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Revisão

# SUPER/SUBCRITICAL FLUID CHROMATOGRAPHY WITH PACKED COLUMNS: STATE OF THE ART AND APPLICATIONS

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Separations using supercritical fluid chromatography (SFC) with packed columns have been re-discovered and explored in recent years. SFC enables fast and efficient separations and, in some cases, gives better results than high performance liquid chromatography (HPLC). This paper provides an overview of recent advances in SFC separations using packed columns for both achiral and chiral separations. The most important types of stationary phases used in SFC are discussed as well as the most critical parameters involved in the separations and some recent applications.

Keywords: Supercritical Fluid Chromatography; Ultra-high Performance Supercritical Fluid Chromatography; packed columns for Supercritical Fluid Chromatography.

## INTRODUCTION

A supercritical fluid can be defined as a highly compressed gas that has a density and solvating power similar to that of a liquid and viscosity and diffusivity similar to that of a gas.[1] These properties, intermediate between liquids and gases, provide the best of each technique and make supercritical fluids unique as mobile phases.

The theory of supercritical fluids applied in the separation sciences can be found in earlier reviews.[2,3] The intent of this paper is to define and contextualize the state of the art of super/subcritical fluid chromatography (SFC) in terms of recent results involving the use of packed high performance chromatographic (HPLC) columns, which has contributed to recent improvements in SFC instrumentation and to serve as a guide to these recent developments, since this separation technique is still in expansion, presenting a different scenario than that described almost 10 years ago.[4] Nowadays the technique is frequently employed in the separation and purification of a diversity of compounds, especially in the pharmaceutical industry, and the principal instrumentation companies for HPLC and GC (gas chromatography) are producing equipment to improve separations using SFC.

The possibility of using modifiers and additives in SFC can be related as an important milestone responsible for the resurgence of the technique in the 1990's, when SFC was recognized as an interesting, useful and powerful separation technique. The 1995 paper of Cui and Olesik[5] used a mixture of $CO_2$, methanol, and water as mobile phase (MP) while the paper of Lee and Olesik[6] in the same year used a mixture of $CO_2$ and *n*-hexane.[4] These papers helped to stimulate the equipment manufacturers to spend time (and, of course, money) on the development of new instruments. Figure 1S of Supplementary Material illustrates the number of publications in SFC published by year from 1965 to 2013, showing how SFC has grown in recent years.

In terms of instrumentation, the 1980's were very promising for SFC, leading to the commercialization of several SFC instruments.[1] These systems were similar to those used in gas chromatography, using the open tubular capillary columns introduced in 1981 by Novotny *et al*.[7] The 50 μm inner diameter glass capillary columns used in this work were fabricated with bonded phenylmethyl polysiloxane films and were connected to a UV detector resistant to high pressures.[7] Since Novotny *et al.*[8] had proposed that packed columns would not provide high-efficiencies at the high linear velocities typical with SFC mobile phases, due to the pressure drop along the chromatographic column as a function of negative density gradients, they proposed that a small pressure drop across an open tubular capillary column would provide higher efficiencies although the basic theory of chromatography is equally valid in both situations.

In open tubular capillary columns for SFC (cSFC), pressure, which is an important parameter related to retention, could not be changed independently of the flow velocity due to instrumental limitations and it was never possible to optimize both flow velocity and pressure.[1] In addition, the flame ionization detector, the most common detector used in GC, did not permit the use of organic modifiers in the MP.

The first use of a packed column for SFC (pSFC) was reported by Gere *et al.*[9] who modified regular HPLC adding a backpressure regulator. The use of pSFC started to increase due to the limitations of cSFC, taking advantage of the numerous improvements in HPLC instrumentation, especially the independent control of flow and pressure that enabled the use of mobile phase gradients in the separations, as well as organic modifiers and additives. The instruments were adapted to work with SFC as MP, resulting in modifications in the pressure limit of the pumps and in the use of UV cells capable of resisting high pressures.[10]

The majority of applications in SFC and in supercritical fluid extraction (SFE) today use carbon dioxide as the principal component of the mobile phase, since its critical conditions[2] of pressure (Pc = 7.4 MPa) and temperature (Tc = 31.3 °C) are mild, combined with other advantages such as being non-toxic and with a non aggressive chemical nature. To enhance solute solubility in the mobile phase, the $CO_2$ is often mixed with a solvent, known as a modifier,[11,12] such as methanol, ethanol, *n*-propanol, isopropanol, acetonitrile, tetrahydrofuran or *n*-hexane, with or without additives such as organic bases or acids.[13,14] This has expanded the technique for use in both the reversed phase mode with non polar stationary phases (SP) and in the normal phase mode with more polar SP, enabling the use of the same stationary phases as in HPLC, including more apolar SP such as octadecylsilane (ODS). The separation of more polar compounds is growing as an alternative to hydrophilic interaction chromatography (HILIC),[15] as discussed by Pereira *et al.*.[16]

As reported by Lesellier,[17] today, for many reasons, and contrary to old visions of this separation technique, pressure and temperature

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are not really used as parameters to modify retention and separation, simplifying method development. Temperature and outlet pressure are usually kept constant at approximately 25 °C and 15 MPa, conditions that are classified as subcritical.[13,18] The values can vary somewhat, depending on the composition of the fluid, that is, on the nature of the modifier added to the carbon dioxide and on its percentage in the mobile phase.[18]

The addition of modifiers to $CO_2$ allows specific new interactions such as the formation of hydrogen bonds or dipole–dipole interactions that significantly change retention factors and, consequently, selectivity.[18,19] On the other hand, the higher the percentage of modifier, the higher are the critical values[19] and phase separation (into one gaseous and one liquid) can occur.[19,20] For this reason the use of modifiers is limited to the range of 5-50%.[21-24] However, a small percentage of modifier (less than 5%) can significantly change the retention factors of compounds, being more useful than changing the polarity of the SP.[18]

Considering that a lot of organic compounds are thermally unstable, the use of subcritical conditions of temperature is an interesting advantage. In this case, if the pressure is maintained over its critical value while temperature is below its critical value, the fluid is not supercritical but subcritical: the fluid density is higher (1 g/cm$^3$) and the fluid properties are closer to those of liquids in terms of diffusion coefficient or eluting strength.[18]

In this context, super/subcritical fluid chromatography on packed columns presents an intermediate behavior between gas chromatography and liquid chromatography, as described in Figure 1, and can "act like a bridge" between these separation techniques, exploiting the best of both. Figure 2 shows a schematic diagram of a supercritical fluid chromatograph designed for use with packed columns. In this system, the $CO_2$ pump needs to be under refrigeration, since the gas is pumped in the liquefied state. The both $CO_2$ and the organic modifier are pumped separately and then mixed before entering the column. Another important accessory is the back-pressure regulator (BPR), which is responsible in maintain the system pressure constant even after the passage of the MP through the chromatographic column.

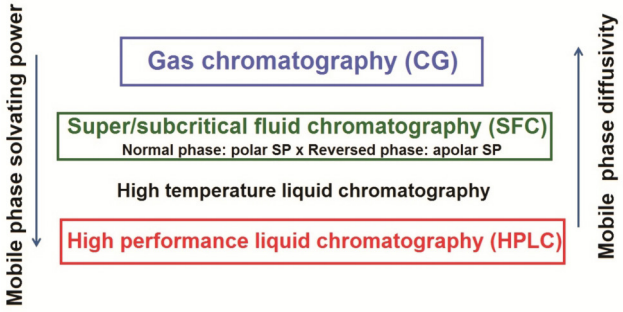


***Figure 1.*** *Schematic description of the three main types of chromatography based in the nature of mobile phase: gas chromatography, liquid chromatography and super/subcritical chromatograph. Adapted from reference 14*

## MOBILE PHASES

The desirable features of a mobile phase for pSFC are: high solute diffusion coefficient ($D_M$) and low viscosity, when compared with the solvents used in liquid chromatography (LC), plus retention control based on physical parameters like temperature, pressure and MP composition. With higher $D_M$, the optimum linear velocity ($\mu_{opt}$) through the column is higher than in LC, so the same number of peaks can be separated in less time, since low viscosity means that the mobile phase linear velocity can be dramatically increased to take advantage of the higher $D_M$ and the lower pressure drop. This permits

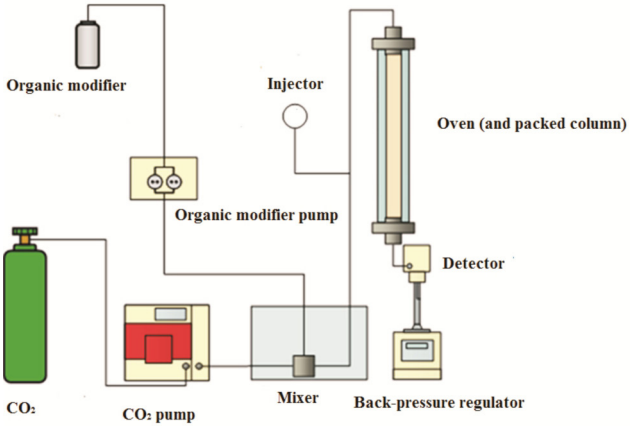


***Figure 2.*** *Schematic diagram of a supercritical fluid chromatograph designed for use with packed columns. Adapted from reference 25*

use of longer and more efficient columns.[23-27] Columns of 25 × 0.46 cm packed with 5 μm particles are generally well suited to provide satisfactory separation performance in pSFC.[17]

Sometimes, when using pure $CO_2$ as the mobile phase in pSFC, tailing and high retention or lack of elution can occur. This can be explained by the presence of active sites on the SP, especially residual silanols which are often present in much higher concentration than with wall bonded capillary columns.[28] The use of modifiers, especially polar ones such as methanol or isopropanol, can cover or deactivate these active sites in the stationary phases and diminish negative interactions with analytes, especially basic ones, even if the effect of modifiers on retention is larger, since they improve MP strength.[13,27,28]

However, the addition of a polar organic modifier to the MP may not be sufficient to yield acceptable chromatographic results.[28] Very polar or basic compounds, such as aliphatic amines or polyfuncional carboxylic acids, may present irreversible retention or elute with tailing peak shapes because of their strong interaction with the residual silanols, or due to the presence of metal ions in the silica supports. For these reasons the addition of more polar compounds, called additives, such as diethylamine, triethylamine, or isopropylamine, to the mobile phase, usually at concentrations of 0.1-2.0%, can improve the separation.[29-33] On the other hand, some recent papers report the use of stronger modifiers, such as 0.1% of a 30% ammonium hydroxide solution, but the use of such basic solutions can prejudice the stability of the SP.[34] Additives such as trifluoroacetic acid or salts such as ammonium acetate can also be encountered.[35,36]

Additives, being polar substances, improve peak shapes by covering up, adsorbing on, or reacting with silanols, especially for non endcapped SP. However, to avoid problems with solubility in the $CO_2$, the best choice is to add the additive to the modifier and then make the mixture in the instrument. Nevertheless, in some cases, when more polar SP are used, the use of additives does not improve peak tailing, suggesting that peak tailing is not always is related to the active sites on the SP.[27] The mechanism of solute-stationary phase/solute-active site interaction is still not well known. However, one thing is well established: additives can cause increases, decreases or even no change in solute retention and always are an important parameter to be tested in the development of a method in pSFC, applying the simple concept that acidic additives may improve the peak shapes of acidic solutes and basic additives may improve the peak shapes of the basic solutes.

## STATIONARY PHASES

Due to the non-polar character of carbon dioxide, the solubility

of hydrophobic compounds when this mobile phase is used is an advantage. For these compounds, pSFC can replace reversed-phase liquid chromatography (RPLC), using bonded alkyl stationary phases.[13] Similarly, also due to the non-polar character of carbon dioxide, numerous separations of polar pharmaceutical compounds are performed on polar stationary phases in pSFC, instead of using normal-phase liquid chromatography (NPLC).[13]

Lesellier[17] indicates that the choice of SP is the most important decision in pSFC, since the absence of water in the mobile phase allows subtle interactions between solutes while the SP and the retention mode depends on the nature of the SP. The use of octadecyl--bonded silica enables the separation of compounds differing by their hydrocarboneous volume (methyl and methylene groups),[17,37] such as steroids,[38] carotenoids,[39] methylated pharmaceutical compounds,[40] chlorophylls[41] or homologous alkyl series.[42] Some of these compounds have even been used for characterization of these SP.[43,44]

Polar-bonded silica stationary phases are widely used in pSFC, including bare silica, diol, aminopropyl, cyanopropyl and polyethylene glycol bonded phases. West and Lesellier[44] studied the behavior and interactions of diol, aminopropyl and bare silica SP towards acidic and basic solutes. Interactions with basic compounds were lower with the aminopropyl phase.

Different aromatic polymeric and silica-based SP, such as polystyrene-divinylbenzene and phenyl-propyl bonded silica, were also studied in pSFC by West and Lesellier.[45] According to this paper, the solute interactions when using $CO_2$ with 10% of methanol as modifier, in the absence of water, depend on the nature of the aromatic group.

Monolithic SP were tested in pSFC by Lesellier *et al.*[22] These authors proved that it is possible to couple several monolithic SP when using SFC, due to the characteristic of monoliths which have bimodal porous structures, that cause drastic decreases in flow resistance, when compared with particulate HPLC SP.

Porous graphitic carbon SP were also tested in pSFC using different modifiers, such as methanol, ethanol, *n*-propanol, isopropanol, acetonitrile, tetrahydrofuran and hexane.[12] The effects of different modifiers in pSFC on interactions between solute and the SP and between solute and the carbon dioxide-modifier mobile phases were studied. With all modifiers tested, the solute-stationary phase interactions were greater than the solute-mobile phase interactions, especially between acidic solutes or solutes having $\pi$ and non-bonding electrons.[46]

Superficially porous particles have been tested in pSFC. The use of superficially porous ODS particles allowed highly efficient separations, with or without column coupling.[47] The high permeability of these particles favors increasing column length, which increases resolution. Kinetic behavior using isocratic mobile phases even improved the efficiency of the most retained compounds, without the need for gradient elution.[47]

Hydrophilic interaction chromatography (HILIC) SP interactions were studied and explored in pSFC by West *et al.*[48] due to the polar nature of these SP, especially as an alternative to normal--phase HPLC. This paper proved that commercial HILIC phases can be used in the isocratic mode, using $CO_2$:methanol (90:10, v/v). Chemometric tools such as hierarchical cluster analysis and principal component analysis grouped classified HILIC SP into three clusters containing phases with similar selectivity: neutral stationary phases like amide and diol phases, basic stationary phases like aminopropyl-bonded silica, and bare silica stationary phases.[48] In the same paper, the applicabilities of HILIC SP also were tested for the separation three mixtures of pharmaceutical compounds: barbiturates, benzodiazepines and propionic acids. The applicability of HILIC SP in pSFC also was tested for the separation of ten drug--like molecules of interest to the pharmaceutical industry using bare silica SP, $CO_2$ modified with methanol (gradient mode; 5 to 50%) and 5% of an additive (trifluoroacetic acid for the separation of acids, isopropylamine for the separation of bases and ammonium acetate as a buffer salt for neutral solutes).[49] Both these papers proved the versatility of HILIC SP when applied in isocratic or gradient mode with or without the use of additives, indicating that even a small quantity of water absorbed onto the SP is enough to provide good retention in pSFC, based on the partition mechanism described by Alpert in 1990,[50] since the HILIC SP has the ability to retain water.[51] However up to now zwitterionic bonded or zwitterionic polymeric SP have not been evaluated for pSFC.

As mentioned before, all stationary phases developed for liquid chromatography are available to use in SFC and are the largest group of stationary phases used in practice, particularly silica-based chemically bonded polar phases. On the other hand, there are a few stationary phases developed and marketed exclusively for the particular needs of supercritical fluid chromatography, which, of course, are also available for use in liquid chromatography.[24] Historically, capillary SP of porous silica particles coated with an immobilized layer of a poly(siloxane) containing alkyl or polar substituent groups can be highlighted.[24] SP with 2-ethylpyridine substituents were introduced to the market to minimize undesirable silanol interactions in the separation of polar compounds, since the hydrogen-bonding between free silanol groups and the nitrogen atom of the pyridine ring, or partial protonation of the pyridine group (due to the acidity of carbon dioxide–methanol mobile phases) would repel positively charged analytes from the stationary phase, reducing interactions with the silanol groups. Steric protection by pyridine groups has led this SP to the first choice for the analysis of some basic solutes, with or without the use of additives, as well as for the separation of neutral or acidic solutes.[24] The same mechanism of interaction (hydrogen--bonding or steric protection) can be encontered with silica-based support having amino, amide, urea and sulfonamide groups.[24]

## Chiral SP

Chiral separations using pSFC were initiated in 1985 by Mourier *et al.*[52] when they separated five enantiomeric pairs of phosphine oxides on the classical Pirkle SP obtained by covalent bonding of (R)-N-(3,5-dinitrobenzoyl)phenylglycine onto aminopropil silica, with $CO_2$ having methanol, ethanol or 2-propanol as organic modifier in both subcritical and supercritical conditions. This work is interesting due to the fact that, in spite of all instrumental and packed column developments over the years since, the analysis parameters were almost the same as those used today. Ever since, pSFC has been used in the separation of chiral mixtures and to determinate the enantiomeric purity of pharmaceuticals, as an alternative to other separation techniques such as GC, HPLC, capillary electrophoresis (CE) and capillary electrochromatography (CEC).[53-61] The use of pSFC as an alternative, especially compared with HPLC, is due to the fact that a significant problem in the performance of chiral stationary phases (CSP) in HPLC relates to efficiency, since many CSP have performances inferior to those of nonchiral stationary phases. Broad peak shapes make quantitative applications difficult and this is particularly problematic when one enantiomer is in excess. In addition, the long equilibration times required when changing CSP in method development are mitigated when liquid mobile phases are replaced by supercritical fluid mobile phases, due to the advantages of this fluid.[62]

### *Chiral polysaccharide SP*

Chiral polysaccharide-based stationary phases are the most widely used SP for pSFC in enantioseparations because of their proven broad enantioselectivity. Polysaccharide CSP such as

tris-(3,5-dimethylphenylcarbamate) absorbed onto amylase or cellulose are the two most successful SP for chiral resolutions of a wide variety of pharmaceutical compounds using pSFC.[63-65]

With the aim of understanding retention and selectivity in chiral polysaccharide-based stationary phases, West *et al*.[65,66] and Khater *et al.*[67] studied the chiral recognition mechanisms in supercritical fluid chromatography using polysaccharide stationary phases with two different polysaccharide backbones: amylose[65,66] and cellulose.[65-67] The chromatographic system was evaluated with 230 achiral compounds and with mixtures of 135 racemates with diverse structures. By the use of statistical and chemometric approaches, these researchers proved that even though being used only as polysaccharide backbones, the presence of amylase or cellulose could provide different enantioseparations and, even with similar structures, the contribution of hydrogen bonding in each phase is one of the possible reasons for the differences in separation.

*Cyclodextrin SP*

Cyclodextrin (CD) CSP have not been used extensively in SFC.[62] CD are naturally occurring cyclic oligosaccharides consisting of several (usually 6, 7 or 8) glucose units connected by α-1,4-linkages. This unique structure give CD the ability to form inclusion complexes with a variety of chiral and achiral compounds.[68-70] The chiral recognition mechanism of native cyclodextrin (CD) remains unclear. On the other hand, for some compounds, such as phosphine oxides or amides, a CD CSP in SFC with $CO_2$:methanol as mobile phase gives selectivity better than that of NPLC using hexane:ethanol as mobile phase. To explain this behavior, the authors postulated that the smaller size of the carbon dioxide molecule, relative to hexane, made it less likely to compete with the analyte for the CD cavity.[71] However, more research should be carried out to better elucidate the mechanism of chiral recognition of these chiral SP in pSFC.

Other separations were performed using cyclodextrin-based SP in pSFC. One example is the work of Williams *et al.,*[72] who compared NPLC and pSFC on a derivatized CD CSP for the separation of chiral compounds of pharmaceutical and agricultural interest. The authors observed improved resolution with pSFC. In other paper by the same authors,[73] the enantiomeric separation of a variety of drugs was performed and resolved on the CD CSP using a simple carbon dioxide/methanol MP where the chiral resolution of cromakalim, a pharmaceutical drug to control blood pressure, was better in SFC when compared with LC. The separation of pharmaceutical compounds using CD CSP for the enantiomeric separations[70] examined the influence of parameters such as the nature of the modifier and the modifier concentration on the enantioselectivity and resolution of these compounds. In some cases, the performance of the separation with pSFC was equivalent to the separation in RPLC.

A recent paper[70] calls attention to the fact that there are limits in chiral pSFC with CD CSP due to the low polarity of the mobile phase, even with the use of modifiers that inhibit the formation of inclusion complexes with the analytes, important to have good separations. On the other hand, SP prepared by the immobilization of cationic perfunctionalized CD onto vinylized silica via a radical co-polymerization demonstrated good enantioselectivity for in the separation of flavanones and thiazides.[70,74-76]

*Brush-type (Pirkle-type) chiral SP*

Brush-type CSP generally involve a combination of dipole–dipole, π-π, steric and hydrogen bonding interactions in the chiral recognition mecanism.[62] There are some reports in the older literature using pSFC but few nowadays. Blum *et al.*[77] compared the separation in RPLC and SFC using brush-type CSP for the chiral separation of ketoprofen and verapamil. The resolution observed was greater in pSFC than in RPLC, which suggests that this kind of chiral SP should be more explored in pSFC.

One interesting and recent application of SFC with dipole–dipole, π-π, steric and hydrogen bonding interactions used a synthetic chiral polymeric SP[78] for the enantioseparation of mitotane or 1,1-dichloro-2-(*o*-chlorophenyl)-2(*p*-chlorophenyl)ethane, a synthetic chiral drug derivative of the insecticide dichlorodiphenyltrichloroethane (DDT).[79] The drug is marketed as the racemic mixture, used in the treatment of adrenocortical carcinoma (ACC). Antelo *et al.*[79] used a diallyl-L-tartardiamide-based stationary phase and $CO_2$ modified with 14% methanol at 160 bar in the temperature range of 303.15–313.15 K, studying the influence of temperature on the retention process and the chiral discrimination mechanism on a semi-preparative scale (250 x 10 mm i.d., column packed with 16 μm particles). The results indicated that enantioseparation process is enthalpically driven under these experimental conditions.

*Macrocyclic antibiotics*

Glycopeptide-based-macrocyclic antibiotic chiral selectors have demonstrated as good enantioselectivity in SFC as is seen in LC, CE, and CEC. Their broad enantioselective power results from a number of inherently favorable properties for chiral recognition, such as the presence of stereogenic centers, an amphoteric character, 'basket-like' geometry that allows interactions by complexation, and both hydrophobic and hydrophilic properties that allow them to be evaluated with a range of different mobile phases and electrolytic systems. The most widely reported and more enantioselective chiral selectors of this class are vancomycin, teicoplanin and ristocetin A, which have been applied effectively in all of the above separation techniques, including pSFC chiral separations.[80]

In pSFC, these CSP tend to exhibit strong retentions and modifier concentrations up to 40% are utilized.[62] Lavison and Thiébaut[81] evaluated a Chirobiotic R CSP for a series of structurally diverse compounds in an attempt to discern the chiral recognition mechanisms in SFC and observed that enantioselectivity was highly analyte dependent. A detailed investigation of three macrocyclic antibiotic CSP was performed by Liu *et al.*,[82] evaluating the performance of these CSP for the enantioseparation of more than 100 chiral compounds, including analgesics, β-blockers and amino acids. Most of the compounds were resolved with at least one of the CSP tested and analysis times were generally less than 15 minutes.[62,82] As with the brush-type CSP, the macrocyclic antibiotic CSP should also more explored in pSFC.

## Characterization of stationary phases by SFC

Much effort by academic scientists and by manufacturers has been spent on understanding retention and selectivity behaviors in SP in order to control and predict chromatographic properties of sometimes nominally identical materials, but which may show very different chromatographic properties.[83] Many techniques are used to characterize SP, such as spectroscopic,[84,85] thermogravimetric[86,87] and physicochemical characterizations that determine some structural properties. However, the best evaluations of stationary phases are based on their retention and selectivity mechanisms, determined by chromatographic characterization, due to differences in the affinities of solute molecules for the stationary phase in the process of solute transfer from the mobile phase onto/into the stationary phase.[85]

Many test procedures have been suggested for column selection and characterization of SP based on their chromatographic properties and parameters such as hydrophobicity, silanol activity, hydrogen bonding capacity and steric selectivity, including the Tanaka,[87,88] Engelhardt,[89] SRM 870[90] and Neue tests.[91-93] In these tests

hydrophobicity is usually measured by the retention of a neutral solute such as pentylbenzene or ethylbenzene, while silanol activity is evaluated by the asymmetry factor of a basic solute such as benzylamine. The other properties are also evaluated with appropriate compounds.

*Carotenoids test*

In 1993, Lesellier *et al.*[94] developed a chromatographic test based on the retention of carotenoids. The test is carried out in a supercritical mobile phase, without water, with the supercritical fluid allowing higher efficiencies. The advantages are:[95]

(i) better characterization of shape selectivity, based on the separation of isomers;
(ii) more intense silanophilic interactions due to the absence of water;
(iii) rapid equilibration time of the columns.

Due to the above advantages, the application of the carotenoid test allows classifications without any loss of information, determining similarities or differences between SP. Tests including all types of phases often lack a fine discrimination within one type of column.[95] This is particularly interesting when chemometric methods, such as HCA (hierarchical cluster analysis) and PCA (principal component analysis), are applied.

The carotenoid test[94,96,97] uses as solutes 13-*cis*-β-carotene, all *trans*-β-carotene and zeaxanthin, using SFC, with $CO_2$:methanol (85:15, v/v) as mobile phase and a temperature of 25 °C. The flow-rate is 3 mL $min^{-1}$, and the outlet pressure is 15 MPa, with UV–vis detection at 440 nm. The parameters obtained are:[95]

- Silanol interaction (separation factor of *trans*-β-carotene and zeaxanthin; $\alpha_{\beta\text{-trans/zea}}$): in comparison to all *trans*-β-carotene, zeaxanthin possesses two additional hydroxyl groups located at the cyclic extremities. The presence of hydroxyl groups favor the interactions between zeaxanthin and the polar modifier of the mobile phase, but also between zeaxanthin and the polar sites on the stationary phase. Working at constant mobile phase composition, the retention of zeaxanthin compared to that of all *trans*-β-carotene (the separation factor) only depends on the silanol activity of the stationary phase.
- Shape selectivity (separation factor of *cis* and *trans*-β-carotene; $\alpha_{cis/trans}$): due to the numerous conjugated double bonds on the central chain of β-carotene, the compound is rigid and linear for the all *trans* conformation, or bent for the *cis* conformations. Because these compounds have similar hydrophobicity but different conformations, the separation factor between the *cis*/*trans* isomers depends on steric or shape recognition.
- Hydrophobicity: ($k_{\beta\text{-carotene}}$): the all *trans*-β-carotene retention factor (k) is used to measure the stationary phase hydrophobicity. For columns having the same bonded chain length, absolute retention depends both on the coverage density and on the specific area of the silica.

*Linear Solvation Energy Relationship (LSER)*

The most common method used for SP characterization, classification and comparison in SFC, in addition to the chromatographic tests used in characterization in RP-HPLC, is the key-solutes quantitative structure-retention relationship (QSRR) based on the linear solvation energy relationship (LSER)[42,44,45,98,99] that uses Abraham descriptors,[100] described by the equation (1): [42,44,101]

$$\log k = c + eE + sS + aA + bB + vV \quad (1)$$

In equation (1), the capital letters represent the solute descriptors, related to particular interaction properties, while lower case letters represent the system parameters, related to the complementary effect of the phases on these interactions. In equation (1), *c* is the model intercept term, E is the excess molar refraction (calculated from the refractive index of the molecule) and models polarizability contributions from n and π electrons; S is the solute dipolarity/polarizability; A and B are the overall solute hydrogen-bond acidity and basicity and V is the McGowan characteristic volume in units of ($cm^3$ $mol^{-1}$)/100, when the retention factor, k, is used as the dependent variable. Figure 3, shows the interactions related to each solute descriptor. The system parameters (*e, s, a, b, v*), obtained through a multilinear regression of the retention data for a certain number of solutes with known descriptors, reflect the magnitude of the differences for that particular property between the mobile and stationary phases. If a particular coefficient is numerically large, then any solute having the complimentary property will interact very strongly with either the mobile phase (if the coefficient is negative) or the stationary phase (if the coefficient is positive). Consequently, the coefficients also reflect the system's relative selectivity towards that particular molecular interaction.[42,44,101]

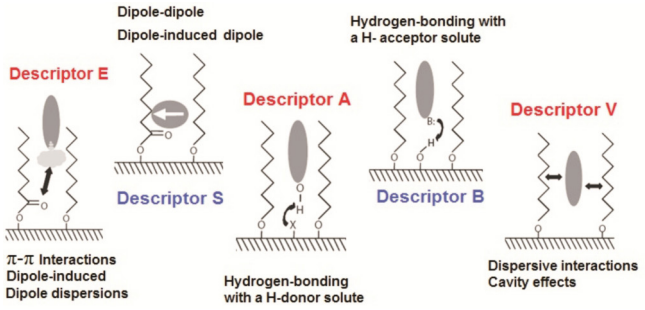


***Figure 3.*** *Principle of the solvation parameter model and the interactions related to each solute descriptor. Adapted from reference 102*

Thus, it is possible to compare different SP, based on the coefficients obtained from the multilinear regressions, as well as plotting graphs of log k versus log k between two systems or presenting the data in five-dimensional plots, called spider diagrams,[98] to evaluate if the parameters obtained are statistically adequate.[102,103]

Since this test was developed, it has been applied in the characterization of many types of packed columns having aliphatic,[42,43,101,104-106] aromatic,[45] polar,[44,101,104,106] chiral[65-67,107] and HILIC[48] SP and has been helpful in understanding and clarifying the types of molecular interactions between solutes and the SP or MP in SFC.

The papers of West *et al.*[42-45,48,101,104-107] are particularly interesting due to the fact that they have characterized a large number of SP using pSFC, introducing new and interesting numeric and graphic tools which help solidify the classification and the characterization of the SP, as, for example, by the use of a spider diagram.[98]

In a spider diagram each SP is represented by a bubble point. Each bubble point is placed in this figure according to the normalized values of the five system parameters (*e*, *s*, *a*, *b* and *v*). The normalized values are obtained by dividing the value of the system parameters by the vector length, $u_i$, defined by Equation 2.[98] It is positioned from an origin defined as the center of a five-branched star. Bubble size is related to $u_i$ and thus related to the overall strength of the interactions in the system.

$$u_i = \sqrt{e_i^2 + s_i^2 + a_i^2 + b_i^2 + v_i^2} \quad (2)$$

Figure 4 shows an example of a spider diagram applied to the characterization of 19 SP based on silica and on silica modified with zirconia and titania, having polysiloxanes with different chain lengths (C1, C8, C14 and C18, phenyl) thermally immobilized onto these

supports, as well as a commercial chemically bonded SP, Kromasil C18.[108] The chemically bonded SP Kromasil C18 (Kr-C18) is positioned very far from the other SP, at the top left of the figure where non-polar phases usually are situated. Among the SP, the least polar phases, with large e and v values, are situated in the upper part of the diagram while the most polar phases, with large a and b values are at the bottom right. The latter are the supports without polymer coatings (Si; Si-Ti; Si-Zr) and the phases with phenyl (Zr-Phe; Si-Phe) or short chain (methyl) ligands (Si-C1). Comparing the positions of the SP confirms the initial information on the similarity of chromatographic behavior between these phases. When replacing one SP by another one with a close position in the figure, small changes of selectivity can be observed.[108] The use of the spider diagram allows selecting specific regions where a given column is more suitable for a particular application based on the properties of these SP. The SP shown in Figure 4 are distributed as follows: more or less polar, more or less retentive, having higher selectivity or better peak symmetry for basic compounds, etc.,[108] proving that pSFC is an interesting and powerful tool to perform the characterization of different types of SP, and can be used to select a stationary phase according to its chromatographic behavior for a specific application.

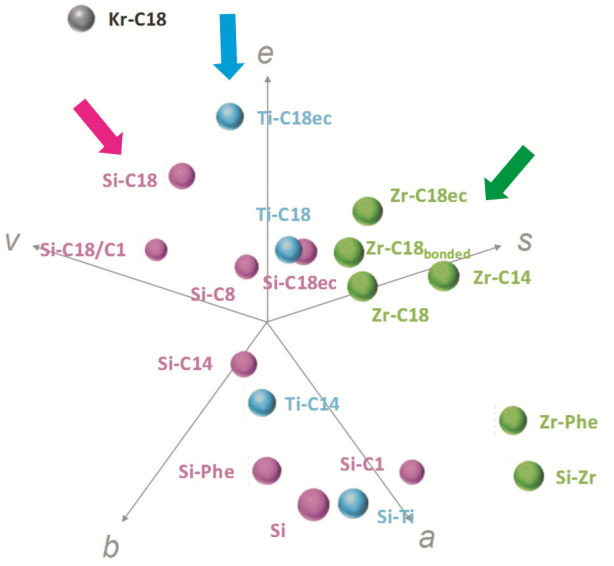


***Figure 4.*** *Spider diagram for a five-dimensional representation of 19 different stationary phases evaluated with the solvation parameter model. Adapted from reference 108*

## Supercritical fluid chromatography coupled with mass spectrometry (SFC-MS)

Before the coupling of SFC with mass spectrometers, SFC systems used UV and flame ionization detectors (FID) for cSFC.[109] On the other hand, since SFC is a hybrid of gas chromatography and liquid chromatography, as described in Figure 1, and the MP is composed principally of $CO_2$ in sub/super critical state, being highly volatile, with molecules pressed closely together but without forming a liquid, the use of MS detectors has become very interesting, since the ionization mechanism required for MS should be easier than in HPLC, even with the higher flow rates (2 to 5 mL $min^{-1}$) typical of SFC.

The most popular ionization sources for SFC-MS are atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), as they allow direct infusion of the chromatographic effluents into the mass analyzer.[109] These are the same ionization sources used in LC-MS.

On the other hand, interfaces for SFC-MS are more challenging when compared with LC-MS, due to the behavior of the $CO_2$. As the SFC mobile phase elutes from the column, the pressure on the gas is released, the density decreases, the $CO_2$ can boil off and any dissolved components can precipitate.[109] It is therefore necessary for the interface that links the ionization source to the mass spectrometer to control or counteract these problems.[109] The pressure and consequently the density of the MP are controlled by the back pressure regulator and its configuration in the MS interface.

Similar to LC-MS, the choice of mass analyzer in SFC-MS depends on the application. The most used mass analyzer for SFC-MS in the pharmaceutical industry is the single quadrupole mass spectrometer due to advantages such as ease of control by the available software being easily adaptable to both sources and the interfaces.[109]

## Preparative SFC

The potential application of sub/supercritical fluid chromatography for preparative purposes (prep-SFC) is also considerable, as it offers several advantages, such as speed and low cost of operation, including:[110,111]

- Faster column equilibration and higher resolution per unit of time, resulting in more rapid method development when compared with HPLC;
- Reduction in solvent consumption;
- Easy separation of analytes from the MP by vaporization following depressurization.
- Use of higher flow rates due to the low viscosity of the supercritical fluid, giving better production rates.

Considering all these reasons prep-SFC is a valuable tool, and in some cases complementary to prep-HPLC, for isolating the different compounds of a mixture, as in the case of producing functional food ingredients; it is frequently used for the fractionation and purification of extracts obtained with SFE. An example is the extraction of pesticides as reported by Rissato *et al.*[112] In the literature, applications of prep-SFC can be found in the analysis and purification of foods, pharmaceuticals compounds, natural products, etc.[3,110]

## Ultra high performance SFC

In recent years, major instrumental improvements have led to the introduction of an ultra-high performance supercritical fluid chromatograph (UHPSFC), developed by the Waters Corporation[63,113] and commercialized with the name Ultra-Performance Convergence Chromatography™ (UPC²). This equipment has been optimized specifically for SFC and presents very low void space volumes and improved detection with flow cell, pump modules and back pressure regulator, including the reliability and precision of the pumping system and backpressure regulation, required for controlling the delivery of mixtures of $CO_2$ and the organic modifier and its additives.[113] These improvements give the separation some interesting advantages such as increases in throughput and in the efficiency of the analyses, especially when used in combination with sub 2-µm particles. However, since the introduction of this equipment is very recent (2012), its actual performance in routine analysis has yet to be confirmed.[63,114,115]

Dr. Davy Guillarme, one of the first users of UHPSFC, discussed the applicability of the equipment in the separation of neutral, acidic and basic compounds.[114,115] According to this researcher, neutral and acidic compounds are not problematic and can be analyzed under SFC/UHPSFC conditions using almost any mobile or stationary phase. On the other hand, basic compounds are problematic and deserve special attention. This may be due to the fact that the mobile phase of SFC (and UHPSFC) has an acidic pH (about 5) related to the transient formation of methylcarbonic acid originating from the reaction

of methanol and $CO_2$ when this modifier is present. To improve the peak shape of basic drugs, particularly those with a pKa higher than 8, it is necessary to change the nature of the stationary phase and work preferentially with a column bonded with a basic group, such as ethylpyridine. On the other hand, it is also possible to change the character of the MP by using other additives (e.g., 20 mmol/L ammonium hydroxide) in the mobile phase.[114,115]

Perrenoud *et al.*,[116] in one of the first publications using UHPSFC, compared the performance, possibilities and limitations of both ultra-high performance liquid chromatography (UHPLC) and ultra-high performance supercritical fluid chromatography (UHPSFC) using columns packed with sub-2 μm particles for the separation of free steroids, benzodiazepines and other pharmaceutical compounds, as shown in Table 1. The results proved the compatibility of these sub-2 μm SP, especially recommended for UHPSFC, for use in the gradient mode, showing results similar to or better than those using UHPLC. In terms of column chemistry this paper tested the performance of different stationary phases for SFC such as bare silica, hybrid silica and silica with ethylpyridine groups (Table 1). The results demonstrated that changes in selectivity are more significant using UHPSFC conditions, when compared with UHPLC conditions, due to the nature of the stationary phase bonding and also the absence of water in the MP, which affects the interaction mechanisms between analytes and the SP. In the same paper, various types of column chemistries with 1.7 μm particles were evaluated for both UHPLC and UHPSFC conditions using a mixture of acidic, neutral and basic compounds. The results showed that more drastic changes in selectivity were obtained using UHPSFC phases, compared to those obtained by changing the UHPLC SP. In addition, there was a good complementarily between the two separation modes. When using small particles in UHPSFC, it was also possible to realize analyses of pharmaceutical compounds in less than 1 minute or to obtain peak capacities of more than 250 in approximately 40 min, both with a high degree of repeatability.[116]

Another paper[117] from the same authors also hyphenated the UHPSFC system with MS/MS, proving it to be an interesting alternative for the analysis of basic compounds, since the use of basic buffers, required in HPLC, was not necessary, even for the separation of basic compounds. Other application notes illustrate the versatility of this new equipment in the separation of lipids, polymers and pharmaceutical compounds.[118]

## APPLICATIONS

Nowadays there are many papers dedicated to reporting applications of packed column SFC and /or UHPSFC, including coupling with MS, in the contrast to past revisions about SFC separations and applications where the emphasis was on capillary columns. The applications have emphasis on separations of pharmaceutical compounds and natural products due to the use of low temperatures in SFC.[119]

West and Lesellier[36] have studied the changes in retention and selectivity that occurred when changing the type of the co-solvent (methanol, ethanol, isopropanol, acetonitrile) in the composition of SFC MP in the separation of four barbiturates, mephobarbital, phenobarbital, glutethimide and primidone, using three types of achiral stationary phases: cyanopropyl-bonded silica, phenyl-oxy-propyl-bonded sílica and pentafluorophenyl-propyl-bonded silica. To compare the results, the isocratic conditions were: $CO_2$–modifier, 90:10 (v/v), 25 °C, 150 bar and 3 mL $min^{-1}$. The results (Figure 5) show different retentions and selectivities when changing the nature of the polar modifier. Acetonitrile was the worst modifier, proven by the peak tailing observed. On the other hand, ethanol provided the best results. In some cases, co-elutions were observed with methanol, when compared with ethanol. Isopropanol was just a little better than acetonitrile. This paper shows that, in some cases, it could be possible to replace methanol by ethanol in SFC separations, leading to a greener separation technique since ethanol is less toxic and can be produced from renewable resources. It is interesting observe as well that all separations were performed in less than 5 minutes, proving one of the main characteristics of SFC separation: production of faster separations when compared to HPLC and GC.

The evaporative light scattering detector (ELSD) is a detector which can be used when analytes are less volatile than the mobile phase. Its mechanism involves three main steps: nebulization, mobile phase evaporation and solute detection when droplets are created and

**Table 1.** Packed columns tested in reference.[116] All SP are manufactured by Waters Corporation

| Stationary phase | SP chemistry | Dimensions | Particle size | Designed for use in |
|---|---|---|---|---|
| Viridis BEH | Silica based ethylene bridged hybrid (BEH) | 150 mm x 4.6 mm | 5 μm | SFC |
| Aquity UPC² BEH 2-EP | Silica based ethylene bridged hybrid (BEH) with 2-ethylpyridine groups | 100 mm x 3.0 mm | 3.5 μm | UHSFC |
| Acquity UPC² BEH | Silica based ethylene bridged hybrid (BEH) | 100 mm x 3.0 mm | 1.7 μm | UHSFC |
| Acquity UPC² BEH 2-EP | Silica based ethylene bridged hybrid (BEH) with 2-ethylpyridine groups | 100 mm x 3.0 mm | 1.7 μm | UHSFC |
| Acquity UPC² CSH fluoro-phenyl | Silica based ethylene bridged hybrid (BEH) functionalized with fluoro-phenyl bonded phase | 100 mm x 3.0 mm | 1.7 μm | UHSFC |
| Acquity UPC² HSS C18SB | Silica particle based C18 phase designed for high mechanical stability, endcapped | 100 mm x 3.0 mm | 1.8 μm | UHSFC |
| XTerra RP18 | Silica hybrid particles with bonded C18, endcapped | 150 mm x 4.6 mm | 5 μm | HPLC |
| XTerra RP18 | Silica hybrid particles with bonded C18, endcapped | 150 mm x 4.6 mm | 3.5 μm | HPLC |
| Acquity BEH Shield RP18 | Silica based ethylene bridged hybrid (BEH) with embedded carbamate group in the bonded C18 phase | 50 mm x 2.1 mm | 1.7 μm | UHPLC |
| BEH phenyl | Silica based ethylene bridged hybrid (BEH) with phenyl groups | 50 mm x 2.1 mm | 1.7 μm | UHPLC |
| CSH fluoro-phenyl | Silica based ethylene bridged hybrid (BEH) functionalized with bonded fluoro-phenyl phase | 50 mm x 2.1 mm | 1.7 μm | UHPLC |
| BEH C18 | Silica based ethylene bridged hybrid (BEH) with C18 groups | 50 mm x 2.1 mm | 1.7 μm | UHPLC |
| HSS C18SB | Silica particle based C18 phase designed for high mechanical stability, endcapped | 50 mm x 2.1 mm | 1.8 μm | UHPLC |

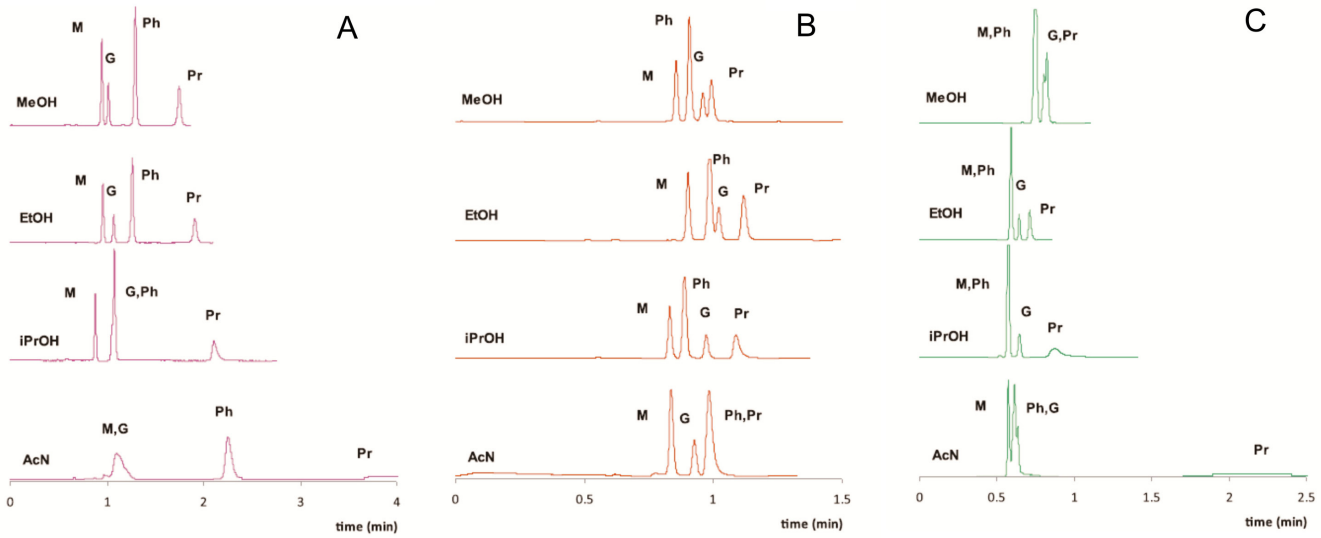


***Figure 5.*** *Chromatograms of four barbiturates on cyanopropyl-bonded silica (A), phenyl-oxypropyl-bonded silica (B) and pentafluorophenyl-propyl-bonded silica (C) as function of the MP modifier. Solutes: M mephobarbital; Ph phenobarbital; G glutethimide; Pris primidone. Chromatographic conditions: $CO_2$-modifier 90:10 (v/v), 25 °C, 150 bar, 3 mL min$^{-1}$ and UV detection 210 nm. Adapted from reference 36*

easily evaporated by a atomization process, due to the addition of a inert gas to the MP at the column outlet in a nebulizing chamber. This detector was developed for compounds that do not show sufficient UV absorption, and can be used in both HPLC and SFC separations.[120,121]

Lesellier *et al.*[121] have studied the effects of the injection volume using SFC with a $CO_2$:MeOH (90:10, v/v) MP and evaporative light scattering detector (ELSD) (Figure 6), where the different detector properties showed a different behavior between the ELSD and the UV detector. A lower increase in peak width when increasing the injected volume for the ELSD peak means it remains sharper, which suggest that the volume injection for the ELSD can be greater than in UV without loss of resolution, increasing the signal/noise ratio and low detection limits, expressed in terms of concentration.

Amides are commonly found within compounds of pharmaceutical importance and were found in more than 25% of the known pharmaceuticals at the beginning of 2000´s.[122,123] Xiang *et al.*[123] have

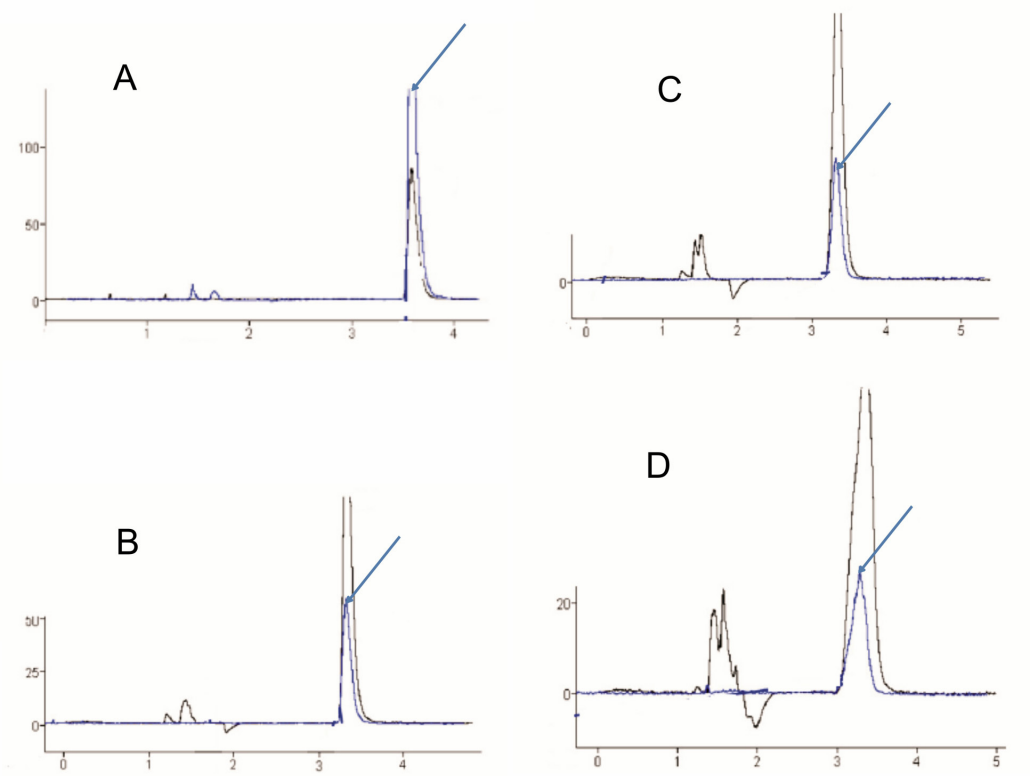


***Figure 6.*** *UV (210 nm) and ELSD chromatograms (3 bar for the nebulizer gas:$N_2$; drift temperature: 40 °C ) obtained for different injected volumes of caffeine: (A) 5µl (500 ppm); (B) 10 µl (100 ppm); (C) 15 µl (50 ppm) and (D) 20 µl (25 ppm). The ELSD chromatograms were slightly shifted on the left side of the UV chromatogram to provide a better view of the right side of the peak width difference between the UV and the ELSD chromatograms. Conditions: $CO_2$–methanol, 90:10 (v/v), 25 °C, outlet pressure: 150 bar, 3 mL min$^{-1}$. The arrows indicate the ELSD peaks. Adapted from reference 121*

reported the chiral separation of amides using chiral polysaccharide-based stationary phases with SFC in the gradient mode of elution. Using a Chiralcel OD-H column (Figure 7 A, B and C), the retention increases using a basic modifier, in this case, isopropylamine (IPA), but the resolution decreases. On the other hand, the mixture of acidic and basic additives, trifluoroacetic acid (TFA) and isopropylamine, does not improve the resolution but increases the retention. The chiral separation with the Chiralcel IC column (Figure 7 D, E and F) showed that the addition of IPA to the MP not only increases the retention of amide, but also improves the resolution. IPA is more effective at increasing the retention and resolution than the combination of IPA and TFA. Another interesting observation is that the eluting order of the enantiomers is reversed in this set of columns, suggesting a different chiral based mechanism for the separation, leading to the conclusion that even with the same chiral SP, the profile of the separation can be different, depending on the MP.

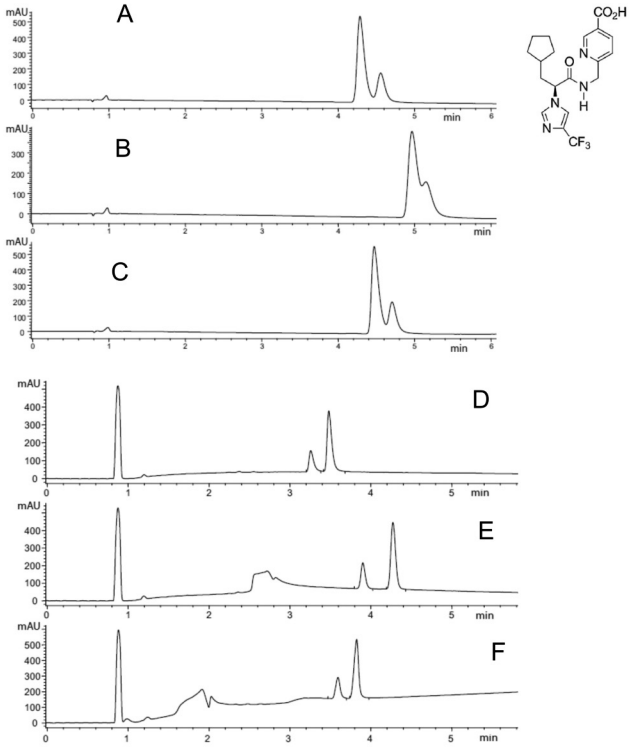


***Figure 7.*** *Separation of a chiral amide on a Chiralcel OD-H column in A, B and C and a Chiralcel IC column in D, E and F. (A,D) methanol; (B,E) methanol with 0.1% of isopropylamine (IPA); (C,F) methanol:ACN (75:25, v/v) with 0.1% of trifluoroacetic acid (TFA) and 0.1% IPA. Chromatographic conditions: Modifier from 5 to 50% gradient at 6.5%/min, hold for 1 min; 40 °C, outlet pressure: 150 bar, 4 mL min$^{-1}$; UV detection at 254 nm. Adapted from reference 123*

## CONCLUSION

Supercritical fluid chromatography (SFC) is an important separation technique for a wide range of compounds. The improvements in instrumentation in recent years and the use of modifiers and additives has provided more versatility and has permitted the use of diverse HPLC SP, especially those used in RPHPLC, expanding the application of SFC to the analysis of many non-polar, polar and ionizable compounds.

Chiral separations using SFC have demonstrated the performance and applicability of SFC as an especially powerful tool for the separation and purification of pharmaceutical compounds.

Following the example of HPLC and UHPLC, ultra high performance SFC (UHPSFC) equipment was commercialized in 2012, permitting the use of sub-2 μm particles, although there are as yet few publications describing these applications.

As perspectives for the future, an understanding of the interaction mechanisms in SFC related to retention and selectivity, the effects of temperature and pressure on the separations, the use of water as modifier, the development of new sub-2μm particles for SFC stationary phases and, as mentioned before, the use of UHPSFC in the separation of achiral and chiral compounds, can be highlighted.

## SUPPLEMENTARY MATERIAL

The Supplementary Figure for this paper is available at http://quimicanova.sbq.org.br, in the PDF format, with free access.

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Supplementary Material

# SUPER/SUBCRITICAL FLUID CHROMATOGRAPHY WITH PACKED COLUMNS: STATE OF THE ART AND APPLICATIONS

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## List of Abbreviations

| Abbreviation | Description |
|---|---|
| ACC | adrenocortical carcinoma |
| ACN | acetonitrile |
| APCI | atmospheric pressure chemical ionization |
| BPR | back-pressure regulator |
| CD | cyclodextrin |
| CE | capillary electrophoresis |
| CEC | capillary electrochromatography |
| cSFC | open tubular capillary column SFC |
| CSP | chiral stationary phase |
| DDT | dichlorodiphenyltrichloroethane |
| $D_M$ | diffusion coefficient |
| ELSD | evaporative light scattering detector |
| ESI | electrospray ionization |
| FID | flame ionization detector |
| GC | gas chromatography |
| HCA | hierarchical cluster analysis |
| HILIC | hydrophilic interaction chromatography |
| HPLC | high performance liquid chromatography |
| IPA | isopropylamine |
| LC | liquid chromatography |
| LC-MS | liquid chromatography coupled with mass spectrometry |
| LSER | linear solvation energy relationship |
| MP | mobile phase |
| MS | mass spectrometry |
| MS/MS | sequential mass spectrometry |
| NPLC | normal-phase liquid chromatography |
| ODS | octadecylsilane |
| PCA | principal component analysis |
| pSFC | packed column SFC |
| prep-SFC | supercritical fluid chromatography for preparative purposes |
| QSRR | quantitative structure-retention relationship |
| RPLC | reversed-phase liquid chromatography |
| SFC | supercritical fluid chromatography |
| SFC-MS | supercritical fluid chromatography coupled with mass spectrometry |
| SFE | supercritical fluid extraction |
| SP | stationary phases |
| TFA | trifluoroacetic acid |
| UHPLC | ultra-high performance liquid chromatography |
| UHPSFC | ultra-high performance supercritical fluid chromatography |
| UPC² | ultra-performance convergence chromatography™ |
| UV | ultraviolet spectrometry |
| $\mu_{opt}$ | optimum linear velocity |

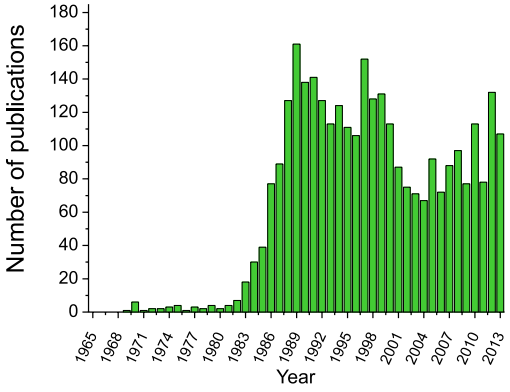


***Figure 1S.*** *Number of publications in SFC by year using the term "supercritical fluid chromatography" based on SciFinder Scholar® in November* 2013

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