How to Design a Column Screening Kit for Preparative SFC Separations?

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Preparative supercritical fluid chromatography (SFC) is a powerful tool for the purification/isolation of both chiral (1-3) and achiral (4,5) compounds. Many chemicals can potentially be used as a supercritical mobile phase (6) in SFC, however virtual all current practitioners of SFC use CO_2 which offers several advantages, particularly when compared to preparative liquid chromatography. 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. In addition, it is miscible with a wide range of organic solvents, non-flammable, and has little UV absorbance at lower wavelengths. CO_2-based SFC is particularly 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.

Other advantages of SFC include the fact that diffusion coefficients of solutes in supercritical mobile phases have been shown to be 3-10 times higher than in normal liquids potentially allowing for very rapid separations through reduction of resistance to mass transfer, although it does not suffer from the solubility issues that are present with GC. The viscosity of mobile phases used in SFC are significantly lower than those used in LC producing much lower pressure drops across the column which 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.

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 (7). In order to modify the elution strength of CO_2 to allow wider use with molecules of increased polarity, organic solvent modifiers are mixed into the CO_2 stream using a second high pressure HPLC pump. Commonly used modifier solvents are methanol or ethanol, but other organic solvents and solvent mixtures are also used. Because of the non-polar behaviour of CO_2 the stationary phase plays a very key role in SFC separations. In addition, many SFC separations require the use of additives such triethylamine in order to diminish peak tailing and maintain acceptable retention factors, particularly when separating amines. Additives are difficult to remove and potentially alter the chemical properties for compounds being purified and isolated using SFC. As a result of these concerns, the use of additives is discouraged when the purifying and isolating compounds using SFC. In recent years SFC optimised stationary phases have been developed (8) to avoid the use of mobile phase additives while delivered desired chromatographic performance of ionisable compounds.

Chromatographers require a degree of flexibility when dealing with difficult to separate mixtures and thus they require a large variety of SFC stationary phases to cover the wide range of molecules encountered in the laboratory. Unfortunately, the large variety of stationary phases greatly complicates the column selection process for any particular separation. The large number of stationary phases available for SFC is the result of several contributing 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, some of the stationary phases used in early practice of SFC are historical and predate the development of SFC optimised stationary phases. These phases were derived from normal phase liquid chromatography and include phases such as cyano, diol, silica and amino. The selection of the best SFC column for a given separation can be a daunting task and it is the focus of this article to design a set of screening columns that will aid the selection of the best preparative SFC column for a given separation.

The Column Screening Kit

Generally, most column screening systems use a set of analytical size columns (column diameters from 2 - 4.6 mm ID and lengths from 100 – 250 mm) to identify the best stationary phase for a particular preparative separation. In addition, most of these screening systems use an automatic switching valve to screen up to six columns. It would be, therefore desirable to limit a column screening kit to six columns. Ideally, one of the six columns in the screening kit would deliver the desired chromatographic performance and could be geometrically scaled up to perform the preparative separation. Preferably, the screening kit would utilise columns that would separate a wide variety of chemical polarities whilst maintaining acceptable peak shape without mobile phase additives being required.

Basic Guidelines for the Column Screening Kit

Defining a column screening kit for SFC is a difficult task given the aforementioned wide variety of SFC stationary phases available and the complexity of mixtures requiring separation. In addition, the screening kit will be used to guide preparative separations and as such it needs to be receptive to the requirements of preparative SFC chromatography. Firstly, the columns should be commercially available and 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 screening kit must be scalable to larger column formats and different particle sizes. There have been SFC stationary phase optimised for analytical column dimensions and unfortunately can't be easily scaled to accommodate larger column formats in an economical fashion. 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. GreenSep™ versions of the historical phases such as diol, cyano and amino are also available.

The Approach to Screening Kit Design

The scientific approach that was developed to build the screening kit is based upon three published articles (9 - 11). 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.

Designing the Screening Kit Study 1

West and Lesellier have published several papers (9, 12-18) to characterise available types of stationary phases and their potential use for a particular SFC separation. 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 (9). The log of the experimentally measured retention factor (k) is then related to specific interactions by the following equation:

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

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 ($(cm^3/mole)/100$)
(e, s, a, b, v are the system constants of LSER Abraham's parameters calculated from the multi-linear regression analysis of the data)	

Table 1 is a representative subset of the chemical probes used in West and Lesellier studies and is included to explain how the terms of the LSER model relate to actual molecules (the probes).

From Table 1 benzene, toluene and ethyl benzene, are small neutral non polycyclic aromatic hydrocarbons that have very low hydrogen bond acidity or basicity, weak polarisable contributions from n and ϖ electrons, weak solute dipolarity/polarisability and a small McGowan characteristic volume (the molecular volume of one mole of a compound when the molecules are stationary divided by 100, basically, the molecular space occupied by one molecule, it is calculated from McGowan's work (19)). While on the other hand, both pyrene and perylene are larger neutral polycyclic aromatic hydrocarbons that have very low hydrogen bond acidity or basicity, strong polarisable contributions from n and ϖ electrons, strong solute dipolarity/polarisability and a large McGowan characteristic volume. Polar molecules that are hydrogen bond acceptors such as pyridine and caffeine have strong hydrogen-bond basicity. Pyridine is also a small molecule similar to benzene with weak polarisable contributions from n and ϖ electrons and a small McGowan characteristic volume. Polar molecules that are hydrogen bond donor such as the phenols have strong hydrogen-bond acidity. They used a total of 109 test probes from their study (9) 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.

Table 1: Subset of Chromatographic solutes and LSER descriptors used by West and Lesellier (8)

Compound	E	S	A	B	V
Benzene	0.610	0.52	0.00	0.14	0.7164
Toluene	0.601	0.52	0.00	0.14	0.8573
Ethylbenzene	0.613	0.51	0.00	0.15	0.9982
Pyridine	0.631	0.84	0.00	0.52	0.6753
Caffeine	1.500	1.60	0.00	1.35	1.3630
Phenol	0.805	0.89	0.60	0.30	0.7751
Coumarine	1.060	1.79	0.00	0.46	1.0620
Resorcinol	0.980	1.00	1.10	0.58	0.8340
Phloroglucinol	1.355	1.12	1.40	0.82	0.8925
o-Chlorophenol	0.853	0.88	0.32	0.31	0.8975
m-Chlorophenol	0.909	1.06	0.69	0.15	0.8975
p-Chlorophenol	0.915	1.08	0.67	0.20	0.8975
o-Nitrophenol	1.045	1.05	0.05	0.37	0.9490
m-Nitrophenol	1.050	1.57	0.79	0.23	0.9490
p-Nitrophenol	1.070	1.72	0.82	0.26	0.9490
Pyrene	2.808	1.71	0.00	0.29	1.5850
Perylene	3.256	1.76	0.00	0.44	1.9536

West and Lesellier have displayed and plotted the LSER generated results in various forms including a five-dimensional Spider diagram (18). They define the vectors of the Spider diagram as the system constants obtained from LSER Abraham's parameters e, s, a, b and v. The calculated normalised model results for each stationary phase tested 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 screening 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 column screen kit. The analysis of the Spider diagram has led to select two of the six columns for the screening kit, GreenSep™ Diol and GreenSep™ PFP. GreenSep™ Diol is between the hydrogen-bond basicity and hydrogen-bond acidity vectors and is not close to the diagram's centre. GreenSep™ PFP is between the solute dipolarity/polarisability and excess molar fraction which is related to polarisable ϖ electrons vectors and is not close to the diagram's centre. We believe that columns associated with the McGowan's volume vector would not be suitable for the screening kit and therefore columns surrounding this region were not selected for the screening kit.

The articles published by West and Lesellier related to stationary phase behaviour in SFC represent immense effort, dedication to the subject, are extremely through and utilise a sound scientific approach. However, to build a column screening kit for the preparative chromatographer based solely on their very well informed efforts would be to some extent deficient. The QSRR approach doesn't emphasise Gaussian elution nor does it comment on the symmetry of the eluted analytes peaks. The ideal symmetry or Gaussian behaviour of the resulting chromatographic peaks is of key importance to preparative SFC separations where the use of mobile additives is particular discouraged.

Designing the Screening Kit Study 2

The column screening kit that is being defined is specifically targeted to the SFC preparative chromatography community and as such the selected columns should produce ideal peak symmetries without the use of mobile phase additives. As a result of this stipulation a second published approach has been selected, the work of McClain and Przybyciel (10), a chemometric approach based on SFC chromatography without mobile phase additives for the separation of structural classes of compounds with a focus on peak symmetry as the key response criteria. 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 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, for the study the Tanimoto dissimilarity (20) was used. The Tanimoto dissimilarity method is a chemoinformatic technique used to query a large chemical library and in this case Tanimoto dissimilarity index was used 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 GreenSep™ Basic (a bonded imidazole derived phase)

From the McClain and Przybyciel study three columns for the screening kit are selected GreenSep™ Ethyl Pyridine II, GreenSep™ Nitro and GreenSep™ 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; maybe these programs can be targeted to the analysis of chromatographic data in conjunction with open source chemical space projects (21), which may make the investigative technique of McClain and Przybyciel more approachable to the general chromatography community.

Designing the Screening Kit Study 3

The published work of Ebinger and Weller (11) provides insight into another pharmaceutically important separation challenge – diastereomers. No specific effort was made in either 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, however it is more stable. GreenSep™ Naphthyl has been included in the screening kit as the sixth column.

Separation Examples Using the Column Screening Kit

The stationary phases selected for the current screening kit are shown in Table 2 and separations using the screening kit are shown in the examples that follow.

Table 2: The Six Columns Selected for the SFC Screening Kit

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™ – pentafluoro phenyl, unique selectivity, electron acceptor
4. GreenSep Nitro™ – nitro aromatic based, unique selectivity
5. GreenSep Napthyl™ – 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

These examples were chromatographed using the newly introduced Shimadzu Nexera UC SFC system. The Shimadzu Nexera UC system had a fixed wavelength UV detector and a 5 µL fixed injection loop. The operating conditions for the examples are contained in the figures.

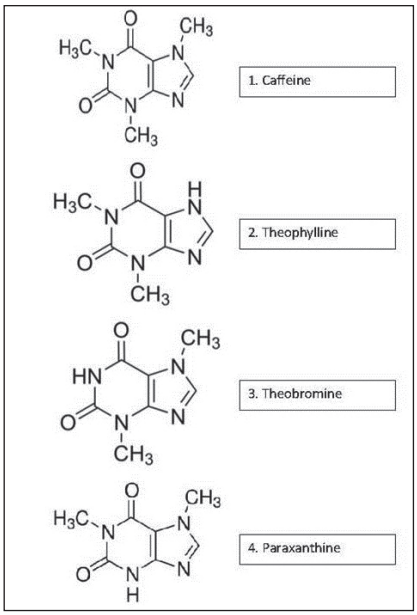

Figure 1: Caffeine Analogue Mixture Structures

The first example is a comparative example to illustrate the chromatographic selectivity of the screening kit for the separation of closely related caffeine analogues (Figure 1).

These analogues are structurally similar heterocyclic xanthine bases and are differentiated by methyl group number and placement. The caffeine mixture was chromatographed on all six screening kit columns using a simple mobile phase consisting of an isocratic mixture of methanol and carbon dioxide with no mobile additive. The only column in the screening kit that completely baseline separated the caffeine mixture was GreenSep™ Basic, the chromatogram shown in Figure 2. The next best column for this separation was GreenSep™ Ethyl pyridine, the chromatogram shown in Figure 3. GreenSep™ Ethyl pyridine did separate the mixture but not a baseline separation.

All other columns in the screening kit failed to separate the caffeine mixture. We also analysed the caffeine mixture on GreenSep™ Silica (bare silica), the chromatogram is shown in Figure 4. Bare silica has been widely used as a stationary phase for SFC (5), but was not included in our screening kit because it exhibits severe tailing for amines without mobile phase additives. GreenSep™ Silica did not completely separate the caffeine mixture (caffeine and theophylline were completely eluted).

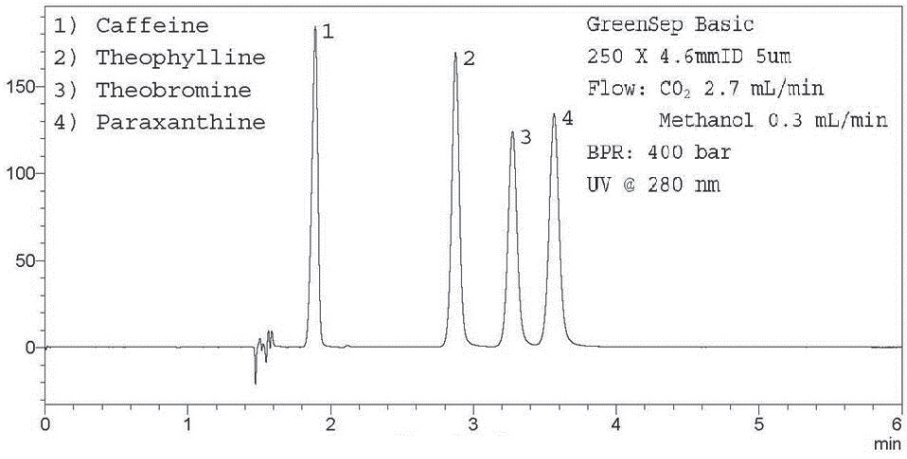

Figure 2: Separation of Caffeine Analogue Mixture on GreenSep™ Basic

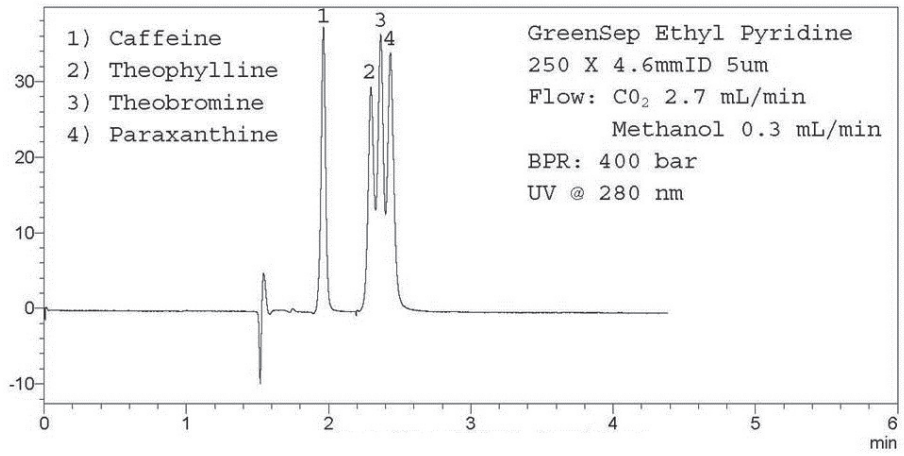

Figure 3: Separation of Caffeine Analogue Mixture on GreenSep™ Ethyl Pyridine

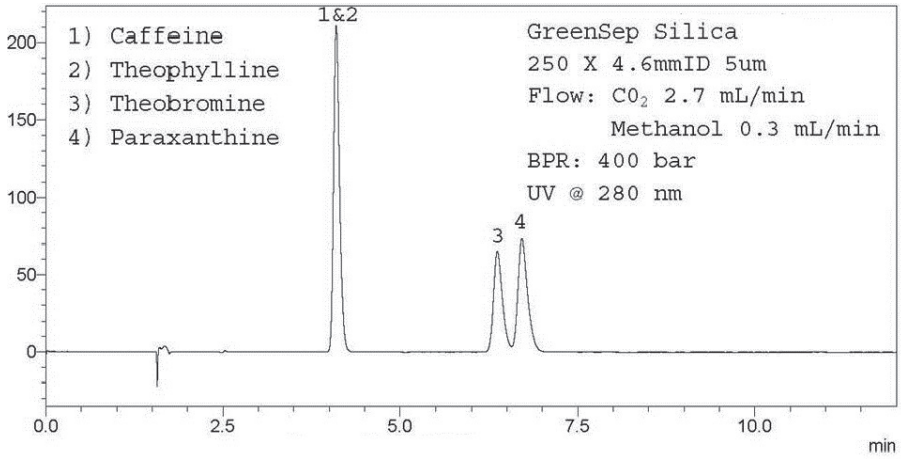

Figure 4: Separation of Caffeine Analogue Mixture on GreenSep™ Silica

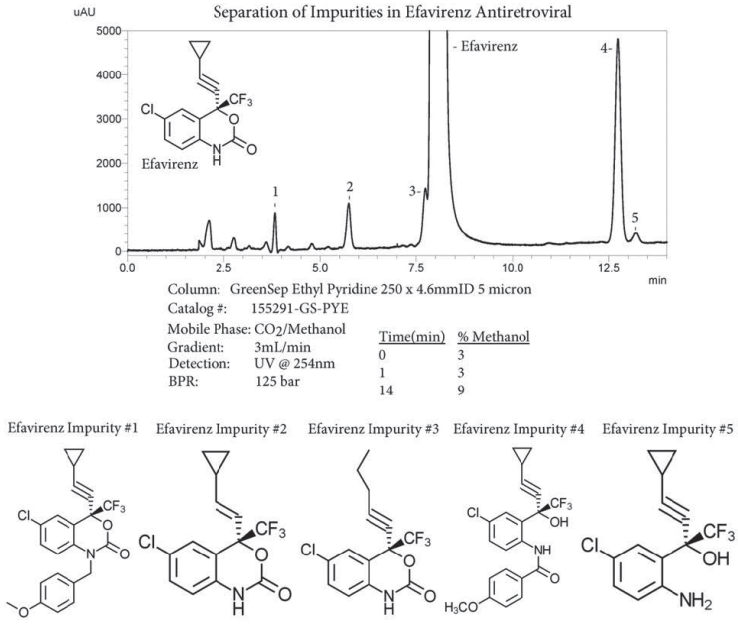

Figure 5: Separation of Efavirenz antiretroviral and its impurities on GreenSep™ Ethyl Pyridine

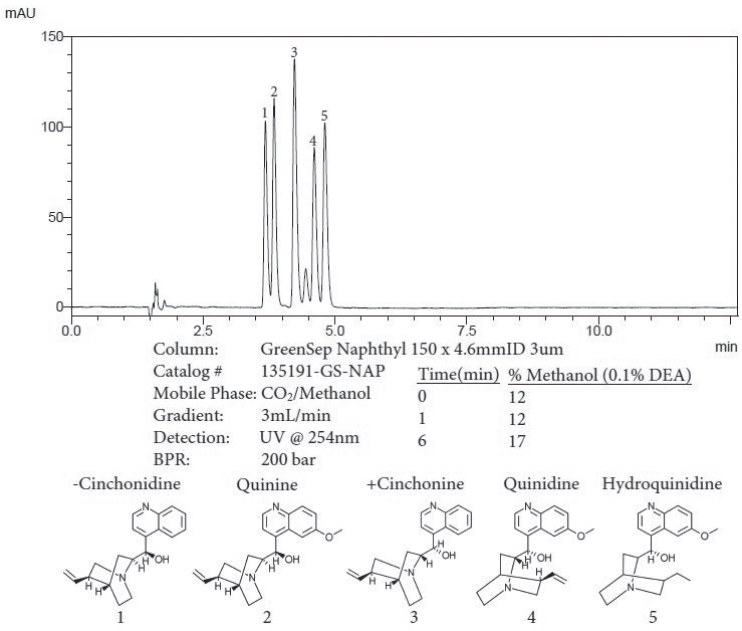

Figure 6: Quinine mixture related compounds on the GreenSep™ Naphthyl.

The next example (Figure 5) is the chromatographic separation of Efavirenz antiretroviral and its impurities on the GreenSep™ Basic. GreenSep™ Basic is inherently deactivated (10) and as such no mobile phase additive was required even though Efavirenz impurity #5 is an aromatic amine. The mobile phase for this separation was just carbon dioxide and methanol.

The final application example is the chromatographic separation quinine mixture related compounds on the GreenSep™ Naphthyl (Figure 6). The quinine mixture is essentially a diastereomeric mixture and shows the separation capabilities of a naphthalene bonded phase. However, this phase is not inherently deactivated like the GreenSep™ Basic or GreenSep™ Ethyl Pyridine. The quinine and its related compounds contain amino groups as well as a base heterocyclic aromatic quinoline structure making them rather basic and so an additive (DEA) was needed improve the peak shape in this case. It should be noted, that the separation of diastereomers can be extremely challenging and in some cases will require the use of additives. In this regard we are investigating inherently deactivated stationary phases for the improved separation of diastereomers without additives. If such a stationary phase is developed it will replace GreenSep™ Naphthyl in the screening kit.

Conclusion

A six column screening kit for SFC has been developed (Table 2), with particular attention being paid to the preparative SFC chromatographer. The columns for the screening kit were selected based on three published studies and the important points from all three studies used to select the columns for the screening 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 screening 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 (22) or new chemometric approaches are introduced (23). However, it is important that any new columns for the screening kit must be scalable to larger column formats and different particle sizes and meet the robustness requirements of the current screening kit.

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