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Leveraging the Power of Supercritical Fluid Chromatography for Eco-Conscious Solutions in Pharmaceutical Analysis

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Abstract

Initially employed primarily at a preparative scale for enantiomer separation of chiral drug candidates, Supercritical Fluid Chromatography (SFC) is nowadays extensively used in the analytical mode. Recent advances in SFC separation science have emphasized its potential for modern and environmentally friendly pharmaceutical analysis.

The aim of this review is to provide a deeper insight into the main fundamental and practical aspects of the SFC technique in order to familiarize readers with its versatile nature and efficiency in creating sustainable chromatographic solutions. All considerations are made primarily in the context of the most widely used mode of operation - achiral SFC. In addition, recent applications of this promising technique are presented at the end of the article to further promote its use in pharmaceutical analytical practice.

Key words: supercritical fluid chromatography, pharmaceuticals, achiral separations, environmental friendliness

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The chromatographic toolbox in modern drug analysis: the place of SFC

Reversed-phase liquid chromatography (RP-LC) is a cornerstone of analytical techniques in the pharmaceutical field due to its simplicity, adaptability, robustness and seamless integration with powerful mass spectrometers (MS). Its widespread use is based on its applicability for analytes with certain hydrophobicity, quantified by a log*P* value ranging from -1 to 6. RP-LC uses a non-polar stationary phase (such as C18, C8 or phenyl) and a polar mobile phase consisting of a mixture of buffered water and organic solvents such as acetonitrile (ACN), methanol (MeOH) or tetrahydrofuran (THF) (1). For pharmaceuticals with lipophilicity falling outside the specified log*P* range, alternative analytical techniques become necessary.

Normal-phase liquid chromatography (NP-LC) is recommended for compounds with log*P* values between 2 and > 10, such as the class III antiarrhythmic drug amiodarone, which exhibits prolonged retention in RP-LC systems. NP-LC employs a polar stationary phase (e.g. bare silica, amino, cyano) with a non-polar organic solvent mobile phase (e.g. hexane). However, NP-LC comes with disadvantages, including the use of toxic and pricey solvents, limited suitability for ionizable compounds and poor compatibility with MS (1).

Conversely, compounds with a log$P < 0$, such as aminoglycoside antibiotic streptomycin, can be analyzed using hydrophilic interaction chromatography (HILIC). HILIC utilizes a polar stationary phase and a mobile phase composed of buffered water (5–40% v/v) mixed with a solvent (usually aprotic ACN). However, HILIC is not as versatile and robust as RP-LC and requires longer equilibration times (1).

In terms of compound polarity, HPLC covers the broadest range of molecules encountered in drug discovery and development, operating in different RP-LC, NP-LC and HILIC elution modes. However, both NP-LC and HILIC modes have limitations, exhibiting inferior resolution, repeatability, and selectivity compared to RP-LC. In addition, there are serious sustainability issues associated with these techniques (2). With regard to the latter, LC practitioners are eagerly seeking strategies to reduce the environmental burden by exploring greener solvent alternatives and optimizing separation conditions to minimize the consumption of toxic solvents. Advances in column technology have also contributed to greater efficiency and resource savings (3−5). However, despite the efforts of analysts, all of the above techniques still face challenges related to the use of harmful solvents and their impact on the environment.

Recently, supercritical fluid chromatography (SFC), which focuses on eco-friendly carbon dioxide (CO_2) as the mobile phase, has moved out of scientific anonymity and into the spotlight in the promotion of sustainable chromatographic approaches (4, 6, 7). The renewed interest in SFC came from the low viscosity of the mobile phase and the high diffusion coefficients which facilitate fast separations at high linear velocities. Besides analytical convenience, these capabilities conserve resources and ultimately ensure compliance with the suitability principles (4, 8). Apart from being environmentally friendly, SFC offers the capability to cover a wider range of (a)chiral active

pharmaceutical ingredients (APIs) and their impurities across varying polarities (1, 9). This is due to the fact that in SFC operating modes are mutually compatible. Consequently, one can begin with conditions tailored for nonpolar analytes and then transition to polar elution modes (7).

The (r-)evolution of SFC

Although the effects of “supercritical fluids” (SFs) were observed by Cagniard de la Tour in 1822 and their usefulness was described more than 50 years later by Hannay and Hogarth in 1879, the first application of this phenomenon in chromatography was not considered until almost 100 years later by Klesper and colleagues (10). In 1962, Klesper et al. (11, 12) described the separation of thermolabile porphyrin derivatives using supercritical chlorofluoromethane at pressures up to 14 MPa and temperatures from 150°C to 170°C. SFC mobile phases carry substances as mobile phases in gas chromatography (GC) and also dissolve these substances as solvents in LC.

Since its debut, SFC has developed into one of the most versatile chromatographic techniques. Its path has been characterized by incremental improvements over more than five decades rather than sudden revolutions (7). In the early times, the pharmaceutical industry showed very limited interest in SFC. Interest begins in 1980 with the discovery of capillary SFC (cSFC) by Novotny et al. (13). For cSFC conditions, capillary or open tubular columns with a mobile phase SF or possibly an SF with the addition of a very low proportion of co-solvent were used. The beginnings of SFC, especially in the 1990s, are exclusively related to chiral separations and preparative chromatography, mainly due to the poor quantitative performance, as well as lack of reproducibility and robustness of the analytical systems.

The recent advancement of SFC separation science looks at the performance of this technique through the prism of new possibilities for modern and environmentally friendly pharmaceutical analyses. Environmental aspects such as less consumption of toxic organic solvents, operator safety, lower cost and faster analysis, as well as reduction in sample preparation, are directly connected to the green aspect of analytical chemistry. However, given the caveats associated with the technique in question, this report intentionally begins by introducing readers to the physical aspects of SFC and listing the instrumentation required for its use. The second part of the article deals with a decision-making process in the selection of mobile phase components and columns when it comes to analyzing pharmaceutical mixtures. Finally, the last part demonstrates the utility of SFC through summarized applications in the pharmaceutical domain.

Supercritical fluids

When discussing SFs, it is necessary to consider the aggregate state being referred to. Substances can exist in three aggregate states: solid, liquid, and gaseous. The state of the substance depends on the environmental conditions, specifically the values of temperature (T) and pressure (P), and changes in these conditions can lead to changes in

the aggregate state. This can be represented by a phase diagram. In Figure 1, a phase diagram corresponding to CO_2 is depicted.

For each substance there is a characteristic combination of pressure and temperature values at which the substance can exist simultaneously in all three states of aggregation, called the triple point. In addition to the triple point, there is another characteristic point at which substances exhibit specific behavior and the distinction between liquid and gas is lost: the critical point. At higher pressure and temperature conditions than the critical point, the substance is in a supercritical state, which is roughly defined as a gas with a high density. There is no clear boundary between the supercritical and subcritical regions; instead, the change of state is continuous. Since there is no clear transition between the supercritical state and gasses and liquids, we do not speak of a fourth aggregate state, although the physical properties of SFs differ from those of liquids and gasses. This can be confirmed by the possibility of transition from gas to liquid and vice versa, by bypassing the critical point by crossing the supercritical region, or by changing the conditions so that there are no clear and sudden changes in physical properties. Due to the possibility of a continuous transition from a liquid to a gas, it can be concluded that there is no significant difference between SFs and gasses or liquids (14).

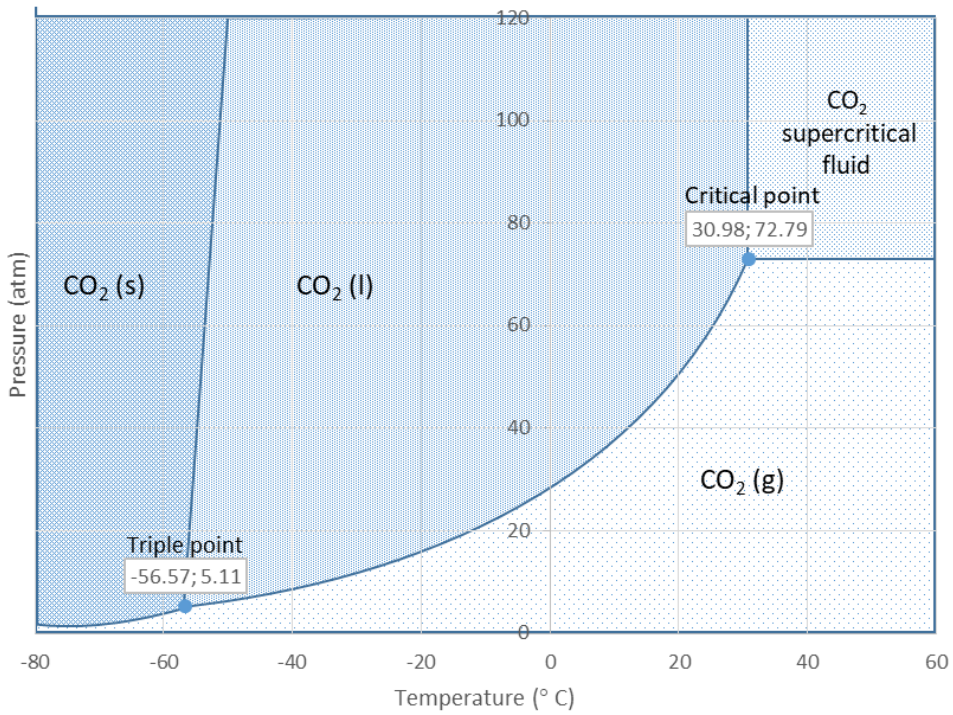

Figure 1. Pressure–Temperature phase diagram CO_2

Slika 1. Fazni dijagram (pritisak–temperatura) za CO_2

Physicochemical characteristics of SFs

Since fluids are regarded as states of matter that have variable forms in space, the physical properties that describe them include viscosity, diffusivity and density. The gaseous state is characterized by molecules with a large spatial distance from each other, which leads to very weak intermolecular interactions (15). When compression is applied, the distance between the molecules decreases. Compressibility is defined as the ability of a fluid to occupy a variable volume or exist at different densities, which is related to the temperature and average pressure in a given volume. This is a feature of gasses and not of liquids (16). During compression, the molecules approach each other, the gas becomes denser and acquires solvent properties. Although the intermolecular distance decreases and the density increases, the intermolecular interactions remain weak and the high diffusivity is maintained (15). The manipulation of density by changing the ambient conditions is easier the further the conditions are from the critical point. The density of SFs can vary from densities liquid-like to gaseous-like. This consequentially causes change in the fluid viscosity and the diffusion rate. SFs with high density and viscosity are less diffusible, and vice versa (14). Finally, SFs have densities and dissolving capacities similar to those of certain liquids, but lower viscosities and better diffusion properties.

Supercritical CO_2 as the main component of the SFC mobile phase

The suitability of CO_2 as a mobile phase is reflected in the values of temperature and pressure at the critical point, which are 304.13 K (31 °C) and 7.38 MPa, respectively. When these values are compared with room temperature and atmospheric pressure, which are 20 °C and 0.1 MPa respectively, it is found that only an order of magnitude increase in pressure above atmospheric pressure is required to reach the critical pressure, while the critical temperature value is close to room temperature, so both conditions are easily achieved. Under these conditions, carbon dioxide has a characteristic density of 0.4676 g/cm^3, which is half that of water under normal conditions, indicating a significant decrease in fluid density. The density of supercritical CO_2 can vary depending on temperature and pressure values, ranging from 0.2 to 1.1 g/cm^3.

When looking at the chemical properties of CO_2, it can be seen that it acts as a Lewis base due to the presence of a lone pair of electrons on the oxygen atom. Due to its basic nature, it can easily combine with Lewis acids such as phenols and amines. Depending on the environment, it can also behave like a Lewis acid due to the electronegativity of the two oxygen atoms, which attract valence electrons and create a partial positive charge on the carbon molecule. This can also be a reactive center of the CO_2 molecule. It is clear from the above that CO_2 is not always inert. Reactivity is observed when it reacts with amines to form carbamides. In some cases, it can also react with alcohol, which is due to the acidic nature of CO_2 (16, 17).

SFC Chromatographs

Previous generations of instrumentation fell short of achieving satisfactory quantitative performance, limiting SFC's adoption primarily to research and development (R&D) environments. The launch of an improved generation of SFC devices has been motivated by the need to ensure wider applicability of the technique. The most important providers of chromatographic instrumentation developed a new generation of analytical SFC instruments, allowing comparable sensitivity and robustness to HPLC (18–20). Modern SFC systems offer the possibility to perform green separations by reducing the usage of toxic solvents, the ability to easily scale up from analytical separation to preparative application, the opportunity to perform (a)chiral separations on one unique system, with the same mobile phase and the capability to achieve very different retention and selectivity compared to RP-LC (21). These analytical SFC instruments have pushed the speed vs efficiency envelope beyond what was achieved with ultra-high performance liquid chromatography (UHPLC) instruments (22). Special attention is paid to the design of a back pressure regulator (BPR) that controls system pressure and a CO_2 delivery system that limits mobile phase density variations (6).

The SFC devices introduced in the early 2010s include the ACQUITY UPC2 from Waters and the 1260 Infinity Hybrid SFC/UHPLC from Agilent Technologies. The former offers improved performance with reduced system dispersion for analytical SFC purposes. Key features include efficient CO_2-pump cooling and a dual-stage BPR to prevent frost formation. This device can handle both liquid and gaseous CO_2, with maximum flow rates of up to 4 mL/min and a pressure of 41.3 MPa. It can work with detectors such as photo diode array (PDA), evaporative light scattering detector (ELSD) and MS. The latter is a hybrid system that enables both SFC and UHPLC separations. As far as the different chromatographic modes are concerned, the device has switching valves and two pumps for more flexibility. All SFC operations are centralized in the 1260 SFC Controller module, which is responsible for the compression of gaseous CO_2 and temperature management. The contained BPR is a single-stage device. It operates at maximum flow rates of 5 mL/min and a pressure of up to 60 MPa. It can utilize detection options such as PDA, ELSD, MS and flame ionization detector (FID). The system has a different interface to the MS than the ACQUITY UPC2 device as it uses a make-up solvent before the BPR (8). In 2014, another instrument, namely Schimadzu's Nexera, was designed to combine properties of earlier mentioned instruments. The CO_2 is supplied using dynamic compression, pumped by two separate pistons in series, at a different temperature. Due to the specific pump construction, it is able to combine a flow of 5 mL/min and pressure of 44 MPa or 3 mL/min and 66 MPa. The mobile phase pump consists of two separate modes, one for the CO_2 and the other for the modifier. The modifier pump can be used solely using the instrument in HPLC mode. This is obtained by turning off the CO_2 pump and BPR. The instrument was designed as on-line as the supercritical fluid extraction (SFE)–SFC–MS system with 2 BPRs, one for pre-column split control and other for outlet pressure control (23).

The strength of modern SFC performance over LC performance is clearly demonstrated in reference (24). Perrenoud et al. investigated the kinetic performance offered by state-of-the-art SFC instruments and dedicated columns packed with small particles considering the system variance, upper pressure limit, and the optimal column dimensions. In terms of sensitivity, many publications demonstrated more sensitivity of modern SFC–MS systems over LC–MS and/or GC–MS technique e.g., for analyzing pesticides (25), doping agents (26), gestagens in bovine and porcine kidney fat (27).

Even though the SFC instrumentation mentioned above represents a valuable innovation in the arsenal of analytical equipment, there is still plenty of room for improvement that would increase the attractiveness of the technique from the analysts' point of view. SFC is still comparatively more complicated than GC due to the large number of parameters that can be set. In addition, the ability to achieve a full gradient range (transition from pure CO_2 to pure liquid solvent) is often limited by the capabilities of the instrument. The underlying assumptions suggest that removing these limitations would encourage analysts to explore the use of SFC, thereby expanding its application and making the most of its potential.

SFC components: Exploring the choice of mobile phase and stationary phase

Originally, various fluids in a supercritical state were used as SFC mobile phases, which led to the current technique's nomenclature. Apart from the advances in instrumentation, the most important changes since the early days of SFC have occurred in the composition of the mobile phase (28). This means that today SFC can be accurately described as a separation technique that utilizes compressed CO_2 (sCO_2) as the main component of the mobile phase, without specific reference to operating conditions. This broader perspective shows a refined understanding of the technique and its suitability to diverse applications across both subcritical and supercritical fluid states (8). In addition to compressed sCO_2, modern SFC mobile phases typically contain a more polar organic modifier and small amounts of additives. Such a composition enhances the SFC analysis of polar and/or ionizable compounds that may not be effectively separated by conventional HPLC methods (29).

In addition to the composition of the mobile phase, the column is a critical factor influencing the chromatographic process, with temperature and pressure playing a lesser role. Factors such as the chemistry of the stationary phase, the particle size and the dimensions of the column are crucial for optimal separations.

Since SFC is comparatively younger than other chromatographic techniques, the upcoming discussion will delve into the specified method parameters, offering both fundamental interpretations and practical considerations related to the method development.

SFC Mobile Phase

Even though CO_2 is favored by most chromatographers today, it is interesting to note that this was not the case in the early stages of SFC development (1960s to 1980s). Back then, analytical scientists were adventurous and experimented with fluorocarbons, nitrous oxide and even ammonia. These fluids were used exclusively in the supercritical state. However, when it became apparent that affordable CO_2 could be easily converted to a supercritical state, all traditional fluids associated with safety concerns, hardware corrosion, and unsuitability for thermolabile compounds (30) were pushed into the scientific background. Compressed CO_2 in particular has gained popularity in modern environmentally conscious circles, being praised for its waste-free use and relatively low toxicity (16, 31).

The ability of the CO_2 to dissolve and elute the sample components from the stationary phase determines the effectiveness of SFC separation. In the case of pure CO_2, fluid density becomes a key factor influencing the compounds' solubility. Increasing the pressure in the instrument improves solubility, leading to a decrease in retention factors (16). However, it's important to bear in mind that CO_2 shares similar polarity with non-polar hexane (32). This means that even with a dense fluid, efficient solubilization of polar molecules encountered in pharmaceutical practice may be challenging. The inefficiency in the solvation of polar molecules can pose obstacles to the effective SFC separation and elution of both these compounds and high molecular weight pharmaceuticals (8).

SFC Mobile Phase: Modifiers (Co-solvents)

The solvating power and strength of sCO_2 can be increased by adding a small amount of a compatible polar solvent to the main fluid. These solvents are referred to as mobile phase's modifiers that are used to elute polar analytes within a reasonable time frame. Frequently used modifiers include alcohols such as MeOH, ethanol (EtOH), isopropanol (IPA) and 1-butanol (1-BuOH) (6, 13). However, a number of less common yet useful solvents such as THF, dichloromethane (DCM) and acetone (Ace) can also be employed in analytical and preparative SFC separations (32–34).

The interaction between polar organic solvents and analytes relies on hydrogen bonds or dipole-dipole interactions, allowing polar analytes to dissolve in the mobile phase. In addition, modifiers not only improve the solubility of analytes in the mobile phase, but also block active sites on the solid support, preventing unwanted interactions and thereby improving peak shape and increasing efficiency. Organic modifiers also change the density of the mobile phase and the mass transfer properties, resulting in faster mass transfer of the analyzed compounds compared to neat CO_2 (8, 28). A final possible mechanism is that solvent molecules are adsorbed on the stationary phase so that the substances can partition between them and the bulk mobile phase (35).

The significance of the contribution of the organic modifier to the chromatographic system is one of the main differences between HPLC and SFC (35). In HPLC and chiral

SFC the mobile phase modifier needs to be selected in the early phases of method development (36, 37)**.** In achiral SFC, the choice of a modifier plays a decisive role in tailoring the properties of the mobile phase and is only made after the choice of the stationary phase. However, for didactic and organizational reasons, we have decided to present the mobile phase as part of an SFC system before the stationary phase.

MeOH is the most commonly employed modifier, mainly due to its widespread availability, cost efficiency, compatibility with CO_2, relatively low toxicity and minimal UV absorption around 205 nm, making it suitable for UV/Vis detection methods. Consequently, a wide range of analytes that are soluble in MeOH can be effectively analyzed with SFC. When alcohols other than MeOH are used as mobile phase modifiers, researchers often encounter irregular peak shapes and extended elution windows. The most notable differences in chromatogram appearance, such as selectivity and peak capacity, are typically obtained with ACN (38). Significantly longer retention times, poorer peak symmetry and lower peak capacity result from the inability of ACN to donate hydrogen bonds and partially block the silanol groups (39).

It is important to note that the addition of an organic modifier to the mobile phase significantly affects its critical point, which varies depending on the type and percentage content of the modifier. For example, the critical point of the commonly used MeOH with parameter values of 512.75 K and 8.12 MPa and a density of 271.6 g/cm³ far exceeds that of CO_2. This means that it is difficult to achieve supercritical conditions in a CO_2–MeOH mixture, except with low concentrations of MeOH (17). The mixtures of organic modifiers with CO_2, as implied, can pose challenges in controlling mobile phase composition in SFC. This is because transitioning from the supercritical to the subcritical region can result in a split into two different fluids at equilibrium, considering that the CO_2 is then in the supercritical state and the modifier in the subcritical state, each in equilibrium with the stationary phase. When phase separation occurs, it alters analyte solubility, retention behavior (jeopardizing separations), and detection behavior (manifesting as noisy UV signals), as a gas–liquid mixture is detected instead of a single phase. However, not all subcritical conditions exhibit phase separation. A two-phase liquid/sCO_2 under the “supercritical region” is more likely if the critical values for pressure and temperature are not reached. Yet, maintaining pressure above supercritical values and keeping the temperature in the subcritical region can sustain a single-phase eluent, making this “subcritical” region operable (16). In addition, it is crucial to consider the density, viscosity and diffusivity of solvent mixtures when using them as a mobile phase. The solvent obtained as a mixture of CO_2 and organic modifier has viscosity and diffusion coefficient values between CO_2 and the modifier. However, they do not show a linear dependence on the concentration of the added modifier. For example, the viscosity of a 40/60 MeOH and CO_2 mixture is equal to 1/3 of the viscosity of MeOH (17). Additionally, by adding only 5.5% MeOH, a near-halving of the diffusion coefficient occurs. This can be explained by the formation of solvated analyte molecules that slows down their movement. However, even with a reduction in diffusion, SFC remains faster than HPLC (15).

SFC Mobile Phase: Additives

Analytes with a high polarity or basicity often engage in robust interactions with unbound silanol groups on the stationary phase. These interactions can be so strong that even a modified mobile phase might have difficulty eluting them effectively or such compounds may elute with severely compromised peak symmetry (15). The root of this challenge lies in the inability of polar organic modifiers to completely suppress unwanted interactions between the analyte and free active sites on the solid support (16). The solution to the deleterious effects of residual silanol groups is the addition of substances called additives, which cover the active sites on the stationary phase. Nature-wise, these additives are generally strong acids, bases, or salts, added in concentrations of 0.1 to 1% of the modifier. Although they are used in small quantities, they can have a significant impact on the quality of SFC separation.

In particular, the additives contribute to an improved peak appearance and can influence selectivity. Most additives have been shown to have a positive effect on efficiency under supercritical conditions, while their effects on analyte retention depend on the structure of the analyte. Essentially, additives interact with the active sites on the column, changing the polarity and pH of the mobile phase, suppressing the ionization of the analyte and leading to ion pairing. The effects of additives may not be noticeable immediately after addition, but only become visible after a certain time, when a dynamic equilibrium has been established (16, 40). Additives are usually poorly soluble in neat CO_2, with their best solubility being quite limited. Therefore, they are usually dissolved in low concentrations (10–20 mM) in the organic modifier. This mixture of modifier and additive is then introduced into the CO_2 to form the mobile phase. Of the three types of components in SFC mobile phases, additives exhibit the greatest diversity (14, 40).

If the elution of organic acids is a problem, it can be overcome by adding formic acid, trifluoroacetic acid or citric acid (41–43). Bases can be eluted more successfully by adding aliphatic amines (isopropylamine, triethylamine) (41, 44) or ammonium hydroxide (45). As far as salts are concerned, ammonium acetate and ammonium formate are commonly used (16, 46). The shift towards volatile ammonium salts stemmed from their compatibility with MS detection (47).

Nevertheless, SFC systems that require little or no additives are an ideal solution, especially for gradient methods, as additives often cause an increased baseline shift (48). Other disadvantages of additives may include their tendency to adsorb on the surface of the stationary phase, which leads to an unwanted change in selectivity (49).

Water can also be used as an additive in SFC experiments. Initially, research groups attempted to use water as a co-solvent alongside neat CO_2 to extend the polarity range of analytes compatible with the technique in question. However, this approach failed due to water's pronounced polarity. With a high dielectric constant of 80.1 at 20 °C, water is sparingly soluble in CO_2 (about 0.1% v/v at ambient temperature and pressure) (17, 50).

In contrast to CO_2, access to the critical point of water (373 °C and 22 MPa) is not so easy. Nevertheless, water in the subcritical state (below 100 °C and 1 MPa), also has practical applications in SFC. Manipulating the temperature can change the viscosity of water. Increasing the temperature reduces the number of hydrogen bonds between the molecules, which in turn lowers the surface tension. Higher temperatures, however, could impair the analysis of thermally labile compounds (32).

Using organic co-solvents together with CO_2, on the other hand, can allow for a greater proportion of water to be incorporated into the SFC system compared to using no co-solvent at all. This approach has proven effective and alleviates concerns about poor miscibility (22, 32). Using higher water content can have several advantages, including shorter analysis time, improved efficiency and a wider range of analytes that can be tested (22). According to (51), the primary mechanism by which added water significantly affects the selectivity and retention of polar analytes is through its adsorption on the surface of the silica stationary phase. This phenomenon leads to a partitioning of the analytes between the aqueous mobile phase and the water adsorbed on the surface, similar to HILIC systems. The influence of adsorbed water on the stationary phase can persist even after the water has been extracted from the mobile phase (17). Regarding the development of the SFC method, it is worth noting that some experts now advocate for using a combination of water and ammonium acetate (typically 2–5% water and 5–20 mM ammonium acetate added to the modifier) as a “universal” additive for CO_2–MeOH mobile phases (14).

Kostenko et al. (52) investigated the unexpected separate elution of cations and anions of ammonium salts in SFC. It is known that amines can react with CO_2 as well as CO_2–alcohol mixtures, which leads to the formation of an ionic compound. This may change the retention behavior of the pharmaceutical compounds in the case of the application of amines as mobile phase additives. So far, the chemical behavior of amines in SFC has been questionable and not fully understood.

SFC−MS Mobile Phases

As with other hyphenated techniques, the coupling of SFC with MS detection is proving to be extremely advantageous, as shown by the growing number of published SFC–MS methods in the pharmaceutical field (Figure 2). In addition to an SFC–MS interface, careful selection of the mobile phase plays a crucial role in SFC–MS analysis. The volatile nature of a CO_2-rich mobile phase enhances the evaporation process during atmospheric pressure ionization, but also brings some challenges. Namely, CO_2 expansion contributes about 5% to the nebulization process. However, as the mobile phase loses control of the back pressure regulator upon entering the MS, the reduction in density can reduce the dissolution capacity and lead to the precipitation of analytes. As a result, chromatographic performance suffers, leading to distorted peak shapes and reduced response. In addition, analytes adhering to the capillary walls can lead to significant carryover effects (28).

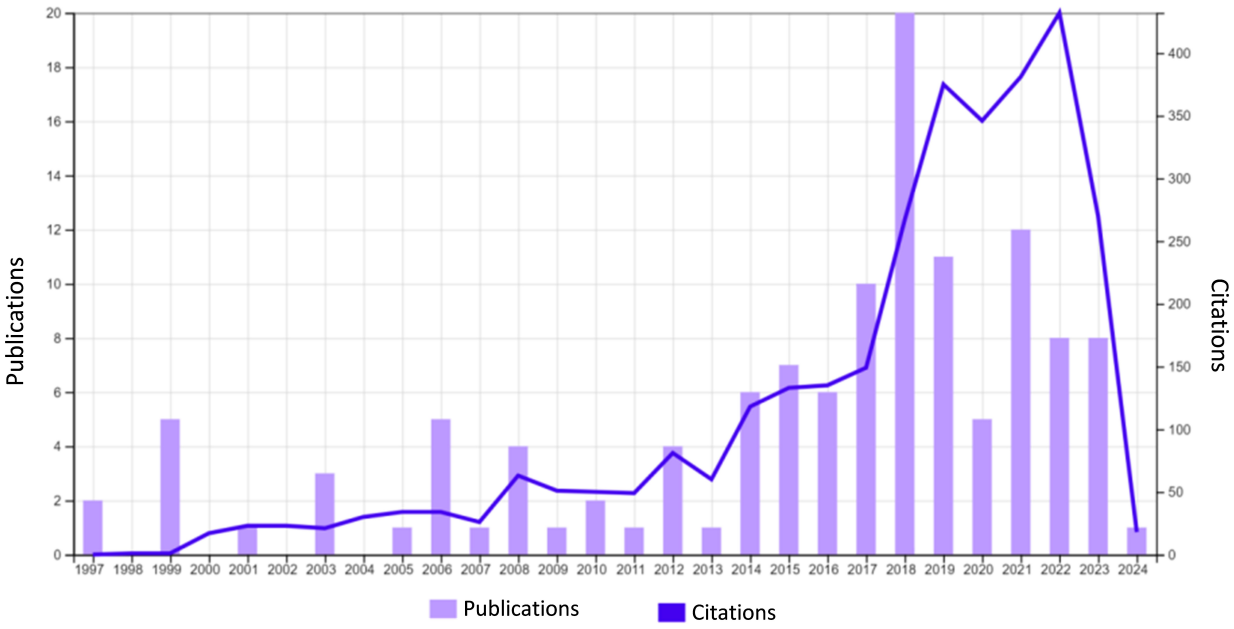

Figure 2. **Web of Science (WoS) results: Distribution of published papers (and citations) on SFC−MS* in the pharma* domain from 1996 to February 2024**

Slika 2. **Web of Science (WoS) rezultati: Distribucija objavljenih radova (i citata) o SFC-MS* u farmaceutskom* domenu od 1996. do februara 2024.**

Considering the SFC–MS coupling, the acid-base properties of the SFC mobile phase play an important role, as well as the possible presence of water, even under anhydrous conditions (28). The choice of additives for a particular SFC separation may be restricted by the detection method used. Additives used with MS detection must be volatile enough to evaporate during the nebulization process. Despite these constraints, a wide range of additives are suitable for the particular detection mode. These include small volatile acids (such as formic and acetic acid), small volatile bases (such as trimethylamine and triethylamine) and even many small volatile salts (such as ammonium formate and ammonium acetate). The presence of volatile salts (i.e., 10 mM ammonium formate) in the SFC–MS mobile phase does not compromise sensitivity in SFC–MS, while it could improve the peak shapes of ionizable compounds (26).

In analytical-scale SFC, the restrictions on the choice of additives are relatively small compared to those in preparative-scale SFC. The additives used in preparative scale SFC must be easily removed from the final product after isolation without degradation or alteration of the product (14).

Stationary Phases for SFC Experiments

Analytical-Scale Stationary Phases in Achiral SFC for Pharmaceutical Applications

The initial milestone in SFC arose from employing cSFC within a setup inspired by GC. The first commercial packed-SFC (pSFC) column, similar to the ones applied in LC,

was introduced in 1983 by Hewlett Packard (53). As technology evolved, featuring packed columns and advanced, next-generation devices, SFC application scope has broadened (8). The pSFC phases provide the addition of an organic modifier to increase the polarity of the mobile phase, which consequently expands the range of analyzable compounds. The potential for replacing RP-LC, NP-LC, HILIC and non-aqueous reversed-phase liquid chromatography (NARP-LC) arises from the fact that all HPLC packed columns can also be used for SFC experiments (6). The only requirement for successful SFC operation is that the analyte of interest should be sufficiently soluble in the co-solvent added to the SF (22). As a result, analysts have a whole range of different stationary phases at their disposal (54–56). In the spectrum from C18-bound silica to bare silica, choosing the optimal stationary phase presents a challenge, but also offers a number of creative opportunities for method development. In addition to the different structures of the bound ligands, the columns that can be used for SFC experiments vary in terms of particle size, pore size, length and diameter, which further complicates the selection process. To ensure optimal performance, it is important to consider parameters such as retention (k), selectivity (α) and efficiency (N). These parameters define the areas in which the working column must excel, so the right choice is crucial for successful experimentation (14).

Column Chemistry: Retention and Selectivity

The first aspect, retention, depends on how the analyte interacts with both the stationary phase and the mobile phase at the molecular level. On the other hand, the chemistry of the stationary phase is the most important factor influencing the selectivity in SFC.

Speaking of the stationary phase, SFC originally used only polar HPLC stationary phases in NP-mode with CO_2-centered mobile phases. Over the last decade, the range of stationary phases available for achiral SFC analysis has expanded considerably. These include a variety of polar stationary phases designed for NP-LC or HILIC (e.g., bare silica gel, propanediol-linked silica, aminopropyl-linked silica and sulfobetaine-linked silica), as well as nonpolar phases tailored for the RP-LC mode (8). Mixed-mode stationary phases have also been evaluated in SFC to disclose their chromatographic potential. These include particularly RP/ion-exchange (IEX) (57) and RP/HILIC modes (58, 59) which were originally used for the rapid separation of pharmaceutical compounds. Currently, there is also a wide range of columns specifically customized for SFC, taking into account both the stationary phase itself and the overall column design (60).

A valuable aid in selecting the optimal stationary phase for SFC separation involves examining the structure of the ligand bound to the silica and understanding the potential molecular interactions it can provide. Miller et al. (14) outlined the structures and molecular interactions of several common and less common stationary phase ligands such as diol, phenyl, 2-ethylpyridine (2-EP), cyano/cyanopropyl, C18, etc. However, for these molecular interactions to be significant, the analytes must have the ability to interact in a

certain way. This means that the choice of stationary phase should be made in accordance with the properties of the analytes contained in the mixture.

Molecules with p*K*a values above 7 are usually problematic for analysis as they often exhibit poor peak shape, which compromises resolution and sensitivity. One of the most popular stationary phases for SFC, 2-EP bonded silica, introduced by Princeton Chromatography, has quickly gained popularity as it provides excellent peak shapes for basic compounds with additive-free mobile phases. Consequently, numerous column manufacturers in the SFC field now offer their own versions of 2-EP bonded silica (16). Perrenoud et al. (61) demonstrated significant variations in peak shapes among five different 2-EP columns, attributed to differences in residual silanol levels, silica pre-treatment, and ligand density. The commercial 2-EP columns were ranked as follows based on their findings: PrincetonSFC 2-EP > Zymor Pegasus 2-EP > Waters Viridis Silica 2-EP > Waters Viridis BEH 2-EP > ES Industries Greensep 2-EP.

Recently, Desfontaine et al. (49) conducted a study in which they investigated three commercially available stationary phases with basic ligands (2-picolylamine (2-PIC), diethylamine (DEA), 1-aminoanthracene (1-AA)) under additive-free conditions. The authors compared the performance of the columns in terms of retention and peak shape and performed additional experiments with ammonium formate in the mobile phase and injection solvent. The compounds of interest in this study included 38 pharmaceutical compounds, mainly with p*K*a > 8. Although some of the columns tested were specifically designed for the analysis of bases (2-PIC and DEA), it was found that the CO_2–MeOH mixture gave unsatisfactory results, with a high incidence of distorted and tailing peaks. However, the addition of ammonium formate to the MeOH significantly improved the results. Surprisingly, the 2-PIC column produced acceptable outcomes when substituting the ammonium formate in the organic solvent to the injection solvent, with DEA showing similar, albeit less pronounced, improvements. Conversely, the 1-AA column turned out to be unsuitable for the analysis of basic pharmaceutical compounds under all conditions tested, leading the authors to conclude that it is better suited for the SFC analysis of neutral compounds. Finally, the three columns in question were compared with established SFC stationary phases, such as bare hybrid silica (BEH), diol, and 2-EP. Of all these columns, BEH and 2-PIC columns showed superior peak appearance in the presence of ammonium formate in the mobile phase. These columns also exhibited distinct retention and selectivity profiles, making them complementary options. Alternatively, DEA or diol columns could be considered, offering similar peak shape performance but with closer selectivity compared to 2-PIC. However, DEA offered shorter retention for analytes than 2-PIC and BEH, which could be advantageous for highly retained compounds, while the observed retention behavior associated with diol was comparable to that of 2-PIC.

In 2021, Wolrab et al. (62) introduced novel mixed-mode ion-exchange stationary phases, featuring a linear hydrocarbon chain divided by an oxygen atom (for strong cation IEX) or a nitrogen atom (for zwitterion IEX), with a sulfonic acid terminal group. These new phases have been intended for basic and zwitterionic analytes.

Due to the diversity of stationary phases and the use of the same main component in the mobile phase (CO_2), careful selection of the stationary phase is crucial for efficient optimization of the SFC analysis (35). The selection of the column can be facilitated by investigating retention mechanisms and predicting the chromatographic behavior of analytes. Several approaches have been proposed for retention prediction, ranging from highly informative methods such as measurement of adsorption isotherms and calculation of adsorption energy distribution (35) — which are effective but time consuming and difficult to apply across multiple chromatography conditions — to simpler ones that rely primarily on retention times of large sets of solutes. The latter approaches include model-based characterization by Snyder et al. (63) or quantitative structure–retention relationship (QSRR) modeling. A simple way to estimate the contribution of partitioning and adsorption to retention is to plot log$k$ against the volume percentage of the co-solvent in the mobile phase and fit the data to the log$k$–φ or log$k$–logφ coordinates representing the partitioning and adsorption regimes respectively (64). The most popular QSRR method for processing retention data of many solutes aimed at column selection is the linear solvation energy relationship (LSER) modeling including Abraham descriptors (65, 66).

The classical Abraham set of five descriptors takes into account the interactions of neutral compounds and is inadequate for ionizable species. However, many molecules from the pharmaceutical sector (a key area for SFC growth) are ionizable, mostly basic. Therefore, to accurately characterize retention and separation of positively and negatively charged solutes in SFC, factors accounting for ionic interactions are necessary. In this regard, soon after proposing the first classification method, West et al. (67) suggested adding two descriptors (describing positive and negative charges (D^- and D^+)) to the typical five-term equation to account for the interactions with cationic and anionic species on different stationary phases. This modified equation was applied to 31 different stationary phases with diverse chemical natures. With the new model, the authors aimed to improve the understanding of chromatographic retention and separation descriptions from a fundamental point of view. They also wanted to refine the column classification to better support SFC users in the analysis of ionizable species. Although the described approach was successfully tested in HPLC and SFC (68, 69), so far it is not entirely clear whether or not free ions can exist in SFC media.

More recently, Gros et al. (70) went one step further and proposed a nine-term LSER model. This model not only considers the Abraham descriptors and the ionic interactions between the analytes and the stationary phase, but also incorporates the shape features of the achiral compounds of interest by using two molecular descriptors: the flexibility (F) and globularity (G). Using this methodological approach, the authors characterized the 14 Shim-pack UC columns specifically designed for SFC and more broadly for unified chromatography (UC). The authors concluded that consideration of both ionic interactions and steric effect improves the understanding of retention mechanisms in achiral SFC. Variations in shape recognition could help in the selection

of columns for method development by revealing different and complementary selectivities within the set of columns.

In addition to the well-known QSRR–LSER approaches, Grooten et al. (71) recently proposed the tailored Phase Optimized Liquid Chromatography (POPLC) algorithm and a commercial kit for SFC method development. This approach aids in selecting the optimal combination of achiral columns, involving the coupling of five stationary phases with different chemistries (aminopropyl, cyanopropyl, diol, EP and bare silica gel) and varying lengths. The authors found that the customized POPLC kit can be effectively applied in SFC experiments. However, due to some discrepancies in the predicted retention times, the POPLC algorithm might be too simple for SFC applications where factors such as pressure and density of the mobile phase play a greater role than in HPLC.

Column Dimensions: Efficiency

When evaluating column efficiency, analytical scientists consider parameters such as the number of theoretical plates (N) or the height equivalent to a theoretical plate (H). Having a large N or a small H indicates a high efficiency, especially when handling similar structures. The most important factors influencing the H include particle size and column length (14). The graphical representations of the van Deemter equation (van Deemter curves) in (19) showed how the size of the particles (3.5 and 1.7 um) influenced column efficiency in analytical-scale SFC and HPLC. As illustrated, the use of a column packed with smaller particles led to higher efficiency in both techniques. As for the SFC, the B term (the left part of the curve) increased due to its proportionality with the diffusion coefficient, which had improved due to the lower viscosity of the SF compared to the hydroorganic mobile phase used in HPLC. Consequently, the optimum of the curve H(µm)/u(mm/s) shifted towards higher linear velocity values. Additionally, the C term (the right-hand side of the curves) decreased, as it is proportional to the ratio between the square of the particle size and the diffusion coefficient, leading to enhanced mass transfer. The situation was similar with columns for ultra-high performance liquid chromatography (UHPLC) and ultra-high performance supercritical fluid chromatography (UHPSFC). The UHPSFC curve embodied the benefits of both SFC and UHPLC.

In SFC, the “UHP” refers to the use of columns packed with small sub-2-µm particles at maximum pressure. It is worth noting that current SFC systems, which offer lower dispersion, typically have a maximum pressure of 40 MPa or 60 MPa (72).

A longer column provides a larger N in a simple linear way, leading to more efficient separations, especially in combination with smaller particles. However, this comes at the cost of higher column backpressure and longer analysis time. For efficiency reasons alone, it is advisable to choose the smallest particles and the longest column compatible with the flow rate and system pressure. In order to determine the correct column dimensions for UHPSFC experiments, Nováková et al. (8) calculated the efficiency loss for several columns with different dimensions, using devices currently available to analysts. The study included only columns packed with 1.7 µm particles, and

only two retention factors (k = 3 and k = 8) were modeled. The authors calculated that the standard UHPLC column, namely 50 x 2.1 mm, 1.7 µm, leads to a 45% loss in efficiency for k = 8, making it poorly compatible with the available UHPSFC instruments. In other words, the current instrumentation struggles with 2.1 mm I.D. columns. Currently, authors suggest that the optimal solution for UHPSFC experiments is to utilize a column with dimensions of 100 × 3 mm, packed with 1.7 mm particles, resulting in an efficiency loss of only 9%. While it is clear that 4.6 mm I.D. columns are superior to their 3 mm I.D. counterparts, authors advocate for the latter as they offer the best balance between efficiency loss, solvent consumption and flow rate adaptability.

Analytical-Scale Stationary Phases in Chiral SFC for Pharmaceutical Applications

Even though recent chiral applications in SFC account for only about a quarter of achiral analyses, chiral analysis remains of crucial importance. Chirality is an important aspect of living systems, as enantiomers of compounds often show remarkable differences in terms of biological activities, pharmacological effects and toxicological properties. In practice, chiral SFC methods development is still based on the screening of different combinations of stationary phases and mobile phases, which requires a considerable number of trial-and-error experiments (29). When it comes to chiral chromatography, different stationary phases are used compared to achiral chromatography. There are more than 200 commercially available chiral stationary phases, including macrocyclic glycopeptides, Pirkle-type, polysaccharides, cyclodextrins, and protein-bound phases. Most of them have been utilized in SFC, except for the protein-based ones. The advantages of applying SFC with chiral stationary phases can be attributed to their stability under appropriate conditions and suitability for preparative applications. Regarding other separation conditions, polar modifiers and additives applied are generally the same as those used in achiral separations, adapting to the examined sample, the chosen stationary phase, and the detection technique (73).

Other method parameters for tuning fluid properties: temperature and backpressure

If the density of pure CO_2 or CO_2 enriched with a very small amount of modifier is not properly handled, solvent strength can vary between analyses. Getting the density just right involves tuning both pressure and temperature (8). In SFC, unlike in LC, raising the temperature (while keeping the pressure constant) initially increases retention, as the density of the mobile phase is reduced. After reaching a peak, however, retention decreases at extremely high temperatures. Within the SFC system, temperature plays a multifaceted role, affecting the vapor pressure of the solute, the density of the SF and the physicochemical parameters of both the SF and the solute. Changes in temperature can also influence the compound's affinity for the stationary phase. Therefore, the impact of temperature on retention in SFC results from a complex combination of mechanisms, making it challenging to explain conclusively. The outcome depends on experimental conditions, solute properties, and the nature of the SF and the stationary phase (74).

To maintain the regular peak appearance in SFC, it is necessary to prevent a single mobile phase from possibly decomposing into two phases. In (75), Berger showed that all binary CO_2–MeOH mixtures form a single phase at 40 °C and a constant pressure of ~8 MPa. Beyond this pressure threshold, the density behaves as a linear function of the MeOH content, regardless of whether the fluid is in the subcritical or supercritical state. Conversely, at lower pressures, all CO_2–MeOH mixtures separate into two phases. In addition, higher temperatures in SFC require higher pressures to prevent a single mobile phase from collapsing. In gradient elution (using MeOH), this effect is mainly observed for early eluting compounds, while later eluting peaks are not affected by the pressure changes. As the organic modifier concentration increases, the compressibility of the mobile phase decreases, making pressure variations have minimal impact on retention. Pressure changes have minor effects on efficiency at commonly used pressures (> 15 MPa) (8).

Design of Experiments in SFC method development

If satisfactory separation is not achieved after the initial experiments, the stationary phase, modifier, additive or column temperature need to be adjusted. While changes to individual variables can improve the separation, comprehensive optimization requires a multivariate approach such as Design of Experiments (DOE) (14).

The use of DoE to develop and optimize SFC methods has increased significantly (76, 77). Designs such as the Full factorial design (FFD) or the Central Composite Design (CCD) have been used extensively to optimize parameters like retention time, retention factors, peak capacity, peak width and resolution. Recently, multiple-stages DoE has been favored for method development. This approach often involves two main steps: the first is the screening of the stationary phase, and the second is the optimization of the chromatographic conditions. For example, Santana et al. (78) proposed a two-step DoE approach to optimize factors such as stationary phase chemistry, volume percent of organic modifier and elution strength (by optimizing temperature, pressure and gradient) for rapid and reliable analysis of eight major cholesterol-lowering pharmaceuticals in Brazil. The DoE methodology confirmed the successful applicability of the SFC mode over non-aqueous HILIC (NA–HILIC) for the separation of structurally related imidazoline and piperazine derivatives (58).

Application space for SFC in drug analysis

Modern SFC has been used in various areas of analytical and preparative separations, especially in the past decade, when its field of application expanded drastically (79). Polar analytes are successfully analyzed on polar stationary phases, using alcoholic solvents (MeOH as a modifier) most often combined with acids, bases or buffer salts as additives dissolved in the mobile phase (58). In comparison to RP-LC, SFC offers improved retention of hydrophilic compounds when selecting a polar stationary phase (e.g., bare silica, diol and amide), the possibility to elute very lipophilic substances (e.g., triglycerides and liposoluble vitamins), and it appears as an orthogonal

separation technique for the analysis of drugs and metabolites, due to the different retention mechanisms (retention mostly occurs through hydrophobic interactions and hydrogen bonding in RP-LC and SFC, respectively) (80). Several publications show the broad scope of SFC for the separation of polar, apolar and ionic analytes. It has been reported that compounds possessing log*P* between -2 and >10 can be successfully analyzed in the SFC mode (81). High selectivity of SFC in relation to NA–HILIC was found in the case of imidazoline and piperazine derivatives (59) and for polynuclear aromatic hydrocarbons in relation to RP-LC (82).

Reference (83) illustrates the versatility of SFC for the separation of spirooxindole alkaloids (SOAs) with two types of mixed-mode reversed-phase/ion-exchange chromatography (RP/IEX) stationary phases. Adequate separation performance of pharmaceuticals and biogenic amines was found in SFC compared to the HPLC (RP, HILIC, IEX) mode with mixed-mode strong cation and zwitterion exchange stationary phases (84). The literature provides information on the use of SFC instrumentation for the evaluation of drug physicochemical properties and characterization of drug formulation. The solubility of a solute in a fluid can be measured directly in the SFC instrument by replacing the SFC column with a saturation cell that is simply pumped with the fluid of interest and the saturated liquid is transported to the SFC detector (85). Vorobei et al. presented a novel method for measuring solubility, designed specifically for multi-component SFs based on an online hyphenation of supercritical antisolvent precipitation and subsequent SFC. Aspirin was used as a model and its solubility was evaluated in CO_2–MeOH, CO_2–EtOH as well as CO_2–Ace. The results were in good agreement with the available literature data (86). Li et al. determined the solubility of chloramphenicol in pure SF–CO_2 using a continuously stirred supercritical solubility vessel (SSV) directly connected to online SFC. The combined SSV–SFC instrumentation and methodology enabled the rapid acquisition of chloramphenicol SF–CO_2 solubility data (87). The excipients in drug product formulations for pharmaceuticals provide the desired pharmacokinetic profile for successful drug delivery. SFC coupled with an ELSD and a MS can be used to characterize three analogs of functionalized polyethylene glycol excipients that allowed a more detailed assessment of material quality (88).

However, there are limitations for the SFC analysis of large biomolecules such as proteins or peptides. SFC provides unique solutions for analytes that degrade in water and where normal phase cannot be used, such as lactones and their hydrolyzed metabolites in biological samples (21). Depending on the test environment, unexpected hydrolysis or reverse dehydration may occur. NP-LC–MS may face challenges because of its relative incompatibility with several ionization techniques.

It is important to note that further studies involving the use of a wider set of pharmaceutical analytes and the evaluation of mobile and stationary phase effects must be conducted in order to obtain a more detailed picture of the applicability of SFC in pharmaceutical analysis. There is great potential for SFC to overcome mixed-mode and classical HPLC in future applications, increasing separation efficiency and reducing analysis time. A better understanding of the retention mechanism of modern SFC will

provide a growing interest in SFC-based applications and insight into their true analytical potential.

Conclusion

In summary, the evolving landscape of pharmaceutical analysis requires the integration of orthogonal techniques to complement traditional LC methods. Recent advances in SFC separation science offer a glimpse into a future where modern, environmentally conscious techniques reduce reliance on toxic organic solvents, increase operator safety, as well as ensure lower costs and faster analysis.

The shift away from the former "science fiction chromatography" that once plagued the reputation of SFC is now underway. Manufacturers should continue to drive this change by further developing SFC devices, while researchers should be more committed to sustainable experimentation.

As advocates of scientific progress, we recognize the central role of knowledgeable personnel in promoting the advancement of SFC. Through this review, our objective was to enable a comprehensive understanding of the fundamentals, system components and current applications of this technique. By doing so, we hope to empower both experienced analysts and aspiring researchers to unlock the full potential of SFC and facilitate its integration into the pharmaceutical field.

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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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Jovana Krmar: Conceptualization, Writing - original draft, Writing - review & editing; **Bojana Svrkota:** Writing - original draft; **Darija Obradović:** Conceptualization, Writing - original draft; **Vladimir Vlatković:** Writing - original draft; **Saša Lazović:** Funding acquisition, Supervision; **Biljana Otašević:** Funding acquisition, Supervision, Writing - review & editing.

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Efikasnost superkritične fluidne hromatografije u kreiranju ekološki prihvatljivih rešenja u farmaceutskoj analizi

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Kratak sadržaj

Na početku pretežno korišćena kao preparativna tehnika u enantioseparaciji hiralnih molekula kandidatâ za lek, superkritična fluidna hromatografija (eng. *supercritical fluid chromatography*, SFC) danas se široko koristi u analitičke svrhe. Noviji naučni napori ukazali su na značaj SFC tehnike u modernoj i ekološki prihvatljivoj farmaceutskoj analizi.

Cilj ovog preglednog rada je pružanje dubljeg uvida u najvažnije fundamentalne i praktične aspekte SFC tehnike, kako bi se čitaocima približio njen svestrani karakter, te efikasnost u kreiranju održivih hromatografskih rešenja. Sva razmatranja prevashodno su data u kontekstu najzastupljenijeg režima rada - ahiralne SFC. Takođe, na kraju rada predstavljene su savremene primene ove obećavajuće tehnike kako bi se dodatno ohrabrilo njeno usvajanje u farmaceutsku analitičku praksu.

Ključne reči: superkritična fluidna hromatografija, lekovi, ahiralna separacija, ekološka prihvatljivost