Column Technology for Achiral SFC Separations

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Supercritical fluid chromatography (SFC) is a powerful tool for the separation of both chiral [1-3] and Achiral [4, 5] compounds. SFC was originally practiced with either open tubular columns [6] or packed columns with an initial emphasis on analytical chromatographic analysis. Presently, SFC using open tubular columns is rarely practiced while the use of packed column SFC is the preferred mode of operation. Packed column SFC can be used for analytical as well as preparative applications. SFC provides for several key advantages for both preparative and analytical chromatographic separations based chiefly on the properties of carbon dioxide (CO_2) which is the most commonly used mobile phase in SFC. The viscosity of a CO_2 based mobile phase is significantly lower than mobile phases used in traditional HPLC which produces a significantly lower pressure drop across the column. The minimised pressure drop allows the use of much smaller particles for both analytical and preparative separations. The use of the smaller particles enables either an increase in chromatographic efficiency in the same chromatographic run time, or a decrease in chromatographic run time while maintaining the same efficiency experienced with larger particles. However, the disadvantage with SFC is the compressibility of the mobile phase generates serious internal pressure/density gradients which may lead to set instrumental conditions deviating more or less strongly from the real conditions in the column. These deviations with appropriate cautionary notes have been carefully explained by E. Forss et. al. [7].

A CO_2 based mobile phase delivers several other key advantages besides lower viscosity. Firstly, the diffusion coefficients of solutes in CO_2 mobile phases have been shown to be 3-10 times higher than in normal liquids, minimising resistance to mass transfer, and thus allowing for use of higher linear velocities without loss of efficiency. The density of CO_2 in the supercritical state is closer to a liquid than a gas, virtually eliminating the solubility issues that are present with GC. Secondly, CO_2 has the potential to act as both a weak Lewis acid and Lewis base, and it can participate in conventional or nonconventional hydrogen bonding interactions. CO_2 is a non-protic solvent with no dipole moment but has also been described as a quadrupolar solvent because of its significant quadrupole moment [(8]. Thirdly, it is miscible with a wide range of organic solvents, nonflammable, and has little UV absorbance at lower wavelengths. CO_2 based SFC is particular well suited to the area of preparative chromatography where it can be easily removed after fractionation, enabling the rapid recovery of isolated, pure compounds. In addition, any residual amounts of CO_2 in isolated products are considered to be non-toxic provided that a toxic co-solvent is not used for the separation. Based on these properties, preparative SFC has become an important tool for pharmaceutical purifications and isolations particularly for chiral compounds [9]. However, over the last several years there has considerable interest in using SFC for Achiral purifications [10] and Achiral analytical methods [11]. It is the increased interest in Achiral SFC separations which is the main focus of this report, with particular emphasis on commercially available Achiral SFC stationary phases.

Role of the Stationary Phase in Achiral SFC

One of the drawbacks of using CO_2 as a mobile phase in SFC is that it is relatively non-polar even though it has been described as a quadrupolar solvent because of its significant quadrupole moment [8]. In order to modify the elutropic strength of CO_2 and allow for the chromatography of polar molecules, organic solvent modifiers must be mixed into the CO_2 mobile phase stream employing a second high pressure HPLC pump. While methanol and ethanol are the most commonly used modifier solvents, users do occasionally try other organic solvents for varied selectivity. Because of the non-polar behaviour of CO_2, the stationary phase plays a key role in SFC separations. Stationary phase selection for SFC can be complicated and influenced by a combination of three major factors: separation selectivity, retention factor, and peak shape concerns. Firstly, separation selectivity, as with any chromatographic technique, is of prime importance and is a major challenge for the chromatographer in utilising SFC. In reversed phase HPLC, C_{18} is viewed as the 'universal' stationary phase; however, the analogous SFC phase is currently unavailable. The current lack of a universal SFC stationary phase has led to development of a large variety of SFC stationary phases which actually complicates the stationary phase selection process for any individual separation. Through addition of a co-solvent such as methanol, the polarity of the mobile phase can be 'tuned' to alter the separation of mixtures containing a wide variety of chemical polarities from non-polar to extremely polar, and in many cases exceeds the separation boundaries of reversed phase HPLC. In addition, it was recently shown that methanol acts not only as a co-solvent but also as an 'additive', i.e. as an adsorbing component, to silica and diol stationary phases [12,13]. However, the chromatography of mixtures containing a wide variety of polarities requires multitude of stationary phase chemistries.

Chromatographic retention times, especially when excessive, can be less than optimal for a separation of diverse molecules presenting another important factor in the SFC stationary phase selection process. For example, underivatised amines can be excessively retained on un-bonded silica columns [10] leading to extremely long retention times, hindering the productivity of both analytical and preparative separations. Another major factor for selecting a SFC stationary phase is the quality of the chromatographic peak shape. It is most desirable that compounds eluted in a chromatographic separation exhibit symmetrical, Gaussian behaviour with a minimal amount of tailing or asymmetry. Symmetrical chromatographic peaks are desirable on both the analytical and preparative scale, ensuring these methods are productive and robust. Many SFC separations require the use of additives such as triethylamine, trifluoroacetic acid (TFA), ammonia or water in order to diminish peak tailing and maintain acceptable retention factors, particular when separating amines. A more complete definition of additive types and their role in SFC separations has been recently investigated [14]. The use of additives for either analytical SFC or HPLC chromatographic methods is generally accepted and is not regarded impediment to the chromatographic method. However, the use of mobile phase additives when purifying and isolating compounds for preparative SFC chromatography are generally discouraged [10]. Many additives used in SFC, such as triethylamine or TFA, are difficult to remove and potentially alter the chemical properties of the compounds being purified. As a result of these concerns a number of SFC optimised stationary phases have been developed [15] to avoid the use of mobile phase additives while delivering the desired chromatographic performance. Several academic researchers have developed a number of these stationary phases developed specifically for SFC separations [17,18] however the focus of this report will be stationary phases that are available commercially.

Stationary Phases Adapted Initially for Packed Column SFC

Stationary phases used in early practice of SFC were often stationary phases and included both polar and non-polar stationary phases. Polar phases including silica and polar bonded phases (such as cyano, diol, and amino) [5, 19-21] have been widely adapted for a number of SFC separations, particularly the separation of polar analytes. The wide application of these polar stationary phases may have been influenced by the opinion that supercritical or sub-critical CO_2 is a non-polar fluid and should be treated as a non-polar mobile phase used for normal phase chromatography [6]. Mobile phases used in normal phase liquid chromatography are composed of non-polar solvents, such as hexane which can be modified with more polar solvents such as dichloromethane. The stationary phases used for normal phase chromatography are polar which can include bare silica, bonded cyano, diol or amino groups. However, as stated earlier CO_2 should not be viewed as strictly a non-polar, aprotic solvent, with no dipole moment possessing a strong quadrupole moment. This quadrupole moment may induce quadrupolar forces in interacting molecules. As a consequence of this complex interactive behaviour, CO_2 can be used with both polar and non-polar (ODS, octyl and C4) stationary phases. ODS and other alkyl based phases have been used for SFC separations [22-24] and provide for retention of aromatic and hydrophobic compounds. However, the vast majority of SFC methods have relied on silica and polar bonded phases [15].

Un-bonded silica is frequently used in SFC separations due to its numerous beneficial attributes. Silica has excellent loading capacity and good selectivity for complex mixtures [25,26]. It is also robust and readily available in a variety of surface areas, pore sizes and particle sizes. It does however have several undesirable characteristics including strong interactions with extremely basic amines leading to excessive retention and poor peak shape of these compounds without the use of mobile phase additives such as diethyl amine [10]. It has been reported that adding a small amount of water to a SFC mobile phase can greatly improve the peak shape for many compounds [4, 27]. The use of water for analytical SFC is generally safe but care must be taken to insure that the carbonic acid formed when water interacts with CO_2 in the mobile phase is compatible with the components of the chromatographic system. In addition, there is a chance the water in the mobile phase may freeze and plug the system during sample collection in preparative SFC.

Diol based columns have proven to be useful for many SFC based separations [5, 28]. Diol based phases deliver similar selectivity to silica without the undesirable strong interactions

leading to excess retention. However, Diol doesn't have the loadability or the sample capacity of silica. Cyano, another popular stationary phase used in SFC, is a polar non-protic stationary phase capable of participating in a degree of pi-pi interactions leading to increased selectivity for many analytes [15,16]. Another commonly used polar stationary phase used both in normal phase and SFC chromatography is the amino phase. Amino is a polar stationary phase that retains basic compounds less than silica while providing increased retention of acidic compounds compared to silica [29].

Stationary Phases Developed Specifically for Packed Column SFC

Currently, there are a variety of stationary phases available for SFC, not only the polar bonded phases 'borrowed' from HPLC, but stationary phases specifically developed for SFC. The motivation to develop SFC optimised phases is the result of several factors. Firstly and chiefly, a 'universal' stationary phase has yet to be developed for SFC; in reversed phase HPLC, C18 is generally accepted as the 'universal' stationary phase. Secondly, SFC can separate mixtures containing a wide variety of chemical polarities from non-polar to extremely polar and in many cases exceeds the separation boundaries of reversed phase HPLC but requires differing phases to accomplish this task. Finally, the development SFC optimised phases have been influenced by the requirements of preparative SFC which include reduction of excessive retention and the isolation of symmetrical peaks without the use of additives such as triethylamine.

Bonded 2-ethyl pyridine *(structure in Figure 1)* was the first commercial stationary phase developed specially for SFC [15] and it has been commercialised by several column manufactures *(see Table 1)*. 2-Ethyl pyridine columns improve the peak symmetry for basic compounds while improving the selectivity for acidic compounds [10,30]. 2-ethyl pyridine has been used in many applications for both analytical methods [31] and preparative methods [30] since its commercial introduction. 2-Ethyl pyridine SFC columns generally don't require mobile additives for moderately basic or neutral compounds, however, many extremely basic compounds (pKa values greater than 9.5) require that an additive such ammonium acetate be used [30].

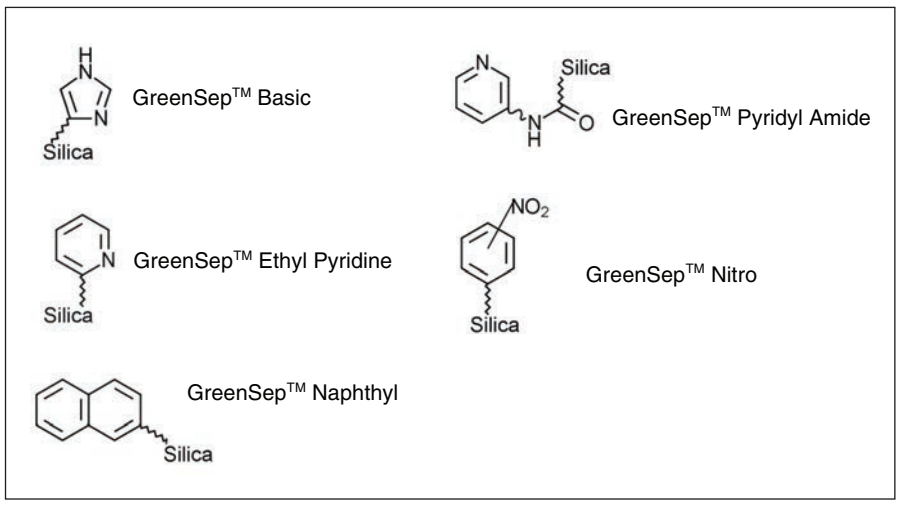

Figure 1. The Chemical Structures of SFC Optimised Stationary Phases Commercially Developed by ES Industries

Table 1. Commercial 2-Ethyl Pyridine SFC Columns

Manufacturer	Trade Name	Particle Size
ES Industries	GreenSep™ Ethyl Pyridine	1.8µ, 3µ, 5µ, 20µ
Akzo Nobel	Kromasil SFC-2EP	2.5µ, 5µ
Nacalai	SunShell 2-Ethylpyridine	2.6µ, 5µ
Princeton Chromatography	SFC 2-Ethylpyridine	3µ, 5µ, 10µ
Regis Technologies	Celeris Ethyl Pyridine	5µ, 10µ
Waters	Viridis BEH 2-Ethylpyridine	1.7µ, 5µ
YMC	2-Ethylpyridine SFC	5µ

The commercial introduction of 2-ethyl pyridine as an SFC stationary phase has lead to the development and subsequent introduction of a number of stationary phases specifically developed for SFC applications. *Table 2* contains a list of some of these stationary phases which have been commercialised specifically for SFC. These optimised SFC stationary phases have been in part formed by the chromatographic behaviour and utility of 2-ethyl pyridine. The Torus™ series introduced by Waters Corporation is based on an initial functionalisation of their ethylene bridged hybrid silica particles with hydrophilic groups, which is further modified with additional functional groups *(See Table 2)*. The initial hydrophilic functionalisation is designed to minimise silanol interactions and possible silyl ether formation [32] possibly leading to improved retention stability. The importance of controlling silanol interaction to obtain stable and reproducible retention times has also been investigated by Ebinger and Weller [33]. Waters unique approach to obtain stable and repeatable retention times is useful but is not the only solution to the problem. Other ways to improve retention time stability include various surface preparation techniques, special end capping procedures and mobile phase additives.

In recent years we have focused our research efforts on developing SFC optimised stationary phases. *Figure 1* shows the structures of some of the SFC optimised stationary phases we have developed and commercialised. An important part of our stationary phase research effort involves development of stationary phases which don't require additives for the chromatography of strongly basic compounds. One of our major stationary phase developments in this regard is GreenSep™ Basic *(listed in Table 2)*. GreenSep™ Basic is a stationary phase based on a bonded imidazole-based functional group and has shown to chromatograph basic compounds, including strong amines, without the use of mobile phase additives while maintaining excellent peak shape [10, 18]. A test mixture containing both acidic and basic compounds chromatographed on GreenSep™ Basic and GreenSep™ Ethyl Pyridine is shown in *Figures 2 and 3*, respectively. Amitriptyline is a very basic compound (pKa = 9.76) contained in the test mixture and delivers improved shape on the GreenSep™ Basic as shown in *Figure 2*, when compared to the amitriptyline peak on GreenSep™ Ethyl Pyridine as shown in *Figure 3*. However, neither column produces a symmetrical peak for amitriptyline which may require the addition of an amine additive to the mobile phase to improve peak shape symmetry for amitriptyline. In addition to this example, there are however many cases, particularly for strongly basic compounds found in the pharmaceutical research laboratories, where basic stationary phases such as GreenSep™ Ethyl Pyridine and GreenSep™ Basic require the use of additives such as ammonium acetate [30].

Both the Basic and Ethyl Pyridine stationary phases *(Table 2)* are selective and show considerable retention for acids, especially benzoic acid. The benzoic acid is actually retained significantly longer on the imidazole-based Basic compared to the Ethyl Pyridine, which is not surprising because imidazole is more basic than ethyl pyridine. Another example of the enhanced acid selectivity and retention for Basic is shown in the chromatogram in *Figure 4* for Cannabidiolic acid (CBDA), a naturally occurring cannabinoid found in cannabis. The retention of CBDA is much shorter and peak is fronting when chromatographed on Ethyl Pyridine *(shown in Figure 5)* using the same conditions. However, two strong acids, salicylic acid (pKa=2.9) and pentafluorobenzoic acid (pKa = 1.48) were included in the test mixture contained in *Figures 2 and* 3 and did not elute on either Ethyl Pyridine or Basic columns. The pKa for these two acids was presumably too low for these basic stationary phases.

Table 2. A List of Some the Stationary Phases Commercialised Specifically for SFC

	Product	Functional Group
ES Industries	GreenSep™ DEAP	Diethylaminopropyl
ES Industries	GreenSep™ Basic	Imidazole based
ES Industries	GreenSep™ Ethyl Pyridine	2-ethyl pyridine
ES Industries	GreenSep™ 4-Ethyl Pyridine	4-ethyl pyridine
ES Industries	GreenSep™ Naphthyl	Naphthalene
ES Industries	GreenSep™ Nitro	Nitroaromatic
ES Industries	GreenSep™ Pyridyl Amide	Pyridyl amide
Princeton Chromatography	PA	Propylacetamide
Princeton Chromatography	PPU	Propylpyridyl urea
Princeton Chromatography	Benzamide	Benzamide
Princeton Chromatography	BeSAM	Benzene Sulphonamide
Princeton Chromatography	4-ethylpyridine	4-ethyl pyridine
Princeton Chromatography	MeSAM	Methane Sulphonamide
Waters Corporation	Torus™ 2-PIC	2-picolylamine
Waters Corporation	Torus™ DEA	Diethylamine
Waters Corporation	Torus™ 1-AA	1-Aminoanthracene

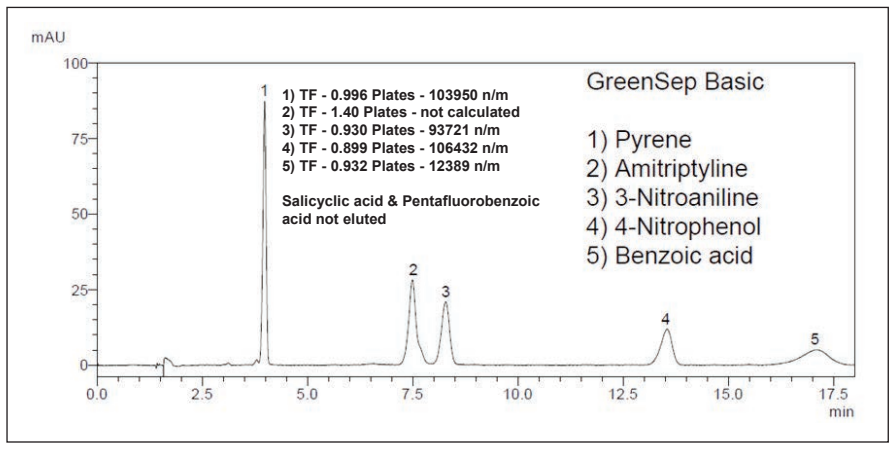

Figure 2. SFC Test Mixture Chromatographed on GreenSep™ Basic. Operating conditions: column dimensions: 250 x 4.6mmID, 5um particles, Total Flow: 3 mL/min, Mobile Phase: 0.3 mL/min methanol and 2.7 mL/min CO_2, Back pressure: 150 bar, Column Temperature: 40ºC, Detection: UV@254nm

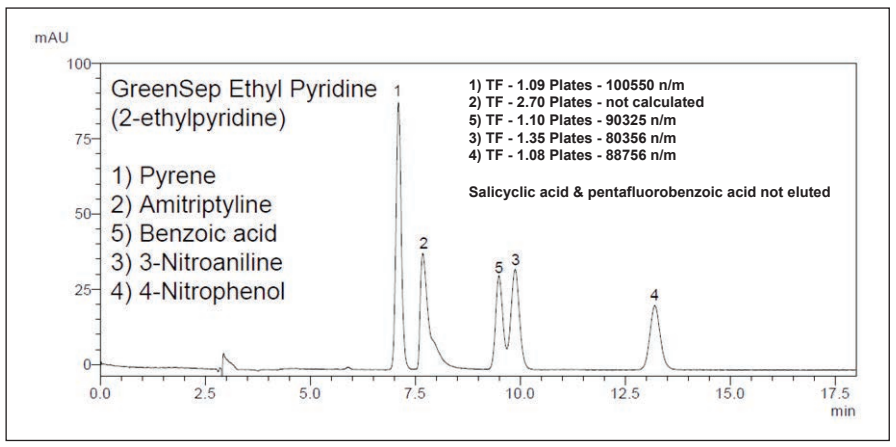

Figure 3. SFC Test Mixture Chromatographed on GreenSep™ Ethyl Pyridine. Operating conditions: column dimensions: 250 x 4.6mmID, 5um particles, Total Flow: 3 mL/min, Mobile Phase: 0.3 mL/min methanol and 2.7 mL/min CO_2, Back pressure: 150 bar, Column Temperature: 40ºC, Detection: UV@254nm

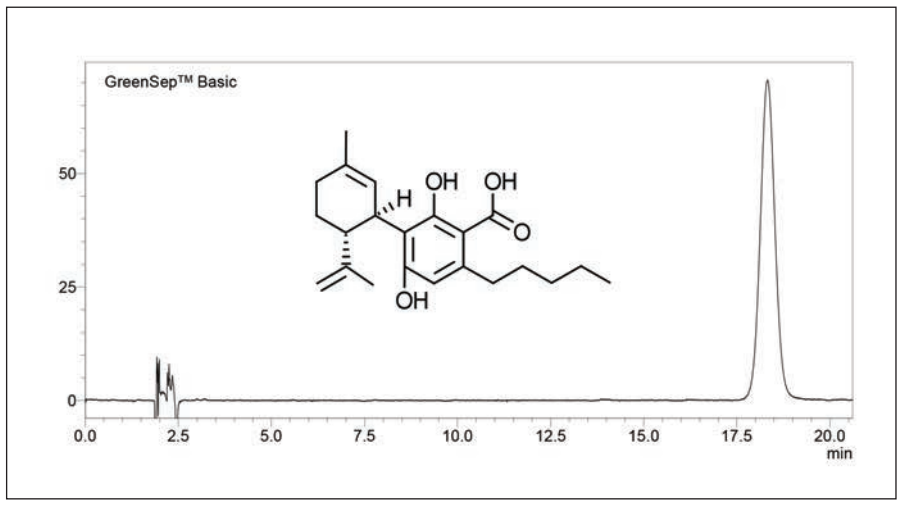

Figure 4. Cannabidiolic acid (CBDA) Chromatographed on GreenSep™ Basic. Operating conditions: column dimensions: 250 x 4.6mmID, 5um particles, Total Flow: 3 mL/min, Mobile Phase: 0.3 mL/min methanol and 2.7 mL/min CO_2, Back pressure: 150 bar, Column Temperature: 40ºC, Detection: UV@254nm

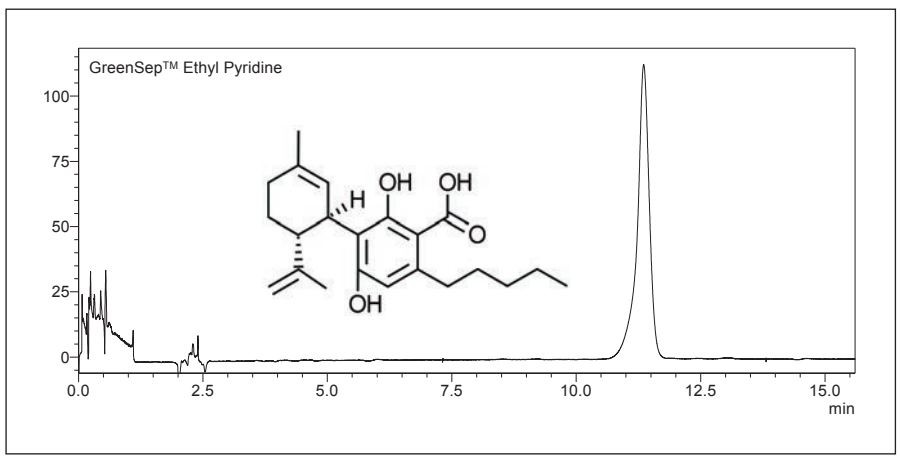

Figure 5. Cannabidiolic acid (CBDA) Chromatographed on GreenSep™ Ethyl pyridine. Operating conditions: column dimensions: 250 x 4.6mmID, 5um particles, Total Flow: 3 mL/min, Mobile Phase: 0.3 mL/min methanol and 2.7 mL/min CO_2, Back pressure: 150 bar, Column Temperature: 40ºC, Detection: UV@254nm

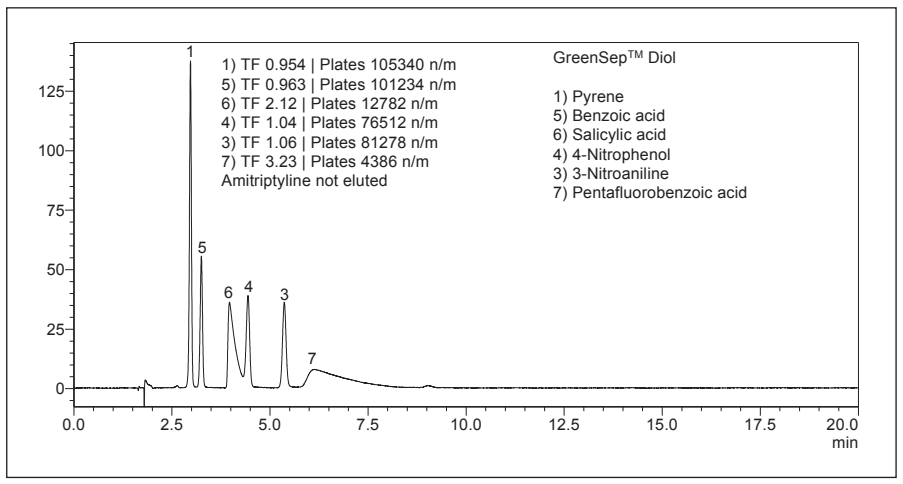

Figure 6. SFC Test Mixture Chromatographed on GreenSep™ Diol. Operating conditions: column dimensions: 250 x 4.6mmID, 5um particles, Total Flow: 3 mL/min, Mobile Phase: 0.3 mL/min methanol and 2.7 mL/min CO_2, Back pressure: 150 bar, Column Temperature: 40ºC, Detection: UV@254nm

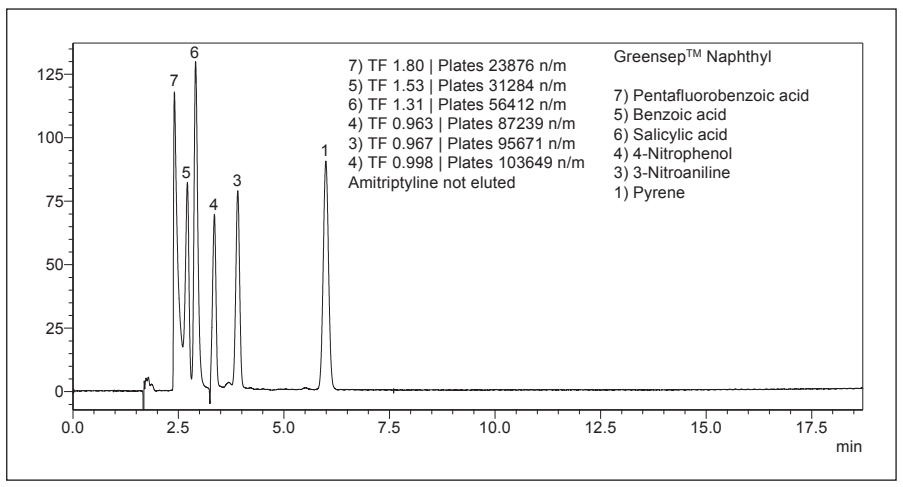

Figure 7. SFC Test Mixture Chromatographed on GreenSep™ Naphthyl. Operating conditions: column dimensions: 250 x 4.6mmID, 5um particles, Total Flow: 3 mL/min, Mobile Phase: 0.3 mL/min methanol and 2.7 mL/min CO_2, Back pressure: 150 bar, Column Temperature: 40ºC, Detection: UV@254nm

SFC Method Development Column Kit

The number of different stationary phases contained in *Table 1 and 2*, along with polar phases (silica, diol, cyano and amino) borrowed from HPLC, provide a considerable challenge for column selection in SFC method development. In order to aid the selection process, we have developed a six-column method development kit. This kit would hopefully provide guidance for both analytical as well as preparative column selection for a particular method development separation or purification. Preferably, the method development kit would utilise columns that would separate a wide variety of chemical polarities whilst maintaining acceptable peak shape without mobile phase additives. However, if necessary the columns contained in the kit are compatible with commonly used SFC mobile phase additives.

Defining a concise method development column kit for SFC is a difficult task given the aforementioned wide variety of SFC stationary phases available and the complexity of mixtures requiring separation. Firstly, the columns should be manufactured using robust support materials, refined chemical bonding procedures, represent stable bonded phases and high performance column packing technology. Secondly, the columns should be engineered to endure the high pressure regime of both analytical and preparative SFC. Finally, any stationary phase chemistry identified for the method development kit must be scalable to larger column formats and different particle sizes for any potential preparative application. Unfortunately, some column manufacturers have introduced SFC optimised stationary phases that are based on chemistries that are not easily or cost effectively scalable for preparative columns dimension. The phases reviewed (GreenSep™, ES Industries, NJ) have all been commercially developed and optimised for SFC and are completely scalable from analytical formats through all sizes of preparative columns.

The method development kit was developed using scientific approach based upon three published articles [10, 34, 35]. These articles have helped to define and quantify how analytes interact with various stationary phases in SFC separations. Each one of the referenced studies informed and has directly influenced the columns selected for the kit. The referenced studies have to a large degree utilised various chemometric based approaches to analyse and postulate how different stationary phases interact with analytes in an SFC regime. West and Lesellier have published several papers [36-39] to characterise available types of stationary phases and their potential use for particular SFC separations. In these papers, they compare stationary phases using a quantitative structure-retention relationship (QSRR) based on the linear solvation energy relationship (LSER) that uses Abraham's parameters as the solvation parameter model. In other words, the retention factor (k) of a selected set of probes is experimentally determined using a set of careful chosen operating conditions [23]. The log of the experimentally measured retention factor (k) is then related to specific interactions by the following equation:

$$Log\ k = c + eE + sS + aA + bB + vV$$

Log k = the log of the measured retention factor
c = the intercept term of the model, which is this case is dominated by the phase ratio
E = excess molar fraction as calculated from the refractive index and is related to polarisability contributions from n and π electrons
S = solute dipolarity/polarisability
A & B = solute overall hydrogen-bond acidity and basicity
V = McGowan characteristic volume ((cm3/mole)/100)
(e, s, a, b, v are the system constants of LSER Abraham's parameters calculated from the multi-linear regression analysis of the data)

The selected probes included polar molecules that are hydrogen bond acceptors such as pyridine and caffeine which possess strong hydrogen-bond basicity. It also included polar molecules that are hydrogen bond donors such as the phenols which possess strong hydrogen-bond acidity. They used a total of 109 test probes from their study [22] and acquired data from a large number of commercially available columns including classic HPLC stationary phases such as ODS (Octadecylsilane), PFP (Pentafluorophenyl) and Diol as well as stationary phases specifically designed for SFC such as EP (ethyl pyridine). All 109 test probes were tested on each column and the retention factor (k) was measured. Using the measured value of k for each test probe on each column, a LSER Abraham's parameters solvation model was generated using multi-linear regression analysis.

They calculated normalised model results for each stationary phase tested and these were plotted on the five-dimensional spider diagram with the various stationary phases placed on the diagram using bubbles of varying sizes depending on the strength of the interactions from the chromatographic system. This spider diagram can be analysed in many ways including where the stationary phases are positioned between vectors lines, distant from the centre of the diagram as well as the size of the bubble. Columns clustered next to each other on the spider diagram have similar system constants and are therefore believed to be similar in chromatographic behaviour. Columns distant from each other on the spider diagram have different system constants and are therefore believed to be different in chromatographic behaviour. The desire to make the method development kit as diverse as possible, to fit a wide variety of samples, entails the selection of columns distant from each other on the spider diagram; by doing this hopefully different chromatographic behaviour would be seen from each column. Given this approach the spider diagram is very useful in building the method development kit. The analysis of the spider diagram has led to the selection of two of the six columns for the method development kit: Diol and PFP. Diol has both hydrogen-bond basicity and hydrogen-bond acidity character. *Figure 6* shows a SFC test mixture was chromatographed on Diol. On this column, acids were retained however there was considerable tailing seen with the strong acids such as salicylic and pentafluorobenzoic and the strongly basic Amitriptyline was not eluted. From the West and Lesellier studies GreenSep™ PFP showed dipolarity/polarisability and excess molar fraction which is related to polarisable π electrons.

The work of McClain and Przybyciel [10] utilised a chemometric approach based on SFC chromatography without mobile phase additives for the separation of various structural classes of compounds with a heavy focus on peak symmetry as the key response criterion. The details of the work can be found in the reference; however, it is important to understand how the basic approach of this work informs to the selection of columns for the method development screening kit.

McClain and Przybyciel used a large and structurally diverse building block library available at Merck, USA representing chemical space to obtain representative compounds in four distinct functional group classes – carboxylic acids, amines, alcohols, and amides. These four functional group classes are important reactive groups for the synthesis of larger molecules. Fifteen chemicals were selected from each functional group class for a total 60 chemical entities. The structure of these proprietary compounds, which served as test probes in the study were not disclosed, however a chromatogram and structures of commercially available amines was shown in the paper. In order to identify the 60 chemical entities, the chemical library was queried by chemoinformatic based computer program developed by Merck. This computer program can utilise various chemoinformatic techniques, but for this study, the Tanimoto dissimilarity [40] was used. The Tanimoto dissimilarity method is a chemoinformatic technique used to query a large chemical library to identify chemicals that are structurally most diverse from each other, thus yielding a molecular diversity model. The Tanimoto dissimilarity index relies on various chemical and physical parameters that are associated with the chemicals in the chemical library such as molecular mass, polar surface area, hydrogen acceptors, and hydrogen donors to name of few of the parameters. The 15 chemicals representing each of the four chemical classes were chosen to have maximal Tanimoto dissimilarity index in other words they were structural most different from each other. Therefore, it was reasoned that chemical space occupied by the Merck building block library at that time was represented by the selected test probes.

The referenced study identified four stationary phases, one for each chemical class as the 'best' from that study.

Acids - Non-endcapped Ethyl Pyridine
Alcohols – Diethyl Amino Propyl (DEAP)
Amides – Non-endcapped Nitro phenyl
Amines – Non-endcapped Basic (a bonded imidazole derived phase)
From the McClain and Przybyciel study three columns for the method development kit are selected:Ethyl Pyridine, Nitro and Basic.

The McClain and Przybyciel study provides a novel approach for selecting columns for the preparative SFC separations based on chemical functional group. Fortunately, for that study they had access to a sophisticated computer program and a large chemical library; unfortunately, the use of a propriety computer program and a large propriety chemical library limits access to many preparative SFC chromatographers. When new stationary phases are introduced or multi-functional chemical compounds need to be purified, the preparative chromatographer does not have access to this approach. However, there are computer programs available for statistical analysis and many of these commercially programs calculate Tanimoto index, Floersheim distance and various other similarity/ dissimilarity factors. It is feasible that these programs can be targeted to the analysis of chromatographic data in conjunction with open source chemical space projects [41], which may make the investigative technique of McClain and Przybyciel more approachable to the general chromatography community.

Ebinger and Weller [34] have provided insight into another pharmaceutically important separation challenge - diastereomers. No specific effort was made by West and Lesellier nor McClain and Przybyciel to specifically address the specific separation of diastereomers. Ebinger and Weller evaluated the separation of 33 synthetic research samples representing a diverse set of diastereomers mixtures against 12 different columns from various vendors. From this study they discovered that 91% of their diastereomers mixtures from their diverse set could be separated using a bonded pyrene stationary phase. They postulate that the good separation performance of the pyrene phase for diastereomer mixtures is attributable to the rigid planar pyrene ring, strong π-π and charge transfer interactions.

The commercial development of a pyrene bonded phase has been explored (ES Industries) however the phase was found to be unstable. A Naphthalene bonded phase, GreenSep™ Naphthyl, was developed which contains many of the properties of the pyrene phase including rigid planar ring, strong π-π and charge transfer interactions, but possesses more stability than the original pyrene phase. Naphthyl has been included in the method development kit as the sixth column. A chromatogram of a test mixture chromatographed on Naphthyl is shown in *Figure 7*. The retention for pyrene, a polyaromatic hydrocarbon, is the highest especially when compared to other examples shown in this report.

The stationary phases selected for the current method development kit are shown below and separations using the kit are shown in the examples that follow.

1. GreenSep™ Basic – imidazole based, best peak shape for amines
2. GreenSep™ Ethyl Pyridine – Good overall selectivity and excellent for acid mixtures
3. GreenSep™ PFP – pentafluorophenyl, unique selectivity, electron acceptor
4. GreenSep™ Nitro – nitro aromatic based, unique selectivity
5. GreenSep™ Naphthyl – naphthalene based, ridged structure, good for diastereomers separation and non-polar compounds, π-π interaction
6. GreenSep™ Diol – the selectivity of silica without reactivity of silica

Conclusion

We have presented an over view of stationary phase progression and development for SFC applications. In recent years there has been increased stationary phase development leading to numerous SFC optimised phases. These optimised phases, along with both polar and non-polar traditional HPLC phases, have given the SFC chromatographer a vast array of column choices. Various applications and different functional groups make the selection of the optimal column a daunting task. In an attempt to aid in column selection for methods development we have established a six column method development kit. The columns for the method development kit were selected based on three published studies and the important points from all three studies used to select the columns for the method development kit. All the stationary phases selected for the kit are scalable to larger column formats and different particle sizes. In addition, the selected columns are manufactured using robust support materials, refined chemical bonding procedures, are stable bonded phases and utilise high performance column packing technology. It is important not to treat the column method kit as 'fixed'; it should be adapted and changed as new stationary phases are developed, new separation challenges occur such as the separation of fluorine-containing pharmaceutical entities [42] or new chemometric approaches are introduced [43,44]. However, it is important that any new column chemistry added to the method kit should be based on robust chemical bonding technology to adequately address the needs of larger column formats and different particle sizes. The SFC as analytical and preparative chromatographic technique will continue to be rapidly adapted for many applications and will continue to lead in the area of green separation technology.

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