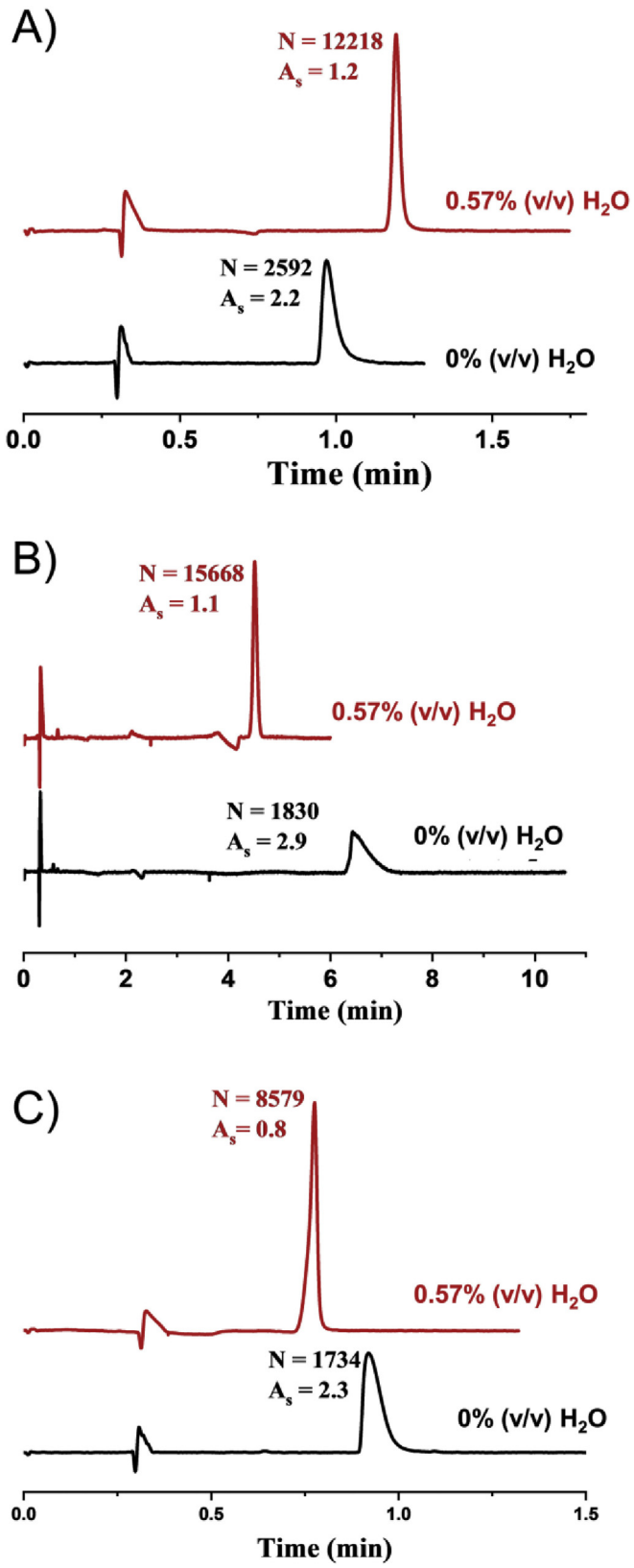

Fig. 2. SFC chromatograms of a) nicotinic acid, b) 1-methyl-3-phenylpropylamine and c) 3,5-dinitrobenzoic acid on FructoShell-N column with or without the addition of water in the mobile phase. Reprinted with permission from Ref. [34].

articles, levels up to 150 mM of salts were reached in the organic modifier (Fig. 3) thanks to the presence of water [23,45,46], while in one work it was possible to use concentrations exceeding 1 M of liquid additives in pure methanol [47]. As shown in these papers, a high amount of additives provided a significant improvement of chromatographic parameters such as peak shapes and peak widths (Fig. 3). However, using concentrations as high as 150 mM could present issues when coupling UHPSFC with mass spectrometry (MS) [23]. Issues such as low MS sensitivity, as well as a higher risk of salt precipitation in the mobile phase might still occur [48]. Nonetheless, concentrations of buffers ranging from 20 to 50 mM have been used to analyze a series of challenging analytes such as amino acids [22,45,46], biosurfactants [49] and a series of polar analytes (log P < −2) present in environmental water samples [50].

As previously mentioned, the choice of the additive has been limited due to potential solubility issues in the $scCO_2$/MeOH mobile phase. However, as water is present in the mobile phase, it enabled either the use of salts which are poorly soluble in organic solvents [23] or the combination of multiple additives in the organic modifier [22,26], due to its improved solvation capability.

2.3. Choice of the gradient profile

Researchers have also focused their efforts on the use of an unconventional ratio of $scCO_2$ to organic modifier, with the goal to further increase the elution strength of $scCO_2$-based mobile phases. One of the potential solutions is the use of innovative gradient profile called “Enhanced Fluidity Liquid Chromatography” (EFLC). With EFLC-type gradients, the amount of $scCO_2$ is reduced and does not reach percentages above 35–40% in the mobile phase. In some of the latest applications involving this technique, it was even demonstrated how an increased presence of water, mixed with the supercritical CO_2, was possible [51,52]. CO_2 is, therefore, used mainly to reduce the mobile phase viscosity. By doing so, the advantages typically observed with the use of $scCO_2$-predominant mobile phases are still observed (i.e. enhanced kinetic performance, reasonable backpressure), while miscibility issues between all components of the mobile phase are reduced [53]. With this approach, it was possible to perform analyses on polysaccharides [53], as well as proteins [52].

Another approach developed by research groups is the “Unified Chromatography” (UC) gradient [15]. The idea with UC-type gradients is quite simple: to ensure the successful elution of the most retained compounds from the stationary phase, the amount of polar co-solvent can be increased, to improve the elution strength, thus ensuring that even highly-retained analytes can be eluted and detected [22,45]. The organic modifier employed in UC-type gradients should not reach high amounts of water, in order to ensure its full miscibility with the $scCO_2$. The advantage of such gradient would be the possibility to perform simultaneous analyses of lipophilic and hydrophilic substances. In one of the first UC gradient application, UHPSFC demonstrated good performance analyzing, at the same time, lipophilic and hydrophilic vitamins [15]. UHPSFC was also successfully used to analyze apolar and polar endogenous metabolites spiked in biological matrices [22].

3. UHPSFC stationary phases

3.1. Impact of high modifier percentages on kinetic performance

The use of EFLC or UC-based gradients comes, however, with different constraints and issues. As the liquid component increases,

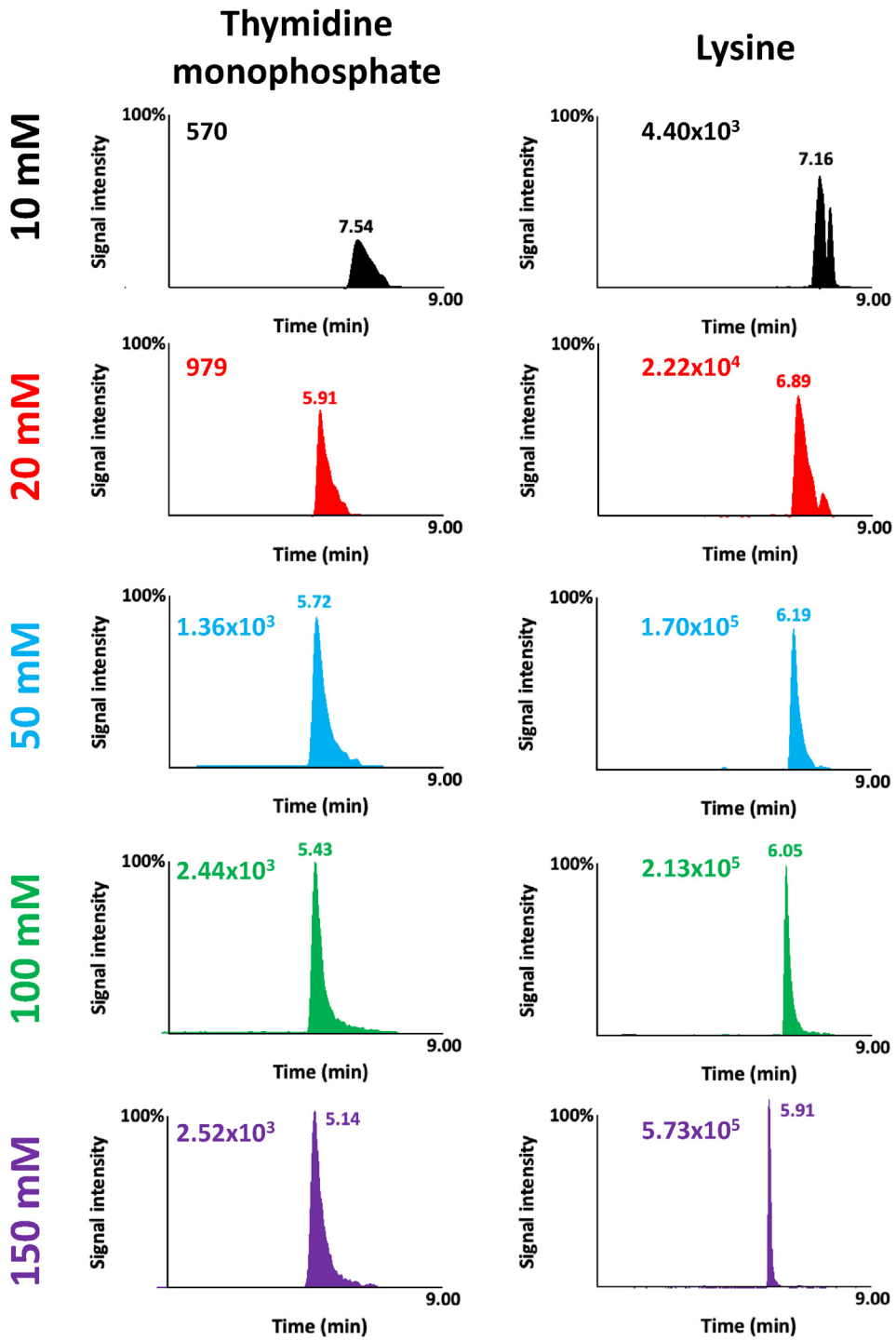

Fig. 3. Chromatograms for thymidine monophosphate (left) and lysine (right) obtained on a Poroshell HILIC column using concentrations up to 150 mM of ammonium formate in the mobile phase. Adapted, with permission, from Ref. [23].

higher column backpressure must be expected [23]. This is particularly relevant for columns packed with sub-2 μm fully porous silica particles having a low permeability [23,54]. Pressure values of 400 bar are quickly reached even at relatively low linear velocities. Unfortunately, the latest generation of UHPSFC equipment is only capable to handle column backpressures up to 400–660 bar. Therefore, if UC or EFLC-type gradients are considered, low flow-rates should be used to avoid system overpressure [23], as well as columns of reduced length [55]. Furthermore, it is also important to consider its impact on the kinetic performance. Some authors have recently attempted to understand how the kinetic performance of a column packed with sub-2 μm particles evolved during a UC-type gradient at four levels of co-solvent, *via* the analysis of van Deemter plots (Fig. 4) [54]. Under supercritical conditions (<5–10% of modifier), UHPSFC was able to provide low plate height values (H) at high velocities, meaning that high flow-rates must be employed to obtain the best performance. This statement is valid in particular for columns packed with sub-2 μm

fully porous silica particles. However, when transitioning progressively from a supercritical to a liquid-like state, the C-term region of the van Deemter curve rapidly increases, resulting in a worsening of the kinetic performance at high flow-rates [54]. This study indicates that, for UC-type gradients, the optimal velocity changes within the course of the analysis. Indeed, relatively low flow-rates are recommended at high co-solvent percentages, while high flow-rates can be used at low organic modifier percentage [23,54].

Unfortunately, as commercial UHPSFC systems do not possess an upper pressure limit as high as to those reached with UHPLC instruments (1200–1500 bar), alternative solutions have to be found to limit pressure and flow-rates issues. One of the most widely used solutions was to employ larger silica particles (3 and 5 μm), as observed in several articles [45,56–58], Another attractive solution is to select stationary phases based on superficially porous silica particles (also known as core-shell or fused-core). Their use is not widespread in UHPSFC, since there is only a limited number of articles using these type of particles, focusing either on chiral [34,36,59,60] or achiral applications [22,23,61]. Nevertheless, they can provide interesting advantages, especially when performing EFLC-type or UC-type gradients. The reduced backpressure, coupled to the possibility of obtaining comparable performance to those generated by fully porous sub-2 μm particles, should make superficially porous particles interesting in the context of UHPSFC [23]. Unfortunately, the commercial offer in terms of polar stationary phases packed with superficially porous particles is still too limited, and alternative strategies must be considered. Recently, Losacco et al. investigated the use of very high temperatures (up to 80 °C) in UHPSFC using a sub-2 μm stationary phase, focusing on the kinetic performance and pressure ranges available using modifier percentages as high as 100% [54]. Their results suggest that, with cosolvent amounts starting from 40 to 50% onwards, high temperatures (>40 °C) helped in maintaining good kinetic performance at high velocities (Fig. 4), as well as in reducing the backpressures generated when the mobile phase was predominantly composed of organic modifier. Besides the impact of column pressure drop, variations in the column temperature can also be helpful in optimizing the achievable selectivity. Finally, the stability of the stationary phase tested at 80°C gave very promising results.

3.2. Choice of UHPSFC stationary phases chemistry

The choice of the stationary phase chemistry in UHPSFC is not as straightforward as in UHPLC, where C_{18} selector is commonly used. Indeed, several UHPSFC column chemistries are available on the market, making the column screening protocol still very important [62]. Nonetheless, it is possible to summarize some common characteristics, to better understand the performance of UHPSFC for highly polar compounds. After its introduction and early development, SFC became an alternative to NPLC, in particular for chiral separation [8]. Hence, a significant part of the columns developed for UHPSFC present a relatively high polarity range with strong H-bonding properties, represented nowadays by bare silica based columns or those functionalized with various selectors such as diol or alkyl-pyridines [19,30,63]. All these stationary phase chemistries have been successfully used to efficiently analyze low to mildly polar compounds, but they should also work with more polar analytes, similarly to what is commonly seen with HILIC applications [19,22]. As previously discussed, the evolution of UHPSFC mobile phases with a more systematic use of water as well as high concentrations of additives made possible to demonstrate how UHPSFC stationary phases performed well also for more hydrophilic compounds. Among them, the most employed ones seem to be those possessing basic selectors (i.e. picolylamine, alkyl-pyridine and alkyl-amine), as they can provide an additional ionic interaction under acidic mobile phase conditions [32,57,64]. Nonetheless, those with a relatively neutral nature (i.e. bare silica, diol) are also widely used by research groups for the analysis of highly polar compounds [28,44,65]. In conclusion, while basic and/or neutral columns can be successfully employed for the analysis of highly polar substances, there is still the need to perform a screening procedure for a wide range of stationary phases to select the most appropriate one for a specific application.

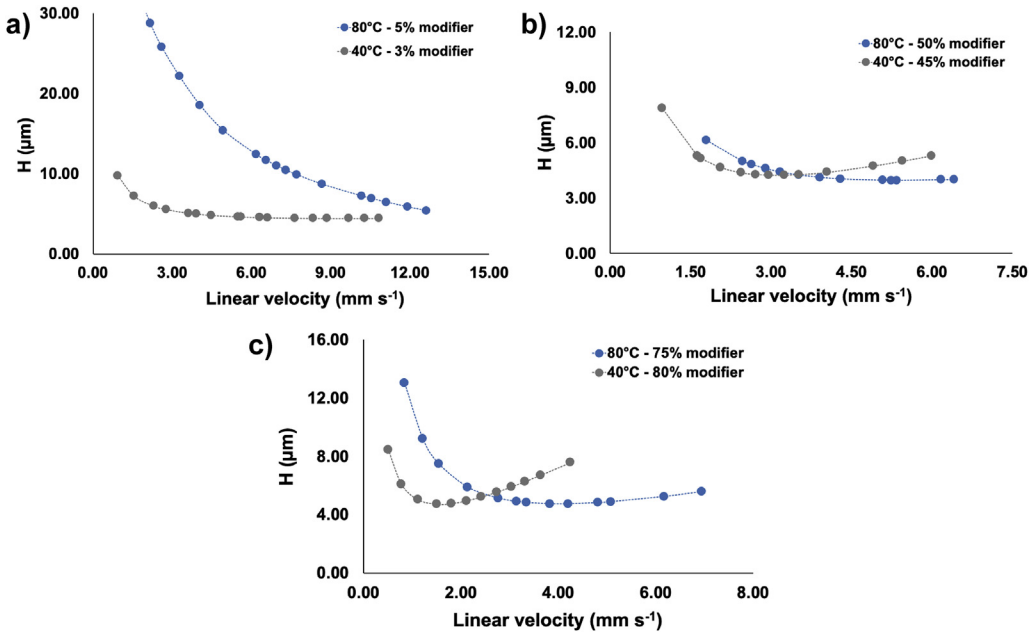

Fig. 4. Van Deemter plots obtained for a) butylparaben, b) maleic acid and c) indoxyl sulphate on the Torus 2-PIC 1.7 μm fully porous silica particles, using either 40°C (grey plots) or 80°C (blue plots) as the column temperature. Modified with permission from Ref. [54].

On the other hand, an increasing number of articles show promising results using alternatives column chemistries. As an example, due to their strong similarity, some researchers have highlighted the potential of HILIC columns under UHPSFC conditions [42,49,53,56,66]. Furthermore, UHPSFC allows an unprecedented flexibility in terms of column choice, compared to UHPLC. As an example, apolar stationary phases such as non endcapped C_{18} coupled to polar stationary phases, have been used for the analysis of saccharides [27]. Weakly apolar columns, such as pentafluorophenyl (PFP) and crown ether-based stationary phases were also used for polar metabolites and peptides analysis [21,31]. Last but not least, columns offering ion-exchange type retention mechanisms were also investigated and successfully used in UHPSFC for polar analytes [26,58].

4. Current applications and future trends

The evolution of UHPSFC in combination with modern stationary phases contributed to expanding the range of applications towards new areas. Previously considered as an analytical tool for apolar and mildly polar compounds, UHPSFC is now increasingly employed for analyzing polar analytes. UHPSFC has been successfully tested in the field of anti-doping analyses for either polar doping agents or related metabolites, in neat solutions or in complex matrices such as urine [20,25,26]. Its routine application has also been verified, demonstrating its complementarity to the more established UHPLC techniques [24,25]. UHPSFC has been also used to analyze amino acids [30,42,45,46] and vitamins in dietary supplements, as well as polar substances present in plant extracts [15,44,64,64,67] and food dyes [68].

More recently, UHPSFC has been more consistently used for the analysis of biomolecules such as nucleosides, peptides and, in few cases, even small proteins (Fig. 5) [22,23,31,32,69,70]. All these applications had already been tested in the past, however with the recent innovations in terms of analytical conditions, a renewed interest emerged. UHPSFC demonstrates interesting performance, leading up, potentially, to its more systematic use for such analytes. More information on the use of UHPSFC for the analysis of biomolecules is available in a recent review [13]. Another important field which has been recently explored in UHPSFC as an alternative analytical tool is metabolomics [19,65,71]. Here, UHPSFC provided good results, and enabled the simultaneous analysis of lipophilic and hydrophilic metabolites, demonstrating a good retention profile for a large library of more than 600 metabolites [22,23]. Finally, UHPSFC also demonstrated its value for the analysis of polar pesticides [72–75].

To further improve the resolving power in the field of UHPLC, a growing number of laboratories have started utilizing a multidimensional approach, by coupling complementary techniques to maximize the amount of information that can be obtained from a single run. Multidimensional chromatography involves, nowadays, the combination of two UHPLC modes such as, for example, RPLC, HILIC or ion-exchange [28,76]. The evolution of UHPSFC has definitely increased its attractiveness as one of the chromatographic dimensions in a multidimensional setup, coupled with a UHPLC technique or, in more extreme cases, to a second UHPSFC dimension [77–79]. Various technical impediments have slowed down the implementation of UHPSFC in multidimensional systems. However, recently there has been a stronger focus on the possibility of coupling UHPLC to UHPSFC in two-dimensional set-ups. Off-line hyphenation of UHPSFC to UHPLC has been, in a first instance, considered, as it presents less technical challenges [80,81]. Subsequently, on-line multidimensional systems with UHPSFC being either in the first or the second dimension have been studied [82–84]. Today, the most convincing application involving UHPSFC

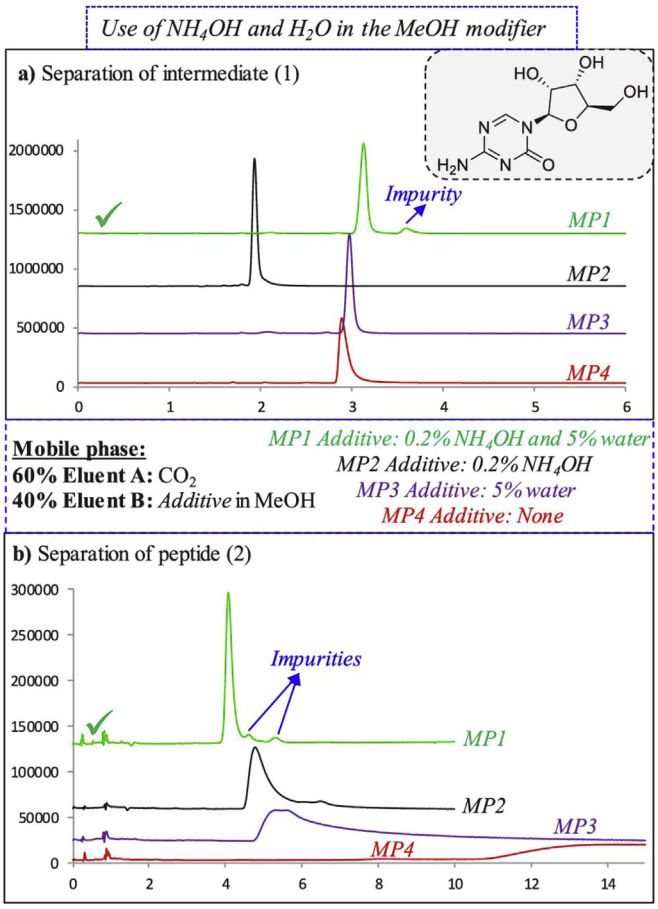

Fig. 5. UHPSFC analysis of a) nucleoside and b) peptide from related impurities using a water-rich modifier. Reprinted, with permission, from Ref. [70].

in one of the on-line dimensions consists in using achiral analysis in the first dimension and chiral separation in the second one, since it can benefit from the well-known advantages that UHPSFC provided in the context of chiral analysis. A more detailed review on this topic can be found elsewhere [77].

5. Conclusion

UHPSFC has witnessed an important transformation to improve its compatibility with strongly polar substances. Most of the work focused in developing alternative mobile phase conditions to boost the elution power in UHPSFC. The systematic addition of water, although in limited quantity, as well as the use of relatively high concentration of additives (>20–30 mM) have greatly contributed to enhance the ability of the $scCO_2$-based mobile phase in successfully eluting highly polar compounds.

Research groups focalized their attention also on the selected gradient profile. Two strategies have consequently emerged. While with EFLC-based gradients, the presence of CO_2 is strongly limited to allow a much higher percentage of water in the modifier, UC-based gradients aim to cover and analyze the widest possible range of molecules, from apolar to highly polar compounds, exploiting the ability of UHPSFC stationary phases to retain an incredible number of analytes, through H-bonds, dipole-dipole and ionic interactions.

As both EFLC-based and UC-based gradients need high percentages of liquid co-solvent, the backpressure values generated by UHPSFC columns, especially those packed with sub-2 μm fully

porous silica particles, are very high and therefore, alternatives have been evaluated. The solutions that have emerged focalize on either the employment of sub-3μm superficially porous silica particles or the use of high temperatures if sub-2 μm particles are chosen. With both approaches, good kinetic performance is maintained and it was possible, at the same time, to greatly reduce the column backpressure.

Finally, various applications involving UHPSFC for highly polar compounds have been developed. Thanks to the latest developments, UHPSFC demonstrated excellent results for different analytes, varying from amino acids, sugars, doping agents, pesticides, to biomolecules. Furthermore, the increased applicability of UHPSFC in different areas can fuel the interest of researchers in using UHPSFC in multidimensional systems, aiming in obtaining an analytical tool capable to generate an increased amount of complementary information within a single run.

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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