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Impact of injection solvents on supercritical fluid chromatography.

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Abstract

Even though there has been a rapid development in instrumentation and applications of supercritical fluid chromatography (SFC), relatively little is known about retention mechanisms compared to high-performance liquid chromatography (HPLC). Much effort has been made to characterize the influence of injection solvents on chromatographic efficiency in HPLC, however has been left rather uninvestigated in the domain of SFC. In this study properties of different injection solvents have been studied and correlated with properties of seven various analytes on three different columns, a C18, a 2-ethylpyridine and a bare-silica column. Aided by calculations of correlation coefficients and principal component analysis (PCA), the physical properties of injection solvents and the interactions between injection solvent, solute and stationary phase were investigated. The findings of this work shows that interactions capable of masking accessible silanol groups on a C18 column are of importance in order to maximize the plate number. While solvents with dipolar and hydrogen bond interaction properties are associated negatively with chromatographic efficiency using polar columns. Properties such as molar density, vapor pressure and boiling point were related to sharper peaks, mostly likely because of solubility issues of the injection solvent into the methanol-modified carbon dioxide. However, no additional solubility due to hydrogen interactions between the injection solvent and the carbon dioxide in SFC was observed. Surface tension and viscosity was not particularly associated with a decrease in plate numbers. By increasing the injection volume a stronger correlation between solubility related properties and plate numbers were obtained. Additional experiments showed that the resistance in solubility became an issue when performing partial-loop injection where additional washing solvent entered the system, thus providing broadened peaks.

1. Introduction

It has been approximately 60 years since carbon dioxide (CO₂) was proposed as a chromatographic eluent, which is today established as supercritical fluid chromatography (SFC). However, much of the instrumental development has actually taken place in recent years owing its rediscovery to the versatility in chiral separations. Nowadays SFC is commonly used at subcritical conditions due to relatively high concentrations of eluent modifiers. Yet the benefits of low viscosities and high diffusivities are maintained providing low pressure drops over the column and efficient separations. The low viscosity also enables the use of several coupled columns as well as high flow rates leading to short analysis times [1].

Thus SFC is becoming a very popular technique for separating particularly chiral analytes but also for achiral separations. Both stationary phases for reversed-phase chromatography (RPLC) and stationary phases for normal-phase chromatography (NPLC) are compatible with modified CO₂ as mobile phase. This enables a wide range of solutes to be separated, also strengthened by option of using real orthogonal column selectivity. Actually, only very polar analytes *i.e.* inorganic ions and proteins are not suitable for SFC [2].

Although the regained popularity of SFC, much of the fundamental understanding remains unfamiliar due to the complexity of the gas expanded liquid. Retention mechanisms are however quite similar to the ones of both RPLC and NPLC [2]. Thus SFC is generally seen as quite closely related to high-performance liquid chromatography (HPLC).

In traditional HPLC it is well known that the choice on injection solvent will affect the efficiency. It is generally recommended that samples should be prepared and diluted in the same solvent used as mobile phase, or even preferably in a solvent of weaker elution strength that is still miscible with the mobile phase. Using a solvent with higher elution strength may cause shifts in retention times and peak broadening. A strong injection solvent will hinder the sample from interacting with the stationary phase as the injection plug will enter column, before getting diluted by the mobile phase. Contrarily, using a weaker injection solvent in liquid chromatography will result in peak focusing, thus decreasing detection limits and improving chromatographic efficiency [3-5]. In liquid chromatography, injection solvents with different viscosity than the mobile phase will cause early eluting peaks to distort due to injection solvents with higher viscosity will less readily dissolved into the mobile phase, while too low viscosity will cause an unstable injection plug front thus also leading to broadened peaks [6].

Transferring this knowledge to SFC is not trivial, due to the fact that predicting solubility is not straightforward. The eluent strength is dependent on density, temperature, pressure and the use of co-solvents, usually methanol, ethanol, iso-propanol or acetonitrile [7]. These variables are not independent of each other and therefore difficult to determine and comprehend. Some initial work has been performed previously on the subject but deeper understanding is needed. Smith and Briggs [8] reported that injected methanol affected peak shape by adsorbing to silanol groups on a cyano-bonded column and also affect subsequent injections until enough CO₂ passed through to wash off the methanol from the stationary phase. However, the authors also showed that this was only the case if less than 0.5% methanol as co-solvent was used in the chromatography. However today SFC is generally used with more than 5% modifier, whilst 2% is enough to mask very polar groups in the stationary phase [2].

Another study investigated the impact of injection volume using methanol on a preparative scale SFC system using a 2-ethylpyridine column (2-EP). The authors stated that the locally increased elution strength based on polarity, which is induced by the injection plug of methanol causes distortion of the peaks and more so at higher injection volumes. However the same authors also stated that the polarities of different injection solvent mixtures were found to have minor impact on the plate number (N) [9].

The aim of this paper was to investigate if the choice of injection solvent has an impact on the chromatographic efficiency, also depending on column choice. Furthermore, by exploratory analysis examine which solvent properties affect peak shape in SFC. A wide range of solvents with different physical properties *i.e.* boiling point and surface tension including molecular interactions through *e.g.* dipole moment and hydrogen interactions, were employed to study their effects on the chromatography. A selection of analytes based on their different molecular characteristics such as hydrophobicity and capabilities of hydrogen interactions, were evaluated on a 2-EP, bare silica or a

C18 column in order to further distinguish important interactions and properties that may affect the chromatography in SFC. In addition the impact of injection solvent on the separation of a more complex sample containing carotenoids, which was separated on two coupled columns, was also studied.

As of our knowledge no attempts have been made to correlate injection solvent properties other than apparent polarity to the chromatographic efficiency in SFC.

2. Materials and methods

2.1. Chemicals

Ethanol (99.7%, Solveco, Rosenberg, Sweden) was used as a co-solvent in SFE. Methanol of HPLC-grade (>99.9%, Honeywell Burdick & Jackson, Seezle, Germany) was used as a co-solvent in SFC. Ultrapure CO₂ was provided by Air Products (Amsterdam, Netherlands) and used for both SFE and SFC.

The dissolution solvents were of analytical grade or higher; 2-propanol, acetone, acetonitrile, heptane (Honeywell Burdick & Jackson, Seezle, Germany), chloroform, methyl tert-butyl ether, toluene (Merck, Darmstadt, Germany), cyclohexane (Acros organics, Pittsburg, PA), cyclopentane, diethyl ether, hexane, pentane, (Fluka, Buchs, Switzerland), dichloromethane, dimethyl sulfoxide and tetrahydrofuran (Fischer Scientific, Pittsburg, PA).

A standard mixture was prepared by dissolving 300 mg L⁻¹ of diclofenac sodium salt, naproxen, fluoranthene, progesterone, sulfanilamide (Sigma-Aldrich, St Louis, MO), caffeine and uracil (Merck, Darmstadt, Germany) in hot ethanol. The solution was subsequently diluted 10 times in each of the 17 solvents, generating 17 solutions containing 30 mg L⁻¹ of each analyte.

SFE extract of *Scenedesmus* sp. was obtained using CO₂ (CO₂ density, 830 g L⁻¹) with 10% ethanol as a co-solvent, at a pressure of 300 bar, a temperature of 60 °C, a flow of 2 mL min⁻¹, and extraction time was 60 minutes, as have previously been described [10]. The extract was diluted 10 times in each of the solvents except in acetone, dichloromethane, chloroform and dimethyl sulfoxide.

2.2. Instrumental and chromatographic conditions

A Thar Investigator semi-preparative SFC (Pittsburgh, PA) was used for separating the analytes of interest, consisting of a cooled fluid delivery module with a 6 co-solvent switching valve, a modified Spark Holland Alias autosampler with a 48-vial plate, an analytical-2-prep oven with a 10 column switching valve, an automated backpressure regulator and a Waters 2998 photodiode array detector (Milford, MA). The fluid delivery module was cooled by a Neslab RTE7 cooling bath controlled by a Digital One thermoregulator. ChromScope (version 1.10, Waters) was used to control the instrument and subsequently analyze the chromatograms.

Three different types of columns were used in the experiments, a SunFire C18 (4.6 x 250 mm, 5 µm particle size, 100 Å pore size, Waters), a Viridis SFC silica 2-ethylpyridine (4.6 x 250 mm, 5 µm particle size, 100 Å pore size, Waters) and a Viridis SFC silica (4.6 x 150 mm, 5 µm particle size, 100 Å pore size, Waters).

Chromatographic conditions were kept constant for each column when separating the mixture of standards. The flow rate was consistently 5 mL min⁻¹ throughout all of the experiments. Methanol was used as modifier to the liquid CO₂. Separation using the C18 column was carried out with 5% modifier, backpressure was 100 bar, temperature was 40 °C and the time of analysis was 3 minutes. Separation using the 2-EP column was carried out with 13% modifier, backpressure was 120 bar, temperature was 40 °C and the time of analysis was 8 minutes. Separation using the silica column was carried out with 10% modifier, backpressure was 140 bar, temperature was 40 °C and the time of analysis was 5 minutes.

The chromatographic analysis of the SFE extract of microalgae containing carotenoids was performed as has been previously described, but with the modification that injection volume was 10 µL instead of 50 µL [10]. The carotenoids were separated on the C18 column coupled in front of the 2-EP column. The SFC method consisted of a gradient starting with 10% methanol increasing to 17% over 8 min subsequently increasing to 25% over 2 min which was then kept for 5 min. The backpressure was 120 bar, the temperature was 32 °C, the flow rate was 5 mL min⁻¹.

2.3. Data analysis

All statistical data processing was carried out using MATLAB R2012b including the statistical toolbox (MathWorks Inc., Natick, MA, USA). Principal component analysis was performed using data standardized by variance.

3. Results and discussion

3.1. Effects of injection solvent

The standard mixtures containing the seven different analytes (**Fig. 1**) diluted in 16 different solvents were injected on three different columns. The elution order of each analyte on each of the columns is presented in **Table 1**. It can also be noticed that the elution order of the analytes using a C18 column is relational to their log P values as would be expected (**Table 2**).

Table 1. Elution order of the analytes using various columns.

Column	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7
2-EP	Caffeine	Progesterone	Fluoranthene	Uracil	Naproxen	Diclofenac	Sulfanilamide
Silica	Fluoranthene	Naproxen, Progesteron, Diclofenac	Caffeine	Uracil	Sulfanilamide		
C18	Uracil, Caffeine, Sulfanilamide	Naproxen	Progesterone	Diclofenac	Fluoranthene		
2-EP + C18	Violaxanthin	Lutein	Beta	Neoxanthin			

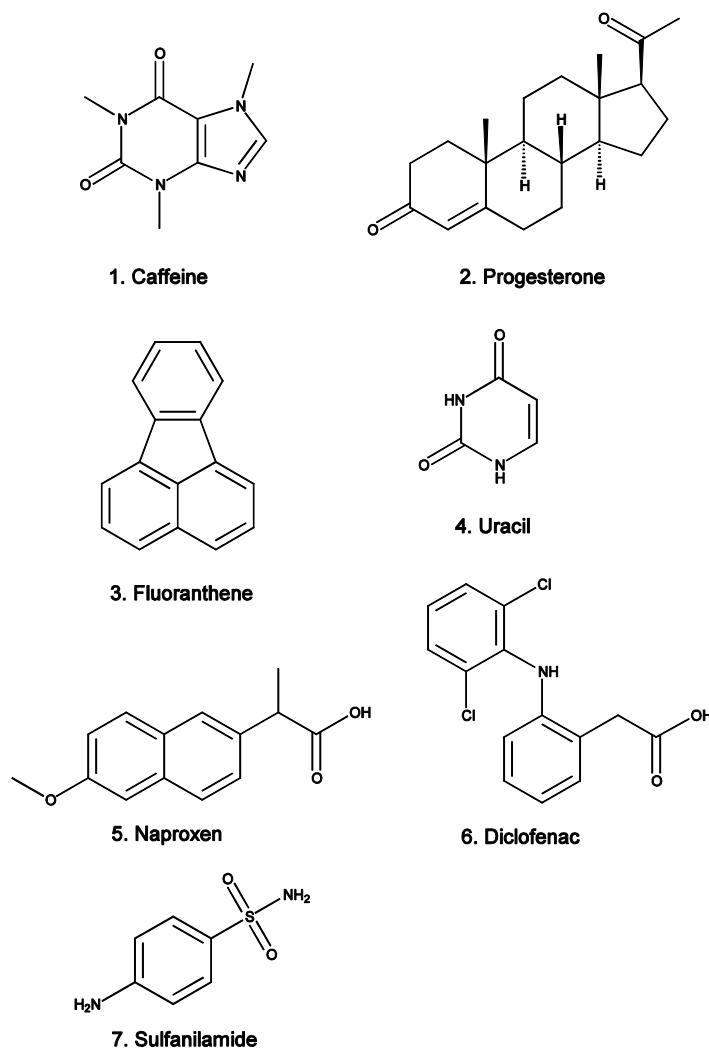


Fig. 1. Analytes injected into the SFC system using various columns. The analytes are presented in the elution order using a 2-EP column.

Table 2. Properties of the solutes used in the mixture of standards. Data obtained through SciFinder (ACS, Columbus, OH).

	Hydrogen acceptors	Hydrogen Donors	Log P	Polar Surface Area (Å ²)	Detection wavelength (nm)
Uracil	4	2	-1.037	58.2	255
Caffeine	6	0	-0.628	58.4	272
Naproxen	3	1	2.876	46.5	260
Diclofenac	3	2	4.548	49.3	275
Sulfanilamide	4	4	-0.667	94.6	257
Progesterone	2	0	3.827	34.1	240
Fluoranthene	0	0	5.004	0	284

Retention times of each analyte were consistently constant while using the same column, however the peak shape and thus the plate number varied depending on choice of injection solvent.

Generally speaking, the non-polar solvents *i.e.* pentane and hexane gave rise to higher chromatographic efficiency in terms of plate number for analytes injected on the 2-EP column, while solvents like methanol and acetonitrile provided the worst peak shapes. The opposite effect was observed using the C18 column. Interestingly on the bare-silica column the highest plate number was observed with 2-propanol and THF for fluoranthene and caffeine, respectively, while contrarily for uracil and sulfanilamide the non-polar solvents provided better peak shapes.

Principal component analysis (PCA) was used to investigate the influence on chromatographic efficiency due to difference in physical properties of solvents (**Table 3**). The chosen properties of interest were boiling point, density, vapor pressure, viscosity, surface tension, eluent strength, dielectric constant, dipole moment, hydrogen donating and accepting capabilities. The eluent strength (ϵ°) in this work is referred to the measure of solvents adsorption energy to bare-silica, using n-pentane as reference value ($\epsilon^\circ=0$) [11].

A PCA was chosen due to the multivariate nature of the data such as properties being highly correlated with each other. The PCA is a standard tool within chemometrics used to reduce the dimensionality to a few principle components (PC) yet minimizing the loss of information. The two axes, the PC are generated to take into account as much of the variability within the original dataset as possible. Thus complex datasets with many variables can be evaluated in a two dimensional manner, usually as a bi-plot containing both loadings and scores. The loadings, principally speaking, measure the importance of each variable in relation to the PC. Therefore, it is possible compare groups of variables in terms of PC loadings. The scores are derived from the individual observations in relation to the PCs. The scores can also be used for comparing groups of observations and in relation to the loadings and thus the studied variables. The interested reader is referred to the review of Bold for a more thorough explanation of PCA and its applications [12].

Among the 17 evaluated solvents, dimethyl sulfoxide (DMSO) was excluded from all statistical analysis due to exceptionally broadened peaks and particular solvent properties. The exclusion is motivated by visual inspection of the chromatograms and also by including the data generated by using DMSO as an injection solvent severely affected the outcome of the PCA, where it also stood out. Therefore, DMSO was considered as an outlier and only 16 injection solvents from here on were studied. The results were not much affected by excluding DMSO and the conclusions remained the same.

The PCA gives some indication that the plate number of relatively non-polar analytes (progesterone, fluoranthene and diclofenac) is less affected than other analytes by molecular interactions (hydrogen bonding, dipole moment *etc.*) in the both cases of using a 2-EP or a C18 column (**Fig. 2**). The same trend was observed for fluoranthene on the bare-silica column, while the other hydrophobic analytes co-eluted and no measurements could be made. However, among the non-polar analytes progesterone was most affected by molecular interactions associated with improved peak shapes and plate numbers using injection solvents with hydrogen donating capabilities.

Table 3. Properties of the injection solvents used to dilute the samples prior to injection onto SFC. All the data is given at 20 °C [16].

Solvent	Boiling point (°C)	Density (g mL ⁻¹)	Vapor pressure (kPa)	Viscosity (cP)	Surface Tension (dyn cm ⁻¹)	Eluent strength (ε°)	Dielectric Constant	Dipole Moment (D)	Hydrogen Donor	Hydrogen Acceptor
Pentane	36	0.626	57.3	0.23	15.48	0	1.84	0	0	0
Cyclopentane	49	0.751	40.0	0.44	22.42	0.04	1.97	0	0	0
Diethyl ether	35	0.713	53.6	0.224	72.8	0.29	4.33	1.3	0	0.47
Toluene	111	0.867	2.90	0.59	28.53	0.22	2.38	0.31	0	0.11
Dichloro-methane	40	1.33	47.0	0.4	26.52	0.3	8.93	1.14	0.13	0.1
Hexane	69	0.655	16.0	0.31	17.91	0	1.88	0.08	0	0
Cyclohexane	81	0.779	10.4	1	24.98	0.03	2.02	0	0	0
Acetone	56	0.791	24.6	0.32	23.7	0.43	20.7	2.7	0.08	0.43
Chloroform	61	1.483	26.2	0.57	26.67	0.31	4.81	1.15	0.2	0.1
Heptane	98	0.684	4.80	0.42	20.3	0	1.92	0	0	0
MTBE	55	0.741	25.0	0.27	19.4	0.48	2.6	1.32	0	0.3
2-Propanol	82	0.785	4.40	2.4	21.79	0.6	20.33	1.66	0.76	0.84
Tetrahydrofuran	66	0.886	20.0	0.55	26.4	0.48	7.58	1.75	0	0.55
Acetonitrile	82	0.786	9.70	0.38	19.1	0.5	37.5	3.44	0.19	0.4
Ethanol	79	0.789	5.90	1.1	22.32	0.68	24.55	1.66	0.86	0.75
Methanol	65	0.791	12.8	0.59	22.55	0.73	32.7	2.87	0.98	0.66
DMSO	189	1.100	0.056	2.24	43	0.48	46.68	4.1	0	0.76

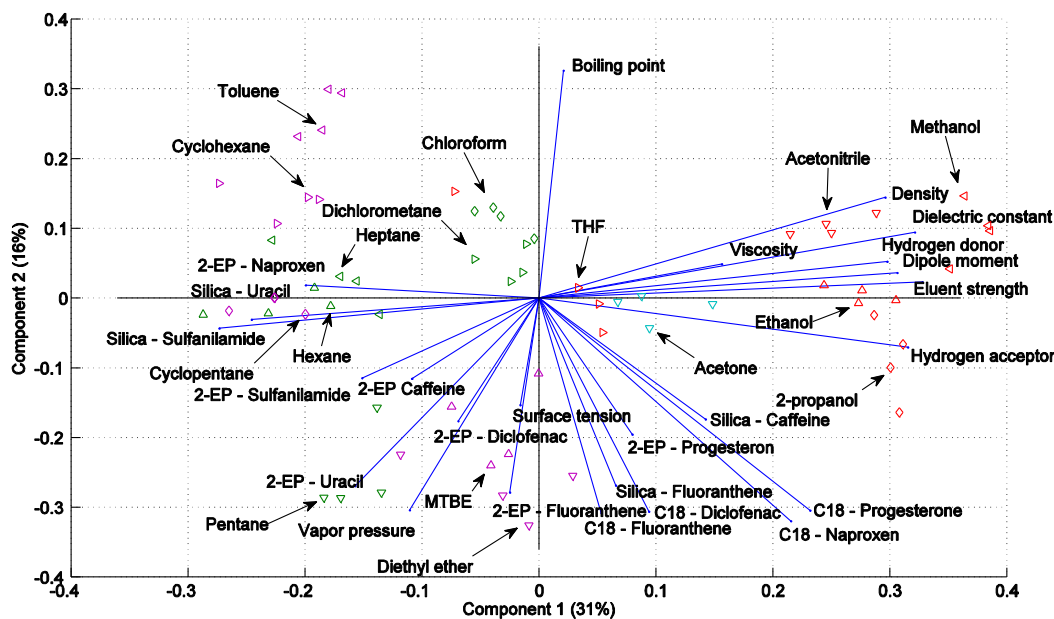


Fig. 2. Principal component analysis based on 16 different injection solvents, their properties and acquired plate number for analytes separated on either a C18, 2-EP or a bare silica column. The injection volumes were 10 μ L and were performed with 4 replicates. All the data was normalized by variance. The scores are represented by various symbols and the solid lines represent the loadings.

In this study we utilized calculated correlation coefficients to determine the correlation between different variables such as properties or acquired plate numbers. Basically, a perfect linear relationship between a variable, *i.e.* an injection solvent property with a response, *i.e.* plate number acquired for one of the analytes will have a correlation coefficient of one. If the variable has a negative impact on the response, the correlation coefficient will be negative and *vice versa*, whilst if there is no linear correlation the correlation coefficient will be determined as zero.

According to the calculated correlation coefficients the plate number of fluoranthene is not affected by hydrogen interactions of the solvent (**Fig. 3**). This is to be expected since fluoranthene has no hydrogen interaction capabilities (**Table 3**). However, on a C18 column the plate number for naproxen, which has three hydrogen-accepting groups and one hydrogen-donating group, is positively correlated with both hydrogen accepting and donating capabilities of the injection solvent. The same tendency could also be seen for diclofenac on the C18 column. Diclofenac has three hydrogen-accepting groups and two donating. No association between hydrogen interactions and plate number could be found for progesterone separated on the same column. Furthermore, the same trend is seen for the eluent strength of the solvent, which could also be derived from the correlation coefficient between eluent strength and hydrogen-donating ($r=0.75$) and hydrogen-accepting ($r=0.91$) capabilities (**Table S-1**).

This suggests that the injection solvent interacts with both the analyte and the stationary phase and thus minimizes the interactions between the analyte and bare silanol groups on a C18 column.

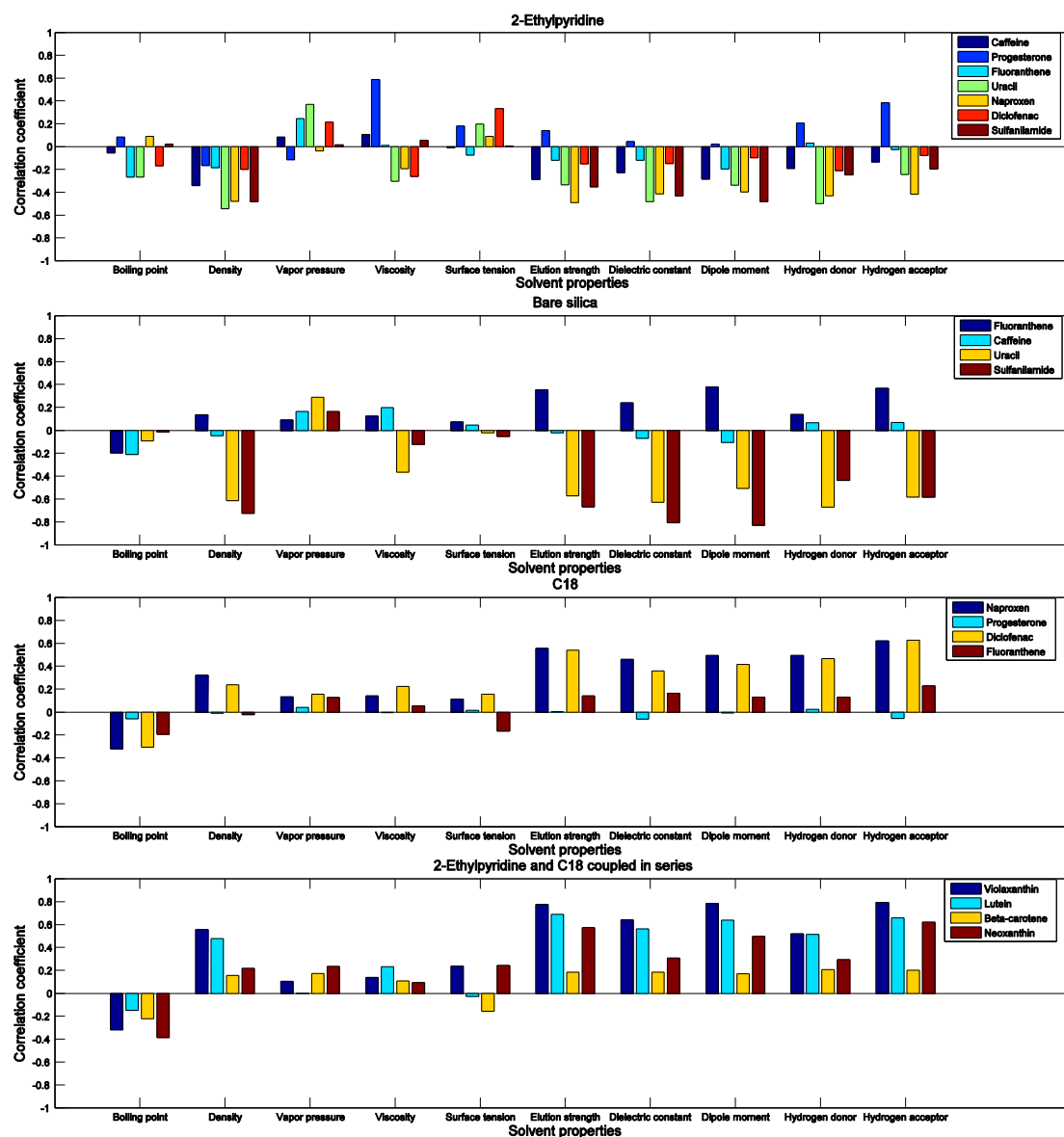


Fig. 3. Correlation coefficient between different injection solvent properties and the plate number using different columns. Three top graphs show injection of a mixture containing 7 different analytes on a (1) 2-EP column, (2) bare-silica column and a (3) C18 column. The bottom graph represents a sample of carotenoids separated on a C18 column in front of a 2-EP column.

The observed interactions in regards to injection solvents have previously been reported for RPLC as well [5]. These findings match previous suggestions that silanol interactions, including dipole – dipole and charge transfer interactions, when utilizing a C18 plays an increased role compared to RPLC due to the lack of water that would otherwise cover the accessible silanol groups of the stationary phase. Also separation on a 2-EP column has been reported to be sensitive towards ionic interactions [2]. Our experiments show an opposite trend when injecting on a 2-EP column, especially for uracil and naproxen. However, higher efficiencies were obtained for progesterone using injection solvents with more capabilities of hydrogen interactions on a 2-EP column. A general overview of the PCA and the correlation coefficients indicate that injection solvents with good capabilities of hydrogen interactions affect the chromatography of relatively hydrophilic analytes negatively on the two polar columns

(Figs. 2-3). Classical solvation effects due to analyte and solvent interactions most probably explain the phenomenon.

In the two polar systems the dielectric constants, dipole moment, hydrogen binding and eluent strength were found to be quite highly correlated to poorer efficiency and this especially for polar analytes such as uracil and sulfanilamide in the case of bare silica ($r > 0.77$). This could also be seen in the PCA. Furthermore, a general indication was given that analytes separated on the C18 column benefited from injection solvents with higher dielectric constant and dipole moments, whilst the opposite was observed on the two polar columns. Once again indicating a blocking of free silanol groups on the C18 and thus generating higher plate numbers.

Fluoranthene is conversely associated with higher plate numbers using injection solvents that are more polar on a bare-silica column, which is most likely an artifact due to very little retention of the very hydrophobic analyte. For all other analytes on each of the three columns the retention factor was larger than 1.5. Solvents with less polar properties generally performed much better on the two polar columns. Progesterone and fluoranthene did not correlate well with the dielectric constant or the dipole moment. This furthermore strengthens the suggestion that coverage of accessible silanol groups on a C18 column generates higher plate numbers for analytes capable of such interactions, while injection solvent interactions with analytes and stationary phases of polar characteristics exhibit lower plate numbers.

A bit counter intuitively the surface tension of the injection solvent does not have any particular association with the chromatographic efficiency when only injecting 10 μL sample. However, this might be explained by the low surface tension and the high diffusivities of the carbon dioxide. Therefore the mobile phase readily penetrates the injected solvent plug and dissolves it. This theory is further more strengthened by investigating the impact of injection solvent viscosity, where besides progesterone injected on a 2-EP column, the plate number generally did not correlate with viscosity. Viscosity had little effect on the plate number for fluoranthene, which should as previously discussed not be affected by polar interactions through neither solvent – solute nor solvent – stationary phase. As seen in the loadings of the PCA, the viscosity of the injection solvent is positively associated with intermolecular interactions including polarity and hydrogen interactions. All of these factors were generally related with lower plate numbers in the chromatography. The correlation coefficients between the viscosity and hydrogen donating and accepting capabilities are quite substantial, 0.60 and 0.54 respectively. Therefore the small variance in plate number might be explained by variable dependency. This suggests that the viscosity of injection solvents while injecting small amounts of 10 μL affects the chromatography to a very little extent in SFC compared to RPLC [6].

The boiling point and the vapor pressure are fairly orthogonally projected against the other solvent properties in the PCA (Fig. 2). At injection volumes of 10 μL , overall the boiling point was negatively associated whilst the vapor pressure was positively associated with higher plate numbers independent of column or analyte. At larger injection volumes this correlation became much more apparent, however discussed further down. This suggests that both these variables may influence the chromatography quite independently in relation to properties such as hydrogen bonding, dielectric constant or viscosity. Some correlation could be hinted that density affects the chromatographic

efficiency negatively when injection relatively small volumes particularly when focusing on injections on the 2-EP column, however this also became more apparent at larger injection volumes. A factor analysis of the same variables used in the PCA shows a rather similar outcome (data not shown). This indicates that generally speaking solvent properties affecting the chromatography could be categorized by either solvent polarity or solvent volatility.

Although density, and boiling point and vapor pressure might be good predictors for improving the number of plates, they are not all closely correlated. Obviously boiling point and vapor pressure are related to each other. However, the correlation coefficient between density of the 16 selected solvents with boiling point and vapor pressure was -0.03 and -0.19, respectively. This suggests that density of the injection solvent by its own might have an impact on chromatographic efficiency. Most likely a resistance in solubility of the injection solvent is the cause. Since retention times of the analytes were constant, an effect of locally high elution strength due to an increase in density was ruled out.

According to the Peng-Robinson equation of state, the solubility is affected by among other factors vapor pressure, molar volume and the critical temperature of the solute, in this case the injection solvent. This relationship and deeper discussions about solubility and models used for prediction are extensively described by Brunner [13] and Clifford [14].

Our observations corresponds well with the theories given in the literature that higher vapor pressure and lower densities of solute, in this given case the injection solvent including the analytes, improves solubility in the carbon dioxide. Analogously the solubility should also be improved in the CO₂ modified with methanol as co-solvent as well.

3.2. Effects of injection volume

By altering the volume injected onto a 2-EP column using the same analyte mixture diluted in the 16 different solvents, we investigated whether the influence of solvent properties on the plate number were consistent over different injection volumes. Injection volumes of 10, 30 and 50 μL , injected using full loop mode were tested. The acquired peak shapes of increasing the injection volume were much poorer. However, the trends of correlation were consequently the same although a bit more pronounced when injecting 30 or 50 μL (**Fig. 4**). Further indicating that the solvation capability of the injection solvent into the mobile phase hampers the chromatographic efficiency.

The peak broadening of fluoranthene that contains no functional group capable of hydrogen interaction is not affected by the hydrogen interacting abilities of the injection solvent. This suggests that there is no improved solubility of the injection solvent in the carbon dioxide through hydrogen interactions, as would otherwise be expected [15]. Hydrogen bonding was also associated negatively on the chromatographic efficiency for the rest of the analytes on the 2-EP column.

Some correlation between the viscosity and the surface tension of the injection solvent and the plate number can be observed using injection volumes of 30 or 50 μL .

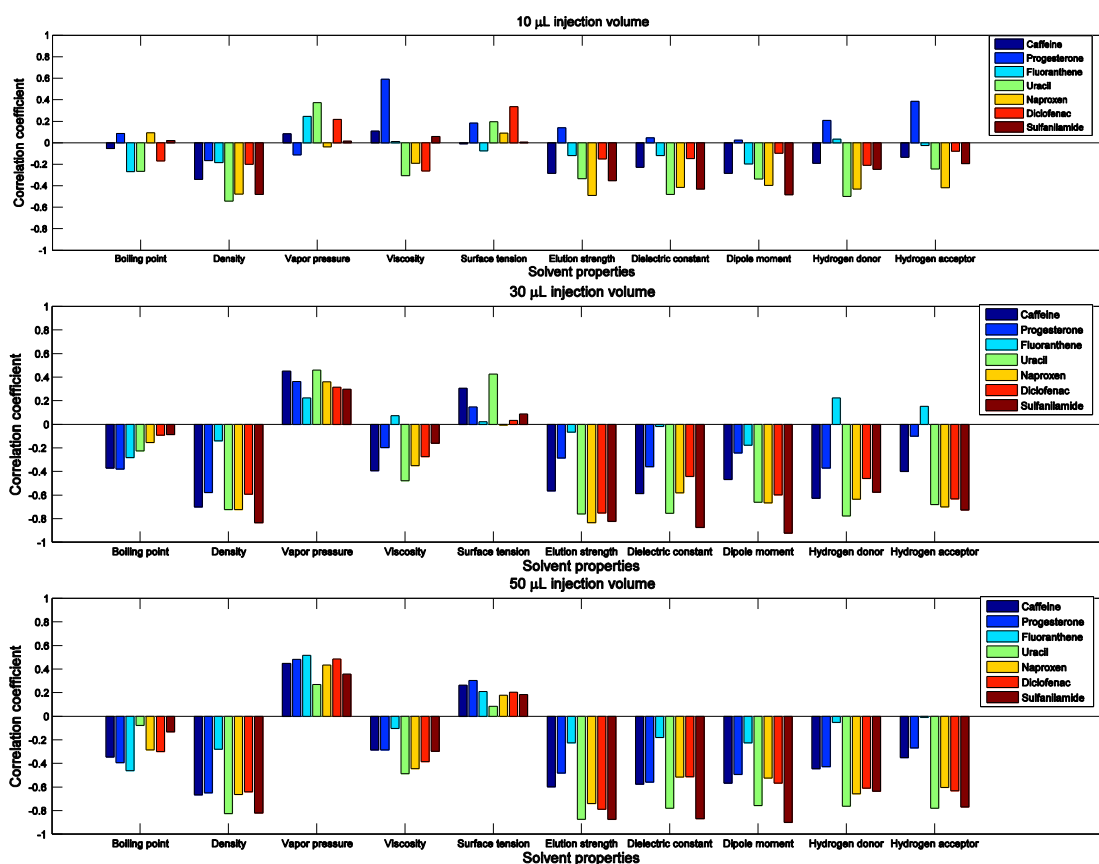


Fig. 4. Correlation coefficient between different injection solvent properties and the plate number using different injection volumes. Injection of a mixture containing 7 different analytes was performed on a 2-ethylpyridine column.

However, these two properties are somewhat correlated with the boiling point and the vapor pressure. Therefore, these findings may be correlated but not directly influential. It is also possible that at large injection volumes that the viscosity and the surface tension might influence the chromatographic efficiency.

By adding an extra 1.5 mL of dead volume by mounting a capillary in front of the column, in order to increase the time for the injected solvent to dissolve in the mobile phase, it was observed that analytes diluted in non-polar and volatile injection solvents generated significantly broader peaks (**Fig. 5**). Caused most probably by dispersion effects. However, peaks of analytes diluted in solvents that were relatively polar and non-volatile were either quite unaffected or had improved plate numbers due to the added dead volume of the system. This further proves that one of the main issues of injecting larger amounts onto a SFC system is the solubility. Based on the previous discussion the volatility and the molar density of the injection solvent may be the most important factor contributing the solubility.

3.3. Effects of injection solvent on two coupled columns

Further investigations were made on a more complex sample to deduce the impact on a real sample. The sample was an microalgae extract of *Scenedesmus* sp. obtained by SFE as described in a previous paper [10].

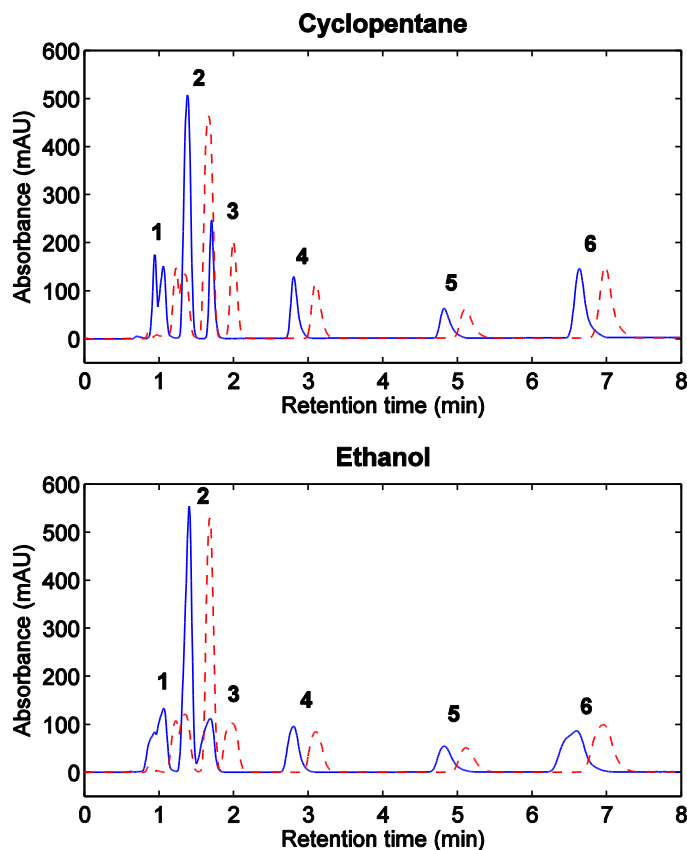


Fig. 5. Injection of analytes (solid line) dissolved in either cyclopentane (top) or ethanol (bottom) overlaid with chromatograms with 1.5 mL added dead volume to the SFC system (dashed line). Injection volume was 50 μ L. Peaks were assigned 1. caffeine followed by progesterone, 2. fluoranthene, 3. uracil, 4. naproxen, 5. diclofenac, 6. sulfanilamide.

The separation method was performed accordingly using a C18 column coupled in front of a 2-EP column, with the difference that the injection volume was 10 μ L instead of 50 μ L. The chromatographic efficiency was determined for beta-carotene, lutein, neoxanthin and violaxanthin. The PCA suggests that highest chromatographic efficiency is obtained using volatile and polar injection solvents capable of masking accessible silanol-groups of the C18 stationary phase (**Fig. 6**). The analysis also shows that beta-carotene is less affected than the other carotenoids. In general the same trend was observed as when utilizing the C18 column in the previous experiment. The carotene was less influenced by polarity or hydrogen bonding capabilities of the injection solvent while the xanthophylls gained improved number of plates. This indicates that the molecular interactions are only important in the start of the chromatography, before the analytes have fully been dissolved into the mobile phase and separated from the injection solvent. The improved peak shapes are most likely due to silanol coverage. It also implies that there is no persistent effect as might be the case when injecting polar solvents and running SFC with low percentages of modifier [8]. Otherwise the effects observed in the previous experiments using a 2-EP column would have occurred as well.

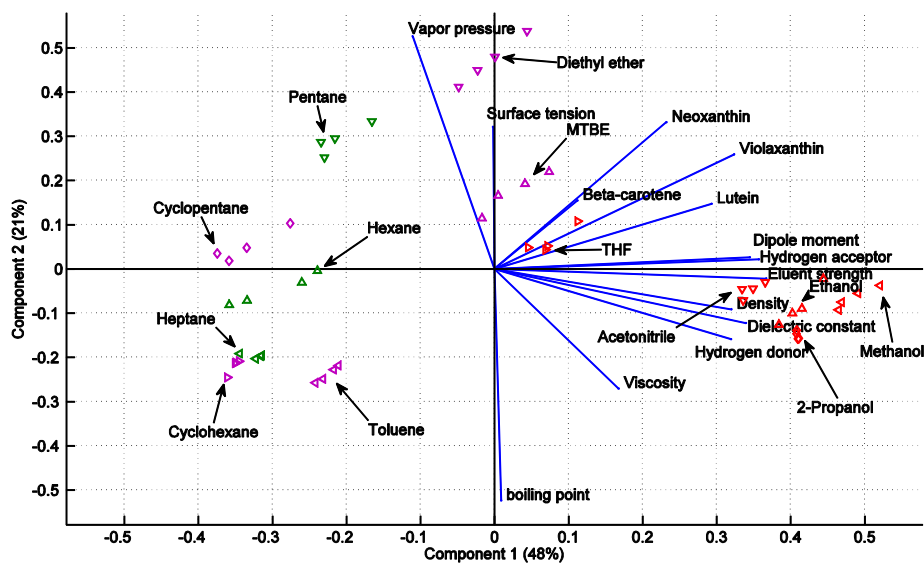


Fig. 6. Principal component analysis based on 13 different injection solvents, their properties and acquired plate number for the carotenoids separated on a C18 coupled in front of a 2-EP column. The injection volume was 10 μL and was performed with 4 replicates. All the data was normalized by variance. The scores are represented by various symbols and the solid lines represent the loadings.

3.4. Partial pick-up injection

In a series of performed experiments utilizing partial sample pickup with either a 30 or 50 μL injection loop. This setting of the autosampler is very useful if valuable sample should not be wasted due to overfilling the injection loop. However, we found that independently of the injection volume in partial pickup mode, the chromatography sustained negative impact on the plate number compared to overfilling an injection loop of desired injection volume (**Fig. S-1**). The phenomenon is most likely caused by the residues from the washing solvent, *i.e.* methanol, used to clean the sample needle and the injection loop between injections. The residues not only dilute the sample causing a longitudinal spread of solute, but also the increased volume hinders the injection plug to solubilize in the mobile phase upon injection. Therefore, injection modes not using loop overfilling should be reconsidered whenever possible.

4. Conclusions

Choice of injection solvent plays a very important role in SFC, particularly if larger volumes are injected. The main factors behind band broadening is suggested to be issues with solubility governed by density and vapor pressure, as well as injection solvent interactions with the column and analytes. Solvent interactions with the column could be positive *i.e.* using a C18 where free silanol groups may be covered, even when using an end-capped column as were the case for the SunFire C18 column.

Dipole moment and dielectric constant of injection solvent affects chromatography, by interacting with analyte and stationary phase and thus minimizing solute interacting with free silanol groups. Overall polarity does not seem to influence solubility in the modified carbon dioxide.

Hydrogen bonding does not measurably improve the solubility of the injection solvent in the mobile phase. However, increased potential of hydrogen interactions is suggested to dampen solute interactions will free silanol groups of C18 columns. These interactions are not desirable upon performing separation on polar columns *i.e.* a 2-EP or a bare-silica column.

Density and vapor pressure affects the solubility of injection solvent in the modified carbon dioxide and thus improves the peak shapes in SFC. A lower molar volume and higher vapor pressure of the injection solvent is positively correlated with higher plate numbers. Viscosity and surface tension did not have substantial effect on the chromatographic efficiency.

The results are consistent independent of injection volumes up to 50 μL , indicating that same level of influence applies regardless injection volume. In cases of more complex samples *i.e.* microalgae extract containing carotenoids, where more than one column is needed to achieve satisfying resolution. Then the impact of the injection solvent is less substantial but also dependent on which column is positioned first.

It should be noted that these conclusions are valid for injection volumes above 10 μL and that the tested range of modifier in this work ranged from 5% to 13% methanol. This contribution does not only provide a source for understanding how to optimize separation by choice of injection solvent, but will hopefully also be useful in understanding the chromatography as a whole through understanding of the molecular interactions inside the column.

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